CHAPTER 1

Identification, cloning and characterization of *Bacillus megaterium*, *Bacillus* sp. BP-6 and *Bacillus* sp. BP-7 lipases

Figure C1.1 Model of the three-dimensional structure of *Bacillus megaterium* LipA.

1 INTRODUCTION AND OBJECTIVES

Bacterial lipases are among the most versatile and widely used enzymes in biotechnological applications due to their unique properties (Jaeger *et al*., 1999; General Introduction 3.3). Thus, there is an increasing interest in isolating new lipases with novel biotechnological potential. *Bacillus* and related genera are one of the most important sources of novel lipases and other industrial enzymes because they have been shown to possess a lipolytic system well suited for biotechnological applications (Zock *et al*., 1994; Dröge *et al*., 2000; General Introduction 3.3.5). Therefore, this chapter is focused on the isolation and characterization of new *Bacillus* lipases which could be of interest for biotechnological applications. The exact aims of this work were:

1. **To analyze the lipolytic system of *Bacillus megaterium* CECT 370, and to isolate, clone, and characterize the lipase(s□of this microorganism.**

    a. To analyze the lipolytic system of *B. megaterium* CECT 370, a strain selected out of 26 *Bacillus* strains due to its high lipolytic activity.

    b. To isolate the gene coding for *B. megaterium* CECT 370 LipA by PCR and inverse PCR techniques, using consensus primers for *Bacillus* lipases.

    c. To clone and express *B. megaterium* CECT 370 LipA in *Escherichia coli* 5K.

    d. To perform the molecular and biochemical characterization of *B. megaterium* CECT 370 LipA in order to evaluate the biotechnological potential of this enzyme, and in order to compare its features with those of other similar lipolytic enzymes from the genus *Bacillus*.

2. To analyze the lipolytic system of *Bacillus* sp. BP-6, and to isolate, clone, and characterize the lipase(s) of this strain.

a. To analyze the lipolytic system of *Bacillus* sp. BP-6, a strain selected out of 26 *Bacillus* strains due to its high lipolytic activity.

b. To isolate the gene coding for *Bacillus* sp. BP-6 LipA by PCR and inverse PCR techniques, using consensus primers for *Bacillus* lipases.

c. To clone and express *Bacillus* sp. BP-6 LipA in *Escherichia coli* 5K.

d. To perform the molecular and biochemical characterization of LipA from *Bacillus* sp. BP-6 in order to evaluate the biotechnological potential of this enzyme, and in order to compare its features with those of other similar lipolytic enzymes from the genus *Bacillus*.

3. To isolate, clone, and characterize the lipase(s) of the previously described strain *Bacillus* sp. BP-7.

a. To isolate the gene coding for *Bacillus* sp. BP-7 LipA by PCR using specific primers for *Bacillus* lipases and to clone the isolated gene. As the isolated gene showed 100% identity to *Bacillus* sp. BP-6 LipA, further enzyme characterization was not performed.

2 MATERIALS AND METHODS

Unless otherwise stated, the materials and methods used were those previously described in the General Materials and Methods section (see Tables M.1, M.2, M.3 and M.7 for a more detailed explanation of the strains, culture media, plasmids and primers used).

2.1 SELECTION AND ANALYSIS OF LIPOLYTIC STRAINS

Detection of the best lipase-producing strains from a collection of 26 strains was previously performed by Dr. A. Blanco on tributyrin- and olive oil-supplemented CeNAN agar plates (see Material and methods 5.1.1). The strains screened were Gram-positive bacterial isolates from rice straw samples collected at the Ebro's Delta River (Spain): *Bacillus* sp. BP-6, *Bacillus* sp. BP-7 (López *et al*., 1998) and *Paenibacillus* sp. BP-23 (Blanco & Pastor, 1993; Sánchez *et al*., 2005); and several *Bacillus* strains from the Spanish Type Culture Collection (CECT): *B. subtilis* 461, *B. subtilis* 216, *B. subtilis* 39T, *B. subtilis* 497, *B. pumilus* 29T, *B. pumilus* 152, *B. pumilus* 510, *B. megaterium* 44, *B. megaterium* 370, *B. firmus* 14T, *B. cereus* 131, *B. cereus* 193, *B. cereus* 4014, *B. cereus* 148T, *B. cereus* 495, *B. cereus* 496, *B. macerans*, *B. coagulans* 12T, *B. alcalophilus* 1T, *B. alvei, B. lentus* 18T, *B. pantothenicus* 42T and *B. brevis* 5. All these strains were grown in Nutrient broth at 30ºC. *B. megaterium* CECT 370 and the natural isolate *Bacillus* sp. BP-6 were the strains chosen for further investigations due to their high lipolytic activity.

The lipolytic activity of the supernatants and crude cell extracts (prepared in solution B as previously described in General Materials and Methods 4.1.1) from the selected strains *B. megaterium* CECT 370 and *Bacillus* sp. BP-6 was determined at room temperature by classical fluorimetric liquid assay (Material and Methods 5.4.2.1).

Moreover, the lipolytic system of each strain was analyzed by zymogram assay (General Materials and Methods 5.2) of supernatants and cell extracts separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and isoelectric focusing (IEF) gels (General Materials and Methods 4.4.1 and 4.6, respectively).

2.2 ISOLATION AND CLONING OF LIPASE-CODING GENES

The whole process of isolation and cloning of *B. megaterium* CECT 370 and *Bacillus* sp. BP-6 lipase-coding genes is summarized in Figure C1.2.

2.2.1 Isolation of lipase-coding genes

Isolation of lipase-coding genes from the selected *Bacillus* strains was performed by PCR amplification using consensus primers for *Bacillus*-related lipase genes, as is described below.

Known lipase amino acid sequences from *B. subtilis* (M74010 and D78508), *B. pumilus* (A34992), *B. licheniformis* (AJ297356), *Bacillus* sp. B26 (AF232707), *Geobacillus (Gb.) thermocatenulatus* (X95309) and *Gb. stearothermophilus* (U78785) were aligned, and the stretches of sequence identity analyzed. According to the identity stretches, the aligned sequences could be divided into two groups: those resembling *B. subtilis lipA*, and those showing homology to *Gb. thermocatenulatus* lipase gene. The regions sharing the highest homologies for the two groups of sequences (Figure C1.5) were chosen to design the following primers: primers for the amplification of the central region of *B. subtilis lipA*-related genes (FWSUB and BKSUB; Figure C1.5), and primers for the amplification of the central region of *Gb. thermocatenulatus* lipase-related genes (FWTHER and BKTHER; Figure C1.5).

The two sets of primers designed were used for the amplification of DNA fragments of any length, using as a template cell suspensions of isolated colonies from

the selected *Bacillus* strains. Amplification by PCR was performed using *Taq* polymerase and low-astringency conditions (Tm = 42−55 ºC). The amplified fragments obtained from *B. megaterium* CECT 370 and *Bacillus* sp. BP-6 were sequenced, and from these sequences, new sets of specific divergent primers starting at both ends of each fragment were designed: BKMEGA and FWMEGA for *B. megaterium* CECT 370, and BKBP6 and FWBP6 for *Bacillus* sp. BP-6. These primers were used to perform inverse PCR (IPCR) amplifications (using *Taq* polymerase and a Tm = 50 ºC) in order to isolate the corresponding complete lipase ORFs. The DNA templates used for IPCR assays were the genomic DNAs of both strains digested with the restriction enzymes *Eco*RI, *Bam*HI, *Cla*I, or *Xba*I, and subsequently re-ligated.

The newly IPCR amplified fragments obtained from both strains were then sequenced using the same set of primers. These fragments showed very high sequence identity to *yfi*P (*lipB*) gene of the *Bacillus subtilis* genome (Kunst *et al*., 1997). Since the complete ORF of the respective genes could not be obtained by this procedure, a new set of convergent primers designed from the 5' and 3' ends of the *yfi*P gene sequence (FWYfiP and BKYfiP; Figure C1.7) was used to perform the direct PCR amplification (using *pfu* Polymerase and low-astringency conditions – Tm = 43 ºC –) of the complete lipase-coding ORFs of *B. megaterium* CECT 370 and *Bacillus* sp. BP-6. The lipase-coding genes obtained were named *B. megaterium* CECT 370 *lipA* (*BMlipA*) and *Bacillus* sp. BP-6 *lipA* (*BP6lipA*). The success obtained using *yfi*P primers led us to perform also the direct amplification of the lipase-coding gene *lipA* (*BP7lipA*) from the previously described (López *et al*., 1998; Prim *et al*., 2001) lipolytic strain *Bacillus* sp. BP-7.

2.2.2 Cloning of the lipase-coding genes

The blunt-ended DNA fragments obtained by amplification using primers FWYfiP and BKYfiP from *B. megaterium* CECT 370, *Bacillus* sp. BP-6 and *Bacillus* sp. BP-7 genomic DNAs were sequenced for analysis of their corresponding nucleotide sequences (General Materials and Methods 3.7), and ligated to *Eco*RV-digested pBR322 plasmid to obtain the recombinant plasmids pBR-BMLipA, pBR-BP6LipA and

pBR-BP7LipA, respectively. These plasmids were transformed into *Escherichia coli* 5K to obtain the respective recombinant clones *E. coli* 5K−pBR-BMLipA, *E. coli* 5K−pBR-BP6LipA and *E. coli* 5K−pBR-BP7LipA. These clones were selected on the basis of their tetracycline sensitivity/ampicillin resistance, and by their activity on MUF-derivative substrates. After confirmation of the sequence of their inserts, the clones containing *BMlipA* and *BP6lipA* were used for the subsequent molecular and biochemical characterization of the enzymes BMLipA and BP6LipA, respectively, whereas the clone containing *BP7lipA* was not further studied since the sequence of *BP7lipA* was 100% identical to that of *BP6lipA*.

Dot and southern blot hybridisation analysis were performed to confirm the presence and to determine the existence of one or more copies of the cloned genes in the parental strains. Digoxigenin probes were prepared through FWSUB/BWSUB amplification of each plasmid DNA in the presence of digoxigenin-labelled dNTPs (General Materials and Methods 3.10).

2.2.3 Search for additional lipase-coding genes

Direct amplification under low-astringency conditions (Tm = 40−55 ºC) was also performed using the specific primers for *B. subtilis lip*A (FWBSLA and BWBSLA) and the genomic DNAs form *B. megaterium* CECT 370, *Bacillus* sp. BP-6 and *Bacillus* sp. BP-7 as templates, in order to obtain additional lipase-coding genes from these strains.

Another approach to isolate additional lipase-coding genes from *B. megaterium* CECT 370 consisted in the construction of a "fast" gene library of the genome of this strain in *E. coli* XL1 blue. For this purpose the strain's genomic DNA was digested with *Eco*RI and *Bam*HI, ligated to the *Eco*RI−*Bam*HI-digested pUC19 plasmid, and transformed into *E. coli* XL1 blue. Transformed cells were directly spread on tributyrin- and olive oil-supplemented CeNAN agar plates containing also ampicillin, tetracycline, IPTG and X-gal. After incubation, white colonies showing lipolytic activity were selected and analyzed.

2.2.4 Nucleotide sequence accession numbers

The DNA sequence of *BMlip*A and *BP6lipA* genes have been submitted to the EMBL under accession numbers AJ430831 and AJ430985, respectively. The DNA sequence of *BP7lipA* was not submitted since it was 100% identical to that of *BP6lipA*.

2.3 CHARACTERIZATION OF THE CLONED ENZYMES

The lipolytic activity of the recombinant clones obtained was screened on lipid-supplemented CeNAN agar plates and by fluorimetric paper assay (General Materials and Methods 5.4.1).

Crude cell extract dilutions of the recombinant clones prepared in 50 mM phosphate buffer (pH 7) or solution B were used for the molecular and biochemical characterization of BMLipA and BP6LipA. Molecular characterization was performed by SDS-PAGE and IEF assays followed by zymogram analysis. Biochemical characterization was performed as described (General Materials and Methods 5.6), using the classical fluorimetric assay to determine the optimum temperature, optimum pH, thermostability, pH stability and enzyme kinetics of the cloned enzymes, and by using the colorimetric assay (General Materials and Methods 5.3.1) to determine their substrate range and specificity.

Inhibition–activation assays in the presence of cations, amino acid modifying agents, and other agents were performed by colorimetric assay using *p*-NP laurate (*p*-NPL) as substrate, as previously described (General Materials and Methods 5.7). The assays were carried out at the optimum temperature and pH of the cloned enzymes (45 ºC and pH 7), with the exception of NBS (*N*-bromosuccinimide), which was assayed at pH 5.5 since this amino acid modifying agent requires acidic conditions to be active (Keskar *et al*., 1989).

Activity and inhibition–activation assays were performed in triplicate, being each replicate the result of an independent assay performed in duplicate.

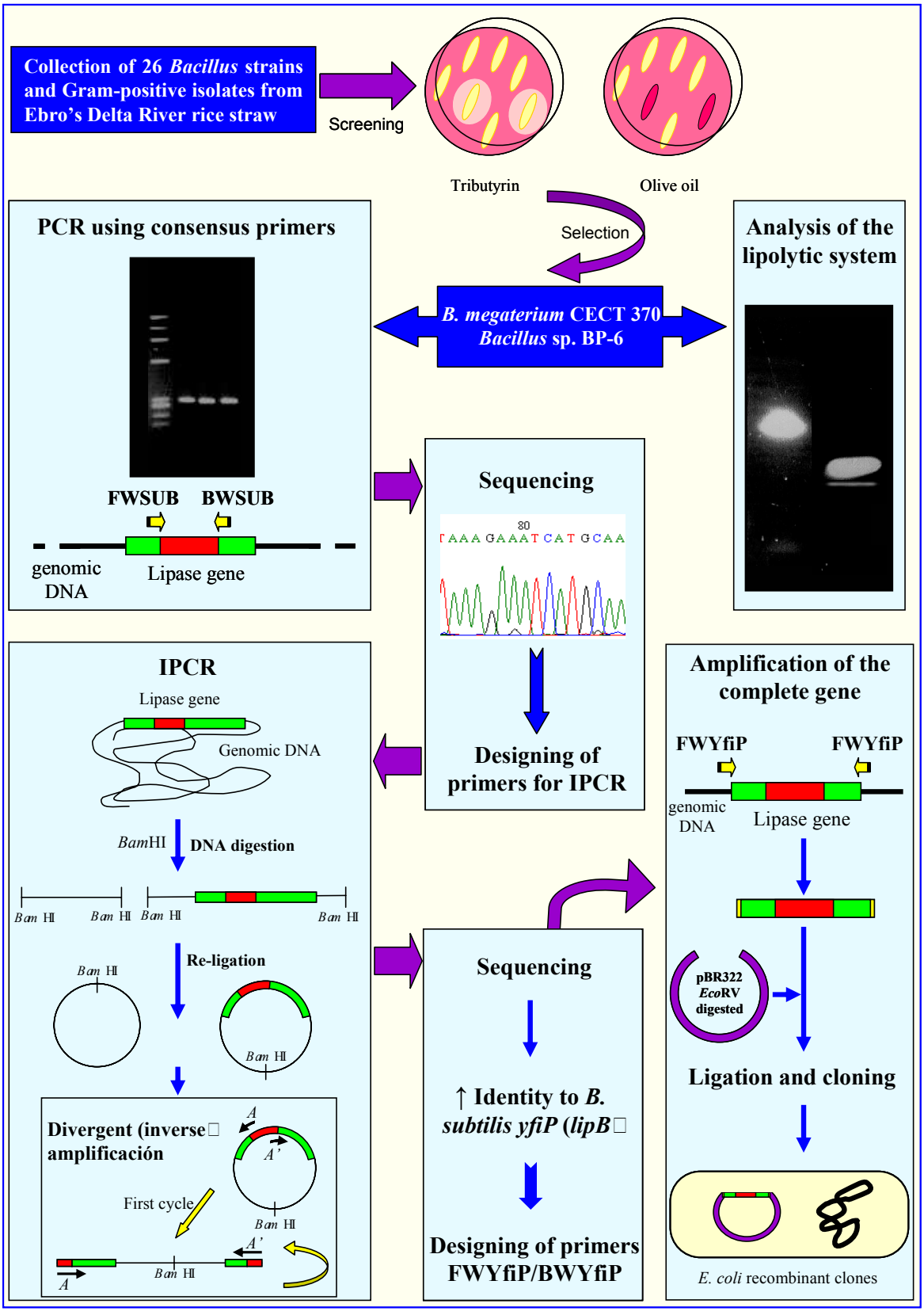


Figure C1.2 General process of isolation and cloning of *Bacillus* lipase-coding genes.

