



UNIVERSITEIT VAN PRETORIA  
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YUNIBESITHI YA PRETORIA

# **Cloning, properties and expression of a novel esterase from *Bacillus coagulans* strain 81-11**

by

**Stephens M. Mnisi**

Submitted in partial fulfilment of the requirements for the degree  
Master of Science  
in the Faculty of Natural and Agricultural Sciences  
University of Pretoria  
Pretoria

**June 2004**



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My family for the continued support and encouragement.

## SUMMARY

### **Cloning, properties and expression of a novel esterase from *Bacillus coagulans* strain 81-11**

by

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Over the past few years, the use of enzymes as catalysts for the preparation of novel organic molecules has received a steadily increasing amount of attention. Lipolytic enzymes are widely distributed in nature and attract great attention because of their biotechnological potential, as they catalyse the enantio- and regioselective hydrolysis and synthesis of a broad range of natural and non-natural esters. Bacteria produce different lipolytic enzymes, such as esterases (EC 3.1.1.1), which hydrolyse ester-containing molecules at least partly soluble in water, and lipases (EC 3.1.1.3), which hydrolyse water-insoluble long-chain triglycerides. In this study, a bacterial isolate, *B. coagulans* strain 81-11, isolated from popcorn seeds, was characterized with the specific aim of isolating and characterizing genes encoding novel lipolytic enzymes.

A genomic library of *B. coagulans* strain 81-11 was screened in *Escherichia coli* JM83 for lipolytic activity by using tributyrin agar plates. A 2.4-kb DNA fragment was subcloned from a lipolytic-positive clone and completely sequenced. Nucleotide sequence analysis predicted a 723-bp open reading frame (ORF), designated *estC1*, encoding a protein of 240 amino acids with an estimated molecular mass of 27 528 Da and a pI of 9.15. The deduced amino acid sequence of the *estC1* gene exhibited significant amino acid sequence identity with carboxyl esterases and sequence analysis showed that the protein contains the signature G-X-S-X-G included in most esterases and lipases. Enzyme assays using *p*-nitrophenyl esters with different acyl chain lengths as the substrate confirmed the anticipated esterase

activity. EstC1 exhibited a marked preference for esters of short-chain fatty acids, yielding the highest activity with *p*-NP butyrate. Maximum activity was found at pH 8 and 50°C, although the enzyme was active in the pH range 7-9 and displayed activity at temperatures up to 55°C.

Since bacterial esterases are potentially important for a variety of biotechnological applications, there is a considerable industrial interest to produce these enzymes at a larger scale. Among the many systems that are available for heterologous protein production, attempts were made to overexpress the newly identified *B. coagulans estC1* esterase-encoding gene in different Gram-positive bacteria, as they are well known for their important contribution to food biotechnology and as production organisms for industrial enzymes. A recombinant expression vector was thus constructed (pMG36-EstC1) and introduced in *Lactococcus lactis*, *Lactobacillus plantarum* and *Bacillus subtilis* strains 154 and 1A297. Of these different bacterial hosts, high levels of intracellular esterase activity were detected in *B. subtilis* 1A297 only. In an attempt to increase extracellular expression of the *B. coagulans* EstC1 esterase, a recombinant secretion plasmid (pNW-EstC1aps) was constructed that contained an alkaline protease promoter and signal sequence from a *Bacillus* species. Following introduction of the construct in *B. subtilis* 1A297, the derived recombinant strain displayed 2.3-fold higher extracellular esterase-activity levels than the parent *B. coagulans* strain, and the extracellular esterase activity represented 82% of the total esterase activity.

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## LIST OF ABBREVIATIONS

A	Absorbance
amp <sup>r</sup>	ampicillin resistance
Asp	aspartic acid (D)
ATP	adenosine triphosphate
bp	base pair
C	carbon
ca.	approximately
cam <sup>r</sup>	chloramphenicol resistance
°C	degrees Celsius
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleoside-5'-triphosphate
e.g.	for example
Fig.	Figure
Gly	glycine (G)
h	hour
His	histidine (H)
IPTG	isopropyl β-D-thiogalactoside
kb	kilobase pairs
kDa	kilodalton
kV	kiloVolt
LAB	lactic acid bacteria
LB broth	Luria-Bertani broth
LGB	low GC Gram-positive bacteria
lt	litre
M	molar
mg	milligram
min	minute
ml	millilitre
mM	millimolar
nm	nanometre
nt	nucleotide
OD	optical density
Ω	ohm
ORF	open reading frame



PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PEG	polyethylene glycol
pmol	picomole
<i>p</i> -NP ester	<i>p</i> -nitrophenyl ester
RNA	ribonucleic acid
rpm	revolutions per minute
s	second
SDS	sodium dodecyl sulphate
Ser	serine (S)
TE	Tris-EDTA
U	units
$\mu$ F	micro-Faraday
$\mu$ g	microgram
UHQ	ultra high quality
$\mu$ l	microlitre
UV	ultraviolet
V	volts
v/v	volume per volume
w/v	weight per volume

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## **CHAPTER ONE**

### **LITERATURE REVIEW**



## 1.1 GENERAL INTRODUCTION

Enzymes catalyse an enormous number of reactions that are necessary for the synthesis, modification and degradation of the organic molecules that make up living organisms. Due to this wide range of different activities, enzymes may provide opportunities for industry to carry out efficient and economical biocatalytic conversions. Indeed, enzymes have been used since the dawn of mankind indirectly via yeasts and bacteria in food manufacturing (Godfrey and West, 1996). Isolated enzymes, however, were first used in detergents in 1914, their protein nature proven in 1926 and their large-scale microbial production started in the 1960s (Godfrey and West, 1996). Since then, industrial enzyme business has grown steadily due to improved production technologies, engineered enzyme properties and new application fields. The estimated value of the world enzyme market in 2001 was about \$1.3 billion and it has been forecasted to grow to almost \$2 billion by 2005 (Schmid *et al.*, 2001). The main industries, which are responsible for using *ca.* 75% of industrially produced enzymes, are the detergents (37%), textiles (12%), starch (11%), baking (8%) and animal feed (6%) industries (Schmid *et al.*, 2001; van Beilen and Li, 2002).

More than 2 000 different enzyme activities have thus far been characterized and the number of reported 3-dimensional (3D) enzyme structures is also increasing (Schmid *et al.*, 2001). In the year 2000, the structure of about 1 300 different proteins were known. The enzymes are classified into six major categories based on the nature of the chemical reaction that they catalyse (Davis and Boyer, 2001). These are: (i) oxidoreductases that catalyse oxidation or reduction of their substrates; (ii) transferases that catalyse group transfer; (iii) hydrolases that catalyse bond breakage with the addition of water; (iv) lyases that remove groups from their substrates; (v) isomerases that catalyse intramolecular rearrangements; and (vi) ligases that catalyse the joining of two molecules at the expense of chemical energy. Only a limited number of all the known enzymes are, however, commercially available and even a smaller number is used in large quantities. More than 75% of industrial enzymes are hydrolases, while protein-degrading enzymes constitute about 40% of all enzyme sales (Schmid *et al.*, 2001; van Beilen and Li, 2002). Hydrolases, inclusive of esterases and lipases, are especially used in laundry detergents, in the food industry and as biocatalysts for the synthesis of fine chemicals.



Since hydrolase enzymes are important for a variety of biotechnological applications, there is a considerable interest to produce these enzymes at a larger scale by making use of appropriate expression systems. Expression systems are comprised of host cells and genetic elements, such as promoters and plasmids, and the choice of each component can be critical for the successful expression of the gene of interest (Old and Primrose, 1994). In addition to *Escherichia coli*, which is usually the principle bacterium of choice for cloning genes and expressing recombinant proteins, several different Gram-positive bacteria are also being used as production organisms for industrial enzymes. Amongst them, lactic acid bacteria, such as *Lactobacillus*, *Lactococcus* and *Streptococcus* spp., are receiving much attention since their “generally regarded as safe” status make them potentially useful organisms for the production of commercially important proteins (de Vos *et al.*, 1997; Leenhouts *et al.*, 1998b; Slos *et al.*, 1998). In contrast to *E. coli*, Gram-positive bacteria are capable of directly secreting proteins in the extracellular medium, which in *Bacilli* have reached a high level of efficiency (Simonen and Palva, 1993). Proteins that are secreted into the extracellular environment present many advantages since the proteins not only tend to be stable, but they can also be readily purified from the culture supernatant (Kitai *et al.*, 1988). In this chapter, literature relevant to hydrolase enzymes and to Gram-negative and Gram-positive bacterial expression systems will thus be reviewed, as they are closely related to the aims of this investigation.

## 1.2 HYDROLYTIC ENZYMES

The hydrolases represent a diverse group of lipolytic enzymes that catalyse the synthesis of ester bonds and the hydrolysis of carboxylic esters into their corresponding carboxylic acids and alcohols (Jaeger *et al.*, 1999; Bornscheuer, 2002). Two major classes of hydrolases, namely esterases (EC 3.1.1.1, carboxyl ester hydrolases) and lipases (EC 3.1.1.3, triacylglycerol hydrolases), have attracted enormous attention because of their biotechnological potential (Faber, 1997; Jaeger and Reetz, 1998; Pandey *et al.*, 1999; Schultze and Wubbolts, 1999). These enzymes are most frequently used, amongst many other applications, for transesterifications and ester synthesis. Despite lipolytic enzymes being widely distributed in animals, plants and microorganisms (Okuda, 1991; Jaeger *et al.*, 1994; MacRae and Hammond, 1992), most of the lipolytic enzymes used in industry are microbial enzymes, of both fungal (from genera such as *Candida*, *Geotrichum*, *Rhizopus* and *Thermomyces*) and bacterial (from genera such as *Bacillus*, *Pseudomonas*, *Staphylococcus*,

*Acinobacter*, *Streptococcus*) origin (Sztajer and Maliszewska, 1988; Jaeger and Reetz, 1998; Pandey *et al.*, 1999).

### 1.2.1 Carboxyl esterases

Esterases represent a diverse group of hydrolases that catalyse the formation or cleavage of ester bonds of water-soluble substrates (Tsujita *et al.*, 1990; Jaeger *et al.*, 1999; Bornscheuer, 2002). Although they are widely distributed in animals, plants and microorganisms, the physiological functions of many esterases are not clear (Okuda, 1991). Many of them show a wide substrate tolerance, which led to the assumption that they have evolved to enable access to carbon sources or to be involved in catabolic pathways (Williamson *et al.*, 1998; Videira *et al.*, 2003). Furthermore, in some plant-pathogenic bacterial and fungal strains, cell wall-degrading esterases, such as acetyl- and cinnamoyl esterases (Ferreira *et al.*, 1993; Dalrymple *et al.*, 1996), are believed to be pathogenic factors (McQueen and Schottel, 1987), while other esterases have been proposed to play a role in the detoxification of biocides (Blackman *et al.*, 1995). For example, fusidic acid resistance of *Streptomyces lividans* is due to an esterase that inactivates the antibiotic (Von den Haar *et al.*, 1997), and a *Bacillus subtilis* esterase that is capable of hydrolysing the phytotoxin brefeldin A has been described (Wei *et al.*, 1999).

In addition to their biological significance, esterases also show high regio- and stereospecificity and are therefore considered attractive biocatalysts for the production of optically pure compounds in fine-chemicals synthesis (Nishizawa *et al.*, 1993; Quax and Broekhuizen, 1994; Zock *et al.*, 1994; Ozaki and Sakimae, 1997; Krebsfanger *et al.*, 1998; Khalameyzer *et al.*, 1999). Moreover, these enzymes do not require co-factors, are usually rather stable and are active in organic solvents (Faber, 1997; Bornscheuer, 2002). However, their widespread industrial use has been limited by the extensive use of many lipases in biocatalysis, as they often show higher enantioselectivity, stability in organic solvents and broader substrate specificity than the esterases.

### 1.2.2 True lipases

A “true” lipase is defined as a carboxyl esterase, which catalyses the hydrolysis and synthesis of long-chain acylglycerols with trioleoylglycerol being the standard substrate (Tsujita *et al.*, 1990; Ferrato *et al.*, 1997; Verger, 1997). Since lipases are indispensable for the

bioconversion of lipids (triacylglycerols) from one organism to another and within the organisms, they are found throughout all kingdoms of life. Although lipases have been found in the pancreas of mammals such as pigs and humans (Horgan *et al.*, 1969), and in higher plants such as castor bean (*Ricinus communis*) and rapeseed (*Brassica napus*) (Fuchs and Hansen, 1994), they are found more abundantly in microbial flora comprising bacteria, fungi and yeast (Jaeger *et al.*, 1994; MacRae and Hammond, 1992). Of the large number of microbial strains, including thermophilic and psychrophilic microorganisms (Demirjian *et al.*, 2001), which have been used as sources of lipases, *Candida* spp., *Pseudomonas* spp. and *Rhizopus* spp. are the most important sources of commercial lipase enzymes (Benjamin and Pandey, 1998b; Jaeger and Reetz, 1998).

Lipases are capable of catalysing a wide range of reactions, which include hydrolysis and interesterification specifically; in addition, they also catalyse alcoholysis, acidolysis, esterification and aminolysis (Jaeger *et al.*, 1999; Davis and Boyer, 2001). Lipases act under extremely mild conditions and they can be used in a variety of organic solvents, whilst still showing selectivity for a specific reaction type. Lipases also do not usually require co-factors nor do they catalyse side reactions (Patel *et al.*, 1996; Gill and Valivety, 1997). Consequently, lipases are the most widely used group of biocatalysts and have been used for a variety of different biotechnological applications (See Section 1.5.2).

### 1.3 MECHANISM OF LIPOLYSIS

Knowledge regarding the structure of lipases and esterases has increased in recent years through the elucidation of many gene sequences and the resolution of several crystal structures (Wei *et al.*, 1995; 1998; Schrag and Cygler, 1997; Kim *et al.*, 1997). All of them exhibit a characteristic folding pattern known as the  $\alpha/\beta$ -hydrolase fold (Ollis *et al.*, 1992; Jaeger *et al.*, 1999). The canonical  $\alpha/\beta$ -hydrolase fold consists of a central, mostly parallel  $\beta$ -sheet of eight strands with the second strand antiparallel (Fig. 1.1). The parallel strands  $\beta_3$  to  $\beta_8$  are connected by  $\alpha$ -helices, which pack on either side of the central  $\beta$ -sheet.

The active site of  $\alpha/\beta$ -hydrolase fold enzymes is formed by a catalytic triad consisting of the amino acids serine, aspartic acid (or glutamic acid for some lipases) and histidine. Usually a consensus sequence Gly-X-Ser-X-Gly (where X represents an arbitrary amino acid residue) is

found around the active site serine (Derewenda and Derewenda, 1991; Ollis *et al.*, 1992; Peterson and Drablos, 1994). Esterases have, however, been identified containing a Gly-X-X-Leu motif (Wei *et al.*, 1995), as well as enzymes showing high homology to class C  $\beta$ -lactamases (Petersen *et al.*, 2001). In the prototype  $\alpha/\beta$ -hydrolase fold enzymes, the nucleophilic Ser residue is located at the C-terminal end of strand  $\beta$ 5 in the highly conserved pentapeptide G-X-S-X-G, forming a characteristic  $\beta$ -turn- $\alpha$  motif termed the “nucleophilic elbow” (Ollis *et al.*, 1992). It positions the nucleophilic residue free of the active site surface and allows easy access on one side by the active site histidine residue and on the other by the substrate. The catalytic acid (Asp or Gly) occurs in a reverse turn after strand 7 of the central  $\beta$ -sheet (Ollis *et al.*, 1992) and is hydrogen-bonded to the active site histidine, which is located in a loop after  $\beta$ -strand 8 of the  $\alpha/\beta$ -hydrolase fold. The length and conformation of this loop are variable (Ollis *et al.*, 1992). The mechanism for ester hydrolysis or formation is essentially the same for lipases and esterases and is composed of four steps (Fig. 1.2). First, the substrate is bound to the active serine, yielding a tetrahedral intermediate stabilized by the catalytic His and Asp (Gly) residues. Next, the alcohol is released and an acyl-enzyme complex is formed. Attack of a nucleophile (water in hydrolysis, alcohol or ester in esterification) forms again a tetrahedral intermediate, which after resolution yields the product (an acid or an ester) and free enzyme (Jaeger *et al.*, 1994; Bornscheuer, 2002).

Lipases are characterized by their increased activity when acting at the lipid-water interface of emulsified substrates (Van Tilbeurgh *et al.*, 1993), a phenomenon called interfacial activation. This increase in enzyme activity is believed to be triggered by structural rearrangements of the lipase active-site region (Derewenda *et al.*, 1992; Van Tilbeurgh *et al.*, 1993). In the absence of lipid-water interfaces, the active site is covered by a so-called “lid”. However, in the presence of hydrophobic substances, *i.e.* a triglyceride phase or a hydrophobic organic, the lid is moved apart, making the catalytic residues accessible to the substrate and exposing a large hydrophobic surface, which is presumed to interact with the lipid interface (Van Tilbeurgh *et al.*, 1993). The lid may consist of a single helix (Brzozowski *et al.*, 1991; Derewenda *et al.*, 1992), or two helices (Schrag *et al.*, 1997) or a loop region (Grochulski *et al.*, 1993). However, not all lipases show this interfacial activation as lipases from *P. aeruginosa* (Schrag *et al.*, 1997) and *Candida antarctica* B (Uppenberg *et al.*, 1994) lack a lid that covers the active site.

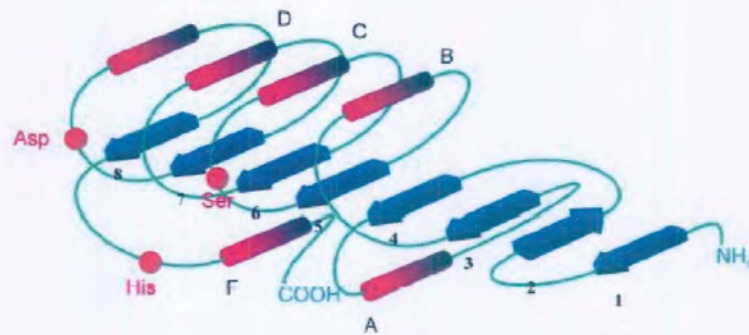


Fig. 1.1 Schematic presentation of the  $\alpha/\beta$ -hydrolase fold.  $\beta$ -Sheets (1-8) are shown as blue arrows,  $\alpha$ -helices (A-F) as red columns. The relative positions of the amino acids of the catalytic triad (serine, aspartic acid and histidine) are indicated as orange circles. (*Adapted from Bornscheuer, 2002*)

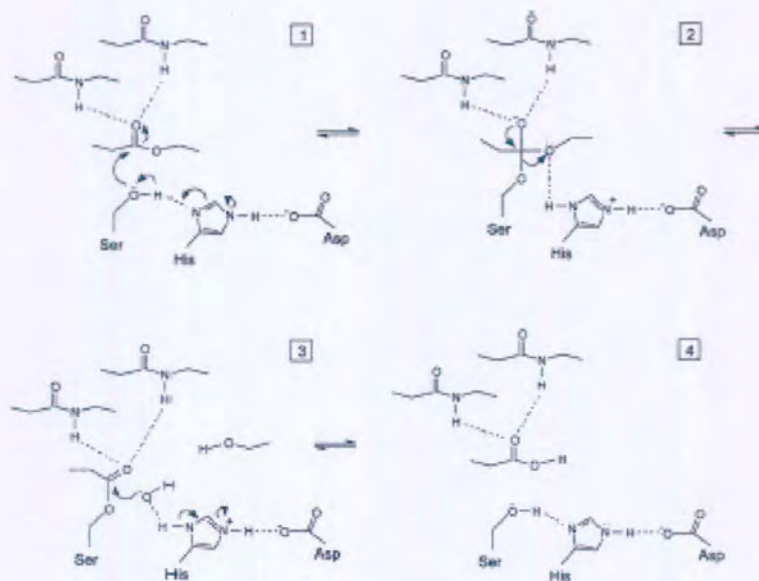


Fig. 1.2 Reaction mechanism of hydrolases. [1] Binding of lipid, activation of nucleophilic serine residue by neighboring histidine and nucleophilic attack of the substrate's carbonyl carbon atom by Ser O<sup>-</sup>. [2] Transient tetrahedral intermediate, with O<sup>-</sup> stabilized by interactions with two peptide NH groups. The histidine donates a proton to the leaving alcohol component of the substrate. [3] The covalent intermediate ("acyl enzyme"), in which the acid component of the substrate is esterified to the enzyme's serine residue. The incoming water molecule is activated by the neighboring histidine residue, and the resulting hydroxyl ion performs a nucleophilic attack on the carbonyl carbon atom of the covalent intermediate. [4] The histidine residue donates a proton to the oxygen atom of the active serine residue, the ester bond between serine and acyl component is broken, and the acyl product is released. (*Adapted from Jaeger et al., 1999*)

Since esterases and some lipases do not show interfacial activation, lipases and esterases may rather be distinguished from each other based on the preference of lipases for water-insoluble substrates, typically triglycerides composed of long-chain fatty acids, whereas esterases preferentially hydrolyse simple esters (*e.g.* ethyl acetate) and usually only triglycerides bearing fatty acids shorter than C<sub>6</sub> (Ferrato *et al.*, 1997; Verger, 1997). More recently, Fojan *et al.* (2000) compared the amino acid sequences and 3D-structures of lipases and esterases and suggested that a pH-dependent “electrostatic” signature can accurately distinguish them. The active site of lipases displays a negative potential in the pH range associated with their maximum activity (typically at pH 8), whereas esterases show a similar pattern, but at pH values around pH 6, which correlates with their usually lower pH-activity optimum (Fojan *et al.*, 2000).

#### 1.4 CLASSIFICATION OF BACTERIAL ESTERASES AND LIPASES

Typically enzymes are classified either based on their substrate specificity or by sequence alignments. The former requires that all enzymes, which need to be compared, have been assayed with the same or at least related substrates, preferentially under similar reaction conditions. By contrast, the comparison of amino acid sequences can provide a clearer picture about the evolutionary relationship between enzymes of different origin. However, high sequence homology cannot always be related to the enzyme properties (substrate specificity, stereoselectivity, pH and temperature optima, solvent stability), and in some cases completely different types of reactions are catalysed (Hecht *et al.*, 1994; Pelletier and Altenbuchner, 1995).

Until recently, it was believed that for all lipases and carboxyl esterases only the consensus sequence motif Gly-X-Ser-X-Gly occurs around the active site serine. More recently, a thorough comparison of 53 amino acid sequences of lipases and esterases revealed that not only do other motifs exist, but has led to a framework to classify bacterial lipases and esterases by grouping them into eight families on the basis of conserved sequence motifs and biological properties (Arpigny and Jaeger, 1999). Several extensions of the original classification scheme have been proposed (Jaeger and Eggert, 2002) and the revised list of lipolytic enzymes is presented in Table 1.1.

**Table 1.1:** Updated classification of bacterial lipolytic enzymes (*Adapted from Arpigny and Jaeger, 1999 and Jaeger and Eggert, 2002*).

Family	Subfamily	Enzyme-producing strain	Accession no.	Properties
I	1	<i>Pseudomonas aeruginosa</i> (LipA)	D50587	True lipases
		<i>Pseudomonas fluorescens</i> C9	AF031226	
		<i>Vibrio cholerae</i>	X16945	
		<i>Pseudomonas aeruginosa</i> (LipC)	U75975	
		<i>Acinetobacter calcoaceticus</i>	X80800	
		<i>Pseudomonas fragi</i>	X14033	
		<i>Pseudomonas wisconsinensis</i>	U88907	
	<i>Proteus vulgaris</i>	U33845		
	2	<i>Burkholderia glumae</i>	X70354	
		<i>Chromobacterium viscosum</i>	Q05489	
		<i>Burkholderia cepacia</i>	M58494	
		<i>Pseudomonas luteola</i>	AF050153	
	3	<i>Pseudomonas fluorescens</i> SIKW1	D11455	
		<i>Serratia marcescens</i>	D13253	
	4	<i>Bacillus subtilis</i> (LipA)	M74010	
		<i>Bacillus pumilus</i>	A34992	
		<i>Bacillus licheniformis</i>	U35855	
		<i>Bacillus subtilis</i> (LipB)	C69652	
	5	<i>Geobacillus stearothermophilus</i> L1	U78785	
		<i>Geobacillus stearothermophilus</i> P1	AF237623	
		<i>Geobacillus thermocatenulatus</i>	X95309	
		<i>Geobacillus thermoleovorans</i>	AF134840	
	6	<i>Staphylococcus aureus</i>	M12715	
		<i>Staphylococcus haemolyticus</i>	AF096928	
		<i>Staphylococcus epidermidis</i>	AF090142	
		<i>Staphylococcus hyicus</i>	X02844	
		<i>Staphylococcus xylosus</i>	AF208229	
		<i>Staphylococcus warneri</i>	AF208033	
	7	<i>Propionibacterium acnes</i>	X99255	
		<i>Streptomyces cinnamomeus</i>	U80063	
	II (GDSL)	<i>Aeromonas hydrophila</i>	P10480	Secreted acyltransferase Secreted esterase Outer membrane-bound esterase Outer membrane-bound esterase Secreted esterase
		<i>Streptomyces scabies</i>	M57297	
		<i>Pseudomonas aeruginosa</i>	AF005091	
		<i>Salmonella typhimurium</i>	AF047014	
		<i>Photobacterium luminescens</i>	X66379	
III	<i>Streptomyces exfoliates</i>	M86351	Extracellular lipase Extracellular lipase Extracellular esterase 1	
	<i>Streptomyces albus</i>	U03114		
	<i>Moraxella</i> sp.	X53053		
IV (HSL)	<i>Alicyclobacillus acidocaldarius</i>	X62835	Esterase Lipase Carboxyl esterase Putative lipase Carboxyl esterase Extracellular esterase 2	
	<i>Pseudomonas</i> sp. B11-1	AF034088		
	<i>Archaeoglobus fulgidus</i>	AE000985		
	<i>Alcaligenes eutrophus</i>	L36817		
	<i>Escherichia coli</i>	AE000153		
	<i>Moraxella</i> sp.	X53868		
V	<i>Pseudomonas oleovorans</i>	M58445	PHA-depolymerase Putative esterase Extracellular esterase Extracellular esterase 3 Esterase Esterase	
	<i>Haemophilus influenzae</i>	U32704		
	<i>Psychrobacter immobilis</i>	X67712		
	<i>Moraxella</i> sp.	X53869		
	<i>Sulfolobus acidocaldarius</i>	AF071233		
VI	<i>Acetobacter pasteurianus</i>	AB013096	Carboxyl esterases	
	<i>Synechocystis</i> sp.	D90904		
	<i>Spirulina platensis</i>	S70419		
	<i>Pseudomonas fluorescens</i>	S79600		
	<i>Rickettsia prowazekii</i>	Y11778		
<i>Chlamydia trachomatis</i>	AE001287			
VII	<i>Arthrobacter oxydans</i>	Q01470	Carbamate hydrolase <i>p</i> -Nitrobenzyl esterase Putative carboxyl esterase	
	<i>Bacillus subtilis</i>	P37967		
	<i>Streptomyces coelicolor</i>	CAA22794		
VIII	<i>Arthrobacter globiformis</i>	AAA99492	Stereoselective esterase Cell-bound esterase Esterase III	
	<i>Streptomyces chrysomallus</i>	CAA78842		
	<i>Pseudomonas fluorescens</i> SIKW1	AA60471		

According to the classification scheme, all “true” lipases are grouped in family I, which is divided into seven subfamilies. Subfamilies I.1 and I.2 encompass the previously described *Pseudomonas* groups I and II lipases (Gilbert, 1993; Jaeger *et al.*, 1994; Svendsen *et al.*, 1995), together with their cognate intramolecular chaperones, which have been designated Lif (lipase-specific foldases). The lipases belonging to subfamily I.3 have in common a higher molecular mass than lipases from subfamilies I.1 and I.2 and the absence of an N-terminal signal peptide. Furthermore, these lipases are secreted via the type I pathway, whereas those belonging to subfamilies I.1 and I.2 use the type II secretion pathway (Rosenau and Jaeger, 2000). The various *Bacillus* lipases constitute subfamily I.4 and have in common that an alanine residue replaces the first glycine in the conserved pentapeptide Ala-X-Ser-X-Gly. The lipases from the two mesophilic strains *B. subtilis* and *B. pumilus* are the smallest true lipases known (*ca.* 20 kDa) and share very little amino acid sequence similarity with the other *Bacillus* lipases (Arpigny and Jaeger, 1999). Subfamily I.5 comprises the lipases from different *Geobacillus* species and these lipases possess similar properties. Their molecular mass is *ca.* 45 kDa and they display maximal activity at pH 9 and 65°C (Schmidt-Dannert *et al.*, 1996; Kim *et al.*, 1998). Subfamily I.6 is comprised exclusively of *Staphylococcus* lipases, which are large enzymes (*ca.* 75 kDa), and they are secreted as precursors that are cleaved in the extracellular medium by a specific protease (Simons *et al.*, 1997; Gotz *et al.*, 1998; Rosenstein and Gotz, 2000). All other remaining lipases are grouped in subfamily I.7.

