# Screening, Nucleotide Sequencing and Biochemical Characterisation of Novel Lipolytic Enzymes from *Bacillus* sp. 01-855 associated with Marine Sponge *Aplysina aerophoba*

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Vorgelegt von

Anna Alexandrovna Karpushova

aus Taschkent (UdSSR)

Hauptberichter: Prof. Dr. R. D. Schmid
Mitberichter: Prof. Dr. D. H. Wolf

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**Eidesstattliche Erklärung**

Ich versichere, daß ich diese Dissertation selbstständig verfaßt und nur die angegebenen Quellen und Hilfsmittel verwendet habe.

Stuttgart, 16.11.2003. Anna Alexandrovna Karpushova

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| Prüfungsvorsitzender: | Prof. Dr. H. Bertagnolli |
| Hauptberichter: | Prof. Dr. R. D. Schmid |
| Mitberichter: | Prof. Dr. D. H. Wolf |
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# 1. Abbreviation

| | |
|---|---|
| Ap | ampicillin resistance |
| AP | alkaline phosphatase |
| APS | ammonium persulfate |
| BCA | bicinchoninic acid |
| *bla* | β-lactamase gene |
| bp | base pairs |
| BPB | bromphenol blue |
| BSA | bovine serum albumin |
| CIAP | calf intestinal alkaline phosphatase |
| °C | degree Celsius |
| d | demineralised |
| dd | double demineralised |
| ddNTP | dideoxynucleoside-5'-triphosphate |
| DMSO | dimethylsulfoxide |
| DNA | deoxyribonucleic acid |
| dNTP | deoxyribonucleoside- 5'-triphospate |
| DTE | dithioerythritol |
| DTT | dithiothreitol |
| EC number | Enzyme Commission number |
| *E. coli* | *Escherichia coli* |
| EDTA | ethylenediaminetetraacetic acid |
| eGFP | enhanced green fluorescent protein |
| g | gram |
| h | hour |
| IMAC | immobilised metal affinity chromatography |
| IPTG | isopropyl-β-D-thiogalactoside |
| kb | kilobases |
| kDa | kilo Dalton |
| l | litre |
| LB | Luria-Bertani |
| LMW | low molecular weight |
| % (m/v) | mass percent |

| | |
|---|---|
| M | mole per litre |
| mg | milligram |
| µg | microgram |
| min | minute |
| ml | millilitre |
| µl | microlitre |
| mM | millimole per litre |
| mmol | millimole |
| mol | mole |
| nm | nanometer |
| $OD_{578}$ | optical density = absorbency at 578 nm |
| ORF | open reading frame |
| PAGE | polyacrylamide gel electrophoresis |
| pH | *pontentia hydrogenii* |
| PBS | phosphate buffered saline |
| PCR | polymerase chain reaction |
| PEG | polyethylenglycole |
| *p*NP | *p*-nitrophenyl (ester) |
| PVDF | polyvinylidene fluoride |
| rpm | rotations per minute |
| RT | room temperature |
| s | second |
| SDS | sodium dodecyl sulfate |
| TAE | Tris/acetate/EDTA buffer |
| TE | Tris/EDTA buffer |
| TEMED | N, N, N′, N′-tetramethyl-ethylendiamine |
| Tris | tris-(hydroxymethyl)-ammoniumethane |
| TSS | transformation storage solution |
| U | unit(s) of protein activity |
| UV | ultraviolet |
| v | volume |
| % (v/v) | volume percent |
| V | volt |
| X-gal | 5-bromo-4-chloro-3-indol-β-D-galactopyranoside |

Additionally, the common codes for nucleotides, amino acids and customary abbreviations for the restriction enzymes, polymerases etc. were used.

# 2. Introduction

## 2.1. Lipases and Esterases

Hydrolases are a large family of enzymes that are responsible for the degradation of amide and ester type bonds in a wide range of substrate molecules. These enzymes accept a wide variety of natural and unnatural substrates and this has led to their use as stereo-selective catalysts in the synthesis of optically pure molecules for the pharmaceutical and agrochemical industries.

Lipases and esterases belong to serine hydrolases, a group of enzymes that catalyses ester bond cleavage by reaction with water (**Figure 2-1**).

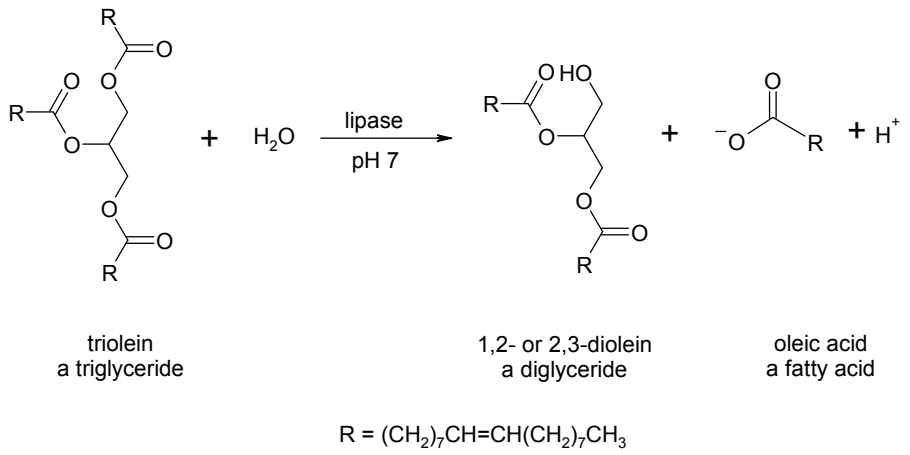


**Figure 2-1** Lipase from *Candida antarctica* catalyses by stepwise hydrolysis of triglycerides (e.g. triolein) to fatty acid and glycerol. The reaction shows only the first step from a triglyceride to a diglyceride.

### 2.1.1. Occurrence and Availability of Esterases

Esterases (carboxylester hydrolases, EC 3.1.1.1) catalyse the hydrolysis and synthesis of carboxyl acid esters and are widely distributed in prokaryotic and eukaryotic organisms. The physiological function of the most esterases is unknown. But many of them show a wide

substrate tolerance which led to the assumption that they are involved in the enabling access to carbon sources or are involved in catabolic pathways [1]. Some acetyl- and cinnamic acid esterases take part in a carbon metabolic pathway and are involved in the degradation of hemicellulose [2]. Acetyl- and butyryl choline esterases hydrolyse *in vivo* neutrotransmitters. Esterases also catalyse the detoxification of biocides: an insecticide resistance, for example, related to an amplification of esterase gene [3]. *Bacillus subtilis* esterase cleavages the phytotoxin Brefeldin A [4]. Esterases from *P. fluorescens* are involved in the formation of ω-hydroxy acids from lactones [5,6], which are produced *in vivo* in an enzymatic Baeyer-Villiger oxidation [7,8]. This might enable growth on carbon sources such as cyclic alkanes or cyclic alkanones or is required for the production of flavour lactones in the industry. Carboxyl esterases from *Acinetobacter* sp. are involved in the catabolism of aryl esters [9]. Esterases do not require cofactors, stable in organic solvents and possess high regio- and stereospecificity, which make them attractive biocatalysts for the production of optically pure compounds in fine-chemical synthesis [10,11,12,13,14].

### 2.1.2. Occurrence and Availability of Lipases

Lipases (triacylglycerol ester hydrolases, EC 3.1.1.3) are defined as carboxylesterases, which catalyse the hydrolysis and synthesis of long-chain acylglycerols with trioleoylglycerol being the standard substrates. In addition, lipases catalyse the hydrolysis of a broad range of natural and unnatural esters, possess high chemo-, regio- or/and stereoselectivity. Besides high selectivity and broad substrate range, lipases act on water-insoluble substrates. Some lipases, for example, pancreatic and the lipoprotein lipases require the presence of another protein based molecules in order to acquire full enzymatic activity. In the case of human pancreatic lipase, the colipase interacts with the small domain of the lipase, and is involved in the major conformational changes around the lid associated with interfacial activation of the lipase. Transcription of lipase genes may be regulated by quorum sensing and two-component systems. Secretion can proceed either via the Sec-dependent general secretory pathway [15] or *via* ABC transporters [16,17,18]. In addition, some lipases need folding catalysts such as lipase-specific foldases and disulfide-bond-forming proteins to achieve a secretion-competent conformation [15]. Lipases are widely distributed in micro-organisms, fungi, plants and animals.

### 2.1.3. Lipases and Esterases are α/β Hydrolases

All serine hydrolases including haloperoxydases, epoxide hydrolases, lipases and esterases have a similar element in their 3 D structure. This element, called α/β-hydrolase fold [19], is composed of a central β sheet consisting of up to eight different β strands (β1-β8) which are connected by up to six α helices (**Figure 2-2**). These elements occur in the same order in all lipase and esterase amino acid sequences and are oriented in the same three-dimensional direction in all structures.

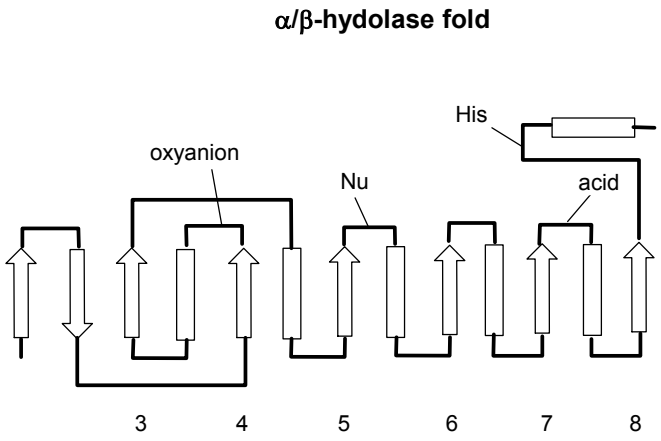


**Figure 2-2** Schematic figure of the α/β-hydrolase fold. Oxyanion: reduces that stabilised the oxyanion. Nu: nucleophilic residue; for the lipases, esterases and serine proteases this is a serine. α-helices are shown as rectangles, β-sheets are arrows.

### 2.1.4. Structure of Lipases and Esterases

The fundamental difference between lipases and esterases is their ability to act on surface display. Esterases act on water soluble carboxylic ester molecules, whereby lipases catalyse the hydrolysis of fats and oils at the water-lipid interface and reversing the reaction in non-aqueous media. The protein structural features underlying these observations have been unravelled with the determination of open and closed structures of *Rhizomucor miehei* lipase in 1990 [20]. The X-ray structures of these lipases indicated the presence of a helical amphiphilic unit (lid or flap) covering the active site of the enzyme in solution. It was shown that a lipid-induced change in the lid orientation causes interfacial activation. Lipases possess

poor activity towards soluble substrates in aqueous solutions because the lid is closed. Upon binding to a hydrophobic interface such as a lipid droplet, the lid opens which leads to the increasing of the catalytic activity of the lipase. In addition, the opening of the lid places one of the oxyanion-stabilising residues into catalytic orientation. More recently, it turned that the presence of a lid-like structure is not necessarily correlated with interfacial activation [21]. Lipases from *Pseudomonas aeruginosa*, *B. glumae* and *Candida antarctica* B, and a coypu pancreatic lipase do not show interfacial activation but nevertheless have an amphiphilic lid covering their active site [21]. Through lipolytic reaction occurs at the water-lipid interface, the lipase kinetics in comparison to kinetic the esterases obey, cannot be described by Michaelis-Menten equations. Lipases need also a minimum substrate concentration before high activity is observed, because of the interfacial activation due to a hydrophobic domain covering the active site. Only in the presence of a minimum substrate concentration, i. e. a triglyceride phase or a hydrophobic organic solvent, the lid moves apart, making the active site accessible. In general, lipases prefer water-insoluble substrates composed of long-chain fatty acids, whereas esterases preferentially hydrolyse substrates bearing fatty acid residues shorter than $C_6$. Lipases and esterases are very similar in terms of their biochemical and structural properties. The catalytic apparatus consists of amino acid triad: serine, histidine and aspartate (or glutamate) and includes also several oxyanion-stabilizing residues.

Lipases and esterases display low sequence homology. The nucleophilic Ser residue from the catalytic triad is located at the C-terminal end of strand β 5 in the conserved pentapeptide Gly-x-Ser-x-Gly, forming a characteristic β-turn-α motif named the "nucleophilic elbow". More recently esterases have been identified containing a Gly-x-x-Leu motif [22] as well as the enzymes with high homology to class C β-lactamases [23]. A thorough comparison of 53 amino acid sequences of lipases and esterases revealed that other motifs also exist. This was reviewed in details in [24]. The most important classes are represented below.

Some lipases and esterases from *Streptomyces scabies* revealed the first example of GDSL consensus sequence. Other esterases from *Pseudomonas aeruginosa*, *Salmonella typhimurium* and *Photorhabdus luminescens* belong also to the GDSL group, whereby the fist two being outer membrane bound esterases. Moreover, the crystal elucidation of the esterase from *Streptomyces scabies* revealed that it contains a Ser-His dyad with the acidic residue being replaced by a neutral hydrogen-bond acceptor (a mainchain oxygen atom) [22]. The enzyme also has an α/β-tertiary fold, which differs substantially from the α/β-hydrolase fold. Other enzymes show homology to the mammalian hormone-sensitive lipase family. Here conserved sequence regions were found, which initially have been related to activity at low

temperatures. However, esterases from psychrophilic (e.g. *Moraxella* sp., *Psychrobacter immobilis*), mesophilic (*Escherichia coli*) and termophilic (*Archeoglobus fulgidus*) origins belong to this family.

The esterases from *Sulfolobus acidocaldarius* and *Acetobacter pasteurianus*, which belong to the family V, show significant homology to non-lipolytic enzymes such as epoxide hydrolases, dehalogenases and haloperoxidases. Esterases from the family VI display approximately 40 % homology to eukaryotic lysophospholipases. Rather small enzymes (23-26 kDa) are found in this family. The esterase from *P. fluorescens* belongs to this family and its structure is known [25]. The esterase is active as a dimer, has a typical Ser-Asp-His catalytic triad and accepts a broad range of substrates. In contrast to the members of family VI, esterases from the family VII are rather large enzymes (ca. 55 kDa). They share significant homology to eukaryotic acetylcholone esterases and intestine or liver carboxyl esterases. The *p*-nitrobenzyl esterase from *Bacillus subtilis* [26,27] and the esterase from *Arthrobacter oxydans*, which is active towards phenylcarbamate herbicides [28], belong to this family. In the last family VIII the high homology to class C β-lactamoses is observed. These enzymes contain a Gly-x-Ser-x-Gly and a Ser-x-x-Lys motives, but it has been recently demonstrated by site-directed mutagenesis studies of the esterase from *Burkholderia gladioli* that the Gly-x-Ser-x-Gly motive does not play a significant role in enzymatic function [29].

### 2.1.5. Catalytic Mechanisms of Lipases and Esterases

The active site in lipases and esterases is very similar to those found in serine proteases [30]. The catalytic mechanism assumed for triacylglyceride lipases and esterases is centred on the active site Ser. First, the ester binds to the enzyme and the catalytic serine attacks the carbonyl forming a tetrahedral intermediate (**Figure 2-3**). Collapse of this tetrahedral intermediate releases the alcohol and leaves an acyl enzyme intermediate. In a hydrolytic reaction, water attacks this acyl enzyme to form a second tetrahedral intermediate. Disaggregation of this intermediate releases the acid. Alternatively, another nucleophile such as alcohol can attack the acyl enzyme thereby yielding a new ester (a transesterification reaction). In most cases, it appears that the formation of the acyl enzyme is fast and the deacylation is the rate-determining step.

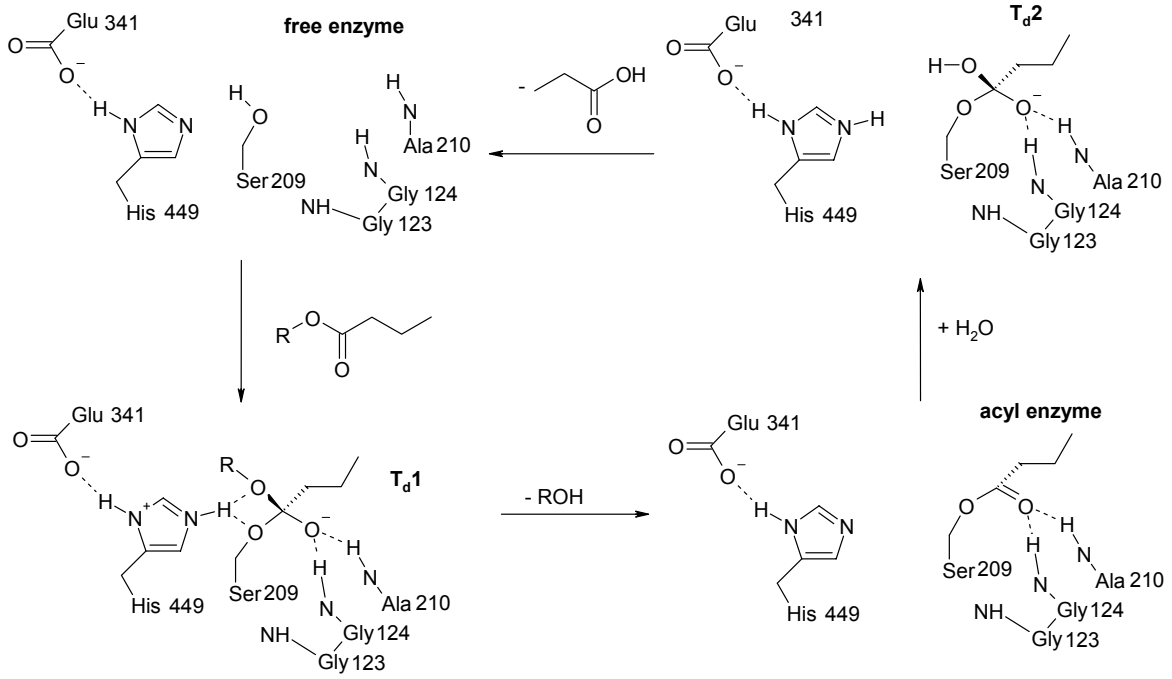


**Figure 2-3** The hydrolysis of butyric acid catalysed by lipase or esterase. During the hydrolysis the formation of the acyl enzyme first involves the formation of tetrahedral intermediate, $T_d1$. In this step the alcohol residue is released, that determines the selectivity of lipases towards alcohols. Release of the acyl enzyme involves the second tetrahedral intermediate, $T_d2$. When deacylation limits the rate, this step determines the selectivity of the lipase towards carboxy acids.

The contribution of the electrostatic interactions to lipase or esterase activity was studied in [31]. Comparison of the amino acid sequences of lipases and esterases and their 3D-structures suggested that they can be distinguished by pH dependent electrostatic "signature". The active site of lipases displays a negative potential in the pH range associated with their maximum activity (usually at pH 8.0), whereas esterases show a similar pattern, but at pH values around 6, which correlates with their usually lower pH activity optimum.

### 2.1.6. Substrate Binding Site in Lipases and Esterases

Lipases and esterases have distinct binding sites in the active site for the alcohol and acid residues. Four substrate binding pockets were identified for triglycerides in lipases: an oxyanion hole and three pockets accommodating the fatty acids bound at position sn-1, sn-2, and sn-3 which examined below in details.

The alcohol binding site is similar in all lipases. It is a crevice containing two regions: a large hydrophobic pocket which is open to the solvents and a small pocket that faces the bottom of the crevice. The shape of this pocket defines the sterioselectivity of lipases towards secondary alcohols.

The binding site for the acid residue of the esters differs considerable among the lipases. But in all structures the α-carbon of an acyl chain binds just below the large hydrophobic region of the alcohol binding site [32]. Substituents at the α-carbon extend into the hydrophobic pocket. The acyl binding region which is formed by the tunnel and the hydrophobic pocket are similar to the S1 site in proteases. According to shape and physico-chemical properties of the fatty acid binding site, the lipases were subdivided into three groups: those with funnel like binding site; lipases with a hydrophobic, crevice like binding site located near the protein surface and lipases with tunnel like binding site [33]. The differences in size and the hydrophilicity/hydrophobicity of these regions determine lipase enantiopreference.

### 2.1.7. Lipases and Esterases in Biotechnology

Lipases and esterases are still the most important group of biocatalysts for biotechnological applications, because they are stable in organic solvents, do not require cofactors, accept a broad range of substrates and often exhibit high enantioselectivity. The commercial use of lipases is a billion dollars business, which comprises a wide variety of different applications in the area of detergents and in the production of food ingredients and enantiopure pharmaceuticals [34]. Lipases are especially useful biocatalysts, because they operate at the water-organic interface and can be applied to water insoluble substrates. Many are inexpensive, stable and commercially available.

Beside lipases a considerable number of esterases have been discovered and characterised. Esterases found wide applications in different spheres of industry. In organic synthesis esterases are used for the resolution of prostereogenic compounds and racemates. Acetylcholine esterases (AChE, EC 3.1.1.7) are used for the hydrolysis of *meso*-diacetates. Mammalian esterases are used for the kinetic resolution of secondary alcohols, lactones or asymmetrisation of prostereogenic or *meso*-dicarboxylic acids. Microbial esterases are used for the resolution of secondary and primary alcohols or carboxylic acids. The esterase from *Arthrobacter globiformis* is used in the synthesis of (+)-trans-(1R,3R) chrysanthemic acid, which is an important precursor of pyrethrin insecticides. In this reaction the formation of the desired enantiomer is >99 % enantiomeric excess (ee), at 77 % conversion. The

carboxylesterases NP from *Bacillus subtilis* show very high activity and stereoselectivity towards 2-arylpropionic acid, which is used in the synthesis of (S)-naroxen-(+)-(S)-2-(6-methoxy-2-naphthyl) propionic acid, which is a non-steroidal anti-inflammatory drug. This reaction yields (S)-naproxen with excellent optical purity (99 % ee) at an overall yield of 95 % [35]. Besides naproxen, various other 2-arylpropionic acids are produced with high enantioselectivity [36,37]. The pig liver esterase has been applied in the asymmetric synthesis of chrysanthemic, permethrinic and caronic acids from the corresponding racemic methyl esters. The esterase from *Pseudomonas fluorescens* shows enantioselectivity towards methyl-3-bromo-2-methylpropionate, a useful chiral synthon. Esterases are also employed in reactions where chemo- or regioselectivity is of interest. Ferulic acid from plant cell wall polysaccharides such as pectin or xylan can be realised by some carboxyl esterases. In xylans ferulic acid is attached to arabinose residues, which are bound to the xylan backbone. In pectins ferulic acid can be linked to side chains of galactose or arabinose. Ferulic acid thus obtained can be converted enzymatically by means of feruloyl esterases [38,39,40] into vanillin, a flavour compound which is used in food industry.

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 industrially relevant lipases and esterases genes and optimisation of already existing enzymes with respect to desired properties are of special interests of industry. Over the years micro-organisms have been a great source for novel enzymes and biologically active compounds. A wide distribution of lipases and esterases in micro-organisms and simple screening assays based either on employing of chromophoric substances or tributyrine-supplemented agar plates allow effective isolation and identification of novel enzymes. For example, several thousand microbial samples isolated from the soil were tested by screening on solid and liquid media for the production of lipases revealing that about 20 % were lipase-producers [41].

The unique properties of the enzymes from extreme environments are of special interest of industry. Therefore, special attention by the isolation of novel microbial enzymes is paid on the source the organisms derived. Nowadays a lot of different lipases and esterases have been isolated from both thermophilic and psychrophilic species. Many of these enzymes were established as recombinant proteins in *Escherichia coli*. The importance of terrestrial bacteria and fungi as a source of hydrolytic enzymes and valuable bioactive compounds has been well established more than half a century ago. On the other hand the expectable enormous

biodiversity of marine micro-organisms and the environment they live can be a reason for the isolation of novel lipases and esterases from them.

## 2.2. Screening for Novel Enzymes for Biocatalytic Processes

There are many different approaches to screen for novel biocatalysts. Wild-type enzymes produced by the host organisms do not always satisfy in terms of stability, activity and enantioselectivity to the requests of industry. These properties can be optimised by modern biotechnologies, such as genetic engineering, protein engineering (including directed and random mutagenesis), metabolic engineering and etc.. Directed evolution nowadays is one of the most powerful tool to optimise the existing lipases and esterases. Another way to discover the new enzymes for commercial applications is based on the genomic sequencing. New genes can be predicted on the bases of the comparative analysis to the known gene sequences.
Many micro-organisms and their enzymes with unique functions have been discovered by means of screening and are now commercially used for the industrial applications. To obtain novel biocatalysts the traditional method of cultivation and subsequent screening of pure strains is used. Large strain collections, particularly when efficiently dereplicated with whole-organism typing procedures such as pyrolysis mass spectrometry (PyMS) [42] to select diverse procedures of biocatalysts and metabolites, are valuable for rapidly accessing a defined pool of natural biodeversity [43]. Screening from the clone-banks of the previously identified micro-organisms that have been recognised as enzyme producers can be extremely rewarding. By setting up the clone banks in a unified or small set of the host organisms like *E. coli*, *Bacillus* or yeast only a limited number of different propagation methods need to be implemented, thus allowing systematic screening methods to be carried out more easily [44]. If the desired gene was identified, it is generally easier to scale it up and to produce it in large quantities. Additionally, the genes of interest from clone banks are often removed from the native regulatory elements that can repress expression and thus facilitates the purification the gene from isoenzymes and other competing activities.
Natural products and novel enzymes from soil microbiota and terrestrial flora and fauna greatly expand the chemical diversity that can be employed directly or modified for biotechnological applications. But molecular ecological studies showed that less then 1 % of microbial diversity of complex environmental samples as soil, which can consist of up to 10 000 different species, is covered by standard cultivation techniques [45]. As a consequence,

the direct isolation of microbial DNA from the environment for the sake of an unbiased genomic representation is used. Direct cloning of such collective genomes of all micro-organisms in a certain habitat (metagenome) with following screening of it, illustrated the potential of isolating any kind of gene from non-cultivated organisms. In the eighties, numerous studies on isolation and cloning of "metagenomic" DNA from a variety of environments have been done. Soil has been the most attractive source for the microbial diversity in spite of difficulties dealing with the co-extraction of inhibitors of molecular manipulations and cloning. Humic acids [46,47,48,49] and the traces of pollution contaminants of organic and inorganic nature were the main problems.

On the other hand, enormous biodiversity of marine micro-organisms might be a novel source for enzymes and different products with little overlap in terms of properties with traditional sources of natural compounds. During the last 10 years marine macro-organisms have provided a large number of new bioactive compounds. But in many cases micro-organisms are known or suspected to be the biosynthetic source of marine invertebrate natural products [50]. The number of secondary metabolites from marine micro-organisms is smaller, but rapidly increase [51,52]. The major challenge in microbial ecology today is that the microbes that predominate in natural environments are not well represented among laboratory strains [53]. The development of methods for making these organisms amenable to laboratory investigations and finally to industrial applications plays an important role in marine biotechnology.

## 2.3. Marine Biotechnology

There is a considerable interest in exploring marine habitats for novel enzymes, biologically active metabolites and biotechnologies. Serious attempts to use the vast potential of marine organisms as a source of bioactive metabolites that may be directly utilised in industry or medicine started in the late 1960s. From 1969 - 1999 approximately 300 patents on bioactive marine naturals products were issued [54]. From humble beginnings the number of compounds isolated from various marine organisms has virtually increased and now exceeds 10.000 [55], with hundreds of new compounds still being discovered every year [56,57]. On the other hand the number of reports dealing with novel enzymes from marine organisms is still limited.

The number of patent applications on marine naturals products is also lower in comparison with those of terrestrial origin. Majority of the isolated "marine" compounds are mainly derived from macro-organisms such as sponges, ascidians, cnidarians, bryozoans and nudibranchs [58]. One explanation of low appeal of marine secondary metabolites for commercial utilisation is the limited availability of large quantities of organisms as starting material for the extraction. Furthermore, the isolation of large amounts of these compounds from animal tissues is not possible because of its devastating impact on the natural environment. Because of enormous difficulties involved in harvesting products from marine animals, and the fact that some of the bioactive compounds are produced by associated bacteria, the advantages of sustainable production of bioactive metabolites by bacteria or fungi, under the protection of natural resources, seems to be very attractive for the future [52].

Studies on marine micro-organisms are facing three main problems. First, the taxonomy of marine bacteria and fungi is very poorly defined, so that the binomial identification of species is frequently difficult. In many publications dealing with marine bacterial strains described metabolites are often isolated from partially or totally undefined organisms. The second question to be considered what are the marine micro-organisms. The requirement for sodium chloride for growth is not a practical solution to define them as bacteria of marine origin. Although the marine micro-organisms may tolerate a wide range of salinities it is speculated, that they are in fact terrestrial organisms that have been swamped into oceans from rivers, estuaries and sewage outfalls. Therefore, the marine micro-organisms are defined as bacteria that have been isolated from marine sources on marine media [59]. The third point refers to

technical problems of cultivation of marine bacteria and fungi. The cultivation conditions for many micro-organisms of marine origin are often unknown.

The major challenge in microbial ecology today is that the microbes that predominate in natural environments are not well represented among laboratory strains [53]. Therefore, isolation and cultivation of suspected microbial producers of bioactive natural products and enzymes either from the seawater or from the tissue of invertebrates though careful design of special media could provide an answer to the supply problem. It is currently presented by many pharmacologically interesting compounds or enzymes and their further development would be very useful. If bacteria are the producers of bioactive metabolites or enzymes of interest, transfer of gene clusters responsible for the biosynthesis of the respective products into a vector suitable for large-scale fermentation could provide an alternative strategy thereby avoiding the foreseeable difficulties in culturing symbiotic bacteria.

### 2.3.1. Marine Sponges: Opportunities for Microbial Biotechnology

Sponges (class Porifera) are one of the oldest living organisms, whose origins date back to the Precambrian more then 600 millions years ago. Today an estimated 9000 sponges species are found mostly on tropical reefs but also at increasing latitudes [60]. Sponges are associated with a large amount of different micro-organisms including cyanobacteria, diverse heterotrophic bacteria, unicelulare algae and zoochlorellae. These organisms, both intra- and extra- cellularly, constituted up to 40 % of the tissue biomass [61]. Sponges are filter feeders and certain amount of transient bacteria are trapped within the vascular system or attached to the sponge surface. Several studies dealing with the diversity of sponge-associated microbial communities by using cultivation based approaches revealed that the microbial communities can be quite different.

Sponges have provided more natural products than any other phylum of marine invertebrate [51]. Moreover, distribution of marine natural products derived from sponges consists 38 % of the total amount of compounds discovered in all marine organisms. The most promising pharmaceuticals with antiviral, antitumor, antimicrobial, anti-inflammatory, immune-suppressive or generally cytotoxic properties and agents for cell biological research belong to these natural compounds. These compounds are endowed not only with high pharmacological potential, but in some cases undergo specific interactions with enzyme systems. Among the anticancer compounds, bryostatin 1 serves as a good example of past and current trends in marine biomedical research [62]. Bryostatin 1 is a macrocyclic metabolite which was isolated

from bryozoan *Bugula neritina* from the Gulf of California. Bryostatin 1 was found to affect protein kinase C activity, which may be the mechanistic basis for both anticancer and immunostimulating activity. Marine chemicals often possess quote novel structures and this in turn leads to pronounced biological activity and novel pharmacology. For example, prostaglandins such as prostaglandin E1, first isolated in 1934 from sheep spermatophore [63] showes exciting uterine and antihypertensive activities. The cost of prostaglandin from this source is high because spermatophores are scarce and synthetic approaches to such compounds are difficult. Clavulactone, isolated from the Okinawan soft coral *Clavularia viridis* [64], is a natural prostanoid possessing a γ-lactone moiety in the α-side chain, and its discovery may provide a new, inexpensive source of prostanoids.

In many cases it will be speculated, that bioactive compounds found in marine macro-organisms can be produced by sponge-microbe symbioses. Therefore, marine sponges symbioses present a fascinating subject for biological and biochemical research. As marine micro-organisms survive under harsh environmental conditions, it can be also expected that they can be a source of novel enzymes which can function at low or high pH values, high salt concentrations or unusual high or low temperatures. In addition, the marine micro-organisms raise the opportunities to develop the marine resources in ways that circumvent environmental and supply problems. Cultivation and basic studies on symbionts completely eliminates the problem of wild collection and can potentially improve the yield and thus the economics of productions.

### 2.3.2. Aplysinidae Family and their Microbial Communities

**Figure 2-4** Marine sponge *Aplysina aerophoba* (photo of Nicola Cadel)

Sponges of the Aplysinidae family inhabit subtropical and tropical waters of the Mediterranean sea and the Pacific and Atlantic oceans. In the past studies on microbial communities of sponges and marine invertebrates were based primarily on the culturability of the respective species and on their observation *in situ* using electron microscope. The characterisation of the microbial communities present in sponges nowadays is performed by applying molecular methods such as fluorescence *in situ* hybridisation (FISH) using specific 16S rRNA targeted oligonucleotide probes [65]. Whereas the morphological plasticity of bacteria does not match their taxonomic diversity, the number of bacteria strains obtained, for example, from sponges is usually exceedingly small compared to the real microbial diversity as exemplified by the Mediterranean sponge *A. aerophoba* [66]. These sponges are especially rich in bacteria. A study on bacteria associated with Mediterranean sponges yield near six hundred microbial strains, which can be isolated by direct inoculation of marine agar plates, a general medium for marine, non-obligatory oligotrophic bacteria. The bacterial communities associated with *A. aerophoba* (**Figure 2-4**) and *A. cavernicola* were found remarkable stable and to be similar. Stable relationship of sponge and associated bacteria was observed even when the specimens of *A. aerophoba* were kept over a week in aquarium with sea water that had been supplemented with antibiotics [67]. The Mediterranean sponges *Aplysina aerophoba* isolates are affiliated with low (*Bacillus*) and high G+C Gram positive bacteria (*Arthobacter*, *Micrococcus*), as well as the α, δ- and γ-Proteobacteria [68]. Among the bacteria the δ-proteobacteria were most the abundant, followed by the high-GC Gram positive bacteria, the γ-proteobacteria and the *Bacteroides* cluster. Whereby no Archaea were detected [65,67]. Gram-positive isolates represent about 10 - 15 % of the total bacteria isolates. Moreover, in comparative studies on uniform of microbial communities in sponges from different oceans [66], the existence of bacteria that are specific to certain host sponges or those that occur only transiently or seasonally was shown. Many of *Chloroflexi* 16S rDNA sequences recovered from *A. aerophoba,* for example, were not shared with any of the investigated sponges. It was assumed, that these bacteria are specifically associated with *A. aerophoba* or with *Aplysina* sponges and they are very abundant in sponge tissues.