3 RESULTS

3.1 SELECTION AND ANALYSIS OF LIPOLYTIC STRAINS

The Gram-positive isolates and *Bacillus* type culture strains mentioned at the Materials and Methods section were screened for lipolytic activity using tributyrin- or olive oil-supplemented CeNAN agar plates. No lipolytic activity was detected for *B. macerans*, *B. firmus*, *B. alvei*, *B. lentus* or *B. pantothenicus*, whereas the rest of strains showed good performance in lipid-based substrate hydrolysis, as confirmed by fluorescence emission from Rhodamine and appearance of hydrolysis areas around the colonies (not shown). Strains *B. megaterium* CECT 370 and *Bacillus* sp. BP-6 were chosen for further analysis due to showing high lipolytic activity.

Table C1.1 Lipolytic activity determination of the selected strains.

Strain	Activity (mU mg^{-1} protein)			
	MUF-butyrate		MUF-oleate	
	Cell extracts	Supernatants	Cell extracts	Supernatants
B. subtilis MB216	96.7	32.1	0.250	6.888
B. megaterium CECT 370	824.6	29.3	1.321	3.200
Bacillus sp. BP-6	114.2	321.6	0.191	3.325

Results were obtained at room temperature. The standard deviations obtained ranged from 3% to 10% of each given value.

First analysis of the lipolytic activity of the selected strains consisted in comparing the activity of their cell extracts and supernatants to that of the lipolytic positive control *B. subtilis* MB216 by means of classical fluorimetric assay. *B. megaterium* CECT 370 cell extracts were by far the most active on both substrates,

whereas strain BP-6 supernatants and control strain supernatants were the most active on MUF-butyrate and on MUF-oleate, respectively (Table C1.1).

Furthermore, the lipolytic system of the selected strains was also analyzed on zymograms after SDS-PAGE and IEF protein separation of 50-fold concentrated cell extracts and 300-fold concentrated supernatants. Several activity bands were obtained for both strains using MUF-butyrate as substrate, whereas no activity could be detected when MUF-oleate was used as substrate (Figures C1.3 and C1.4).

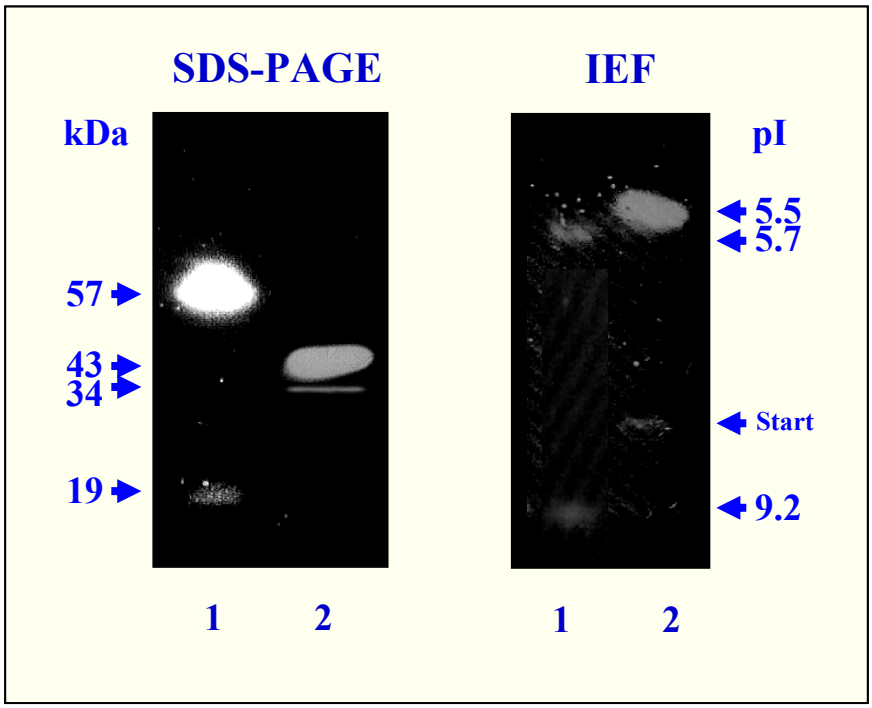


Figure C1.3 Zymogram analysis of *B. megaterium* CECT 370 lipolytic system.

The activity on MUF-butyrate of *B. megaterium* CECT 370 supernatants (1) and cell extracts (2) was analyzed by SDS-PAGE and IEF followed by zymogram. The MW and pI of the activity bands obtained are indicated.

A prominent band of ca. 57 kDa and a fainter band of ca. 19 kDa appeared after zymogram analysis of concentrated supernatants from *B. megaterium* CECT 370 Study of the supernatants separated on IEF gels confirmed the presence of two activity bands, indicating that the strain produces at least two extracellular enzymes with activity on

MUF-butyrate. The most prominent band on IEF gels had a pI of 5.7, whereas the pI of the faintest band was 9.2 (Figure C1.3).

Zymogram analysis of crude cell extracts from *B. megaterium* CECT 370 separated on SDS-PAGE gels showed the presence of two bands with activity on MUF-butyrate, one of ca. 43 kDa (more intense) and another of ca. 34 kDa (fainter). IEF zymogram analysis of this strain cell extracts showed the presence of a single prominent activity band with a calculated pI of 5.5. These bands or their processed forms were not found in the supernatant, suggesting that the strain would code for one or two additional non-secreted lipolytic enzymes (Figure C1.3).

Analysis of *Bacillus* sp. BP-6 concentrated culture supernatants on SDS-PAGE using MUF-butyrate as substrate showed an activity band of 56 kDa and faint activity band of ca. 19 kDa. However, only a faint band with a pI of 9.2 was found at the IEF gels (Figure C1.4), suggesting that this strain produces one or two extracellular lipases.

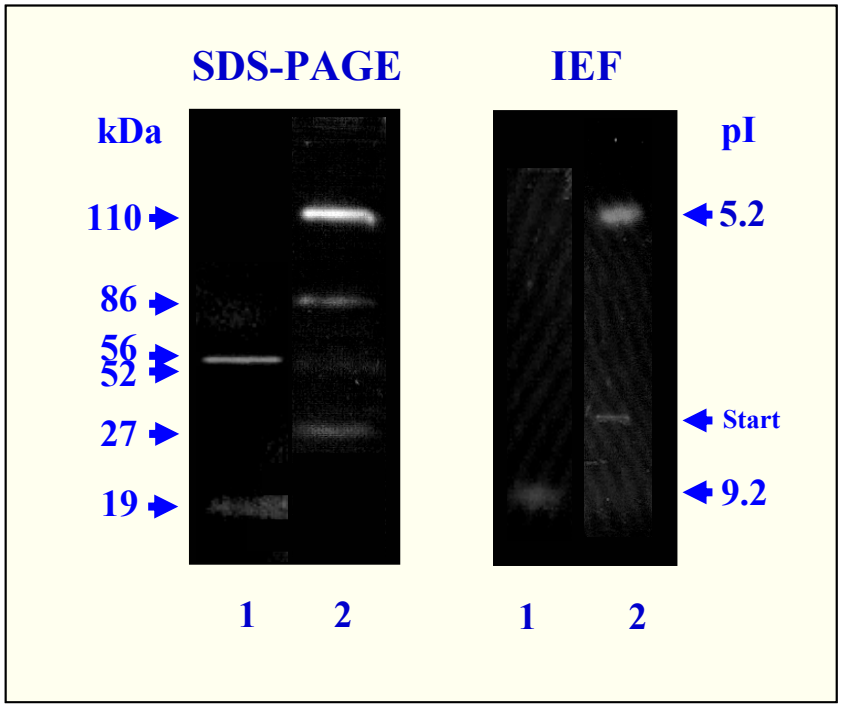


Figure C1.4 Zymogram analysis of *Bacillus* sp. BP-6 lipolytic system.

The activity on MUF-butyrate of *Bacillus* sp. BP-6 supernatants (1) and cell extracts (2) was analyzed by SDS-PAGE and IEF followed by zymogram. The MW and pI of the activity bands obtained are indicated.

Zymogram analysis after SDS-PAGE separation of *Bacillus* sp. BP-6 crude cell extracts showed the presence of several activity bands with calculated MW of 110, 86, 52 and 27 kDa, suggesting that the strain would code for one to four additional non-secreted lipolytic enzymes. However, when cell extracts of this strain were analyzed by IEF, a single activity band with a pI of 5.2 could be detected (Figure C1.4).

3.2 ISOLATION AND CLONING OF LIPASE-CODING GENES

3.2.1 Isolation of lipase-coding genes

The amino acid and nucleotide sequences of several known *Bacillus*-related lipases were aligned, and the stretches of sequence identity were located. Figure C1.5 shows some portions of the aligned nucleotide sequences of *B. subtilis* (M74010 and D78508), *B. pumilus* (A34992), *B. licheniformis* (AJ297356), *Bacillus* sp. B26 (AF232707), *Gb. thermocatenulatus* (X95309), and *Gb. stearothermophilus* (U78785) lipolytic genes where maximum identity was found. Analysis of the aligned sequences showed the existence of two lipase families in the genera *Bacillus* and *Geobacillus*: the small lipases of subfamily I.4 resembling *B. subtilis lipA*, and the larger lipases resembling *Gb. thermocatenulatus* lipase (subfamily I.5) and bearing some motifs of family I.6 staphylococcal lipases. While this work was being carried out, coincident results were found, and the grouping of bacterial lipase families was published (Arpigny & Jaeger, 1999). Some of the differences between both families of lipase genes can be appreciated in Figure C1.5. The two upper rows correspond to the regions where the highest identity between *Gb. thermocatenulatus* lipase gene family (**T**) was found, while the two lower rows correspond to the areas where lipase genes resembling that of *B. subtilis lip*A (**S**) showed the most identical stretches. Thus, the consensus sequences shown at the bottom of each set of alignments correspond to the identity nucleotides found for *Gb. thermocatenulatus* lipase subfamily (upper rows, Consensus**T**) and *B. subtilis* lipase subfamily (lower rows, Consensus**S**), at two different locations of the respective genes.

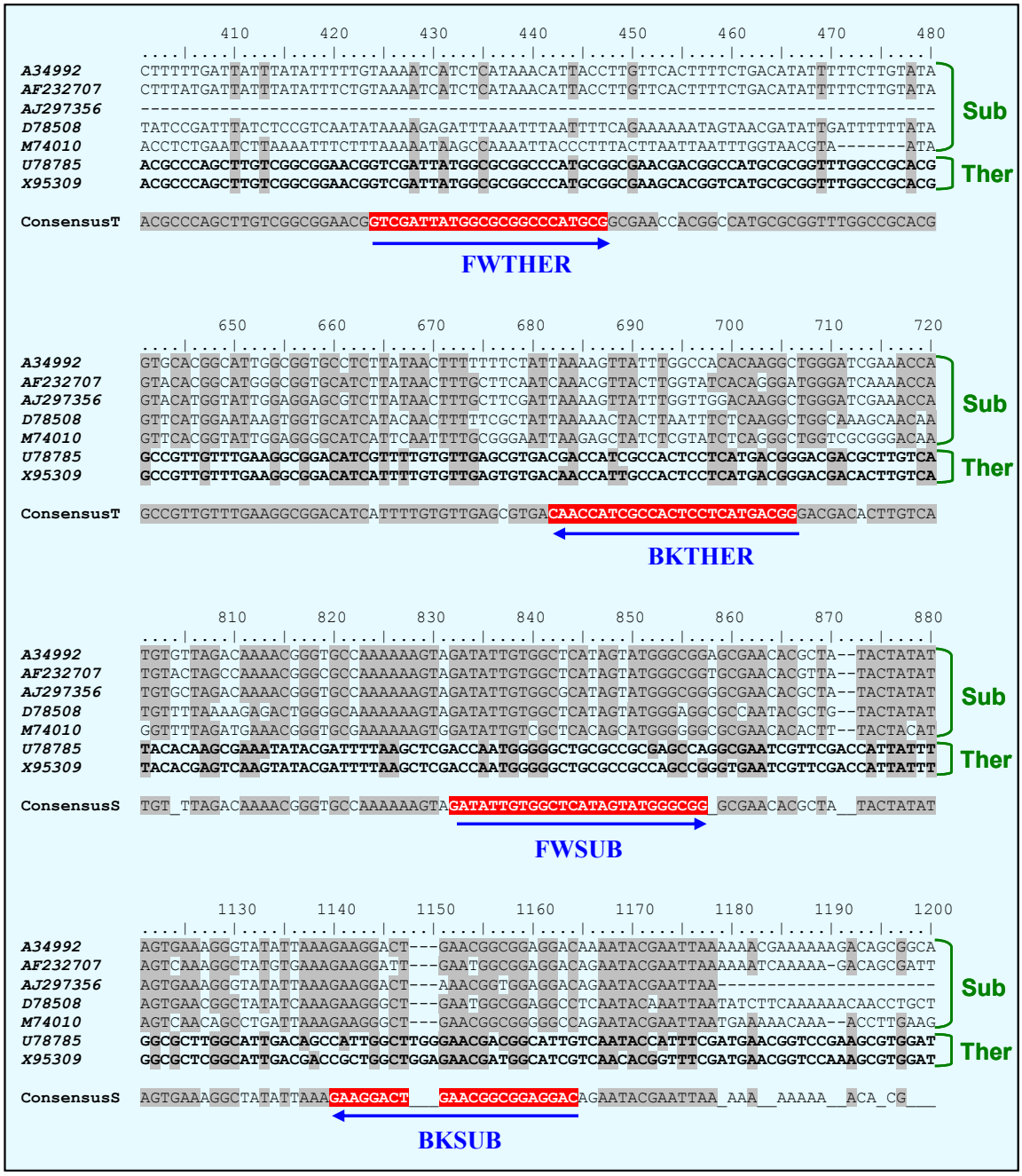


Figure C1.5 Consensus primers for the central region of *(Geo)Bacillus* lipases.

Partial nucleotide sequence alignments of *B. subtilis* (M74010 and D78508), *B. pumilus* (A34992), *B. licheniformis* (AJ297356), *Bacillus* sp. B26 (AF232707), *Gb. thermocatenulatus* (X95309), and *Gb. stearothermophilus* (U78785) lipase coding genes. Sequences grouped by "Sub" correspond to *B. subtilis* lipase subfamily, while sequences grouped by "Ther" are those of *Gb. thermocatenulatus* lipase subfamily. The consensus sequences obtained for *B. thermocatenulatus* (ConsensusT) and *B. subtilis* (ConsensusS) lipase families are shown below each alignment. FWTHER/BKTHER and FWSUB/BKSUB correspond to the primers obtained from the corresponding sequence identity stretches and used for convergent PCR amplification of new lipase coding genes.

From the consensus sequences found, two sets of convergent primers (FWTHER/BKTHER and FWSUB/BKSUB) were designed for each lipase gene subfamily, and used to amplify the internal region of the lipase gene or genes present in the selected strains *B. megaterium* CECT 370 and *Bacillus* sp. BP-6.

Amplification using *Gb. thermocatenulatus* lipase-family primers (FWTHER/BKTHER) produced no PCR bands, whereas a prominent band of 320 bp (Figure C1.6A) appeared for both strains when amplification using *B. subtilis lipA* family primers (FWSUB/BKSUB) was performed. These results suggested that the lipolytic system of each strain contained at least a lipase-coding gene belonging to the *B. subtilis lipA* family. The DNA fragments amplified were sequenced, and their similarity to known genes studied. In both cases, the sequences obtained showed the presence of an uninterrupted ORF with high identity to previously described lipase genes of subfamily I.4 of bacterial lipases (Arpigny & Jaeger, 1999).