The esterases grouped in family II do not exhibit the conventional pentapeptide Gly-X-Ser-X-Gly but rather display a Gly-Asp-Ser-Leu [GDSL] consensus sequence. In these enzymes, the active-site serine residue lies much closer to the N-terminus than in other lipolytic enzymes (Upton and Buckley, 1995). Moreover, crystallographic analyses of the esterase from *Streptomyces scabies* revealed that the catalytic centre of the enzyme forms a Ser-His dyad instead of the common Ser-Asp-His triad (Wei *et al.*, 1995). The acidic side chain, which usually stabilizes the positive charge of the active-site histidine residue, is replaced by the backbone carbonyl of Trp<sub>315</sub> located three positions upstream of the His itself. The enzyme also has an  $\alpha/\beta$  tertiary fold, which differs substantially from that of the  $\alpha/\beta$ -hydrolase family. Another novel feature of the GDSL esterases from *Pseudomonas aeruginosa*, *Salmonella typhimurium* and *Photobacterium luminescens* is the presence of an additional C-terminal domain that is similar to that of autotransporting bacterial virulence factors (Loveless and Saier, 1997; Henderson *et al.*, 1998). In these autotransporting proteins, the C-terminal domain is folded into several amphipathic  $\beta$ -sheets forming an



aqueous pore in the bacterial outer membrane. The catalytic N-terminal domain transits through this pore and the enzyme remains bound to the outer membrane (*P. aeruginosa* and *S. typhimurium*) or is released into the extracellular medium by a specific proteolytic process (*P. luminescens*) (Rosenau and Jaeger, 2000).

Much less is known regarding the lipolytic enzymes grouped in families III through VI. Family III contains extracellular lipases and an esterase of which information is available for the *Streptomyces* spp. lipases (Cruz *et al.*, 1994; Wei *et al.*, 1998). The enzymes display the canonical fold of  $\alpha/\beta$ -hydrolases and contain a typical catalytic triad. Lipases and esterases displaying high amino acid sequence similarity to the mammalian hormone-sensitive lipase (HSL) family (Hemila *et al.*, 1994) have been grouped into family IV and contains esterases from psychrophilic (*Moraxella* sp.), mesophilic (*Escherichia coli*), as well as thermophilic (*Archaeoglobus fulgidus*) origins. Enzymes grouped in family V, like proteins in the HSL family, originate from mesophilic bacteria (*Pseudomonas oleovorans*, *Acetobacter pasteurianus*), as well as from cold-adapted (*Moraxella* sp.) or heat-adapted (*Sulfolobus acidocaldarius*) organisms. However, in contrast to lipolytic enzymes grouped in family IV, these enzymes share significant amino acid sequence similarity to various non-lipolytic enzymes, *e.g.* dehalogenases and haloperoxidases (Verschuere *et al.*, 1993; Misawa *et al.*, 1998). The enzymes grouped in family VI are among the smallest esterases known and have a molecular mass in the range of 23-26 kDa. Very little is known about the enzymes in this family, except for a carboxyl esterase from *P. fluorescens*, of which the 3D structure has been solved (Kim *et al.*, 1997). The esterase is active as a dimer, has a typical Ser-Asp-His catalytic triad and hydrolyses small substrates with a broad specificity, but displays no activity towards long-chain triglycerides (Hong *et al.*, 1991).

Esterases from family VII are large enzymes (*ca.* 55 kDa) and share significant amino acid sequence homology to eukaryotic acetylcholine esterases and intestine or liver carboxyl esterases (Arpigny and Jaeger, 1999). The esterase from *B. subtilis* was found to efficiently hydrolyse *p*-nitrobenzyl esters (Zock *et al.*, 1994; Moore and Arnold, 1996) and has consequently been used in the final removal of the *p*-nitrobenzyl ester used as a protecting group in the synthesis of  $\beta$ -lactam antibiotics (Zock *et al.*, 1994). The esterase from *Arthrobacter oxydans* is plasmid-encoded and is therefore potentially transmissible to other strains or species. It is, however, active against phenylcarbamate herbicides by hydrolysing their central carbamate bond (Pohlenz *et al.*, 1992; Jaeger *et al.*, 1994).

Enzymes belonging family VIII show high homology to several class C  $\beta$ -lactamases (Galleni *et al.*, 1988). These enzymes contain a Gly-X-Ser-X-Gly motif and a Ser-X-X-Lys motif, which is an active site found in the class C  $\beta$ -lactamases (Lobkovsky *et al.*, 1993; Nishizawa *et al.*, 1995). However, it has recently been demonstrated by site-directed mutagenesis studies of an esterase (EstB) from *Burkholderia gladioli* that the Gly-X-Ser-X-Gly motif does not play a significant role in enzyme function (Petersen *et al.*, 2001). In addition, the Gly-X-Ser-X-Gly motif is not conserved in the *Arthrobacter globiformis* esterase (Nishizawa *et al.*, 1993), which stereoselectively forms an important precursor of pyrethrin insecticides (Nishizawa *et al.*, 1995).

## 1.5 INDUSTRIAL APPLICATIONS OF HYDROLYTIC ENZYMES

Hydrolases are considered excellent alternatives to classical organic techniques in the selective transformation of complex molecules, as they impart specificity to a reaction in which a chemical process is typically more non-specific. In addition, enzyme-catalysed processes offer cost-effectiveness in comparison with traditional downstream processing in which energy consumption and toxic by-products might often present a problem (Davis and Boyer, 2001). The industrial deployment of hydrolase enzymes can either be *in situ* by cultivating the desired microorganism in the medium with a suitable substrate (especially in the food industry) (Benjamin and Pandey, 1997; 1998a; Matsumoto *et al.*, 1998), or by *ex situ* application by using purified commercial enzymes (particularly in the making of fine chemicals) (Tengerdy, 1998; Liese *et al.*, 2001).

### 1.5.1 Applications of esterases

Although a considerable number of microbial carboxyl esterases are known of which some have been overexpressed in suitable hosts (Zock *et al.*, 1994; Nishizawa *et al.*, 1995; Khalameyzer and Bornscheuer, 1999; Manco *et al.*, 2000), only a few of them have been used for the synthesis of optically pure compounds. The major reasons for this are their limited commercial availability and their frequently observed moderate enantioselectivity (Faber, 1997). Nevertheless, esterases are starting to make an impact in the pharmaceutical and fine chemical industries where they are used in the resolution of chiral drugs and the production of optically pure compounds. Esterases have furthermore found application in the food industry for the development of flavour in foodstuffs.

### 1.5.1.1 Synthesis of optically pure compounds

Microbial esterases have been used for the kinetic resolution of secondary alcohols by enantioselective hydrolysis of the corresponding acetate. An esterase from *Bacillus subtilis* var. *niger* has been employed in the preparation of chiral cyclohexyl and racemic alkynyl alcohols in a process that involves enantioselective hydrolysis of the corresponding acetates. This has yielded optically-active alcohols of which the optical purity ranges between 7 to 90% (Phytian, 1998). A recombinant esterase from *P. fluorescens* has been reported to exhibit very high enantioselectivity in the resolution of  $\alpha$ -phenyl ethanol (Krebsfanger *et al.*, 1998), which is used as a chiral auxiliary or reagent in its optical pure form.

Many biologically-active compounds exist as a mixture of stereoisomers, but in most cases the activity is restricted to only one of the isomers. For example, Naproxen, a non-steroidal anti-inflammatory drug, exists as an enantiomer with an (*R,S*) configuration, but the *S*-isomer is up to 150-times more active than the *R*-isomer and the *R*-isomer also gives rise to unwanted gastro-intestinal disorders (Quax and Broekhuizen, 1994). An esterase originating from *B. subtilis*, carboxyl esterase NP (NP from naproxen), has been used to hydrolyse the (*R,S*)-naproxen methylester yielding (*S*)-naproxen with excellent optical purity (99% enantiomeric excess) at an overall yield of 95% (Quax and Broekhuizen, 1994). Carboxyl esterase NP has also been used in the resolution of the (*R,S*)-ibuprofen methylester and showed higher selectivity compared to a *Candida rugosa* lipase (Mustranta, 1992). Using an esterase from *A. globiformis*, a similar high efficiency in kinetic resolution was achieved in the synthesis of (+)-*trans*-(1*R*,3*R*)-chrysanthemic acid, which is an important precursor of pyrethrin insecticides (Nishizawa *et al.*, 1993). The esterase was reported to catalyse the sole formation of the desired enantiomer with high optical purity (>99% enantiomeric excess at 77% conversion).

### 1.5.1.2 Food Industry

Structural biomolecules of plant cell walls, including xylan, pectin and lignin, occur naturally as esterified forms. Not only are microbial esterases capable of degrading or modifying the polysaccharides, which are important in the extraction and utilization of plant material for biotechnology and in the texture and digestibility of food (McKay, 1993), but they have also been used for the development of flavour in foods (Kugimiya *et al.*, 1992). The most prominent example of the use of esterases in these processes is the application of carboxyl

esterases to release ferulic acid from cell wall polysaccharides such as pectin or xylan. In xylan, ferulic acid is attached to arabinose residues, which are bound to the xylan backbone. In pectins, ferulic acid can be linked to galactose or arabinose in side chains. Ferulic acid thus obtained can be converted enzymatically into vanillin, a major flavour compound (Lesage-Meessen *et al.*, 1996; Gasson *et al.*, 1998). Feruloyl esterases, used in the above process, have been isolated from a wide range of microorganisms, including *Penicillium* sp., *Aspergillus niger*, *Orpinomyces* sp. and *Clostridium thermocellium* (Christov and Prior, 1993; de Vries and Visser, 1999; Blum *et al.*, 2000; Kroon *et al.*, 2000). In addition, the 2-*O* or 3-*O* positions of arabinose are often acetylated and the acetyl group can be removed by the action of specific acetyl xylan esterases (Lorenz and Wiegel, 1997; Blum *et al.*, 1999; Degrassi *et al.*, 2000).

### 1.5.2 Applications of lipases

Although lipases have been studied for many years, the use of lipases was initially confined mainly to the products and processes of oleochemistry and dairy-based industries (MacRae and Hammond, 1992). The last two decades have, however, witnessed an increased usage of lipases in the pharmaceutical and pesticide industries and, more recently, in the production of cosmetics.

#### 1.5.2.1 Application of lipases as hydrolases

Lipases have become an integral part of the food industry (McKay, 1993) and have been used for the production of desirable flavours in cheese (Stead, 1986), concentrated milk and creams (Kilcawley *et al.*, 1998), and for the interesterification of fats and oils to produce modified acylglycerols, which cannot be obtained by conventional chemical interesterification (Ziller, 1994). Lipases have also been used for the transesterification of cheap edible oil, *e.g.* palm oil mid-fraction, to produce cocoa butter equivalents that are used in chocolate (Harwood, 1989). By making use of a mixed hydrolysis and synthesis reaction, a cocoa butter substitute can be produced using an immobilized *R. miehei* lipase, which carries out a transesterification reaction replacing palmitic acid with stearic acid in cocoa butter to give the desired stearic-oleic-stearic triglyceride.

Although lipases of different origins have been used in bioremediation of oil spills during rigging and refining, as well as in the disposal of lipid-tinged wastes in lipid processing

factories and restaurants (Salleh *et al.*, 1993; Marin *et al.*, 1995), the most commercially important field of application for hydrolytic lipases is, however, their addition to detergents (Grant *et al.*, 1990; Pandey *et al.*, 1999). Lipases are generally added to the detergents primarily in combination with proteases and cellulases. At present, lipases are in extensive use in household detergents, which are used mainly for household laundry and dishwashers, and in industrial cleaners (Misset *et al.*, 1994; Jaeger and Reetz, 1998). To be a suitable additive in detergents, lipases should be both thermophilic and alkalophilic and resistant to the effects of chemical denaturation and/or proteolytic degradation caused by detergent additives such as surfactants and proteases (MacRae and Hammond, 1992). Since most lipases have been reported to be optimally active at neutral pH in the temperature range 35-48°C (Jaeger *et al.*, 1999), protein engineering has been used to enhance lipase properties. Lipomax<sup>TM</sup> and Lipolase<sup>TM</sup>, which were introduced by Novo Nordisk, have been developed by genetic and protein engineering of the *Pseudomonas alcaligenes* lipase (Misset *et al.*, 1994) and the lipase from *Thermomyces lanuginosus* (formerly known as *Humicola lanuginosa*) (Omar *et al.*, 1987; Maheshwari *et al.*, 2000), respectively. These engineered enzymes display elevated activity at washing conditions, such as alkaline pH (7-11), high temperature (up to 60°C) and in the presence of non-ionic and anionic detergents.

#### 1.5.2.2 Application of lipases as synthetases

Key intermediates in the synthesis of therapeutics, agrochemicals and flavour compounds are usually complex and/or chiral compounds, which are difficult to synthesize with chemical methods. Furthermore, just one out of two enantiomers is functional, making the synthesis of enantiopure building blocks an important task for the pharmaceutical (Patel, 2001), pesticide (Ngooi *et al.*, 1990) and cosmetic (Jaeger and Reetz, 1998) industries.

Lipases have been used successfully in the biomedical field to catalyse synthetic reactions, which lead to the production of life-saving drugs. Efficient kinetic resolution processes have been developed for the preparation of optically active homochiral intermediates for the synthesis of non-steroidal anti-inflammatory drugs (ibuprofen, suprofen and ketoprofen), the anti-viral agent lamivudine and for the enantiospecific synthesis of anti-tumour agents, alkaloids and antibiotics (Itoh *et al.*, 1993; Yoshinari *et al.*, 1994; Milton *et al.*, 1995; Goto *et al.*, 1996; Kato *et al.*, 2001; Ono *et al.*, 2001).



In the field of pesticide biotechnology, *P. fluorescens* lipase has been used successfully for the enantioselective transesterification of a racemate (*R,S*)-4-methyl-1-heptyn-4-en-3-ol, which is a component of the insecticide S-2852 (Pandey *et al.*, 1999). The lipase-catalysed resolution of 2-(4-propan-2-yloxy)-phenylpropionic acid, an intermediate in the synthesis of a chiral acaricide, was described by Bosetti *et al.* (1994), using lipase from yeast, fungi and bacteria. The stereoselective enantio-discrimination of *C. rugosa* lipase yielded an optically pure propionic acid derivative in the (*S*)-form. The (*S*)-form was then converted to the corresponding *R*-form, which was found to be very effective as an ovicide against the glasshouse spider mite, *Tetranychus urticae* (Bosetti *et al.*, 1994). Avdagic *et al.* (1994) reported the production of enantiomerically pure (*S*)-(-)-fenpropimorph, a systematic fungicide, with *P. cepacia* lipase-mediated kinetic resolution. Lipases are also used in the efficient production of enantiopure (*S*)-indanofan. Only the (*S*)-enantiomer showed herbicidal activity and it is being used as a novel herbicide against grass weeds in paddy fields (Tanaka *et al.*, 2002).

Due to its activity in the presence of surfactants, which is one of the main ingredients in cosmetics and perfumes, lipases are used in aroma production in the cosmetic and perfume industry. The most prominent flavour and fragrance compound in perfumes is (-)-menthol. Enantiomerically pure (-)-menthol esters can be isolated by a process involving a transesterification step with ( $\pm$ )-menthol using a *Burkholderia cepacia* lipase. The final product, menthylmethacrylate, has the potential to be used as a sustained release perfume (Athawale *et al.*, 2001). Furthermore, rose oxide, which is another important fragrance ingredient in the perfume industry, has been prepared by the transesterification of 3,7-dimethyl-4,7-octadien-1-ol with lipases from various microbial sources (Izumi *et al.*, 1997). The plant growth factor (-)-methyl jasmonate is also an important perfumery constituent and has been synthesized with a lipase-catalysed reaction using the commercially available Lipase P (Amano) to yield the chiral key intermediate (+)-(6*S*)-methyl-7-epicucurbate (Kiyota *et al.*, 2001).



## 1.6 RECOMBINANT PROTEIN EXPRESSION

The objective of gene cloning for biotechnological purposes is often the expression of the cloned gene in a selected host organism. Although *Escherichia coli* is usually the principle bacterium of choice for cloning genes and expressing recombinant proteins, it is unlikely that a single organism, such as *E. coli*, will be suitable for every application and the choice of host bacterium is thus primarily influenced by the application at hand. Consequently, many different bacteria have been developed as alternative hosts to *E. coli*, but the availability of tools for their genetic manipulation has generally dictated the extent of their utilization. In this part of the literature review, the focus will be on prokaryotic expression systems and experimental approaches that may be useful for the overproduction of proteins will be highlighted.

### 1.6.1 Transcriptional regulation

#### 1.6.1.1 Promoters

The minimum requirement for an effective gene expression system is the presence of a promoter sequence upstream of the cloned gene. Such a promoter should, however, have several properties to render it suitable for high-level protein synthesis (Goldstein and Doi, 1995). The promoter must be strong, resulting in the accumulation of protein making up 10 to 30% or more of the total cellular protein. The promoter should exhibit a minimal level of basal transcriptional activity as large-scale gene expression usually employs cell growth to high density and minimal promoter activity, followed by induction or derepression of the promoter. The tight regulation of a promoter is also essential for the synthesis of proteins that may be detrimental to the host cell (Wülfling and Plückthum, 1993; Suter-Crazzolaro and Unsicker, 1995). Furthermore, incompletely repressed expression systems may cause plasmid instability, a decrease in cell growth rate and consequently, may prevent the efficient production of recombinant protein (Chen *et al.*, 1991; Mertens *et al.*, 1995).

Promoters often consist of a hexanucleotide sequence located *ca.* 35 bp upstream of the transcription initiation base (-35 region) and is separated by a short 17-bp spacer sequence from another hexanucleotide sequence (-10 region) (Hawley and McClure, 1983; Old and Primrose, 1994). Many promoters used to transcribe heterologous genes in *E. coli* have been constructed from *lac*-derived regulatory elements. Although the *lac* promoter is rather weak and rarely used for high-level production of recombinant proteins, they are valuable tools to



achieve graded expression of helper or toxic proteins (Hashemzadeh-Bonehi *et al.*, 1998). The synthetic *tac* and *trc* promoters, which consist of the -35 region of the *trp* promoter and the -10 region of the *lac* promoter, only differ by 1 bp in the length of the spacer domain separating the two hexamers (Friesen and An, 1983). Both promoters are quite strong and routinely allow the accumulation of polypeptides to about 15-30% of the total cell protein. Additional promoters that are nutritionally inducible, *e.g.* *trp* and *araBAD*, which are induced by tryptophan limitation and the inexpensive sugar L-arabinose, respectively, have also been used, but they are somewhat weaker than the *tac* promoter (Guzman *et al.*, 1995; Newman and Fuqua, 1999). These promoters are recognized by the sigma ( $\sigma$ ) 70-RNA polymerase holoenzyme, which is present in much larger quantities than other minor sigma factors in bacterial cells (Record *et al.*, 1996). Consequently, transcription is not limited because of a shortage of available sigma factor.

Although the development of systems for high-level controlled expression of genes in *Bacillus* is not as well advanced as it is in *E. coli*, promoters that are constitutively expressed, as well as promoters whose expression may be controlled by chemical or temperature induction, have been developed for *B. subtilis* (Henner, 1990a; 1990b; Le Grice, 1990; Conrad *et al.*, 1996). These include promoters from well-characterized *Bacillus* genes such as the stationary phase-induced  $\alpha$ -amylase gene from *B. amyloliquefaciens* and the sucrose-inducible *sacR* control region of *B. subtilis* levansucrase. In addition to promoters isolated from Gram-positive bacteria and their phages, a series of promoters has been developed for use in *Bacillus* strains that are either entirely of Gram-negative origin or which contain elements of both Gram-positive and Gram-negative origin. Such promoters have been used for high-level, controlled synthesis of heterologous proteins and include the hybrid P25/0 promoter system, consisting of the phage T5 promoter P<sub>N25</sub> and the *E. coli lac* operator (Williams *et al.*, 1981; Osbourne *et al.*, 1985; Le Grice, *et al.*, 1986), and the hybrid *spac-1* promoter system, consisting of the phage SPO1 promoter and the *E. coli lac* operator (Yansura and Henner, 1984). Both of these promoters are induced by isopropyl  $\beta$ -D-thiogalactoside (IPTG).

### 1.6.2 Transcriptional terminators

In prokaryotes, transcription termination is effected by either a Rho-dependent or Rho-independent mechanism (Richardson, 1993). Rho-dependent transcription termination



depends on the hexameric protein Rho, which causes the release of the nascent RNA transcript from the template. In contrast, Rho-independent termination depends on signals encoded in the template, specifically, a region of dyad symmetry that encodes a hairpin or stem-loop structure in the nascent RNA and a second region that is rich in dA and dT and is located 4 to 9 bp distal to the dyadic sequence (Richardson, 1993; Wilson and von Hippel, 1995). Efficient transcription terminators are indispensable elements of expression vectors, because they serve several important functions. Transcription through a promoter may inhibit its function, a phenomenon known as promoter occlusion (Adhya and Gottesman, 1982). This interference can be prevented by the proper placement of a transcription terminator downstream of the coding sequence to prevent continued transcription through another promoter. Similarly, a transcription terminator placed upstream of the promoter that drives expression of the gene of interest minimizes background transcription (Nishihara *et al.*, 1994).

### **1.6.3 Translational regulation**

#### **1.6.3.1 Translational issues**

Putting a cloned gene under control of a regulatable, strong promoter, although essential, may not be sufficient to maximize the yield of the cloned gene product. Other factors, such as the efficiency of translation and the stability of the newly transcribed mRNA may also affect the amount of product. Because of the close coupling between transcription and translation in prokaryotes, engineering of the translation initiation region is considered a powerful tool for modulating gene expression in a promoter-independent fashion (Ringquist *et al.*, 1992). Initiation of translation of prokaryotic mRNAs requires a Shine-Dalgarno (SD) sequence complementary to the 3' end of 16S rRNA, followed by an initiation codon, which is most commonly AUG (Shine and Dalgarno, 1974; Gualerzi and Pon, 1990). Although the optimal spacing between these two features is 8 nt, translation initiation is only severely affected if this distance is reduced below 4 nt or increased above 14 nt (Chen *et al.*, 1994; Ringquist *et al.*, 1992). Stable mRNA secondary structures encompassing the SD sequence and/or the initiation codon have been reported to dramatically reduce gene expression by interfering with ribosome binding (Hall *et al.*, 1982; de Smit and van Duin, 1990). It is believed that occlusion of the SD region and/or the AUG codon by a stem-loop structure precludes the accessibility to the 30S ribosomal subunits and inhibits translation (Ramesh and De Nagaraja, 1994). This problem can be circumvented by increasing the homology of SD regions to the

consensus (Coleman *et al.*, 1985; Stanssens *et al.*, 1985) and by raising the number of A residues in the initiation region through site-directed mutagenesis (Olsen *et al.*, 1989; Chen *et al.*, 1994).

#### 1.6.4 mRNA stability

The process of mRNA degradation provides a major control point of gene expression in virtually all organisms (Ross, 1995). Prokaryotic mRNAs are rather unstable, with half-lives ranging between 30 s and 20 min. The major enzymes involved in mRNA degradation are two 3' → 5' exonucleases (RNase II and polynucleotide phosphorylase) and the endonuclease RNase E (Carpousis *et al.*, 1999; Coburn and Mackie, 1999). The catalytic activity of RNase E is located at the N-terminus, whereas the C-terminus serves as a scaffold for the assembly of a highly efficient “degradasome” involving PNPase, the RNA helicase RhlB and endolase. Two classes of protective elements are known to stabilize mRNAs in bacteria and may subsequently be used to prolong the half-life of heterologous mRNAs (Duvoisin *et al.*, 1986; Chen *et al.*, 1991; Emory *et al.*, 1992). One class consists of sequences in the 5' untranslated regions (UTRs) of mRNAs, and the other class consists of 3' UTR sequences that can form stem-loop structures, thereby blocking exonucleolytic degradation of the transcripts from the 3' terminus (Wong and Chang, 1986). In either case, stable secondary structures present in the 5' UTR of certain transcripts, as well as 3' Rho-independent terminators can both increase mRNA stability and consequently increase the level of protein production (Wong and Chang, 1986; Ehretsmann *et al.*, 1992; Carpousis *et al.*, 1999).

#### 1.6.5 Protein targeting

In contrast to *E. coli*, *Bacillus* species have been regarded as attractive hosts for the production of homologous and heterologous secretory proteins. In *E. coli* and other Gram-negative bacteria, secretion of extracellular proteins involves passing through the periplasm in addition to two membranes, the inner or cytoplasmic membrane, which surrounds the cytoplasm, and the outer membrane, which encloses the periplasm and acts as a barrier to the environment. The cell envelope of *B. subtilis* is, however, much simpler than that of *E. coli*, since it has no outer membrane, associated lipopolysaccharides (LPS) or a membrane-enclosed periplasm (Simonen and Palva, 1993). The absence of lipid A moieties and an outer



membrane thus simplify the secretion process, since exoproteins need only be translocated through a single membrane (and the cell wall) before reaching the culture medium.

#### 1.6.5.1 Production and secretion of heterologous proteins in *E. coli*

Overproduction of heterologous proteins in the cytoplasm of *E. coli* is often accompanied by their misfolding and segregation into insoluble aggregates known as inclusion bodies (Rudolph and Lilie, 1996). In addition, protein degradation is more likely to occur in the cytoplasm than in other cell compartments, because of the greater number of proteases located there (Talmadge and Gilbert, 1982; Gottesman, 1996). Another difficulty that affects cytosolic gene expression is the need to purify the soluble target protein from the pool of intracellular proteins. Although inclusion body formation can greatly facilitate protein purification, there is no guarantee that the subsequent *in vitro* refolding will yield large amounts of biologically active product. Some traditional approaches to reduce protein aggregation include growth of bacterial cultures at lower temperatures (Cabilly, 1989; Schein, 1993), substitution of selected amino acid residues (Rinas *et al.*, 1992; Dale *et al.*, 1994), the co-production of chaperones to facilitate the correct folding of the protein (Battistoni *et al.*, 1993) and the use of *E. coli* thioredoxin as a fusion partner (La Vallie *et al.*, 1993; Yasukawa *et al.*, 1995).