*Aplysina* sponges contain up to 13 % of the dry weight of brominated metabolites with antimicrobial activity [69,70], repellent properties against predators [71] and cytotoxic activity in human breast cancer cell lines [72]. The localisation of these compounds in the sponge indicates that they are probably produced by the sponge itself, whereby the involvement of bacteria in the secondary metabolism is still conceivable owing to the structural similarities to microbially produced analogies. During the past in the search for new

pharmaceutical, agrochemical or food industry needs increasing attention is being given to marine micro-organisms as a source of novel enzymes and biologically active compounds. Though the number of publications dealing with novel enzymes from marine micro-organisms used for the industrial applications is relatively small in comparison to the reports dealing with the utilisation of marine chemicals.

## 2.4. Bacillus

### 2.4.1. *Bacillus* in Biotechnology

The genus *Bacillus* has a long history of importance, both from an economic point of view and as a source of experimental micro-organisms [73]. Different *Bacillus* strains have commercial importance in the area of enzymes production for food, drink, chemical synthesis and detergent markets. Nowadays *Bacillus* strains are used for the production of antibiotics, fine biochemicals including flavour enhances, food supplements and insecticides. The ability of *Bacillus* to secret proteins, coupled with its regulatory acceptability, has resulted in application of *Bacillus* as a host for the production of heterologous proteins. That is why it is important for the biotechnologist interested in novel microbial systems and products, to isolate bacteria with desired properties in terms of temperature or pH tolerance, or simply novel bacteria.

### 2.4.2. Classification of *Bacillus* Genus

Bacteria of the genus *Bacillus* are aerobic or facultative anaerobic, endospore-forming and Gram positive rods. The genus *Bacillus* comprises a phylogenetically and phenotypically heterogeneous group of species. Recently, the systematic of the *Bacillus* group has been modified. On the basis of extensive studies of the small-subunit ribosomal RNA sequences, the species of the genus *Bacillus* were split into four distinct clusters and several ungrouped species. Group 1 (*Bacillus sensu strico)* includes *B. subtilis*, the type species of genus, and 27 other species. Group 2 includes the round spore-forming bacilli, together with some asporogenous taxa (the genera *Caryophanon, Exiguobacterium, Kurthia* and *Planococcus*) the group constitutes a distinct cluster, only remotely related to *B. subtilis*. Group 3 with ten representatives, comprise *B. polymyxa* and *B. macerans*, which have been reclassified in the

new genus *Paenibacillus*. And group 4, with strains classified into two newly created genera, *Aneurinibacillus* and *Brevibacillus*. Besides, a new genus *Virgibacillus* was recently created to accommodate former *B. pantothenticus*. Additionally, several new isolated *Bacillus* species including *B. mojavensis* and *B. vallismortis*, *B. ehimensis* and *B. chitinolyticus*, *B. infernus*, *B. carboniphilus* and *B. horti* have been described.

### 2.4.3. *Bacillus* Strains of Marine Origin

*Bacillus* species are very common in nature and inhibit soil, water and air. *Bacillus* species are associated with animals, insects, plants and foods. *Bacillus* strains may constitute up to 20 % of the total heterotrophic flora in seawater and, although even higher figures have been quoted, the average population seems to be around 9 % [73]. Marine sediments contain a wider range of bacilli than the water and may account for between 14 and 80 % of the total number of the heterotrophic bacteria. The most marine strains of *Bacillus* conform to established species and have no special characteristics. Species of *B. badius*, *B. subtilis*, *B. cereus*, *B. licheniformis*, *B. firmus*, *B. pulmilus, B. mycoides* and *B. lentus* are often isolated from marine habitats. Although according to Ivanova *et al*., 1992 [74] and Ivanova *et. al*., 1999 [75] the strains *B. subtilis* and *B. pumilus* are the most abundant species among those associated with marine sponges and soft corals. This *Bacillus* species were found to be present in seawater as well. There are true marine species, such as *B. marinus*, *B. salexigens, B. dipsosauri*, and the species of the newly created genus *Halobacillus* (*H. halophilus*, *H. litoralis* and *H. trueperi*) that were isolated from marine environment on marine media and require NaCl ions for growth. A heterogeneous group of moderately halophilic bacteria, which comprise *B. salexigens*, and three species of the new genus *Halobacillus*, *H. halophilus, H. litoralis* and *H. trueperi* may grow at 10 to 20 % of total salts and display an unusual type of murein. However, some isolates of marine macro-organisms pose a taxonomic problem at present. These bacteria are often pigmented orange, yellow, or pink and are probably variants of *B. firmus* and *B. lentus* [76,77].

A few bacilli of marine origin have been reported to produce unusual metabolites including antibiotic [78,79] and antifungal compounds [80], new glucanase [81] and cyclic acylpeptides [80,82,83] differ from those isolated from terrestrial bacteria [84]. For that reason the physiologically uncharacterised *Bacillus* strains may be candidates for the production of novel enzymes and biocompounds.

## 2.5. Aim of this Work

Until nowadays the terrestrial microbiota have proven to be extraordinary repositories of diverse compounds and enzymes that can be employed directly or modified for biotechnological exploitation. The marine micro-organisms promise to be another excellent source with little overlap with traditional sources of natural products. As the marine micro-organisms are adapted to survive in ecological niches at low temperatures, high pressure, extremes pH and high salt concentrations, they can be a source for the unique biocatalysts that function under extreme conditions comparable to those prevailing in various industrial processes.

Most marine invertebrates harbour diverse bacteria, cyanobacteria and fungi within their tissues where they can reside in the extra- and intra- cellular space [61,85]. In same cases these associated micro-organisms may constitute up to 40 % of the biomass, for example, of sponges such as the Mediterranean *Aplysina aerophoba* [65,86]. Therefore this marine invertebrate can be viewed as a promising source of novel, physiological uncharacterised bacteria producing natural products which may greatly expand the chemical and biochemical diversity available for applications.

Hydrolytic enzymes such as esterases and lipases are the most important biocatalysts because they accept a broad range of non-naturals substrates, are usually very stable in organic solvents and exhibit good to excellent stereoselectivity, e. g. in the kinetic resolution of racemates or the desymmetrization of prostereogenic compounds [1]. The commercial use of esterases and lipases nowadays is a billion dollars business. Due to exponential increase in the use of lipases and esterases in biotechnology and growing demand in their applications, the identification and characterisation of novel esterase/lipase genes is of prime importance.

Therefore, the objectives this study were screening, cloning, sequencing and biochemical characterisation of novel esterases and lipases of marine origin, which can have potential interest for biotechnological applications. Lipolytic active bacteria were isolated from the Mediterranean sponge *Aplysina aerophoba* according to their ability to hydrolyse tributyrin [87]. The strongest lipolytic activity was observed with the *Bacillus* species. Based on the comparative 16S rDNA sequence analysis it was assumed that this micro-organisms belongs to a novel *Bacillus* species, as its 16S rDNA sequence similarity to the previously described species of this genera was below 97 %. Because this strain is physiologically uncharacterised, it was used in this work as a candidate for the isolation of novel enzymes.

The closer identification of the novel *Bacillus* species should be performed in DSMZ. Different chromosomal DNA libraries of *Bacillus* sp. should be constructed in pUC18 vector and screened in *E. coli* for lipolytic activity by plate assay towards tributyrin. The genes encoding the esterase or lipase activity should be subcloned into the expression vector pET-22b(+) and functionally overexpressed in *E. coli*. Biochemical characterisation including the substrate specificity, determination of storage stability, temperature and pH optimum as well as the influence of different salt concentrations, metal ions and organic solvents on the purified recombinant proteins should be done.

# 3. Materials and Methods

## 3.1. Materials

If compounds are indicated in molarity, mass or volume percentage without any further explanation, this refers to their amount in aqueous solution.

### 3.1.1. Chemicals and Enzymes

**Table 3-1**

| Company | Chemicals and Enzymes |
|---|---|
| Amersham Pharmacia Biotech AB, Uppsala, Sweden | Electrophoresis Calibration Kit |
| Amresco, Solon, USA | PAGE-PLUS CONCENTRATE |
| Applied Biosystems, Weiterstadt, Germany | Big Dye Terminator Sequencing Kit (v 1.1) |
| BioRad Laboratories, Richmond, USA | BioRad protein assay kit, SDS-PAGE protein standard (LMW) |
| Clontech, Palo Alto, USA | TALON™ Superflow™ Metal Affinity Resin |
| DIFCO-Laboratories, Detroit, USA | tryptone |
| Fluka Chemie, Buchs, Switzerland | agar, ampicillin sodium salt, 6-aminohexanoic acid, ammonium chloride, ammonium hydroxide, ammonium peroxo disulphate, ammonium sulphate, bromphenol blue, γ-buterolactone, calcium chloride dehydrate, cholic acid, Coomassie Brilliant Blue R-250, DMSO, di-potassium hydrogen phosphate, di-sodium hydrogen phosphate dehydrate, ethanol, ethidiumbromide, EDTA, D (+)-glucose monohydrate, glycine, gum arabic, hydrochloric acid, imidazole, lysozyme from hen egg white, magnesium chloride hexahydrate, magnesium sulphate anhydrous, 2-mercaptoethanol, methyl acetate, methyl propionate, methyl butyrate, methyl caproate, |

| | |
|---|---|
| | methyl caprylate, methyl caprate, methyl laurate, methyl myristate, methyl palmitate, methyl stearate, 4-nitroacetanilide, *p*NP acetate, *p*NP butyrate, *p*NP myristate, sodium acetate anhydrous, sodium chloride, sodium dehydrogen phosphate monohydrate, sodium hydroxide, D (+)-sucrose, sulphuric acid, taurocholic acid sodium salt hydrate, TEMED, trichloracetic acid, yeast extract, zinc sulphate heptahydrate |
| Gibco BRL GmbH, Eggenstein Germany | agarose, standard for agarose gel (1 kb, 1 kb plus ladder), urea |
| Kodak, New Haven, USA | ammonium persulphate |
| Lancaster, Muelheim am Main, Germany | 4-nitrophenyl caprylate, δ-hexanolactone |
| MBI Fermentas GmbH, St. Leon-Rot, Germany | CIAP, DNA Polymerase I Large Fragment, T4 DNA ligase, restriction nucleases |
| Merck, Darmstadt, Germany | |
| New England Biolabs GmbH, Frankfurt am Main, Germany | restriction nucleases |
| Pierce, Rockford, USA | 1,4-dithiothreitol, cobaltous (II) chloride hexahydrate |
| QIAGEN, Hilden, Germany | QIAquick Gel Extraction Kit, QIAprep Spin Miniprep Kit, QIAGEN Plasmid Midi Kit |
| Riedel-de-Haen, Seelze, Germany | BCA™ Protein Assay, hydrochloric acid Fixanal ®, sodium hydroxide Fixanal ® |
| Roche, Mannheim, Germany | acetic acid, acetone, glycerol, n-hexane, methanol, tris-(hydroxymethyl)-aminomethane, Tween 20, Protease Inhibitor Cocktail Tablets |
| Roth, Karlsruhe, Germany | Roti®-Phenol/Chloroform, complete™ |
| Sigma-Aldrich, Taufkirchen, Germany | Casein, GenElute ™ Plasmid Miniprep Kit, copper (II) sulphate pentahydrate, 1,4-dithioerythritol, Fast Red TR, guanidine hydrochloride, monochlorodimedon, *p*NP caprate, *p*NP caproate, *p*NP laurate, *p*NP palmitate, *p*NP stearate |
| Wilhelma Zoo and Botanical Garden, Stuttgart, Germany | seawater |

### 3.1.2. Instruments

**Table 3-2**

| Purpose | Instrument | Company |
|---|---|---|
| absorbance measurement | Ultrospec 3000 UV/Visible Spectrophotometer | Amersham Biotech, Freiburg, Germany |
| | BioPhotometer | Eppendorf, Hamburg, Germany |
| agarose gel electrophoresis | DNA Sub Cell™, Mini Sub DNA Cell™, Mini Sub Cell GT™, Power Pac 300 | BioRad, München, Germany |
| | Video Copy Processor P66E | Mitsubishi, Cambridge, USA |
| | BWM 9X Monitor | Javelin Electronics, Schaumburg, USA |
| | UV-lamp table | MWG-Biotech, Ebersberg, Germany |
| centrifugation | Centrifuges 5417 C, 5417R, 5810R | Eppendorf, Hamburg, Germany |
| | Universal 30 F | Hettich, Tuttlingen, Germany |
| | Sorvall® RC-5B (rotors: SLA 3000, SA 600) | Du Pont Instruments, Leipzig, Germany |
| DNA concentration | Concentrator 5301 | Eppendorf, Hamburg, Germany |
| DNA sequencing | ABI Prism 377 DNA Sequencer with ABI Prism 377 software Version 3.2 | Applied Biosystems, Weiterstadt, Germany |
| homogenisation | Ultra-turrax T25 | Janke & Kunkel, IKA® Labortechnik, Germany |
| incubation | WTE incubators shaking incubators | Binder, Tuttlingen, Germany<br>Infors AG, Bottmingen, Switzerland |
| | Thermomixer 5436/Comfort<br>Heidolph REAX 3 orbital | Eppendorf, Hamburg, Germany |

| | | |
|---|---|---|
| | shaker<br><br>water bath<br><br>cryostat RMS, RM6 | Heidolph, Kelheim, Germany<br><br>Haake, Karlsruhe, Germany<br><br>MGW Lauda, Hannoversch-Muenden, Germany |
| microscopy | Leica DMIRBE microscope<br><br>HCX PL Apo 63x/1.32-0.6 oil immersion objective Polychrome 2<br><br>dichroic mirror: F53-009 bandpass filter 535/50 nm<br><br>Orca C4742-95 camera<br><br>Openlab2 | Leica Microsystems, Wetzlar, Germany<br><br>TILL Photonics, München, Germany<br><br>AHF Analysentechnik, Tuebingen, Germany<br><br>Hamamatsu, Japan<br><br>Improvision, Coventry, England |
| PAGE | Minigel-Twin G42<br><br>Power Pac 3000, power Pac 300 Model 583 Gel Dryer | Biometra, Goettingen, Germany<br><br>BioRad, München, Germany |
| PCR | Robocycle gradient 40<br><br>Mastercycler Gradient | Stratagene, Amsterdam, Netherlands<br><br>Eppendorf, Hamburg, Germany |
| pH measurement | Digital pH Meter pH 500<br>665 Dosimat | WTW, Weilheim. Germany<br>Metrohm Ltd., Herisau, Switzerland |
| pipetting | 0.5-10, 10-100, 100-1000, 500-2500, 500-5000 µl pipettes, 30-300 µl multi-channel pipettes, multi-stepper pipettes | Eppendorf, Hamburg, Germany |
| protein blotting | Trans-Blot SD, semidry transfer cell | BioRad, München, Germany |
| protein sequencing | 491 Procise Protein Sequencer<br>785 Programmable | Applied Biosystems, Weiterstadt, Germany |

| | | |
|---|---|---|
| | Absorbance Detector<br>140 C Microgradient System | |
| rotary evaporator | Rotavapor R-134,<br>Waterbath B-480 | Buechi, Flawil, Switzerland |
| scales | Basic<br>MC1 Research RC 210 D<br><br>Precision Advanced | Sartotius, Goettingen,<br>Germany<br><br>OHAU ®, Florham Park,<br>N.J., USA |
| sonication | Sonifier W-250 | Branson, Dietzenbach,<br>Germany |
| ultrafiltration | Amicon 8050, 80200 | Millipore, Bedford, USA |

### 3.1.3. Consumables

**Table 3-3**

| **Consumables** | **Company** |
|---|---|
| pipette tips, 15, 50 ml reaction tubes | Greiner, Nuertingen, Germany |
| pipette tips, 0.5, 1.5, 2.0 ml reaction tubes | Eppendorf, Hamburg, Germany |
| sterile filters | Millipore, Molsheim, France |
| ultrafiltration membranes | Millipore, Bedford, USA |
| filter paper<br>Sequi-Blot™ PVDF membrane<br>Zeta-Probe® GT Genomic Tested Blotting Membrane | Biorad, Hercules, USA |
| PS microplate 96 well | Greiner Bio-one, Frickenhausen, Germany |

### 3.1.4. Micro-organisms

**Table 3-4**

| Organism | Genotype/Features | Reference/Company |
|---|---|---|
| *Escherichia coli* DH5α | F′gyrA96(Nal$^r$)recAA1 relA1 endA thi-1 hsdR17 ($r_k$-$m^k$+) glnV44 deoRΔ(lacZYA-argF)U169[ϕ80dΔ(lacZ)M15] | Clontech, Heidelberg, Germany |
| *Escherichia coli* BL21(DE3) | F-optT hsd$S_b$($r_b$-$m_b$-)gal dcm (DE3) | Novagen, Madison, USA |
| *Bacillus* sp. 01-855 | | Fatma Kabaoglu, Master Thesis, University of Stuttgart, Institute of Technical Biochemistry, Germany, 2001 [87]. |

### 3.1.5. Plasmids

**Table 3-5**

| Plasmid | Promoter | Copy number | Features | Company |
|---|---|---|---|---|
| pET-22b(+) | T7 | medium copy number | *pelB* coding sequence, $His_6$·Tag coding sequence, *lacI* coding sequence, pBR322 origin, *bla* coding sequence, f1 origin | Novagen, Madison, USA |
| pUC18/pUC19 | *lac* | high copy number | CAP protein binding site, LacZ gene, *bla* coding sequence, *lac* repressor binding site | MBI Fermentas GmbH, St. Leon-Rot, Germany |

### 3.1.6. Synthetic Oligonucleotides

#### 3.1.6.1. Primers for DNA Sequencing

**Table 3-6**

| Name | Binding Site | Sequence |
|---|---|---|
| M13 /pUC sequencing primer (-20), 17-mer | M13 /pUC sequencing primer (-20) | 5'-GTAAAACGACGGCCAGT-3' |
| M13 /pUC reverse sequencing primer (-26), 17-mer | M13 /pUC reverse sequencing primer (-26) | 5'-CAGGAAACAGCTATGAC-3' |
| T7 promoter sequencing primer, 20-mer | T7 promoter primer | 5'-TAATACGACTCACTATAGGG-3' |
| T7 terminator sequencing primer, 19-mer | T7 terminator primer | 5'-GCTAGTTATTGCTCAGCGG-3' |
| AKA-pUC18NdeIF | *est*B1 | 5'-CAGACAAGCCCGTCAG-3' |
| EstB1F2 | *est*B1 | 5'-CGTTTGCATGCAGTTTG-3' |
| EstB1F3 | *est*B1 | 5'-CGATGATTGTGATCAG-3' |
| EstB1F4 | *est*B1 | 5'-CTATGGTTGCCGCAG-3' |
| EstB1R2 | *est*B1 | 5'-CAGCTGTATAGCTTTATC-3' |
| EstB1R3 | *est*B1 | 5'-GCAGCATACTGTCTTTTG-3' |
| EstB1R4 | *est*B1 | 5'-CTACATAAGCTTCATAACG-3' |
| EstB2F2 | *est*B2 | 5'-GTTGCAACAAACGCTGGAC-3' |
| EstB2F3 | *est*B2 | 5'-GGAGCAATCACTGGTG-3' |
| EstB2F4 | *est*B2 | 5'-CTAGTACATACAGGTCC-3' |

| | | |
|---|---|---|
| EstB2R2 | *est*B2 | 5’-TAAGAAATTCGAGCACATCCTC-3’ |
| EstB2R3 | *est*B2 | 5’-CATGAAGCATCAATACCG-3’ |
| EstB2R4 | *est*B2 | 5’-CGTAAACTGCAAGGTGC-3’ |
| AmdB1F2 | *amd*B1 | 5’-GAACGCATTTGCTTGCAAC-3’ |
| AmdB1F3 | *amd*B1 | 5’-CATAGTTGCTACTGAAG-3’ |
| AmdB1F4 | *amd*B1 | 5’-GGACTACGGTGTTCAGGTTAG-3’ |
| AmdB1R2 | *amd*B1 | 5’-TGGACATAAAGGTACAACGG-3’ |
| AmdB1R3 | *amd*B1 | 5’-GATTACAGGCTTCAGCAAG-3’ |
| AmdB1R4 | *amd*B1 | 5’-CGTCATCAATTTAGCTC-3’ |

### 3.1.6.2. Primers for Cloning of the *est*B1, *est*B2 and *amd*B1 into pUC18/19 and pET-22b(+) vectors

**Table 3-7**

| **Gene** | **Primer Name** | **Sequence** |
|---|---|---|
| *est*B1 | AKA-NPetF | 5’-GGAATTCCATATGATGGGGAGCAACAACG-3’ |
| *est*B1 | AKA-NPetHisTag | 5’-ATAAGAATGCGGCCGCTTTCTCTAAAAAGTCAG-3’ |
| *est*B2 | aka-E7pUC19F | 5’-AACTGCAGAATGAAAGTTGTTGCACCAAAG-3’ |
| *est*B2 | aka-E7pUC19RHis Tag | 5’-CCGGAATTCTTAGTGGTGGTGGTGGTGGTTCTTTCCA-3’ |

| | | |
|---|---|---|
| *est*B2 | AKA-E7bluntpET22-b(+)1 | 5’-ATGAAAGTTGTTGC ACCAAAGCC-3’ |
| *est*B2 | AKA-E7bluntpET-22b(+)R | 5’-CGCGGATCCTTATTC TTTCCAGTCAAGTCC-3’ |
| *est*B2 | AKA-eECORI7_F | 5’-CGCGGATCCATGAA AGTTGTTGCACCAAAG-3’ |
| *est*B2 | AKA-E7RHisTag | 5’-ATAAGAATGCGGCC GCTTCTTTCCAGTCAAG TCCGTT-3’ |
| *amd*B1 | AKA-E14pUC18F | 5’-CCGGAATTCAATGA GTGCCAAAGAAATGG C-3’ |
| *amd*B1 | AKA-E14pUC18R | 5’-CGCGGATCCTTAGTG GTGGTGGTGGTGGTGTA AGCTTGGTGAGCTGGT G-3’ |

## 3.2. Methods

### 3.2.1. Molecular-Genetic Methods

#### 3.2.1.1. Isolation of Plasmid DNA from *Escherichia coli*

Plasmid DNA isolation from *E. coli* was performed with QIAprep Spin Miniprep Kit (Qiagen), GenElute ™ Plasmid Miniprep Kit (Sigma) and Giagen Plasmid Midi Kit (Qiagen). Isolation of plasmid DNA was performed according to the manual instructions supplied by manufactures. An overnight recombinant *E. coli* culture was harvested by centrifugation and subjected to the modified alkaline-SDS lysis procedure according to the method of Birnboim and Doly, 1979 [88] with following adsorption of DNA onto silica gel membrane in the presence of high salts. Finally the bound DNA was eluted in water or TE buffer (10 mM Tris·HCl, pH 8.0 and 0.1 mM EDTA).

#### 3.2.1.2. Extraction and Purification of Plasmid DNA from *Escherichia coli* for Colony Screening

For the applications such as restriction enzyme digestion, where the yield and purity of plasmid DNA were not critical, a method based on modified alkaline lysis of Birnboim and Doly, 1979 [88] was used. Five ml of overnight recombinant *E. coli* culture were harvested by centrifugation for 2 min at 4°C and 20.800 g and resuspended in 200 µl ice-cold buffer containing 100 mM Tris·HCl, pH 7.5, 10 mM EDTA and 400 µg/ml RNase I by vigorous vortexing. The cells were lysed by the addition of 200 µl freshly prepared 1 M NaOH and 5.3 % (m/v) SDS solution. After addition of 200 µl ice-cold 5 M sodium acetate solution, precipitated proteins and genomic DNA were removed by centrifugation for 5 min at 4°C and 20.800 g. Plasmid DNA in the supernatant was precipitated with 0.7 volume 2-propanol at –20°C, air dried and finally resuspended in water or TE buffer.

#### 3.2.1.3. Precipitation of Chromosomal DNA and Plasmid DNA with Ethanol

Chromosomal DNA or plasmid DNA were mixed with 1/10 volume of 3 M sodium acetate, pH 4.5 and 5 volumes of high-quality ethanol pre-chilled to –20°C. The tube was then

vortexed and kept at RT for 20 min or at –20°C overnight to allow DNA precipitation. If handling very small amounts of nucleic acid, addition of 1/20 volume of a 2 mg/ml glycogen solution as carrier point prior to the addition of ethanol was done. After centrifugation for 30 min at RT and at 20.800 g, the supernatant was removed, the pellet was washed with 90 % (v/v) ethanol, air dried and finally resuspended in sterile water or TE buffer.

#### 3.2.1.4. Precipitation of Chromosomal DNA and Plasmid DNA with 2-Propanol

For the precipitation, chromosomal DNA or plasmid DNA were mixed with 1/10 volume of 3 M sodium acetate, pH 4.5 and 0.7 volumes of 2-propanol. After incubation at RT for 20 min or at –20°C overnight, DNA was pelleted by centrifugation for 30 min at RT and at 20.800 g. The supernatant was discarded, the DNA pellet was washed with 90 % (v/v) ethanol, air dried and resuspended in sterile water or TE buffer.

#### 3.2.1.5. Agarose Gel Electrophoresis of DNA

Agarose gel electrophoresis of DNA was performed as described by Sambrook *et al*., 1989 [89]. To pour 0.8 – 1.5 % gels, agarose powder was mixed with one fold of electrophoresis buffer, consisting of 40 mM Tris base, 20 mM glacial acetic acid and 2 mM EDTA, pH 8.3 to the desired concentration and then heated in a microwave oven until completely melting. Ethidium bromide (final concentration 0.5 µg/ml) was added to the gel at this point to facilitate visualisation of DNA after electrophoresis. After cooling the solution to about 60°C, it was poured into a casting tray containing a sample comb and allowed to solidify at RT. After the gel had solidified, the comb was removed, using care not to rip the bottom of the wells. The gel, still in its plastic tray, was inserted horizontally into the electrophoresis chamber and just covered with one fold of electrophoresis buffer. Samples containing DNA were mixed with six fold of loading buffer consisted of 30 % (v/v) glycerine, 0.2 % (m/v) BPB and 25 mM EDTA, pH 7.5 and then pipetted into the sample wells. For the electrophoretic run current of 120 V for 30 min was applied. The distance DNA migrated has been judged by visually monitoring migration of 10 µl of kb ladder from Gibco. After the run the gel was examined under UV and photographed.

### 3.2.1.6. Isolation of DNA Fragments from Agarose Gel

To recover DNA fragments from standard agarose gels in TAE (Tris-acetate/EDTA) buffer and cleanup from enzymatic reactions the QIAquick Gel Extraction Kit from Qiagen was used according to the manual instructions of manufacturer. The QIAquick system utilises the selective binding properties of DNA on selica-gel membrane in the presence of high salts.

### 3.2.1.7. Isolation of Chromosomal DNA from *Bacillus* sp. 01-855

For the chromosomal DNA isolation 5 g of fresh or stored at –80°C cells were resuspended in 40 ml TE buffer, pH 8.0. After centrifugation for 10 min at 4°C and 2500 g supernatant was discarded. The pellet was resuspended in 5 ml 50 mM Tris·HCl, pH 8.0 buffer, containing 0.7 M sucrose and then 0.6 ml of freshly-prepared lysozyme solution (20 $mg \cdot ml^{-1}$) was added. To allow lysis, the cell suspension was stored on ice for 30 min. After addition of 0.6 ml 0.5 M EDTA, pH 8.0 and 10 % (m/v) SDS the mixture was gently mixed and stored on ice for 10 min. The fluid sample was adjusted to 1 % (m/v) SDS and 0.5 mg $ml^{-1}$ proteinase K. The samples were incubated at 55°C for 1 h with very gentle shaking. The mixture was finally transferred into a Falcon tube and centrifuged at 4000 g for 10 min at 25°C. The pellet was discarded and 1.0 volume of phenol-chloroform-isoamyl alcohol (25:24:1) solution was added to the supernatant, gently inverted to mix and finally centrifuged at 3000 g for 10 min at RT. The wash procedure was repeated three times, the rests of phenol were extracted with 1.0 volume of chloroform. The upper aqueous layer containing chromosomal DNA was transferred to a fresh tube and precipitated with ethanol or 2-propanol.

### 3.2.1.8. Enzymatic Modifications of DNA

Restriction enzymes bind specifically to and cleave double-stranded DNA at specific sites within or adjacent to a particular sequence known as the recognition sequence [89]. Restriction analysis and DNA modifications were performed using enzymes purchased from MBI Fermentas according to the recommendations of the manufactures. To remove of 5'-phosphate residues from DNA calf intestinal alkaline phosphatase (CIAP) was used. For filling-in of recessed 3'- termini of double-stranded DNA, DNA Polymerase I Large Fragment (Klenow Fragment) was applied. Ligation reactions were performed with

bacteriophage T4 DNA ligase. For joining DNA molecules with compatible cohesive termini ligation was done at RT for 1 h. In the case of genomic DNA library of *Bacillus* sp. 01-855, ligation was performed for 12 h at 16°C. Ligation of blunt-ended double-stranded DNA molecules to one another was carried out in a presence of 4 % (m/v) PEG for 12 h at 16°C.
If necessary, enzymes were inactivated by heating, buffer compounds were removed by dialysis and the desired DNA fragments were purified from standard agarose gels by means of QIAquick Gel Extraction Kit (Qiagen).

### 3.2.1.9. Automated DNA Sequencing

DNA sequencing was performed by dideoxy chain termination method of Sanger *et al*., 1977 [90], using a 377 Prism DNA Sequencer (Applied Biosystem) and a Big Dye Terminator Sequencing Kit with *Taq* DNA polymerase (Perkin Elmer). This method involves the use of four differently fluorescent dye-labelled ddNTPs. These fluorophors were excited with two argon lasers at 488 nm and 514 nm correspondently, when the respective bands passed the lasers during electrophoresis. The specific emissions were detected and the data collected for analysis [91,92]. All four termination reactions were prepared in a single tube as shown below:

| | |
|---|---|
| Terminator Ready Reaction Mix | 4 µl |
| sequencing primer | 20 pmol |
| DNA | 300 - 500 ng |
| $ddH_2O$ | ad 20 µl |

**Table 3-8** Thermal cycling conditions for the sequencing reactions

| Step | Denaturation | Annealing | Extension | Cycle Number |
|---|---|---|---|---|
| 1<br>2<br>3 | 95°C, 4 min<br>95°C, 40 s | 55°C, 30 s | 60°C, 4 min<br>72°C, 4 min | 1<br>25<br>1 |

Purification of the reaction products was performed in microtiter plates by ion-exchange chromatography using Sephadex™ G-50 Superfine (Amersham Pharmacia) according to the instructions of the manufacturer. The eluates were dried at 80°C, resuspended in 3 µl of loading buffer (80 % (v/v) 25 mM EDTA, pH 8.0 and 20 % (v/v) formamide), denatured for 2 min at 95°C and cooled to RT. 0.7 µl of this mixture were loaded in a single lane at a polyacrylamide slab gel for electrophoresis. A 5.25 % (m/v) polyacrylamide gel consisted of:

| | |
|---|---|
| urea | 10.8 g |
| PAGE-PLUS CONCENTRATE (Amresco) | 6.6 ml |
| 10 fold TBE buffer (0.9 M Tris, 0.9 M boric acid, 20 mM EDTA) | 5.0 ml |
| $ddH_2O$ | ad 50 ml |
| 10 % (m/v) APS solution | 250 µl |
| TEMED | 25 µl |

### 3.2.1.10. Polymerase Chain Reaction (PCR)

The polymerase chain reaction (PCR) was used to amplify the specific DNA fragments of the known sequences [93]. Standard PCRs were carried out in 0.5 ml Eppendorf tubes as follows:

| | |
|---|---|
| 10 fold polymerase buffer | 5 µl |
| dNTP mixture (2.5 mM of each one) | 4 µl |
| forward primer | 20 pmol |
| reverse primer | 20 pmol |
| template DNA | ± 100 ng |
| *PfuTurbo* DNA polymerase | 1 - 2 U |
| or *Taq* DNA polymerase | 0.5 - 1 U |
| 25 mM $MgCl_2$ (for *Taq* DNA polymerase) | 4 µl |
| ad $ddH_2O$ | 50 µl |

**Table 3-9** PCR conditions

| Step | Denaturation | Annealing | Extension | Cycle Number |
|---|---|---|---|---|
| 1 | 95°C, 2 min | | | 1 |
| 2 | 95°C, 1 min | $A_t$, 2 min | 72°C, 3 min | 30 |
| 3 | | | 72°C, 10 min | 1 |

Annealing temperature ($A_t$) was 5°C below the melting temperature of the primer.

### 3.2.1.11. Statistical Calculation for Genomic Library

The probability of having any given DNA sequence in the genomic DNA library of *Bacillus* sp. 01-855 was calculated from the equation of Clarke and Carbon, 1992 [94]:

$$N = \frac{\ln(1-P)}{\ln(1-f)}$$

where $P$ is desired probability, $f$ is the fractional proportion of the genome in a single recombinant, and $N$ is the necessary number of recombinants. According to it, to achieve a 99% probability of having any 3-kb DNA fragment represented in a library of a *Bacillus* genome (4, 2 x $10^6$ bp) 20.000 colonies must be tested.

### 3.2.1.12. Computer Analysis of Nucleotide and Amino Acid Sequences

Computer analysis of DNA sequences and the deduced amino acid sequences was performed with Expert Sequence Analysis Software SeqMan™ II and Clone Manager Version 5 for Windows 95, correspondently. Search for nucleotide and amino acid sequence similarities was done on the National Centre for Biotechnology Information web server (http://www.ncbi.nlm.nig.gov) in the noredundant nucleotide (nr-nt) or amino acid (nr-aa) databases using BLASTX, BLASTP and BLASTN programmes [95].

### 3.2.2. Microbiological Methods

#### 3.2.2.1. Growth Media for *Escherichia coli*

*E. coli* was grown in Luria-Bertani (LB) media [96] prepared as described below:

| | |
|---|---|
| tryptone | 10 g/l |
| yeast extract | 5 g/l |
| sodium chloride | 5 g/l |
| agar (optional for agar plates) | 15 g/l |
| pH | 7.0 |

The media was sterilised by autoclaving at 121°C for 20 min at 2.2 bar on the liquid cycle. If required, 100 μg/ml (end concentration) of sterile filtrated ampicillin and 1 % (v/v) tributyrin were added.