From the known sequences of the DNA fragments obtained, new sets of specific divergent primers were designed to amplify by inverse PCR (IPCR) the portions of DNA flanking the known central region of the respective genes, in order to obtain the complete ORF sequences of the lipase-coding genes from *B. megaterium* CECT 370 and *Bacillus* sp. BP-6. A single common band of ca. 950 bp was obtained from both strains (not shown). Sequencing of the 950-bp bands revealed two stretches of 599 nucleotides of the corresponding lipase coding genes. However, a small portion of sequence was missing at the 5'- and 3'-ends of the ORF due to the formation of truncated lipase-coding genes in the circular DNA generated as template. Thus, the same strategy was used for IPCR from *Bam*HI, *Xba*I or *Cla*I digested and re-ligated genomic DNAs, but neither with these enzymes could the complete ORFs of the lipase-coding genes be obtained.

From the analysis of the partial sequences obtained by PCR and IPCR, the highest identity was found to *yfi*P gene from *B. subtilis* (Yamamoto *et al*., 1996), also described later as *lipB* (Kunst *et al*., 1997; Eggert *et al*., 2000). As only a few nucleotides were missing for the complete knowledge of the ORFs of *B. megaterium* CECT 370 and *Bacillus* sp. BP-6 lipase-coding genes, and on the view of the high identity found to *B. subtilis* LipB (>98%), we performed a direct PCR amplification of

the lipase genes of both strains using the specific primers FWYfiP/BKYfiP designed from the 5' and 3' ends of *yfi*P (Figure C1.7). Both strains produced a common amplification band of ca. 650 bp (Figure C1.6B). In addition, *Bacillus* sp. BP-6 produced some minor bands of ca. 3 Kb, 1 Kb and 450 bp, corresponding to repeats or fragments of the same gene, as demonstrated after nucleotide sequencing of the corresponding DNA fragments. An amplification band of 650 pb (Figure C1.6B) was also found when these primers were used to perform the direct amplification of lipase-coding genes from the previously described (López *et al*., 1998; Prim *et al*., 2001) lipolytic strain *Bacillus* sp. BP-7.

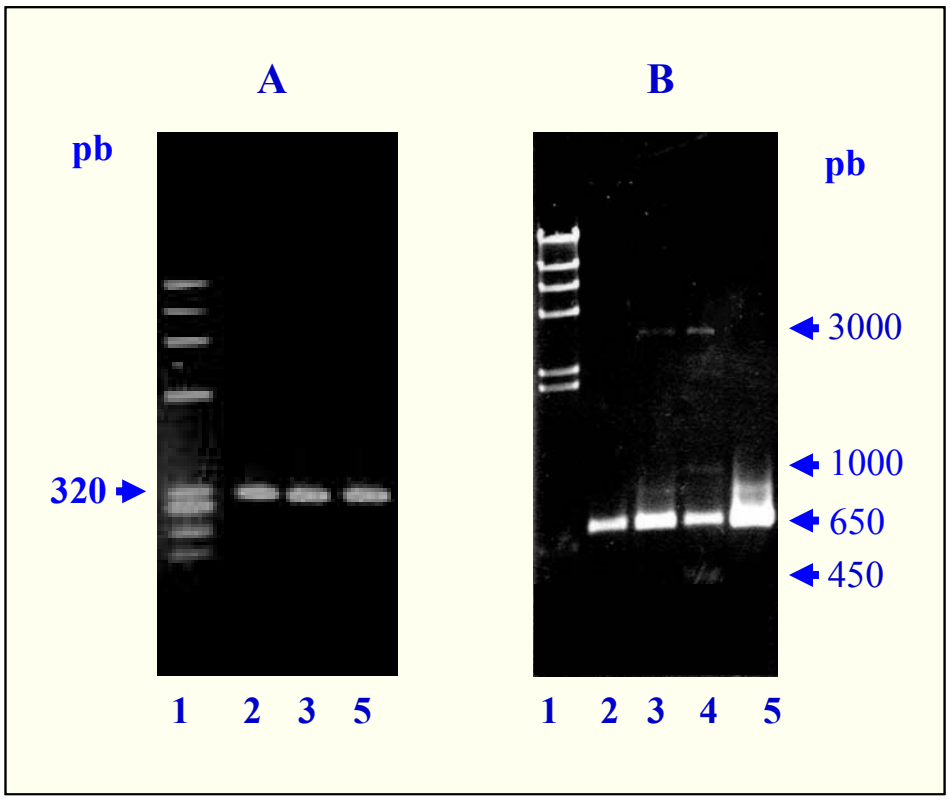


Figure C1.6 Amplification of *Bacillus* lipase-coding genes.

A: amplification using *B. subtilis lipA* family primers (FWSUB/BKSUB); B: amplification using *lipB* primers (FWYfiP/BWYFiP). 1: MW-marker (A: Φ DNA/*Hae*III marker; B: λ DNA/*Hind*III marker); 2: *B. megaterium* CECT 370; 3: *Bacillus* sp. BP-6; 4: *Bacillus* sp. BP-7; 5: positive control *B. subtilis* MB216.

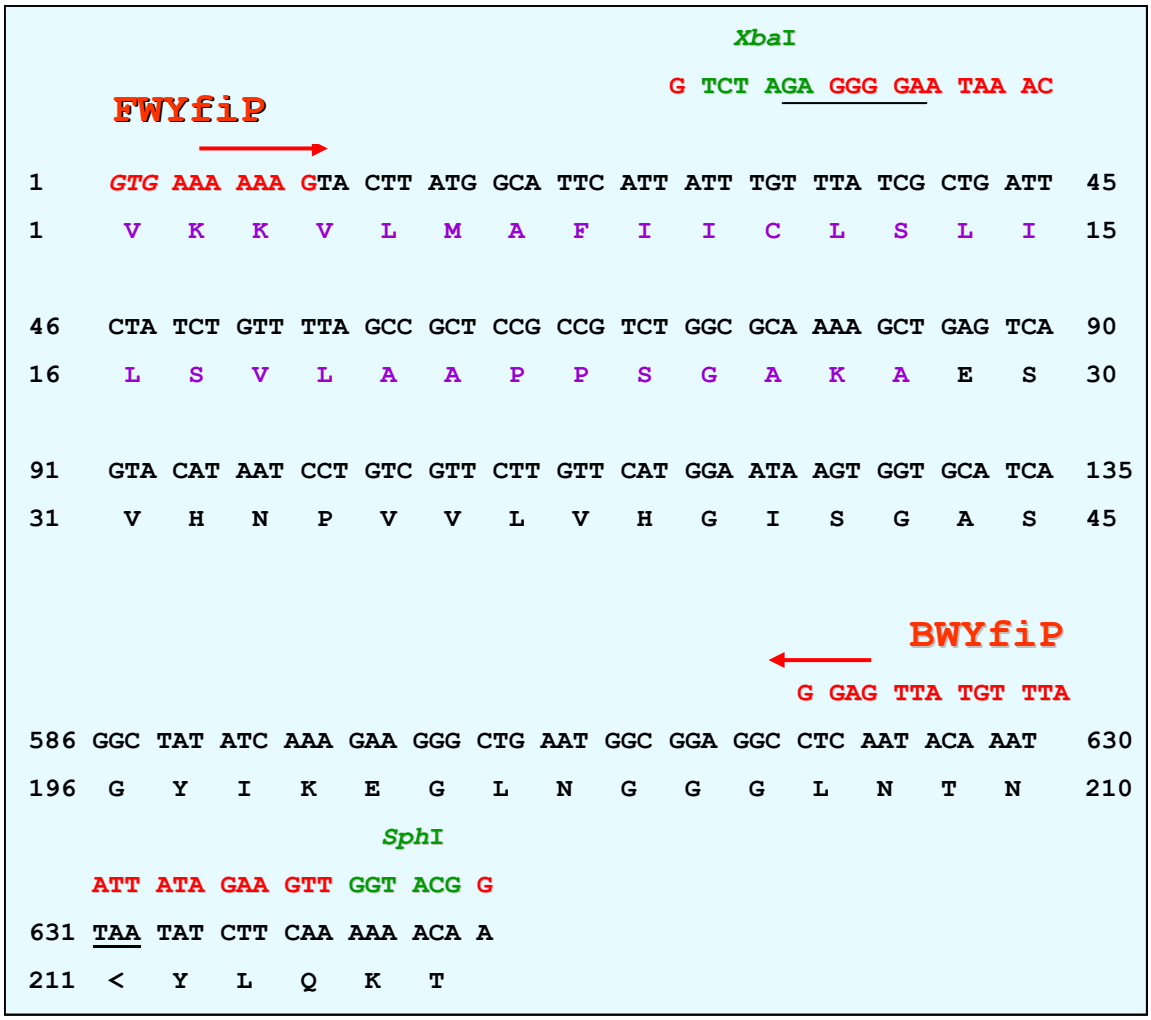


Figure C1.7 Primers designed from the 5' and 3'ends of the *yfi*P gene sequence.

Primers FWYfiP and BWYfiP (in red) were designed from the 5' and 3' ends of the *yfi*P (*lipB*) gene of the *B. subtilis* genome (Kunst *et al*., 1997). The sequences for the restriction enzymes *Xba*I and *Sph*I were added to these primers (in green). *yfi*P 5' region: Shine-Dalgarno region is underlined, start codon is in italics and the putative signal peptide of the corresponding protein (*B. subtilis* LipB, Eggert *et al*., 2000) is in lilac. *yfi*P 3' region: stop codon is underlined.

3.2.2 Cloning of the lipase-coding genes

The bands of 650 bp amplified were isolated, ligated to *Eco*RV-digested pBR322 plasmid, and cloned into *E. coli* 5K. The recombinant clones were selected on the basis of their tetracycline sensitivity/ampicillin resistance, and their high activity on MUF-derivative substrates (Figure C1.8). Restriction analysis of the recombinant plasmids showed that the cloned lipase-coding genes were under the control of the

tetracycline promoter from pBR322. As confirmed by Dot blot hybridization (Figure C1.9), the isolated recombinant clones contained a DNA insert from *B. megaterium* CECT 370, *Bacillus* sp. BP-6 and *Bacillus* sp. BP-7, respectively. A faint single hybridization band (not shown) was also obtained for each strain genomic DNA by Southern blot, indicating that the cloned genes were present as a single copy in the genome of their corresponding parental strains.

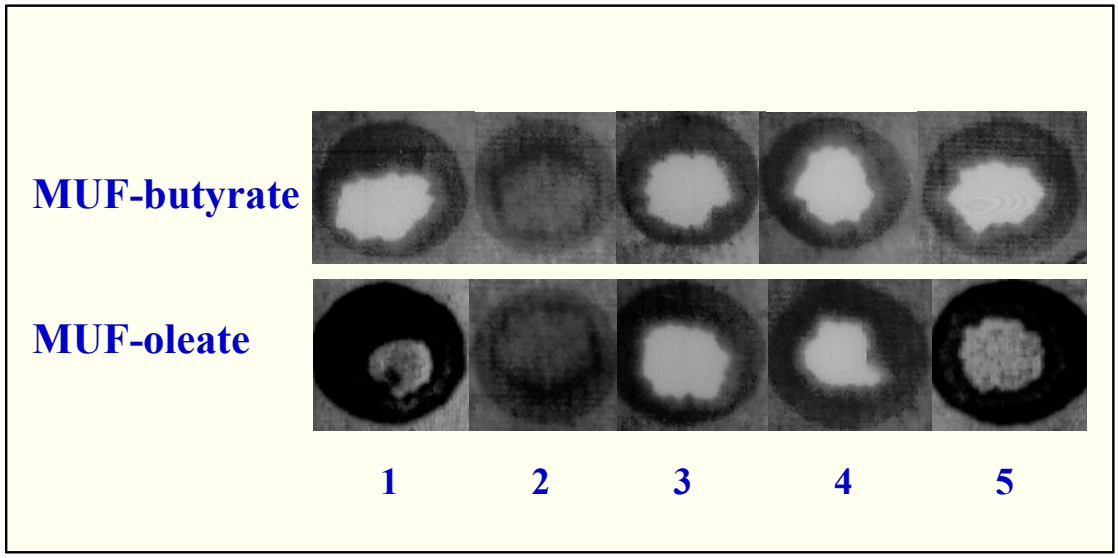
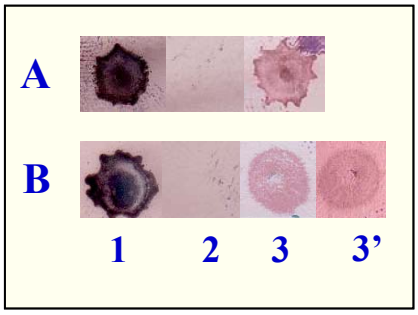


Figure C1.8 Activity on MUF-derivatives of the recombinant clones obtained.

The *E. coli* 5K recombinant clones obtained were analyzed on MUF-butyrate and MUF-oleate (fluorimetric paper assay) to select those containing the lipase-coding genes from the parental strains. 1: Positive control (*E. coli* 5K−pL37; Prim *et al*., 2000); 2: negative control (*E. coli* 5K−pBR322); 3: *E. coli* 5K−pBR-BMLipA; 4: *E. coli* 5K−pBR-BP6LipA; and 5: *E. coli* 5K−pBR-BP7LipA.

Figure C1.9 Confirmation by Dot blot of the presence of the cloned genes in the parental strains.



DNA hybridization using the FWSUB/BWSUB amplified fragment obtained from 5K−pBR-BMLipA (A) or from *E. coli* 5K−pBR-BP6LipA (B) (100% identical to that obtained from 5K−pBR-BP7LipA). 1: positive control plasmid DNA (A1: pBR-BMLipA; B1: pBR-BP6LipA). 2: negative control (*E. coli* 5K genomic DNA plus pBR322 plasmid DNA). 3: parental strain genomic DNA (A3: *B. megaterium* CECT 370; B3: *Bacillus* sp. BP-6; B3': *Bacillus* sp. BP-7).

3.2.3 Search for additional lipase-coding genes

Direct amplification under low-astringency conditions (Tm = 40−55 ºC) using specific primers for the amplification of *B. subtilis lipA* (FWBSLA and BWBSLA) was also attempted in order to obtain additional putative lipase-coding genes from the selected strains *B. megaterium* CECT 370, *Bacillus* sp. BP-6 and *Bacillus* sp. BP-7 *lipA*, although no amplification bands were obtained.

Another approach to isolate additional lipase-coding genes from *B. megaterium* CECT 370 consisted in the elaboration of a "fast" gene library of the genome of this strain in *E. coli* XL1 blue performed as explained in the materials and methods section. Active clones on tributyrin- or triolein-supplemented plates were obtained, although their subsequent culture or plasmid isolation could not be achieved.

3.2.4 Analysis of the isolated lipase-coding genes

The lipase-coding genes obtained were named *B. megaterium* CECT 370 *lipA* (*BMlipA*), *Bacillus* sp. BP-6 *lipA* (*BP6lipA*) and *Bacillus* sp. BP-7 *lipA* (*BP7lipA*). The nucleotide sequence of the cloned genes was determined and their sequences analyzed (see Figure C1.10). *BMlipA* showed 99% identity to both *BP6lipA* and *BP7lipA* (100% identical among them). All three genes contained an ORF of 633 nucleotides encoding for predicted proteins of 210 amino acids (Figure C1.10) 99–100% identical among them. The predicted proteins had a deduced molecular weight of 22379 (BMLipA) or 22331 (BP6LipA/BP7LipA) Da, and a deduced pI of 9.56 (BMLipA) or 9.43 (BP6LipA/BP7LipA). As expected, the deduced amino acid sequence of the enzymes showed the highest identity (99%, 100% and 100%, respectively) to *B. subtilis* LipB (YfiP) (Yamamoto *et al*., 1996; Eggert *et al*., 2000), an enzyme whose cloning and characterization was reported while this work was in progress. Lower identity (70−75%) was found to the secreted enzymes *B. subtilis* LipA (Dartois *et al*., 1994), *B. pumilus* lipase (Moeller *et al*., 1991), and *B. licheniformis* lipase (Nthangeni *et al*., 2001), all of them grouped into subfamily I.4 of bacterial lipases (Arpigny & Jaeger, 1999). Similarity to other Gram-positive or Gram-negative bacterial lipases was much

lower (15−37%), indicating that the cloned enzymes are members of a reduced cluster of lipases grouped into subfamily I.4 of bacterial lipases, exclusive from the mesophilic or moderately thermophilic members of the genus *Bacillus*.