By contrast to the cytoplasm, the periplasm offers several advantages for protein targeting. The periplasm contains only 4% of the total cell protein (Pugsley and Schwartz, 1985). The target protein can thus be effectively concentrated and its purification is considerably less tedious. The oxidizing environment of the periplasm facilitates the proper folding of proteins and protein degradation in the periplasm is thus less extensive (Talmadge and Gilbert, 1982; Missiakas and Raina, 1997). Despite these advantages, protein transport to the bacterial periplasm is a complex process and requires a signal sequence (Thanassi and Hultgren, 2000). Most signal sequences derived from naturally occurring secretory proteins support the efficient translocation of heterologous polypeptides across the inner membrane when fused to their N-termini. In some cases, preproteins are not readily exported and either become lodged in the inner membrane, accumulate in precursor inclusion bodies or are readily degraded within the cytoplasm (Wilcox and Studnicka, 1988; Cheah *et al.*, 1994; Gottesman, 1996). In such instances, translocation of proteins to the periplasm may be improved by

overproduction of components involved in protein transport and processing (Van Dijn *et al.*, 1991).

The targeting of synthesized proteins for secretion to the culture medium presents advantages such as a low level of proteolysis, simpler purification strategies and improved folding (Kitai *et al.*, 1988). However, *E. coli* normally secretes very few proteins and the manipulation of the various transport pathways to facilitate secretion of foreign proteins remains a formidable task (Pugsley, 1993). Nevertheless, the methodological approaches to protein secretion can be divided into two categories, *i.e.* the exploitation of existing pathways for “truly” secreted proteins (Stader and Silhavy, 1990), and the use of signal sequences, fusion partners, permeabilizing proteins or other agents that may effect protein secretion as a result of “leakage” or selective and limited permeability of the outer membrane (Suominen *et al.*, 1987; Ko *et al.*, 1995).

#### **1.6.5.2 Production and secretion of heterologous proteins in *Bacillus* species**

Exoprotein genes of Gram-positive bacteria are usually expressed in *Bacillus* species with their own promoters. The proteins can be secreted to the medium by the aid of their own secretion signals, and the use of specific secretion vectors is therefore not necessary (Bolhuis *et al.*, 1999). In contrast, the proteins of Gram-negative bacteria are generally secreted in *Bacillus* species by the aid of secretion vectors based on promoters and signal sequences of various *Bacillus* exoenzymes. Promoters and ribosome-binding sites of Gram-positive origin must be used, since those of Gram-negative bacteria are often non-functional in *Bacillus* species. The yield of the secreted protein, however, depends mainly on the expression system applied and on the efficiency of the means used to protect the foreign protein against the exoproteases of the host.

The proteases secreted by *Bacillus* species severely affect the production and secretion of foreign proteins. *B. subtilis* produces at least seven extracellular proteases, which cause substantial degradation of secreted foreign proteins (Wu *et al.*, 1991). Several approaches have been used to overcome the problem of degradation of the secreted proteins. Protease inhibitors, *e.g.* PMSF and EDTA, have been used to protect the foreign proteins. These increase the yield slightly, but their use in large-scale production is not feasible owing to their toxicity and high cost, and their adverse affects on the growth and physiology of the bacteria



(Coxon *et al.*, 1991). *Bacillus* strains lacking all but one of the protease enzymes have also been constructed, thereby reducing the level of protease activity in the culture medium to less than 0.5% of that of the wild-type (Wu *et al.*, 1991). However, protease-deficient strains are still able to degrade foreign secretory proteins (Nakamura *et al.*, 1991; Bolhuis *et al.*, 1999) and have been reported to lyse in the stationary phase much more readily than protease-proficient strains (Joliff *et al.*, 1989; Coxon *et al.*, 1991), possibly as a consequence of decreased protease action on autolytic enzymes. A solution might therefore be to use *Bacillus* strains that naturally secrete either very small amounts of proteases or none at all. For example, the extracellular protease activity of *B. brevis* 47 is only 1.6% of the level of *B. subtilis* (Udaka and Yamagata, 1993). Alternatively, the foreign proteins can be produced in the exponential growth phase, when relatively small amounts of proteases are secreted (Edelman *et al.*, 1988; Dion *et al.*, 1989).

Proteins exiting on the external surface of the *B. subtilis* cytoplasmic membrane encounter the cell wall before they are free to enter the culture medium. The cell wall of *B. subtilis* is a highly crosslinked semi-porous structure, comprised primarily of peptidoglycan and teichoic or teichuronic acid, which form a negatively-charged network around the cell (Harwood *et al.*, 1990). Although some exoproteins may be able to diffuse freely through the cell wall, other exported proteins may remain in the wall for a long period (Simonen and Palva, 1993). Such proteins may be entrapped in the cell wall because of their size, shape or charge, and they are possibly pushed outwards from the cell membrane by the growth of the cell wall, which occurs outwards from the cytoplasmic membrane (Merad *et al.*, 1989). *Bacillus* species other than *B. subtilis* or strains with slightly different cell wall structures could be more suitable hosts for the secretion of proteins that remain entrapped in the cell wall. *B. brevis*, for example, has a much thinner peptidoglycan layer than *B. subtilis*, and this could facilitate the secretion of proteins through the cell wall (Udaka and Yamagata, 1993).

## 1.6.6 Other factors of importance

### 1.6.6.1 Metabolic load

The introduction and expression of foreign DNA in a host organism often change the metabolism of the organism in ways that may impair normal cellular functioning. The phenomenon, which is a multifaceted biological response, is due to a metabolic load that is imposed upon the host by the foreign DNA. A metabolic load can occur as the result of a

variety of conditions (Glick and Whitney, 1987; Glick, 1995). For example, increasing plasmid copy number and/or size requires increasing amounts of cellular energy for plasmid replication and maintenance, and the limited amount of dissolved oxygen in the growth medium is often insufficient for both host cell metabolism and plasmid maintenance and expression. In such cases, plasmid-bearing cells grow more slowly than untransformed cells, often resulting in the loss of the recombinant plasmid or a portion of the plasmid DNA. Since cells growing in the presence of a metabolic load generally have a decreased level of energy available for a variety of cellular functions, the cell's energy-intensive metabolic processes such as protein synthesis are invariably adversely affected by a metabolic load (Glick and Whitney, 1987). Furthermore, overproduction of foreign proteins may deplete the pools of certain aminoacyl-tRNAs and/or drain the host cell of its energy in the form of ATP or GTP. This may result in the initiation of a cellular stress response, which includes increased synthesis of cellular proteases, so that the overexpressed recombinant protein is rapidly degraded (Glick, 1995).

#### **1.6.6.2 Plasmid copy number and maintenance**

To achieve high gene dosage, the genes to be expressed are typically cloned into plasmids that replicate in a relaxed fashion and are present at high copy numbers, ranging from 15 to a few hundred copies per cell. Under laboratory conditions, such multicopy plasmids are randomly distributed during cell division and, in the absence of selective pressure, are lost at low frequency, primarily as a result of multimerization (Summers, 1998). However, plasmid loss can increase in the case of very high copy number plasmids, when plasmid-borne genes are toxic to the host or otherwise significantly reduce its growth rate, or when cells are cultivated at high density or in continuous processes (Summers, 1998).

The simplest way to address this problem is to take advantage of plasmid-encoded antibiotic resistance-markers and supplement the growth medium with antibiotics to kill plasmid-free cells. The drawbacks of this approach are loss of selective pressure as a result of antibiotic degradation, or inactivation and the contamination of the product or biomass by antibiotics, which may be unacceptable in the production of human therapeutic proteins (Glick and Whitney, 1987; Old and Primrose, 1994). A number of alternative strategies have therefore been developed to ensure that plasmid-free cells will not overtake a culture. In most cases, cloning vectors are engineered to carry gene(s) or repressors that cause cell death upon



plasmidloss (Williams *et al.*, 1998). Alternatively, the heterologous genes may be directly inserted within the chromosome of the host organism (Chopin *et al.*, 1989; Olson *et al.*, 1998; Hinds *et al.*, 1999). In this case, problems associated with plasmid instability may be overcome and the transformed host cell will also not waste its resources synthesizing unwanted and unneeded antibiotic resistance-marker gene products.

## 1.7 AIMS OF THIS STUDY

Carboxyl esterases (EC 3.1.1.1) and lipases (EC 3.1.1.3) are enzymes that hydrolyse carboxylate esters and are widespread in various organisms including animals, plants and microorganisms. As many of these enzymes exhibit activity in organic solvents, they have become two of the most widely used enzymes in organic synthesis (Jaeger and Reetz, 1998; Phytian, 1998; Bornscheuer and Kazlauskas, 1999; Jaeger *et al.*, 1999; Bornscheuer, 2002). These enzymes are able to perform a number of valuable biotransformations, *e.g.* resolution of racemic mixtures, synthetic reactions, blocking or unblocking of catalytic groups in peptide chemistry, and the modification of sugars (Davis and Boyer, 2001). As more and more enzymatic reactions are applied in industry, an increasing demand for novel biocatalysts is being generated. Approaches whereby such novel enzymes may be obtained include exploitation of the natural biodiversity (Miller, 2000), improvement of enzymes by rational design and directed evolution (Bornscheuer and Pohl, 2001; Chen, 2001), and exploration of the enormous amount of sequence information generated by the various genome sequencing projects (Eggert *et al.*, 2000). However, for biotechnological applications, overexpression of the corresponding gene of interest is often required in an effort to allow the development of economical production processes.

Although the overproduction of enzymes by recombinant cells is widely used for research and commercial purposes, expression systems are quite diverse and there does not exist a single universal strategy for achieving maximal expression of every gene in all bacteria. Despite *E. coli* having been used extensively as a host for the expression of foreign genes (Balbas and Bolivar, 1990; Old and Primrose, 1994), it may not necessarily be the microorganism of choice for the expression of all foreign proteins. Consequently, a number of alternative prokaryotic gene expression systems in a variety of other organisms such as *Bacillus* spp., *Streptomyces* spp. (Binnie *et al.*, 1997) and industrial Gram-positive bacteria with low guanine and cytosine content (*e.g.* *Lactobacillus*, *Lactococcus*, *Staphylococcus* and

*Streptococcus*) (Pouwels and Leer, 1993; Mercenier *et al.*, 1994), have been developed into hosts for heterologous protein production.

Towards the isolation and characterization of novel lipolytic enzymes, the aims of this investigation were the following:

- To screen different *Bacillus* isolates for the production of lipolytic enzymes by growth on tributyrin agar plates.
- To construct and screen a genomic library from a selected *Bacillus* strain in order to isolate gene(s) encoding lipolytic activity.
- To genetically characterize the gene(s) encoding lipolytic activity and to characterize the biochemical properties of the lipolytic enzyme(s).
- To express the gene(s) encoding lipolytic enzyme(s) in *Bacillus* strains and lactic acid bacteria in order to identify the most appropriate host for overexpression of the newly identified enzyme(s).



## **CHAPTER TWO**

# **MOLECULAR CLONING OF A CARBOXYL ESTERASE GENE AND BIOCHEMICAL CHARACTERIZATION OF THE ENCODED PROTEIN FROM *Bacillus* sp. 81-11**

## 2.1 INTRODUCTION

Hydrolases are the most predominant group of enzymes employed in biocatalysis research and in the production of fine chemicals by biocatalytic resolution (Faber, 1997; Schultze and Wubbolts, 1999; Davis and Boyer, 2001). Most important in this class of enzymes are hydrolytic enzymes cleaving carboxylic ester bonds, such as lipases (EC 3.1.1.3) and carboxyl esterases (EC 3.1.1.1), as they accept a broad range of natural and non-natural substrates, are usually stable in organic solvents and exhibit good to excellent stereoselectivity, *e.g.* in the kinetic resolution of racemates or the desymmetrization of prostereogenic compounds (Faber, 1997; Gill and Valivety, 1997). The carboxyl esterases hydrolyse water-soluble or emulsified esters with relatively short fatty acid chains, whereas lipases preferentially act on emulsified substrates with long-chain fatty acids (Tsujita *et al.*, 1990; Verger, 1997; Jaeger *et al.*, 1999). The hydrolytic mechanism of most esterases and lipases resembles that of serine proteases and they all contain a similar catalytic triad, generally consisting of a nucleophilic serine residue that acts in conjunction with a histidine and an aspartic acid residue (Derewenda and Derewenda, 1991; Ollis *et al.*, 1992; Peterson and Drablos, 1994).

Although hydrolases are widely distributed in animals, plants and microorganisms (Okuda, 1991; MacRae and Hammond, 1992), several mesophilic and thermophilic *Bacillus* species have been reported to possess hydrolytic systems well suited for biotechnological applications (Jaeger *et al.*, 1999). Bacteria of the genus *Bacillus* are aerobic, endospore-forming Gram-positive rods and has 48 validly described species (Claus and Fritze, 1989; Holt, 1993). The genus is one of the most ubiquitous and diverse, with representatives being found in the soil, plants and different water and food sources. The metabolic diversity of *Bacilli* has led to members of this group being used for a wide range of industrial processes, including the production of hydrolytic (and other) enzymes, antibiotics, fine biochemicals and insecticides (Harwood, 1992; Yamada *et al.*, 1995; Bolhuis *et al.*, 1999). Lipases from *B. subtilis* (Dartois *et al.*, 1992), *B. licheniformis* (Nthangeni *et al.*, 2001), *B. thermocatenuatus* (Schmidt-Dannert *et al.*, 1996), *Geobacillus thermoleovarans* (Lee *et al.*, 1999) and *G. stearothermophilus* (Kim *et al.*, 1998) have been biochemically characterized, purified or cloned. Due to the growing interest in esterases, as an alternative to the use of lipases, esterases have been purified and partially characterized from *B. subtilis* (Quax and Broekhuizen, 1994; Zock *et al.*, 1994), *G. stearothermophilus* (Wood *et al.*, 1995; Henke and

Bornscheuer, 2002), *B. coagulans* (Molinari *et al.*, 1996), *B. acidocaldarius* (Manco *et al.*, 1994), *B. circulans* (Kademi *et al.*, 2000), *Paenibacillus* sp. BP-23 (Prim *et al.*, 2000) and *Bacillus* sp. BP-7 (Prim *et al.*, 2001).

Research on thermophilic *Bacillus* spp., as promising sources for highly stable enzymes, is an active research subject (Davis, 1998). Thermophiles represent an obvious source of thermostable enzymes, it being reasonable to assume that such character will confer on their proteins a high thermal stability (Adams and Kelly, 1998). Enzymes isolated from these microorganisms are not only thermostable and active at high temperature, but are also often resistant to and active in the presence of organic solvents and detergents (Jaenicke *et al.*, 1996). However, moderate thermophilic *Bacillus* spp. may also be good sources of new thermostable enzymes. The enzymes isolated from such microorganisms can present significant activity at moderate temperatures, a range where most industrial processes are run. By contrast, this is not usually the case for enzymes from thermophiles as their activity at room temperature is usually very low (Kademi *et al.*, 2000).

As hydrolytic enzymes are important biocatalysts in several biotechnological applications, and as more and more enzymatic reactions are being applied in industrial processes, there is an increasing demand for new biocatalysts adapted to special needs. Thus, attempts have been made to improve existing enzymes by rational design and directed evolution (Bornscheuer and Pohl, 2001; Chen, 2001) or, alternatively, to isolate and characterize novel enzymes from the natural biodiversity (Miller, 2000). The objectives of this part of the study were therefore to (i) screen different *Bacillus* isolates for hydrolytic enzymes, (ii) to clone and characterize the gene(s) encoding hydrolytic activity and (iii) to characterize the biochemical properties of the enzyme(s).

## **2.2 MATERIALS AND METHODS**

### **2.2.1 Bacterial strains, plasmids and culturing conditions**

The bacterial strains and plasmids used in this part of the study are listed in Table 2.1. *Escherichia coli* JM83 (Vieira and Messing, 1982) was used as the host in all of the cloning procedures. The strain was cultured at 37°C with rotary shaking aeration at 150 rpm in Luria-Bertani broth (LB; 0.5% [w/v] yeast extract, 1% [w/v] tryptone, 1% [w/v] NaCl; pH

7.4) or maintained on LB agar plates at 4°C. For selection and maintenance of plasmid DNA in *E. coli* JM83, the medium was supplemented with 20 µg/ml of chloramphenicol (Roche Diagnostics) or 100 µg/ml of ampicillin (Roche Diagnostics). *Bacillus* sp. 81-11, which showed lipolytic activity (see below), was cultured in LB broth at 44°C. Plasmids pBS19 and pBC(SK) were used as cloning vectors.

## 2.2.2 Identification of a *Bacillus* isolate displaying lipolytic activity

### 2.2.2.1 Screening of *Bacillus* isolates for the production of lipolytic enzymes

Fifteen *Bacillus* isolates, previously isolated from different foods, seeds and fermenting brews (B. Fabian, unpublished results), were screened for lipolytic enzyme production by streaking a loopful of growth, following overnight incubation at 44°C in LB broth, onto tributyrin agar plates (1% [w/v] tryptone, 0.5% [w/v] yeast extract, 1% [w/v] NaCl, 1% [w/v] tributyrin, 0.1% [w/v] polyvinyl alcohol; pH 7.2) (Mourey and Kilbertus, 1975). The agar plates were incubated at 44°C for 3 days and colonies were inspected daily for a clear zone around their margins. An isolate, designated *Bacillus* sp. 81-11, producing the largest hydrolysis zone was selected and used throughout this study.

### 2.2.2.2 16S rDNA gene analysis

Chromosomal DNA of *Bacillus* sp. 81-11 (Section 2.23.1) was used as template to amplify a 1695-kb region of the 16S rDNA gene by PCR using universal oligonucleotide primers fD1 and rP2 (Table 2.1). The PCR reaction mixture (50 µl) contained 1 × PCR buffer (50 mM KCl, 10 mM Tris-HCl [pH 9.0], 0.1% [v/v] TritonX-100), 1.5 mM MgCl<sub>2</sub>, 0.2 mM of each dNTP, 25 pmol of each primer and 1 U of *Taq* DNA polymerase (Whitehead Scientific). Following an initial denaturation at 94°C for 3 min, the samples were subjected to 30 cycles of amplification in a Techne Progene thermal cycler using the following cycle conditions: denaturation at 94°C for 1 min, primer annealing at 55°C for 1 min and extension at 72°C for 1 min followed by a final extension step at 72°C for 5 min. As a control, a reaction mixture containing distilled water and all other reagents but no template DNA was included in the analysis. Following agarose gel electrophoresis of an aliquot of the reaction mixtures, the PCR product amplified from *Bacillus* sp. 81-11 chromosomal DNA was purified from the gel, sequenced and databases were then searched, as described below (Section 2.2.8.3), for sequences similar to the obtained 16S rDNA sequence.



**Table 2.1:** Bacterial strains, plasmids and primers used

Strain, plasmid or primer	Relevant properties	Source
<b><u>Strains:</u></b>		
<i>E. coli</i> JM83 <i>Bacillus</i> sp. 81-11	{F <sup>-</sup> <i>ara</i> ( <i>lac-proAB</i> ) <i>rpsL</i> (Str <sup>r</sup> ) [80 <i>dlac</i> ( <i>lacZ</i> )M15] <i>thi</i> } Wild-type	Vieira and Messing, 1982 This study
<b><u>Plasmids:</u></b>		
pBS19	<i>E. coli-Bacillus</i> shuttle vector, pBR322- <i>ori</i> , pUB110- <i>ori</i> , Cam <sup>r</sup>	Ferrari, Personal communication
pBC(SK) pSEI	Cloning vector, ColE1, Amp <sup>r</sup> pBS19 with a cloned 3.2-kb <i>Sau3AI</i> DNA fragment from <i>B. coagulans</i> strain 81-11, confers lipolytic activity in <i>E. coli</i> JM83	Stratagene This study
pSEI-PH	pBS19 carrying a 2.4-kb <i>PstI-HindIII</i> DNA fragment from pSEI containing the <i>estC1</i> gene of <i>B. coagulans</i> strain 81-11	This study
pSEI-SA	pBS19 carrying a 1.9-kb <i>SacI-AatII</i> DNA fragment from pSEI	This study
pSEI-AH	pBS19 carrying a 1.3-kb <i>HindIII-AatII</i> DNA fragment from pSEI	This study
pSEI-EE	pBC(SK) carrying a 2.2-kb <i>EcoRI-EcoRI</i> DNA fragment from pSEI	This study
pSEI-NN	pBC(SK) carrying a 1.2-kb <i>NaeI-NaeI</i> DNA fragment from pSEI	This study
<b><u>Primers:</u></b>		
fD1	5' - AGAGTTTGATCCTGGCTCAGT - 3'	Weisburg <i>et al.</i> , 1991
rP2	5' - AGGGCTACCTTGTTACGACTT - 3'	Weisburg <i>et al.</i> , 1991
pUC/M13 Forward	5' - GTTTCCCAGTCACGAC - 3'	Promega
pUC/M13 Reverse	5' - GTAAAACGACGGCCAGT - 3'	Promega

### 2.2.3 Construction of a genomic DNA library from *Bacillus* sp. 81-11

#### 2.2.3.1 Isolation and purification of chromosomal DNA

Chromosomal DNA from *Bacillus* sp. 81-11 was isolated according to the method of Lovett and Keggins (1979), with the following modifications. The bacterial cells from 200 ml of an overnight culture of *Bacillus* sp. 81-11 were collected by centrifugation at 7 000 rpm for 15 min, washed once with TES buffer (30 mM Tris, 5 mM EDTA, 50 mM NaCl; pH 7.5) and then suspended in 50 ml TES buffer. Lysozyme and RNase A were added to the cell suspension to final concentrations of 500 µg/ml and 100 µg/ml, respectively, followed by incubation at 37°C for 25 min. The suspension was then diluted with an equal volume of TES buffer and Proteinase K was added to a final concentration of 100 µg/ml, and Sarkosyl to a final concentration of 0.8% (w/v). Following incubation at 37°C for 30 min, the DNA sample was deproteinized by repeated phenol/chloroform extractions. The chromosomal DNA was precipitated from the final aqueous phase by the addition of two volumes of ice-cold 96% ethanol and incubation at -20°C overnight. The precipitated chromosomal DNA was spooled out on a hooked pasteur pipette, air-dried and suspended in 5 ml TE buffer (10 mM Tris, 1 mM EDTA; pH 8.0) before being dialysed in 3 lt of TE buffer with three buffer changes for 24 h. The DNA concentration was determined at an absorbance of 260 nm, while the purity of the DNA sample was assessed by determining the OD<sub>260</sub>:OD<sub>280</sub> ratio using a WPA Model S2000 spectrophotometer (Labotec).

#### 2.2.3.2 Size fragmentation of chromosomal DNA

The extracted *Bacillus* sp. 81-11 genomic DNA was partially digested with *Sau3AI* (Roche Diagnostics) by incubating 1 µg of genomic DNA with 3 U of enzyme in the appropriate buffer as supplied by the manufacturer. The reaction mixtures were incubated at 37°C for 30 min after which the enzyme was inactivated by heating to 65°C for 10 min in a water bath. The reaction mixtures were analyzed by agarose gel electrophoresis in the presence of an appropriate DNA molecular weight marker (phage lambda DNA digested with *PstI*, *EcoRI* and/or *HindIII*; Roche Diagnostics). The restriction fragments of between 3 to 5 kb were purified from the agarose gel and then used in subsequent cloning procedures. For agarose gel electrophoresis, horizontal 1% (w/v) agarose slab gels were cast and electrophoresed at 85 V in 1 × TAE buffer (40 mM Tris-HCl, 20 mM Acetic acid, 2 mM EDTA; pH 8.5). The

agarose gels were supplemented with ethidium bromide (0.5 µg/ml) in order to allow visualization of the DNA on an UV transilluminator (Sambrook and Russel, 2001).

#### **2.2.3.3 Purification of restriction DNA fragments**

The DNA fragments of interest were excised from ethidium bromide-stained agarose gels with a razor blade and purified using a GeneClean™ kit (Bio101, Inc.). Briefly, three volumes of the supplied 3 M NaI solution were added to the gel slice followed by incubation in a water bath at 55°C for 5 min. After complete dissolution of the agarose, 5 µl glassmilk® was added, mixed and incubated at room temperature for 5 min to allow the DNA to bind to the silica matrix. The silica-bound DNA was recovered by brief centrifugation (10 000 rpm, 30 s) and washed three times with ice-cold 500 µl NEW wash (supplied in the kit). The pellet was air-dried and the DNA was eluted from the silica matrix at 50°C for 2-3 min in a final volume of 10 µl TE buffer. A small aliquot of the eluted DNA was electrophoresed on an agarose gel to assess both its purity and yield.

#### **2.2.3.4 Preparation of pBS19 vector DNA**

To obtain plasmid DNA of high purity, large-scale plasmid DNA extractions were carried out from a 200-ml culture of *E. coli* transformed with plasmid pBS19, using the Qiagen Midiprep kit, according to the manufacturer's instructions. Purified plasmid pBS19 was linearized by digestion with *Bam*HI and subsequently dephosphorylated using calf intestinal alkaline phosphatase (CIAP; Roche Diagnostics) to prevent recircularization of the vector DNA during subsequent ligation reactions. Briefly, 5 µg of the linearized vector DNA was incubated at 37°C for 1 h in a 20-µl reaction mixture containing 1 × CIAP buffer (50 mM Tris-HCl, 0.1 mM EDTA; pH 8.5) and 1.5 U calf intestinal alkaline phosphatase (1 U/µl). Following incubation, the alkaline phosphatase enzyme was inactivated by heating the reaction mixture to 75°C for 15 min. The linearized, dephosphorylated vector DNA was then purified from an agarose gel using the GeneClean procedure described above.

#### **2.2.3.5 Ligation of vector and insert DNA**

The purified size-fractionated genomic DNA from *Bacillus* sp. 81-11 was ligated to the linearized, dephosphorylated pBS19 vector DNA in a reaction mixture containing 2.5 µl of 10 × ligation buffer (660 mM Tris-HCl [pH 7.5], 10 mM DTT, 50 mM MgCl<sub>2</sub>, 10 mM ATP),

1 U of T4 DNA ligase (1 U/ $\mu$ l) and distilled water to a final volume of 25  $\mu$ l. Following incubation at 15°C overnight, the contents of the tube was collected by brief centrifugation and dialysed for 1 h at room temperature before being used in transformation of *E. coli* JM83 cells.