#### 3.2.2.2. Growth Media for *Bacillus* sp. 01-855

**Seawater-based Media**

*Bacillus* sp. 01-855 was cultivated in seawater-based media (SWM) prepared as shown below:

| | |
|---|---|
| peptone | 5 g/l |
| yeast extract | 1 g/l |
| sterile filtrated seawater | 0.750 l |
| agar (optional for agar plates) | 15 g/l |

If required, 1 % (v/v) tributyrine was emulsified in Ultra-turax at 9000 rpm for 10 min with peptone-yeast solution and autoclaved at 121°C for 20 min at 2.2 bar on the liquid cycle. 0.750 l of sterile filtrated seawater was finally added to the pre-chilled to RT peptone-yeast solution.

### 3.2.2.3. Cultivation and Storage of *Bacillus* sp. 01-855

50 ml of SWM media were inoculated 1:100 with a *Bacillus* sp. 01-855 culture and incubated at 30°C and 250 rpm for 48 h. The *Bacillus* strain was maintain on SWM agar plates at 4°C or as a glycerol stock at –80°C. The culture, which was maintained on SWM agar plates was re-inoculated once a month.

### 3.2.2.4. Preservation of *Escherichia coli* and *Bacillus* sp. 01-855 Cultures

To preserve the cultures if frozen, 15 % (v/v) glycerol or 5 - 7 % (v/v) DMSO were added. The culture was mixed with cryoprotectants, immediately frozen in liquid nitrogen and stored at –80°C.

### 3.2.2.5. Preparation and Transformation of Competent *Escherichia coli*

Chemically competent *E. coli* cells were prepared according to Chung *et al*., 1989 [97] and transformed by heat shock. 50 ml of fresh LB media were inoculated 1:100 with an overnight *E. coli* culture and incubated at 37°C at 200 rpm until $OD_{568}$ 0.2 - 0.5. The cells were collected by centrifugation (4°C, 10 min, 3020 g) and resuspended in 2 ml of pre-chilled to 4°C TSS buffer (10 % (m/v) PEG 600, 5 % (v/v) DMSO and 50 mM $MgCl_2$ in LB media, pH 6.5). After 5 min incubation on ice the cells were frozen in 200 µl aliquots in liquid nitrogen or transformed with at least 5 ng of plasmid DNA. The cells were incubated for 30 min on ice, subjected to heat shock for 45 s at 42°C and filled up to 1 ml with LB media. After incubation at 37°C for 1 h the cells were spread on LB agar plates and incubated overnight at 37°C.

### 3.2.2.6. α-Complementation of Defective β-Galactosidase

For the identification of recombinants constructed in vector pUC 18/19 colour test based on α-complementation of the defective β-galactosidase was used [98]. The $Lac^+$ bacteria that results from α-complementation are easily recognised because they form blue colonies in the presence of the chromogenic substrate 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) [99]. For testing bacteria for α-complementation 40 µl of a stock solution of X-gal (20 mg/ml in dimethylformamide) were added to LB agar plates containing 100 µl/ml ampicilline. The plates were then inoculated with the bacteria to be tested and incubated for 12 - 16 h at 37°C.

### 3.2.2.7. Replication of Colonies onto Flax Filters

Transferring of *E. coli* colonies which are spersed over the master agar plates to the flax filters and finally to the surface of the second agar plates was performed according to Sambrook *et. al*., 1989 [89].

### 3.2.2.8. Expression of the *est*B1, *est*B2 and *amd*B1 in *Escherichia coli*

The *est*B1, *est*B2 or *amd*B1 genes were either expressed under the control of T7 promoter in *E. coli* BL21(DE3) or under the control of *lac* promoter in *E. coli* DH5α strain.

#### 3.2.2.8.1. Expression under the Control of T7 Promoter

The pET system is the most powerful system yet developed for the cloning and expression of recombinant proteins in *E. coli* [100]. Target genes are cloned in pET vectors under the control of bacteriophage T7 promoter, which has a strong transcription initiation signal and highly efficient ribosome binding site from the T7 major capside protein. Expression is induced by providing the source of T7 RNA polymerase in the host cells [101]. In the host cells like *E. coli* BL21(DE3) and BL(DE3)pLysS the T7 RNA polymerase gene is encoded in the genomic DNA under the control of the *lac*UV5 promoter and its expression can be induced with IPTG [102]. The *est*B1 and *est*B2 genes were cloned into medium copy number vector pET-22b(+), which carries the T7 promoter, a *pelB* leader sequence at the C-terminus for periplasmatic expression and a poly-histidine tag at the N-terminus for easy purification. After the plasmids were established in a non-expression *E. coli* DH5α host, they were transformed into *E. coli* BL21(DE3) strain bearing the T7 RNA polymerase gene for expression of the target DNA.

Expression in Shake Flasks

*E. coli* BL21(DE3) were freshly transformed with the expression pET-22b(+) plasmids bearing the *est*B1 or *est*B2 and grown at 37°C in 400 ml LB media supplemented with ampicillin (100 µl/ml) until the early exponential phase ($OD_{568}$ 0.4 - 0.8). In the case of the *est*B1, esterase prodjuction was induced with 1 mM IPTG and cultivation was continued at 37°C for 4 h. In the case of the *est*B2, induction of the target DNA was performed with 0.1 - 1 mM IPTG. After induction cultivation was continied at different temperatures (25°C - 37°C) for 1 - 5 h. *E. coli* cells containing the overexpressed EstB1 and EstB2 esterases were harvested by centrifugation (4000 g) and washed twice with 50 mM potassium phosphate buffer, pH 7.5, 4°C. *E. coli* cells were finally disrupted by sonication (3 times for 2 min, the power level set between 4 - 5, 50 % output, Branson Sonifier 250) and used for purification or to assay esterase activity.

### 3.2.2.8.2. Expression under the Control of *lac* Promoter

For the construction of genomic DNA library of *Bacillus* sp. 01-855 and expression of the *est*B2 and *amd*B1 high copy number vectors pUC18/19 were used. The most characteristic feature of the pUC18/19 vectors is the presence of a part of the *E. coli* l*ac*Z' fragment with multiple cloning site downstream of the translation-initiation codon ATG, which allows α-complementation by screening procedure. *E. coli* DH5α strain was used for the transformation and expression experiments.

Expression in Shake Flasks

50 ml or 100 ml of LB media were inoculated 1:100 with a recombinant overnight *E. coli* culture and incubated at 37°C and 250 rpm. Induction was done with 1 mM IPTG at $OD_{568}$ = 0.6 and incubation was continued at 30°C and 150 rpm. After 4 h induction the cells were harvested by centrifugation, washed twice with 50 mM potassium phosphate buffer, pH 7.5, 4°C and disrupted by sonication.

### 3.2.3. Protein-biochemical Methods

#### 3.2.3.1. SDS-Polyacrylamide Gel Electrophoresis of Proteins

Proteins from the crude extract and from various purification steps were analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli, 1970 [103]. The 4 % stacking gel was prepared with

| | |
|---|---|
| Rotiphorese® Gel 30 | 0.53 ml |
| four fold Upper Tris (Tris 12.11 g, SDS 0.8 g, ad 200ml dd$H_2O$, pH 6.8) | 1.00 ml |
| d$H_2O$ | 2.47 ml |
| 10 % (m/v) APS | 40 µl |
| TEMED | 4 µl |

The 12.5 % resolving gel consisted of:

| | |
|---|---|
| Rotiphorese® Gel 30 | 3.33 ml |
| four fold Lower Tris (Tris 36.46 g, SDS 0.8 g, ad 200 ml dd$H_2O$, pH 8.8) | 2.00 ml |
| d$H_2O$ | 2.67 ml |
| 10 % (m/v) APS | 40 µl |
| TEMED | 4 µl |

The protein samples were mixed with one volume of two fold SDS loading buffer prepared according to Sambrook *et al*., 1989 [89] and heated for 5 min at 95°C. After cooling to RT the samples were loaded under the running buffer consisting of 25 mM Tris, 192 mM glycine and 0.1 % (m/v) SDS, pH 8.4 on stacking gels. Two protein standards were applied onto the SDS-polyacrylamide gels as references. A low molecular weight (LMW) standard from BioRad was used for the qualitative analysis. For the quantitative purposes a LMW Electrophoresis Calibration Kit from Amersham Pharmacia Biotech was used. The LMW standard from BioRad was prepared as suggested by manufacturer. The content of one vial of the Amersham Pharmacia Biotech LMW standard was dissolved in 200 µl dd$H_2O$ and 200 µl SDS loading buffer. The protein composition of both standards including the molecular masses and for Amersham Pharmacia Biotech standard also the concentration of each protein is shown in **Table 3-10**.

**Table 3-10** Protein standards used for SDS-PAGE

| **Protein** | **BioRad LMW** Moleculare mass/kDa | **LMW Electrophoresis Calibration Kit** Moleculare mass/ kDa | μg Protein/μl |
|---|---|---|---|
| phosphorylase B | 97.4 | 94.0 | 160.0 |
| bovine serum albumin | 66.2 | 67.0 | 207.5 |
| ovalbumin | 45.0 | 43.0 | 367.5 |
| carbonic anhydrase | 31.0 | 30.0 | 207.5 |
| trypsin inhibitor | 21.5 | 20.1 | 220.0 |
| lysozyme/α-lactalbumin | 14.4 | 14.4 | 302.5 |

The gels were run at 120 V until the BPB band reached the bottom of the resolution gel. The run followed the staining with Coomassie Dye (0.1 % (m/v) Coomassie Brilliant Blue R250, 30 % (v/v) methanol and 10 % (v/v) acetic acid). The staining procedure was performed on shaker for at least 1 h at RT with agitation. The distaining of the gels was done in the solution consisting of 30 % (v/v) methanol, 10 % (v/v) acetic acid and 60 % $dH_2O$. For storage the gels were dried at 80°C under vacuum in BioRad Gel Dryer Model 583.

### 3.2.3.2. N-terminal Sequencing of Proteins

N-terminal amino acid sequencing of the purified 29BS and 32BS was based on the degradation method developed by Edmann, 1950 [104] and carried out with an Applied Biosystems model 470A protein sequencer. The samples containing the proteins of interest were loaded onto 12.5 % SDS-polyacrylamide gels and electrophoresed according to Laemmli, 1970 [103]. The electroblotting of the 29BS and 32BS onto PVDF membrane was performed according to Matsudaira, 1998 [105] as described below. After electrophoresis, the gels were soaking in transfer buffer for 5 min to reduce the amount of Tris and glycine. During that time a PVDF membrane was rinsed with 100 % methanol and stored in transfer buffer. The gel, sandwiched between a sheet of PVDF membrane and several sheets of blotting paper, was ordered as shown in **Scheme 3-1**, assembled into a blotting apparatus and elecroeluted for 45 min at 15 V.

**Scheme 3-1** The components of the sandwich arranged on the anode platform of the semi-dry transfer cell (BioRad)

1. three filter papers incubated in anode buffer 1
2. three filter papers incubated in anode buffer 2
3. a PVDF membrane
4. the SDS-polyacrylamide gel equilibrated in cathode buffer
5. three filter papers incubated in cathode buffer

| | |
|---|---|
| anode buffer 1 | 0.3 M Tris, 10 % (v/v) methanol, pH 10.4 |
| anode buffer 2 | 25 mM Tris, 10 % (v/v) methanol, pH 10.4, |
| cathode buffer | 25 mM Tris, 40 mM 6-aminohexanoic acid, 10 % (v/v) methanol, pH 9.4 |

After electroblotting the PVDF membrane was washed in $dH_2O$, stained with 0.1 % Coomassie Brilliant Blue R250 in 50 % methanol for 5 min and then distained in 50 % (v/v) methanol at RT. The membrane was finally rinsed in $dH_2O$ and air dried. The bands corresponding to the proteins of interest were excised and applied to the protein sequencer or, alternatively, stored at –20°C.

### 3.2.3.3. Immunological Detection of Proteins (Western Blot)

Immunological detection of the recombinant proteins fused to a 6xHis-tag was performed with QIAexpress Detection System (Qiagen) according to the instructions of the manufacturer. The detection is based on the affinity of Ni-NTA conjugates consisting of Ni-NTA coupled to calf intestinal alkaline phosphatase (AP) for affinity tags bearing consecutive histidine residues. The proteins immobilized to a Zeta-Probe® membrane were detected with Ni-NTA conjugates, which make secondary binding reagents such as enzyme-conjugates antibodies unnecessary and allow detection of 2 - 5 ng protein.

### 3.2.3.4. Purification of Polyhistidine-tagget Proteins by Immobilised Metal Affinity Chromatography (IMAC)

The proteins containing sequences of consecutive histidine residues were purified under native conditions by means of IMAC [106] using TALON™ Superflow Resin (Clontech) according to the manufacturer recommendations. The principle of the IMAC is based on the reversible interaction between $His_6$-tagged proteins and $Co^{2+}$-carboxylmethylaspartate, which is coupled to a solid-support resin. The wash and elution steps were performed at pH 8.0. For the elution 150 mM imidazole was used. The purified proteins were finally dialysed overnight at 4°C with 50 mM potassium phosphate buffer, pH 7.5 or alternatively with $ddH_2O$ and stored for at least one week at 4°C until biochemical characterisation.

### 3.2.3.5. Refolding of Inclusion Bodies

Refolding was done to obtain soluble, active EstB2 protein from inclusion bodies. 15 g wet cells, obtained from the batch cultivation were washed and resuspended in lysis buffer (50 mM Tris·HCl, pH 8.0) containing 1 μg/ml lysozyme. After 30 min incubation on ice, the cells were disrupted by sonication. After centrifugation of the cell lysate at 4°C for 20 min at 20.000 g (SS-34 rotor, Sorvall) the pellet containing the cell debris and inclusion bodies was resuspended in 10 ml wash buffer (50 mM Tris·HCl, pH 7.5, 150 mM NaCl, 1 % Triton X-100, 0.5 M urea per 1 g cell wet weight) to remove loosely associated contaminants. After incubation at RT for 10 min the suspension was centrifuged at 4°C for 20 min at 20.000 g (SS-34 rotor, Sorvall). 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 the final protein concentration 1 mg/ml. After 3 h incubation at RT with gentle stirring the solubilazed IBs were dialysed at 4°C for 8 - 10 h with 50 mM potassium phosphate buffer, pH 7.5 containing 1 mM EDTA or Protease Inhibitor Cocktail from Roche.

### 3.2.3.6. Concentration of Proteins

Samples with low protein concentrations were concentrated by ultrafiltration using Amicon® Stirring Cells and Centricon® Centrifugal Filter Devices from Millipore YM 10, 30, 50 filters according to the instructions supplied by manufacturer. Amicon Stirring Cells were supplied with Millipore Ultrafiltrarion Membrames (YM 10, 30 and 50).

### 3.2.3.7. Determination of the Protein Concentration

Detection and quantitation of total protein was performed with BC Assay: protein quantitation kit from Uptima and BCA Protein Assay Kit from Pierce according to the instructions of manufacturers. The determination of protein concentration was performed by spectrometric method based on bicinchoninic acid (BCA) assay introduced by Smith *et. al*., 1985 [107]. This reaction involves the reduction of $Cu^{+2}$ by protein to $Cu^{+1}$ in alkaline media. The resulting $Cu^{+1}$ formed by the chelation of two molecules of BCA purple-coloured complex with exhibiting a strong absorbency at 562 nm.

### 3.2.3.8. Densitometric Determination of Protein Concentration

Different concentrations of the sample containing the protein of interest and the LMW standard from LMW Electrophoresis Calibration Kit (**Table 3-10**) were separated by means of SDS-PAGE. The gels were stained with Coomassie Dye, dried and finally scanned. For the densitometric analysis ScionImage software (http://www.scioncorp.com) was used. To avoid saturation of the density, not more then 800 ng of protein per slot was used. This method is very useful when the protein has not been purified.

### 3.2.3.9. Measurement of Lipolytic Activity

#### 3.2.3.9.1. Plate Assay with Tributyrin

Esterases produced from colonies were directly tested on LB agar plates supplemented with 1% (v/v) of emulsified tributyrin. The appearance of clear zones (halos) around colonies resulting from release of tributyrin was taken as a positive indication of lipolytic activity.

#### 3.2.3.9.2. Esterase Assay in Microtiter Plates

For the determination of esterase activity in samples, where amount and purity of the enzymes are not critical, activity tests in microtiter plates with α-naphtphyl acetate and Fast Red were performed. To prepare substrate solution 20 mg of α-naphtphyl acetate were dissolved in 5 ml acetone and added to 45 ml 50 mM Tris·HCl buffer, pH 7.5. Fast Red TR solution was

prepared by dissolving of 50 mg of colour reagent in 50 ml 50 mM Tris·HCl buffer, pH 7.5. For the activity tests 100 µl of substrate solution were piped with 100 µl of the sample into 96 - wells micrototer plates. After incubation of the microtiter plates for 30 min at 37°C 50 µl of Fast Red solution were added. The development of purple colour in the wells was taken as a positive indication of esterase activity.

#### 3.2.3.9.3. Zymogram

For the identification, calculation of molecular weight and estimation of the purity of esterases on polyacrylamide gels activity staining with α-naphthyl acetate and Fast Red (zymogram) was performed. The protein samples containing esterases were separated by SDS-PAGE and renaturated 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 with developing solution consisting of 3 mM α-naphthyl acetate, 1 mM Fast Red TR and 100 mM sodium phosphate buffer, pH 7.5 for 5 min at RT. The esterase activity was detected by the appearance of purple coloured bands in the SDS-polyacrylamide gels.

#### 3.2.3.9.4. Spectrometric Assay towards *p*-Nitrophenyl Esters

The esterase activity of the crude extract and purified enzymes was measured spectrometric towards *p*-nitrophenyl (*p*NP) esters. In the spectrometric assay 0.01 - 0.1 mg of the purified enzyme was added to 1.0 ml of 50 mM sodium phosphate buffer, pH 7.5 in a 1.5 ml cuvette. The cuvette was placed in the jacket holder of Pharmacia Biotech Ultraspec 3000 spectrophotometer. The reaction temperature was maintained at 35°C ± 0.1°C by a circulation waterbath. After the reaction solution was thermal equilibrated (5 min), an aliquot of 100 mM stock solution of *p*NP esters in dimethyl sulfoxide was added to initiate the reaction. The reaction progress was monitored by reading of absorbance at 410 nm (ε 1.45 $10^{-4}$ $M^{-1}cm^{-1}$ at pH 7.5) that accompanies hydrolysis of *p*NP esters and release *p*-nitrophenol. One unit of esterase activity was defined as the amount of enzyme that released 1 µmol of *p*-nitrophenol per min under assay conditions.

$$A = \frac{\Delta E'}{\varepsilon \cdot d \cdot c_{enzyme}}$$

| | |
|---|---|
| A [U·mg$^{-1}$] | specific activity |
| ΔE′ [min$^{-1}$] | slope at 410 nm |
| ε [1·M$^{-1}$·cm$^{-1}$] | extinction coefficient $1.45 \cdot 10^{-4}$ at pH 7.5 |
| d [cm] | cuvette diameter (1 cm) |
| $c_{enzyme}$ [mg·l$^{-1}$] | concentration of enzyme |

### 3.2.3.9.5. pH-stat Assay

A modified pH-stat assay of Peled and Krenz, 1981 [108] was used for the determination of esterase activity of the crude extract or purified enzymes. For the pH-Stat assay 20-ml aliquots of a substrate emulsion (200 mM of methyl esters, 2 % (m/v) gum arabic in water) were added to the thermostated reaction chamber of pH-Stat equipment, preheated to 35°C and adjusted to pH 7.5. After the addition of 0.2 mg purified enzyme pH was automatically kept constant by addition of 0.1 M NaOH solution. The NaOH consumption, which is proportional to the conversion, was recorded and used for the calculation of activity. One unit of esterase activity was defined as the amount of enzyme that released 1 µmol of acetic acid per min under assay conditions.

$$D = \frac{f_x \cdot f_y \cdot c_{NaOH}}{m_{enzyme}} \cdot \left( \frac{\Delta x}{\Delta y} \right)$$

| | |
|---|---|
| D [U·mg$^{-1}$] | specific activity |
| $f_x$ [µ$l_{NaOH}$·mm$^{-1}$] | parameter correlated to the consumption of NaOH |
| $f_y$ [mm·min$^{-1}$] | parameter correlated to the reaction time |
| $C_{NaOH}$ [mol·l$^{-1}$] | molar NaOH concentration |
| $m_{enzyme}$ [mg] | amount of esterase |
| Δx/Δy [mm·mm$^{-1}$] | slope |

#### 3.2.3.9.6. Haloperoxidase Assay

The haloperoxidase activity of the EstB1 was measured spectrometric according to Wiesner *et. al*, 1985 [109] towards monochlorodimedone as a substrate. 100 mM sodium acetate buffer, pH 6.5 containing 82 mM bromide, chloride or iodide ions, 7.2 mM $H_2O_2$ and 44 μM monochlorodimedone was incubated at 35°C for 3 min. The reaction was started by the addition of 0.1 mg of the purified enzyme with following absorption measurements at 290 nm (ε 1.99 $10^{-4}$ $M^{-1}cm^{-1}$, pH 5.5). One unit of haloperoxidase activity was defined as the amount of enzyme catalysing the consumption of 1 μmol monochlorodimedone per min under assay conditions.

#### 3.2.3.9.7. Lactonase Assay

The lactonase activity of the EstB1 and EstB2 was measured with pH stat apparatuses. 20 ml of 50 mM sodium phosphate buffer, pH 7.5, 2 % (m/v) gum arabic and 200 mM lactone were preincubated at 35°C for 5 min and adjusted to pH 7.5. The reaction was stated by adding 0.2 mg of the purified enzyme. One unit of lactonase activity was defined as the amount of enzyme catalysing the consumption of 1 μmol substrate per min under assay conditions.

### 3.2.3.10. Biochemical Characterisations

#### 3.2.3.10.1. Storage Stability of the EstB1 and EstB2

The storage stability of the purified from *E. coli* EstB1 and EstB2 was examined in 50 mM buffers: potassium- and sodium phosphate buffer, Tris·HCl buffer and phosphate buffered saline (PBS) and at different temperatures: –20°C, 4°C and 37°C. The purified enzyme at the concentration of 1 mg/ml was stored in the appropriate buffer and at the corresponding temperature until measurements. The activity measurements were perfomed in 12 h intervals by standard *p*NP acetate assay. In each experiment 0.2 mg of the purified EstB1 and EstB2 were used.

#### 3.2.3.10.2. Temperature and pH Optimum

The temperature and pH optimum of the purified EstB1 and EstB2 were determined by a pH-Stat assay towards 200 mM methyl acetate, which ensured that there was a large excess of the substrate. The temperature optimum was determined after 5 min incubation of the enzyme at a given temperature.The following measurements of the esterase activity was performed at pH 7.5. The pH optimum was measured after 5 min incubation of the protein at 35°C towards substrate emulsion consisting of 200 mM methyl acetate, 2 % (m/v) gum arabic and the appropriate buffer. 50 mM acetate buffer, sodium phosphate buffer and Tris·HCl buffer were used for the measurements in the pH 2 - 5, 5 - 7.5 and 7.5 - 10 ranges, respectively. In each experiment 0.2 mg of the purified EstB1 and EstB2 were used.

#### 3.2.3.10.3. Influence of Organic Solvents, NaCl and KCl Salts and Metal Ions

The influence of 10 - 50 % (v/v) organic solvents, 0.1 - 2.5 M NaCl and KCl salt concentrations and metal ions at the 1 - 100 mM concentrations on the EstB1 and EstB2 was measured by standard spectrometric assay towards *p*NP acetate as a substrate. In each experiment 0.2 mg of the purified EstB1 and EstB2 were used.

#### 3.2.3.10.4. Influence of PMSF, EDTA, β-mercaptoethanol and DTT

The influence of 1 - 10 mM PMSF and EDTA on the EstB1 and EstB2 esterases was tested according to standard spectrometric assay towards *p*NP acetate, whereby the influence of 1 - 10 mM β-mercaptoethanol and DTT was measured with methyl acetate by standard pH-stat assay. In each experiment 0.2 mg of the purified EstB1 and EstB2 were used.

#### 3.2.3.10.5. Kinetic Measurements

The kinetic constants $K_m$ and $V_{max}$ for the hydrolysis of *p*NP acetate and *p*NP butyrate followed Michaelis-Menten reaction were measured by standard spectrometric assay at pH 7.5. 0.05 mg of the purified EstB1 and EstB2 were used for the kinetic measurements. *p*NP esters were used at concentrations ranging from 1 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 %.

# 4. Results

## 4.1. Identification of *Bacillus* strain

The *Bacillus* strain used in this work was isolated from mesohyl of the homogenised marine sponge *Aplysina aerophoba* according to its ability to hydrolyse tributyrin [87]. It was assumed that this bacterial isolate represents a novel *Bacillus* species as its 16S rDNA sequence similarity to the previous described species of this genus is below 97 % [110]. For the closer identification *Bacillus* species (**Figure 4-1**) was sent to DSMZ (German Collection of Micro-organisms and Cell Cultures, Braunschweig, Germany). This micro-organism was identified as a new *Bacillus* species (RNA Group VI in *Bacillus*) by physiological assays, partial 16S rDNA sequensing and fatty acid analysis. According to the DSMZ nomenclature this *Bacillus* strain was named *Bacillus* sp. 01-855. Its morphological features are shown in **Table 4-1**.

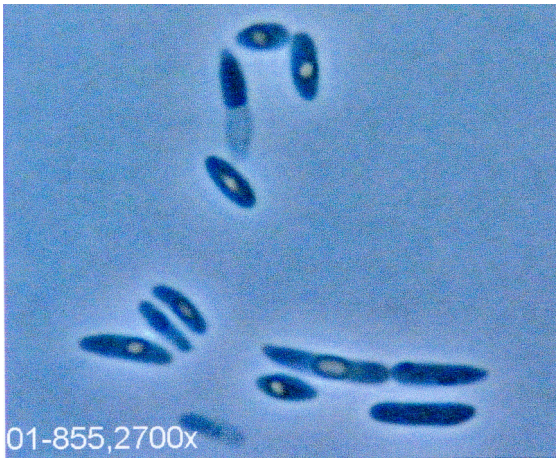


**Figure 4-1** *Bacillus* sp. 01-855 identified in DSMZ

*Bacillus* species isolated from the marine sponge *Aplysina aerophoba* with 2700 x magnification.

**Table 4-1** Morphological features of the new *Bacillus* sp. 01-855

| **Features** | **Results** |
|---|---|
| cell form | coccoid rod |
| width, µm | 0.7 - 0.9 |
| length, µm | 2.0 - 4.0 |
| spore | +, oval |
| swollen sporangium | + |
| growth under anaerobic conditions | - |
| Voges-Proskauer reaction | - |
| pH in VP-media | 5.6 |
| growth at 30°C<br>45°C<br>50°C | +<br>+<br>- |
| gas formation from glucose | - |
| acid formation from D-glucose<br>L-arabinose<br>D-xylose<br>D-mannose<br>D-fructose | little<br>-<br>-<br>little<br>little |
| hydrolysis of gelatine<br>starch<br>Tween 80<br>casein | +<br>+<br>+<br>+ |
| phenylalanindesaminase | - |
| arginindihydrolase | - |
| reduction of nitrate | - |
| utilisation of citrate<br>propionate | -<br>- |
| growth at pH 5.7 | - |
| growth in the presence of 0.001 % lysozyme | + |

The characterisation of *Bacillus* sp. 01-855 revealed that it is an aerobe, gram-positive coccoid rod shaped bacteria, which forms red or orange coloured colonies. The new *Bacillus* sp. 01-855 is a spore former with spores of oval shape. A width of *Bacillus* sp. 01-855 cells is 0. 7 - 0.9 µm with a length of 2.0 - 4.0 µm. The growth temperature optimum lies between 30°C and 45°C. *Bacillus* sp. 01-855 can utilise glucose, mannose and fructose as a carbon source. It displays the ability to hydrolyse starch, casein, gelatine and Tween-20. The analysis of fatty acids composition showed a typical muster for *Bacillus* genus. The 95.1 % 16S rDNA sequence similarity was found to *Bacillus pseudofirmus*, whereby the physiological features were found not sufficient for the closer identification between the known spices.

## 4.2. Construction of a *Bacillus* sp. 01-855 Genomic DNA Library and Screening for Lipolytic Activity

Chromosomal DNA from *Bacillus* sp. 01-855 was isolated (**Figure 4-2**) and digested with *Eco*RI, *Pst*I, *Hind*III or *Nde*I nucleases (**Figure 4-3**). 1.5 to 6.0 kilobases pair (kbp) fragments were excised from 1 % agarose gels and DNA was recovered. The pUC18 vector was digested with *Eco*RI, *Pst*I, *Hind*III or *Nde*I, dephosphorylated with CIAP and ligated with the chromosomal fragments, which were cut with the same enzymes. Chemically competent *E. coli* cells were transformed with the ligation products individually into library efficiency and plated onto LB agar plates containing ampicillin, X-Gal and IPTG such that 50 to 100 colonies were visible after overnight growth. In the case of the genomic DNA libraries constructed with *Eco*RI, *Hind*III and *Nde*I approximately 20 % of the total colony number were religants. About 40 - 50 % of the recerculated plasmid DNA molecules were obtained in the case of the genomic DNA library constructed with *Pst*I. After overnight growth the colonies were transferred onto sterile flax filters and replicated onto LB agar plates supplemented with tributyrin to detect lipolytic activity. The putative positive clones were picked from the replica plates and grown overnight in LB media containing ampicillin. The identity of the clones was confirmed by restriction analysis and DNA sequencing.

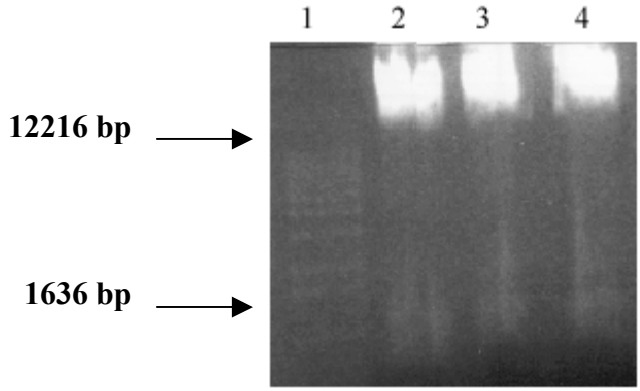


**Figure 4-2** Isolation of chromosomal DNA from *Bacillus* sp. 01-855

1 % Agarose gel

*Lane 1* - 1 kb Ladder; *lanes 2 - 4* - 20 ng of chromosomal DNA of *Bacillus* sp. 01-855. Arrows on the left indicate the size of DNA standard.

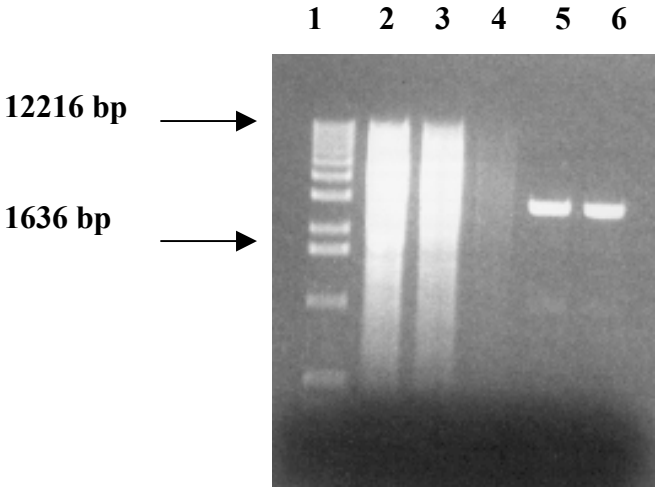


**Figure 4-3** Digestion of chromosomal DNA of *Bacillus* sp. 01-855 with *Eco*RI

1 % Agarose gel

*Lane 1* - 1 kb Ladder; *lanes 2 - 4* - 20 ng, 15 ng and 1 ng of chromosomal DNA digested with *Eco*RI respectively; *lanes 5* and *6* - 20 ng of pUC18 vector digested with *Eco*RI. Arrows on the left indicate the size of DNA standard.

## 4.3. Isolation of Lipolytic Positive Clones from *Bacillus* sp. 01-855 Genomic DNA Library

In the DNA library pool consisting of 93.000 positive clones (approximately 20.000 in each library) one *Nde*I and two *Eco*RI clones, which exhibited lipolytic activity were detected. No positive clones were found in the libraries constructed with *Pst*I and *Hind*III. The plasmids called pBS1 (*Nde*I clone), pBS2 (*Eco*RI clone) and pBS3 (*Eco*RI clone) were isolated from the lipolytic active colonies and analysed by restriction analysis (**Figure 4-4**). The chromosomal DNA insertions of the pBS1, pBS2 and pBS3 were designated *ins*BS1 (2.2 kb), *ins*BS2 (1.9 kb) and *ins*BS3 (2.3 kb), respectively and sequenced by primer walking method on both strands. The lipolytic activity encoded in the *ins*BS1, *ins*BS2 and *ins*BS3 could be not induced by IPTG, which indicated that the genes were transcribed from their own promoters. The scheme of DNA sequencing and nucleotide sequence analysis of the *ins*BS1, *ins*BS2 and *ins*BS3 are given in **chapters 4.3.1**, **4.3.2** and **4.3.3** in details.

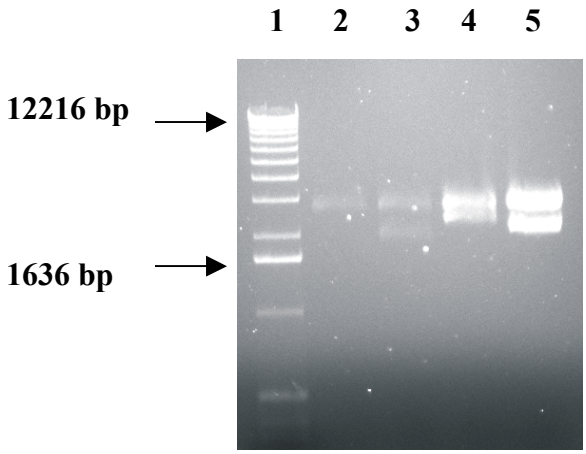


**Figure 4-4** Restriction analysis of lipolytic positive clones

1.5 % Agarose gel

*Lane 1* - 1 kb Ladder; *lane 2* - 5 ng of pUC18 digested with *Eco*RI; *lane 3* - 5 ng of pBS2 digested with *Eco*RI; *lane 4* - 15 ng of pBS3 digested with *Eco*RI; *lane 5* - 20 ng of pBS1 digested with *Nde*I. Arrows on the left indicate the size of DNA standard.