In view of these results, only *BMlipA* and *BP6lipA* (identical to *BP7lipA*) were selected for further studies. The amino acid sequence of the corresponding deduced proteins was analyzed using different proteomic tools (Table C1.2; Figure C1.10). Analysis by SignalP software revealed an identical 28 amino acid stretch at the N-terminal region of both proteins that showed the features of the signal peptides found in *Bacillus* proteins secreted by the Sec−Xcp system (Tjalsma *et al*., 2000), suggesting the extracellular location of both proteins. The resulting BMLipA and BP6LipA mature lipases showed a deduced molecular weight of 19542 and 19493 Da, and a pI of 9.42 and 9.2, respectively. These differences were produced by the existence of two amino acid substitutions at the C-terminal region of both mature proteins: position 169 was tyrosine (Tyr; Y) in BMLipA and asparagine (Asn; N) in BP6LipA, and position 172 was lysine (Lys; K) in BMLipA and glutamate (Glu; E) in BP6LipA.

From the amino acid sequence alignments, the catalytic apparatus of lipases involving the triad serine, glutamate or aspartate and histidine was placed in the mature proteins at positions 78 (Ser), 134 (Asp) and 157 (His). The catalytic serine was found in both proteins at the motif AH**S**^{78}MG, corresponding to the consensus pentapeptide Ala-Xaa-**Ser**-Xaa-Gly common in all known lipases from subfamilies I.4 and I.5 (Fojan *et al*., 2000).

Both proteins showed also a high content in short non-polar residues (47% of the total; Table C1.2), many of them located in the primary sequence near the catalytic residues (Figure C1.10), as is usual in enzymes acting on hydrophobic substrates that that can be found in an aggregated state (Fojan *et al*., 2000).

```
1     GTG AAA AAA GTA CTT ATG GCA TTC ATT ATT TGT TTA TCG CTG ATT   45
1      V   K   K   V   L   M   A   F   I   I   C   L   S   L   I   15

46    CTA TCT GTT TTA GCC GCT CCG CCG TCT GGC GCA AAA GCT GAG TCA   90
16     L   S   V   L   A   A   P   P   S   G   A   K   A   E   S   30

91    GTA CAT AAT CCT GTC GTT CTT GTT CAT GGA ATA AGT GGT GCA TCA   135
31     V   H   N   P   V   V   L   V   H   G   I   S   G   A   S   45

136   TAC AAC TTT TTC GCT ATT AAA AAC TAC TTA ATT TCT CAA GGC TGG   180
46     Y   N   F   F   A   I   K   N   Y   L   I   S   Q   G   W   60

181   CAA AGC AAC AAA CTG TAC GCA ATT GAT TTT TAT GAT AAA ACA GGA   225
61     Q   S   N   K   L   Y   A   I   D   F   Y   D   K   T   G   75

226   AAC AAC CTA AAT AAC GGC CCG CAG CTT GCT TCA TAT GTT GAC CGT   270
76     N   N   L   N   N   G   P   Q   L   A   S   Y   V   D   R   90

271   GTT TTA AAA GAG ACT GGG GCA AAA AAA GTA GAT ATT GTG GCT CAT   315
91     V   L   K   E   T   G   A   K   K   V   D   I   V   A   H   105

316   AGT ATG GGA GGC GCC AAT ACG CTG TAC TAT ATT AAA TAT TTA GGC   360
106    S   M   G   G   A   N   T   L   Y   Y   I   K   Y   L   G   120

361   GGG GGC AAT AAG ATT CAA AAT GTC GTA ACG CTT GGA GGG GCT AAT   405
121    G   G   N   K   I   Q   N   V   V   T   L   G   G   A   N   135

406   GGT TTA GTG TCA TCA ACC GCG CTG CCG GGC ACA GAC CCT AAT CAA   450
136    G   L   V   S   S   T   A   L   P   G   T   D   P   N   Q   150

451   AAG ATC CTC TAT ACA TCT ATT TAC AGT CTC AAT GAT CAA ATT GTC   495
151    K   I   L   Y   T   S   I   Y   S   L   N   D   Q   I   V   165

496   ATC AAT AGC TTG TCT CGG TTA CAA GGA GCG CGA AAC ATC CAG CTT   540
166    I   N   S   L   S   R   L   Q   G   A   R   N   I   Q   L   180

541   TAT GGC ATC GGT CAT ATT GGC TTG CTT TCT AAT AGC CAA GTG TAC   585
181    Y   G   I   G   H   I   G   L   L   S   N   S   Q   V   Y   195

586   GGC TAT ATC AAA AAA GGG CTG AAT GGC GGA GGC CTC AAT ACA AAT   630
196    G   Y   I   K   K   G   L   N   G   G   G   L   N   T   N   210

631   TAA   633
211   STOP
```

Figure C1.10 Nucleotide and amino acid sequence of *B. megaterium* CECT 370 *lipA* ORF.

The 28 residues corresponding to the deduced signal peptide of the protein are in lilac. The residues of the catalytic triad are in red: Ser^{78}, Asp^{134} and His^{157}. The residues of the conserved pentapeptide of subfamilies I.4 and I.5 of bacterial lipases flanking the catalytic serine are in blue. The sequence of *BP6lipA*/*BP7lipA* was the same as for *BMlipA* with the exception of the nucleotides/residues in yellow: $T^{583}\rightarrow A^{583}$ $(Y(Tyr)^{169}\rightarrow N(Asn)^{169})$; $A^{598}\rightarrow G^{598}$ $(K(Lys)^{172}\rightarrow E(Glu)^{170})$. All residue positions are indicated with respect to the mature protein.

Table C1.2 Amino acid composition of BMLipA and BP6LipA.

Amino acid composition of BMLipA

Ala (A)	15	7.1%
Arg (R)	3	1.4%
Asn (N)	**20**	**9.5%**
Asp (D)	6	2.9%
Cys (C)	1	0.5%
Gln (Q)	9	4.3%
Glu (E)	**2**	**1.0%**
Gly (G)	25	11.9%
His (H)	4	1.9%
Ile (I)	18	8.6%
Leu (L)	25	11.9%
Lys (K)	**14**	**6.7%**
Met (M)	2	1.0%
Phe (F)	4	1.9%
Pro (P)	6	2.9%
Ser (S)	18	8.6%
Thr (T)	8	3.8%
Trp (W)	1	0.5%
Tyr (Y)	**13**	**6.2%**
Val (V)	16	7.6%
Asx (B)	0	0.0%
Glx (Z)	0	0.0%
Xaa (X)	0	0.0%

Total number of negatively charged residues (Asp + Glu): **8**

Total number of positively charged residues (Arg + Lys): **17**

Short non-polar residues (Ala + Gly + Ileu + Leu + Val) = **99 (47%**□

Amino acid composition of BP6LipA

Ala (A)	15	7.1%
Arg (R)	3	1.4%
Asn (N)	**21**	**10.0%**
Asp (D)	6	2.9%
Cys (C)	1	0.5%
Gln (Q)	9	4.3%
Glu (E)	**3**	**1.4%**
Gly (G)	25	11.9%
His (H)	4	1.9%
Ile (I)	18	8.6%
Leu (L)	25	11.9%
Lys (K)	**13**	**6.2%**
Met (M)	2	1.0%
Phe (F)	4	1.9%
Pro (P)	6	2.9%
Ser (S)	18	8.6%
Thr (T)	8	3.8%
Trp (W)	1	0.5%
Tyr (Y)	**12**	**5.7%**
Val (V)	16	7.6%
Asx (B)	0	0.0%
Glx (Z)	0	0.0%
Xaa (X)	0	0.0%

Total number of negatively charged residues (Asp + Glu): **9**

Total number of positively charged residues (Arg + Lys): **16**

Short non-polar residues (Ala + Gly + Ileu + Leu + Val) = **99 (47%**□

The high identity of BMLipA and BP6LipA to *B. subtilis* LipA (73−74%), whose crystal structure has previously been described (van Pouderoyen *et al*., 2001; Figure C1.12A), allowed us to construct an automated comparative protein 3D–model for the mature form of the cloned lipases (Figures C1.11, and C1.12B). The protein models obtained were compared also with the prediction model obtained for *B. subtilis* LipB by Eggert *et al*. (2001), using a different software (Figure C1.12C). The structural model of the three proteins showed a globular shape very similar to that of *B. subtilis*

LipA, and constituted by a compact domain displaying the α/β-hydrolase fold characteristic of lipases (Jaeger *et al*., 1999). This domain consisted of 6 parallel β sheets surrounded by 5 α helices, three on one side of the β sheet, and two on the other side. As in the case of *B. subtilis* LipA, the active site of the proteins was freely accessible from the solvent, since no "lid" for interfacial activation existed. In fact, the active site of *B. subtilis* LipA and those of the cloned lipases were identical since the nearest amino acid substitutions occurred at the rim of the putative substrate-binding surface, at a distance of about 9 Å in the model of Eggert *et al*. (2001). When BMLipA and BP6LipA models were compared, the two amino acid substitutions that differentiate these proteins were found at the lower part of the α helix preceding the C-terminal end of the molecules (helix 5, see Figure C1.11).

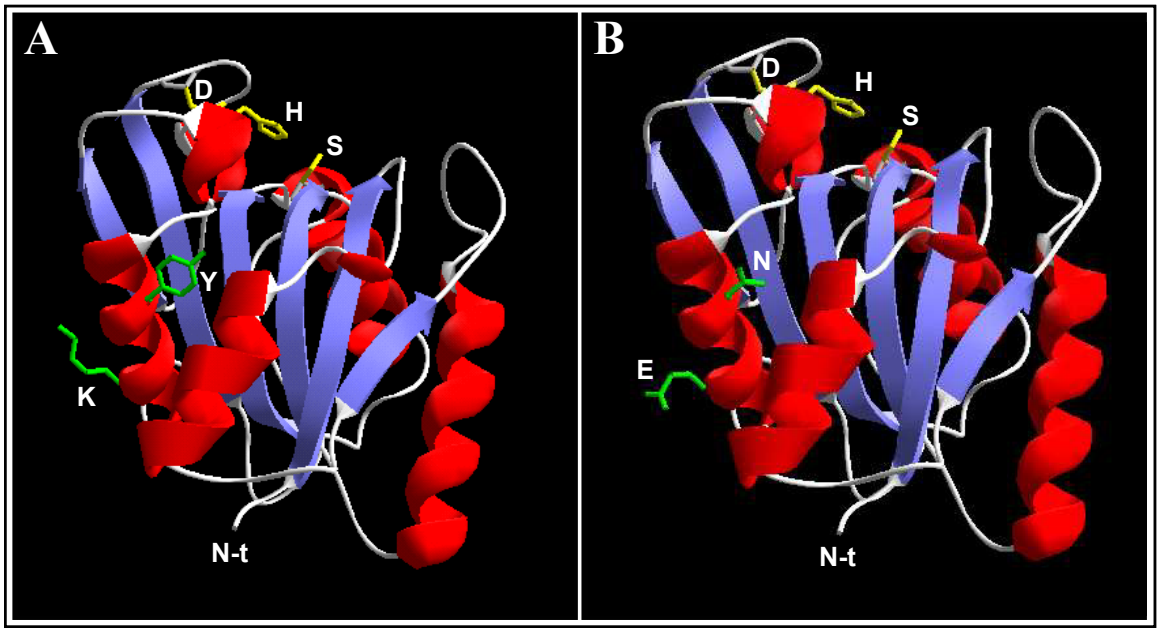


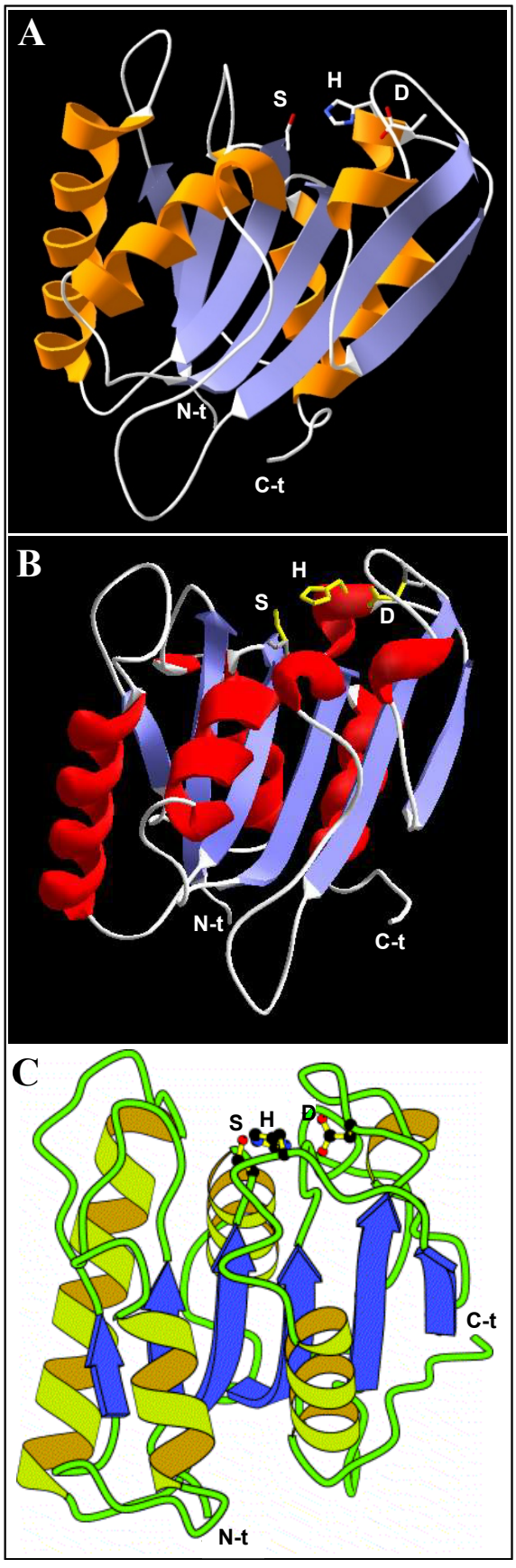
Figure C1.11 Automated comparative protein model of BMLipA and BP6LipA.

The protein models obtained for the mature form of BMLipA (A) and BP6LipA (B) are shown in the same orientation. The residues of the catalytic triad are in yellow: S (Ser^{78}), D (Asp^{134}) and H (His^{157}). The residues different for both proteins are in green: Y(Tyr^{169})/N(Asn^{169}) and K(Lys^{172})/E(Glu^{172}), respectively. N-t: N-terminal end.

Figure C1.12 Three-dimensional structure of several *Bacillus* lipases.

Structure of *B. subtilis* LipA (A; van Pouderoyen *et al*., 2001), the comparative protein model obtained for BMLipA (B; this study), and the comparative protein model obtained for *B. subtilis* LipB (C; Eggert *et al*., 2001) are displayed in the same orientation. The model obtained for BP6LipA (this work) is not shown since it was almost the same as that obtained for BMLipA in this orientation, and due to the fact that BP6LipA sequence was the same as that of *B. subtilis*.

All three proteins show a very similar structure, constituted by a globular compact domain with the α/β-hydrolase fold characteristic of lipases. In all three lipases, this domain consists of 6 parallel β sheets surrounded by 5 α helices, three on one side of the β sheet, and two on the other side. The active site is freely accessible from the solvent in all three proteins, since no "lid" for interfacial activation exists. These proteins show also a similar three-dimensional disposition of the Ser-Asp-His (S-D-H) catalytic triad (in yellow). However, some differences can be observed in the disposition of the C-t (C-terminal end) loop, whereas the disposition of the N-t (N-terminal end) loop is highly similar for all three enzymes.