### **2.2.3.6 Transformation of *E. coli* JM83 cells**

#### **2.2.3.6.1 Preparation of competent *E. coli* JM83 cells**

Competent *E. coli* JM83 cells were prepared according to the method of Armitage *et al.* (1988), with the following modifications. An aliquot (2 ml) of an overnight *E. coli* JM83 culture was inoculated into 100 ml of preheated (at 37°C) sterile LB broth and the culture was then incubated at 37°C with shaking until an OD<sub>600</sub> of 0.6 was reached. The culture was chilled on ice for 30 min prior to the cells being harvested by centrifugation at 5 000 rpm for 15 min at 4°C in a Sorvall RC-5B centrifuge. The cells were washed twice with 100 ml of an ice-cold 10% (v/v) glycerol solution and then suspended in 0.4 ml ice-cold GYT medium (10% [v/v] glycerol, 0.125% [w/v] yeast extract, 0.25% [w/v] tryptone; pH 7.3). Aliquots (100  $\mu$ l) of the competent cells were pipetted into 1.5-ml Eppendorf tubes and either used immediately or stored at -70°C until use, with minimal reduction in competency.

#### **2.2.3.6.2 Electroporation of *E. coli* JM83 cells**

DNA was introduced into the *E. coli* JM83 cells by electroporation using the method described by Tung and Chow (1995). For this purpose, aliquots (3  $\mu$ l) of the ligation reaction mixture were added to 100  $\mu$ l of the competent cells, and then transferred to an ice-cold electroporation cuvette (0.1-cm electrode gap; BioRad). The cells were exposed to a single electrical pulse of 1.7 s using a Gene-Pulser™ (BioRad) set at 1.8 kV, 25  $\mu$ F and 100  $\Omega$ . Immediately following the electrical discharge, 1 ml of SOC recovery medium (0.5% [w/v] yeast extract, 2% [w/v] tryptone, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl<sub>2</sub>, 20 mM MgSO<sub>4</sub>, 20 mM glucose; pH 7.0) was added to the cells. The transformation mixtures were incubated at 37°C for 1 h and the transformed cells were selected by plating the cells onto LB agar plates containing 20  $\mu$ g/ml chloramphenicol. The agar plates were incubated at 37°C overnight after which the transformants were screened for lipolytic activity.



#### **2.2.4 Screening of the genomic DNA library for lipolytic activity**

Transformants were replica-plated onto tributyrin agar plates and incubated at 37°C for up to 72 h. The agar plates were investigated daily for halo-formation. One of the transformants displayed a large clearing zone and the recombinant plasmid DNA, designated pSEI, was characterized by restriction enzyme digestion with both *EcoRI* and *HindIII*, which cut in the multiple cloning site of the vector flanking the cloned DNA fragment. Agarose gel electrophoresis of the reaction mixture indicated that in addition to a 4.1-kb band corresponding in size to the vector DNA, two bands of *ca.* 2.2 and 1.0 kb, respectively, were also present. These results thus indicated that the total length of the cloned insert DNA is 3.2 kb.

#### **2.2.5 Restriction endonuclease mapping of plasmid pSEI**

The recombinant plasmid pSEI was digested with various different restriction endonucleases, either singularly or in combination, to identify which enzymes cut the cloned DNA fragment and to map their relative positions in the cloned DNA fragment. All restriction endonuclease digestions were performed in sterile Eppendorf tubes and contained the appropriate concentration of salt (using the 10 × buffer supplied by the manufacturer) for the specific enzyme and 5-10 U of enzyme per µg of plasmid DNA. The reaction volumes were small (15-20 µl) and incubation was typically for 1-1.5 h at 37°C, except for *SmaI*, which was incubated at 25°C. When digestion entailed the use of two enzymes requiring different salt concentrations for optimal activity, the plasmid DNA was first digested with the enzyme requiring a lower salt concentration, after which the salt concentration was adjusted and the second enzyme added. All restriction enzymes were supplied by Roche Diagnostics. The digestion products were typically analyzed on a 1% (w/v) agarose gel and sized according to their migration in the gel compared to that of standard DNA molecular weight markers (phage lambda DNA digested with *EcoRI* and *HindIII*; Roche Diagnostics).

#### **2.2.6 Construction of subclones to identify the lipolytic-encoding region in pSEI**

By making use of the compiled restriction enzyme map, three different subclones were constructed in pBS19 to facilitate isolation of the lipolytic-encoding region within the cloned insert contained in pSEI (Fig. 2.1). Recombinant plasmid pSEI was digested with *HindIII*, which cuts once in the multiple cloning site of the vector, and *PstI*, which cuts once within

the cloned DNA fragment and once in the multiple cloning site of the vector, to excise DNA fragments of *ca.* 2.4 and 0.8 kb, respectively. The 2.4-kb DNA fragment was subsequently purified from the agarose gel and cloned into the vector pBS19, which had been digested with the same enzymes, to yield plasmid pSEI-PH. Recombinant plasmid pSEI was furthermore digested with *AatII*, which cuts once only within the cloned DNA fragment. The ends of the linearized recombinant plasmid DNA were blunt-ended by incubation at 25°C for 16 h after addition of 5 mM MgCl<sub>2</sub>, 12.5 μM of each dNTP and 2 U of Klenow polymerase (2 U/μl) in a final reaction volume of 20 μl. The purified blunt-ended DNA fragment was used as the source for construction of two additional subclones. To construct subclone pSEI-SA, the prepared DNA was digested with *SacI* and the resultant 1.9-kb DNA fragment was purified from the agarose gel and cloned into vector pBS19, which had been digested with both *SmaI* and *SacI*. To construct subclone pSEI-AH, the linearized blunt-ended DNA fragment was digested with *HindIII*, and a 1.3-kb DNA fragment was purified from the agarose gel and cloned into vector pBS 19, which had been digested with both *SmaI* and *HindIII*.

#### 2.2.7 Characterization of recombinant subclones

Putative recombinant transformants resulting from transformation of competent *E. coli* JM83 with the different ligation reaction mixtures were randomly selected and further characterized by agarose gel electrophoresis and restriction enzyme digestion following plasmid DNA extraction. As an alternative to the plasmid DNA extraction procedure in Section 2.2.3.4, the plasmid DNA was extracted using an alkaline lysis protocol (Sambrook and Russel, 2001) to facilitate the rapid screening of a large number of transformants. Single colonies picked from the agar plates with sterile toothpicks were inoculated into 5 ml LB broth containing chloramphenicol and then incubated at 37°C overnight. Cells from 2 ml of the overnight cultures were harvested by centrifugation at 14 000 rpm for 1 min and the bacterial cell pellets were suspended in 200 μl of cell resuspension solution (50 mM Tris-HCl [pH 7.5], 10 mM EDTA, 100 μg/ml RNase A). The cells were lysed by the addition of 400 μl of cell lysis solution (0.2 N NaOH, 1% [w/v] SDS) and the contents of the tubes were mixed by inverting the tubes several times until the suspension had cleared. Following the addition of 300 μl of neutralization solution (2.55 M potassium acetate, pH 4.8), the bacterial lysates were centrifuged at 10 000 rpm for 5 min. The plasmid DNA was precipitated from the recovered supernatant by the addition of 450 μl isopropanol and collected by centrifugation at 10 000

rpm for 10 min. The plasmid DNA was rinsed once with 70% ethanol, air-dried and suspended in 20  $\mu$ l TE buffer.

## 2.2.8 Nucleic acid sequencing

### 2.2.8.1 Construction of subclones for sequencing

In addition to clones pSEI-PH, pSEI-SA and pSEI-AH, the strategy used for nucleotide sequencing of the cloned genomic fragment involved the construction of two additional subclones in plasmid pBC(SK), which together would span the full-length of the cloned insert DNA (Fig. 2.1). All molecular cloning techniques employed in the construction of the recombinant subclones were performed according to the procedures described in the preceding sections. Plasmid pSEI was digested with *Eco*RI and following agarose gel electrophoresis, a DNA fragment of *ca.* 2.2 kb was isolated and purified from the agarose gel by the GeneClean method (Section 2.2.3.3) and then cloned into vector pBC(SK), which had been similarly prepared. The derived subclone was designated pSEI-EE. Plasmid pSEI was also digested with *Nae*I, which cuts 5 times in the cloned genomic fragment, and a 1.9-kb DNA fragment was purified from the agarose gel and subcloned into the *Nae*I site of vector pBC(SK) to generate pSEI-NN. All plasmid constructs were confirmed by restriction endonuclease digestion using agarose gel electrophoresis.

### 2.2.8.2 Sequencing reactions

The nucleotide sequence of the cloned insert DNA in each of the constructed subclones was determined using an ABI PRISM™ BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, Perkin-Elmer) together with the universal pUC/M13 forward and reverse sequencing primers (Table 2.1). Each reaction mixture contained 80-100 ng of purified plasmid DNA, 12.5 pmol of sequencing primer, 2  $\mu$ l Terminator Ready Reaction Mix and UHQ water in a final volume of 5  $\mu$ l. Cycle sequencing reactions were performed in a Perkin-Elmer GeneAmp 2400 thermal cycler with 25 of the following cycles: denaturation at 96°C for 30 s, primer annealing at 50°C for 15 s and extension at 60°C for 4 min. Following brief centrifugation, the extension products were precipitated by the addition of 2  $\mu$ l of 3 M NaOAC (pH 4.6) and 50  $\mu$ l of 95% ethanol in a final volume of 70  $\mu$ l. The tubes were incubated at room temperature for 20 min in the dark, centrifuged at 15 000 rpm for 30 min and the supernatants carefully aspirated. The pellets were rinsed twice with 250  $\mu$ l of 70% ethanol, dried under vacuum for 10 min and stored at 4°C. Prior to electrophoresis, the

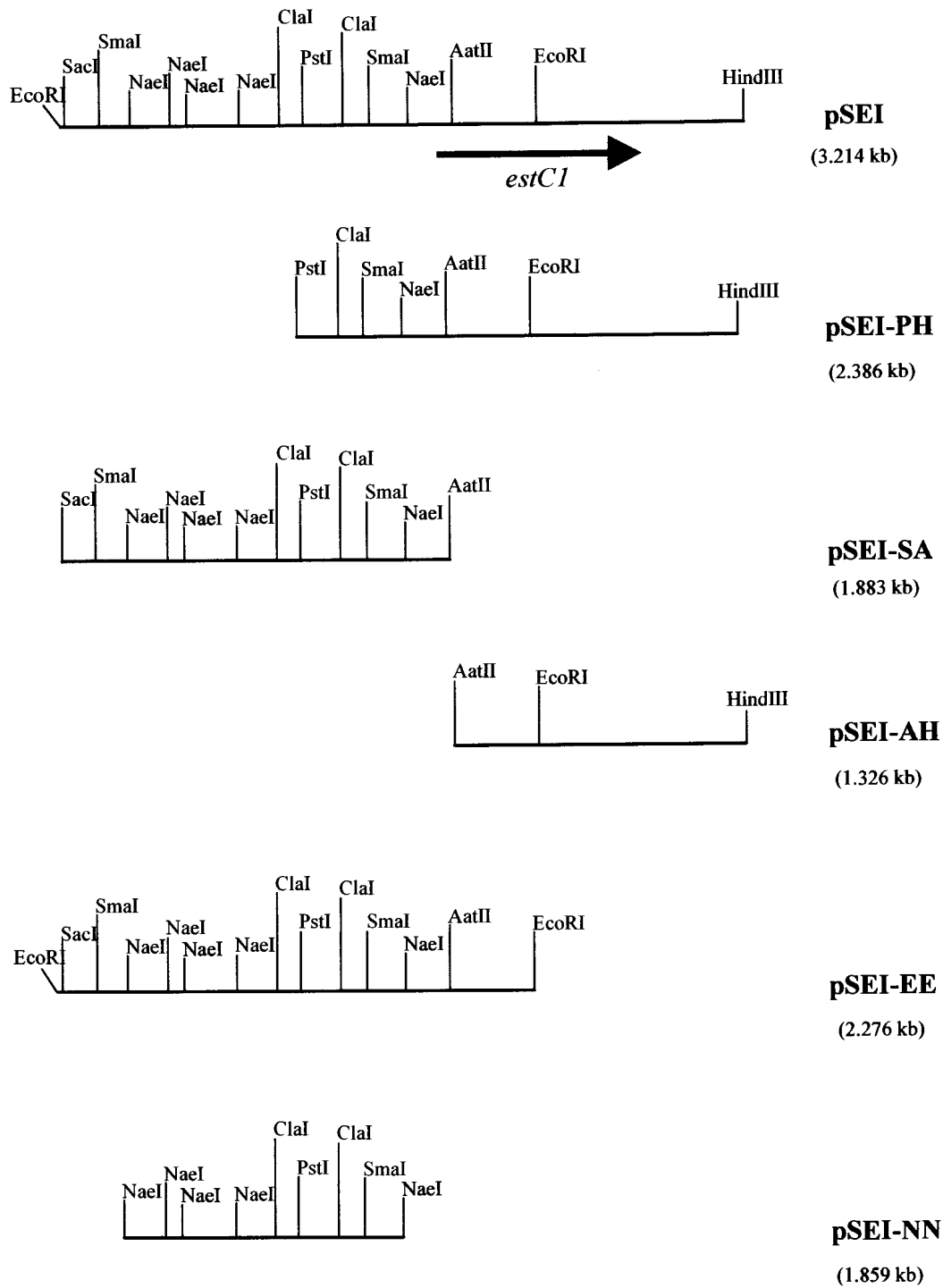


Fig. 2.1 Restriction map of the 3.2-kb *EcoRI-HindIII* insert of plasmid pSEI and the nucleotide sequencing strategy. The restriction sites used to construct overlapping subclones of the DNA fragment harbouring the *estC* gene are indicated. The orientation of the *estC* gene is indicated by a blue arrow.

purified extension products were resuspended in 3.5  $\mu$ l Blue dextran/EDTA loading buffer, denatured for 2 min at 90°C and loaded in a Model 377 Perkin-Elmer automated DNA sequencer.

### 2.2.8.3 Sequence analysis

The obtained nucleic acid sequences were assembled and analysed using DNAMAN (Lynnon BioSoft). The nucleic acid and deduced amino acid sequences were compared against the entries in the nonredundant GenBank Database by using the BLASTN and BLASTP programs (Altschul *et al.*, 1997) available on the National Centre for Biotechnology Information web page (<http://www.ncbi.nlm.nih.gov/BLAST/>). Pair-wise alignments were performed using LALIGN (Pearson *et al.*, 1997), while multiple alignments were carried out using CLUSTALW (Thompson *et al.*, 1994). The physico-chemical properties of the deduced amino acid sequence and presence of defined protein patterns were determined using the PROSITE and PRODOM databases at the ExPASy server (<http://www.expasy.org>). Searches for consensus promoter sequences were performed using the NEURAL NETWORK PROMOTER PREDICTION PROGRAM (at <http://www-hgc-lbl.gov/projects/promoter.html>), as well as the SEQSCAN program (at <http://www.bmb.psu.edu/seqscan>).

### 2.2.9 Preparation of cell extracts and fractions

For preparation of cell fractions, *Bacillus* sp. 81-11 and recombinant *E. coli*/pSEI was cultured overnight in LB broth at 44°C and 37°C, respectively, after which the cells were harvested by centrifugation at 5 000 rpm for 10 min and both the supernatant and the cell pellet kept. The cell pellet was suspended in 5 ml sonication buffer (25 mM Tris-HCl, 10 mM EDTA, 25 mM glucose, 50 mM NaCl; pH 8) and the cells lysed by sonication at 5-min intervals for 15 min using a Bandelin Sonoplus sonicator at an output of 70%. The cell lysate was cleared by centrifugation (10 000 rpm, 10 min) and the supernatant, considered as the cytoplasmic fraction, recovered for further analysis. The remaining pellet, consisting mainly of membrane proteins, was suspended in 5 ml of the mentioned buffer. The three fractions (cell-free culture medium, cytoplasmic extract and cell debris) were subsequently assayed for enzyme activity as described below.



### 2.2.10 Enzyme assays

Enzyme activity was determined spectrophotometrically by measuring the liberation of *p*-nitrophenol from *p*-nitrophenyl butyrate (*p*-NPB; Sigma) according to the method of Cho *et al.* (2000). The enzyme substrate was prepared by mixing 1 ml of 10 mM *p*-nitrophenyl butyrate (dissolved in acetonitrile) with 4 ml absolute ethanol and 95 ml of 50 mM potassium phosphate (pH 8). To 4.5 ml of this mixture, 500  $\mu$ l of the cell extract was added and the reaction mixture incubated at 30°C for 5 min. The absorbance at 405 nm was read against a reagent blank and the relative activity was calculated by regarding the maximum activity as 100%.

### 2.2.11 Protein concentration determination

The protein concentration of the crude enzyme extracts was determined by the method of Bradford (1976) using a commercial kit (BioRad Protein Assay kit) with bovine serum albumin (BSA) as standard. Aliquots (50  $\mu$ l) of each sample was added to 2.5 ml protein assay reagent (Bradford reagent), mixed well and the absorbance at 595 nm was determined following incubation at room temperature for 5 min. Distilled water was used to zero the absorbancy readings and the protein concentration (mg/ml) was then determined from a standard curve.

### 2.2.12 Influence of pH and temperature on enzyme activity

The influence of pH on enzyme activity was determined at 30°C in the following buffers: pH 4-7, 50 mM potassium phosphate; pH 7-8, 50 mM Tris-HCl; and pH 8-10, 50 mM glycine-NaOH. The reaction mixtures at the above pH values were incubated at 30°C for 5 min. To determine the influence of temperature on enzyme activity, samples were incubated for 5 min in 50 mM potassium phosphate buffer (pH 8) at various temperatures ranging from 30°C to 60°C. The thermal stability was investigated by measuring the residual activities after incubation of the enzyme for 3 h at temperatures of 30, 50, 60 and 65°C, respectively, in 50 mM potassium phosphate buffer (pH 8). In all cases, the enzyme activity was determined using the standard photometric assay described above and *p*-NP butyrate as substrate.

### 2.2.13 Effect of chain length on enzyme specificity

The hydrolytic activity of the enzyme towards various *p*-nitrophenyl esters was investigated according to the method of Zhang *et al.* (2003) by making use of *p*-nitrophenyl esters with a chain length of between C<sub>3</sub> and C<sub>18</sub>, *i.e.* *p*-NP propionate (C<sub>3</sub>), *p*-NP butyrate (C<sub>4</sub>), *p*-NP caproate (C<sub>6</sub>), *p*-NP laurate (C<sub>12</sub>), *p*-NP myristate (C<sub>14</sub>), *p*-NP palmitate (C<sub>16</sub>) and *p*-NP stearate (C<sub>18</sub>). All of these *p*-NP esters were purchased from Sigma. The substrates were prepared by dissolving 2.625 mM of the respective *p*-nitrophenyl esters in 4% (v/v) TritonX-100, to which 8 ml distilled water and 2 ml 0.1 M phosphate buffer (pH 8) were added to a final volume of 20 ml. The assay was conducted by mixing 950 µl of the substrate and 50 µl of the crude enzyme preparation followed by incubation in a water bath at 50°C for 10 min. The assay was terminated by addition of 2 ml acetone and the enzyme activity was measured spectrophotometrically at 410 nm against a reagent blank. The obtained results were expressed as a relative activity of the substrate that gave maximal activity.

### 2.2.14 Electrophoresis and zymography

Proteins from the crude extracts were analysed by 12% SDS-PAGE, essentially as described by Laemmli (1970), using a LKB 2001 vertical slab electrophoresis unit (Hoefer Scientific Instruments). Samples were loaded onto the gel without prior heating and electrophoresis was performed in 1 × TGS buffer (25 mM Tris, 250 mM glycine [pH 7.5], 0.1% [w/v] SDS) at 20 mA for 1 h and then at 40 mA until the dye front had migrated to the bottom of the gel. The proteins in a pre-stained molecular weight marker (Roche Diagnostics) were used as reference proteins. After electrophoresis, the gels were either stained with 3% Coomassie brilliant blue R-250 (Sigma) to visualize the protein bands, or activity staining (zymography) was performed. For zymography (Takahashi *et al.*, 1998), the gel was renatured in 25 mM Tris-HCl (pH 7.5) buffer containing 2.5% (v/v) TritonX-100 for 20 min at room temperature. The gel was subsequently equilibrated for 20 min in 25 mM Tris-HCl (pH 7.5) and then stained for esterase activity by incubating the gels in 50 mM Tris-HCl (pH 7.5) containing 0.1% (w/v) 1-naphthylacetate (Sigma) and 0.2% (w/v) Fast Red TR salt (Sigma). Esterase-active protein bands can be detected as red-stained bands. Alternatively, the samples were applied to a 12% nondenaturing polyacrylamide gel. Following electrophoresis, the gel was transferred to a glass plate containing an agar overlay, the same size as the gel, consisting of 1% (w/v) tributyrin, 25 mM Tris-HCl (pH 7.5) and 1.3% (w/v) agar (Henne *et al.*, 2000).

The gel-agar overlay was incubated at room temperature and hydrolysis of tributyrin was detected by zones of clearing.

#### 2.2.15 Nucleotide sequence accession number

The sequence data reported in this study have been submitted to GenBank and assigned the accession number AY634688.

### 2.3 RESULTS

#### 2.3.1 Screening of *Bacillus* isolates for lipolytic activity

Several *Bacillus* isolates, previously isolated from different food sources, seeds and fermenting brews, were streaked onto tributyrin-containing agar plates and incubated at 44°C for up to 72 h. Among the 15 *Bacillus* isolates screened for lipolytic activity, one isolate was found to hydrolyse tributyrin significantly, whilst three other strains showed slight or low degradative activities (Fig. 2.2). The strain with highest tributyrin-hydrolyzing activity, originally isolated from popcorn seeds, was selected for further study. The isolate was identified as a strain of *Bacillus coagulans* based on 99% sequence identity of the 16S rRNA sequence to several other *B. coagulans* strains.

#### 2.3.2 Screening for genes conferring lipolytic activity

To screen for lipolytic enzymes at the gene level, a genomic library was constructed by partially digesting the total DNA from *B. coagulans* strain 81-11 with *Sau3AI* and ligating the agarose gel-purified fragments between 3 and 5 kb into the dephosphorylated *Bam*HI site of vector pBS19, a multipurpose *B. subtilis*/*E. coli* shuttle vector. The ligation reaction mixtures were subsequently electroporated into *E. coli* JM83, which exhibits no esterase activity (Fig. 2.3), and plated onto LB agar plates supplemented with chloramphenicol. For detection of *E. coli* clones exhibiting esterase activity, the chloramphenicol-resistant transformants were plated onto LB agar plates containing 1% [w/v] tributyrin and positive *E. coli* clones were detected by zones of clearing around the colonies.



From the *ca.* 9 000 chloramphenicol-resistant transformants screened, only one clone showed hydrolysis of tributyrin and was selected for characterization. To confirm that the lipolytic phenotype of the clone is plasmid encoded, the recombinant plasmid was isolated and retransformed into *E. coli* JM83, and the *E. coli* strains were screened again on indicator plates as above. The recombinant plasmid, designated pSEI, conferred a stable lipolytic phenotype on the resulting recombinant *E. coli* strains (Fig. 2.3). Since plasmid pBS19 lacks a promoter, the results furthermore indicated that the gene was transcribed from its own promoter and probably contained the full-length enzyme-encoding gene.

The pSEI plasmid DNA was subsequently characterized by restriction enzyme analysis. Digestion of pSEI with both *Eco*RI and *Hind*III indicated that it harboured a 3.2-kb DNA insert. In order to identify the gene(s) on pSEI that are responsible for the lipolytic activity of the corresponding recombinant *E. coli* strain, the insert DNA was subcloned, as described under Materials and Methods (Section 2.2.6), by restriction digestion with various restriction enzymes and fragments were inserted into pBS19. The transformants resulting from electroporation of *E. coli* JM83 cells were then screened for lipolytic activity on indicator plates containing tributyrin as substrate. In this way, a 2.4-kb *Pst*I-*Hind*III fragment that encoded the lipolytic activity was identified. The corresponding plasmid was designated pSEI-PH (results not shown).

### 2.3.3 Nucleotide sequence analysis of pSEI-PH

In order to identify the gene(s) that are responsible for the lipolytic activity of the recombinant *E. coli*/pSEI-PH strain, the nucleotide sequence of the 2.4-kb *Pst*I-*Hind*III fragment was determined by automated sequencing procedures following subcloning of different restriction fragments into pBC(SK) (Fig. 2.1).

Analysis of the nucleotide sequence of the 2.4-kb *Pst*I-*Hind*III fragment of pSEI-PH by using the ORF finder program revealed the existence of two open reading frames (ORFs). The amino acid sequence deduced from one of these ORFs exhibited significant homology to known esterase enzymes. The *B. coagulans* strain 81-11 enzyme was thus designated EstC1 (for esterase 1 of *B. coagulans*), and the corresponding gene was designated *estC1* (Fig. 2.4). The *estC1* gene starts at an ATG start codon at nucleotide 971 and terminates at a TGA stop

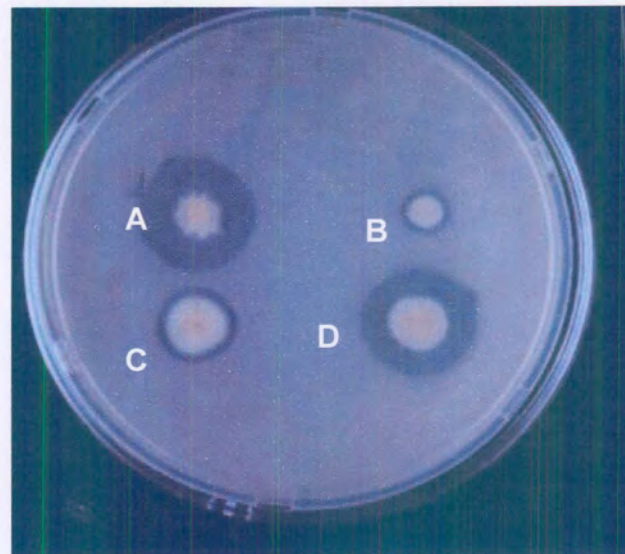


Fig. 2.2 Screening of different *Bacillus* isolates for lipolytic activity on an agar plate with tributyrin as substrate. Of the different *Bacillus* isolates screened, strain 81-11 (A) hydrolysed the tributyrin significantly, whilst strains 66-11 (B), 85-9 (C) and 65-31 (D) displayed lower degradative activities.