### 4.3.1. DNA Sequencing and Sequence Analysis of the *ins*BS1

The scheme of DNA sequencing of the *ins*BS1 and its nucleotide sequence are shown on **Figure 4-5** and **Figure 4-6**, correspondently. The computer analysis of the 2283 bp *ins*BS1 fragment revealed three open reading frames (ORFs) (**Figure 4-7**). A 942-bp ORF1 was found to extend from ATG start codon at position 192 to TAG stop codon at position 1134. A putative ribosomal binding site was identified 15 bp upstream the ATG codon, which most probably represents the translation initiation site. The second possible ATG start codon followed at a distance of 18 bp, but it was not preceded by a ribosomal binding site. Two possible –10 and –35 promoter regions were found at positions 120 - 125 and 97 - 102; 89 - 93 and 58 – 63, respectively. The ORF1, designated *est*B1, encodes a putative 314 amino acids protein (EstB1) with a predicted molecular weight of 35.30 kDa. The deduced amino acid sequence of the EstB1 from *Bacillus* sp. 01-855 is shown on **Figure 4-8**. The protein sequence search using BLAST revealed that the EstB1 exhibited sequence similarity to the 3-oxoadipate enol-lactonase from *Bacillus cereus* ATCC 14579 (44 % identical amino acids), abhydrolase α/β hydrolase fold from *Bacillus anthracis* A2012 (45 % identical amino acids) and the putative chloroperoxidase from *Clostridium acetobutylicum* (26 % identical amino acids). The amino acid sequence Gly-Trp-Ser-Thr-Gly, starting at residue 114 to 117, was found in the EstB1, that fits the Gly-X1-Ser-X2-Gly motif found in most bacterial lipases and esterases [124]. Two other ORFs (*orf2* and *orf3*) found in the *ins*BS1 were located downstream from the *est*B1 in the same orientation of those the *est*B1. The *orf2* encodes a putative protein of 213 amino acids with a predicted molecular weight of 22.69 kDa. The deduced amino acid sequence of this protein shared similarity to the 3-oxyacyl reductase from *Thermotoga maritime* (51 % identical amino acids) and adh_short chain dehydrogenase from *Bacillus anthracis* A2012 (47 % identical amino acids).

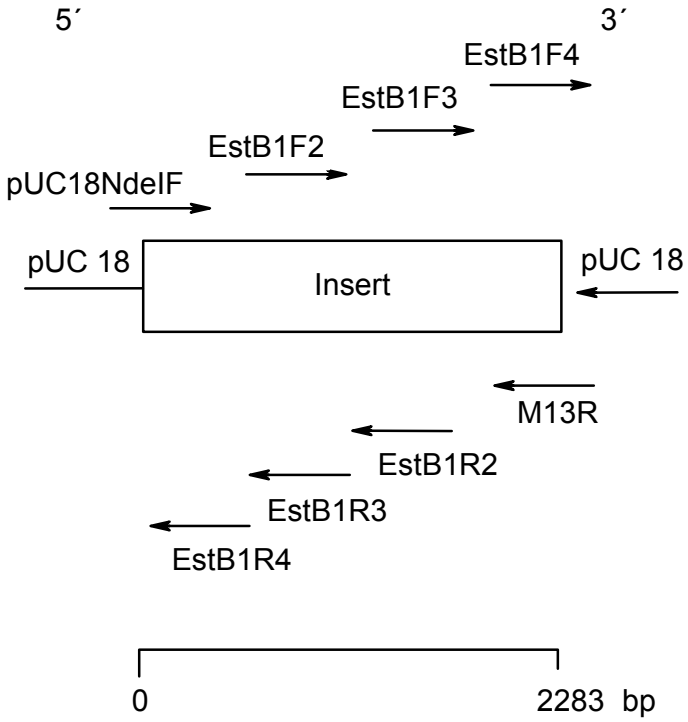


**Figure 4-5** Construction of primers used for the complete DNA sequencing of the *ins*BS1 by primer walking method. Arrows show the sequence direction. The names of the primers used for DNA sequencing are indicated above and below the arrows. The length of the *ins*BS1 fragment in base pares (bp) is indicated below the line.

CATATGAAACGATCTGCACACGATTATGTCCTAAGTGAGCTTGGCCT**CACCGA**AGAACATTC
-35
GATTTACTTGGAGGAA**TATGG**ACA**CATCGG**ACAAATCGATCAGAT**TATCG**CTGAAGCTAGCT
-10 -35 -10
GAAGCACAAGGCAAGATTCAAGCAGGTGATGTAGTCGTTCTAGTATCTGC**AGG**AATTGGCTA
rbs
TGC***ATG***GGGAGCAACAACGATAATATGGGGAAACGAGGAGGAAATTTAATGATTACAATACC
AACTGTTCATAAAGTCAGCCTTCCTAATGGAGAAGTGATGGGATATCGAAAACGAGATGGAG
GAGAAAAAACAATCTTACTCGTTCATGGTAATATGACGTCTTCAAAACATTGGGACCTTTTC
TTCGAAACATTTCCTGCTTCTTATACGCTGGTTGCGATCGATATGAGAGGGTTTGGTGAATC
ATCTTATAACAAAAGAGTTGAGGGAATAGAGGATTTTGCTCAAGATCTTAAATTCTTTGTAG
ATCAGCTAGGATTAAACGATTTTACGATGATCGGCTGGTCCACTGGAGGAGC*CGTTTGCATG*
*CAGTTTG*AAGCGCAGTACCCTGGATATTGCGATAAGATCGTCCTCATTTCTTCTGCTTCAAC
AAGGGGTTATCCTTTCTTTGGGACGCATTCTGACGGGACGCCAGATCTTAACCAAAGGCTGA

*AAACAGTTGACGATATAGAAAAAGATCCGATGAGAACGATCCCGATTCAACAGGCCTATGAT*
*ACGGGCAATCGAGCGCTTCTAAAAACGATTTGGAACTCTCTTATCTACACCCACAATCAGCC*
*TGAAGAGAAGCGTTATGAAGCTTATGTAGATGATATGATGACACAGCGAAATCTTGCTGATG*
*TTTACCATGCATTAAATACATTCAATATTAGTAGTGTGACAAATGGGTTAACTGAAGGAACA*
*AATCAGGCAAATCTCATACGTATCCCTGTTCTCGTTTTGCGTGGAGAACGAGATCTTGTTAT*
*CTCAAAAGAAATGACAGAAGAAATCGTCGAAGACCTTGGTACGAATTCAACGTACAAGGAGT*
*TGTCTGCATCCGGTCACTCTCCATTCATCGATGATTGTGATCAGCTTACGAATATTATTACT*
*GACTTTTTAGAGAAA**TAG***GAGGAATATTATGAGGTTGCAAGATAAGGTGGCTGTCATTACGG
GAGGAGCGAGCGGTATTGGTTTAGCTGCGACGGAACGTTTTGCCAAAGAAGGCGCGCGCGTT
GCTATGCTAGATTTACAAGAGGAAATTGGCAAGCAAAAAGCGGAGGAATTACAAGCTGAAGG
ATACCGTGTTACGTTTTATCAGCTTGATGTATCGAAGGAAGATGAAGTACAAAGGACGATAA
ACGAGATCGCCGAGGTAAACGGAACAATCGATATCTTAATTAATAATGCCGGCATTACAAAA
GACAGTATGCTGCACAAAATGAGCGGTGAAGCGTTTAAGAACGTGATGGATGTTAATGTGAA
TGGTGTTTTCTACTGCACGCAGACTACTGTACCTTATATGGTAAAACAGGGTAAAGGAAAGA
TCATCAATACCTCTTCTGTTAGCGGTGTGTATGGAAATGTTGGTCAAACGAATTATGCTGCT
TCAAAAGCAGCTGTCGTAGGAATGACCAAGACATGGGCAAAAGAATTGGGTGGCAAAGGCAT
TAACGTGAATGCCGTGGTTCCAGGCTTTACTGAAACAACTATGGTTGCCGCAGTGCCCGGAG
AAAGTAATCGAAAAAATGGTTGCGATGGTGCCTGTAAAGCGTCTTGGTAAACCAGAGGACAT
CGCAGATGCATACTTGTTTCTGGCGAGTGAAGAAGCGGATTACATTAATGGAACGGCGCTTC
ATGTAGATGGCGGTATTATGATGTAAAGTCTCATGCTTACGGTTGGGAGGGATGAAGCGAAG
TGGAATTGGATTGGATGGAAAAACGAGCAAATCTATTCCCAGATGATTGCGCGTTAATCGAT
GGTGTTTCACGTGAAGAATGGACGTATAAGCAGTTGAATGATAAAGCTATACAGCTGGCGGC
GTTTTTACAAAAGAACGATGTGGGGAAAGGTGATCGGGTTCTGGTGCTCTCCCATAACTCAC
TAGACATGTTTGCTTTGTTATTTGCTTGTAGAAAAACTGGATCAGTATTTGTTCCACTAAAC
TGGCGTCTTTCTAATAAGGAAATTGCTGAGTTGATTGAGGATTCTGATCCTTCCCTCCTCAT
TTTCGATCGTCATACGAAAGAACTTGCAAAGTTATGTGTAGATCATATG

**Figure 4-6** Nucleotide sequence of the esterase gene *est*B1 from *Bacillus* sp. 01-855 and flanking regions. The ribosomal binging 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 esterase gene *est*B1 is marked in grey (*italic*). The oligonucleotides used for the DNA sequencing are shown in frames.

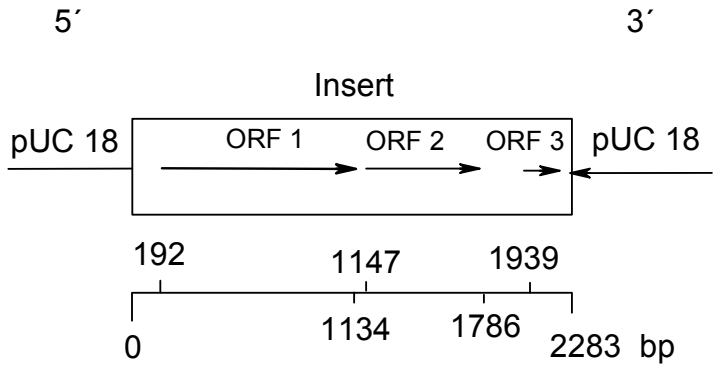


**Figure 4-7** Computer analysis of the chromosomal fragment *ins*BS1 from *Bacillus* sp. 01-855 encoding the lipolytic activity. Arrows show the putative *est*B1, *orf2* and *orf3*. The relative positions and the length of the fragments in base pares (bp) are indicated at the bottom.

MGSNNDNMGKRGGNLMITIPTVHKVSLPNGEVMGYRKRDGGEKTILLVHGNMTSSKHWDLFF
ETFPASYTLVAIDMRGFGESSYNKRVEGIEDFAQDLKFFVDQLGLNDFTMI**GWSTGG**AVCMQ
FEAQYPGYCDKIVLISSASTRGYPFFGTHSDGTPDLNQRLKTVDDIEKDPMRTIPIQQAYDT
GNRALLKTIWNSLIYTHNQPEEKRYEAYVDDMMTQRNLADVYHALNTFNISSVTNGLTEGTN
QANLIRIPVLVLRGERDLVISKEMTEEIVEDLGTNSTYKELSASGHSPFIDDCDQLTNIITD
FLEK

**Figure 4-8** The deduced amino acid sequence of the putative esterase EstB1 from *Bacillus* sp. 01-855. The amino acid sequence is given in 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.

### 4.3.2. DNA Sequencing and Sequence Analysis of the *ins*BS2

The scheme of DNA sequencing of the *ins*BS2 and its nucleotide sequence are shown on **Figure 4-9** and **Figure 4-10**, respectively. One 744-bp ORF assigned as *est*B2 with ATG start codon at 1085 position and TAA stop codon at 1829 position was detected in the 1977 bp *ins*BS2 (**Figure 4-11**). A putative ribosomal binding site was located 15 bp upstream of the translation codon. The putative -10 and –35 promoter consensus was detected at 1117 - 1021 and 986 - 990 positions, correspondently. Because the second putative ATG start codon situated in a distance of 54 bp was not preceded by –10 and –35 promoter consensus, it was

assumed that this ATG does not represent the translation initiation site. The *est*B2 encodes a putative protein called EstB2 of 248 amino acids with a deduced molecular weight of 28.32 kDa. The deduced amino acid sequence of the EstB2 is represented on **Figure 4-12**. The comparison of the amino sequence of the EstB1 with proteins in databases revealed similarity to the following enzymes: carboxylesterase from *Bacillus cereus* ATCC 14579 (74 % identical amino acids) and proteins from dienelactone hydrolase family from *Bacillus anthracis* A2012 (73 % identical amino acids). The consensus sequence Gly-X1-Ser-X2-Gly which is perfectly conserved among many lipases and esterases, was found in the EstB2 at position 91 to 94, where X1 and X2 are represented by the amino acid Leu. The upstream and downstream flanking sequences of the *est*B2 were also analysed for the putative ORFs, but no significant similarity to the published sequences was found.

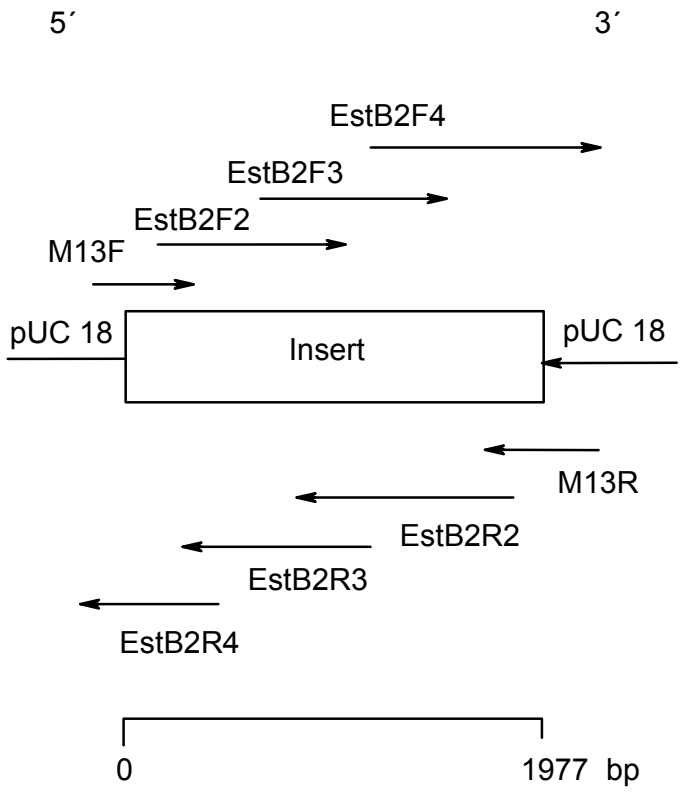


**Figure 4-9** Construction of primers used for the complete DNA sequencing of the *ins*BS2 by primer walking method. Arrows show the sequence direction. The names of the sequence primers are indicated above and below the arrows. The length of the *ins*BS2 fragment in base pares (bp) is indicated below the line.

GAATTCTATAGCCAACTTTGCGAGAAGTACCCAATCATCTCGATCGAAGACGGTCTAGATGA
AAACGATTGGGAAGGCTGGGAGAAGCTAACTCAAGCACTTGGTGAGAAAGTACAGCTTGTTG
GTGACGATCTATTTGTTACAAACACAAACAAACTTTCTGAAGGTATCGAGCGCAGTGTTGGT
AACTCAATCCTAATTAAAGTGAACCAAATCGGTACACTTACTGAAACGTTCGAAGCAATCGA
AATGGCGAAGCGCGCTGGTTACACAGCAGTTATCTCTCACCGTTCTGGTGAAACAGAAGATA
CAACGATCGCTGACATCGCTGTTGCAACAAACGCTGGACAAATTAAAACAGGCGCACCGTCT
CGTACGGACCGCGTTGCGAAGTACAACCAGCTTCTTCGTATCGAAGATGAGCTTGAGCACCT
TGCAGTTTACGGTGGTAAGACAAGCTTCTATAACCTTGCGAACAAGTAAGGTCATTTACTCC
CCCTCATGTTAATCATGAGGGGGTTTTTGTGTTAGGCTTAAAGTAACGAGGGTTTCCGAAAT
TTATTGCTTTTATTGAAATTTTTGTGGTAAATTTATAGTATTGGAAGGTTAATGTCCGGGAG
GTGGAATCATGCATCTAGCAGCATCTATTGCGTTAATCATCGTATCGATCTTACTAATCACT
GTCGTATTGCTTCAATCAAGTAAAAGCGCAGGTTTATCGGGAGCAATCACTGGTGGCGCTGA
ACAGTTATTCGGAAAGCAAAAAGCTCGCGGTTTTGAAGCCGTATTAAATAAAGTGACGGTCG
TACTCGCCGTATTATTCTTTGTTCTTTCTATTTTAGTTGCGTACCTCGTGTAACGCAGGAGG
CAGCAAGTGATGAACCACTTGCTGCTTTTTTATTTCTCAAAAGCCAAAAAAACTCGATGGTT

TTCTACATAACCATACAGGGTAGGAAATTGTAAGGAGCACTTTCCTTTAGCCTCC**CTGAT**GG
-35
AAAAGGTAAAATACAGATAGAATT**TATAA**TCAAACTAATCAAAGGAAAAGCTACATGAGAGA
-10
GGTAGAGCTTTCGA**AGGAGG**AAACACATC***ATG***AAAGTTGTTGCACCAAAGCCTTTTACATTC
rbs
*GAAGGAGGCAATAGGCGGTATTGATGCTTCATGGTTTTACAGGAAATTCAGCCGACGTCCG*
*AATGATGGGCAGGTATTTACAAGAGCAGGGCTACACTTGTCATGCACCGCAATACAAAGGAC*
*ACGGCGTACCGCCTGAAGAACTAGTACATACAGGTCCGTCTGATTGGTGGAAAGATGTTCAA*
*GAAGGATATAACAAGCTCAAGGAAATGGGCCATGACGAGATTGCAGTAGTGGGATTATCTCT*
*CGGAGGCGTATTTTCCTTAAAGCTCGGCTATACTGTTCCTGTAAAGGGAATCGTACCCATGT*
*GTGCGCCGATGGATATGAAAGACGAAGAAACGATGTATGAGGGCGTACTCGCATATGCGAGA*
*GATTATAAAAAATACGAGCGTAAATCTCCTGAACAGATTGAAGAGGAAATGGAAGCGTTCAA*
*AGAAACACCGATGAATACACTAGGTGAATTACGAGATTTGATCTACGATGTCAGAGATAACA*
*TTGATATGATCTATGCACCTACTTTCGTCGTCCAAGCGAGACACGATGAAATGATCAATACG*
*GATAGTGCGAATGTCATTCATGACAATATCGAATCTGATGAGAAAAGCTTAAAATGGTATGA*
*AAATTCAACTCATGTCATCACGTTAGGAAAAGAAAAAGAAGTCCTTCATGAGGATGTGCTCG*
*AATTTCTTA*ACGGACTTGACTGGAAAGAA***TAA***TACGTTAATTTGTTTGAAGGAAGGTGAAAC

GATATGGCAGAAGAACATGTACAAAAAATATTGAGCTACATGAAAGACGAGGCATATAAACC
ATTAACGGTTAAAGAACTTGAAGAAGTGTTCGGCATTCAAGATTCGAGTGAATTC

**Figure 4-10** Nucleotide sequence of the esterase gene *est*B2 from *Bacillus* sp. 01-855 and flanking regions. The ribosomal binging 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 esterase gene *est*B2 is marked in grey (*italic*). The oligonucleotides used for the DNA sequencing are shown in frames.

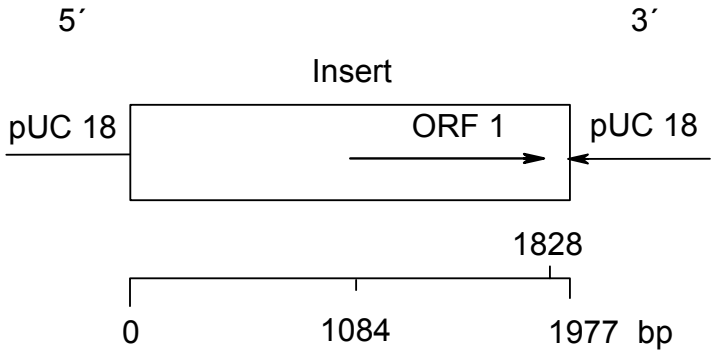


**Figure 4-11** Computer analysis of the chromosomal fragment *ins*BS2 from *Bacillus* sp. 01-855 encoding the lipolytic activity. Arrow shows the putative *est*B1. The relative position and the length of the fragment in base pares (bp) are indicated at the bottom.

MKVVAPKPFTFEGGNRAVLMLHGFTGNSADVRMMGRYLQEQGYTCHAPQYKGHGVPPEELVH
TGPSDWWKDVQEGYTKLKEMGHDEIAVV**GLSLG**GVFSLKLGYTVPVKGIVPMCAPMDMKDEE
TMYEGVLAYARDYKKYERKSPEQIEEEMEAFKETPMNTLGELRDLIYDVRDNIDMIYAPTFV
VQARHDEMINTDSANVIHDNIESDEKSLKWYENSTHVITLGKEKEVLHEDVLEFLNGLDWKE

**Figure 4-12** The deduced amino acid sequence of the putative esterase EstB2 from *Bacillus* sp. 01-855. The amino acid sequence is given in 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.

### 4.3.3. DNA Sequencing and Sequence Analysis of the *ins*BS3

The scheme of DNA sequencing of the *ins*BS3 and its nucleotide sequence are shown on **Figure 4-13** and **Figure 4-14**, correspondently. The analysis of the 2318 bps fragment revealed the presence of two ORFs, which were found sequentially on the complementary DNA strands and were transcribed in the opposite orientation to each other (**Figure 4-15**). The first ORF1 called *orf1* with ATG start codon at position 229 and TAA stop codon at position 670 encoded a putative protein consisting of 147 amino acids with a deduced molecular weight of 16.41 kDa. This protein showed similarity to the nucleoside diphosphate kinase from *Bacillus halodurans* (72 % identical amino acids) and *Oceanobacillus iheyensis* HTE831 (74 % identical amino acids). The second ORF2 designated *amd*B1 with ATG as the initial codon and TAA as the termination codon spanned a region of 1398 bps (position from 180 to 1578). The *amd*B1 start codon was preceded by a putative ribosomal binding site (position from 152 to 156) and putative –10 (position from 138 to 143) and –35 (position from 106 to 111) promoter regions. The second possible ATG start codon followed at a distance of 15 bp but it was not preceded by a ribosomal binding site. The *amd*B1 encoded a putative protein named AmdB1 which consists of 466 amino acids with a molecular mass of 50.43 kDa. The deduced amino acid sequence of the AmdB1 is represented on **Figure 4-16**. The sequence similarity search showed that the AmdB1 exhibited similarity to the putative amidases from *Mesorhizobium loti* (32 % identical amino acids) and *Pseudomonas putida* KT2440 (33 % identical amino acids).

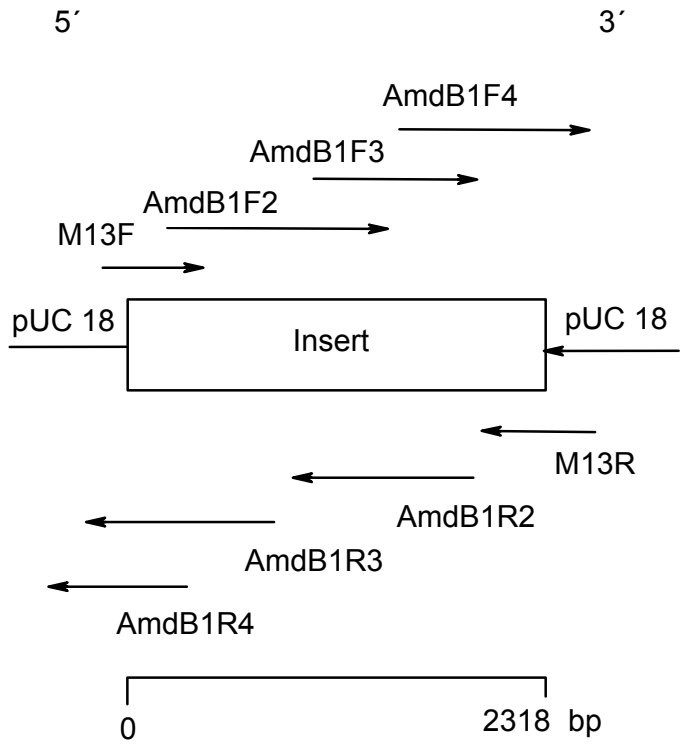


**Figure 4-13** Construction of primers used for the DNA sequencing of the *ins*BS3 by primer walking method. Arrows show the sequence direction. The names of the sequence primers are indicated above and below the arrows. The length of the *ins*BS3 fragment in base pares (bp) is indicated below the line.

GAATTCCTATTAAAATTCAAAAAACACCGTAGACGTCTTCTTTATTGAAGCTGGTGTACCTT

CATGTTAACAAAAATGTTTTCTTACGAGCTCGTCTAATAAATT**GCTCA**CTCCCTTATTTTTC

-35

GCTAAAGTTAAAC**TCATA**GACCATTTT**AGGA**AGTGATACAGTGAACATTATAGAC***ATG***AGTG

-10 rbs

*CCAAAGAAATGGCGAGGCTGATCGCTCACAAAGAGATTTCCCCAGTCGATGTTGTTTCAGCA*

*CACTTAGATCGAATCGACCAGCTTAATGGGAAACTGAATATGTTTGTTTCTGTCTTTAGAAA*

*CGAAGCATTACAAGAAGCAAAATTAGCAGAAGACCGTTTGATGAGAGGAGAAAGCCTCGGTC*

*CACTTCACGGCGTTCCGATCGCACTAAAAGACCTGACACCCGTTCGAGGAAGACGTACAACG*

*TTTGGCTCACAGCTCTTTAAAGACCATATTGCGACACATGAACCGACAATCGTCAAACGTAT*

*TAAACATTCGGGAGCGATCATCGTTGGAAAAACGAACACACCTGAATTTGGACATAAAGGTA*

*CAACGGATAACGAAATTGTAGGCCCTGCCAAGAATCCTTGGAATCCCTCTATGACTGCAGGT*

*GGATCGAGCGGTGGTTCAGGAGCTGCCGTTGCAAGCAAATGCGTTCCACTAGCAGAAGGAAG*

*CGACGGAGGCGGTTCAATACGTATTCCAGCGAGCTTTAACGGAATTTTCGGGTTAAAGCCTA*
*CTTTCGGTCGAGTTCCATTCGATAGTTCACCTATTAATCGATTTGGTACAACACAGCCATAC*
*GTTCATTTTGGACCTTTGAGTAGAAGGGTAGAAGATTCCGCTCTCCTTCTCTCAATACTTGA*
*AGGGTATGAGCCCAACGACCCTTATTCAGTGCCTCTTGTAAAAGAAAATTTATATGAAGAAG*
*CGCTTAAAGGAATAAAAGGGATGACATTTGCTTACACGCGAAATTTTGGATTATATGAGTGT*
*GATTCTGAGGTAAGCAGCGTTATTGAGAGCGTTCTACCTATTTTAGAAGAGTACGGTGCAAA*
*AATAGTAGAGGTACCAATTGACTTTGGGTTAGATCTCAGAGAATTAATATCTTTCTTTAATA*
*GAATGTGGTATGCGGGTCTCGCTACAGCGTACGGCGATCTCTATGACCGAAACCCCGCATCC*
*TTTGGTTCTTCAGTAGCAACTATGATCGATGAGGGGAGAACGATCAGTGCGGTCGAACTTCG*
*TAGAACGGAGGAGCTGCGAACTGTTATTTGGAATACGTTACAGCATATTTACAGCGAATATG*
*ATGCTTTGCTATCCCCTGTCCTAGGCGTTACCGCTTTCCCATACGAAATAGAAGGTCCTGAT*
*CGAATAAACGGTAAGGATGCAGCGCCCATCTCTGACTGGATGATGACACAGCTCTACAATTG*
*TACAGGACATCCAGCTGCCTCGATCCCAGCAGGGTTTTCAGGTGGTTTGCCCGTTGGATTAC*
*AGGCTTCAGCAAGTAAATTTAACGATTCGATCCTTCTTCGCATTGCAAGCGTAATTGAAACT*
*GCAAACGGCACCAGCTCACCAAGCTTATAAA*AAAAACAACCGACCGGACAAATTGTCCGGTCG
GTTGTATATAAGCGCTTTCATAAGACTTAAAAAATTAAACCCACTTAGAGATGGTCTTTGAG
TACTCATTTACTTCATTTTCGTTGAAGAACAGTGATACTTCACGCTCAGCACTTTCAGGAGA
ATCTGAACCATGGATAACGTTCATGCTAACCTGAACACCGTAGTCCCCACGAATTGTACCAG
GAGCAGCATCCTTCGGGTTTGTTGCACCCATCATCGTACGAGCTGTTGAGATTACGTTCTCA
CCTTCCCATACCATTGCAAAGACAGGTCCAGAAGTAATGAAATCAACGAGTTCTCCAAAGAA
AGGACGCTCTTTATGTTCACCATAATGGTTTTCTGCTAGCTCTTTTGAAACCGTCATCAATT
TAGCTCCCACCAAATTAAAACCTTTTTTCTCGAAACGAGATACGATTTCACCGATAAGGTTT
CTTTGAACGCCGTCAGGCTTTACCATCAAAAACGTTTTCTCCATGTAAATCTCCTCACTTTC
AACAAGTTATATGTATTGGGCAGAAAGCGTTTTCTGCCCTCCAAATTCTATCAAATTTTCGA
AAAAATAACAACATTACTTTTAATGTTTTCTTTTCCCTATGTATTCTGCGATTTGCTTGAGT
GAGGTTCTCGAAGAAGTTGATGGTAGATGCTCTAGCGCTTGAAATGCTTTTTGAAGGTAGCG
ATCACTAATCTCTCGAGCGAATTC

**Figure 4-14** Nucleotide sequence of the amidase gene *amd*B1 from *Bacillus* sp. 01-855 and flanking regions. The ribosomal binging 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 amidase gene *amd*B1 is marked in grey. The oligonucleotides used for the DNA sequencing are shown in frames.

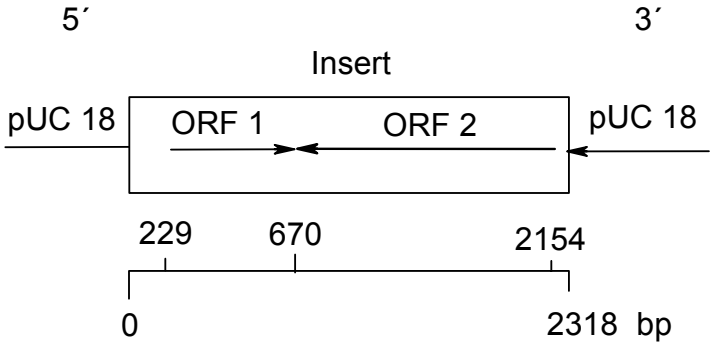


**Figure 4-15** Computer analysis of the chromosomal fragment *ins*BS1 from *Bacillus* sp. 01-855 encoding the lipolytic activity. Arrows show the putative *amd*B1 and *orf1*. The relative positions and the length of the fragments in base pares (bp) are indicated at the bottom.

```
MSAKEMARLIAHKEISPVDVVSAHLDRIDQLNGKLNMFVSVFRNEALQEAKLAEDRLMRGES
LGPLHGVPIALKDLTPVRGRRTTFGSQLFKDHIATHEPTIVKRIKHSGAIIVGKTNTPEFGH
KGTTDNEIVGPAKNPWNPSMTAGGSSGGSGAAVASKCVPLAEGSDGGGSIRIPASFNGIFGL
KPTFGRVPFDSSPINRFGTTQPYVHFGPLSRRVEDSALLLSILEGYEPNDPYSVPLVKENLY
EEALKGIKGMTFAYTRNFGLYECDSEVSSVIESVLPILEEYGAKIVEVPIDFGLDLRELISF
FNRMWYAGLATAYGDLYDRNPASFGSSVATMIDEGRTISAVELRRTEELRTVIWNTLQHIYS
EYDALLSPVLGVTAFPYEIEGPDRINGKDAAPISDWMMTQLYNCTGHPAASIPAGFSGGLPV
GLQASASKFNDSILLRIASVIETANGTSSPSL
```

**Figure 4-16** The deduced amino acid sequence of the putative amidase AmdB1 from *Bacillus* sp. 01-855. The amino acid sequence is given in one-letter code according to the codon usage of *E. coli*.

## 4.4. Cloning, Expression and Purification of the EstB1

### 4.4.1. Molecular Cloning of the Esterase Gene *est*B1

The *est*B1 gene coding for the EstB1 esterase was cloned by PCR from the pBS1 plasmid isolated from genomic DNA library of *Bacillus* sp. 01-855 using the AKA-NPetF and AKA-NPetR primers. The forward AKA-NpetF primer was used to insert a *Nde*I restriction site exactly at the ATG start codon. A *Not*I site was introduced at the 3'- terminus of the *est*B1 gene by the reverse AKA-NpetR primer. Additionally, the 3'- end of the *est*B1 gene was fused to the $His_6$-tag coding sequence of the vector. The prepared by these means PCR fragments were digested with *Nde*I and *Not*I and inserted into the expression vector pET-22b(+), which was previously cut with the same enzymes, resulting pET-*est*B1 plasmid. The designed medium copy vector allowed the expression of the cloned sequences in *E. coli* BL21(DE3) under the control of T7 promoter. The DNA sequence analysis of the pET-*est*B1 confirmed that the *est*B1 was cloned in frame with the downstream $His_6$-encoding sequence of the vector pET-22(b+) and no mutations have occurred in the nucleotide sequences during routine propagation in *E. coli*.

#### 4.4.1.1. Expression of the *est*B1 in *E. coli* BL21(DE3) Strain in Shake Flask Cultures

The expression of the esterase gene *est*B1 was done in *E. coli* BL21(DE3) strain in shake flask cultures for 0.1 - 2 l. The *E. coli* cells were transformed with the expression plasmid pET-*est*B1 and the esterase production was induced with IPTG. After induction, the incubation was continued at 30°C for 4 h and harvested cells were disrupted by sonication. The expression was confirmed by SDS-PAGE and zymogram and activity measurements with *p*NP acetate assay.