3.3 CHARACTERIZATION OF THE CLONED ENZYMES

The cloned lipases from *B. megaterium* CECT 370 and *Bacillus* sp. BP-6 were characterized and their biochemical and molecular properties determined as described in the materials and methods section, using the previously obtained recombinant clones and their corresponding cell extract dilutions.

3.3.1 Biochemical characterization

The lipolytic activity of the recombinant clones containing *BMlipA* or *BP6lipA* genes was screened on lipid-supplemented CeNAN agar plates (not shown) and on MUF-derivatives (fluorimetric paper assay, see Figure C1.8). While clear hydrolysis zones were detected using tributyrin as substrate, poor degradation was found on plates containing triolein or olive oil, as is described for carboxylesterases (CEs) (Jaeger *et al*., 1999). However, the recombinant clones produced high activity on both MUF-butyrate and MUF-oleate.

Dilutions of cell extracts from the recombinant clones containing BMLipA or BP6LipA were tested for determining their lipolytic activity on several *p*-NP- and MUF-derivatives (Table C1.2, Figure C1.13). Both enzymes showed a very similar behaviour. The highest activities (100%) were found on *p*-NP butyrate (844 ± 16 and 1410 ± 98 mU mg^{-1} protein for BMLipA and BP6LipA, respectively) and on MUF-butyrate (822.1 ± 41 and 656.1 ± 33 mU mg^{-1} protein for BMLipA and BP6LipA, respectively). Furthermore, the cloned enzymes efficiently hydrolyzed other esters of short-chain fatty acids (activity higher than 50 % up to C_8-derivatives), while activity decreased as the length of the fatty acid chains increased (activity lower than 10% on C_{10-18}-derivatives), a behaviour typical of CEs (Jaeger *et al*., 1999). Interestingly, both total and residual (%) activity of the cloned enzymes were much higher on MUF-oleate ($C_{18:1c\Delta9}$) than on *p*-NP stearate ($C_{18:0}$), the corresponding saturated *p*-NP-derivative (relative activities 9.8−9.9 and 0.1−0.5, respectively) (Table C1.2, Figure C1.13).

Table C1.3 Substrate profile of BMLipA and BP6LipA.

Substrate	BMLipA		BP6LipA	
	mU mg^{-1} protein	%	mU mg^{-1} protein	%
p-NP butyrate ($C_{4:0}$)	844.5	100.0	1413.3	100.0
p-NP valerate ($C_{5:0}$)	669.6	79.4	1040.6	73.8
p-NP caproate ($C_{6:0}$)	620.2	73.5	810.9	57.5
p-NP caprylate ($C_{8:0}$)	547.2	64.8	924.2	65.4
p-NP caprate ($C_{10:0}$)	82.4	9.7	75.1	5.7
p-NP laurate ($C_{12:0}$)	41.1	4.9	41.6	1.8
p-NP palmitate ($C_{16:0}$)	7.3	0.8	11.2	0.8
p-NP stearate ($C_{18:0}$)	3.5	0.5	2.1	0.1
MUF-butyrate ($C_{4:0}$)	822.1	100.0	656.1	100%
MUF-oleate ($C_{18:1c\Delta9}$)	82.2	9.9	64.2	9.8

The standard deviations obtained ranged from 2% to 10% of the corresponding mean values.

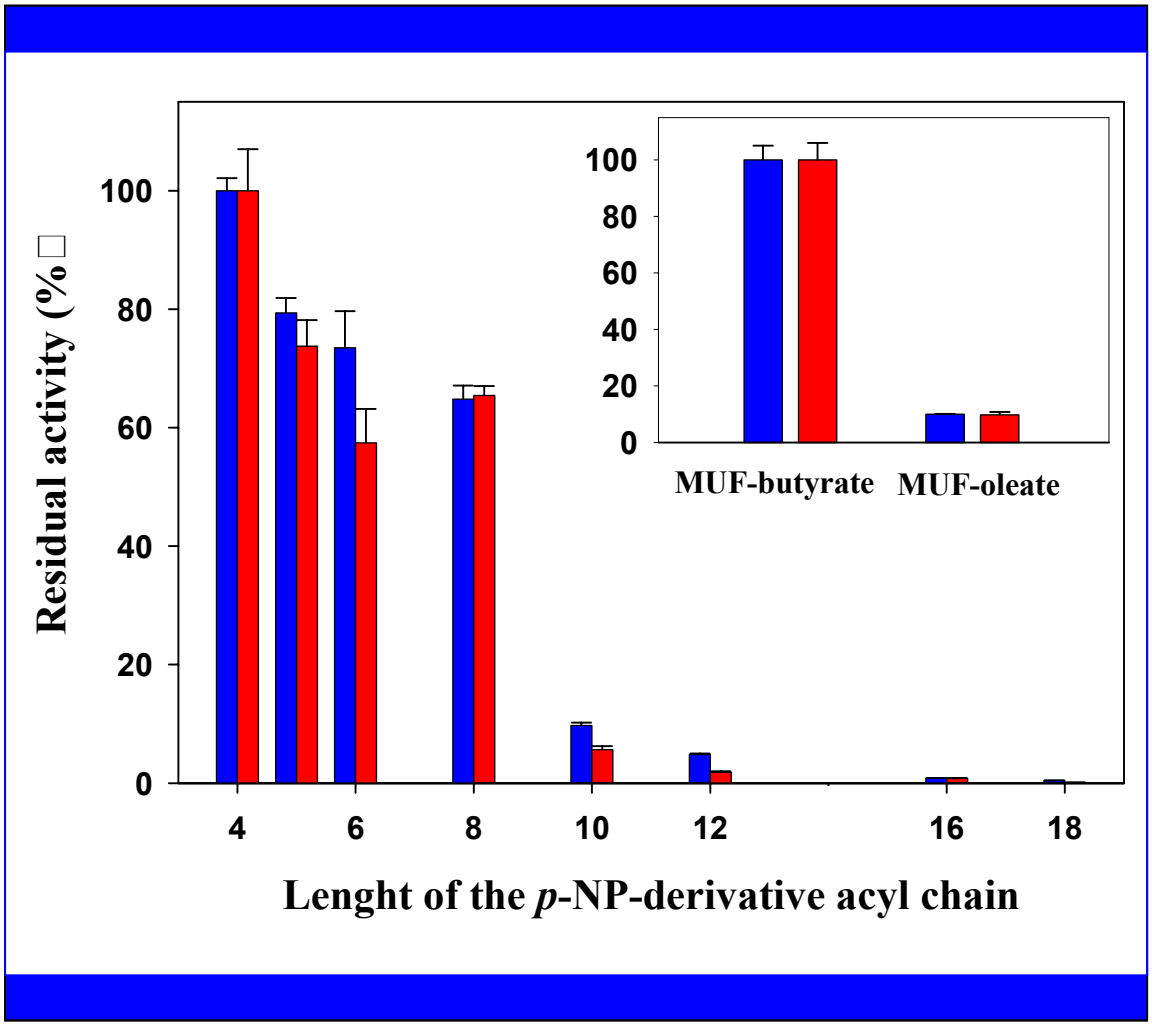


Figure C1.13 Substrate range of BMLipA and BP6LipA.

Relative activity with respect to the maximum activity (activity on *p*-NP butyrate) *vs*. length of the *p*-NP-derivative acyl chain is plotted for BMLipA (blue) and BP6LipA (red). Activities on MUF-butyrate (C_4; 100%) and MUF-oleate (C_{18}) are also compared.

The effect of temperature and pH on the activity and stability of the cloned lipases BMLipA and BP6LipA was determined using MUF-butyrate as substrate. Both enzymes showed a very similar behaviour. The highest activity (100%) on this substrate was found at 45 ºC and pH 7 for both lipases. Moreover, BMLipA and BP6LipA showed high activity under a wide range of temperatures and pH, since the activities were higher than 50% from 20 to 50 ºC, and higher than 65% from pH 5.5 to pH 8.5 (Figure C1.14).

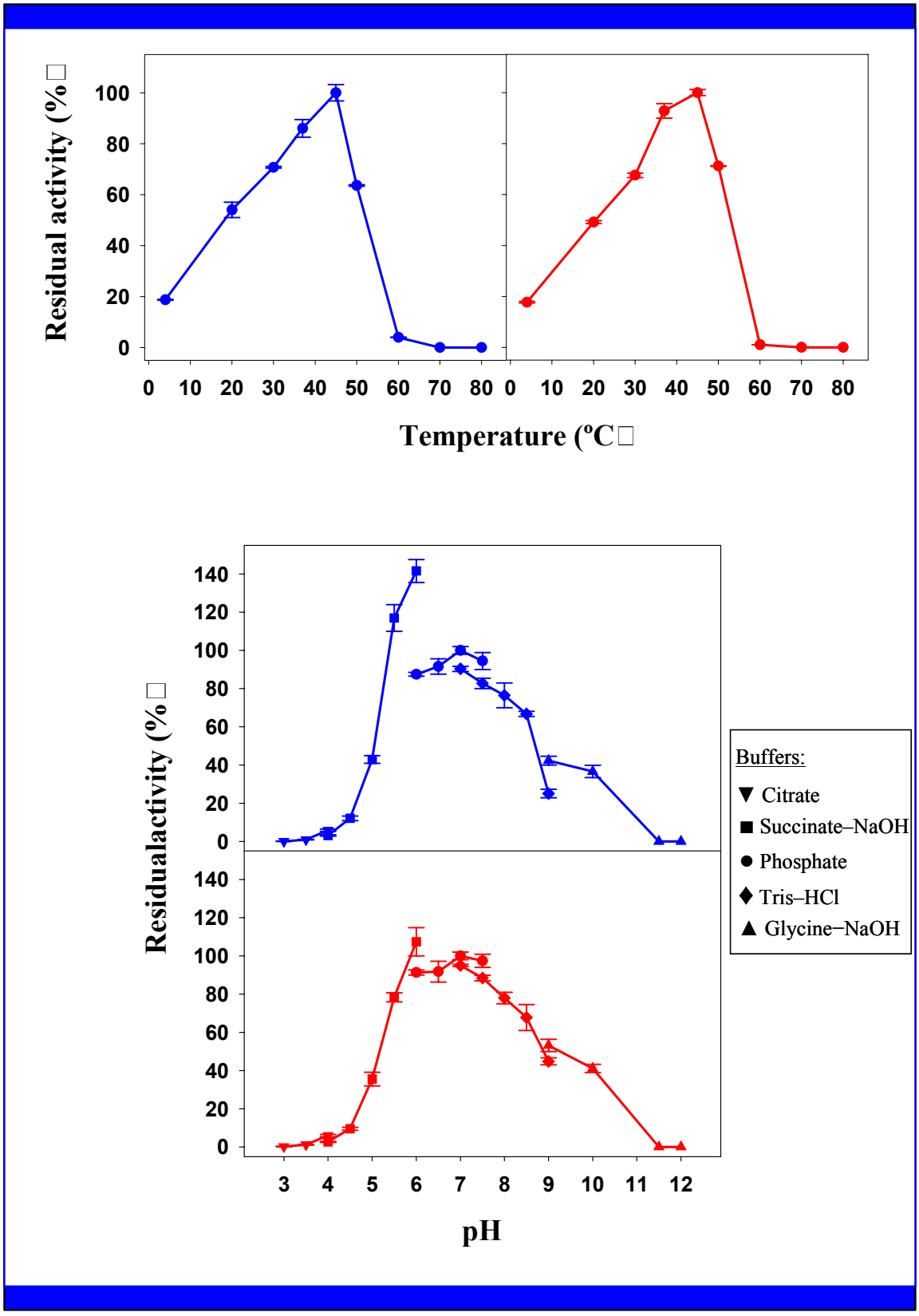


Figure C1.14 Optimum temperature and pH of BMLipA (blue□and BP6LipA (red□

The two cloned enzymes showed also a high stability in a wide range of temperatures and pH. Both enzymes were stable in a pH range from 4 to 12 when incubated for 1 h at room temperature (relative activity higher than 80%; Figure C1.15). BMLipA and BP6LipA remained also stable at temperatures from 4 ºC to 45 ºC when incubated at pH 7 for 1 h (relative activity higher than 80%). However, a dramatic decrease of activity (relative activity lower than 5%) was found after 1 h-incubation at temperatures above 50 ºC (Figure C1.15).

When the stability at pH 7 of both enzymes was analyzed at time periods other than 1 hour, interesting results were found (Figure C1.16). After 20-min incubation, activity at 4−45 ºC was equal or even higher (100–160%) than the activity at time zero (100%), probably as a result of a better solubilization of the enzymes. However, even in such short periods, the activity of both enzymes incubated at 50 ºC decreased dramatically (20% residual activity). Considering incubation periods longer than 1 h, the activity of both enzymes at 4−30 ºC remained stable for the first 24 h (90–120% relative activity). Furthermore, the enzymatic activity at 4−30 ºC was higher (120−220%) for the following 40 days, probably as result of the previously mentioned increase in enzyme solubility. In fact, occurrence of aggregation is a frequent process among cloned lipases that makes difficult their access to the substrate, and which can sometimes be reversed by light heat-treatments (Khalameyzer *et al*., 1999; Prim *et al*., 2000).

On the contrary, activity at 45 ºC decreased up to a 60% in 2 hours, and up to a 15−20% in 8 h, whereas no activity could be detected at this temperature for incubation periods of 10 days or longer. Inactivation at 50 ºC was even faster since the enzymatic activity was 4−6% after 2 h-incubation, and was undetectable after 4 h-incubation at this temperature (Figure C1.16).