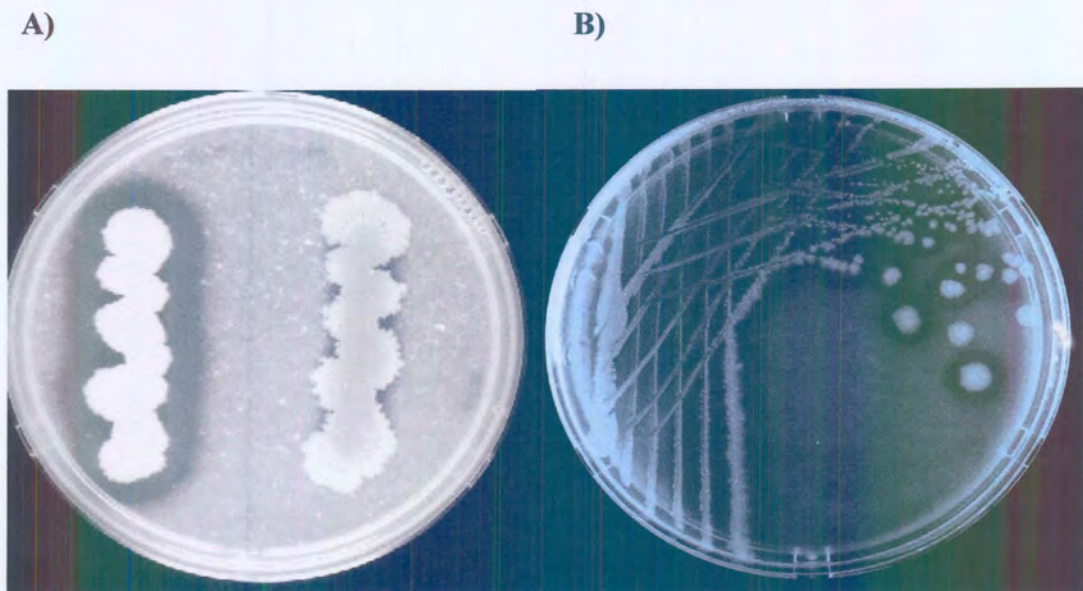


Fig. 2.3 Screening on tributyrin-containing agar plates for genes from *B. coagulans* strain 81-11 conferring lipolytic activity to *E. coli* JM83. (A) During screening of a *B. coagulans* strain 81-11 genomic DNA library in *E. coli* JM83, a clone was identified that displayed hydrolysis of tributyrin (colony to the left). As a control, untransformed *E. coli* JM83 was also streaked on the agar plate (colony to the right). (B) Following transformation of *E. coli* JM83 with the isolated recombinant plasmid, pSEI, the recombinant *E. coli*/pSEI strain displayed lipolytic activity on a freshly prepared tributyrin agar plate.

codon at nucleotide 1694, yielding an ORF of 723 nucleotides that encodes a predicted protein of 240 amino acids, with a molecular mass of 27 529 Da and a pI of 9.15. No signal peptide was found, suggesting that the cloned enzyme was a cell-bound esterase. Preceding the ATG start codon, a putative ribosome binding sequence (Shine and Dalgarno, 1974), AAGAGG, as well as potential -10 (TAAAAT) and -35 (TTCCGA) promoter elements could be identified. No inverted repeat sequence for a possible transcription terminator with a sufficient  $\Delta G$  value (kilocalories per mole) was found downstream of the termination codon (TAG). The overall G+C content in the esterase-encoding region was 48%. The G+C contents in first, second and third positions of the *estC1* codons are 52%, 36% and 51%, respectively, values which are in agreement with the codon usage of *Bacillus coagulans* (Nakamura *et al.*, 2000).

A second, apparently incomplete ORF (*orf2*) was located downstream from *estC1*. Despite the deduced amino acid sequence derived from this ORF being truncated, it nevertheless exhibited significant levels of homology to the amino acid sequence of ribonuclease R enzymes. The highest level of homology was observed with the exoribonuclease R of *B. cereus* ATCC 14579 (GenBank accession no. NP834797.1; 69% amino acid sequence identity over 201 amino acids) and *B. halodurans* C-125 (GenBank accession no. NP244420.1; 55% amino acid sequence identity over 198 amino acids), while lower homology to the cognate protein from *Staphylococcus epidermidis* ATCC 12228 (GenBank accession no. NP764120.1; 48% amino acid sequence identity over 199 amino acids) was observed.

#### 2.3.4 Identification of homologues by sequence comparisons

Comparison of the deduced amino acid sequence of *estC1* from *B. coagulans* strain 81-11 by a BLASTP search to the sequences in the GenBank database revealed similarity to several esterase enzymes from bacterial origin. EstC1 showed the highest amino acid sequence identity (70-72%) to the esterases of *G. stearothermophilus* (Kugimiya *et al.*, 1992) and *G. thermoleovorans* (GenBank Accession no. AF327065), while lower identity (66%) was found to the BsubE esterase, formerly known as YvaK, from *B. subtilis* strain 168 (Kunst *et al.*, 1997). EstC1 also displayed significant homology (61-67% amino acid sequence identity) to putative esterases of several bacteria of which the completed genome sequences have recently been published. These included the enzymes from *B. halodurans* C-125 (Takami *et al.*, 2000), *B. anthracis* A2012 (Read *et al.*, 2002) and *B. cereus* ATCC 14579 (Ivanova *et al.*,

1 ctgcaggctgcccgggtttaccgggcagtttttgggtgtacgggaaaaatggggctattcgttttgctctgg  
 76 ctgacaaaaacgcggaacggggtgagggacatgaaggacaaaccaggaccggtaactagagaaatggttcttca  
 151 gggggcttatgaaagacaaaacgaggggctgaggtgaaagaaatgtccttcatagggttcatgaaagacaaaaca  
 226 cgggctgagaaacaaggaaaggttatgaaggacaaaccaggaccggtaaccagagaaatggttcttcaaggggct  
 301 tacggaagacaacgaagtacctggatgaaagagatatttttccattagccacgtgtaagacaaaacaggtaccgg  
 376 aaatcaaaaaacatccttcatcatcgagcccataaagacaaaacgggggcccggaccaataagatactgttctt  
 451 tcggacaattattccaatgtagtgaaccctgcccccaataggtacactcaggattaggattgcccgtctca  
 526 ggaggtgtgcagttgtgcatacattgttattaacattactaatcatcgattcaattctgttgattgcagtcatac  
 601 tggctccagcccgggaaaagcacggggattgtccggcgccatttccggcgcgctgaacagcttttcgggaaaca  
 676 aaaaagtgcgcggaattgacctgattttacacaggattacgattgtgctcgtgttttatttttctgtggcca  
 751 tcggacttgcatatataaacctataagggtatgcaaatcaggcaactataaagaagccttatccgcacatgcct  
 826 gtaaaccttatgacttccatcccgggtctcaaaatccaggccgggtatttttattgctggattgctggcat**ttccgaa**  
 901 acgactttgttacaaa **taaaat**gaacaaaggccccaaagaaaaacagcaggaagaggcgtttttaccg  
  
*estCI*  
 971 **ATG**ATGAGAATTGTACCACCAAAACCGTTTTTATTTGAAGCCGGCAAAAGGGCTGTACTGCTTTTGACGGATTT  
 M M R I V P P K P F L F E A G K R A V L L L H G F 25  
 1046 ACCGGAAGCAGCGCCGACGTCAGGATGCTCGGGCGAAAAAAGGATACACTGCCATGCCCGCAATACAAAGGG  
 T G S S A D V R M L G R K K G Y T C H A P Q Y K G 50  
 1121 CACGGGGTACCGCCTGAAAACTTTTGCATACGGGTCCAAGCGACTGGTGGCAAGATGTTATGAATGGCTACCAG  
 H G V P P E N L G H T G P S D W W Q D V M N G Y Q 75  
 1196 TTATTAAGAAAAAGGGTATCATGAAATTGCAGTAGCGGGATTGTCATTGGGGGGGGTATTTTCTTAAATG  
 L L K E K G Y H E I A V A **G L S L G** G V F S L K L 100  
 1271 GGTTACTACTGTACCTGTAAAGGGAATTATCACCATGTGCGGCCCATGTACATAAAAAGCGAGCAAACGATGTAT  
 G Y T V P V K G I I T M C A P M Y I K S E Q T M Y 125  
 1346 GAAGGCGTGGTTGCGTATGCCCGCAAATACAAACAGTTCGAAGGGAAAAGCGAAGCGCAAATCGAACAGGAAATG  
 E G V V A Y A R K Y K Q F E G K S E A Q I E Q E M 150  
 1421 CGGGAATTCAAAAAGACGCCGATGCATACGCTGCAATCGCTGCAAGAGCTTATTCAGGATGTGCGCAACCATGTC  
 R E F K K T P M H T L Q S L Q E L I Q D V R N H V 175  
 1496 GATCACATTTATGCGCCCGTTTTTGTGCTACAAGCGGCCATGACGAAATGATCCGCCCGGAAAGCGCCAATATT  
 D H I Y A P V F V V Q A R H D E M I R P E S A N I 200  
 1571 ATTTATTCAGAAGTGAAACAGACCATAAGCAACTCAAATGGTATGAGAACTCCGGCATGTTATTACTCGAGA  
 I Y S E V E T D H K Q L K W Y E N S G M L L H S R 225  
 1646 AGAAAAGATCACTTCACGAAGATGTTGTTCCGTTCTGGAGTCACT**TGA**ctggtcgggtttaaatccccgaaa  
 R K D H F T K M L F R S G V T \* 240  
 1721 aaggagggaaataaatggaacaagaccaactacggcaagatggtgaaaaatcttctgtatatgaaagaagag  
 1796 acgtacaagccgctgacggttcaggaactgaaaaagcgttcggtattgacgattcctccgagtttaagcattt  
 1871 gtgaaagtgtcgttttatatggaggaaaaaggcctcattgtccgcacgcaagcaaccgttacgctgacgctggaa  
 1946 aaaatgaatctcgtacggggaaaactgatcggccatccaaaagggttttgcgtttgtcgcggccgagcctggg  
 2021 atggacgatattttatcccgcgcatgaaactaacatgcgttaaatggagacaccgttattgtccgtgtgtcc  
 2096 accaaaagctcaggccagcggcaggaaggaaccggttgtcgtattcttgagcggggccagacagaacttgtcggc  
 2171 acatacgtcgaagcaaacatttcggatttgcgtaccgatgacaaacgggttcggcagcgatattttataccg  
 2246 aaaacagcaaacggcggtgctgtcgaaggccataaagtattgtccatattaccgcctaccggaaggcagcaaa  
 2321 aatgcggaaggcgaaatcatcaaaattcttgccataaaaatgatcctctagagtcgacctgcagcaagctt

Fig. 2.4 The nucleotide sequence of the 2 392-bp *PstI-HindIII* fragment containing the esterase-encoding gene of *Bacillus coagulans* strain 81-11 and the deduced amino acid sequence in one-letter code is shown. The ATG start and TGA stop codons of the predicted ORF are shown in bold, the putative Shine-Dalgarno sequence is underlined and the putative -35 and -10 nucleotides upstream of the *estCI* ORF are indicated in italics. The amino acids that are in the boxed region represent the signature of the esterase common sequence.

2003), while 48% amino acid sequence identity was observed with the putative esterase from *Oceanobacillus iheyensis* strain HTE831 (Takami *et al.*, 2000). Furthermore, notable levels of homology (48-51% amino acid sequence identity) were also found with putative carboxyl esterases from other Gram-positive bacteria such as *Listeria monocytogenes* strain EGD-e (Glaser *et al.*, 2001), *Listeria innocua* strain Clip 11262 (Glaser *et al.*, 2001), *Staphylococcus epidermidis* ATCC 12228 (GenBank accession no. AAO05971) and *Staphylococcus aureus* subsp. *aureus* N315 (Kuroda *et al.*, 2001). The percentage identity between the closest matching sequences was calculated in pair-wise alignments for the full-length proteins using LALIGN (Pearson *et al.*, 1997) and is presented in Table 2.2, while multiple sequence alignment of the above proteins is shown in Fig. 2.5.

Analysis of the deduced EstC1 amino acid sequence revealed a potential serine hydrolase motif (G-L-S<sub>94</sub>-L-G). The motif corresponds to the consensus sequence G-X-S-X-G for the active site of most serine esterases. In addition, the surrounding residues resembled the signature motif of lipases and esterases ([LIV]-X-[LIVFY]-[LIVMST]-G-[HYWV]-S-X-[GSTAC]) present in the PROSITE motif database. The putative catalytic apparatus of esterases, involving the triad serine, glutamate or aspartate, and histidine was located in the protein, by similarity, at positions 94 (Ser), 190 (Asp) and 229 (His). However, the validity of these predictions will have to be experimentally verified by site-directed mutagenesis analysis.

### 2.3.5 Localization of esterase activity

The production of lipolytic activity in both *B. coagulans* strain 81-11 and *E. coli*/pSEI was studied. Cells from the strains were harvested and fractionated to obtain cytoplasm, membrane and extracellular fractions, and their lipolytic activity was determined in 50 mM potassium phosphate buffer (pH 8) at 30°C. For both strains, the highest enzyme activity was observed in the cell debris fraction, where *E. coli*/pSEI exhibited 0.1 U/ml and *B. coagulans* strain 81-11 displayed 0.3 U/ml. Activity in the supernatants was much lower (0.04 U/ml for both), and was probably a result of cell lysis during growth of the culture. No activity was detectable in the cytoplasmic fraction. These results therefore suggested that EstC1 is a cell-bound esterase, a result that agrees well with the *in silico*-predicted absence of a signal peptide. By contrast, a control nonrecombinant *E. coli* JM83 culture did not show lipolytic activity in identically prepared fractions (results not shown). Consequently, crude cell extracts of recombinant *E. coli*/pSEI was selected and used as the source for further enzyme characterization.

**Table 2.2:** Pair-wise identity between esterase amino acid sequences

Carboxyl esterases sequences* from:	Identity value (%) for esterase sequences* from:											
	<i>Bacillus coagulans</i> strain 81-11	<i>Geobacillus stearothermophilus</i>	<i>Geobacillus thermoleovorans</i>	<i>Bacillus subtilis</i> 168	<i>Bacillus cereus</i>	<i>Bacillus anthracis</i>	<i>Bacillus halodurans</i>	<i>Oceanobacillus iheyensis</i>	<i>Listeria monocytogenes</i>	<i>Listeria innocua</i>	<i>Staphylococcus epidermidis</i>	<i>Staphylococcus aureus</i>
<i>Bacillus coagulans</i> strain 81-11	100	72.1	70	66.1	67.2	66.4	61.8	47.8	51	51.4	51	48.6
<i>Geobacillus stearothermophilus</i>		100	96.4	74.6	79.8	78.9	69.9	59.1	53.4	53.4	57.4	57.4
<i>Geobacillus thermoleovorans</i>			100	72.2	77.7	77.7	76.9	57.5	51.8	51.4	56.6	56.2
<i>Bacillus subtilis</i> 168				100	76.6	76.6	67.2	55.6	54	54.4	55.2	52.8
<i>Bacillus cereus</i>					100	98.8	68.7	58.3	53	52.6	53.4	51.4
<i>Bacillus anthracis</i>						100	68.5	58.9	52.8	52.4	53.6	51.6
<i>Bacillus halodurans</i>							100	55.2	54.4	54.4	56.9	55.6
<i>Oceanobacillus iheyensis</i>								100	49.2	50	53.6	52.4
<i>Listeria monocytogenes</i>									100	98	52.4	51.2
<i>Listeria innocua</i>										100	52.8	51.2
<i>Staphylococcus epidermidis</i>											100	84.1
<i>Staphylococcus aureus</i> subsp. <i>aureus</i>												100

\* The sequences are from the following sources: *B. coagulans* strain 81-11 EstC1 (this study), *Geobacillus stearothermophilus* IFO12550 (GenBank accession no. BAA02182), *Geobacillus thermoleovorans* (GenBank accession no. AF327065), *Bacillus subtilis* subsp. *subtilis* 168 (GenBank accession no. CAB15367), *Bacillus cereus* ATCC 14579 (GenBank accession no. NP834798), *Bacillus anthracis* A2012 (GenBank accession no. NP653555), *Bacillus halodurans* C-125 (GenBank accession no. NP244421), *Oceanobacillus iheyensis* HTE831 (GenBank accession no. NP693350), *Listeria monocytogenes* EGD-e (GenBank accession no. AB1381), *Listeria innocua* Clip 11262 (GenBank accession no. AC1750), *Staphylococcus epidermidis* ATCC 12228 (GenBank accession no. AAO05971) and *Staphylococcus aureus* subsp. *aureus* N315 (GenBank accession no. NP373989).





EstC1_Bc	Y I K S E Q T M Y E G V V A Y A R K Y K Q F E G K S E A Q I E Q E M R E F K K T P M H T L Q S L Q E L I Q D V R N H V D	176
Est_Bc	Y I K S E E T M Y Q G I L A Y A R E Y K K R E Q K S P E Q I E Q E M L E F Q K T P M N T L K A L Q Q L I A D V R N N V D	179
Est_Ba	Y I K S E E T M Y Q G I L A Y A R E Y K K R E Q K S P E Q I E Q E M L E F Q K T P M N T L K A L Q Q L I A D V R N N V D	178
Est_Bs	H I K S E E V M Y Q G V L S Y A R N Y K K F E G K S P E Q I E E E M K E F E K T P M N T L K A L Q D L I A D V R N N V D	180
Est_Bh	R P K T D D A I Y K G V L E Y A E E Y K R R E K K S D E Q I E E E M E R F K S A P Q T T L F G L K Q L I E D V R D H L D	178
Est_Oi	F F D N E Q E L T K G F R Q F S T E F K Q L E K K D K E V I E E E V N Q L M D Q S K D L F K G V A T T I N E V K T N I D	178
Est_Se	E N K T E G S I Y E G F L E Y A R N F K K Y E G K D Q Q T I D Q E M E Q F H - - P T E T L R E L S D T L N G V K E H V D	176
Est_Sa	G G K T E G A I Y E G F L E Y A R N F K K Y E G K N Q E T I D N E M D H F K - - P T E T L K E L S E A L D T I K D Q V D	176
Est_Lm	R M D S S S P I I Q G F L D Y V R N Y K K L E G K T T E Q I D A E M V A Y K D A P M N T I A K L K D E I S G V V A E I D	178
Est_Li	R M D S S S P I I Q G F L D Y V R N Y K K L E G K T P E Q I D A E M V A Y K D A P M N T I A K L K D E I N G V V S E I D	178

Est_Gs	L I Y A P T F V V Q A R H D E M I N P D S A N I I Y N E I E S P V K Q I K W Y E Q S G H V I T L D Q E K D Q L H E D I Y	239
Est_Gt	L V Y A R T F V V Q A R H D K M I N P D S A N I I Y N E I E S P V K Q I K W Y E Q S G H V I T L D Q E K D Q L H E D I Y	238
EstC1_Bc	H I Y A P V F V V Q A R H D E M I R P E S A N I I Y S E V E T D H K Q L K W Y E N S G M L L H S R R - K D - - H F T K M	233
Est_Bc	M I Y A P T F V V Q A R H D E M I N T D S A N I I Y N G V E S T L K D I K W Y E D S T H V I T L D K Q R D E L H E D V Y	239
Est_Ba	M I Y V P T F V V Q A R H D E M I N T D S A N I I Y N G V E S T L K D I K W Y E D S T H V I T L D K Q R D E L H E D V Y	238
Est_Bs	M I Y S P T F V V Q A R H D H M I N T E S A N I I Y N E V E T D D K Q L K W Y E E S G H V I T L D K E R D L V H Q D V Y	240
Est_Bh	H I Y A P V F V V Q A R H D E M I D V E S A N V I H D T V E S D E K S L K W Y E D S T H V I T L D K E K E Q L H E D V Y	238
Est_Oi	M I Y T P T F V V Q A K N D K M I N T D S A N Y I Y E N V E A S K K D M K W Y E E S G H V I T M D K E K D Q L H E D I Y	238
Est_Se	E V I D P I L V V Q A E Q D T M I D P Q S A N Y I Y N H V D S D E K E I K W Y Q H S G H V I T I D K E K E K V F E D V Y	236
Est_Sa	E V F D P I L V I Q V E N D N M I D P Q S A N Y I Y D H V D S D D K D I K W Y S Q S G H V I T I D K E K E Q V F E D I Y	236
Est_Lm	M I Y A P I M V V Q G E K D D M V D V S G A Q L I Y D T V E S T K K E L H W F K E S G H V I T L D K E R K E V N Q A I L	238
Est_Li	M I Y A P I M V V Q G E K D D M V D V S G A Q L I Y D T V E S T K K E L H W F K E S G H V I T L D K E R K D V N Q A I L	238

Est_Gs	A F L E S L D W - -	247
Est_Gt	A F L E S L D W - -	246
EstC1_Bc	L F R S G V T - - -	240
Est_Bc	N F L E Q L D W - -	247
Est_Ba	N F L E Q L D W - -	246
Est_Bs	E F L E K L D W - -	248



Est_Bh	RFLE <u>GL</u> NWSE	248
Est_Oi	QFLES L DW - -	246
Est_Se	QFLES L <u>EW</u> TE	246
Est_Sa	QFLES L DWSE	246
Est_Lm	TFL <u>DS</u> L DWQE	248
Est_Li	TFL <u>DS</u> L DWQE	248

Fig. 2.5 Alignment of the amino acid sequence of *B. coagulans* strain 81-11 EstC1 esterase with the amino acid sequences of other bacterial esterases. Amino acid residues are indicated by single-letter codes. Alignment was maximized by introducing gaps, which are indicated by dashes. The numbers indicate the multiple alignment positions from the N terminus. Identical amino acid residues are shown in inverted text, while similar amino acids are shaded. The consensus active site sequence of serine esterases is underlined. Abbreviations: Est\_Gs, *Geobacillus stearothermophilus*; Est\_Gt, *Geobacillus thermoleovorans*; EstC1\_Bc, *B. coagulans* strain 81-11 EstC1; Est\_Bc, *Bacillus cereus* ATCC 14579; Est\_Ba, *Bacillus anthracis* A2012; Est\_Bs, *Bacillus subtilis* subsp. *subtilis* 168; Est\_Bh, *Bacillus halodurans* C-125; Est\_Oi, *Oceanobacillus iheyensis* HTE831; Est\_S, *Staphylococcus epidermidis* ATCC 12228; Est\_Sa, *Staphylococcus aureus* subsp. *aureus* N315; Est\_Lm, *Listeria monocytogenes* EGD-e; and Est\_Li, *Listeria innocua* Clip 11262. The GenBank accession numbers are as indicated in Table 2.2.



### 2.3.6 Effect of pH and temperature on enzyme activity

Since *E. coli* JM83 lacked detectable esterase activity and *E. coli* harboring pSEI-PH displayed lipolytic activity indistinguishable from *E. coli*/pSEI, the effect of pH and temperature on enzyme activity was determined using crude cell extracts prepared from *E. coli*/pSEI-PH and *p*-nitrophenyl butyrate as substrate. The activity of the esterase at various pH values was measured following adjustment of the reaction pHs from 4 to 10 with various buffers (Fig. 2.6). The enzyme showed maximal activity at pH 8. However, more than 80% of the maximal activity was detected at pH 7 and 9, respectively. The optimum temperature of the enzyme was determined by varying the reaction temperature at pH 8 (Fig. 2.7). The enzyme had maximal activity at 50°C. The enzyme remained active over a range of temperatures varying from 40 to 55°C, with *ca.* 90% relative activity at 40 and 55°C, respectively. The thermal stability of the enzyme was determined by incubating the enzyme extract at pH 8 for 3 h at different temperatures and then measuring the residual esterase activity (Fig. 2.8). Heat treatment at 30°C and 50°C did not result in significant loss of esterase activity, but incubation at higher temperatures, however, induced activity loss. Approximately 35% of the initial activity was lost after incubation at 60°C and almost all activity was lost after incubation at 65°C.

### 2.3.7 Substrate specificity

The substrate specificity of the lipolytic enzyme in crude cell extracts of *E. coli*/pSEI-PH was quantified spectrophotometrically using *p*-nitrophenyl esters with chain lengths ranging from C<sub>3</sub> to C<sub>18</sub> as substrate. The enzyme assays indicated that the enzyme exhibited a marked preference for short-chain fatty acids, yielding the highest activity against *p*-NP butyrate (C<sub>4</sub>). High hydrolytic activities were also obtained with *p*-NP propionate (C<sub>3</sub>) and *p*-NP caproate (C<sub>6</sub>), which exhibited relative activities of 77 and 82%, respectively. The hydrolytic activity of the protein dropped abruptly toward *p*-nitrophenyl esters with chain lengths ranging from C<sub>12</sub> to C<sub>18</sub> and less than 10% relative activity was detected against these long-chain fatty acids (Fig. 2.9). Since lipases are, by definition, carboxyl esterases that have the ability to hydrolyse long-chain acylglycerols ( $\geq C_{10}$ ), whereas esterases hydrolyse ester substrates with short-chain fatty acids ( $\leq C_{10}$ ) (Verger, 1997; Ferrato *et al.*, 1997), the decrease of activity as the length of the fatty acid chains increased, is behaviour typical for true esterases. These results therefore serve to confirm that the enzyme produced by *E. coli*/pSEI-PH is indeed an esterase.

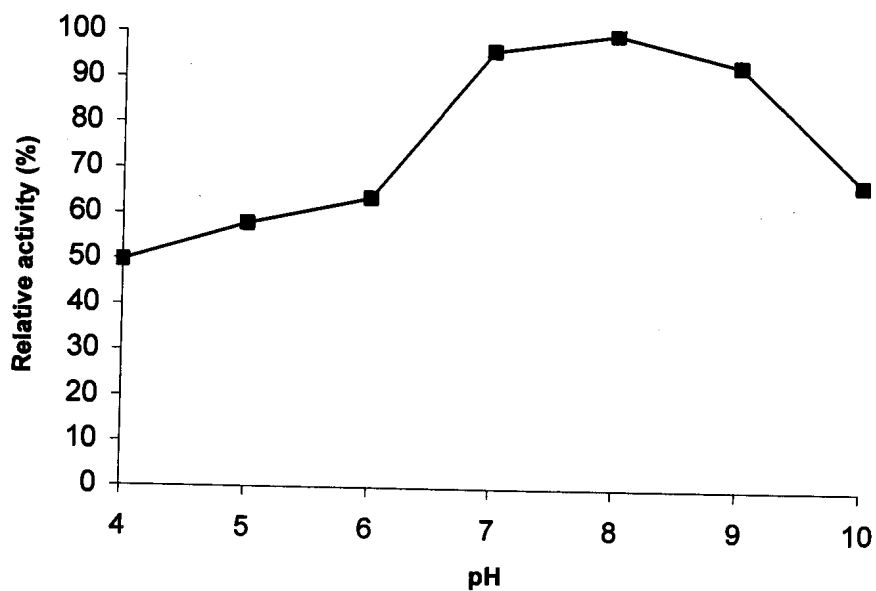


Fig. 2.6 Influence of pH on the esterase activity of EstC1 of *B. coagulans* strain 81-11. For the pH profile, activity was measured at 30°C in buffers of different pH values. Values are the means of results of duplicate experiments.

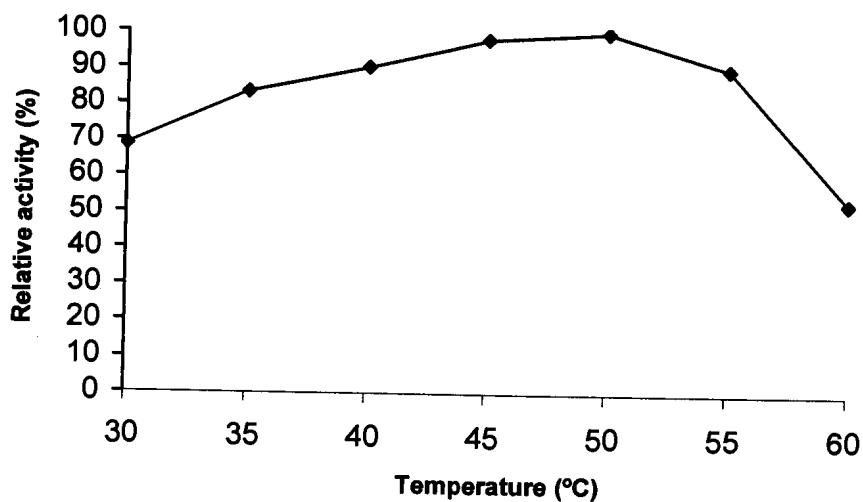


Fig. 2.7 Influence of temperature on the esterase activity of EstC1 of *B. coagulans* strain 81-11. For the temperature profile, activity was measured in 50 mM potassium phosphate (pH 8) at different temperatures. Values are the means of results of duplicate experiments.