The SDS-PAGE expression analysis of *E. coli* BL21(DE3)(pET-*est*B1) indicates the presence of a 35 kDa protein, which corresponds to the calculated mass of the mature EstB1 protein (**Figure 4-17**). In controls containing the cell lysate of *E. coli* BL21(DE3) transformed with the pET-*est*B1 without induction (*lane 4*) and of *E. coli* BL21(DE3) bearing the empty vector (*lanes 2* and *3*) no distinct bands were visible at the respective positions. Zymogram with α-naphthyl acetate and Fast Red showed that the EstB1 was expressed in an active form (**Figure 4-17,** *lanes 8 - 10*). From SDS-PAGE it can be seen that the induction time does not play a

crucial role in the recombinant EstB1 production. The amount of the recombinant protein increased rapidly after 1 h induction and was found to be constant during the rest of induction time. When measuring the enzymatic activity (U/mg total protein) gained by expression of the *est*B1, a tendency to slightly increased values with longer induction times was recognized. The highest activity was observed in the cell lysate obtained from a 500-ml culture of *E. coli* BL21(DE3)(pET-*est*B1) after 4 h induction that consisted 9.8 U/mg total protein as measured spectrometric towards *p*NP acetate.

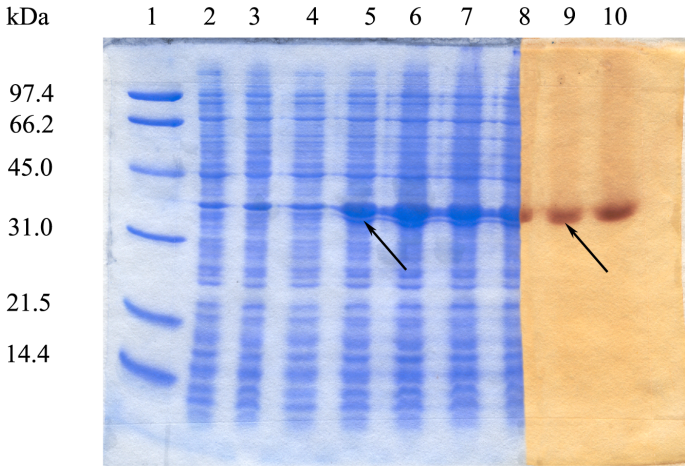


**Figure 4-17** Overexpression of the esterase EstB1 from *Bacillus* sp. 01-855 in *E. coli* BL21(DE3). Protein samples were separated in 12.5 % SDS-polyacrylamide gels and stained with Coomassie blue (*lanes 1 - 8*) and α-naphtyl acetate and Fast Red (zymogram) (*lanes 8-10*). Cell lysate of *E. coli* BL21(DE3) bearing the empty vector without induction (*lane 2*) and after 4 h induction (*lane 3*); and of *E. coli* BL21(DE3) transformed with pET-*est*B1 without induction (*lane 4*); after 1, 2, 3 and 4 h induction (*lanes 5 - 8*, respectively). *Lane 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.

### 4.4.1.2. One–step Purification of the EstB1 Esterase by Means of Immobilized Metal Affinity Chromatography (IMAC)

A total wet cell weight of 3.0 g was obtained from a 500-ml *E. coli* BL21(DE3)(pET-*est*B1) culture after centrifugation. The EstB1 was purified from the crude raw extract of *E. coli* in a single step under native conditions by cobalt-based IMAC on TALON ™ Superflow Resin, which yielded an almost homogeneous esterase. The adsorption of weakly bound unspecific proteins was eliminated by using of 5 mM imidazole in the equilibration and loading buffers. The purity of the purified EstB1 were confirmed by SDS-PAGE (**Figure 4-18**). The specific activity of the purified EstB1 was determined by a spectrometric assay towards *p*NP acetate and consisted 95.7 U/mg, that corresponds to a purification factor of 34. 50 mg of the active EstB1 esterase was obtained from 1 l of cultural broth. On the basis of catalytic activity of the purified esterase, the expression level of the active enzyme in the recombinant *E. coli* BL21(DE3) transformed with the pET-*est*B1 was estimated to be 20 % of the total soluble proteins. The purified and concentrated EstB1 was used for further biochemical characterisation.

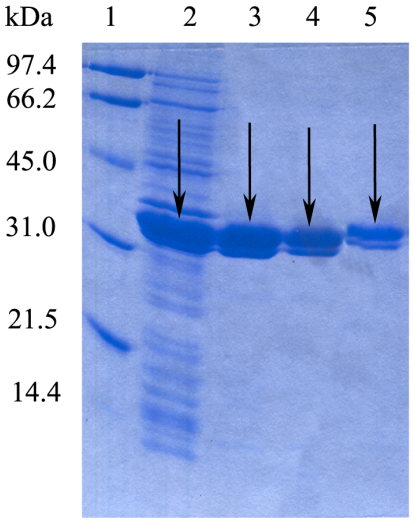


**Figure 4-18** Purification of the esterase EstB1 from *Bacillus* sp. 01-855 expressed in *E. coli* BL21(DE3). Protein samples were separated in 12.5 % SDS-polyacrylamide gels and stained with Coomassie blue. Soluble fraction of *E. coli* BL21(DE3) transformed with pET-*est*B1 after 4 h induction (*lane 2*); fractions after IMAC purification (*lanes 3 - 5*). *Lane 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.

## 4.5. Biochemical Characterisation of the EstB1 Esterase

### 4.5.1. Substrate Specificity of the EstB1 Esterase and Determination of Kinetic Data

The substrate specificity of the purified from *E. coli* EstB1 was studied by using various *p*NP esters and methyl esters of straight-chain fatty acids ranging in chain length from $C_2$ (acetate) to $C_{14}$ (myristate). The substrate specificity of the EstB1 is represented in **Table 4-2** and **Table 4-3**. $K_m$ and $V_{max}$ of the EstB1 were determined with *p*NP acetate and *p*NP butyrate and calculated from Lineweaver-Burk plots using a least-squares best fit of the Michaelis-Menten equation (**Table 4-4**). In the case of *p*NP esters the EstB1 showed the highest activity towards *p*NP acetate (95.7 U/mg), *p*NP butyrate (147.1 U/mg) and *p*NP caproate (22.9 U/mg). The higest activity of the EstB1 towards acyclic aliphatic esters was observed with methyl acetate (111.1 U/mg), methyl butyrate (109.0 U/mg) and methyl caproate (76.3 U/mg). The EstB1 was active on substrates with carboxylic acids of short chain length. *p*NP and methyl esters with acyl chain length bigger then $C_8$ were poor substrates for the EstB1. *p*NP esters were hydrolysed by the EstB1 at lower rates in comparison with methyl esters. Since the amino acid sequence analysis revealed that the EstB1 is similar to the 3-oxoadipate enol-lactonase and putative chloroperoxidase, the EstB1 was additionally tested for the lactonase and haloperoxidase activity. No detectable lactonase or haloperoxidase activity was measured. These results provide evidence for the EstB1 being a putative carboxylesterase.

**Table 4-2** Substrate specificity of the purified EstB1 towards *p*NP esters.

| **Substrate** | **Specific activity (U/mg)** |
|---|---|
| *p*NP acetate | 95.7 |
| *p*NP butyrate | 147.1 |
| *p*NP caproate | 22.9 |
| *p*NP caprylate | 13.6 |
| *p*NP caprate | 12.4 |
| *p*NP laurate | 3.1 |

**Table 4-3** Substrate specificity of the purified EstB1 towards methyl esters.

| **Substrate** | **Specific activity (U/mg)** |
|---|---|
| methyl acetate | 111.1 |
| methyl butyrate | 109.0 |
| methyl caproate | 76.3 |
| methyl caprylate | 30.3 |
| methyl caprate | 16.8 |
| methyl laurate | 4.5 |

**Table 4-4** Kinetic data determined from hydrolysis of *p*NP acetate and *p*NP butyrate with the purified EstB1.

| **Enzyme/substrate** | **$K_m$ (M)** | **$V_{max}$ (M min$^{-1}$)** |
|---|---|---|
| *p*NP acetate | $3.8 \cdot 10^{-3}$ | $7.6 \cdot 10^{-5}$ |
| *p*NP butyrate | $1.9 \cdot 10^{-4}$ | $2.7 \cdot 10^{-5}$ |

### 4.5.2. Storage Stability of the EstB1 Esterase

The storage stability of the EstB1 esterase at different temperatures (–20°C, 4°C and 37°C) and in different buffers (potassium- and sodium phosphate buffers, Tris·HCl buffer and PBS) is represented on **Figure 4-19**, **Figure 4-20**, and **Figure 4-21**.

The best storage stability of the EstB1 esterase was observed in potassium- and sodium phosphate buffers at –20°C and 4°C. The activity of the EstB1 decreased continually in potassium- and sodium phosphate buffers after 2 days of storage at –20°C. The EstB1 activity stored at –20°C was 90.2 % and 84.7 % after 3 days of incubation in potassium- and sodium phosphate buffers, correspondently. 45.1 % and 31.9 % of the EstB1 activity were measured in potassium- and sodium phosphate buffers, respectively after 7 days of storage at –20°C. Potassium- and sodium phosphate buffers had no influence on the EstB1 activity during 3 days of storage at 4°C. After 3 days of storage at 4°C the activity of the EstB1 decreased slowly and was 65.8 % and 60.1 % on the 7th day in potassium- and sodium phosphate buffers, correspondently. During the storage of the EstB1 in potassium- and sodium phosphate buffers at 37°C the activity decreased after 1 day of storage and was 79.4 % and 64.2 % after 3 days of incubation, correspondently. 19.8 % and 15.8 % of the activity were measured for the EstB1 esterase in potassium- and sodium phosphate buffers, respectively at 37°C after 5 days of storage. The complete inactivation of the protein was detected after 6 days of storage.

Tris·HCl buffer and PBS were found to be not appropriate for the long-term storage of the EstB1 esterase. The best results during storage of the EstB1 in Tris·HCl buffer and PBS were obtained at –20°C and 4°C. The best storage stability of the EstB1 esterase in Tris·HCl buffer and PBS was measured at –20°C. The activity of the EstB1 in Tris·HCl buffer decreased slowly and reached 74.9 % and 30.8 % after 2 and 4 days of incubation, correspondently. 85.1 % and 42.0 % of the EstB1 activity were measured in PBS after 2 and 4 days of storage at –20°C, respectively. The storage of the EstB1 at 4°C in Tris·HCl buffer led to the deactivation of the protein already after 1 day incubation and consisted 35.1 % and 11.4 % activity after 2 and 3 days of storage, correspondently. The activity of the EstB1 esterase measured in PBS after 2 and 3 days of storage at 4°C was 46.2 % and 18.9 %, respectively. The rapid inactivation of the EstB1 was observed in Tris·HCl buffer and PBS already after 2 days of storage at 37°C. The EstB1 activity was 11.5 % and 18.4 % after 2 days of storage in Tris·HCl buffer and PBS at 37°C, correspondently.

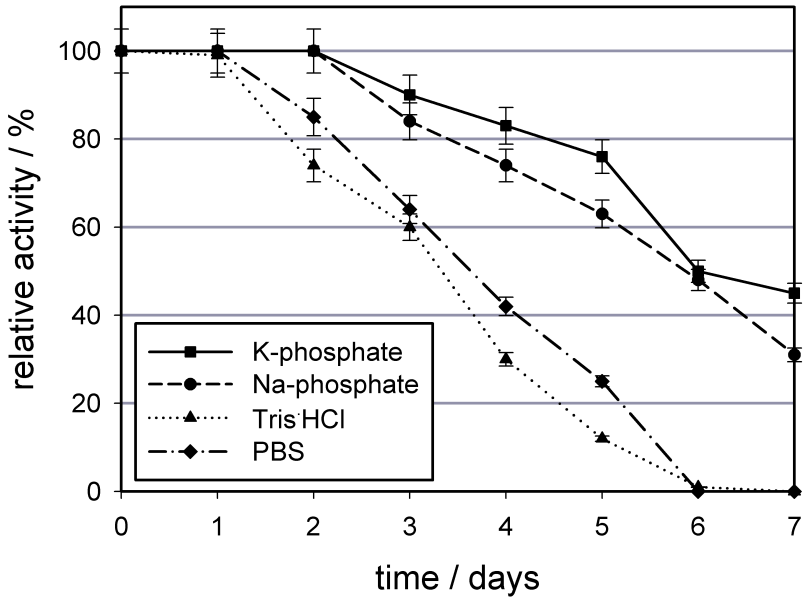


**Figure 4-19** Storage stability of the EstB1 in potassium- and sodium phosphate buffers, Tris·HCl buffer and PBS at –20°C. The enzymes activity was measured towards *p*NP acetate at 35°C and pH 7.5.

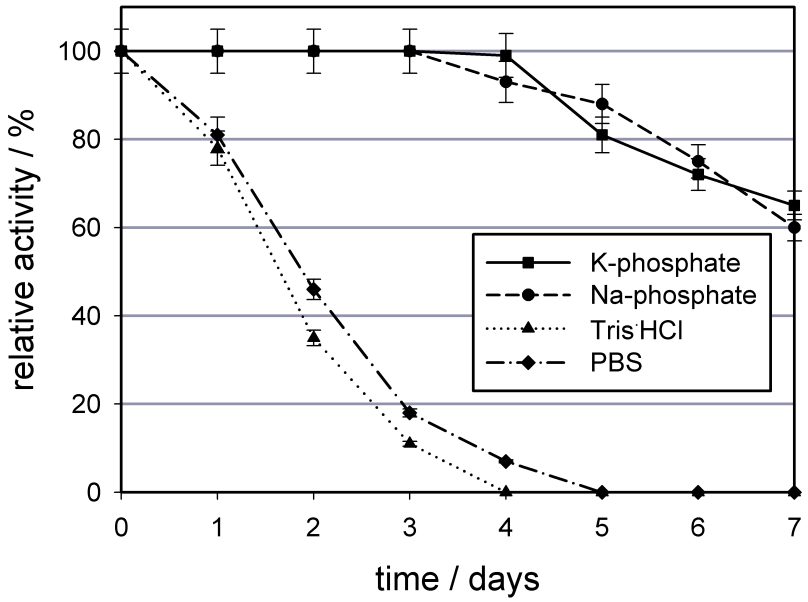


**Figure 4-20** Storage stability of the EstB1 in potassium- and sodium phosphate buffers, Tris·HCl buffer and PBS at 4°C. The enzymes activity was measured towards *p*NP acetate at 35°C and pH 7.5.

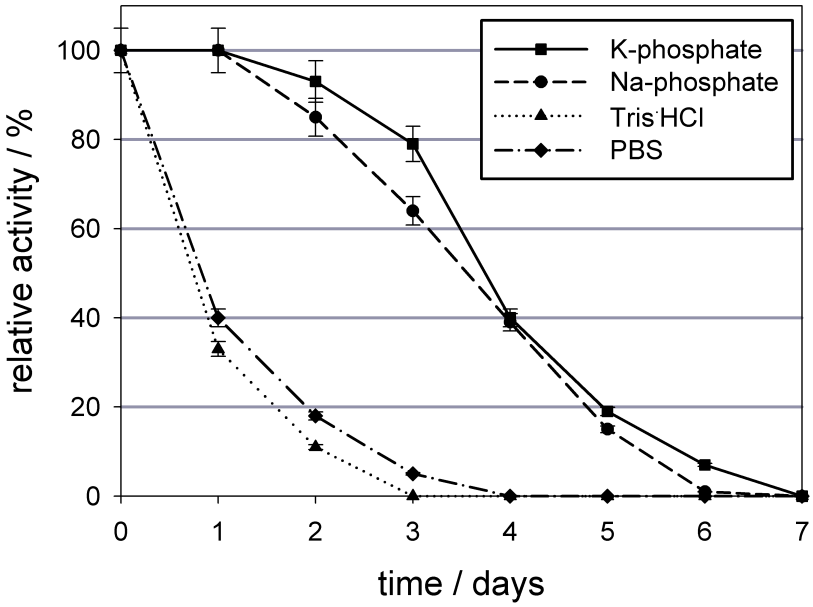


**Figure 4-21** Storage stability of the EstB1 in potassium- and sodium phosphate buffers, Tris·HCl buffer and PBS at 37°C. The enzymes activity was measured towards *p*NP acetate at 35°C and pH 7.5.

### 4.5.3. Temperature Optimum of the EstB1 Esterase

The temperature optimum of the EstB1 esterase is shown on **Figure 4-22**. The EstB1 exhibited the temperature optimum in a broad temperature range between 30°C (84.1 %) and 50°C (61.5 %), which indicates the light termophilic properties of the enzyme. With the increasing of the temperature from 20°C to 40°C the enzyme activity increased exponent. The EstB1 activity measured at 10°C, 20°C and 30°C was 34.7 %, 37.8 % and 84.1 %, correspondently. At 40°C the EstB1 activity reached its maximum. The further increasing of the temperature led to the slow decreasing of the EstB1 activity. The activity of the EstB1 measured at 50°C and 60°C was 61.5 % and 39.3 %, correspondently.

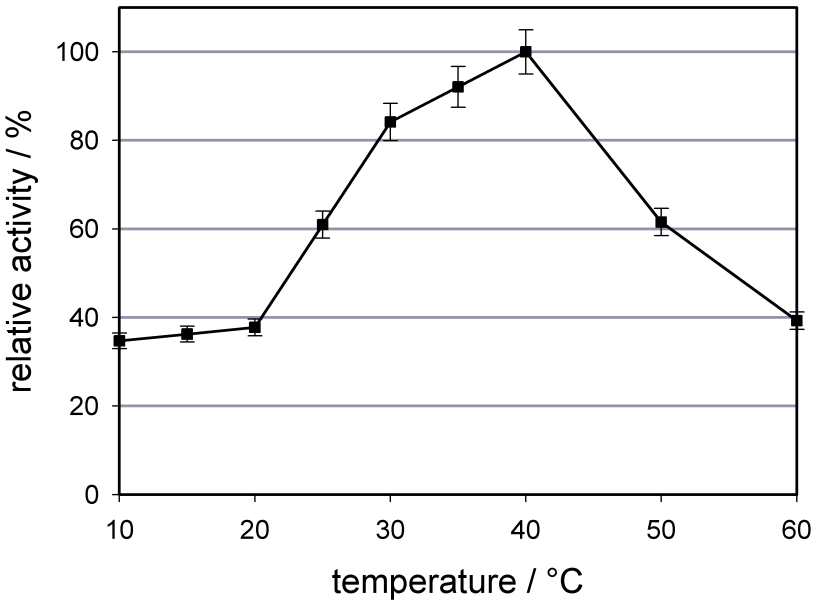


**Figure 4-22** Temperature optimum of the EstB1 esterase. The enzymes activity was measured towards methyl acetate at various temperatures at pH 7.5.

### 4.5.4. pH Optimum of the EstB1 Esterase

The effect of different pH values on the EstB1 activity is represented on **Figure 4-23**. The EstB1 displays the pH optimum in a range from pH 6.5 to pH 7.5. The increasing of pH values from pH 6.0 to pH 7.5 resulted in an exponential growth of the EstB1 activity. The activity of the EstB1 esterase measured at pH 5.0, pH 6.0 and pH 6.5 was 15.1 %, 19.7 % and 75.3 %, correspondently. At pH 7.5 the activity of the EstB1 reached its maximum and decreased rapidly with the increasing of pH values. So, the activity of the EstB1 esterase was 14.5 % and 7.5 % at pH 8.0 and pH 9.5, correspondently.

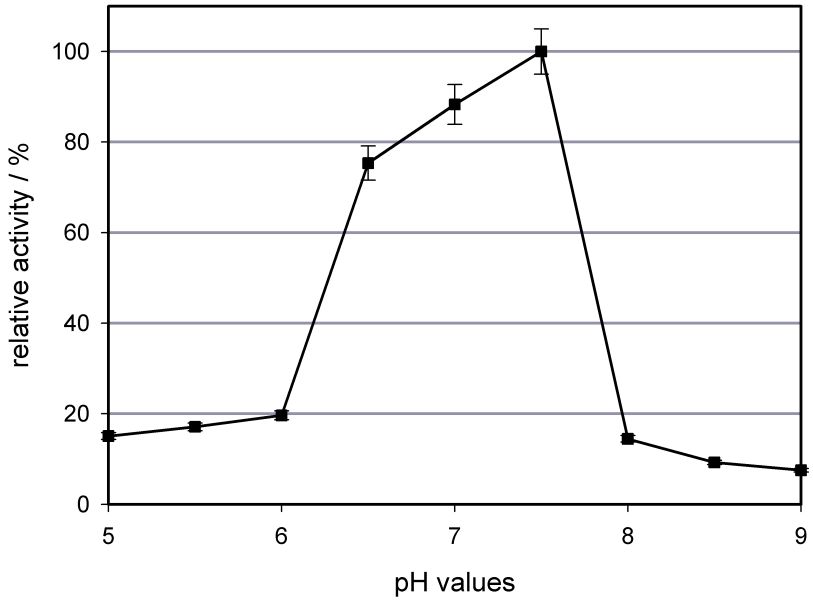


**Figure 4-23** pH optimum of the EstB1 esterase. The enzymes activity was measured towards methyl acetate at various temperatures at 35°C.

### 4.5.5. Influence of NaCl and KCl on the EstB1 Esterase

Since the EstB1 esterase was isolated from the marine micro-organism, the influence of NaCl and KCl salts on the enzyme activity was determined. The effect of 0.1-2.5 M NaCl and KCl on the EstB1 activity is shown on **Figure 4-24**. Additionally, the enzyme stability was determined in the presence of 1 M NaCl or KCl salts during 1 h incubation at RT. The EstB1 was found to be stable in up to 2.5 M NaCl and KCl salts concentration. It was shown that in the presence of 0.1-1.5 M NaCl and KCl the EstB1 activity was influenced in a similar way. Up 1.5 M salts concentration KCl had less influence on the EstB1 activity in comparison with NaCl. No influence on the EstB1 activity was measured in the presence up to 0.25 M of NaCl salt, whereby 0.1 M and 0.25 M KCl decreased the EstB1 activity to 98.4 % and 89.0 %, respectively. The further increasing of the salts concentration led to the slightly decreasing of the EstB1 activity. The measured activity of the EstB1 in the presence of 0.5 M NaCl and KCl was 93.6 % and 88.0 %, respectively. In the presence of 1 M NaCl and KCl the EstB1 activity was 89.2 % and 86.6 %, correspondingly. The incubation of the EstB1 esterase with 2 M NaCl and KCl resulted in 69.0 % and 79.9 % activity, correspondently. 46.3 % and 65.1 % of the EstB1 activity was measured in the presence of 2.5 M NaCl and KCl, respectively. No

influence of 1 M NaCl or KCl salts on the EstB1 activity was measured after 1 h incubation at RT.

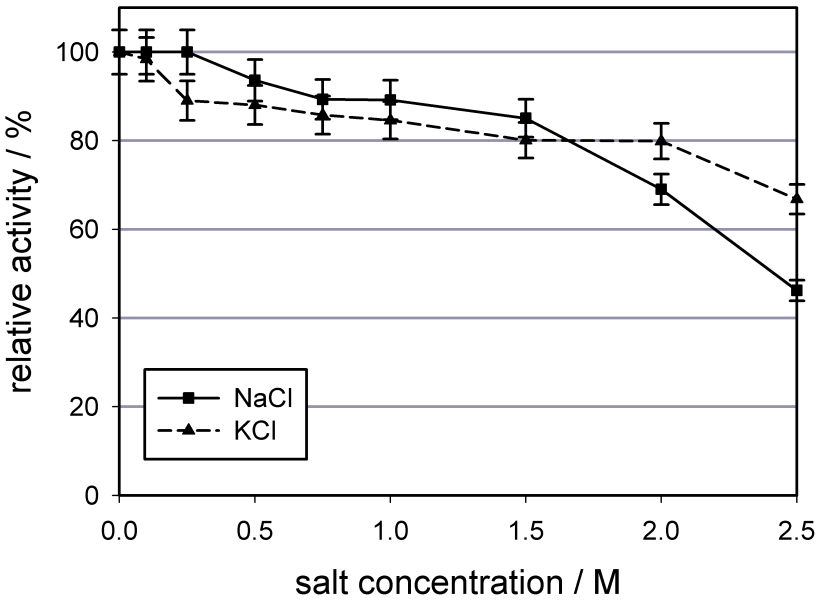


**Figure 4-24** Influence of NaCl and KCl on the EstB1 esterase. The enzyme activity was measured towards *p*NP acetate at 35°C and pH 7.5.

### 4.5.6. Influence of Metal Ions on the EstB1 Esterase

Since many esterases or lipases are activated or inhibited by metal ions, the influence of 1 - 100 mM $Zn^{2+}$, $Mg^{2+}$ and $Ca^{2+}$ ions on the EstB1 activity was determined. The effect of metal ions on the EstB1 esterase is represented on **Figure 4-25**. The EstB1 activity was strongly inhibited by $Zn^{2+}$ ions. The inhibition of the EstB1 was observed already with 1 mM $Zn^{2+}$ ions that consisted 25.9 % activity. 10 mM $Zn^{2+}$ ions decreased the EstB1 activity to 12.2 % and 50 mM $Zn^{2+}$ ions complete deactivated the EstB1 esterase. $Mg^{2+}$ and $Ca^{2+}$ ions have less influence on the EstB1 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. 50 mM $Mg^{2+}$ and $Ca^{2+}$ ions concentration demitted the EstB1 activity to 88.7 % and 51.2 %, correspondently. 78.8 % and 28.1 % of the EstB1 activity were measured with 100 mM $Mg^{2+}$ and $Ca^{2+}$ ions, respectively.

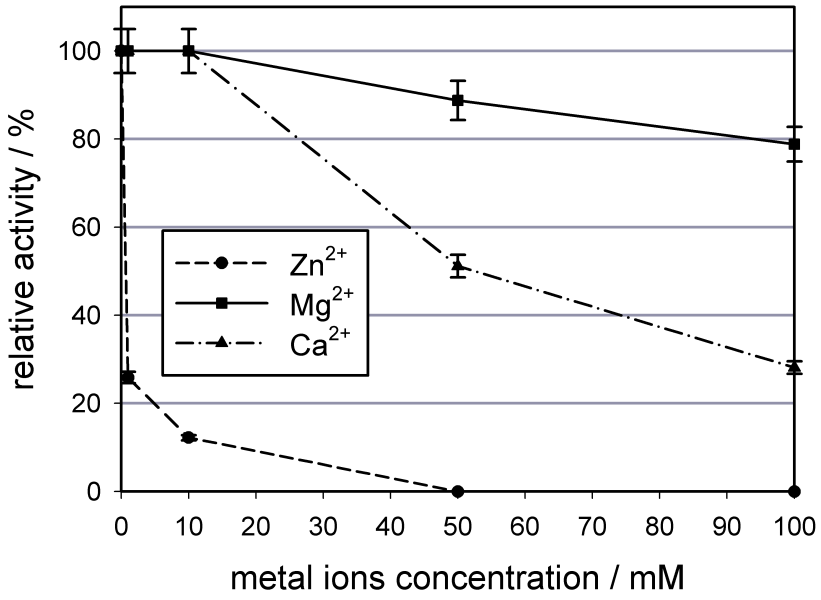


**Figure 4-25** Influence of $Zn^{2+}$, $Mg^{2+}$ and $Ca^{2+}$ ions on the EstB1 esterase. The enzyme activity was measured towards *p*NP acetate at 35°C and pH 7.5.

### 4.5.7. Influence of PMSF, EDTA, β-Mercaptoethanol and DTT on the EstB1 Esterase

Of the inhibitors analysed, PMSF was the most effective in the inhibition of the EstB1. 57.3 % of the EstB1 activity was measured in the presence of 1 mM PMSF. The full inactivation of the EstB1 esterase was observed after the addition of 5 mM PMSF. The addition of 1 - 5 mM EDTA, β-mercaptoethanol and DTT had no influence on the EstB1 activity.

### 4.5.8. Influence of Organic Solvents on the EstB1 Esterase

Because many biotechnological applications using esterases and lipases are performed in non-aqua solutions, the influence of different organic solvents such as DMSO, methanol, ethanol and 2-propanol on the EstB1 esterase was measured. The influence of organic solvents on the EstB1 activity is represented on **Figure 4-26**. Additionally, the enzyme stability in 10 % (v/v) DMSO was measured towards *p*NP acetate in 20 min intervals during 1 h incubation at RT. The best stability of the enzyme in organic solvents was measured in DMSO. In the presence of 20 % (v/v) DMSO the activity of the EstB1 was 70.6 %. The increasing of the DMSO concentration to 30 % (v/v) and 40 % (v/v) led to the decreasing of the EstB1 activity to

54.9 % and 27.6 % correspondingly. 50 % (v/v) DMSO lessened the activity of the EstB1 esterase to 7.3 %. One hour incubation of the EstB1 esterase in the presence of 10 % (v/v) DMSO had no influence on the protein activity.

Methanol was found to influence the EstB1 more then DMSO. 47.3 % of the EstB1 activity was measured in the presence of 10 % (v/v) methanol, whereby 30 % (v/v) methanol led to the complete deactivation of the EstB1 esterase.

Ethanol and 2-propanol strongly inhibited the EstB1 activity. The addition of 10 % (v/v) ethanol decreased the activity of the EstB1 esterase to 25.3 %. Only 2.3 % of the EstB1 activity remains in the presence of 20 % (v/v) ethanol. 14.9 % and 8.2 % of the enzyme activity were measured in 10 % (v/v) and 20 % (v/v) 2-propanol, correspondently.

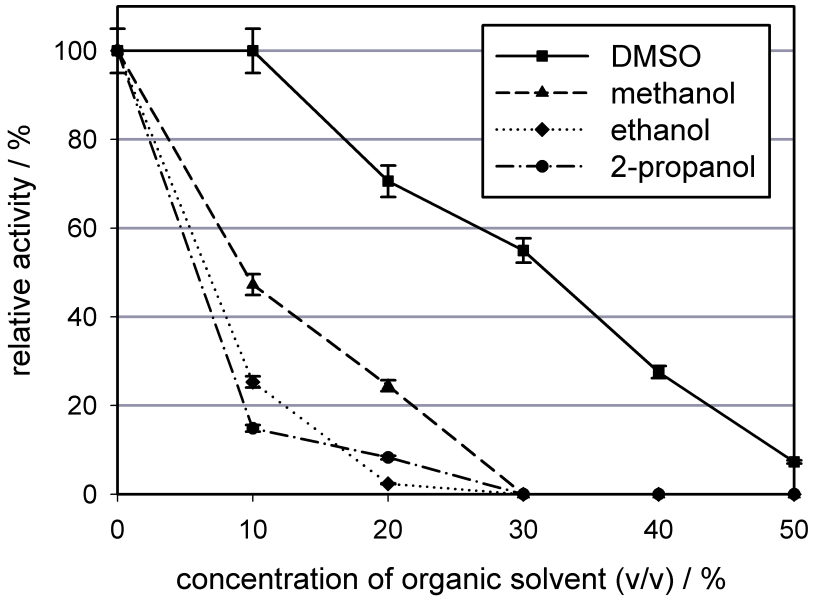


**Figure 4-26** Influence of DMSO, ethanol, methanol and 2-propanol on the EstB1 esterase. The enzyme activity was measured towards *p*NP acetate at 35°C and pH 7.5.

## 4.6. Cloning, Expression and Purification of the EstB2

### 4.6.1. Molecular Cloning of the Esterase Gene *est*B2

#### 4.6.1.1. Construction of Plasmids for Expression of the *est*B2 under the Control of *lac* Promoter

The *est*B2 was first expressed in *E. coli* under the control of *lac* promoter using a high copy number vector pUC19. The 744 bp *est*B2 gene encoding the EstB2 esterase was amplified by PCR from the pBS2 plasmid isolated from genomic DNA library of *Bacillus* sp. 01-855. A *Pst*I site was introduced at the ATG start codon with the aka-E7pUC19F primer. Additionally, the 3' - terminal end of the *est*B2 gene was fused to the His6-tag coding sequence by means of PCR with the aka-E7pUC19RhisTag primer. The primer includes the 3'- end of the *est*B2 gene, His6-tag coding sequence and a *Eco*RI restriction site. The PCR fragments were finally cleaved with *Pst*I and *Eco*RI and inserted into the vector pUC19, which was cut with the same enzymes to give pUC-*est*B2. The pUC19-*est*B2 was verified by DNA sequencing for possible mutations and frameshifts during cloning procedures.

#### 4.6.1.2. Construction of Plasmids for Expression of the *est*B2 under the Control of T7 Promoter

For the expression of the *est*B2 under the control of T7 promoter, it was cloned into a medium copy number vector pET-22b(+). The pET-22b(+) vector carries the N-terminal *pelB* signal sequence for potential periplasmatic localisation and the C-terminal $His_6$-tag sequence, which can be used for easy detection of the fusion proteins on Western blots and IMAC purification using TALON resin. To optimise expression of the target gene three different plasmids encoding the *est*B2 were constructed in pET-22b(+) vector (**Table 4-5**).

**Table 4-5** Three different type of constructs encoding the *est*B2 in pET-22b(+) vector were used in this work.

| **Name** | **Presence of *pelB* leader sequence** | **Presence of $His_6$-tag coding sequence** |
|---|---|---|
| pET-*est*B2-*pelB*His | + | + |
| pET-*est*B2-*pelB* | + | - |
| pET-*est*B2-His | - | + |

The *est*B2 encoding in the pET-*est*B2-*pelB*His berries the N-terminal *pelB* leader sequence and the C-terminal sequence of six histidine codons. The *est*B2 gene was first amplified by PCR from the pBS2 found in genomic DNA library of *Bacillus* sp. 01-855 with the AKA-eEcoRI_7F and AKA-LEcoRI7_R primers. A *Bam*HI site was introduced at the 5'- end and a *Not*I at the 3'- end of the gene. Thereby the 3'- end of the *est*B2 gene was fused to the $His_6$-tag coding sequence of the vector. The prepared by this means PCR fragments were finally digested with *Bam*HI and *Not*I and inserted into the pET-22(b+) vector which was cut with the same enzymes.

The pET-*est*B2-*pelB* was used to investigate the influence of the N-terminal *pelB* sequence on the inclusion bodies formation. The *est*B2 encoding in the pET-*est*B2-*pelB* berries the N-terminal *pelB* leader sequence that allows potential periplasmatic localisation of the recombinant EstB2. To generate the pET-*est*B2-*pelB*, the *est*B2 gene was PCR amplified from the pBS2 isolated from genomic DNA library of *Bacillus* sp. 01-855 using the AKA-eEcoRI_7F and AKA-LEcoRI7_R primers. A *Bam*HI site was introduced at the ATG start codon and a *Not*I site just after stop codon. The PCR fragments were digested with *Bam*HI and *Not*I and inserted into the pET-22(b+) vector, which was previously cut with the same enzymes.