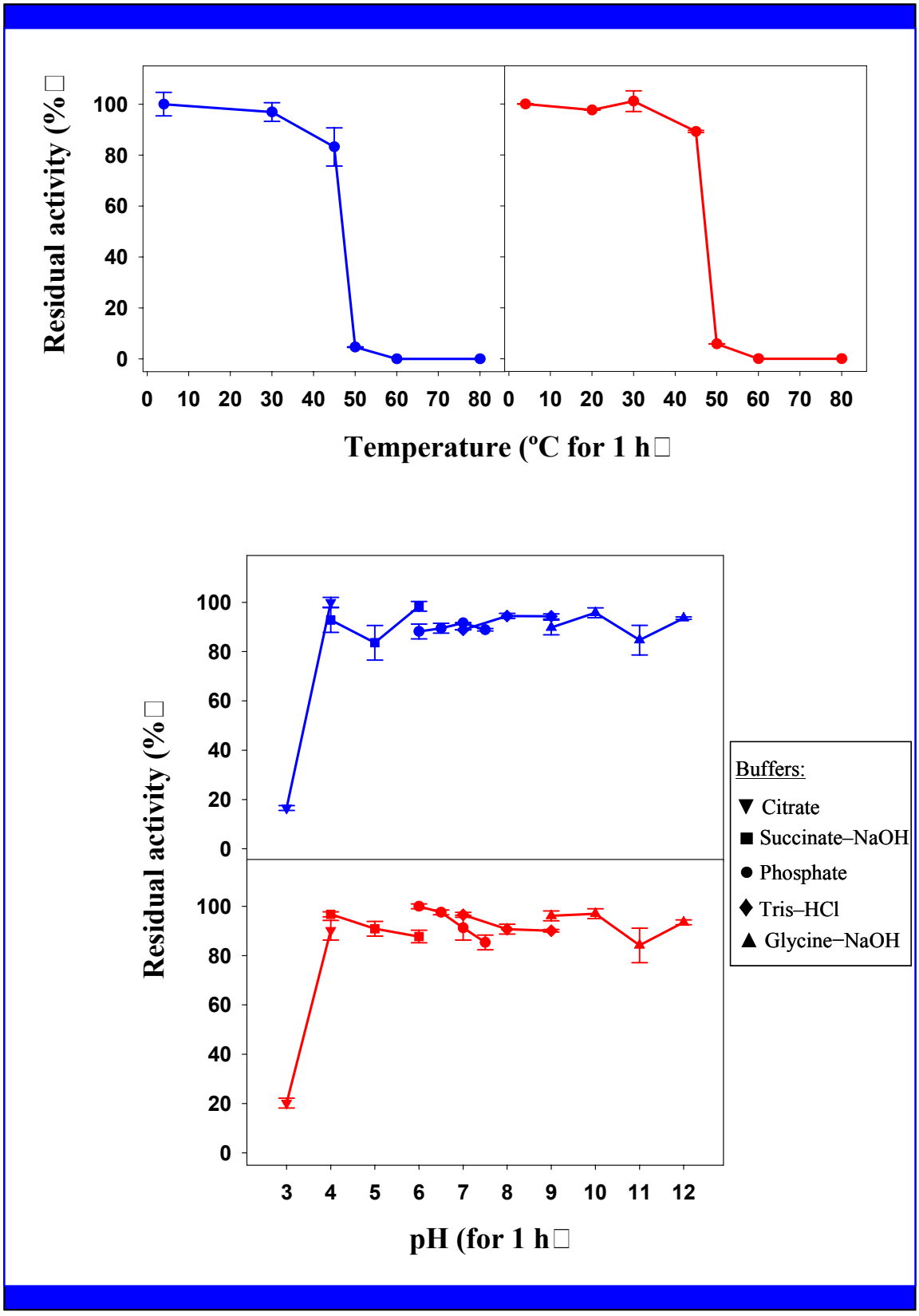
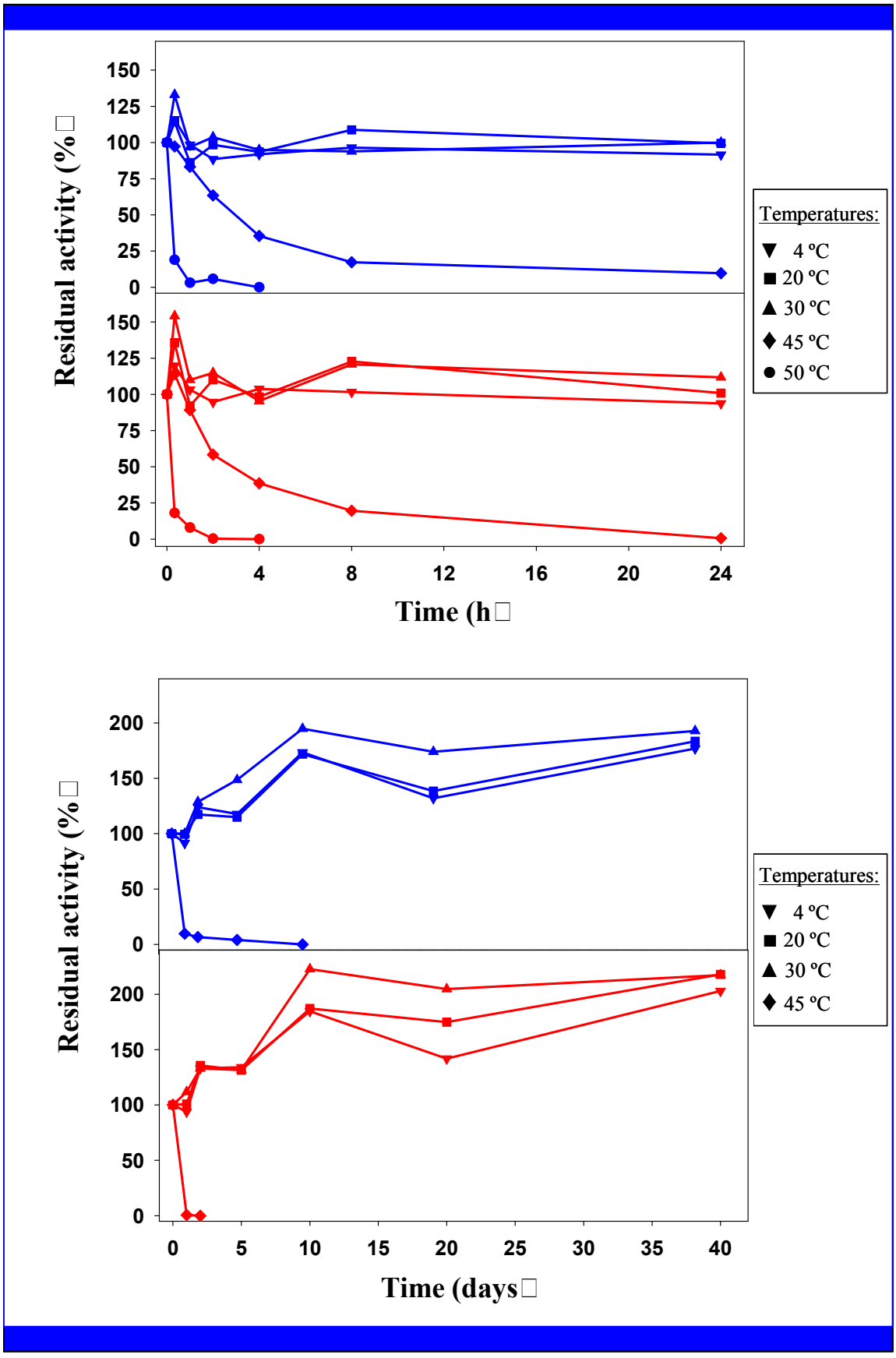


Figure C1.15 Temperature and pH stability of BMLipA (blue□and BP6LipA (red□after 1 h-incubation.


Residual activity (%
150
125
100
75
50
25
0
0
4
8
12
16
20
24
Time (h
Temperatures:
4 ºC
20 ºC
30 ºC
45 ºC
50 ºC
Residual activity (%
200
150
100
50
0
0
5
10
15
20
25
30
35
40
Time (days
Temperatures:
4 ºC
20 ºC
30 ºC
45 ºC

Previous page: Figure C1.16 Long-term temperature stability of the cloned lipases.

The stability at several temperatures of BMLipA (blue) and BP6LipA (red) was measured for forty days. The standard deviations (from 1 to 10% of the given values) are not shown for clarity reasons.

When we analyzed the kinetic behaviour of the cloned enzymes on MUF-butyrate and MUF-oleate, they showed the kinetics of typical CEs (Jaeger *et al*., 1999), with higher affinity for short-chain length substrates, and displaying no interfacial activation (Figure C1.17). A Michaelis-Menten plot with the maximum activity over $2 \cdot 10^{-4}$ M, and showing a decreasing activity at higher concentrations was obtained when both enzymes were assayed on increasing concentrations of MUF-butyrate. On the contrary, a standard Michaelis-Menten plot without reaching a maximum or a decrease in activity was obtained for both enzymes at the concentrations of MUF-oleate analyzed (Figure C1.17). The activity *vs*. substrate concentration plots were used to calculate the kinetic parameters of both enzymes (Table C1.4).

Table C1.4 Kinetic parameters of BMLipA and BP6LipA.

Parameter	BMLipA		BP6LipA	
	MUF-butyrate	MUF-oleate	MUF-butyrate	MUF-oleate
V_{max}^{app} (mU mg^{-1} protein)	904.6 ± 23.6	98.6 ± 1.6	737.2 ± 19.9	76.1 ± 0.8
K_M^{app} (M)	$3.7 \cdot 10^{-5}$ ± $1.1 \cdot 10^{-6}$	$7.7 \cdot 10^{-5}$ ± $3.5 \cdot 10^{-6}$	$2.5 \cdot 10^{-5}$ ± $1.6 \cdot 10^{-6}$	$7.0 \cdot 10^{-5}$ ± $2.3 \cdot 10^{-6}$

The ± refers to the standard deviations obtained from regression curves with Rsquare coefficients higher than 0.99.

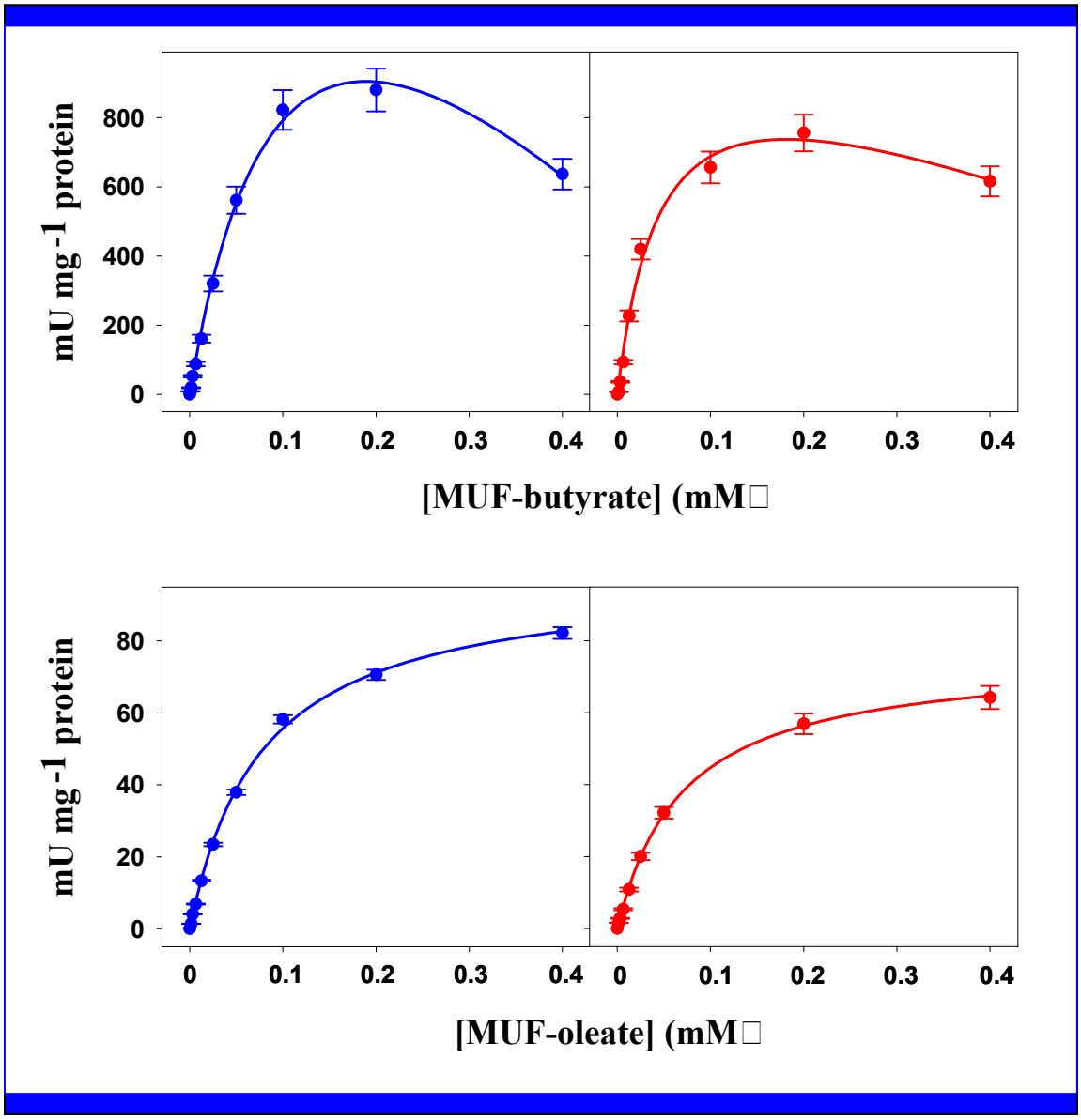


Figure C1.17 Kinetic behaviour of BMLipA (blue and BP6LipA (red.

The effect of different agents on the activity of the cloned enzymes was determined using *p*-NP laurate, and the results obtained are summarized in Table C1.5. Among the cations analyzed, only Ag^{+} and Hg^{2+} caused high inhibition (residual activity lower than 30% and 52%, respectively) at the two concentrations assayed (1 mM and 10 mM). Fe^{2+} caused also a high inhibition at 10 mM (residual activity lower than 50%), whereas partial inhibition (residual activity 70−86%) was caused by 10 mM concentrations of Cu^{2+}, Mn^{2+}, Pb^{2+} and Zn^{2+}. On the contrary, Fe^{2+} (at 1 mM) and NH^{4+} (at both concentrations) activated both enzymes (residual activity higher than 115%).

The rest of ions (residual activity 86–115%) did not significantly affect the activity of the cloned lipases (Figure C1.18; Table C1.5).

The influence of the amino acid-modifying agents NAI (*N*-acetylimidazole; tyrosine), NBS (*N*-bromosuccinimide; tryptophan), PHMB (*p*-hydroxymercuribenzoic acid; cysteine) and PMSF (phenylmethylsulfonyl fluoride; serine) was also tested (Figures C1.18–19; Table C1.5). Only NBS and PMSF caused a significant reduction of activity, suggesting that tryptophan and serine, but not cysteine nor tyrosine are involved in the functional or structural domains of the cloned enzymes. PMSF, whose effect was analyzed in more detail (Figure C1.19 and Table C1.5), was by far the most active compound (IC_{50} = $2.5 \cdot 10^{-4}$ M for both enzymes; Table C1.5), confirming the importance of the serine amino acids in the catalytic activity of the cloned enzymes.

The effect of EDTA, phytic acid, SDS, Triton X-100, and urea was also analyzed in detail for the cloned lipases (Figure C1.19). The highest inhibition was caused by phytic acid (IC_{50} = $1.7-1.8 \cdot 10^{-4}$ M; Table C1.5), whereas EDTA, Triton X-100 and urea caused a lower inhibition on both enzymes at high concentrations. On the contrary, low concentrations of EDTA, Triton X-100 and urea produced a slight activation of the cloned enzymes (residual activity 100−110%), probably due to the fact that Triton X-100 and urea produced an increased solubilization of both enzymes.

The most surprising effect was that caused by SDS on both lipases (Figure C1.20). An extraordinary activation (residual activity higher than 800%) of the hydrolytic activity occurred at 1.7 mM SDS. The activation decreased at higher SDS concentrations, and the activity levels shown in the absence of the tensioactive were restored when the SDS concentration was raised to 14 mM values. Higher concentrations caused a moderate inhibition of both enzymes (IC_{50} = 20−28 mM; Table C1.5). The observed effect could be due to the solubilization of the protein aggregates occurring in the concentrated cell extracts that make difficult the interaction between the enzyme and the substrate molecules, as mentioned before for the increased activity found at low concentrations of Triton X-100 and urea, or after enzyme incubation at 4−30 ºC. In fact, in many occasions aggregation can be reversed by addition of low concentrations of detergents such as Triton X-100 or SDS (Khalameyzer *et al*., 1999; Prim *et al*., 2000).