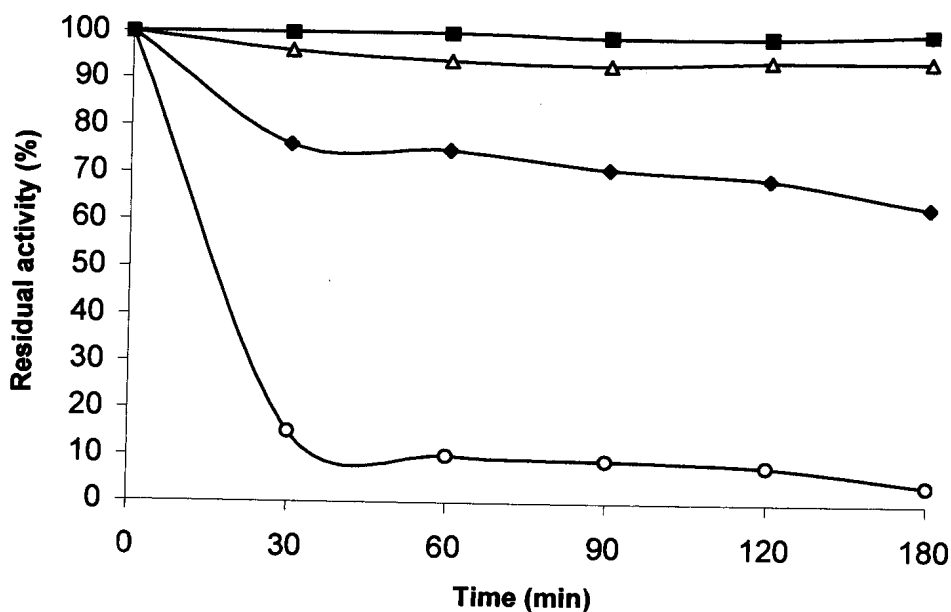


Fig. 2.8 Effect of temperature on the stability of the EstC1 esterase of *B. coagulans* strain 81-11. The residual enzyme activity was measured, using *p*-nitrophenyl butyrate as substrate, after incubation of the crude enzyme extract for the indicated times at temperatures of 30°C (■), 50°C (△), 60°C (◆) and 65°C (○). Values are the means of results of duplicate experiments.

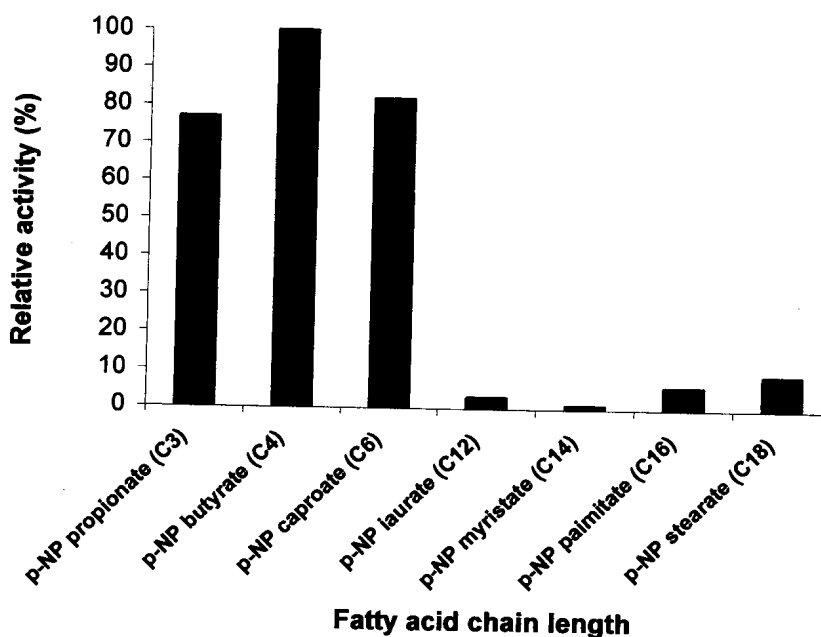


Fig. 2.9 The substrate specificity of the EstC1 esterase of *B. coagulans* strain 81-11 as measured by hydrolysis of *p*-nitrophenyl esters (*p*-NP) with different carbon chain lengths as substrates at pH 8 and 50°C. Values are the means of results of duplicate experiments.

### 2.3.8 SDS-PAGE analysis and activity staining of crude enzyme extracts

In order to determine the molecular mass of the enzyme, an unboiled sample of the crude enzyme extract from *E. coli*/pSEI-PH was subjected to SDS-PAGE and then stained for activity with 1-naphthylacetate and Fast Red. In contrast to the control lipase from *Chromobacterium viscosum*, no esterase-active protein could be observed in the crude enzyme extract of *E. coli*/pSEI-PH (results not shown). However, by making use of a nondenaturing polyacrylamide gel followed by activity staining using a tributyrin agar overlay, a clearing zone could be observed from the *E. coli*/pSEI-PH enzyme extract, and no activity was observed with the crude extract of the negative control *E. coli*/pBS19, which contained only the nonrecombinant cloning vector (results not shown). Since a nondenaturing polyacrylamide gel was used, the exact size of the protein could, however, not be determined accurately from the gel.

## 2.4 DISCUSSION

Several *Bacillus* isolates were screened for lipolytic activity on tributyrin agar plates in order to isolate lipolytic enzymes potentially useful for biotechnological purposes. An isolate, strain 81-11, which had originally been isolated from popcorn seeds, produced the greatest hydrolysis zone and was thus selected for further characterization. Strain 81-11 was subsequently identified as a strain of *Bacillus coagulans* by 16S rDNA gene analysis and used throughout this study.

In order to identify the lipolytic enzyme(s) produced by this strain, a genomic library was constructed from which a clone (pSEI) with high activity against tributyrin was selected and characterized. The *B. coagulans* strain 81-11 esterase-encoding gene, designated *estCI*, was identified in a 2.4-kb *Pst*I-*Hind*III fragment; it encodes a protein of 240 amino acids with a calculated molecular mass of *ca.* 28 kDa. Interestingly, the protein showed no significant similarity with most previously characterized *Bacillus* esterases; however, it displayed the highest identity with esterases from thermophilic *G. stearothermophilus* (72%) and *G. thermoleovorans* (70%) followed by a slightly lower identity (66%) to the BsubE esterase isolated from the mesophile *B. subtilis*. Significant levels of sequence identity to the putative esterases from other Gram-positive bacteria (*Listeria* and *Staphylococcus* spp.) were also

observed (Table 2.2). Multiple sequence comparison of the deduced amino acid sequence of EstC1 with the above structural homologues indicated that the proteins each contain the consensus active site sequence of serine esterases (G-X-S-X-G) located at a similar position (Fig. 2.5). It was also observed that all the proteins are of similar size and display regions of identity throughout the whole protein (Fig. 2.5). Notably, only a low percentage of all amino acids are completely different between the esterases of *B. coagulans*, *G. stearothermophilus* and *G. thermoleovorans* (12%). These small changes may, however, have a strong influence on the properties of each esterase. For example, Spiller *et al.* (1999) demonstrated, using directed-evolution studies, that only seven amino acid changes were necessary to increase the thermostability of a *p*-nitrobenzyl esterase from *B. subtilis* by 17°C.

Biochemical characterization of the *B. coagulans* strain 81-11 EstC1 esterase indicated that although the substrate profile and pH optimum of the enzyme is quite similar to that reported for esterases from different mesophilic and thermophilic bacteria, the EstC1 enzyme differs from these enzymes in features such as temperature optimum for catalytic activity and thermostability. The pH profile of *B. coagulans* strain 81-11 esterase showed highest activity under neutral and slightly basic conditions (pH 7-8), but was also active in basic conditions, around pH 9. The substrate specificity profile of the *B. coagulans* EstC1 enzyme is similar to that reported for most esterases. The enzyme shows maximal activity on short-chain fatty acid esters (C<sub>4</sub>), while activity on long-chain esters was very low (C<sub>12</sub> to C<sub>18</sub>). The esterase hydrolyzed *p*-NP butyrate in the temperature range of 30-55°C, with a maximum at 50°C. Notably, the increase in activity from 30 to 50°C is low (1.43-fold) and the activity dropped slowly for temperatures above the maximum activity, suggesting that the enzyme may be thermostable. This was subsequently investigated by incubating the enzyme for 3 h at temperatures ranging from 30-65°C. The EstC1 esterase activity was stable up to 60°C, and only a 35% loss of activity was observed after 3 h of incubation at this temperature. The optimum temperature and thermostability of EstC1 is higher than most of the known *Bacillus* esterases (Prim *et al.*, 2000; Prim *et al.*, 2001; Henke and Bornscheuer, 2002), but similar to that of *B. circulans* (Kademi *et al.*, 2000) and lower than that of *B. acidocaldarius* (Manco *et al.*, 1998) and *G. stearothermophilus* strains NCA 2184 (Henke and Bornscheuer, 2002) and Tok19A (Wood *et al.*, 1995). In this study, attempts at detecting esterase activity on renatured SDS-PAGE gels using 1-naphthylacetate and Fast Red were unsuccessful. These results are in agreement with the lack of observable activity in SDS-PAGE gels for the esterases from *B. subtilis* and *G. stearothermophilus* (Henke and Bornscheuer, 2002), and *B.*

*circulans* (Kademi *et al.*, 2000). However, activity-staining using a tributyrin agar overlay on a nondenaturing polyacrylamide gel resulted in the detection of an esterase-active band, but precluded the accurate determination of the molecular mass of the *B. coagulans* strain 81-11 esterase enzyme. It is therefore likely that the lack of activity of the EstC1 esterase on SDS-PAGE gels is due to improper folding of the protein after denaturing with SDS.

It has previously been reported that *B. coagulans* possesses esterase activity (Aragozzini *et al.*, 1991) and the purification and partial biochemical characterization of two intracellular carboxyl esterases, carboxyl esterase A and B, from *B. coagulans* NCIMB 9365 has subsequently been reported by Molinari *et al.* (1996). The purified monomeric carboxyl esterase A and B has apparent molecular weights of 70 and 60 kDa, respectively, on SDS-PAGE. Whereas esterase A hydrolyzes short-chain fatty acid esters (C<sub>6</sub>) optimally at pH 9 and 50°C, esterase B is unable to hydrolyze esters with an acyl portion of 4 or more C atoms. The esterase EstC1 isolated and characterized in this study therefore appears to be novel and extends the number of esterase enzymes present in *B. coagulans* to three. The newly identified EstC1 enzyme differs from the previously reported esterase A and B enzymes by having a predicted molecular mass of 28 kDa, preference for *p*-NP butyrate (C<sub>4</sub>) and displays maximum activity at pH 8 and 50°C. Unfortunately, the lack of sequence data for esterase A and B prohibits comparative sequence analysis.

The production of different esterases in the same bacterial species has been reported for other microorganisms as well. In addition to *B. coagulans* (Molinari *et al.*, 1996; this study), both *B. subtilis* (Higerd and Spizizen, 1973) and *B. acidocaldarius* (Manco *et al.*, 1994; 2000) produce two intracellular esterases with different properties. Functional screening of a genomic library of *Burkholderia gladioli* resulted in the identification of at least five different esterases (Schlacher *et al.*, 1998; Petersen *et al.*, 2001). Furthermore, at least four kinds of esterases have been identified in *P. fluorescence* strains, which differ in substrate specificity and cellular location (Choi *et al.*, 1990; Khalameyzer *et al.*, 1999). Since esterases from bacteria and fungi are not essential for growth (Okuda, 1991), their physiological functions are not clear. Nevertheless, it is tempting to speculate that the presence of different esterases in a single bacterium may function to hydrolyse ester compounds (short-chain), carboxylic acids and/or alcohols that are to be assimilated by the cells.



In conclusion, although the biological function of EstC1 and the natural substrate for EstC1 remains unknown, the indications obtained in this part of the study suggest a possible role for the new *B. coagulans* EstC1 esterase in biotechnology. Specifically, EstC1 exhibits considerable similarity to the biochemically characterized BstE of *G. stearothermophilus* (Amaki *et al.*, 1992; Henke and Bornscheuer, 2002) and *B. subtilis* BsubE (*yvak*) esterase (Eggert *et al.*, 2000; Henke and Bornscheuer, 2002). Both these enzymes show high enantioselectivity in the kinetic resolution of menthyl acetate (Henke and Bornscheuer, 2002). The high enantioselectivity towards menthyl acetate may be of particular importance since it allows access to (-)-menthol, which is a key substance in the fragrance industry and is currently being produced by fractionated crystallization from racemic menthyl benzoate.



## CHAPTER THREE

### EXPRESSION OF THE EstC1 ESTERASE FROM *Bacillus coagulans* strain 81-11 IN DIFFERENT GRAM-POSITIVE BACTERIAL HOST STRAINS

### 3.1 INTRODUCTION

Esterases (EC 3.1.1.1) and lipases (EC 3.1.1.3) are enzymes with a great potential for biotechnological applications such as ester synthesis as flavours for the food industry (McKay, 1993), modification of the physico-chemical properties of triglycerides in the fat and oil industry (Ziller *et al.*, 1994), aroma production in the cosmetic industry (Jaeger and Reetz, 1998), and preparation of optically pure bioactive molecules for the pharmaceutical and pesticide industries (Jaeger *et al.*, 1999; Davis and Boyer, 2001; Bornscheuer, 2002). Although there are several industrial processes that employ these enzymes as biocatalysts, both as whole-cell systems and in the form of isolated enzymes (Benjamin and Pandey, 1997; Tengerdy, 1998; Liese *et al.*, 2001), there are, however, fewer industrial applications for esterases, compared to lipases. This is mainly because of the lack of availability of these biocatalysts in sufficient amounts (Faber, 1997). One way of obtaining sufficient amounts of protein products is to clone and overexpress the relevant genes in an appropriate expression host.

Among the many systems that are available for heterologous protein production, the Gram-negative bacterium *Escherichia coli* has remained one of the most attractive due to its ability to grow rapidly and at high density on inexpensive substrates, its well-characterized genetics and the availability of a large number of cloning vectors and mutant host strains (Balbas and Bolivar, 1990; Old and Primrose, 1994). However, in spite of the extensive knowledge on the genetics and molecular biology of *E. coli*, not every gene can be expressed efficiently in this organism and the recombinant gene product may accumulate in *E. coli* at high levels in a truncated and biologically inactive form (Gottesman, 1996; Rudolph and Lilie, 1996). This may be due to the unique and subtle structural features of the gene sequence, the stability and translational efficiency of mRNA, the ease of protein folding, degradation of the protein by host cell proteases, major differences in codon usage between the foreign gene and native *E. coli*, and the potential toxicity of the protein to the host (Old and Primrose, 1994).

Gram-positive bacteria, such as *Bacillus* spp., are well known for their important contribution to medical, agricultural and food biotechnology and as production organisms for industrial enzymes (Harwood, 1992; Bolhuis *et al.*, 1999). The latter capacity is largely based on the common architecture of their cell envelope, which allows for direct secretion of proteins into the extracellular medium (Simonen and Palva, 1993). *Bacillus* species have therefore been



regarded as attractive hosts for the production of both homologous and heterologous secretory proteins and a great number of exoprotein genes from different organisms have been cloned and expressed in *Bacillus* species (Wu *et al.*, 1991; Yamada *et al.*, 1995; Bolhuis *et al.*, 1999). In addition, *Bacillus* strains offer many potential advantages in the production of cloned gene products, *e.g.* (i) *Bacilli* are non-pathogenic and do not synthesize endotoxins, (ii) many of the gene products are secreted to the growth medium, in contrast to *E. coli*, which retains most of the proteins, and (iii) *Bacilli* have been widely used for production of industrial enzymes in large-scale fermentation processes (Lam *et al.*, 1998; Jan *et al.*, 2001). Furthermore, the development of improved genetic tools for *B. subtilis* has made it an attractive host for recombinant protein production (Henner, 1990a; 1990b; Le Grice, 1990; Conrad *et al.*, 1996). However, endogenous proteases degrade recombinant products (Palva, 1989), even in mutant *Bacillus* strains with little proteolytic activity (Nakamura *et al.*, 1991). Furthermore, protease-deficient *Bacillus* strains tend to be autolytic (Joliff *et al.*, 1989; Coxon *et al.*, 1991) and consequently the ideal strains of *B. subtilis* for industrial production have yet to be developed. Another potentially useful host for the production of heterologous proteins is *Bacillus brevis* (Yamagata *et al.*, 1985; Udaka and Yamagata 1993; Ichikawa *et al.*, 1993). *B. brevis* has a superior secretion capacity to *B. subtilis* and has no detectable intracellular proteases.

Other than *B. subtilis* expression hosts, lactic acid bacteria (LAB) is also of importance because of their widespread use in industrial food fermentation processes. These food-grade microorganisms have “generally regarded as safe” status, making them potentially useful organisms for the production of commercially important proteins. Based on the increased knowledge regarding their gene structure and expression and of protein secretion, much progress has been made in recent years in the development of gene expression systems for bacteria that belong to the genera *Clostridium*, *Lactococcus*, *Lactobacillus*, *Staphylococcus* and *Streptococcus* (de Vos *et al.*, 1997; Leenhouts *et al.*, 1998b; Slos *et al.*, 1998). By contrast to *B. subtilis*, food-grade bacteria such as *Lactococcus lactis* (Wells *et al.*, 1993; Steidler *et al.*, 1995) and *C. glutamicum* (Billman-Jacobe *et al.*, 1995) have no detectable extracellular proteases or toxins and are therefore attractive hosts for secretion based on their potential to produce pure, high-quality recombinant products. Moreover, genes of bacterial, plant and fungal origin have been expressed successfully in these food-grade bacteria (Mercenier *et al.*, 1994; Savijoki *et al.*, 1997; Slos *et al.*, 1998).

Based on the above, it follows that an “ideal” expression host does not exist but rather that the most appropriate host depends on the application at hand. Since overexpression of the EstC1 esterase from *B. coagulans* strain 81-11 may enable evaluation of its potential applicability and utility in biotechnological processes, the aims of this part of the investigation were therefore (i) to express the esterase-encoding *estC1* gene from *B. coagulans* strain 81-11 in different *Bacillus subtilis* strains and lactic acid bacteria in order to identify the most appropriate host for overexpression of the newly identified enzyme, and (ii) to evaluate the use of a *Bacillus* species alkaline protease promoter and signal sequence as a means whereby the level of extracellular esterase expression might be increased.

## 3.2 MATERIALS AND METHODS

### 3.2.1 Bacterial strains, plasmids and growth conditions

The bacterial strains and plasmids used in this part of the study are listed in Table 3.1. *Escherichia coli* JM83 and *Bacillus subtilis* 154 were used as cloning and expression hosts, while *Bacillus subtilis* 1A297, *Lactobacillus plantarum* NCIB1193 and *Lactococcus lactis* IL403 were used as additional Gram-positive expression hosts. The *E. coli* and *Bacillus* strains were cultured at 37°C with rotary shaking aeration at 175 rpm in Luria-Bertani broth (LB: 0.5% [w/v] yeast extract, 1% [w/v] tryptone, 1% [w/v] NaCl; pH 7.4), or maintained at 4°C on LB agar plates. *Lactobacillus plantarum* was cultured in MRS broth (Oxoid; Code CM0359), whereas *Lactococcus lactis* was cultured in GSM17 medium consisting of 38 g M17 broth (Oxoid; Code CM0817), 171 g sucrose and 25 ml 20% (w/v) glucose per litre. The *Lactobacillus plantarum* and *Lactococcus lactis* strains were cultured at 30°C without rotary shaking aeration. To select for recombinant transformants and to maintain plasmid DNA in the host cells, the culture media were supplemented with the appropriate antibiotics, as indicated in the text. All antibiotics were purchased from Roche Diagnostics. Plasmids pMG36e (van de Guchte *et al.*, 1989) and pNW33N (De Rossi *et al.*, 1994) were used for cloning and expression of the *B. coagulans* strain 81-11 esterase enzyme.



**Table 3.1: Bacterial strains and plasmids used**

Strain or plasmid	Relevant properties	Source
<b>Strains:</b>		
<i>E. coli</i> JM83	{F <sup>-</sup> <i>ara</i> ( <i>lac-proAB</i> ) <i>rpsL</i> (Str <sup>r</sup> ) [80 <i>dlac</i> ( <i>lacZ</i> )M15] <i>thi</i> }	Vieira and Messing, 1982
<i>B. coagulans</i> strain 81-11	Wild-type	This study (Chapter 2)
<i>B. subtilis</i> 154	( $\Delta apr$ , $\Delta npr$ , <i>amy</i> , <i>spo</i> )	Laboratory collection
<i>B. subtilis</i> 1A297	<i>AmyE</i> , <i>aspTI</i> , <i>TrpC2</i>	Laboratory collection
<i>Lactobacillus plantarum</i> NCIB1193	Type strain	Laboratory collection
<i>Lactococcus lactis</i> IL1403	Plasmid-free strain NCDO712	Laboratory collection
<b>Plasmids:</b>		
pBC(SK)	Cloning vector, ColE1, Amp <sup>r</sup>	Stratagene
pMG36e	Expression vector for <i>L. lactis</i> , Em <sup>r</sup> , pWV01- <i>ori</i> , P32 promoter	van de Guchte <i>et al.</i> , 1989
pNW33N	<i>E. coli-B. subtilis</i> shuttle vector, Cm <sup>r</sup>	De Rossi <i>et al.</i> , 1994
pSK-Approm	pBC(SK) harbouring an alkaline protease promoter and signal sequence from <i>Bacillus</i> species	M. Louw, unpublished
pSEI	pBS19 with a cloned 3.2-kb <i>Sau3AI</i> DNA fragment from <i>B. coagulans</i> strain 81-11, confers lipolytic activity in <i>E. coli</i> JM83	This study (Chapter 2)
pSEI-PH	pBS19 carrying a 2.4-kb <i>PstI-HindIII</i> DNA fragment from pSEI containing the <i>estC1</i> gene of <i>B. coagulans</i> strain 81-11	This study (Chapter 2)
pMG36-EstC1	pMG36e carrying a 2.4-kb <i>PstI-HindIII</i> DNA fragment from pSEI containing the <i>estC1</i> gene of <i>B. coagulans</i> strain 81-11	This study
pBC-EstC1aps	pBC(SK) harbouring the <i>estC1</i> gene of <i>B. coagulans</i> strain 81-11 cloned under control of the alkaline protease promoter and signal sequence from <i>Bacillus</i> species	This study
pNW-EstC1aps	pNW33N carrying a 2.7-kb <i>BamHI-SalI</i> DNA fragment comprising the promoter-signal sequence- <i>estC1</i> cassette from pBC-EstC1aps	This study

### 3.2.2 Construction of expression vectors

For expression of the *B. coagulans* EstC1 esterase in *Bacillus*, *Lactobacillus* and *Lactococcus* species, plasmid pMG36e was used as the source for construction of the desired expression vector. The broad-host-range vector pMG36e contains the strong lactococcal promoter P32, transcriptional terminators derived from the *L. lactis* Wg2 proteinase gene and a pWV01 origin of replication, thus enabling replication of the plasmid DNA in different Gram-positive bacteria (van de Guchte *et al.*, 1989). Plasmid pNW33N, a shuttle vector that stably replicates in *B. subtilis*, *G. stearothermophilus* and *E. coli* (De Rossi *et al.*, 1994), was used as the source for construction of a secretion vector whereby the level of extracellular EstC1 esterase expression could possibly be increased. All molecular cloning techniques employed in the construction of the recombinant vectors were performed in accordance with the procedures described in Chapter 2. All plasmid constructs were confirmed by restriction endonuclease digestion using agarose gel electrophoresis following plasmid DNA extraction (Section 3.2.8). The cloning strategies used in the construction of the respective vectors are shown in Fig. 3.1.

#### 3.2.2.1 Construction of expression vector pMG36-EstC1

To construct the recombinant pMG36-EstC1 expression vector, plasmid pSEI, harbouring a 3.2-kb genomic DNA fragment from *B. coagulans* strain 81-11 (Chapter 2), was digested with both *Pst*I and *Hind*III to excise a 2.4-kb DNA fragment harbouring the full-length *estC1* ORF, together with the upstream promoter and regulatory sequences. The DNA fragment was purified from the agarose gel using the GeneClean method (Section 2.2.3.3) and ligated into identically prepared pMG36e vector DNA. Ligation reactions were performed using the Fast-Link™ DNA ligation kit (Epicentre) and the ratio of insert to vector was typically in excess of 2:1. Briefly, the ligation reaction mixture consisted of 160 ng of insert DNA, 60 ng of vector DNA, 1 × Fast-Link™ ligation buffer (33 mM Tris-acetate [pH 7.8], 66 mM KOAc, 10 mM MgOAc, 0.5 mM DTT), 1 mM ATP and 1 U of DNA ligase in a final volume of 15 µl. Following incubation for 1 h at room temperature, the DNA ligase enzyme was inactivated by heating to 70°C for 15 min and the ligation reaction mixture was then transformed into *B. subtilis* 154 protoplasts, as described below (Section 3.2.3).

### 3.2.2.2 Construction of secretion vector pNW-EstC1aps

Recombinant plasmid pSK-APprom, harbouring the alkaline protease promoter and signal sequence from *Bacillus* species (M. Louw, unpublished results), was digested with both *Hind*III and *Pst*I to excise the promoter, together with its signal sequence. Recombinant plasmid pSEI was digested with both *Pst*I and *Sal*I to excise a 2.4-kb DNA fragment harbouring the *B. coagulans* strain 81-11 *estC1* gene. The DNA fragments were purified from an agarose gel using the GeneClean method (Section 2.2.3.3) and cloned into plasmid pBC(SK), which had been digested with both *Hind*III and *Sal*I, to yield recombinant plasmid pBC-EstC1aps. The ligation reactions were performed using the Fast-Link™ DNA ligation kit (Epicentre), as described above, except that the reaction mixture contained 100 ng of the *estC1* DNA fragment, 100 ng of the alkaline protease promoter and signal sequence DNA fragment, and 35 ng of digested pBC(SK) vector DNA. The cloned expression/secretion cassette was subsequently recovered from pBC-EstC1aps by digestion with *Bam*HI and *Sal*I, agarose gel-purified and cloned into identically digested pNW33N. Following transformation of *B. subtilis* 1A297, using the procedures described below (Section 3.2.4), the extracted plasmid DNA was characterized by restriction enzyme digestion. One of the resulting recombinant clones was selected for further use and designated pNW-EstC1aps.