The pET-*est*B2-His was used to investigate the influence of the C-terminal sequence of six histidine codons on the inclusion bodies formation. In comparison with the pET-*est*B2-*pelB*His and pET-*est*B2-*pelB*, the *est*B2 gene encoding in the pET-*est*B2-His plasmid lacks the 5' - terminal *pelB* leader sequence and carries the 3' - terminal sequence of six histidine codons. The *est*B2 was PCR-amplified from the pBS2 found in genomic DNA library of *Bacillus* sp. 01-855 using the AKA-E7bluntpET-22b(+)1 and AKA-E7RHisTag primers. The AKA-E7bluntpET-22b(+)1 primer includes the 5'- end of the *est*B2 gene, whereby the AKA-

E7RHisTag primer introduces a *Not*I restriction site at the 3'- end of the gene. To discriminate the periplasmatic translocation of the heterologous protein EstB2, the pET-22b(+) vector was digested with *Nde*I nuclease followed by fill-in of recessed 3'-termini with DNA Polymerase I Large Fragment (Klenow Fragment). After the treatment with Klenow Fragment the vector was digested with *Not*I to delete the *pelB* leader sequence and finally ligated with the PCR fragments, which were digested with *Not*I.
All plasmids used for expression of the *est*B2 were verified by DNA sequencing for possible frameshifts and mutations.

### 4.6.2. Expression of the *est*B2 in *Escherichia coli* in Shake Flask Cultures

#### 4.6.2.1. Expression of the *est*B2 in *E. coli* DH5α under the Control of *lac* Promoter

The small scale expression of the *est*B2 under the control of *lac* promoter was performed in *E. coli* DH5α in shake flask cultures for 0.1 - 1 l. *E. coli* DH5α transformed with the pUC-*est*B2 were grown until the early exponential phase. The esterase production was induced with IPTG and the incubation was continued at 37°C for 4 h. The cells were collected by centrifugation, disrupted by the combination of lisozyme treatment and sonication and the expression level of the EstB2 was analysed by SDS-PAGE and activity assay with *p*NP acetate as a substrate.
The SDS-PAGE analysis of *E. coli* DH5α transformed with the pUC-*est*B2 indicates a 32 kDa protein, which corresponds to the theoretically calculated mass of the EstB2 (**Figure 4-27**). No protein bands at the respective positions were visible in a negative control containing the cell lysate of *E. coli* DH5α bearing the empty vector pUC19 (*lane 2*). It was demonstrated that the induction time does not play a critical role in the recombinant protein production. The amount of the EstB2 increased after 1 h induction and was constant during the rest of the induction time. The esterase activity obtained from a 500-ml of *E. coli* DH5α(pUC-*est*B2) culture after 4 h induction was 0.2 U/mg total protein as determined spectrometric by *p*NP acetate assay. The amount of the EstB2 was not sufficient for further biochemical characterisation.

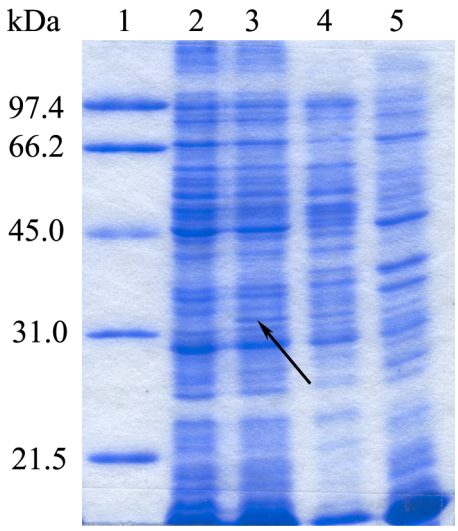


**Figure 4-27** Expression of the esterase EstB2 from *Bacillus* sp. 01-855 in *E. coli* DH5α. Protein samples were separated in 12.5 % SDS-polyacrylamide gels and stained with Coomassie blue (*lanes 1 - 5*). Cell lysate of *E. coli* DH5α bearing the empty vector (*lane 2*); and of *E. coli* DH5α transformed with pET-*est*B2 after 1, 2 and 4 h induction (*lanes 3* - 5, respectively). *Lane 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.

### 4.6.2.2. Expression of the *est*B2 in *E. coli* BL21(DE3) under the Control of T7 Promoter

The pET system from Novagen is one of the most powerful system yet developed for the cloning and expression of recombinant proteins in *E. coli*. The desired product can comprise more than 50 % of the total cell protein a few hours after induction. To optimise the expression level of the EstB2 esterase, the *est*B2 gene was cloned into the pET-22b(+) vector and expressed under the control of T7 promoter. The expression of the *est*B2 under the control of T7 promoter was performed in *E. coli* BL21(DE3) cells in shake flask cultures for 0.1 - 2 l. Different temperatures (from 25°C to 37°C), concentrations of IPTG (between 0.1 mM and 1mM) and induction times (from 1 h to 5 h) were tested to optimise the production of the EstB2 in *E. coli* by batch cultivation. The induced *E. coli* BL21(DE3) cells carrying the expression pET-*est*B2-*pelB*His plasmid were harvested and the cell extracts were prepared to

investigate the amount and accumulation of the EstB2 in different cell compartments by SDS-PAGE and zymogram. To confirm the expression of the EstB2 the Ni-NTA-AP conjugate assay on western blots was also done. The densitometric evaluation of the SDS-polyacrylamide gels stained with Coomassie Dye was used for the rough estimation of the protein content. The activity measurements of the expressed esterase were performed by *p*NP acetate assay.

The SDS-PAGE expression analysis (**Figure 4-28**) and the Ni-NTA-AP conjugate assay (**Figure 4-29**) of *E. coli* BL21(DE3)(pET-*est*B2-*pelB*His) cell extracts revealed two proteins called 29BS and 32BS at the levels about 29 kDa and 32 kDa, respectively. The appearance of two proteins on western blot (**Figure 4-29**) indicates that the 29BS and 32BS are in frame to $His_6$-tag coding sequence and to each other. As it is shown on zymogram (**Figure 4-28,** *lanes 8 - 10*) the recombinant EstB2 was expressed in an active form although only one band corresponding to the 32BS was active. It was demonstrated that the amount of the EstB2 increased rapidly after 1 h induction and was constant until the cell harvesting. The expression level of the EstB2 esterase consisted about 30 % of the total amount of the cell protein as estimated densitometric. Although the EstB2 overexpression, 0.1 U/mg total protein of maximum esterase activity was recovered from a 500-ml *E. coli* BL21(DE3)(pET-*est*B2-*pelB*His) culture after 4 h induction. Although expression the gene under various conditions (temperature, IPTG amount and induction time), the biggest part of the EstB2 (about 98 - 99 % as estimated densitometric) was consistently produced in an insoluble form as inclusion bodies.

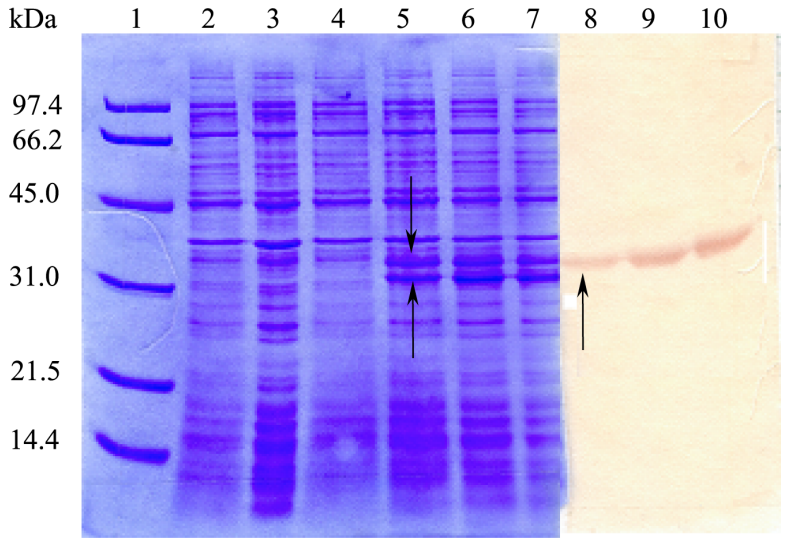


**Figure 4-28** Overexpression of the esterase EstB2 from *Bacillus* sp. 01-855 in *E. coli* BL21(DE3). Protein samples were separated in 12.5 % SDS-polyacrylamide gels and stained with Coomassie blue (*lanes 1 - 7*) and α-naphtyl acetate and Fast Red (zymogram) (*lanes 8-*

*10*). Cell lysate of *E. coli* BL21(DE3) bearing the empty vector (*lane 2*); and of *E. coli* BL21(DE3) transformed with pET-*est*B2-*pelB*His without induction (*lane 3*); after 1, 2, 3 and 4 h induction (*lanes 4 - 7*, respectively). *Lane 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.

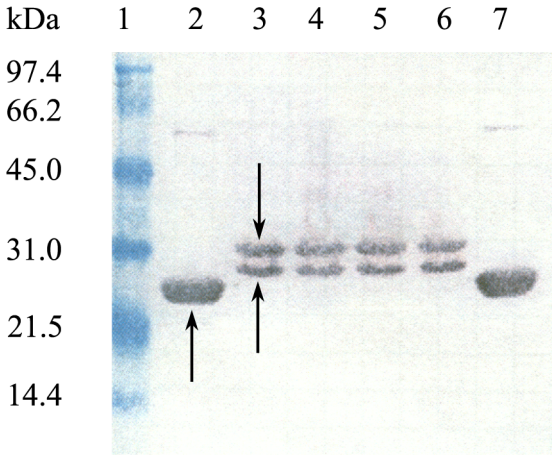


**Figure 4-29** Ni-NTA-AP conjugate assay on western blot with the His-tagged EstB2 was performed to confirm the expression of the 29BS and 32BS. Purified from *E. coli* DH5α His-tagged eGFP was used as a positive control (*lanes 2* and *7*). Cell extracts of *E. coli* BL21(DE3) transformed with pET-*est*B2-*pelB*His after 4 h induction (*lanes 3 - 6*). *Lane 1* – prestained 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 eGFP and EstB2 esterase.

### 4.6.3. N-terminal Protein Sequencing of the 29BS and 32BS

N-terminal amino acid sequencing of the purified 29BS and 32BS was performed to determine the nature of these proteins. The samples containing the proteins of interest were separated by SDS-PAGE, electrobloted onto PVDF membrane and sequenced. The N-terminal sequencing yielded MDIGINSDPMKVVAPKPFTFE for the 29BS and MKYLLPT AAAGLLLLAAQPAMAMDIGINSDPMKV for the 32BS, respectively. Based on the N-terminal sequence results two EstB2 protein species with and without *pelB* leader sequence were detected. The EstB2 with *pelB* leader sequence was first expressed to the cytoplasm (32BS) and then it was exported to the periplasm (29BS) where the leader sequence was

deleted by the signal peptidase. It was shown that the EstB2 esterase displays domains for the periplasmatic expression.

### 4.6.4. Inclusion Bodies

#### 4.6.4.1. Influence of $His_6$-tag and *pelB* Leader Sequences on Formation of Inclusion Bodies

To investigate the influence of the $His_6$-tag and *pelB* leader sequences on the inclusion bodies formation two plasmids pET-*est*B2-*pelB* and pET-*est*B2-His coding for the *est*B2 were used (**Chapter 4.6.1.2.**; **Table 4-5**). The *est*B2 encoding in the pET-*est*B2-*pelB* plasmid contains the 5' - terminal *pelB* sequence and no $His_6$-tag coding sequence. Thereby the *pelB* leader sequence, which is responsible for the periplasmatic expression, was eliminated in the pET-*est*B2-His during cloning procedures. Additionally, the *est*B2 encoding in the pET-*est*B2-His contains the 3' - terminal sequence of six histidine codons. The expression of the *est*B2 encoding in the pET-*est*B2-*pelB* and pET-*est*B2-His was performed in *E. coli* BL21(DE3) cells in shake flask cultures for 0.1 - 2 l. Different temperatures (25°C - 37°C), concentrations of IPTG (0.1-1mM) and induction times (1 - 5 h) were tested. The induced cells were separated from the media by centrifugation and disrupted by sonication. Soluble and insoluble fractions were prepared by centrifugation of the cell extracts and evaluated by SDS-PAGE and activity measurements with *p*NP acetate assay. The protein content in the soluble and insoluble fractions applied on the SDS-polyacrylamide gels was estimated densitometric after staining with Comassie Dye.

The SDS-PAGE expression analysis of *E. coli* BL31(DE3)(pET-*est*B2-*pelB*) revealed that the expression level of the EstB2, accumulation of the protein in the cells and the amount of the soluble, active protein were identical with the results compared with expressed *est*B2 in the pET-*est*B2-*pelB*His under similar induction conditions.

The SDS-PAGE expression analysis of *E. coli* BL31(DE3)(pET-*est*B2-His) indicates the presence of a protein of about 32 kDa (**Figure 4-30**). No bands at the respective positions were detected in a negative control containing the cell lysate of *E. coli* BL31(DE3) bearing the empty pET-22(b+) vector (*lane 2*). The expression level of the EstB2 consisted about 28 % of the total amount of cell protein as estimated densitometric. The biggest part of the EstB2 (about 98 % as estimated densitometric) was accumulated in inclusion bodies. The maximal esterase activity recovered from a 500-ml of *E. coli* BL31(DE3)(pET-*est*B2-His) culture after

4 h induction was 0.05 U/mg total protein. The esterase activity recovered did not correlated with the varying of temperature, concentration of IPTG and induction time as measured by *p*NP acetate assay.

It was demonstrated that in the case of the *est*B2 expressed in *E. coli* BL31(DE3) under the control of T7 promoter neither the $His_6$-tag nor the *pelB* leader sequences influenced the folding of the protein and thus the formation of inclusion bodies. In both cases the biggest part of the protein (about 98 %) was accumulated in the insoluble fraction. It was also shown that the EstB2 expressed to the cytoplasm can be found in an active, soluble form as measured by *p*NP acetate assay. The activity measurements revealed that the expression of the *est*B2 encoding in the pET-*est*B2-His led to a 3 times lower extent of product compared with the *est*B2 expressed in the pET-*est*B2-*pelB* and pET-*est*B2-*pelB*His. Based on the catalytic activity of the EstB2, it was assumed that the oxidizing environment of the periplasmatic space of *E. coli* facilitates the formation of functional EstB2 protein.

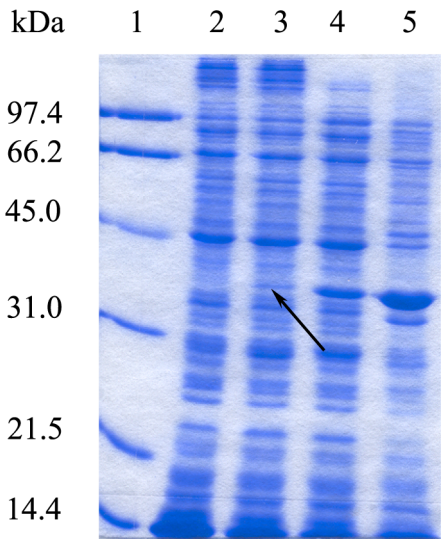


**Figure 4-30** SDS-PAGE analysis of the influence of N-terminal *pelB* sequence on the formation of inclusion bodies. Overexpression of the esterase EstB2 from *Bacillus* sp. 01-855 in *E. coli* BL21(DE3). Protein samples were separated in 12.5 % SDS-polyacrylamide gels and stained with Coomassie blue (*lanes 1 - 5*). Cell lysate of *E. coli* BL21(DE3) bearing the empty vector (*lane 2*); and of *E. coli* BL21(DE3) transformed with pET-*est*B2-His after 4 h induction (*lane 5*). Soluble (*lane 3*) and insoluble (*lane 4*) fractions resulting centrifugation of the cell extracts of *E. coli* BL21(DE3)(pET-*est*B2-His). *Lane 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.

### 4.6.4.2. Influence of Detergents on the Solubility of the EstB2

It was assumed that the EstB2 esterase contains hydrophobic or membrane-associated domains which may be partition into insoluble fraction, but may not actually be present in inclusion bodies. Different nonionic, anionic and zwitterionic detergents such as Triton X-100, cholic acid or CHAPS were tested to solubilize cell walls and membrane components, thereby realising cellular proteins without denaturation. 0.1 - 10 % (m/v) amounts of the detergents required for the solubilization and delipidation of the target protein were tested to overcome the problem of critical micelle concentration. The *E. coli* BL21(DE3)(pET-*est*B2-*pelB*His) cells containing the expressed EstB2 esterase were harvested by centrifugation, washed in 50 mM sodium phosphate buffer (pH 7.5), resuspended in the same buffer with or without the corresponding detergent and then disrupted by sonication. Soluble and insoluble fractions were separated by centrifugation and analysed by SDS-PAGE. The influence of the nonionic detergent Triton X-100 on the solubility of the EstB2 esterase is shown on **Figure 4-31**. The comparative analysis of the *lane 2* to *3*, *4* to *5* and *6* to *7* revealed no influence of Triton X-100 on the solubility of the EstB2. In general, the addition of nonionic, ionic or zwitterionic detergents was not conducive to the conversion of the insoluble fraction to the soluble one. It was demonstrated that the EstB2 esterase associated neither with lipids nor with cell membranes.

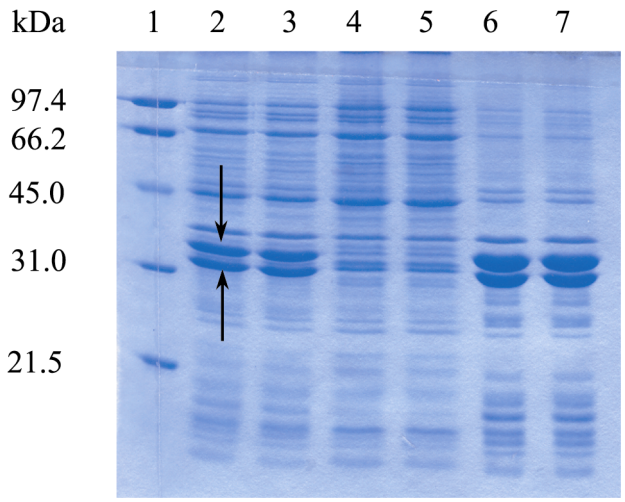


**Figure 4-31** Influence of the nonionic detergent Triton X-100 on the solubility of the EstB2. Protein samples were separated in 12.5 % SDS-polyacrylamide gels and stained with Coomassie blue (*lanes 1 - 7*). Cell lysate of *E. coli* BL21(DE3) transformed with pET-*est*B2-*pelB*His without (*lane 2*) and with (*lane 3*) Triton-X100 treatment; soluble fraction without

(*lane 4*) and with (*lane 5*) Triton-X100 treatment; insoluble fraction without (*lane 6*) and with (*lane 7*) Triton-X100 treatment. *Lane 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.

#### 4.6.4.3. Solubilization of Inclusion Bodies with Urea

Different urea concentrations were tested to denaturate the EstB2 inclusion bodies. Induced *E. coli* BL21(DE3)(pET-*est*B2-His) containing the overexpressed EstB2 were harvested by centrifugation and disrupted by sonication. Inclusion bodies were separated by centrifugation of the cell extracts, solubilized with 0.5 - 8 M urea and analysed by SDS-PAGE (**Figure 4-32**). The full solubilization was observed with 5 M urea (*lane 7*) that indicates the formation of inclusion bodies and not inactive protein agglomerates due to overexpression of the *est*B2 under the control of T7 promoter.

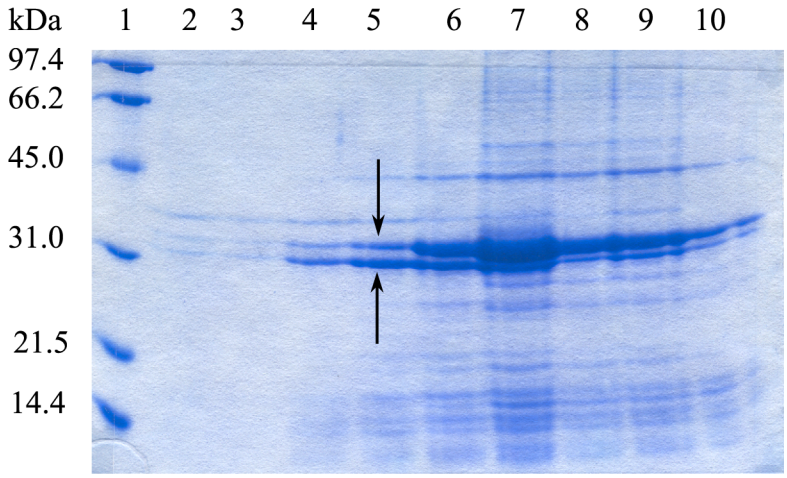


**Figure 4-32** SDS-PAGE analysis of influence of different concentrations of urea on the solubilization of inclusion bodies. Insoluble fraction resulting centrifugation of the cell extracts of *E. coli* BL21(DE3)(pET-*est*B2-His) treated with 0.5 M, 1 M, 2 M, 3 M, 4 M, 5 M, 6 M, 7 M and 8 M urea (*lanes 2 - 10*, respectively). *Lane 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 from *Bacillus* sp. 01-855.

### 4.6.5. One–step Purification of the EstB2 Esterase by Means of Immobilized Metal Affinity Chromatography (IMAC)

A total wet cell weight of 2.5 g was obtained from a 500-ml *E. coli* BL21(DE3)(pET-*est*B2-*pelB*His) culture after centrifugation. The EstB2 esterase expressed in *E. coli* under the control of T7 promoter was accumulated in a soluble and insoluble form (inclusion bodies). The soluble, active EstB2 was purified in a single step by IMAC under native conditions. Since it is preferable to use native conditions for extraction if only 5-10 % of the target protein is soluble, a batch purification using TALON resin was performed. The resin was incubated with soluble fraction of *E. coli* containing the overexpressed EstB2 for 2 - 3 h at 4°C with slightly agitation. The wash and elution steps were performed according to the instructions of the manufacturers. The enzyme purification was monitored by SDS-PAGE. The activity of the purified EstB2 was evaluated by *p*NP acetate assay.

Two protein bands 29BS and 32BS were visible after purification of the EstB2 on SDS-polyacrylamide gels that is an evidence of the soluble, active protein accumulated not only in the periplasm but also in the cytoplasm (**Figure 4-33**). The intensity of the protein bands corresponding to the 29BS compared with those of the 32BS shows that the amount of the soluble EstB2 accumulated in the periplasm is higher then in the cytoplasm. The specific activity of the purified EstB2 determined by *p*NP acetate assay was 30.6 U/mg which corresponds to the concentration factor 105. Based on the catalytic activity of the purified esterase, the expression level of the soluble enzyme in the recombinant *E. coli* BL21(DE3)(pET-BSL2-*pelB*His) was estimated to be 1 % of total soluble protein. That is the production level of the active esterase reached 0.1 mg/l of cultural broth. As the amount of the EstB2 gained after IMAC purification was not sufficient for biochemical characterisation, refolding of the EstB2 was performed to obtained soluble, active protein.

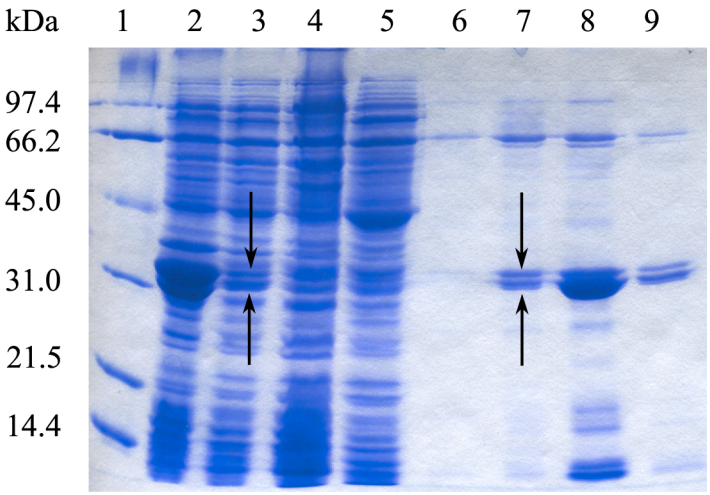


**Figure 4-33** Purification of the esterase EstB2 from *Bacillus* sp. 01-855 expressed in *E. coli* BL21(DE3). Protein samples were separated in 12.5 % SDS-polyacrylamide gels and stained with Coomassie blue. Cell lysate of *E. coli* BL21(DE3) transformed with pET-*est*B2-*pelB*His after 4 h induction (*lane 2*) and soluble fraction applied to purification (*lane 3*). Purification fractions: the first flow (*lane 4*), the first wash step (*lane 5*), the last wash step (*lane 6*); elution steps (*lanes 6 - 9*) containing the purified EstB2 (*lanes 7 - 9*). *Lane 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.

### 4.6.6. Refolding of the EstB2

The *est*B2 esterase gene from *Bacillus* sp. 01-855 was expressed in *E. coli* under the control of T7 promoter. Although expression of the *est*B2 under various conditions, the protein was consistently accumulated in an insoluble form as inclusion bodies. Therefore, the EstB2 was denaturated in 5 M urea, and refolding conditions were examined. It was found that the fractional dialysis of the EstB2 in potassium phosphate buffer containing DTT and EDTA led to the efficient refolding of the enzyme. The homogeneity of the protein was analysed by SDS-PAGE and the esterase activity was assayed spectrometric towards *p*NP acetate.

As it can be seen on the SDS-PAGE (**Figure 4-34**) two protein species 29BS and 32BS were visible after refolding at the level of approximately 29 kDa and 32 kDa, respectively. The appearance of two proteins 29BS and 32BS indicates that inclusion bodies were formed not only in the cytoplasm but also in the periplasm of *E. coli*. Thereby the higher intensity of the band corresponding to the 32BS protein compared with those of the 29BS shows that the biggest part of inclusion bodies was accumulated in the cytoplasm. The specific activity of the

refolded EstB2 was 30.6 U/mg as determined towards *p*NP acetate. That corresponds to the concentration factor 20. 40 mg of the active EstB2 could be obtained after refolding from 1 l of cultural broth. The refolded EstB2 was used for further biochemical characterisation.

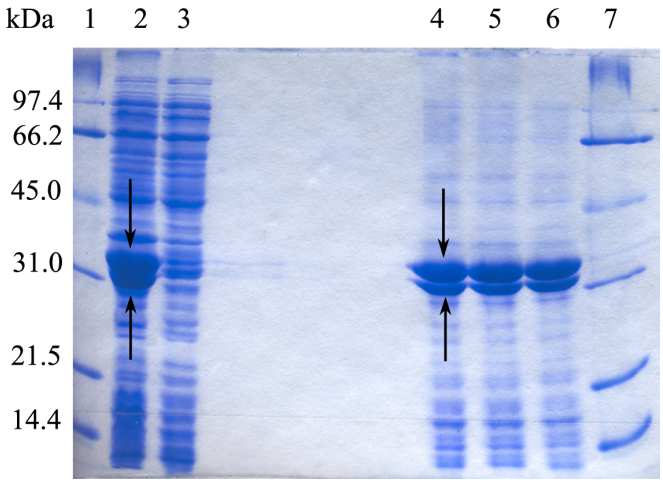


**Figure 4-34** Refolding of the esterase EstB2 from *Bacillus* sp. 01-855 expressed in *E. coli* BL21(DE3). Protein samples were separated in 12.5 % SDS-polyacrylamide gels and stained with Coomassie blue (*lanes 1 - 7*). Insoluble fraction (*lane 2*) and soluble fraction (*lane 3*) of *E. coli* BL21(DE3) transformed with pET-*est*B2-*pelB*His after 4 h induction; fraction containing the EstB2 after refolding (*lane 4 - 6*). *Lanes 1*, *7* - 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.

## 4.7. Biochemical Characterisation of the EstB2 Esterase

### 4.7.1. Substrate Specificity of the EstB2 Esterase and Determination of Kinetic Data

The substrate specificity of the purified from *E. coli* EstB2 was determined by use of *p*NP esters and methyl esters of straight-chain fatty acids ranging in chain length from $C_2$ (acetate) to $C_{14}$ (myristate). The substrate specificity of the purified EstB2 is represented in **Table 4-6** and **Table 4-7**. The $K_m$ and $V_{max}$ values were determined from the hydrolysis of *p*NP acetate and *p*NP butyrate and calculated from Lineweaver-Burk plots using a least-squares best fit of the Michaelis-Menten equation (**Table 4-8**). The highest activity of the EstB2 protein in the case of *p*NP esters was measured towards *p*NP acetate (30.6 U/mg), *p*NP butyrate (52.1 U/mg) and *p*NP caproate (25.0 U/mg). The maximum specific activity of the EstB2 towards methyl esters was observed with methyl acetate (48.1 U/mg), methyl butyrate (43.5 U/mg) and methyl caproate (22.0 U/mg). The activity of the EstB2 decreased with the acyl chain length, whereby the EstB2 preferentially hydrolyses *p*NP acetate and methyl butyrate. *p*NP and methyl esters with acyl chain length bigger then $C_8$ were poor substrates for the EstB2. The EstB2 activity towards *p*NP laurate and methyl laurate consisted 3.1 U/mg and 1.7 U/mg, respectively. No preferences on *p*NP esters and methyl esters were detected for the EstB2. Since the comparative amino acid sequence analysis of the EstB2 revealed that the enzyme is related to the proteins of dienlactonase family, lactonase activity towards γ-butyrolactone and δ-hexanolactone was also measured. The lactonase activity of the EstB2 purified from *E. coli* was 1.8 U/mg and 2.3 U/mg towards γ-butyrolactone and δ-hexanolactone, correspondently. These results indicate that the EstB2 is a putative carboxylesterase.

**Table 4-6** Substrate specificity of the purified EstB2 towards *p*NP esters.

| Substrate | **Specific Activity (U/mg)** |
|---|---|
| *p*NP acetate | 30.6 |
| *p*NP butyrate | 52.1 |
| *p*NP caproate | 25.0 |
| *p*NP caprylate | 10.1 |
| *p*NP caprate | 9.1 |
| *p*NP laurate | 3.1 |

**Table 4-7** Substrate specificity of the purified EstB2 towards methyl esters.

| **Substrate** | **Specific Activity (U/mg)** |
|---|---|
| methyl acetate | 48.1 |
| methyl butyrate | 34.5 |
| methyl caproate | 22.0 |
| methyl caprylate | 14.7 |
| methyl caprate | 5.9 |
| methyl laurate | 1.7 |
| methyl myristate | 0.8 |

**Table 4-8** Kinetic data determined from hydrolysis of *p*NP acetate and *p*NP butyrate with the purified EstB2.

| **Enzyme/substrate** | $K_m$ **(M)** | $V_{max}$ **(M min$^{-1}$)** |
|---|---|---|
| *p*NP acetate | $1.1 \cdot 10^{-3}$ | $1.5 \cdot 10^{-4}$ |
| *p*NP butyrate | $1.5 \cdot 10^{-4}$ | $3.5 \cdot 10^{-5}$ |

### 4.7.2. Storage Stability of the EstB2 Esterase

The storage stability of the EstB2 esterase was determined in four different buffers: potassium- and sodium phosphate buffers, Tris·HCl and PBS and at different temperatures: –20°C, 4°C, 37°C. The storage stability of the EstB2 esterase is shown on **Figure 4-35**, **Figure 4-36** and **Figure 4-37**.

Among tested buffers the best storage stability of the EstB2 was observed in potassium- and sodium phosphate buffers at –20°C and 4°C. The EstB2 esterase displayed less stability in potassium- and sodium phosphate buffers stored at –20°C in comparison with those of at 4°C. Moreover, potassium- and sodium phosphate buffers influenced the EstB2 enzyme in a similar way during the storage at –20°C. The complete inactivation of the EstB2 esterase was observed in potassium- and sodium phosphate buffers after 7 days of storage at –20°C. 58.6 % and 7.7 % of the EstB2 activity was detected in potassium phosphate buffer at –20°C after 3 and 5 days of storage, correspondently. In the case of the storage of the EstB2 in sodium phosphate buffer at –20°C 50.3 % and 5.8 % activity was measured after 3 and 5 days, respectively. The activity of the EstB2 esterase stored in potassium- and sodium phosphate buffers at 4°C decreased slowly after 2 days incubation. The EstB2 activity measured in potassium phosphate buffer was 95.7 % and 75.8 % after 3 and 4 days of storage at 4°C, correspondently. 54.2 % and 48.2 % of the activity were detected for the EstB2 esterase stored in potassium phosphate buffer at 4°C after 5 and 7 days of incubation, respectively. 85.2 %, 50.2 % and 21.3% of the EstB2 activity was observed in sodium phosphate buffer at 4°C after 3, 5 and 7 days of storage, correspondently. The storage of the EstB2 in potassium- and sodium phosphate buffers at 37°C led to the complete deactivation of the protein already after 5 days of storage. The EstB2 activity measured at 37°C in potassium- and sodium phosphate buffers consisted 81.2 % and 75.2 %, correspondently after 1 day of storage. 10.1 % and 7.9 % of the EstB2 activity was observed in potassium- and sodium phosphate buffers after 4 days of storage at 37°C, respectively.

Tris·HCl buffer and PBS were found to be not appropriate for the long-term storage of the EstB2 esterase. The best storage stability of the EstB2 in Tris·HCl buffer and PBS were observed at –20°C and 4°C. 73.2 % and 15.3 % of the EstB2 activity remained after 1 and 3 days of storage in Tris·HCl buffer at 4°C, correspondently. 93.2 % and 16.8 % activity was measured for the EstB2 esterase in PBS at 4°C after 1 and 3 days of storage, respectively. The inactivation of the protein was observed after 4 days of storage in Tris·HCl buffer and PBS at 4°C. The rapid deactivation of the EstB2 esterase was measured in Tris·HCl buffer and PBS

during the storage at –20°C. So, the EstB2 activity was found to be 4.4 % and 5.7 % in Tris·HCl buffer and PBS after 3 days storage at –20°C, correspondently. The storage of the EstB2 esterase in Tris·HCl buffer and PBS at 37°C led to the rapid deactivation of the protein already after 1 day of storage. The EstB2 activity consisted 3.1 % and 4.8 % after 2 days of storage in Tris·HCl buffer and PBS at 37°C, correspondently.