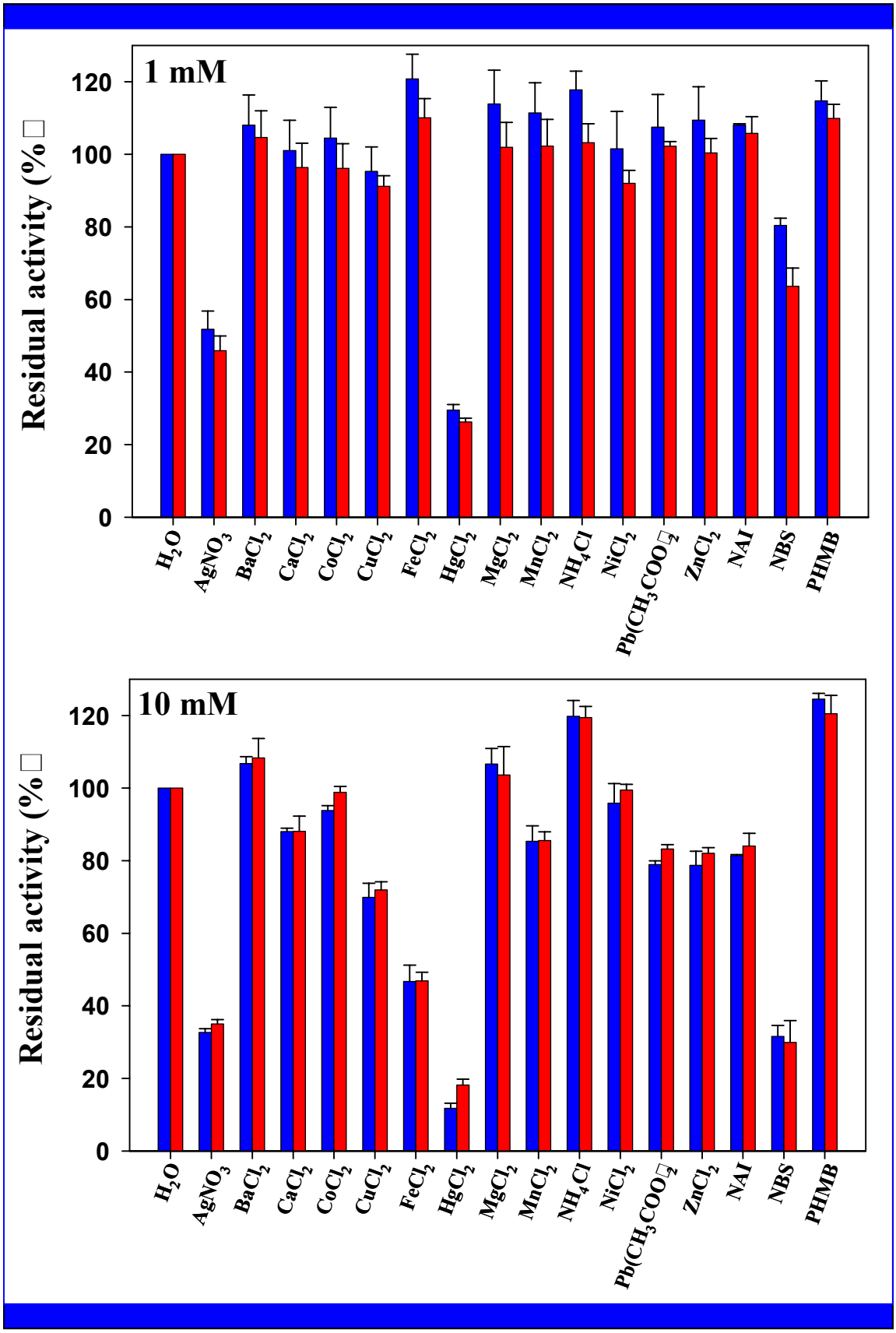


Figure C1.18 Effect of cations and amino acid modifiers on BMLipA (blue and BP6LipA (red

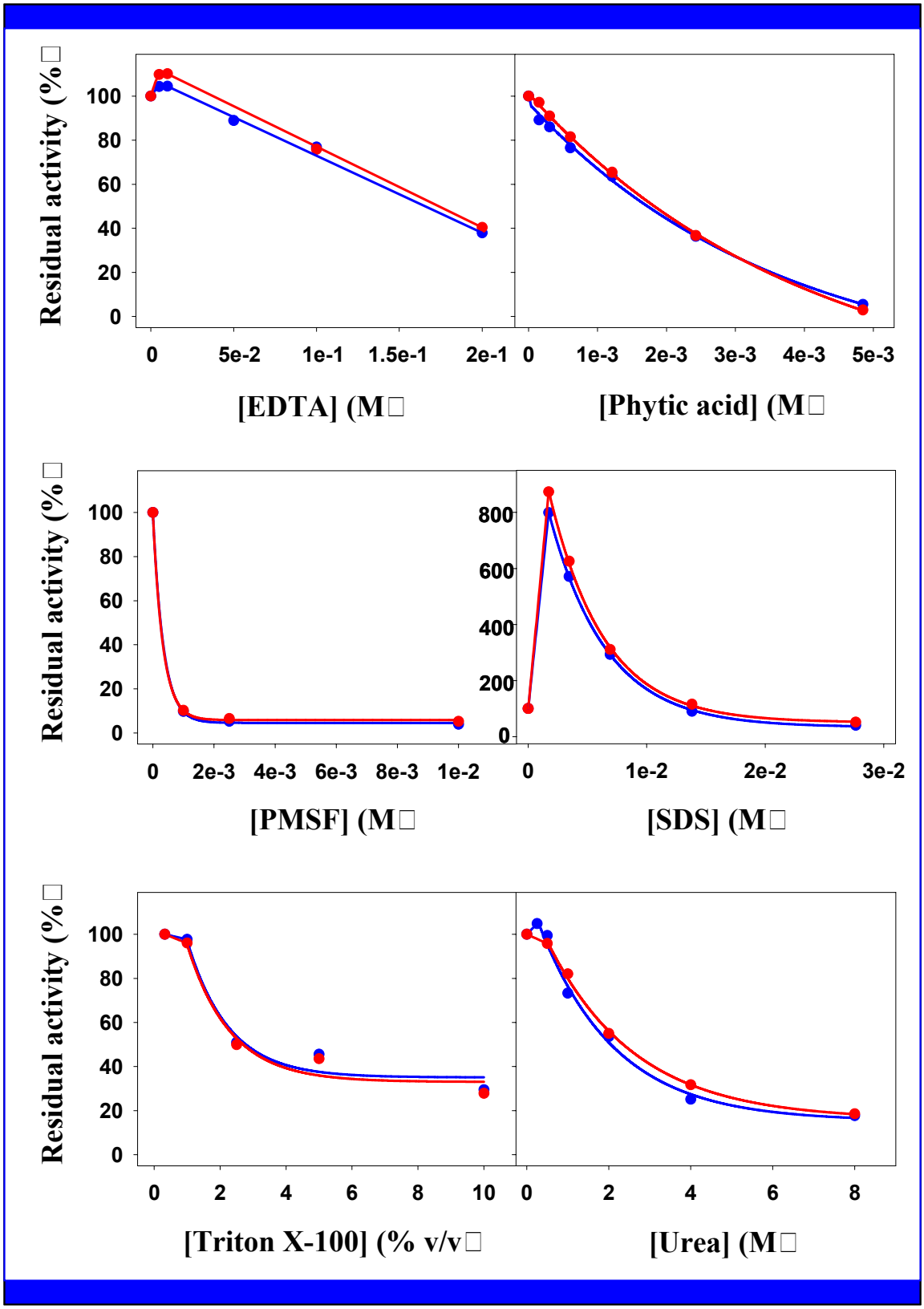


Figure C1.19 Effect of PMSF and other agents on BMLipA (blue□and BP6LipA (red□

The standard deviations ranged from 1 to 10% of the given values, and are not shown for clarity reasons.

Table C1.5 Effect of several agents on BMLipA and BP6LipA activity.

Agent	Residual activity (%□			
	BMLipA		BP6LipA	
	1 mM	10 mM	1 mM	10 mM
H_2O	100.0	100.0	100.0	100.0
$AgNO_3$	51.8	32.7	48.9	35.0
$BaCl_2$	108.1	106.8	104.6	108.3
$CaCl_2$	101.1	88.1	96.4	88.1
$CoCl_2$	104.1	93.8	96.1	98.8
$CuSO_4$	95.3	69.9	91.2	71.9
$FeCl_2$	120.8	46.7	101.1	46.9
$HgCl_2$	29.5	11.8	26.3	18.2
$MgCl_2$	113.9	106.6	102.0	103.6
$MnCl_2$	111.4	85.4	102.3	85.6
NH_4Cl	117.8	119.8	103.2	119.5
$NiCl_2$	101.6	95.9	92.0	99.5
$Pb(CH_3COO)_2$	107.5	79.0	102.2	83.2
$ZnCl_2$	109.5	79.7	100.4	82.1
NAI	108.1	81.4	105.7	84.1
NBS	84.4	31.6	63.7	29.9
PHMB	114.8	124.5	108.9	120.6
PMSF	9.8	4.0	10.2	5.2
	IC_{16} (M□	IC_{50} (M□	IC_{16} (M□	IC_{50} (M□
EDTA	$6.5 \cdot 10^{-2}$	$1.6 \cdot 10^{-1}$	$7.4 \cdot 10^{-2}$	$1.7 \cdot 10^{-1}$
Phytic acid	$3.9 \cdot 10^{-4}$	$1.7 \cdot 10^{-3}$	$5.1 \cdot 10^{-4}$	$1.8 \cdot 10^{-3}$
PMSF	$6.0 \cdot 10^{-5}$	$2.5 \cdot 10^{-4}$	$6.0 \cdot 10^{-5}$	$2.5 \cdot 10^{-4}$
SDS	$1.5 \cdot 10^{-2}$	$2.0 \cdot 10^{-2}$	$1.7 \cdot 10^{-2}$	$2.8 \cdot 10^{-2}$
Triton X-100	1.3 (v/v)	2.8 (v/v)	1.3 (v/v)	2.7 (v/v)
Urea	$7.8 \cdot 10^{-1}$	2.0	$8.7 \cdot 10^{-1}$	2.3

The standard deviations obtained ranged from 2% to 10% of the corresponding mean values.

3.3.2 Molecular characterization

SDS-PAGE and IEF analysis of crude cell extracts from the recombinant clones bearing *BMlipA* and *BP6lipA* did not show the presence of any additional protein band to those detected in control extracts from *E. coli* 5K-pBR322. Nevertheless, when analyzed as zymograms, cell extracts of the two clones showed the presence of a prominent band of ca. 22 kDa and pI 9.5 that hydrolyzed MUF-butyrate and MUF-oleate, and not present in control strain *E. coli* 5K-pBR322 cell extracts (Figure C1.20). This activity bands probably correspond to the unprocessed form of the cloned enzymes BMLipA and BP6LipA.

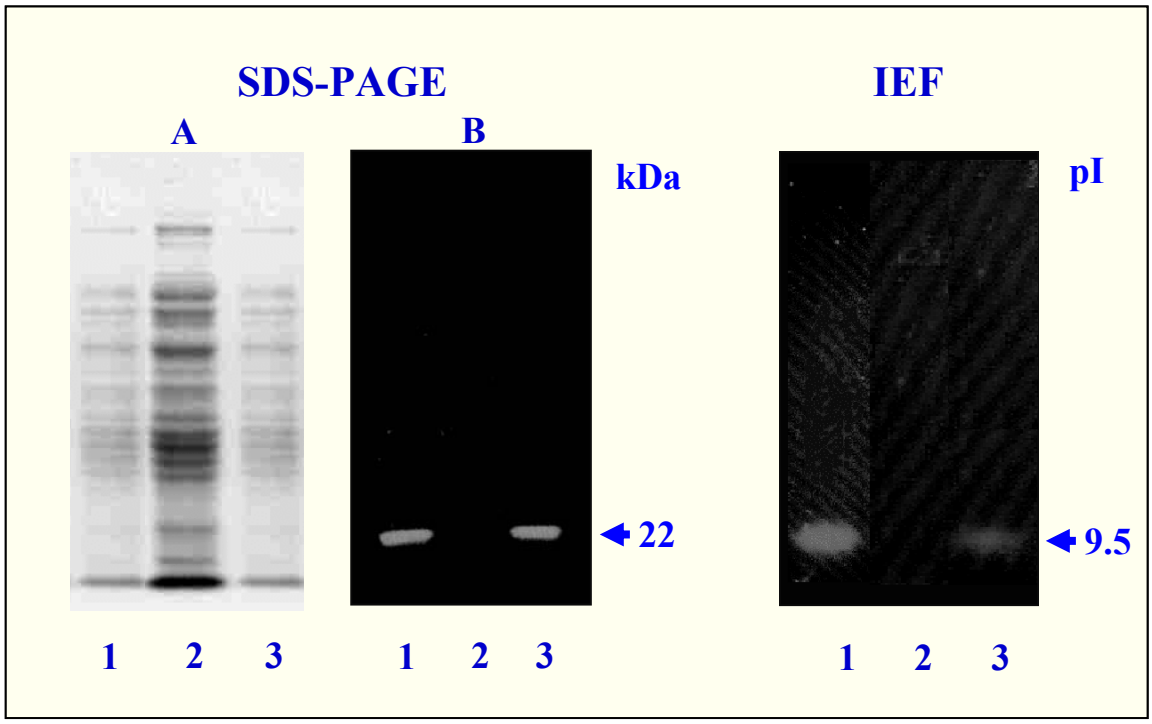


Figure C1.20 Zymogram analysis on MUF-butyrate of BMLipA and BP6LipA.

E. coli 5K−pBR-BMLipA (1), *E. coli* 5K−pBR322 (2; negative control) and *E. coli* 5K−pBR-BP6LipA cell extracts were analyzed by SDS-PAGE (A: Coomassie staining; B: zymogram) and IEF (zymogram). The MW and pI of the activity bands obtained on MUF-butyrate are indicated. Similar bands (but fainter) were obtained on MUF-oleate (not shown).

4 DISCUSSION

4.1 SELECTION AND ANALYSIS OF LIPOLYTIC STRAINS

Strains *B. megaterium* CECT 370 and *Bacillus* sp. BP-6 were chosen from a collection of 26 Gram-positive isolates and *Bacillus* type culture strains due to showing high lipolytic activity on tributyrin- and olive oil-supplemented culture media, and because of having a completely unknown lipolytic system.

The high lipolytic activity of these strains was confirmed when the activity of their cell extracts and supernatants was determined in MUF-butyrate and MUF-oleate. *B. megaterium* CECT 370 cell extracts (on both substrates) and strain BP-6 supernatants (on MUF-butyrate) showed higher activity than the lipolytic positive control strain *B. subtilis* MB216 (Table C1.1).

Thus, the lipolytic system of these strains was analyzed in more detail by SDS-PAGE and IEF gels followed by zymogram. *B. megaterium* CECT 370 supernatants showed to posses at least two secreted enzymes active on MUF-butyrate, one with a MW of 57 kDa and a pI of 5.7, and another with a MW of 19 kDa and a pI of 9.2, whereas cell extract analysis revealed the presence of two additional bands active on MUF-butyrate and showing a MW of 43 and 37 kDa, although only a single band with a pI of 5.2 was found at the IEF analysis (Figure C1.3). The supernatant band of 57 kDa probably corresponds to an unknown secreted lipolytic enzyme whose isolation would be of high interest since a secreted lipase with such a MW and pI has not been found before in a strain from the genus *Bacillus* or from a related genus. In fact, these molecular properties are typical of cell-bound carboxylesterases (CEs) from family VII of bacterial lipases (Arpigny & Jaeger, 1999), which show a MW of 50−65 kDa and a pI of 4.5−6). Among secreted lipases, only those from *Geobacillus* strains (MW = 46.2 kDa and pI = 6.3−6.8) show some similarity to this lipase. The band of 19 kDa and pI 9.2 probably corresponds to the mature form of the cloned lipase BMLipA since the molecular properties shown are highly similar to those deduced for the mature form of

this enzyme. However, this band could also correspond to a secreted lipase similar to *B. subtilis* LipA (Dartois *et al*., 1992), whose MW and pI is very similar to those of *B. subtilis* LipB, and therefore to BMLipA, although PCR amplification using *B. subtilis lipA* primers did not reveal the existence of such a lipase.

With respect to cell extracts, three possibilities exist two explain the results obtained: (1) the pI of the bands of 43 and 37 kDa is the same (5.5), (2) these two bands correspond to different forms of the same protein that cannot be differentiated by their pI, or (3) one of the lipolytic enzymes results inactivated under the IEF conditions. Anyhow, production of one or two non-secreted lipases by *B. megaterium* CECT 370 would be in agreement with the presence of cell-bound CEs in most *Bacillus*-related species, including PnbA from *B. subtilis* (Zock *et al*., 1994; Kunst *et al*., 1997), EstA from *Paenibacillus* sp. BP-23 (Prim *et al*., 2000) and EstA1 from *Bacillus* sp. BP-7 (Prim *et al*., 2001). The bacterial CEs mentioned, grouped into family VII of bacterial lipases (Arpigny & Jaeger, 1999), show a similar pI but a higher MW to those of the activity bands found at the cell extracts of *B. megaterium* CECT 370 strain.

From the results obtained, we suggest that the lipolytic system of *B. megaterium* CECT 370 involves at least two secreted lipases, one of them being BMLipA cloned in this work, and probably two non-secreted lipases with a pI similar to those of family VII cell-bound CEs. However, additional lipolytic enzymes could exist in this strain in view of its high lipolytic activity. These putative enzymes probably require different assay conditions for their detection by zymogram analysis, such as more concentrated cell extracts or supernatants, presence of inducing lipids in the culture medium, other temperature or pH conditions in the cultures used or during the assay, native conditions to avoid lipase inactivation by SDS or by some chemical agents present in the SDS-PAGE or IEF gels, etc.