### 3.2.3 Transformation of *B. subtilis* 154

#### 3.2.3.1 Preparation of protoplasts

Protoplasts of *B. subtilis* 154 were prepared according to the method of Chang and Cohen (1979). A single colony of *B. subtilis* 154 was inoculated into 60 ml LB broth and the culture was incubated at 37°C with shaking to an OD<sub>540</sub> of 5.5. The culture was then used to inoculate 60 ml LB broth to an OD<sub>540</sub> of 0.05, and the flask was incubated at 37°C until an OD<sub>540</sub> of 0.3 had been reached. The cells were harvested by centrifugation at 8 000 rpm for 8 min and the cell pellet suspended in 5 ml SMMP medium. To obtain protoplasts, lysozyme was added to a final concentration of 2 mg/ml and the suspension was incubated for 45 min at 37°C with gentle shaking (40 rpm). Following incubation, the protoplasts were harvested by centrifugation at 4 000 rpm for 15 min at 20°C, washed once in 5 ml SMMP medium and resuspended gently in 3 ml of the SMMP medium. The SMMP medium was prepared by mixing 100 ml of 2 × SMM buffer (34% [w/v] sucrose, 0.46% [w/v] maleic acid, 3.98 mM MgCl<sub>2</sub>; pH 6.5) with an equal volume of 4 × PAB (0.3% [w/v] beef extract, 0.3% [w/v] yeast

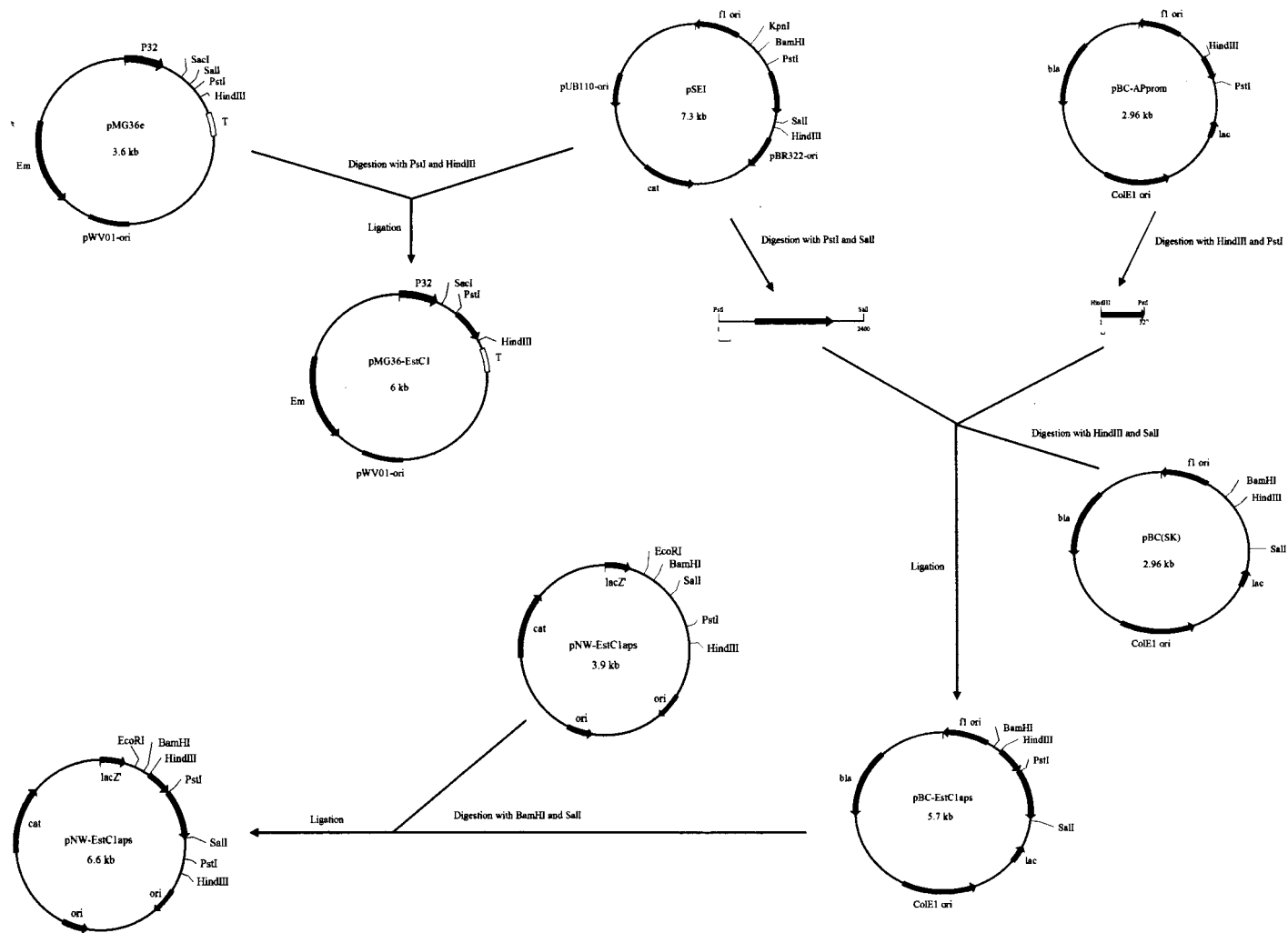


Fig. 3.1 Construction of the expression vector pMG36-EstC1 and the secretion vector pNW33-EstC1.



extract, 1% [w/v] peptone, 0.2% [w/v] glucose, 0.7% [w/v] g NaCl, 4.19 mM K<sub>2</sub>HPO<sub>4</sub>, 1.98 mM KH<sub>2</sub>PO<sub>4</sub>; pH 7).

### 3.2.3.2 Transformation

The prepared protoplasts were transformed using a polyethylene glycol (PEG) method as described by Chang and Cohen (1979). Prior to transformation, the volume of the pMG36e ligation reaction mixture was adjusted to 50 µl with TE buffer and an equal volume of 2 × SMM buffer was added. The ligation reaction mixture was subsequently mixed with 500 µl of the prepared protoplast suspension and following the addition of 1.5 ml of 30% (w/v) PEG-4000 (prepared in 1 × SMM buffer), the suspension was incubated at room temperature for 2 min. Following incubation, 5 ml of SMMP medium was added and the protoplasts were collected by centrifugation at 4 000 rpm for 25 min. The collected protoplasts were suspended in 1 ml SMMP medium and incubated at 37°C for 90 min in a shaking incubator (80 rpm) to allow regeneration of the cell walls. The transformed cells were selected by plating 100-µl aliquots of the suspension onto DM3 agar plates (13.5% [w/v] sodium succinate, 1% [w/v] cas-amino acids, 0.5% [w/v] yeast extract, 0.5% [w/v] glucose, 10 mM K<sub>2</sub>HPO<sub>4</sub>, 5.5 mM KH<sub>2</sub>PO<sub>4</sub>, 21.5 mM MgCl<sub>2</sub>, 0.6% [w/v] agar; pH 7.3) supplemented with 2% (w/v) filter-sterilized bovine serum albumin (BSA) and 10 µg/ml erythromycin. The agar plates were subsequently incubated at 37°C for 48 h.

### 3.2.4 Transformation of *B. subtilis* 1A297

#### 3.2.4.1 Preparation of competent cells

Plasmid DNA was introduced into *B. subtilis* 1A297 cells by electroporation of competent cells (Xue *et al.*, 1998). To prepare competent cells, a single colony of *B. subtilis* 1A297 was inoculated into 10 ml LB broth containing 9.1% (w/v) sorbitol (LB+S). After incubation at 37°C overnight, the culture was used to inoculate 50 ml preheated LB+S broth to an OD<sub>540</sub> of 0.3. The culture was grown at 37°C to an OD<sub>540</sub> of between 0.85 and 0.95 whereafter the cultures were cooled on ice for 10 min to inhibit further growth. The cells from 40 ml of the culture were harvested by centrifugation at 6 000 rpm for 5 min at 4°C, washed four times (7 000 rpm, 10 min) with electroporation medium (9.1% [w/v] sorbitol, 9.1% [w/v] mannitol, 10% [v/v] glycerol) and finally suspended in 1 ml of the medium.

### 3.2.4.2 Electroporation

For transformation of the prepared competent *B. subtilis* 1A297 cells, 60  $\mu$ l of the cells were pipetted into sterile 1.5-ml Eppendorf tubes and mixed with 1  $\mu$ g of the recombinant pMG36-EstC1 plasmid DNA. The transformation mixtures were then transferred to a 0.1-cm electrode gap electroporation cuvette (BioRad) and exposed to a single electrical pulse of 4.6 s using a BioRad Gene-Pulser™ set at 2.1 kV, 25  $\mu$ F and 200  $\Omega$ . Immediately following the electrical discharge, the cells were transferred to 1 ml recovery medium (1% [w/v] tryptone, 0.5% [w/v] yeast extract, 0.5% [w/v] NaCl, 9.1% [w/v] sorbitol, 6.9% [w/v] mannitol; pH 7.3) and incubated for 3 h at 37°C in a shaking incubator. To select for transformed cells, aliquots (100  $\mu$ l) of the cells were plated onto LB agar plates supplemented with 10  $\mu$ g/ml erythromycin and 100  $\mu$ g/ml lincomycin, and incubated at 37°C overnight.

### 3.2.5 Transformation of *Lactococcus lactis* IL1403

#### 3.2.5.1 Preparation of competent cells

Competent *L. lactis* IL403 cells were prepared and transformed using the procedures of Bron (1991). A single colony of *L. lactis* IL403 was inoculated into 5 ml of SMGG17 medium consisting of 38 g M17 broth (Oxoid; Code CM0817), 171 g sucrose, 25 ml 20% (w/v) glucose and 2% glycine per litre. Following incubation at 30°C overnight, 1 ml of the culture was inoculated into 100 ml preheated SMGG17 broth and the culture grown to an OD<sub>600</sub> of 0.2 to 0.7. The cells were collected by centrifugation at 6 000 rpm for 5 min at 4°C and washed twice in an ice-cold solution consisting of 0.5 M sucrose and 10% (v/v) glycerol, before being suspended in 1 ml SMGG17 medium.

#### 3.2.5.2 Electroporation

For transformation, recombinant pMG36-EstC1 plasmid DNA (1.2  $\mu$ g) was mixed with 40  $\mu$ l of the competent *L. lactis* IL403 cells in a 0.2-cm electrode gap electroporation cuvette (BioRad) and exposed to a single electrical pulse of 8.5 s using a BioRad GenePulser™ set at 2.0 kV, 25  $\mu$ F and 200  $\Omega$ . Following the electrical discharge, the electroporated cells were added to 960  $\mu$ l ice-cold SMGG17 medium and incubated on ice for 5 min. After addition of a further 1 ml SMGG17 medium and erythromycin at a concentration of 0.5  $\mu$ g/ml, the cells were incubated at 30°C for 2 h. The cells were then concentrated by centrifugation at 1 000 rpm for 1 min and suspended in 0.5 ml SMGG17 medium. To select for transformed cells,

aliquots (100  $\mu$ l) of the cells were plated onto LB agar plates containing 5  $\mu$ g/ml erythromycin and incubated overnight at 30°C.

### **3.2.6 Transformation of *Lactobacillus plantarum* NCIB1193**

#### **3.2.6.1 Preparation of competent cells**

The recombinant pMG36-EstC1 plasmid DNA was introduced into competent *L. plantarum* NCIB1193 cells by electroporation, as described by Josson *et al.* (1989), with the following modifications. A single colony of *L. plantarum* NCIB1193 was inoculated into 50 ml MRS broth and cultured at 30°C overnight without shaking. Following incubation, the culture was diluted 1:50 in sterile 50 ml MRS broth and incubated at 30°C until an OD<sub>600</sub> of 0.5 had been reached. The cells were harvested by centrifugation at 6 000 rpm for 5 min at 4°C, washed twice with distilled water at room temperature and then suspended in 1 ml of 30% (w/v) PEG-1000.

#### **3.2.6.2 Electroporation**

Recombinant plasmid pMG36-EstC1 (0.1  $\mu$ g) was added to 100  $\mu$ l of the prepared *L. plantarum* NCIB1193 competent cells in a 0.2-cm electroporation cuvette (BioRad) and exposed to a single electric pulse of 8.5 s using a BioRad GenePulser™ set at 1.7 kV, 25  $\mu$ F and 400  $\Omega$ . The electroporated cells were kept on ice for 30 min, diluted in 1 ml MRS broth and incubated for 3 h at 37°C. To select for transformed cells, 100- $\mu$ l aliquots of the cells were plated onto MRS agar plates supplemented with 10  $\mu$ g/ml erythromycin and 100  $\mu$ g/ml lincomycin, and the agar plates were incubated overnight at 37°C.

### **3.2.7 Characterization of plasmid DNA**

#### **3.2.7.1 Plasmid DNA extractions**

##### **3.2.7.1.1 Plasmid DNA extractions from *E. coli***

Plasmid DNA was isolated from selected colonies using a modified alkaline lysis method (Sambrook and Russel, 2001). Colonies were picked from the agar plates with sterile toothpicks, inoculated into 5 ml of LB broth containing the appropriate antibiotic and then incubated at 37°C overnight with shaking. After incubation, cells from 2 ml of the cultures were collected by centrifugation for 1 min at 14 000 rpm. The bacterial cell pellet was



suspended in 400  $\mu$ l of Solution 1 (50 mM glucose, 25 mM Tris-HCl, 10 mM EDTA, 10 mg/ml lysozyme; pH 8.0) before incubation at room temperature for 10 min. The spheroplasts were lysed following the addition of 400  $\mu$ l of freshly prepared Solution 2 (0.2 N NaOH, 1% [w/v] SDS). After incubation on ice for 10 min, 300  $\mu$ l of Solution 3 (7.5 M ammonium acetate; pH 7.6) was added and incubation was continued on ice for a further 10 min. The cellular debris was removed by centrifugation for 10 min at 10 000 rpm, after which the plasmid DNA was precipitated from the recovered supernatants by the addition of 650  $\mu$ l isopropanol at room temperature for 10 min. The precipitated plasmid DNA was collected by centrifugation, as above, rinsed with 70% ethanol, air-dried and suspended in 10  $\mu$ l TE buffer.

#### **3.2.7.1.2 Plasmid DNA extractions from *Bacillus* strains**

*B. subtilis* transformants were cultured at 37°C overnight in 5 ml of LB broth supplemented with 10  $\mu$ g/ml erythromycin. The overnight cultures were then diluted 1:50 in 30 ml sterile LB broth and grown to an OD<sub>540</sub> of 0.6, after which the cells from 20 ml of the culture were harvested by centrifugation at 6 000 rpm for 10 min. The cell pellets were suspended in 200  $\mu$ l of Solution 1 and following incubation at 37°C for 30 min with shaking at 40 rpm, 200  $\mu$ l of Solution 2 was added and the tubes incubated at room temperature for 5 min. After addition of 200  $\mu$ l of Solution 3, the lysates were incubated on ice for 20 min and then cleared by centrifugation at 14 000 rpm for 5 min. Plasmid DNA was precipitated from the recovered supernatant by addition of 600  $\mu$ l isopropanol and the precipitated plasmid DNA was pelleted by centrifugation at 14 000 rpm for 10 min. The DNA pellet was rinsed once with 70% ethanol, air-dried and suspended in 10  $\mu$ l TE buffer.

#### **3.2.7.1.3 Plasmid DNA extractions from *Lactococcus lactis* and *Lactobacillus plantarum***

*Lactococcus lactis* transformants were cultured at 30°C overnight in 5 ml of SMGG17 medium supplemented with 20% (w/v) glucose after which the cells from 2 ml of the culture were collected by centrifugation at 14 000 rpm for 1 min. For *Lactobacillus plantarum* transformants, the cultures were grown at 30°C overnight in MRS broth and then diluted 1:25 in 50 ml sterile MRS broth. Following incubation at 30°C to an OD<sub>600</sub> of 0.4, the cells were harvested by centrifugation at 6 000 rpm for 5 min at 4°C and washed once with 10 ml sterile distilled water. The obtained *L. lactis* and *L. plantarum* cell pellets were suspended in 500  $\mu$ l of Solution 1 followed by addition of 25  $\mu$ l mutanolysin (Sigma). The suspensions were



incubated at 37°C for 30 min after which 500 µl of Solution 2 was added, and the tubes incubated at room temperature for 3 min. Following incubation, 500 µl of Solution 3 was added to the cell lysates and the tubes incubated on ice for 15 min. The cell lysates were then centrifuged at 14 000 rpm for 15 min and the supernatants were aliquoted into new Eppendorf tubes. The plasmid DNA was precipitated by addition of 500 µl isopropanol and collected by centrifugation (14 000 rpm, 15 min). The plasmid DNA was washed once with 70% ethanol, air-dried and suspended in 15 µl TE buffer.

### **3.2.7.2 Agarose gel electrophoresis**

DNA was analyzed on 1% (w/v) agarose slab gels (Sambrook and Russel, 2001) and electrophoresis was performed at 80 V in 1 × TAE buffer (40 mM Tris-HCl, 20 mM Acetic acid, 2 mM EDTA; pH 8.5). The agarose gels were supplemented with ethidium bromide (0.5 µg/ml) and the DNA fragments visualized by UV fluorescence. Where appropriate, the DNA fragments were sized according to their migration in the gel as compared to that of a standard DNA molecular weight marker, namely phage lambda DNA digested with *EcoRI* and *HindIII* (Roche Diagnostics).

### **3.2.7.3 Restriction endonuclease digestions**

Purified plasmid DNA (1 µg) was typically digested at 37°C for 90 min with 5 U of restriction enzyme (Roche Diagnostics) in the appropriate concentration salt (using the 10 × buffer supplied by the manufacturer). Following inactivation of the enzyme by heating to 65°C for 20 min, the restriction fragments were analyzed by agarose gel electrophoresis as described above.

## **3.2.8 Expression of the *B. coagulans* strain 81-11 esterase in Gram-positive bacterial hosts**

### **3.2.8.1 Esterase plate assays**

Transformants were streaked onto freshly prepared tributyrin-containing agar plates (Mourey and Kilbertus, 1975), supplemented with the appropriate antibiotics. Following incubation of the agar plates at the appropriate temperatures, the bacterial growth was inspected for a clear zone around their margins.



### 3.2.8.2 Preparation of crude enzyme extracts

Recombinant strains used as hosts for expression of the *B. coagulans* strain 81-11 EstC1 esterase were cultured overnight in the appropriate media supplemented with the appropriate antibiotics (*Lactobacillus* was cultured to exponential phase). The cells were harvested by centrifugation at 6 000 rpm for 15 min at 4°C and the cell-free culture supernatants were recovered and kept to assay the extracellular enzyme activity. The pellets were each suspended in 20 ml sonication buffer (25 mM Tris-HCl, 10 mM EDTA, 25 mM glucose, 50 mM NaCl; pH 8), lysed by sonication of 5-ml volumes for 1 h at 5-min intervals and used in subsequent assays (intracellular). The protein concentration of the extracellular and intracellular extracts was determined according to the method of Bradford (1976), using a commercial kit (BioRad Protein Assay kit), as described in Section 2.2.11.

### 3.2.8.3 Enzyme assay

The enzyme activity was determined spectrophotometrically by measuring the liberation of *p*-nitrophenol from *p*-nitrophenyl butyrate (*p*-NPB; Sigma) according to the method of Cho *et al.* (2000). Briefly, 500 µl of the cell extract was added to 4.5 ml of the enzyme substrate, prepared as described in Section 2.2.10, and the reaction mixture was incubated at 30°C for 5 min. The absorbance at 405 nm was read against a reagent blank using a WPA Model S2000 spectrophotometer (Labotec). One activity unit was defined as the amount of enzyme capable to release 1 µmol of *p*-NPB per minute under the reaction conditions used. Mean results of duplicate, independent samples are reported.

## 3.3 RESULTS

### 3.3.1 Construction of recombinant expression vector pMG36-EstC1

Towards identifying the most appropriate Gram-positive host for overexpression of the EstC1 esterase from *B. coagulans* strain 81-11, the *estC1* gene, together with the upstream putative promoter and regulatory sequences, was recovered from plasmid pSEI (Chapter 2) as a 2.4-kb *Pst*I-*Hind*III DNA fragment and cloned into the identically digested broad-host-range plasmid pMG36e. Following transformation of protoplasts prepared from *B. subtilis* 154 cells, plasmid DNA was isolated from randomly selected transformants and characterized by agarose and restriction endonuclease analysis. Digestion of recombinant pMG36-EstC1 (Fig. 3.2a) with both *Pst*I and *Hind*III resulted in the excision of a 2.4-kb DNA fragment, which



corresponded with the expected size of the cloned DNA insert (Fig. 3.2b). The recombinant pMG36-EstC1 vector construct, harbouring the cloned esterase-encoding gene of *B. coagulans* strain 81-11, was subsequently introduced into *Lactococcus lactis* IL403, *Lactobacillus plantarum* NCIB1193 and *Bacillus subtilis* 1A297 by electroporation.

### 3.3.2 Expression of the *B. coagulans* strain 81-11 EstC1 esterase in different Gram-positive prokaryotic hosts

Following introduction of the recombinant pMG36-EstC1 plasmid DNA into different Gram-positive bacteria, the derived recombinant strains (*L. lactis*/pMG36-EstC1, *L. plantarum*/pMG36-EstC1, *B. subtilis* 154/pMG36-EstC1 and *B. subtilis* 1A297/pMG36-EstC1) were examined for esterase activity by first streaking the respective strains onto agar plates supplemented with 1% (w/v) tributyrin and the appropriate antibiotics. Following incubation, the agar plates were examined for zones of hydrolysis and recombinant strains displaying esterase activity were selected for further analyses. To obtain quantitative data, the esterase activity of both the cell-free culture supernatant (extracellular) and prepared cell extracts (intracellular) were assayed using *p*-nitrophenyl butyrate as substrate. Recombinant strains harbouring the nonrecombinant pMG36e plasmid DNA were included in the assays as controls whereby the endogenous esterase activity of the respective host strains could be determined, whilst *B. coagulans* strain 81-11 was included in these assays for comparative purposes.

#### 3.3.2.1 Expression in different lactic acid bacteria

Following streaking of the recombinant *L. lactis* and *L. plantarum* strains harbouring either the recombinant or nonrecombinant pMG36e plasmid DNA onto tributyrin-containing agar plates, no zones of substrate hydrolysis could be observed. To determine whether the lack of extracellular esterase activity observed for the *L. lactis*/pMG36-EstC1 and *L. plantarum*/pMG36-EstC1 strains could have been due to intracellular accumulation of the esterase, the esterase activity of intracellular and extracellular fractions was examined by cup plate assays. No esterase activity was observed in either the culture supernatants or intracellular enzyme extracts, even after 48 h of incubation (results not shown).

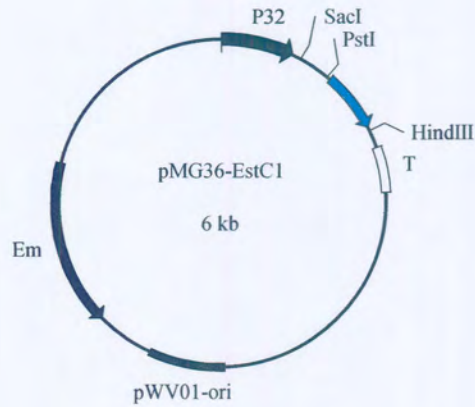


Fig. 3.2a Plasmid map of the recombinant plasmid pMG36-EstC1 containing a cloned copy of the *B. coagulans* strain 81-11 *estC1* gene.

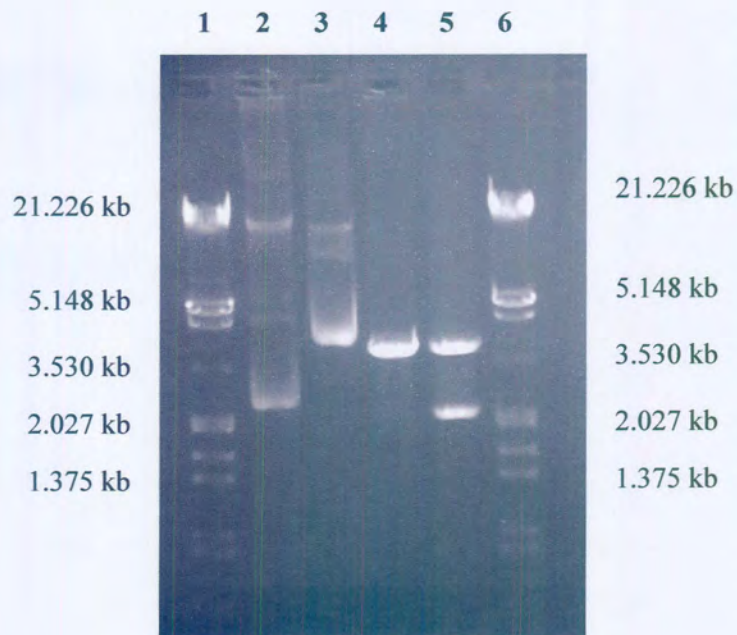


Fig. 3.2b Agarose gel electrophoretic analysis of the recombinant plasmid pMG36-EstC1. Lanes 1 and 6, DNA molecular weight marker; lane 2, uncut parental pMG36e vector DNA; lane 3, uncut pMG36-EstC1 plasmid DNA; lane 4, pMG36e vector DNA digested with *PstI* and *HindIII*; lane 5, recombinant plasmid pMG36-EstC1 digested with *PstI* and *HindIII*. The sizes of the DNA molecular weight marker, phage  $\lambda$  DNA digested with both *EcoRI* and *HindIII*, are indicated to the sides of the figure.



To determine whether the lack of detectable esterase activity may have been due to plasmid loss, plasmid DNA extractions were performed on overnight cultures of the recombinant *L. lactis* and *L. plantarum* strains harbouring the recombinant and nonrecombinant pMG36e vector DNA. Although plasmid DNA could be extracted from *L. lactis*/pMG36e, the recombinant plasmid DNA could not be extracted from *L. lactis*/pMG36-EstC1 strains. By contrast, plasmid DNA could be extracted from recombinant *L. plantarum* cultures harbouring the recombinant or nonrecombinant pMG36e plasmid DNA. Digestion of the recombinant plasmid DNA with both *Pst*I and *Hind*III yielded two DNA fragments corresponding in size to the vector DNA (3.6 kb) and *estC1*-containing DNA fragment (2.4 kb).

Based on these results, it was concluded that the lack of EstC1 esterase activity in *L. lactis* may be due to instability of the recombinant plasmid DNA and that the lack of EstC1 esterase activity in *L. plantarum* may be due to the *estC1* gene not being expressed in this bacterial strain.

### 3.3.2.2 Expression in different *Bacillus subtilis* strains

Following streaking of the recombinant *B. subtilis* 154 and *B. subtilis* 1A297 strains harbouring either the recombinant or nonrecombinant pMG36e plasmid DNA onto tributyrin-containing agar plates, zones of hydrolysis could be seen surrounding the bacterial growth. These results suggested that both *Bacillus subtilis* strains produce an endogenous esterase(s). Thus, to determine whether the *B. coagulans* EstC1 esterase was indeed expressed in these bacterial hosts, the esterase activity of cell-free culture supernatants (extracellular) and cell extracts (intracellular) prepared from the different recombinant *B. subtilis* strains was assayed using *p*-nitrophenyl butyrate as substrate and the specific activity expressed as units per milligram protein.

Analysis of the results, presented in Table 3.2, indicated that the recombinant *B. subtilis* 154 strains harbouring either the recombinant or nonrecombinant pMG36e plasmid DNA displayed similar low intracellular and extracellular esterase activities, albeit that the intracellular activities were slightly higher than the extracellular activities. By contrast, the *B. subtilis* 1A297 strain harbouring the recombinant pMG36-EstC1 plasmid DNA displayed a 1.7-fold increase in intracellular esterase activity compared with the intracellular esterase

activity of *B. subtilis* 1A297 harbouring the nonrecombinant plasmid DNA. The levels of extracellular esterase activity displayed by these respective strains were, however, comparable to each other and also similar to the levels of extracellular activity observed for the recombinant *B. subtilis* 154 strains (Table 3.2). Plasmid DNA could be extracted from the recombinant *B. subtilis* strains harbouring the recombinant and nonrecombinant pMG36e plasmid DNA and characterization of the extracted recombinant pMG36-EstC1 plasmid DNA by restriction enzyme digestion with both *Pst*I and *Hind*III yielded DNA fragments of the expected sizes.