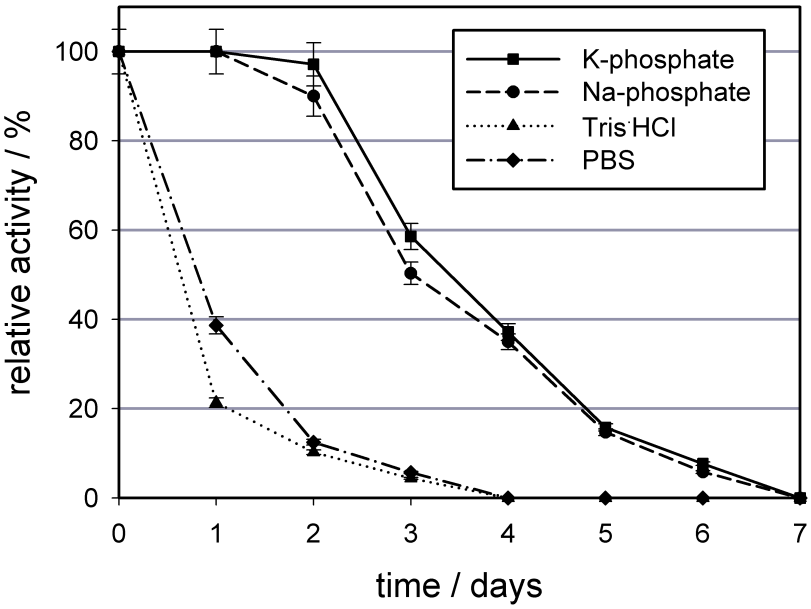


**Figure 4-35** Storage stability of the EstB2 in potassium- and sodium phosphate buffers, Tris·HCl buffer and PBS at –20°C. The enzymes activity was measured towards *p*NP acetate at 35°C and pH 7.5.

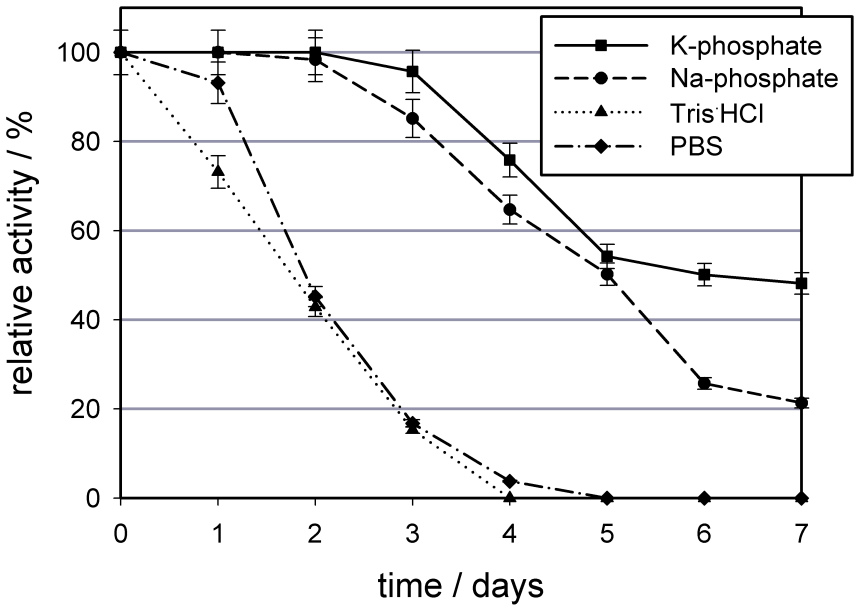


**Figure 4-36** Storage stability of the EstB2 in potassium- and sodium phosphate buffers, Tris·HCl buffer and PBS at 4°C. The enzymes activity was measured towards *p*NP acetate at 35°C and pH 7.5.

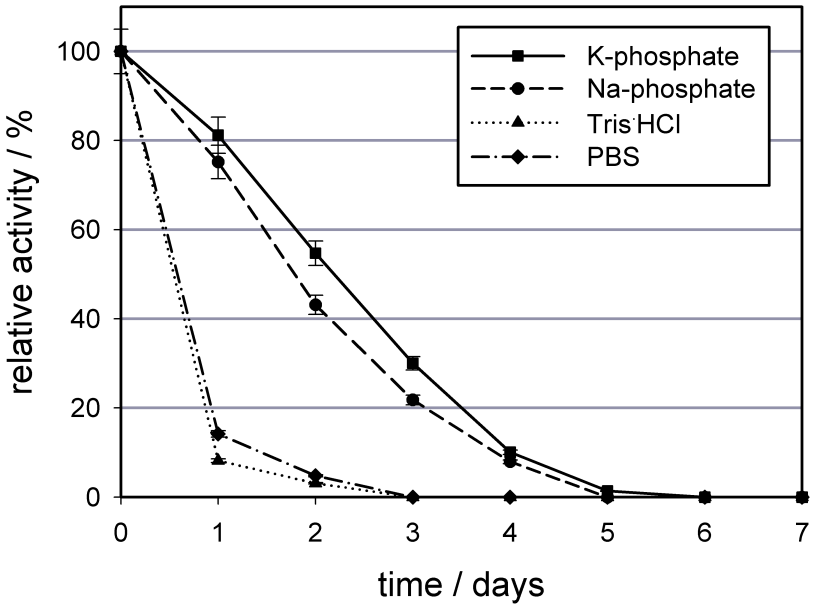


**Figure 4-37** Storage stability of the EstB2 in potassium- and sodium phosphate buffers, Tris·HCl buffer and PBS at 37°C. The enzymes activity was measured towards *p*NP acetate at 35°C and pH 7.5.

### 4.7.3. Temperature Optimum of the EstB2 Esterase

The temperature optimum of the EstB2 esterase is represented on **Figure 4-38**. 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 the steady increasing in activity up to 35°C. 32.9 %, 64.4 % and 97.9 % of the EstB2 activity was measured at 10°C, 20°C and 30°C, respectively. The further increasing of the temperature led to the rapid inactivation of the enzyme. The activity of the EstB2 esterase measured at 40°C and 50°C was 34.7 % and 26.5 %, correspondently.

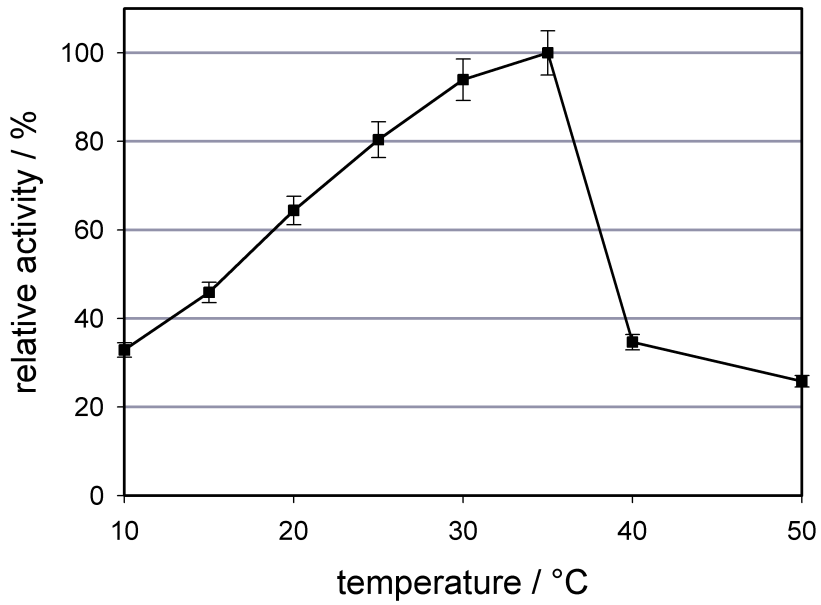


**Figure 4-38** Temperature optimum of the EstB2 esterase. The enzymes activity was measured towards methyl acetate at various temperatures at pH 7.5.

### 4.7.4. pH Optimum of the EstB2 Esterase

The effect of different pH values on the EstB2 activity is shown on **Figure 4-39**. The EstB2 esterase has the pH optimum in a range between pH 6.5 and pH 8.0. The lowest activity of the EstB2 esterase was measured at pH 5.0 and pH 5.5 that consisted 0.4 % and 2.6 %, correspondently. The activity of the protein increased continually with the increasing of pH values and reached its maximum at pH 7.0. 27.1 % and 54.2 % of the EstB2 activity was

measured at pH 6.0 and pH 6.5, respectively. Up pH 7.0 the exponent decreasing of the EstB2 activity was observed. So, at pH 8.0, pH 8.5 and pH 9.0 41.7 %, 25.4 % and 13.0 % of the EstB2 activity were measured.

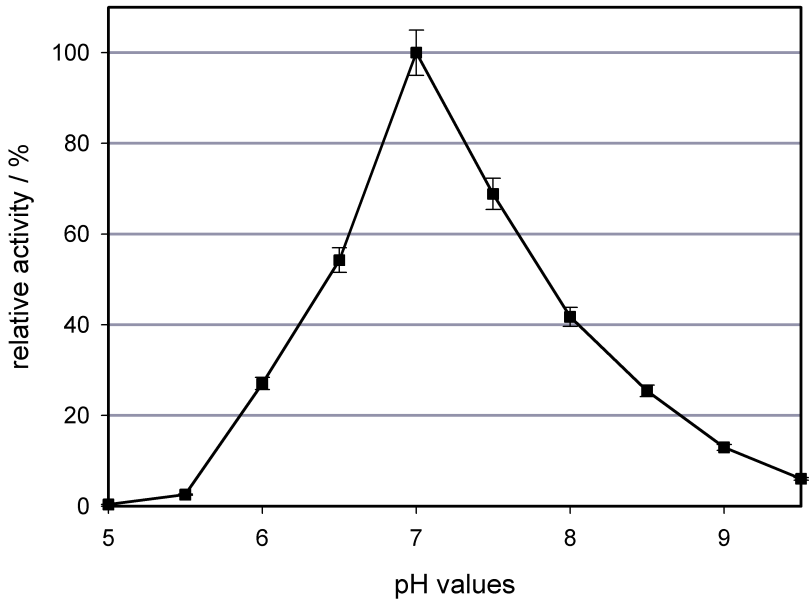


**Figure 4-39** pH optimum of the EstB2 esterase. The enzymes activity was measured towards methyl acetate at various temperatures at 35°C.

### 4.7.5. Influence of NaCl and KCl on the EstB2 Esterase

The influence of 0.1 - 2.0 M NaCl and KCl concentrations on the activity of the EstB2 esterase was tested because of marine origin of *Bacillus* sp. 01-855. The effect of different NaCl and KCl concentrations on the EstB2 is represented on **Figure 4-40**. The stability of the EstB2 in the presence of 1 M NaCl and KCl salts during 1 h incubation at RT was additionally tested. Up to 0.5 M NaCl and KCl influenced the activity of the EstB2 in a similar way. With the further increase of the salt concentrations KCl had less influence on the EstB2 activity in comparison with NaCl. The EstB2 activity measured in the presence of 0.25 M NaCl or KCl consisted 93.3 % and 90.1 %, correspondently. In the presence of 1 M NaCl or KCl salts the activity of the EstB2 esterase lowed to 52.1 % and 68.2 %, respectively. 2 M NaCl and KCl concentrations decreased the activity of the EstB2 esterase to 28.9 % and 53.4 %, correspondingly. The incubation of the EstB2 esterase in the presence of 1 M NaCl or KCl at RT for 1 h had no influence on the protein activity.

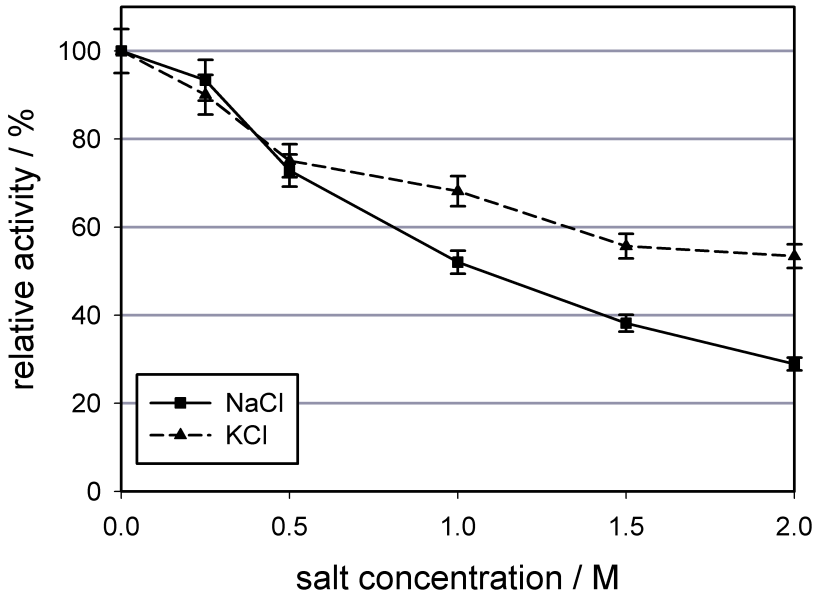


**Figure 4-40** Influence of NaCl and KCl on the EstB2 activity. The enzyme activity was measured towards *p*NP acetate at 35°C and pH.

### 4.7.6. Influence of Metal Ions on the EstB2 Esterase

The influence of 1 - 100 mM $Zn^{2+}$, $Mg^{2+}$ and $Ca^{2+}$ ions on the EstB2 esterase was measured, because many esterases and lipases are activated or inhibited by metal ions. The effect of different metal ions on the EstB1 activity is represented on **Figure 4-41**. The strongest inhibition effect on the EstB2 activity was observed in the presence of $Zn^{2+}$ ions. 11.1 % of the EstB2 activity was measured with 1 mM of $Zn^{2+}$ ions, whereby 10 mM concentration of $Zn^{2+}$ ions fully inactivated the protein. In comparison with $Zn^{2+}$ ions, $Mg^{2+}$ and $Ca^{2+}$ ions had less influence on the EstB2 activity. Moreover, $Mg^{2+}$ and $Ca^{2+}$ ions influenced the EstB2 activity in a similar way. 50 mM and 100 mM of $Mg^{2+}$ ions concentration resulted in the decreasing of the EstB2 activity to 30.8 % and 11. 6 %, respectively. The increasing of $Ca^{2+}$ ions concentration to 10 mM, 50 mM and 100 mM decreased the EstB2 activity to 57.2 %, 34.5 % and 8.5 %, correspondingly.

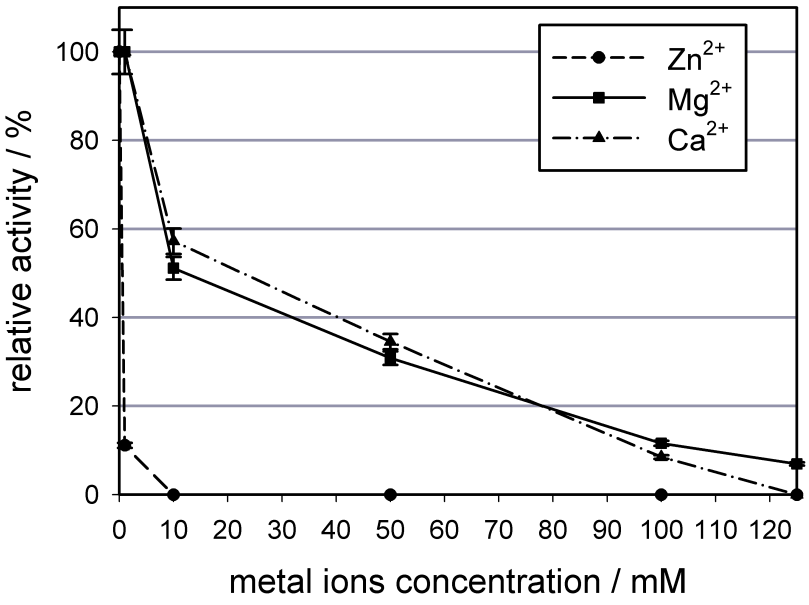


**Figure 4-41** Influence of $Zn^{2+}$, $Mg^{2+}$ and $Ca^{2+}$ions on the EstB2 esterase. The enzyme activity was measured towards *p*NP acetate at 35°C and pH 7.5.

### 4.7.7. Influence of PMSF, EDTA, β-Mercaptoethanol and DTT on the EstB2 Esterase

The influence of 1 - 10 mM PMSF, EDTA, β-mercaptoethanol and DTT on the EstB2 activity was tested. PMSF was found to be the most effective inhibitor among the tested chemicals. 1 mM PMSF led to the complete deactivation of the EstB2 esterase. EDTA, β-mercaptoethanol or DTT at the concentrations of 1 - 10 mM had no influence on the EstB2 activity.

### 4.7.8. Influence of Organic Solvents on the EstB1 Esterase

The effect of DMSO, methanol, ethanol and 2-propanol on the EstB2 activity was tested, because many biotechnological processes using esterases and lipases are performed in the presence of diverse organic solvents. The influence of organic solvents on the EstB2 activity is represented on **Figure 4-42**. Additionally, the enzyme stability in the presence of 10 % (v/v) DMSO was measured in 20 min intervals during 1 h incubation at RT. The EstB2 esterase showed the best stability in DMSO followed by ethanol, methanol and 2-propanol. The EstB2 esterase was remarkable stable in up to 50 % (v/v) concentrations of DMSO. 75.3 %, 62.2 % and 53.7 % of the EstB2 activity were measured in the presence of 20 %

(v/v), 30 % (v/v) and 40 % (v/v) DMSO, correspondently. 50 % (v/v) of DMSO decreased the EstB2 activity to 49.4 %. The incubation of the EstB2 esterase for 1 h in the presence of 10 % (v/v) DMSO had no influence on the protein activity. In the presence of 10 % (v/v) and 20 % (v/v) methanol the activity of the EstB2 was diminished to 72.8 % and 24.2 % respectively. 30 % (v/v) methanol fully inactivated the EstB2 esterase. 54.2 % and 33.5 % of the EstB2 activity was observed in the presence of 10 % (v/v) and 20 % (v/v) ethanol, correspondingly. 30 % (v/v) ethanol decreased the activity of the EstB2 to 8.9 %. The strongest inhibition effect on the EstB2 esterase showed 2-propanol. 20 % (v/v) 2-propanol lowered the EstB2 activity to 2.4 %.

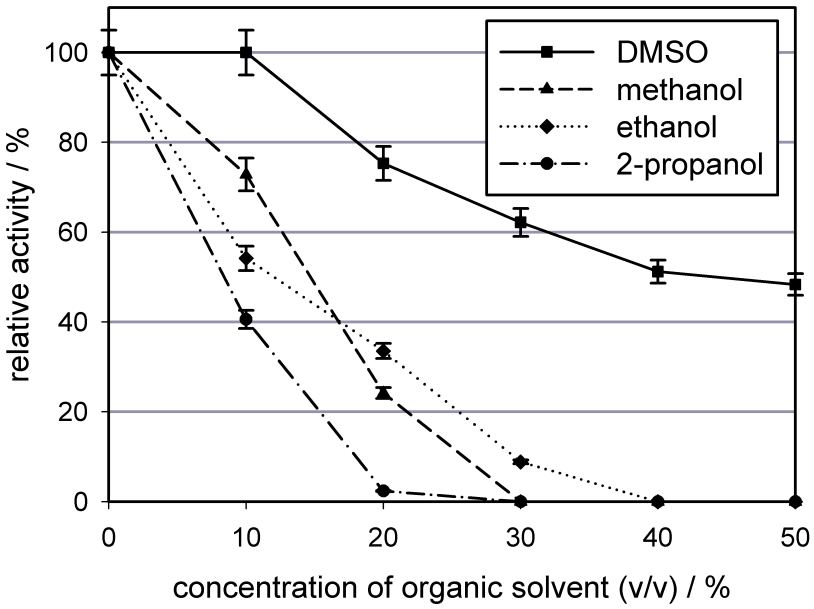


**Figure 4-42** Influence of DMSO, ethanol, methanol and 2-propanol on the EstB2 esterase. The enzyme activity was measured towards *p*NP acetate at 35°C and pH 7.5.

## 4.8. Cloning and Expression of the AmdB1

### 4.8.1. Molecular Cloning of the Putative Amidase Gene *amd*B1

The gene coding for the putative amidase AmdB1 from *Bacillus* sp. 01-855 was expressed under the control of *lac* promoter using a high copy number vector pUC18. The 1398 bp *amd*B1 gene was PCR-amplified from the plasmid pBS3 isolated from genomic DNA library of *Bacillus* sp. 01-855 using the AKA-PUC18F and AKA-pUC18R primers. By this means a *Eco*RI site was introduced at the ATG start codon. In order to fuse the $His_6$-tag coding sequence to the 3'-terminal end of the amidase gene, it was amplified with the AKA-pUC18R primer including the 3'-end of the *amd*B1, the six histidine codons and a *Bam*HI restriction site just after the translation stop codon. The prepared by this means PCR fragments were digested with *Eco*RI and *Bam*HI and inserted into the IPTG-inducible pUC18 vector, which was cut with the same enzymes, resulting pUC-*amd*B1 plasmid. The results of the cloning procedure were verified by DNA sequencing.

### 4.8.2. Expression of the *amd*B1 in *Escherichia coli* DH5α Strain in Shake Flask Cultures

The gene coding for the AmdB1 putative amidase was expressed in shake flask cultures for 0.05 - 0.25 l. *E. coli* DH5α were transformed with the pUC-*amd*B1 plasmid and induced with IPTG. After induction, the cultivation was continued for 4 h at 30°C. The harvested cells were disrupted by sonication, yielding 1.3 U/mg of the active enzyme in the supernatant as measured spectrometric towards *p*NP acetate. The expression which was confirmed by the SDS-PAGE analysis (**Figure 4-43**) and zymogram (**Figure 4-44**), indicates the presence of an active 50 kDa protein (calculated: 50.43 kDa). The amount of the expressed protein increased continually after 1 h induction and reached its maximum after 4 h induction (**Figure 4-43**; *lane 5*). No bands at the respective positions were detected in a control bearing the empty pUC18 vector (**Figure 4-43**; *lane 6*). Soluble recombinant proteins have usually attained their native state and consequently their functionality. Therefore, the amount of the functional AmdB1 was estimated by comparing bands of the protein after SDS-PAGE in soluble and insoluble fractions. Soluble and insoluble fractions of *E. coli* DH5α(pUC-*amd*B1) after 4 h induction were prepared by centrifugation of the cell lysate and separated by SDS-PAGE (**Figure 4-45**). The recombinant AmdB1 protein was accumulated in the cytoplasm in a

soluble form. No formation of inclusion bodies was detected during expression of the *amd*B1 in *E. coli* DH5α under the control of *lac* promoter. The densitometric evaluation of SDS-polyacrilamide gels stained with Coomassie Dye was done for a rough estimation of the protein content after 4 h induction. The band corresponding to the AmdB1 was found to contribute about 5 - 8 % of the total cell protein.

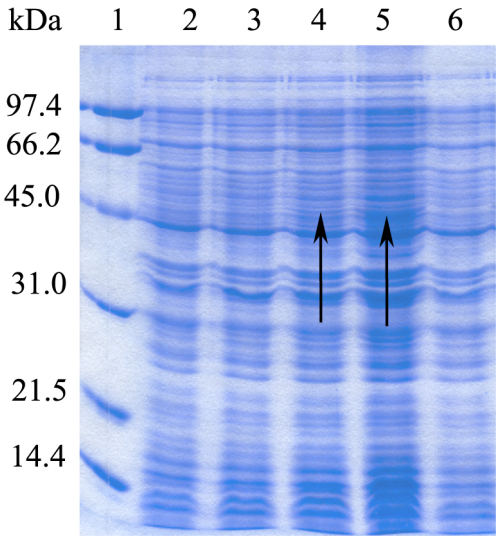


**Figure 4-43** Expression of the putative amidase AmdB1 from *Bacillus* sp. 01-855 in *E. coli* DH5α. Protein samples were separated in 12.5 % SDS-polyacrylamide gels and stained with Coomassie blue. Cell lysate of *E. coli* DH5α transformed with pUC-*amd*B1 after 1, 2, 3 and 4 h induction (*lanes 2 - 5*, respectively); and *E. coli* DH5α bearing the empty vector after 4 h induction (*lane 6*); *Lane 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 AmdB1 putative amidase.

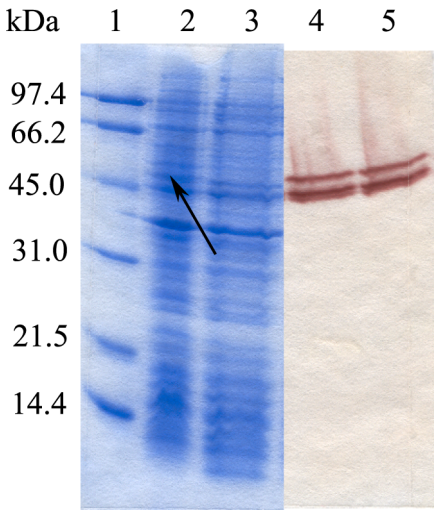


**Figure 4-44** Expression of the putative amidase AmdB1 from *Bacillus* sp. 01-855 in *E. coli* DH5α. Protein samples were separated in 12.5 % SDS-polyacrylamide gels and stained with Coomassie blue (*lanes 1 - 3*) and α-naphtyl acetate and Fast Red (zymogram) (*lanes 4 - 5*). Cell lysate of *E. coli* DH5α transformed with pET-*amd*B1 after 4 h induction (*lanes 2-5*). *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 AmdB1 putative amidase.

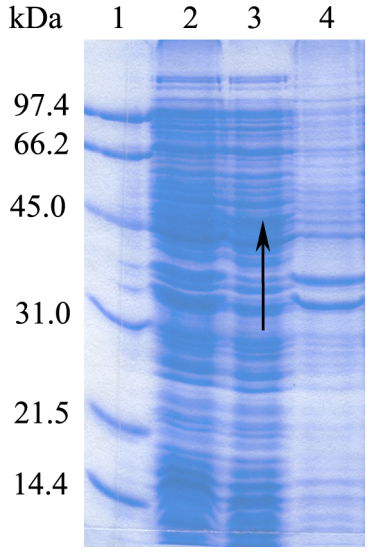


**Figure 4-45** Analyse of soluble and insoluble fractions of *E. coli* DH5α expressing the putative amidase AmdB1 from *Bacillus* sp. 01-855. Soluble and insoluble protein samples were separated in 12.5 % SDS-polyacrylamide gels and stained with Coomassie blue (*lanes 2-4*). Cell lysate of *E. coli* BL21(DE3) transformed with pET-*amd*B1 after 4 h induction (*lane*

*2*) and soluble and insoluble fractions (*lane 3* and *4* respectively). *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 AmdB1 putative amidase.

# 5. Discussion

## 5.1. Screening for Novel Lipolytic Enzymes from Marine *Bacillus* sp. 01-855 associated with Marine Sponge *Aplysina aerophoba*

The aim of this study was identification and characterisation of novel lipolytic enzymes of marine bacterial origin, which can have potential interest for biotechnological applications. In this work a new *Bacillus* sp. 01-855 which is associated with the marine sponge *Aplysina aerophoba* was established as a new source of novel lipolytic enzymes with unique properties and little overlap of amino acids sequences to those of terrestrial micro-organisms origin. According to Amann *et. al.,* 1995 [111] about 99 % of the world micro-organisms could be not covered by traditional cultivation techniques. Though of the intensive studies focused on the terrestrial micro-organisms it is still very little known about the micro-organisms inhabit marine environments. Therefore the special interest of marine biotechnology is paid on the isolation and studies of novel marine micro-organisms which are suspected to be producers of biologically active compounds. The approach of isolation of marine micro-organisms is not only conductive in expanding of knowledge on bacterial physiology but also develops the marine resources in ways that circumvent environmental and supply problems.

## 5.2. Identification of Novel *Bacillus* sp. 01-855 of Marine Origin

The novel micro-organisms represent not only a source of novel products, they serve as models for the understanding of structure and function that will facilitate the genetic manipulations of organisms and advance their ability to engineer novel enzymes. The isolation of micro-organisms from novel or extreme environments holds tremendous promise in biotechnology. The special interest of marine biotechnology today is paid on the sponges because they are especially rich in different bacteria and cyanobacteria in the mesohyl. In this study the Mediterranean sponge *Aplysina aerophoba* was collected in March and September 2000 by the *Oberservatoire Oceanologique de Banyuls-sur-mer* in France from the depth of 5.3-13.7 m [87]. *Bacillus* sp. was isolated from mesohyl of the homogenised sponge on seawater based media under aerobic conditions. The attempts to cultivate *Bacillus* sp. on different from marine media were unsuccessful. Moreover the novel *Bacillus* sp. belongs to

the seldom micro-organisms isolated from *Aplysina aerophoba* with ability of independent from the host organism growth under laboratory conditions. Among bacteria found in marine macro-organisms *Bacillus* species are very common [73]. According to Mohapatra *et. al*., 2002 [112] among 57 bacterial isolates from 8 marine sedentary organisms, 6 sponges, 1 soft coral and 1 alga 20 % of bacteria of the genus *Bacillus* were predominated. It was also shown that the Mediterranean sponges *Aplysina aerophoba* associated with bacteria of the *Bacillus* genus [79,113]. Because the bacterial communities of sponges from Aplysinidae family were stable even during incubation in aquariums supplied with antibiotics for a couple of weeks [67], *Bacillus* sp. can be considered as a natural symbiont of the marine sponge *Aplysina aerophoba*. According to the definition of Faulkner [70] this bacterial isolate can be recognised as a true marine micro-organism, which derived from marine environment.

The identification of the strain has been done in DSMZ. The partial 16S rDNA sequence analysis, Gram, KOH and AP basic staining tests, determination of fatty acids composition and physiological tests revealed that this micro-organism belongs to the new *Bacillus* species (RNA group VI in *Bacillus*). According to the DSMZ nomenclature this novel *Bacillus* species was named *Bacillus* sp. 01-855. *Bacillus* sp. 01-855 displays the typical for *Bacillus* genera fatty acids composition. In a phylogenetic tree based on the comparative 16S rDNA sequence analysis most of the bacilli of marine origin are clustered with *B. subtilis*, *B. pumilus*, *B. firmus*, *B. lentus*, *B. licheniformis* and *B. horti* [75,112,114]. The partial 16S rDNA sequence analysis of *Bacillus* sp. 01-855 showed the 95.1 % similarity to seldom in marine environment *Bacillus pseudofirmus*. Because *Bacillus* sp. 01-855 is physiologically uncharacterised, it can be considered as a potential producer of useful enzymes and biologically active compounds.

## 5.3. Screening of Genomic DNA Library from *Bacillus* sp. 01-855 for Novel Lipolytic Enzymes

The screening of micro-organisms for the production of useful biologically active products and enzymes is an important aspect of biotechnology. Although the progresses in genetics methods, expanded knowledge in microbial physiology and in instrumentation, screening programmes are still primarily based on classical techniques including the screening of DNA libraries. In this work identification and isolation of novel lipolytic enzymes was performed by screening of genomic DNA library of *Bacillus* sp. 01-855 in *E. coli* by plate assay towards

tributyrin. This screening method allows easy identification of positive clones and do not require the knowledge of sequence information prior to cloning. So, 20 % of the lipolytic clones could be detected by plate assay screening of soil metagenome libraries [41].
A number of colonies which should be tested in order to find a 3.5 kb DNA fragment in a *Bacillus* genom was calculated according to Clarke and Carbon, 1992 equation [94]. One *Nde*I and two *EcoR*I lipolytic active clones were found in the genomic DNA library pool of *Bacillus* sp. 01-855. The lipolytic active clones were isolated from the DNA library pool consisting of 93.000 of positive colonies (approximately 20.000 colonies in each library), that corresponds to the theoretically calculated probability of 1.

## 5.4. DNA Sequencing and Sequence Analysis

Two novel esterases and a novel putative amidase were isolated from the genomic DNA library of *Bacillus* sp. 01-855. The nucleotide sequencing of the first esterase from *Bacillus* sp. 01-855, designated *est*B1, revealed a 945 bp ORF which could encode a putative protein called EstB1 of 35.30 kDa. A 744 bp ORF called *est*B2 encoded the second putative esterase designated EstB2 with a deduced molecular weight of 28.32 kDa. A putative amidase AmdB1 consisting of 50.43 kDa was encoded in a 1398 bp ORF called *amd*B1. The presence of putative transcription regions detected upstream of the *est*B1, *est*B2 and *amd*B1, suggests that the genes are transcribed from original *Bacillus* promoters. It was assumed that the *est*B2 is transcribed monocistronically, because of lack of other ORFs immediately upstream or downstream of the *est*B2.
The protein sequence alignment of the EstB1 revealed low similarity to the 3-oxadipate enol-lactonase from *Bacillus cereus* ATCC 14579, α/β hydrolase fold from *Bacillus anthracis* A2012 and the putative chloroperoxidase from *Clostridium acetobutylicum*. The presence of the proteins like 3-oxoadipate enol-lactonase in marine macro- and micro-organisms have not been described. On the other hand, haloperoxidases are widespread in marine environment and have been found in a variety of seaweeds [115]. The presence of especially high level of lipid-extractable halometabolites in some species of sponges and red algae was also described [116]. The involvement of peroxidases in the halogenation reactions began with the discovery of thyroid peroxidase role in the thyroxine biosynthesis. It is presently known that many peroxidases are capable of oxidizing halide ions, expect fluoride, to an electrophilic species which then reacts with an electron-rich acceptor thereby forming carbon-halogen bonds [117].

This fact in combination with the ever increasing number of halogenated organic compounds isolated from marine algae [118] suggests that peroxidase catalysed halogenation may be important in the biogenesis of the halogenated compounds in marine environment. The physiological roles of chloroperoxidases are still unknown. It was suggested that *in vivo* the haloperoxidases actually have esterase or other hydrolytic activities with unknown substrates [119].

The protein alignment of the EstB2 showed high similarity to the carboxylesterase from *Bacillus cereus* ATCC 14579 and to the dienelactone hydrolase from *Bacillus anthracis* A2012. According to Ivanova *et al*., 2003 [120] and Read *et al*., 2002 [121] the carboxylesterase from *Bacillus cereus* ATCC 14579 and the protein from dienelactone hydrolase family from *Bacillus anthracis* A2012 were predicted based on the genome project and physiological roles of these enzymes are still unknown. On the amino acid level, the AmsB1 displays low similarity to the known enzymes. The AmsB1 displays the highest amino acid sequence similarity to the amidases from *Mesorhizobium loti* and *Pseudomonas putida* KT2440. The amidase from *Mesorhizobium loti* was detected based on the comparative DNA sequence analysis of the *Mesorhizobium loti* strain R7A symbiosys island, being a chromosomally integrated element which transfers to nonsymbiotic mesorhizobia in the environment, covering them *Lotus* symbionts [122]. The shared DNA regions containing the gene of putative amidase is likely to be required for nitrogen fixation. According to Nelson *et. al.,* 2002 [123] the amidase from *Pseudomonas putida* KT2440 was predicted based on the genome sequencing and is not yet characterised.

The EstB1 and EstB2 share protein similarity to the enzymes which possess α/β hydrolase fold. The members of this protein superfamily catalyse a wide variety of hydrolytic reactions and are suggested to have diverged from a common ancestor sharing similar overall topology, a conserved arrangement of the catalytic triad residues and oxyanion hole even in the absence of sequence conservation [19]. The activity of esterases and lipases is dependent upon a charge relay system involving an active-site Ser-Asp/Glu-His triad [124]. The active-site serine of esterases and lipases is commonly conserved in a Gly-X-Ser-X-Gly motif in which X is a variable residue [124]. 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. The Asp/Glu and His members of the catalytic triad of the EstB1 and EstB2 are still not identfied. Phenylmethylsulfonyl fluoride (PMSF) is a potential inhibitor of serine hydrolases. The hydroxyl group of serine nucleopholocally attacks the sulfonyl group of PMSF which leads to the irreversible sulfonylation of the active serine. The

inhibition of the EstB1 and EstB2 by 5 mM and 1 mM PMSF correspondently, imply that Ser residues are important for their activity.

The low protein similarity of the AmdB1 to the published protein sequences and not complete biochemical characterisation of this protein, does not allow to identify the enzym class the AmdB1 belongs. Further biochemical characterisation of the AmdB1 including the determination of its substrate spectra is necessary.

The insufficient information about *Bacillus* sp. 01-855 limits the elucidation of the physiological functions of the EstB1, EstB2 and AmdB1 in the cells.

## 5.5. Expression Analysis of the *est*B1, *est*B2 and *amd*B1

The high copy number vectors pUC18/pUC19 with a strong *lac* promoter and a medium copy vector pET22-b(+) with a strong bacteriophage promoter T7 were used for the cloning and expression of the *est*B1, *est*B2 and *amd*B1. The *est*B1 and *est*B2 genes from *Bacillus* sp. 01-855 were efficiently expressed in *E. coli* under the control of T7 promoter. The expression level of the EstB1 esterase consisted about 20 % of total cell protein. The *amd*B1 gene was expressed in *E. coli* under the control of *lac* promoter at the substantially high level (5 - 8 % of total cell protein). The EstB1 esterase and the putative AmdB1 amidase were expressed in an active soluble form, whereby only a small part of the recombinant EstB2 was found to be soluble. Approximately 98 - 99 % of the EstB2 esterase expressed in *E. coli* BL21(DE3) cells under the control of T7 promoter was accumulated in a form of inclusion bodies. The optimisation of expression conditions by varying of temperature or amount of IPTG during induction had no influence on the formation of soluble, active protein. The expression of the EstB2 in *E. coli* was performed to the periplasm and to the cytoplasm. The periplasm of *E. coli* in contrast to the cytoplasm has an oxidising environment and contains enzymes that catalyse the formation and isomerisation of disulfide bonds [125,126,127]. It was shown that the expression of the EstB2 to the periplasm influenced the formation of the soluble, active protein. So, expression of the EstB2 to the periplasm and cytoplasm led to a 3 times greater extent of product compared with those of expressed only to the cytoplasm.

In general the mechanism behind inclusion bodies formation is not well understood. Early hypotheses included solubility limitations, protein size, type of promoter, and improper disulfide bonds formation. But the comparison of the literature data shows no simple relationship between these factors and the formation of inclusion bodies. It has been

suggested that inclusion bodies are formed from intermediates of the folding pathway [128]. Chaperons and proteases are major components of the cellular protein quality control system devoted to prevent the occurrence of misfolded polypeptides [129]. The folding of the lipase from *Pseudomonas glumae*, for example, expressed in *P. aeruginosa*, *B. subtilis* and *E. coli* needs a lipase-specific foldase LipB for obtaining of the active protein. Traub *et. al*., 2001 [130] demonstrated that for the correct folding of the lipases from *Pseudomonas* sp. KWI 56 and *Chromobacterium viscosum* in *E. coli* three specific chaperones are required.

The addition of non-ionic (Triton X-100) and zwitterionic detergents (CHAPS) to *E. coli* cells containing the expressed EstB2 was performed during lysis procedure. It was shown that the EstB2 does not contain hydrophobic or membrane-associated domains which may be partition the EstB2 into insoluble fraction due to association with lipid or cell membranes. It was demonstrated that the EstB2 esterase forms insoluble complexes neither with lipids nor with cell membranes.

## 5.6. Purification of the EstB1 and EstB2 Esterases

Different methods for the purification of *Bacillus* lipases and esterases expressed both extra- and intracellular were described in a great number of articles and reviews. The EstB1 and EstB2 could be efficiently purified from *E. coli* to homogeneity in a single step by IMAC. In the case of the EstB1 esterase 50 mg of the protein could be obtained from one litre of cultural broth. The 36-fold concentrated enzyme was used for further biochemical characterisation. It was demonstrated that the EstB2 esterase expressed in *E. coli* under the control of T7 promoter is accumulated in soluble and insoluble fractions. Soluble fraction of the EstB2 esterase purified by means of IMAC was concentrated in 105 fold and consisted 0.05 mg of the active protein from one litre of cultural broth.

For the conversion of inactive inclusion bodies into the soluble active protein the refolding method for the EstB2 esterase was established. A variety of methods concerning the refolging of insoluble proteins were published [131,132,133,134,135,136]. Most protocols describe the isolation of inclusion bodies by centrifugation with following solubilization under denaturing conditions. The purified proteins are finally dialysed or diluted into a non-denaturing buffer where refolding occurs. Depending on the target protein, expression conditions and intended applications, proteins solubilized from the washed inclusion bodies may be more then 90 % homogeneous and may not require further purification. 40 mg of the nearly homogenous

EstB2 esterase could be obtained from one litre of cultural broth. The active EstB2 esterase obtained after refolding was used for further biochemical characterisation.

## 5.7. Comparative Biochemical Characterisation of the EstB1 and EstB2 Esterases

The determination of substrate spectra of a newly identified enzyme is often a tedious and time-consuming task. The EstB1 and EstB2 purified from *E. coli* showed a substrate specificity on a set of typical for esterases and lipases substrates including *p*NP esters and methyl esters. The highest activity of the EstB1 and EstB2 was observed towards *p*NP esters and methyl esters of C2 - C6 that is the evidence of esterase nature of the both enzymes. The EstB1 showed the higher hydrolysis rate for methyl esters rather then *p*NP esters, whereas no preferences in hydrolysis of *p*NP and methyl esters were detected for the EstB2. For the EstB1 increase of carboxylic acid chain length from C2 to C4 resulted in decreasing of $K_m$, whereas declines in respective $V_{max}$ values did not occur. The increasing of carboxylic acid chain length from C2 to C4 resulted in the decreasing of $K_m$ for the EstB2 and in the decreasing in corresponding $V_{max}$ values. These trends suggest that the EstB1 and EstB2 esterases showed the preference for the *p*NP esters with acyl chain length of C4 and the ability of the EstB1 to stabilise the transition state of C2 and C4 substrates is similar. Because the isolated EstB1 and EstB2 showed similarity to the proteins of chloroperoxidase and lactonases families respectively, haloperoxidase and lactonase activity was additionally tested. No haloperoxidase and lactonase activity was detected for the EstB1. It was demonstrated that the EstB2 displays very low activity towards tested lactones in comparison with *p*NP esters and methyl esters.

It was shown that the EstB1 esterase displays the better storage stability in comparison with the EstB2. The optimum storage conditions for the EstB1 and EstB2 esterases were observed in 50 mM potassium- or sodium phosphate buffers at –20°C or 4°C. The EstB1 and EstB2 could be stored under these conditions up to 1 week. Tris·HCl and PBS buffers were found to inactivate the EstB1 and EstB2 already after 2 - 4 days of storage at –20°C or 4°C and therefore are not recommended for a long-term storage of both enzymes.

The temperature optimum of the EstB1 and EstB2 esterases was found surprising different. The EstB1 esterase displays a wide temperature optimum between 30°C and 50°C that indicates the light termophilic properties of the enzyme. The temperature optimum of the

EstB2 esterase was found to be in a range between 20°C and 35°C. The enzymes, which function at 20°C or at lower temperatures offer economic benefits through energy saving for the expensive heating steps, provide increased reaction yields, accommodate a high level of stereospecificity, minimise undesirable chemical reactions that occur at high temperatures and exhibit thermal lability for rapidly and easily inactivating the enzyme when required [137,138,139]. The cold-active lipase from *Aspergillus nidulans* found a wide application in food, detergent and cosmetic industy [125], as well as the lipase from *Typhicia ishibariensis*. The Norwegian company Biotec ASA has exploited the marine environment of the North Sea to isolated cold-adapted enzymes. The list of enzymes from Biotec includes also a novel lipase which is used for the production of detergents and flavourings [137].

The pH optimum of the EstB1 and EstB2 was found to be similar and in a narrow neutral pH range. The pH optimum of the EstB1 esterase was between pH 6.5 and pH 7.5 and the pH optimum of the EstB2 esterase was measured in a range of pH 6.5 to pH 8.0.

Because the EstB1 and EstB2 were isolated from the marine micro-organism the influence of different NaCl and KCl concentrations on the enzyme activities was tested. It was demonstrated that the EstB1 and EstB2 display a high stability in high NaCl and KCl concentrations, whereby the EstB1 was found to be more stable in high salts in comparison with the EstB2. It was shown that the activity of both EstB1 and EstB2 was less influenced with KCl then with NaCl. Nearly 80 % and 50 % of the EstB1 and EstB2 activity remained in the presence of 2 M KCl, correspondently.

From the literature data it is known that many lipolytic enzymes are regulated or activated by metal ions. In the secretory pathway of the phopholipase A2, calcium ions are involved to the binding of the substrate and to the polarization of the scissile ester bond [140,141]. Several lipases have been reported to be calcium-dependent [142,143]. Additionally, it has been reported in a number of publications that different metal ions activated (or inhibited) lipases and esterases of prokaryotic and eukaryotic origins. For example, the intracellular esterase from *Serratia marcescens* 345 was activated by $Mg^{2+}$ ions [144], the acetylcholinesterase was activated by monovalent $Na^{+}$, $K^{+}$ and divalent $Ca^{2+}$ and $Mg^{2+}$ cations [145] or in the case of pancreatic lipase both alkali and alkali earth metal ions, especially $Na^{+}$, $Ca^{2+}$ and $Mg^{2+}$ stabilized the enzyme [143]. Therefore the influence of different metal ions on the EstB1 and EstB2 activity was tested. The activity of the EstB1 and EstB2 esterases could not be activated by tested metal ions. In both cases small concentrations (10 - 50 mM) of $Zn^{2+}$ ions strongly inhibited the EstB1 and EstB2 activity, whereby the addition of $Ca^{2+}$ and $Mg^{2+}$ ions slowly decreased the activity of both esterases.

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.

The influence of diverse organic solvents was tested on the EstB1 and EstB2 esterases because nearly all lipase- or esterase-catalysed esterification reactions used for biotechnological applications are performed in non-aqueous medium. Among tested organic solvents remarkable stability in up to 50 % (v/v) of DMSO was shown for the EstB1 and EstB2 esterases. The strongest inhibition effect on the EstB1 and EstB2 activity was measured for 2-propanol followed by ethanol and methanol.

# 6. Summary

The particular microbial ecology of the sponge mesohyl with respect to the high number of taxonomically diverse bacteria provides vast potential for biotechnology in terms of novel enzymes and bioactive compounds. The uncharacterised micro-organisms can be novel sources for the enzymes and biologically active compounds with little overlap to those of terrestrial origin. In this work identification and preliminary physiological characterisation of a novel *Bacillus* sp. 01-855 isolated from the marine sponge *Aplysina aerophoba* was performed. Two novel esterases (EC 3.1.1.1) called EstB1 and EstB2 and a new putative amidase (EC 3.5.1. ) AmdB1 were isolated from genomic DNA library of *Bacillus* sp. 01-855 by means of screening using plate assay. The *est*B1, *est*B2 and *amd*B1 were cloned and functionally expressed in *E. coli*. The purification of the EstB1 and EstB2 to homogeneity was done in a single step by IMAC. Refolding method for the EstB2 esterase, which forms inclusion bodies was established. Preliminary biochemical characterisation of the EstB1 and EstB2 esterases was done. The biochemical characterisation revealed the unique properties of the EstB1 and EstB2 esterases, that could be potentially used for different biotechnological applications. Further studies on the biochemical properties of the AmdB1 amidase are necessary.

# 7. Zusammenfassung

Terrestrische Mikroorganismen sind bis in die heutige Zeit eine der wichtigsten Quellen für verschiedenste chemische Verbindungen und Enzyme, die direkt oder modifiziert in der Biotechnologie Anwendung finden. Marine Mikroorganismen sind eine vielversprechende alternative Quelle für solche natürlichen Substanzen. Marinen Naturprodukte zeichnen sich besonders dadurch aus, daß sie sich in der Regel deutlich in ihren chemischen und physikalischen Eigenschaften von denen aus terrestrisch Mikroorganismen isolierten Verbindungen unterscheiden. Meeresorganismen haben sich an ein Leben unter extremen Bedingungen, wie z.B. niedrige Temperaturen, hohe Drücke, besonders hohe bzw. niedrige pH-Werte und hohe Salzionenkonzentrationen, angepaßt. Dies führt dazu, daß auch die in ihnen enthaltenen Biokatalysatoren unter solchen Bedingungen funktionieren. Diese Eigenschaften prädestinieren solche Verbindungen für industrielle Anwendungen, bei denen ebenfalls häufig extreme Reaktionsbedingungen auftreten.

Die meisten im Meer lebenden Makroorganismen beherbergen eine Vielzahl verschiedener Bakterien, Cyanobakterien und Pilze [61,68]. Ihr Lebensraum ist dabei sowohl das Zellinnere als auch der extrazelluläre Bereich des Wirtes. Bei Schwämmen, wie z.B. dem im Mittelmeer vorkommenden Schwamm *Aplysina aerophoba,* können die mit ihm assoziierten Mikroorganismen bis zu 40 % der gesamten Biomasse ausmachen [65,85]. Die große Anzahl an assoziierten Mikroorganismen macht *Aplysina aerophoba* zu einer vielversprechenden Quelle für neuartige und bis jetzt physiologisch uncharakterisierte Bakterien, deren bioaktive Metaboliten und Enzyme großen biotechnologischen Nutzen haben können.

Esterasen (Carboxylester Hydrolasen, EC 3.1.1.1) und Lipasen (Triacylglycerin Ester Hydrolasen, EC 3.1.1.3) gehören zu der Klasse der Serin-Hydrolasen, welche die Hydrolyse und Synthese von Carbonsäureestern katalysieren. Sie sind in prokariotischen und eukariotischen Lebewesen weitverbreitet. Der grundsätzliche Unterschied zwischen Lipasen und Esterasen ist, daß Lipasen sowohl spaltende als auch bildende Wirkungen haben können. Esterasen hingegen katalysieren die Spaltung von kurzkettigen wasserlöslichen Carbonsäureester. Lipasen können die Spaltung von Fetten und Ölen an der Wasser-Öl-Grenzfläche bewirken, verwendet man sie hingegen in nicht-wässrigen Medium, können sie die Bildung von Estern fördern. Das Phänomen der Grenzflächenaktivierung von Molelülen durch Lipasen beruht auf dem einzigartigen Molekülbau dieser Enzymklasse. Lipasen enthalten helixartig gebaute Oligopeptideinheiten, die das katalytisch aktive Zentrum wie mit einem Deckel („lid“) abschirmen. Durch Wechselwirkung mit einer hydrophoben Grenzfläche

kann dieser „Deckel“ weggeschoben werden, was dazu führt, daß das aktive Zentrum nun für das Substrat frei zugänglich ist. Das katalytisch aktive Zentrum von Esterasen und Lipasen ist durch eine Triade, die aus Serin, Histidin und Aspartat gebildet wird, gekennzeichnet. Der entscheidende Schritt bei allen Esterasen- bzw. Lipasen-katalysierten Reaktionen ist die Bildung eines Substrat-Enzymkomplexes. Esterasen und Lipasen sind die wichtigsten Biokatalysatoren, da sie zum einen bei einer Vielzahl von nicht in der Natur vorkommenden Substraten wirken, andererseits sehr stabil in organischen Lösungsmitteln sind und bei stereospezifischen Reaktionen, wie z.B. der kinetischen Auftrennung von racemischen Gemischen, gute bis hervorragende Enantioselektivitäten liefern. Die kommerzielle Nutzung von Esterasen und Lipasen ist heutzutage ein Milliarden-Dollar Geschäft [34]. Aufgrund der exponentiell steigenden biotechnologischen Verwendung von Lipasen und Esterasen, ist die Identifikation und Charakterisierung von neuartigen Esterasen/Lipase-Genen von besonderer Bedeutung.

Die vorliegende Arbeit konzentriert sich daher auf das Screenen nach neuartigen Esterasen und Lipasegenen aus Meeresbakterien, sowie deren Klonierung und Sequenzierung und der biochemische Charakterisierung deren Produkte, welche von potentiellem Interesse für biotechnologische Anwendungen sein können. Die in dieser Arbeit untersuchten lipolytisch aktiven Bakterien wurden aus dem Mittelmeer stammenden Schwamm *Aplysina aerophoba* isoliert. Die lipolytische Aktivität wurde durch die Hydrolysereaktion mit Trbutyrin bestimmt, wobei die stärkste lipolytische Aktivität bei einer *Bacillus* Spezies festgestellt wurde [87]. Basierend auf vergleichender 16S rDNA Sequenzanalyse, lag die Übereinstimmung zu bereits bekannten Spezies unter 97 %. Dies ließ den Schluß zu, daß es sich um eine bislang unbekannte *Bacillus* Spezies handelte, die physiologisch uncharakterisiert ist. Augrund dieser Tatsache wurde dieser Stamm für die Isolierung neuartiger Enzyme ausgewählt.

Die eingehendere Bestimmung der Bacillus Spezies wurde von der DSMZ (Deutsche Sammlung für Mikroorganismen und Zellkulturen GmbH, Braunschweig, Deutschland) vorgenommen. Die Zugehörigkeit des Bakteriums zu einer neuen Bacillus Spezies (RNA Gruppe VI in *Bacillus*) wurde durch physiologische Assays, partielle 16S rDNA Sequenzierung und Fettsäureanalyse bestätigt. Gemäß der DSMZ Nomenklatur wurde dieser *Bacillus* Stamm *Bacillus* sp. 01-855 genannt.

Nach der Verdauung mit den Nukleasen *Eco*RI, *Nde*I, *Pst*I and *Hind*III wurden vier verschiedene chromosomale Bibliotheken des *Bacillus* sp. 01-855 im Vektor pUC18 erstellt. Danach wurde sie durch Tributyrin-Plate-Assay auf lipolytische Aktivität in *E. coli* getestet. Im Pool der DNA-Bibliotheken, der insgesamt aus 93.000 positiv getesteten Klonen (ca.

20.000 Klone pro Bibliothek) bestand, wurde ein *Nde*I-Klon und zwei *Eco*RI-Klone mit lipolytischer Aktivität bestimmt. Die Plasmide wurden aus den lipolytisch aktiven Kolonien isoliert und mittels Restriktionsanalyse untersucht. Drei chromosomale DNA-Fragmente, die als *ins*BS1 (2.2 kb), *ins*BS1 (1.9 kb) and *ins*BS3 (2.3 kb) bezeichnet wurden, wurden durch die „Primer Walking Methode“ an beiden Strängen ansequenziert.

Zwei neuartige Esterasen und eine putative Amidase wurden auf diese Weise aus den chromosomalen DNA-Bibliotheken des *Bacillus* sp. 01-855 isoliert. Die Nukleotid-sequenzierung der ersten Esterase, die den Namen *estB*1 erhielt, zeigte einen ORF mit 945 bp, der ein Protein (EstB1) der Masse 35.30 kDa kodiert. Der ORF *estB*2 enthielt 744 bp und codiert eine zweiten Esterase (EstB2) mit der Masse 28.32 kDa. Die putative Amidase (AmdB1) hatte eine Masse von 50.43 kDa und wurde durch einen ORF (*amd*B1) mit 1398 bp kodiert. Die Anwesenheit von putativen Transkriptionsbereichen in den „Stromaufwärts“-Bereichen von *est*B1, *est*B2 und *amd*B1 sowie die Tatsache, daß die lipolytische Aktivität, welche in *ins*BS1, *ins*BS2 und *ins*BS3 kodiert ist, nicht mit IPTG induzierbar ist, läßt darauf schließen, daß die Gene unter den ursprünglichen *Bacillus* Promotoren transkribiert sind. Die Proteinsequenzanalyse, die mittels der Datenbank BLAST durchgeführt wurde, zeigte, daß das Protein EstB1 eine Sequenzübereinstimmung mit der 3-Oxoadipate Enol-Lactonase des *Bacillus cereus* ATCC 14579 (44 %ige Übereinstimmung), mit der Abhydrolase $\alpha/\beta$ Hydrolase des *Bacillus anthracis* A2012 (45 %ige Übereinstimmung) und eine 26 %ige Übereinstimmung mit einer Chloroperoxidase von *Clostridium acetobutylicum* besitzt. Ein Vergleich der Aminosäuresequenzen von EstB2 mit denen von Proteinen der Datenbank BLAST erbrachte die Übereinstimmung mit den folgenden Enzymen: der Carboxylesterase von *Bacillus cereus* ATCC 14579 (74 %ige Übereinstimmung) und Proteinen der Dienlactonhydrolasefamilie von *Bacillus anthracis* A2012 (73 %ige Übereinstimmung). Die Aktivität von Lipasen und Esterasen hängt von der Ladungsverteilung am aktiven Zentrum, der Triade Ser-Asp/Glu-His, ab. Das aktive Serin-Zentrum von Esterasen und Lipasen wird normalerweise durch das Motiv Gly-X-Ser-X-Gly konserviert. In diesem Motiv stellt X einen variablen Rest dar. Es wird angenommen, daß die putativ aktiven Serine der Proteine EstB1 und EstB2 in den Aminosäuresequenzen Gly-Trp-Ser-Thr-Gly und Gly-Leu-Ser-Leu-Gly sitzen. Da Phenylmethylsulfonylfluorid (PMSF) als bekannter Inhibitor für Serin-Hydrolasen sowohl EstB1 als auch EstB2 bei Zugabe von 5 bzw. 1 mM deaktiviert, läßt dies den Schluß zu, daß Serin eine entscheidende Rolle für die Aktivität beider Proteine spielt.

Der Sequenzübereinstimmungsanalyse für AmdB1 zeigte eine 32%ige Übereinstimmung mit einer Amidase von *Mesorhizobium loti* und eine 33%ige Übereinstimmung mit einer Amidase von *Pseudomonas putida* KT2440. Aufgrund der geringen Übereinstimmung mit Proteinen der Datenbank ist es im Moment schwierig, AmdB1 einer bestimmten Enzymklasse zuzuordnen. Weitere biochemische Untersuchungen, welche die Bestimmung des Substratspektrums miteinschließt, sind für dieses Protein notwendig.

Die Gene *estB1* und *estB2* von *Bacillus* sp. 01-855 wurden mittels PCR in den Expressionsvektor pET-22b(+) subkloniert und danach unter Kontrolle des Promoters T7 in *E. coli* BL21(DE3) überexprimiert. Das durch PCR amplifizierte *amd*B1 Gen von *Bacillus* sp. 01-855 wurde in den Venktor pUC18 subkloniert und anschließend unter Kontrolle des *lac* Promoters in *E. coli* DH5α expremiert (ca. 5 - 8 % des gesamten Zellproteins).

Die Esterase EstB1 als auch die putative Amidase AmdB1 wurden in löslicher Form expremiert, wohingegen bei der Esterase EstB2 festgestellt wurde, daß nur ein kleiner Teil des rekombinanten Proteins löslich ist. Etwa 98 - 99 % der in *E. coli* expremierten Esterase EstB2 wurde in Form von sogenannten „Inclusion Bodies“ erhalten. Es konnte gezeigt werden, daß durch Optimierung der Expressionsbedingungen, wie z.B. Variation der Temperatur und Änderung der Menge an für die Induktion zugesetztem IPTG, kein Einfluß auf die Bildung des aktiven löslichen Proteins ausgeübt werden kann. Die Expression von EstB2 in *E. coli* BL21(DE3) wurde in das Periplasma und in das Zytoplasma durchgeführt. Das Periplasma von *E. coli* weist im Gegensatz zum Zytoplasma oxidierende Bedingungen auf und enthält Enzyme, welche die Bildung und Isomerisierung von Disulfidbrücken katalysieren. Es zeigte sich, daß die Expression von EstB2 in das Periplasma die Bildung des löslichen aktiven Proteins beeinflußt. So wurde bei der Expression von EstB2 in das Periplasma und in das Zytoplasma eine dreifach höhere Menge an Produkt festgestellt, als wenn nur in das Zytoplasma exprimiert wurde. Die Lysierung der *E. coli* Zellen, welche die expremierte Esterase EstB2 enthielten, wurde mit Zusatz von nicht-ionischen (Triton X-100) bzw. zwitter-ionischen Detergentien (CHAPS) durchgeführt. Es konnte gezeigt werden, daß das Protein zum einen keine hydrophoben Domänen enthält und es auch nicht die Tendenz zeigt, sich an Zellmembranen anzulagern und dadurch eine unlösliche Fraktion zu bilden. Somit kann der Schluß gezogen werden, daß die Bildung von „Inclusion Bodies“ nicht auf Wechselwirkung der Esterase EstB2 mit Lipden oder Zellmembranen zurückzuführen ist.

Die Enzyme EstB1 und EstB2 konnten sehr effizient und homogen in einem einzigen Arbeitsgang durch IMAC mit TALON$^{TM}$ (Clontech) aus *E. coli* aufgereinigt werden. Im Fall von EstB1 wurden 50 mg Esterase pro Liter Kulturansatz erhalten. Beim Enzym EstB2,

welches in *E. coli* unter der Kontrolle des Promoter T7 expremiert wurde, wurden lösliche als auch unlösliche Fraktionen erhalten. Die lösliche Fraktion der Esterase EstB2 wurde analog zur Vorgehenensweise bei EstB1 aufgereinigt, wobei 0.05 mg aktives Protein pro Liter Kulturansatz erhalten wurden. Für die Umwandlung der inaktiven „Inclusion Bodies" in ein lösliches aktives Protein wurde ein „Refolding" für EstB2 entwickelt und angewandt. 40 mg an nahezu homogenen aktives Protein wurden pro Liter Ansatz erhalten.

Die biochemische Charaktersisierung der aus *E. coli* aufgereinigten Esterasen EstB1 und EstB2 wurde im Rahmen der vorliegenden Arbeit durchgeführt. Im Einzelnen wurde die Substratspezifität, die Stabilität bei Lagerung, das pH- und Temperaturoptimum sowie der Einfluß von organischen Lösungsmitteln, Salzen und Metallionen verschiedener Konzentration auf die Wirkung der beiden aufgereinigten rekombinanten Enzyme getestet und überprüft.

Beide Esterasen zeigten eine katalytische Aktivität auf eine Reihe von für Esterasen und Lipasen typische Substrate. Sowohl für EstB1 als auch EstB2 konnte die höchste Aktivität gegenüber *p*NP Estern und aliphatischen Methylestern mit C-Kettenlängen zwischen zwei und sechs Kohlenstoffatomen beobachtet werden. Damit ist gezeigt, daß beiden Enzymen das Verhalten von Esterasen aufweisen. Die Esterase EstB1 zeigte eine höhere Hydrolyseaktivität gegenüber Methylestern, wohingegen beim Enzym EstB2 keine Bevorzugung zwischen *p*NP Estern und Methylestern beobachtet wurde. Bei der Esterase EstB1 nimmt der Wert der Michaelis-Konstante $K_m$ mit steigender Kohlenstoffzahl der Carbonsäurekettenlänge von $C_2$ nach $C_4$ ab, wohingegen der Wert der maximalen Reaktionsgeschwindigkeit $V_{max}$ keine Kettenlängenabhängigkeit aufweist. Im Falle des Enzyms EstB2 nimmt sowohl die Konstante $K_m$ als auch die maximale Reaktionsgeschwindigkeit $V_{max}$ mit wachsender C-Kettenlänge ab.

Da die Sequenzanalyse der Proteine EstB1 und EstB2 ergab, daß sie sowohl Übereinstimmung mit Chloroperoxidasen als auch mit Lactonasen zeigen, wurde zusätzlich die Haloperoxidase und Lactonase-Aktivität untersucht. Das Enzym EstB1 zeigte dahingehend keine Aktivitäten. Bei der Esterase EstB2 wurde eine leichte Aktivität gegenüber Lactonen festgestellt.

In dieser Arbeit konnte gezeigt werden, daß das Protein EstB1 eine besser Lagerungsstabilität aufweist als die Esterase EstB2. Das Optimum der Lagerungsstabilität kann erreicht werden, wenn die Enzyme in einem 50 mM Kalium- bzw. Natriumphoshatpuffer bei –20°C bzw. 4°C gehalten werden. Unter diesen Lagerungsbedingungen sind beide Enzyme bis zu einer Woche haltbar. Die Verwendung von Tris·HCl oder PBS Puffern führt schon nach 2 - 4 Tagen bei

Lagerung bei –20°C bzw. 4°C zur Deaktivierung beider Enzyme. Diese Puffer sind daher für eine längere Lagerung der Enzyme ungeeignet.

EstB1 und EstB2 weisen überraschenderweise unterschiedliche Temperaturoptima auf. Die höchste Enzymaktivität wird bei EstB1 in einem Temperaturbereich zwischen 30°C und 50°C beobachtet, was auf leicht thermophile Eigenschaften des Enzyms schließen läßt. Bei EstB2 liegt das Temperaturoptimum im Bereich zwischen 20°C und 35°C. Das pH-Optimum wurde bei EstB1 im neutralen Bereich zwischen pH 6.5 und 7.5 bestimmt, bei EstB2 lag es zwischen pH 6.5 und 8.0.

Da beide Esterasen aus Meeresorganismen isoliert wurden, wurde auch der Einfluß der Salzkonzentration von NaCl und KCl auf die Enzymaktivität überprüft. Für beide Esterasen gilt, daß sie eine hohe Stabilität bezüglich hohen Salzkonzentrationen besitzen, wobei EstB1 stabiler als EstB2 ist. KCl beeinflußt hierbei die Aktivität sowohl von EstB1 als auch EstB2 weniger als NaCl. Bei Zugabe von 2 M KCl Lösung blieb die Aktivität von EstB1 zu 80 % bei EstB2 zu 50 % erhalten.

Es wurde auch der Einfluß anderer Chemikalien, wie EDTA, β-Mercaptoethanol und DTT, auf EstB1 und EstB2 untersucht. Es zeigte sich, daß alle drei Substanzen keinen Einfluß auf die Enzymaktivität haben.

Der Einfluß von $Zn^{2+}$-, $Ca^{2+}$- und $Mg^{2+}$-Ionen auf die Aktivität beider Enzyme wurde aus dem Grund überprüft, da viele lipolytische Enzyme durch Metallionen reguliert oder aktiviert werden. Es wurde festgestellt, daß Zink-, Calcium- und Magnesiumionen keine aktivierende Wirkung auf die Enzyme EstB1 und EstB2 haben, sondern das der Zusatz von kleinen Mengen an $Zn^{2+}$-Ionen (10 - 50 mM) eine stark inhibierende Wirkung auf beide Esterasen ausübt. Der Zusatz von $Ca^{2+}$- und $Mg^{2+}$-Ionen führt zu einer langsamen Erniedrigung der Enzymaktivität bei beiden Esterasen.

Bei biotechnologischer Anwendung von Esterasen ist eine Forderung an das jeweilige Enzym, daß es sich stabil in organischen Lösungsmitteln verhält, da praktisch alle Esterasen-katalysierten Reaktionen in nicht-wässrigen Medien durchgeführt werden. Eine besonders gute Stabilität zeigten EstB1 und EstB2 gegenüber DMSO, welches bis zu 50 Volumenprozente zugesetzt werden konnte, ohne das die Enzyme ihre Aktivität verloren. Beide Esterasen werden gleichermaßen durch den Zusatz von Alkoholen (Methanol, Ethanol und 2-Propanol) beeinflußt, wobei der Inhibitionseffekt mit sinkender C-Kettenlänge des Alkohols abnimmt.

Zusammenfassend kann gesagt werden, daß die biochemische Charakterisierung der aus den *Bacillus* sp. 01-855 des Schwamms *Aplysina aerophoba* isolierten Esterasen zeigte, daß sowohl EstB1 als auch EstB2 einzigartige biochemische Eigenschaften besitzen, die bei verschiedene biotechnologische Anwendungen genutzt werden könnten. Bei der Amidase AmdB1 sind weitere Untersuchungen zu ihren biochemischen Eigenschaften notwendig.

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# 9. Curriculum vitae

Anna Alexandrovna Karpushova

| | |
|---|---|
| **Geburtsdatum, Geburtsort:** | 13. Juli 1975, Taschkent (UdSSR) |
| **Bildungsweg:** | |
| 1992: | Allgemeine Hochschulreife, Moskau |
| 1992-1997: | Studium der Lebensmitteltechnologie an der Moskauer Universität der Nahrungsmittelindustrie, Fakultät Technologie und Industriemanagement<br>Studium der englischen Sprachen in der Abteilung Referenten-Übersetzung beim Lehrstuhl für Fremdsprachen der Moskauer Universität der Nahrungsmittelindustrie |
| 10/1995: | DAAD Stipendium: Praktikum an der Universität Hohenheim, Institut für Lebensmitteltechnologie |
| 01/1997: | Diplomarbeit zum Thema „Untersuchung der antioxidativen Eigenschaften der Naturbestandteile der Zellen und deren Gemische bei der Autooxidation von Lipiden“ |
| 19.06.1997:<br>17.07.1997: | Diplomverteidigung<br>Titel:<br>- Dipl. Ingenieur für Verfahrenstechnik<br>- Dipl. Übersetzerin für wissenschaftliche Literatur in englischer Sprache |
| 2000-2004: | Dissertation am Institut für Technische Biochemie der Universität Stuttgart zum Thema „Screening, Cloning and Biochemical Characterisation of Novel Lipolytic Enzymes from *Bacillus* sp. 01-855 associated with Marine Sponge *Aplysina aerophoba“* |
| **Berufserfahrung:** | |
| 1998-2000: | wissenschaftliche Mitarbeiterin am Institut für Lebensmittelchemie der Universität Hohenheim |
| 2000-2004: | wissenschaftliche Mitarbeiterin am Institut für Technische Biochemie der Universität Stuttgart |

## Publikationsliste

Karpushova A., Brümmer F., Lange S., Schmid R.D.:
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