Based on the above results, it was concluded that although both *B. subtilis* 154 and *B. subtilis* 1A297 produce an endogenous esterase(s), the *B. coagulans estC1* gene was expressed intracellularly in *B. subtilis* 1A297, but not in *B. subtilis* 154. Since the plasmid DNA was stably maintained in both *B. subtilis* strains, the difference in EstC1 esterase expression observed between the respective strains may be due to strain-specific differences.

### **3.3.3 Extracellular expression of the *B. coagulans* strain 81-11 EstC1 esterase in *B. subtilis* 1A297**

#### **3.3.3.1 Construction of recombinant secretion vector pNW-EstC1aps**

Exported proteins are synthesized initially as preproteins with an amino-terminal extension, the signal peptide. This signal peptide, which distinguishes the secreted proteins from cytoplasmic ones, is needed for targeting to the export pathway (Simonen and Palva, 1993). Consequently, a secretion vector was constructed in which the *B. coagulans* strain 81-11 *estC1* gene was cloned downstream of an alkaline protease promoter and its associated signal sequence from a *Bacillus* species. This promoter and signal sequence has been used to successfully direct the high-level synthesis and export of several different enzymes in *Bacillus* hosts (M. Louw, unpublished results). DNA fragments consisting of the alkaline protease promoter and signal sequence, the *estC1* gene and digested plasmid pBC(SK) were thus ligated to yield the intermediate vector pBC-EstC1aps (Fig. 3.1). The cassette was subsequently cloned into pNW33N vector DNA as a *Bam*HI-*Sa*II DNA fragment to complete construction of the recombinant pNW-EstC1aps secretion plasmid. Following transformation of *E. coli* JM83, the isolated plasmid DNA was characterized by agarose gel electrophoresis and by restriction endonuclease digestion.



**Table 3.2:** Intracellular and extracellular esterase activities of wild-type and recombinant *Bacillus* strains

Strain	Intracellular activity (Units/mg)	Extracellular activity (Units/mg)
<i>B. coagulans</i> strain 81-11	0.8	2.9
<i>B. subtilis</i> 154/pMG36e	3.2	2.1
<i>B. subtilis</i> 154/pMG36-EstC1	3.3	2.6
<i>B. subtilis</i> 1A297/pMG36e	2.4	2.3
<i>B. subtilis</i> 1A297/pMG36-EstC1	4.1	2.6
<i>B. subtilis</i> 1A297/pNW33N	1.5	2.5
<i>B. subtilis</i> 1A297/pNW-EstC1-aps	1.5	6.9

Digestion of the recombinant pNW-EstC1aps plasmid DNA (Fig. 3.3a) with both *Hind*III and *Pst*I resulted in three DNA fragments of ca. 3.9, 2.4 and 0.327 kb (Fig. 3.3b, lane 5). These corresponded with the expected size of the parental plasmid (pNW33N), the *B. coagulans* strain 81-11 esterase-encoding DNA fragment and the alkaline protease promoter and signal sequence DNA fragment, respectively. The 327-bp band was barely visible due to diffusion of the DNA fragment from the agarose gel during electrophoresis. Subsequent digestion of the recombinant plasmid DNA with both *Hind*III and *Sal*I resulted in the excision of the 2.7-kb expression/secretion cassette, whereas digestion with both *Pst*I and *Sal*I resulted in the excision of the 2.4-kb *B. coagulans* strain 81-11 esterase-encoding DNA fragment (Fig. 3.3b, lanes 6 and 7, respectively). These results served to confirm the successful cloning of the alkaline protease promoter-signal sequence-*estC1* secretion cassette in plasmid pNW33N.

### 3.3.3.2 Extracellular expression of *B. coagulans* strain 81-11 EstC1

Since the aforementioned results indicated that the *B. coagulans* strain 81-11 EstC1 esterase was expressed intracellularly in *B. subtilis* 1A297 (Section 3.3.2.2), this strain was chosen for subsequent experiments in an attempt to increase extracellular EstC1 expression. Recombinant plasmid pNW-EstC1aps was introduced in *B. subtilis* 1A297 and the specific esterase activity of both the cell-free culture supernatant (extracellular) and prepared cell extracts (intracellular) were assayed using *p*-nitrophenyl butyrate as substrate. *B. subtilis* 1A297 harbouring the nonrecombinant pNW33N plasmid DNA was included in the assays as a control whereby the endogenous esterase activity of the host strain could be determined, whilst *E. coli* JM83/pBC-EstC1aps and *B. coagulans* strain 81-11 were also included in these assays for comparative purposes.

Analysis of the results (Table 3.2) indicated that expression of the *B. coagulans estC1* gene resulted in a high level of extracellular esterase activity in *B. subtilis* 1A297. The level of extracellular esterase activity was 4.6-fold higher compared to the intracellular esterase activity and displayed 2.3-fold higher extracellular esterase-activity levels than the *B. coagulans* 81-11 strain (Table 3.2). As a control, the alkaline protease promoter-signal sequence-*estC1* cassette cloned into plasmid pBC(SK) did not result in secretion of EstC1 in *E. coli* JM83. This was not unexpected, since the protein is of Gram-positive origin and thus cannot be secreted through the two membranes of Gram-negative bacteria.

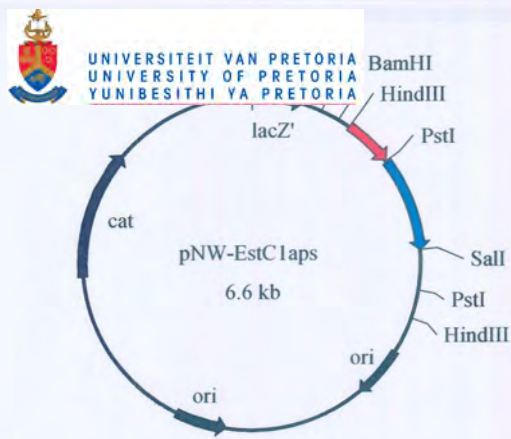


Fig. 3.3a Plasmid map of the recombinant plasmid pNW-EstC1aps containing a cloned copy of the *B. coagulans* strain 81-11 *estC1* gene downstream of an alkaline protease promoter and signal sequence from *Bacillus* species.

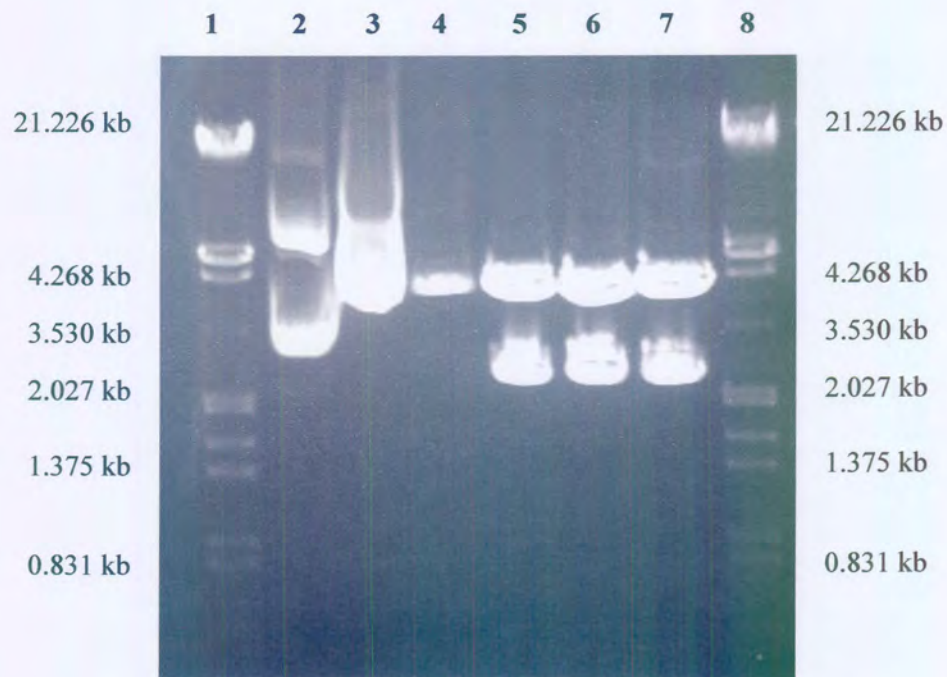


Fig. 3.3b Agarose gel electrophoretic analysis of the recombinant plasmid pNW-EstC1aps. Lanes 1 and 8, DNA molecular weight marker; lane 2, uncut parental pNW33N vector DNA; lane 3, uncut pNW-EstC1aps plasmid DNA; lane 4, pNW33N vector DNA digested with *HindIII* and *Sall*; lane 5, recombinant plasmid pNW-EstC1aps digested with *HindIII* and *PstI*; lane 6, recombinant plasmid pNW-EstC1aps digested with *HindIII* and *Sall*; lane 7, recombinant plasmid pNW-EstC1aps digested with *PstI* and *Sall*. The sizes of the DNA molecular weight marker, phage  $\lambda$  DNA digested with both *EcoRI* and *HindIII*, are indicated to the sides of the figure.

### 3.4 DISCUSSION

Since bacterial esterases are potentially important for a variety of biotechnological applications, there is a considerable industrial interest to produce these enzymes at a larger scale (Faber, 1997). The first step needed to isolate an esterase for biotechnological applications is usually overexpression of the corresponding gene of interest. Frequently, this step is considered to be trivial, because proteins can easily be overexpressed in *E. coli* using commercially available systems (Balbas and Bolivar, 1990). Although *E. coli* has remained one of the most attractive systems for heterologous protein production, recent years have seen an increase in the development of gene expression systems for industrial Gram-positive bacteria with a low guanine and cytosine content (LGB) that belong to the genera *Bacillus*, *Clostridium*, *Lactococcus*, *Lactobacillus*, *Staphylococcus* and *Streptococcus* (Mercenier *et al.*, 1994; de Vos *et al.*, 1997; Slos *et al.*, 1998). In this part of the study, the usefulness of *L. lactis*, *L. plantarum* and *B. subtilis* as expression hosts for the production of the *B. coagulans* strain 81-11 EstC1 esterase was specifically investigated. The DNA fragment containing the *estC1* ORF from *B. coagulans* strain 81-11 (Chapter 2) was thus cloned into the *L. lactis* expression vector pMG36e and the derived recombinant expression vector was subsequently introduced in *L. lactis* IL403, *L. plantarum* NCIB1193, as well as in *B. subtilis* strains 154 and 1A297.

Surprisingly, after transfer of the recombinant pMG36-EstC1 plasmid to the *L. lactis* and *L. plantarum* strains, no detectable intracellular or extracellular esterase activity could be observed by cup plate assays on tributyrin indicator plates. Plasmid DNA extractions from the transformed *L. lactis* cultures indicated that the parental pMG36e, but not the recombinant pMG36-EstC1 plasmid, could be extracted, thereby suggesting that the presence of the cloned DNA fragment containing the *B. coagulans* strain 81-11 *estC1* gene destabilized the vector. The nature of the instability of the recombinant pMG36-EstC1 plasmid remains a subject of speculation, but it may be that the high G+C content of the cloned DNA fragment (48%) compared to the G+C content of *L. lactis* genomic DNA (35%), interfered with optimal plasmid replication. Plasmid pMG36e replicates via a rolling circle, single-stranded mode of replication, which can result in deletions and single-stranded intermediates, and in many cases, the extent of the plasmid instability depends on the nature and size of the cloned DNA fragment (Harwood, 1992). Similar plasmid instability phenomena have also been reported by Leenhouts *et al.* (1998a) whilst attempting to express

the *pip* gene from *Propionibacterium shermanii*, containing a similar G+C-content as the *estC1* gene, in *L. lactis* using pMG36e.

By contrast with *L. lactis*, the plasmid DNA (parental and recombinant pGM36e) was stably maintained in *L. plantarum*, yet no esterase activity was observed. These results suggested a lack of either transcription or translation of the *estC1* gene in this host strain. It has been reported that lactococcal promoters do not always work in related genera such as lactobacilli and staphylococci (Brurberg *et al.*, 1994; Somkuti *et al.*, 1995), and within the LGB the efficiency of transcription initiation may also vary in a species-dependent way, as was observed when transcriptional *gusA* fusions with the lactococcal *lacA* promoter were studied in different lactic acid bacteria (Platteeuw *et al.*, 1994). Moreover, *Lactobacillus* has been reported to display a high selectivity for promoters (Pouwels and Leer, 1993). This phenomenon may be due to the fact there may be a bias in *Lactobacillus* against the high fraction of AT-rich sequences found in lactococcal and *Bacillus* promoters, which include the conserved -35 and -10 hexamers found in *E. coli*, a TG dinucleotide at position -15 and an AT-rich region immediately upstream from the -35 sequence (Harwood, 1992; Pouwels and Leer, 1993; Waterfield *et al.*, 1995; de Vos *et al.*, 1997). Although the P32 promoter can function in *L. plantarum*, it has been reported to function optimally in translationally coupled constructs (van de Guchte *et al.*, 1991) and that the relative positions of the transcription/translation signals strongly affected heterologous gene expression in *L. plantarum* (Axelsson *et al.*, 2003). Based on the above, and taking into account that transcriptional fusions (*i.e.* P32 promoter followed by the native ribosome-binding site and then by the AUG start codon of the *B. coagulans* 81-11 *estC1* gene) were not prepared in this study, it is likely that the *estC1* gene was not efficiently transcribed in *L. plantarum*. Such a conclusion is, however, subject to confirmation by Northern blot analysis.

Recombinant strains of *B. subtilis* 1A297 displayed an increase in intracellular esterase activity compared with the control strain harbouring the nonrecombinant pMG36e plasmid. By contrast, no increase in either the intra- or extracellular esterase activity could be detected upon introduction of the recombinant pMG36-EstC1 vector into *B. subtilis* 154 (Table 3.2). Since the same recombinant plasmid construct was used in both *B. subtilis* strains, it was expected that the level of esterase activity should therefore be at least the same or similar. The obtained results may thus suggest that this respective *B. subtilis* strains possess different strain-specific control mechanisms for gene expression. The lower esterase activity of *B.*

*subtilis* 154 may be due to it suppressing the activity of the transferred gene by an as yet unknown mechanism or, alternatively, there may be a positive regulation of EstC1 production in *B. subtilis* 1A297 by other gene products.

Because of their non-pathogenicity and high secretion capacity, *Bacillus* species have been regarded as attractive production hosts, especially for the secretion of endogenous and heterologous proteins. Secretion as a mode of production provides several advantages over intracellular production, *e.g.* facilitated purification of the product, theoretically higher yield, no aggregation of the product, as well as the possibility for continuous cultivation and production (Kitai *et al.*, 1988; Harwood, 1992; Lam *et al.*, 1998; Bolhuis *et al.*, 1999). Since *Bacilli* do not have an outer membrane, the proteins that are translocated across the cytoplasmic membrane either are liberated to the culture medium or remain associated with the outer membrane either transiently or for a longer period (Simonen and Palva, 1993). Most of the endogenous proteins secreted into the external medium by *Bacilli* are degradative enzymes such as proteases, amylases and levansucrases (Wu *et al.*, 1991; Simonen and Palva, 1993). Therefore, a secretion vector, pNW-EstC1aps, was constructed containing the promoter and signal sequence of an alkaline protease from a *Bacillus* species; the rationale being that the use of a *Bacillus*-derived promoter may result in more efficient gene expression compared to the lactococcal P32 promoter previously used and that the inclusion of the signal sequence might facilitate efficient targeting of the heterologous protein to the secretion pathway for extracellular export.

Following introduction of the newly constructed recombinant pNW-EstC1aps secretion plasmid into *B. subtilis* 1A297, the results indicated that the esterase activity (intracellular plus extracellular) in *B. subtilis* 1A297 was higher (8.4 U/mg) than that observed in *B. subtilis* 1A297 when the *estC1* gene was cloned downstream of the lactococcal P32 promoter (6.7 U/mg) and higher than the esterase activity in the parent *B. coagulans* 81-11 strain (3.7 U/mg) (Table 3.2). Notably, the level of extracellular esterase activity was 2.3-fold higher in the recombinant *B. subtilis* 1A297/pNW33-EstC1aps strain when compared to that of *B. coagulans* strain 81-11 and represented 82% of the total esterase activity. The increase in esterase expression may be ascribed to the generalized increase in transcription starting from the P32 and alkaline protease promoters on the respective plasmids, in addition to the expression obtained under control of the *B. coagulans estC1* promoter and ribosome-binding site. Moreover, the increased expression may also be due to a gene dosage effect. As the





level of expression is generally proportional to the number of transcribed gene copies in the host cell (Summers, 1998), it can therefore be expected that by increasing the plasmid copy number, there will be a concomitant increase in the amount of protein that is being synthesized. In the parent *B. coagulans* strain, the *estC1* gene is present on the chromosome and under transcriptional control of the native promoter only, whilst *B. subtilis* 1A297 carrying the autonomously replicating recombinant pMG36e and pNW33N plasmid DNAs would result in strains carrying multiple *estC1* gene copies. Thus, it is possible that these strains may produce more active EstC1 than the single copy parent strain.

In conclusion, of the different Gram-positive bacterial hosts evaluated for expression of the esterase-encoding *estC1* gene from *B. coagulans* strain 81-11, *B. subtilis* 1A297 appeared to be most efficient. However, note should be taken that if this strain is to be used in future for overexpression of the *B. coagulans* EstC1 esterase, it would be necessary to construct an esterase-deficient strain since it produces endogenous esterase(s). Regarding the different plasmid vectors used in this investigation, the use of the pMG36e plasmid resulted in intracellular accumulation of the esterase, whereas the constructed pNW33N-based secretion vector, as detailed in this study, harbouring the promoter and signal sequence of an alkaline protease from a *Bacillus* species, resulted in high levels of expression and efficient secretion of the esterase to the extracellular environment. Despite the levels of esterase activity obtained in this study being lower than that reported in other studies using *E. coli* as expression host (Nishizawa *et al.*, 1995; Khalameyzer *et al.*, 1999; Henke and Bornscheuer, 2002), the results can nevertheless be used as a starting point for further optimization of *estC1* gene expression. Several options for optimization are possible and these will be discussed in the following Chapter.



## **CHAPTER 4**

### **CONCLUDING REMARKS**



The increasing demands for especially optically pure products have drawn much attention to methods for converting organic compounds into desirable products using various enzymes. Hydrolytic enzymes, inclusive of lipases and esterases, are of particular importance in such bioconversion reactions (Faber, 1997; Jaeger and Reetz, 1998; Pandey *et al.*, 1999; Davis and Boyer, 2001; Bornscheuer, 2002). Distinguished from lipases, esterases show a preference toward short-chain acetyl esters and can be found in many organisms including animals, plants and microorganisms (Okuda, 1991). The physiological functions of bacterial esterases are, however, not clear; although some may be involved in plant pathogenicity (McQueen and Schottel, 1987; Dalrymple *et al.*, 1996), carbon source provision (Williamson *et al.*, 1999; Videira *et al.*, 2003) and biocide detoxification (Blackman *et al.*, 1995; Wei *et al.*, 1999). In addition to ester hydrolysis, esterases are capable of catalysing interesterification, aminolysis and peracid formation (Faber, 1997; Krebsfanger *et al.*, 1998; Davis and Boyer, 2001). These biochemical reactions are applicable in pharmaceutical and food industries. Despite their commercial value, esterases are used less frequently in industrial processes compared with lipases due to their lack of availability. Accordingly, this investigation was primarily aimed at identifying and characterizing novel lipolytic enzymes from *Bacillus* species and to overexpress the enzyme-encoding gene(s) in an appropriate Gram-positive prokaryotic host.

In the first part of the study, several *Bacillus* isolates were screened for the production of lipolytic enzymes. One of the isolates, *B. coagulans* strain 81-11, which had been isolated from popcorn seeds, displayed the highest lipolytic activity on tributyrin agar plates. A genomic library of the bacterium was subsequently constructed and screened for lipolytic activity in *E. coli* using tributyrin-containing medium. One positive transformant was isolated and analyses of the plasmid DNA by restriction mapping revealed a 2.4-kb DNA fragment potentially carrying an esterase gene. The nucleotide sequence of the DNA was determined and found to contain an ORF encoding a carboxyl esterase, which was designated *estC1*. Notably, the deduced amino acid sequence of the *B. coagulans* EstC1 esterase displayed a high level of amino sequence identity with esterases from *G. stearothermophilus* (72%) and *G. thermoleovorans* (70%). Amino acid sequence analysis of EstC1 revealed the presence of the serine esterase consensus motif G-X-S-X-G located between residues 89 and 93, and Ser<sub>91</sub>, together with Asp<sub>190</sub> and His<sub>229</sub>, may comprise the catalytic triad. Investigations regarding the biochemical properties of the *B. coagulans* EstC1 esterase indicated that the carboxyl esterase functioned optimally at 50°C in the pH range 7 to 9. The



enzyme exhibited greater preference toward short-chain ( $\leq C_6$ ) than medium- and long-chain fatty acids. In addition, the enzyme is characterized by high stability at temperatures around 50°C and no significant loss of activity could be observed following incubation for 3 h at 30°C and 50°C, respectively.

Despite the high level of amino acid sequence identity observed between the *B. coagulans* EstC1 esterase and those from *G. stearothermophilus* and *G. thermoleovorans*, the esterases differ substantially in their biochemical properties. Whereas the esterase enzymes from the thermophilic *Geobacillus* spp. are active at temperatures in excess of 65°C (Wood *et al.*, 1995; Henke and Bornscheuer, 2002), the *B. coagulans* esterase showed activity in a range typical for mesophilic enzymes. Enzymes from thermophiles are often considered to be advantageous for use in industrial-level biotransformations, since they exhibit high thermostability that can be correlated with enhanced resistance to denaturation in organic solvents (Jaenicke *et al.*, 1996; Adams and Kelly, 1998). However, thermophilic enzymes have a significant operational disadvantage. One of the major advantages of enzymes over industrial catalysis is their potential of high activity at low temperatures, often a critical factor in protecting labile substrates from deleterious reactions. The activity of thermophilic enzymes at room temperature is often relatively low, and the apparent high temperature requirements for thermophilic enzymes would appear to impart some limitation on their applications as industrial biocatalysts. Ideal enzyme characteristics might thus include high molecular stability (to temperature, solvents, etc.) and high mesophilic activity. The temperature activity and stability of the *B. coagulans* strain 81-11 EstC1 esterase may therefore provide a good basis for enzyme utilization at moderate temperatures such as 30-50°C. In this range, the enzyme is very active (> 70% of the maximal activity) and would be highly thermostable.

In the second part of the study, the newly characterized *estC1* gene from *B. coagulans* was cloned into the expression vector pMG36e and expressed in several different Gram-positive bacterial hosts that included *L. lactis*, *L. plantarum* and *B. subtilis* strains 154 and 1A297. Of these different expression hosts, the EstC1 esterase was expressed intracellularly in *B. subtilis* 1A297 only. However, following construction of a recombinant secretion vector containing the *estC1* gene cloned downstream of an alkaline protease promoter and signal sequence from *Bacillus* species, high levels of extracellular esterase activity was attained. Despite the observed efficient expression and export of the enzyme, *B. subtilis* 1A297 produces



endogenous secreted esterase(s) that could complicate recovery and purification of the *B. coagulans* EstC1 esterase from the extracellular medium. By contrast, *L. lactis* and *L. plantarum* did not display detectable endogenous intracellular or extracellular esterase activity. Although it may be possible to construct an esterase-deficient *B. subtilis* 1A297 strain, it may be preferable to rather optimise *L. lactis* and/or *L. plantarum* as expression hosts for the overexpression of the *B. coagulans* EstC1 esterase. In addition to the construction of transcriptional and/or translational gene fusions, several other factors should, however, also be taken into account when attempting to optimise these strains as expression hosts.

Despite structural instability during cloning and expression in *L. lactis* not having been frequently reported (de Vos *et al.*, 1997), it has, however, been observed in several cases, especially when a strong promoter such as *lacA* or P32 was used (Platteeuw *et al.*, 1994; Platteeuw *et al.*, 1996; Leenhouts *et al.*, 1998a; this study). Moreover, the use of a constitutively transcribed strong promoter, such as P32, may have detrimental effects on host cells. Not only may it create an energy drain thereby impairing essential host functions, but it may also lead to counter-selection of expressing cells and the population in culture loses the foreign gene of interest (Glick and Whitney, 1987; Glick, 1995). Continuous high-level synthesis of a recombinant protein could also lead to intracellular accumulation, aggregation or degradation of the protein in the cytoplasm (Gottesman, 1996). Furthermore, low-copy-number plasmids, such as pMG36e, are reported to lose stability at higher frequencies compared to plasmids with higher copy numbers. Solutions to these problems might be to use an alternative *L. lactis* strain as expression host, e.g. *L. lactis* L108, which overproduces the plasmid replication initiation protein RepA *in trans* and maintains pWV01 derivatives (such as pMG36e) in 5- to 10-fold higher copy numbers (Leenhouts *et al.*, 1998a), thereby improving plasmid stability and maintenance in the host. Alternatively, integration plasmids may be used in which only the expression cassette is inserted into the genome by double crossover events (Leenhouts *et al.*, 1998a; Olson *et al.*, 1998), but the EstC1 activity may be lower compared to multicopy strains due to a gene dosage effect. To prevent the above-mentioned negative effects caused by high levels of heterologous protein production, it may also be preferable to use inducible promoters to coordinate expression with cultural growth so that recombinant proteins are only expressed at times when maximum yields can be obtained. Several different inducible promoters have been developed whereby gene expression can be controlled by an inducer or repressor (de Ruyter *et al.*, 1996; O'Sullivan *et al.*, 1996; Diep *et*

*al.*, 2000; Axelsson *et al.*, 2003), as well as by environmental factors, such as pH, temperature or ion concentrations (Sanders *et al.*, 1997; Saunders *et al.*, 1998; Madsen *et al.*, 1999). Since *L. lactis* and *L. plantarum* do not produce any extracellular proteases (Wells *et al.*, 1993; Steidler *et al.*, 1995; de Vos *et al.*, 1997), they can be used for secretion of heterologous proteins. Protein secretion from these hosts can be enhanced using upstream cassettes based on the signal sequence of the Usp45 protein, an unknown secreted protein of 45 kDa (van Asseldonk *et al.*, 1993), which has been used to direct the efficient secretion of various different prokaryotic and eukaryotic proteins in *L. lactis* and *Lactobacillus* spp. (Arnau *et al.*, 1997; Hols *et al.*, 1997; Drouault *et al.*, 2000).

Finally, as the *B. coagulans* EstC1 esterase may potentially be used in the kinetic resolution of menthyl acetate to allow access to (-)-menthol, which is a key substance in the fragrance industry (Chapter 2), future studies may also involve the use of directed evolution to generate improved *B. coagulans* EstC1 esterase variants. This technology has been used to develop esterases with improved enantioselectivity (Bornscheuer *et al.*, 1998; Henke and Bornscheuer, 1999), as well as improved stability and activity (Moore and Arnold, 1996; Spiller *et al.*, 1999). The approach relies upon introducing mutations into the gene by error-prone PCR or by random recombination of DNA fragments, such as DNA shuffling or random primer recombination. After expression of the mutated genes in a prokaryotic host, screening procedures are used to identify the “best” mutant enzyme in a large library of potential candidates, and the procedure is repeated several times until the desired catalytic features have been attained.



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