The analysis of *Bacillus* sp. BP-6 supernatants revealed the presence of two bands with activity on MUF-butyrate in the SDS-PAGE gels (56 and 19 kDa), although only a band with a pI of 9.2 was found at the IEF gels (Figure C1.4). As for *B. megaterium*, the band of 19 kDa probably corresponds to the band of pI 9.2 at the IEF gels, and could correspond to the mature form of the cloned lipase BP6LipA. However, this band could also correspond to an additional lipase similar to *B. subtilis* LipA,

although it was not found by amplification using *B. subtilis* lipA primers. On the contrary, the band of 56 kDa could be an additional lipolytic enzyme having the same pI than the band of 19 kDa or not showing activity on IEF gels, or could be a trimmer of the 19 kDa band, since aggregation is a phenomenon frequently described for lipolytic enzymes (Khalameyzer *et al*., 1999). Anyhow, further experiments are necessary to find out if this band corresponds to an additional secreted enzyme of this strain or not.

Analysis of *Bacillus* sp. BP-6 cell extracts showed the presence of several activity bands with sizes ranging from 110 to 27 kDa, although a single activity band with a pI of 5.2 was observed at the IEF gels. In this case, these bands could be also the result of a variable aggregation of a single enzyme, or could correspond to different lipases with the same pI, or not showing activity on IEF gels. Anyhow, this strain possesses at least one non-secreted enzyme active on MUF-butyrate and showing the pI (and MW in the case of the band of 52 kDa) of cell-bound CEs from family VII of bacterial lipases (Arpigny & Jaeger, 1999).

From the results obtained, we suggest that the lipolytic system of *Bacillus* sp. BP-6 involves at least one or two secreted lipases, one of them being BP6LipA cloned in this work, and at least one non-secreted lipase with a pI similar to those of family VII cell-bound CEs. However, additional lipolytic enzymes not detected under our assay conditions could also exist in this strain, as explained before for *B. megaterium* CECT 370.

4.2 ISOLATION AND CLONING OF LIPASE-CODING GENES

In view of the lipolytic enzymes detected in the analysis of the lipolytic systems of the strains *B. megaterium* CECT 370 and *Bacillus* sp. BP-6, we attempted the isolation and cloning of their corresponding lipase-coding genes. For this purpose, previously designed consensus primers for the central region of *Bacillus* and *Geobacillus* lipases were used to perform the amplification under low astringency conditions of putative similar genes in the selected strains. Amplification using primers

Gb. thermocatenulatus family primers produced no PCR bands, whereas a prominent band of 320 bp (Figure C1.6A) appeared for both strains using *B. subtilis lipA* family primers, indicating that both strains possessed at least one lipase-coding gene belonging to subfamily I.4 of bacterial lipases (Arpigny & Jaeger, 1999). Subsequent IPCR amplifications revealed that the lipase-coding genes detected were highly similar to *B. subtilis lipB* (*yfiP*), thus specific primers for the amplification of *lipB* were designed and used to perform the complete amplification of the lipase-coding genes *lipA* from *B. megaterium* CECT 370 (*BMlipA*) and *lipA* from *Bacillus* sp. BP-6 (*BP6lipA*). Success with these primers led us to perform also the direct amplification of the gene *lipA* from *Bacillus* sp. BP-7 (*BP7lipA*), a lipolytic strain previously analyzed in our group. A gene library of the genomic DNA of this strain had previously allowed the isolation of the gene *estA1*, coding for the cell-bound CE EstA1, but not the isolation of any other putative lipase-coding gene of this strain (Prim *et al*., 2001). The genes obtained were ligated into pBR322 plasmid and transformed into *E. coli* 5K. The recombinant clones obtained were used for the molecular and biochemical characterization of the enzymes encoded by these genes, and for confirming the existence and copy number of these genes in the parental strains.

On the contrary, the search for additional lipase-coding genes from *B. megaterium* CECT 370, *Bacillus* sp. BP-6 and *Bacillus* sp. BP-7 using specific primers for the amplification of *B. subtilis lipA* produced no results, suggesting that these strains do not posses a lipase equivalent to *B subtilis* LipA, or that if they possess it, its similarity to *B. subtilis* LipA is low.

4.3 ANALYSIS OF THE LIPASE-CODING GENES

The analysis of the cloned genes *BMlipA*, *BP6lipA* and *BP7lipA* revealed that *BMlipA* was 99% identical to both *BP6lipA* and *BP7lipA* (100% identical among them). All three genes contained an ORF of 633 nucleotides encoding for predicted proteins of 210 amino acids, 99–100% identical among them, and showing a small deduced

molecular weight (22.3−22.4 kDa) and a very basic deduced pI (9.43−9.56). As expected, the deduced amino acid sequence of the enzymes showed the highest identity (99%, 100% and 100%, respectively) to *B. subtilis* LipB (YfiP) (Yamamoto *et al*., 1996; Kuntz *et al*., 1997; Eggert *et al*., 2000), whereas lower identity (70−75%) was found to *B. subtilis* LipA (Dartois *et al*., 1994) and other secreted enzymes belonging to subfamily I.4 of bacterial lipases (Arpigny & Jaeger, 1999). Finding such a high degree of identity to LipB in all these strains was surprising, even more considering that a lipase similar to *B. subtilis* LipA was not found in any of them. This fact seems to indicate that the cloned enzymes are members of a reduced cluster of lipases grouped into the subfamily I.4 of bacterial lipases, exclusive from the mesophilic or moderately thermophilic members of the genus *Bacillus*, and being responsible for an essential function that requires their high conservation. Further experiments performed in other chapters seem to reinforce this hypothesis, as is explained in the General Discussion.

In view of these results, only *BMlipA* and *BP6lipA* (identical to *BP7lipA*) were selected for further studies. The biochemical and molecular characterization of their corresponding enzymes resulted of high interest since knowledge about *B. subtilis* LipB or similar enzymes was very low at that moment. In fact, LipB was assigned as a lipase by similarity alignments (Yamamoto *et al*., 1996; Kuntz *et al*., 1997), and the only activity experiments performed using this enzyme corresponded to detection of its activity on 1-naphthyl acetate and dimercaptobutyrate after its expression as a fusion protein attached to the cell wall of *B. subtilis* (Kobayashi *et al*., 2000). Nevertheless, when the characterization of the cloned enzymes was almost completed, the cloning and partial characterization of *B. subtilis* LipB was reported (Eggert *et al*., 2000), which allowed us to compare and confirm our results.

In general, the results of protein analysis and biochemical characterization we obtained for BMLipA and BP6LipA were very similar to those obtained by Eggert *et al*. (2000). Analysis of the amino acid sequence of the deduced proteins BMLipA and BP6LipA revealed the existence of an identical 28 amino acid signal peptide at the N-terminal region of both proteins responsible for their secretion through the Sec–Xcp system (Tjalsma *et al*., 2000). These results are in agreement with the results obtained by Eggert *et al*. (2000), which confirmed that LipB possesses a signal peptide of 28 amino acids responsible for the secretion of the mature lipase to the culture medium.

Moreover, the resulting BMLipA and BP6LipA deduced mature lipases (19.4−19.5 kDa and pI of 9.2−9.4) showed a MW very similar to that of the mature LipB recovered by Eggert *et al*. (2000). In addition, the mature lipases BMLipA and BP6LipA probably correspond to the secreted lipases of similar MW and pI detected in the concentrated supernatants of the parental strains *B. megaterium* CECT 370 and *Bacillus* sp. BP-6, as mentioned before.

From the amino acid sequence of the cloned enzymes, the catalytic apparatus of lipases was placed in the mature proteins at positions 78 (Ser), 134 (Asp), and 157 (His), with the catalytic serine located at the motif AH**S**^{78}MG, corresponding to the consensus pentapeptide Ala-Xaa-**Ser**-Xaa-Gly common in all known bacterial lipases from subfamilies I.4 and I.5 (Fojan *et al*., 2000). These results are in agreement with the results obtained by Eggert *et al*. (2000) using sequence alignments and site-directed mutagenesis. Interestingly, these authors also describe the effect of replacing the first alanine of the mentioned pentapeptide by the typical glycine found in lipases belonging to other families. Such a substitution produced a reduction in the stability of LipB at 45 ºC and at pH 11−12, but an increase of its stability at pH 5−7. Moreover, this substitution resulted in a shift in the substrate specificity of the variant enzyme from a CE-type to a monoacylglycerol hydrolase-type.

The high identity of BMLipA and BP6LipA to *B. subtilis* LipA allowed us to construct an automated comparative protein model for the mature form of the cloned lipases (Figures C1.11, and C1.12B) once the crystal structure of *B. subtilis* LipA was determined (van Pouderoyen *et al*., 2001; Figure C1.12A). These proteins model showed a globular structure very similar to that of *B. subtilis* LipA, constituted by a compact globular domain with the α/β-hydrolase fold characteristic of lipases (Jaeger *et al*., 1999). This domain consisted of 6 parallel β strands surrounded by 5 α helices, three on one side of the β sheet and two on the other side, and with the active site freely accessible from the solvent, since no "lid" for interfacial activation exists. This fact would explain the lack of interfacial activation found for the cloned enzymes, that showed a kinetic behaviour typical of carboxylesterases despite of having an amino acid composition more characteristic of "true" lipases (TLs), very rich (47% of the total) in short non-polar residues, to facilitate their activity in aggregated hydrophobic substrates

(Fojan *et al*., 2000). The models obtained were in agreement with that obtained by Eggert *et al*. (2001) using a different software. These authors found that *B. subtilis* LipA and LipB shared a conserved core, having 43 out of the 45 different residues in the protein-surface. The substitutions found in the core only slightly affected the volume of the side chain, and probably have a low effect on the 3D structure of both enzymes, whereas the substitutions at the surface of the proteins were located far from the active site, since the nearest amino acid substitutions occurred at the rim of the putative substrate-binding surface, at a distance of about 9 Å. Therefore, these changes seemed to not affect the active site, which would be located at the bottom of a small cleft between two loops, and freely accessible from the solvent.

Interestingly, the two amino acid substitutions that differentiate the cloned proteins (position 169 was tyrosine in BMLipA and asparagine in BP6LipA, and position 172 was lysine in BMLipA and glutamate in BP6LipA) were also found in the surface of the proteins, at the lower part of the α helix preceding the C-terminal end of the molecules (helix 5, see Figure C1.11), confirming that the surface structure of all these proteins allows a certain degree of variation, probably responsible for the different adaptation of these enzymes, whereas the structure of the core is essential for their activity and stability.

4.4 CHARACTERIZATION OF THE CLONED ENZYMES

The cloned enzymes displayed the typical behaviour of CEs, showing maximum activity on short chain substrates, and displaying a Michaelis-Menten kinetic behaviour. The highest activity was found on tributyrin, *p*-NP butyrate and MUF-butyrate, respectively, although both enzymes showed also high activity on substrates with a chain length C_4−C_8. These results are in part in agreement with those obtained by Eggert *et al*. (2000), who found the maximum activity of *B. subtilis* LipA and LipB on triacylglycerols (TAGs) of C_4−C_8-chain length, and on *p*-NP-derivatives with a chain length from 6 to 14 (*p*-NP-derivatives with a chain length lower than 6 were not assayed

by these authors). The main difference between our results and those of Eggert *et al*. (2000) corresponds to the highest activity they found on intermediate *p*-NP-derivatives (C_8−C_{14}), which could be explained by the different assay conditions used by these authors (they performed the assays using a different buffer and detergent, and incubating at 37 ºC and pH 8), or by the fact that they worked with a purified and mature protein, whereas our assays were performed with the non-purified dilutions of the whole cell-extract proteins.

The kinetic behaviour of the cloned enzymes on MUF-butyrate and MUF-oleate confirmed the assignation of the cloned enzymes to the group of CEs. Both enzymes showed a higher $V_{\max}^{app}$ (almost 10-fold more active) and a lower K_M^{app} (substrate affinity more than 2-fold higher) on MUF-butyrate than on MUF-oleate. Moreover, the cloned lipases produced Michaelis-Menten plots when assayed on both substrates instead of the interfacial activation typical of most TLs. These results are in agreement with those obtained by Eggert *et al*. (2000), who also classified LipB as a CE on the basis of the enzyme kinetics they obtained by the monomolecular film technique, and due to the lack of activity of LipB on triolein, the standard substrate for TLs, in pH-stat assays.

With respect to the effect of temperature and pH on BMLipA and BP6LipA, both enzymes showed their maximum activity at 45 ºC and pH 7 a result very similar to the assay conditions used by Eggert *et al*. (2000), who reported 37 ºC as the optimum temperature of LipB, although they did not showed any curve with the temperature points checked. Moreover, these authors did not report an optimum pH for LipB although they performed the assays at pH 8.

Nevertheless, the most interesting results obtained from temperature and pH assays correspond to high stability found for the cloned enzymes in a wide range of temperatures and pH. Both enzymes kept most of their activity up to 45 ºC for 1 h, and up to 30 ºC for more than 40 days, whereas they were stable at pH from 4 to 12 for 1 h (at room temperature). These results are also in agreement with those obtained by Eggert *et al*. (2000), who reported a high stability of LipB for 30 min at temperatures below 45 ºC, and for 48 h at room temperature and pH 5−12. The fast inactivation reported by these authors at 45 ºC as well as the lower pH stability they describe, could be explained by the fact that they worked with a purified and mature protein. In this

sense, keeping the signal peptide could increase the stability of BMLipA and BP6LipA with respect to the mature LipB, since it has been described that the N-terminal pro-region of lipases produced by the genus *Staphylococcus* acts as a folding catalyst, facilitates the translocation of these lipases through the cytoplasmic membrane, and also protects them from proteolytic attacks (Götz *et al*., 1998). Furthermore, purification of a protein can produce also a reduction in the stability of some enzymes, either due to the process itself, or due to the removal of other proteins that help in the stabilization, solubilization or enzymatic activity of the purified enzymes (Scopes, 1994). Anyhow, the high activity and stability observed for BMLipA and BP6LipA would be in part expected for secreted enzymes belonging to mesophilic microorganisms adapted to rich environments with high competence.

BMLipA and BP6LipA showed also a high stability against a wide variety of cations/inorganic salts and other agents. In fact, among the cations analyzed only Ag^{+}, Hg^{2+}, and high concentrations of Fe^{2+} caused high inhibition of the cloned enzymes, whereas partial inhibition was caused by 10 mM concentrations of Cu^{2+}, Mn^{2+}, Pb^{2+}, and Zn^{2+}. Moreover, BMLipA and BP6LipA did not require cofactors for being active, although Fe^{2+} (at 1 mM) and NH^{4+} slightly activated their enzymatic activity. In general, these results are in agreement with the typical traits of many bacterial lipases, although the high inhibition caused by Ag^{+}, as well as the lack of effect produced by Ca^{2+}, Co^{2+}, Mg^{2+} and Ni^{2+} is less usual among bacterial lipases (Patkar & Björkling, 1994; Gupta *et al*., 2004).

On the other hand, assays with amino acid-modifying agents allowed us to confirm the importance of the amino acid serine in the catalytic activity of both enzymes, in agreement with the fact that the Ser^{78} was assigned (comparative alignments) as a member of the catalytic triad of the cloned enzymes, and with the fact that three-dimensional protein modelling showed that the active site of BMLipA and BP6LipA was exposed to the solvent independently of the presence of a substrate. In fact, the serine inhibitor PMSF, a potent inhibitor of most lipases (Gupta *et al*., 2000), was the strongest inhibitor among all the substances analyzed. Moreover, these assays revealed the importance of tryptophan, but not cysteine nor tyrosine, in the functional or structural domains of the cloned enzymes.

EDTA, phytic acid, SDS, Triton X-100 and urea also inhibited both enzymes, in agreement with previous data about inhibition of lipolytic enzymes by these substances (Prim *et al*., 2000; Prim *et al*., 2001). Nevertheless, SDS caused an extraordinary activation (residual activity higher than 800%) of both enzymes at low concentrations, suggesting that a good solubilization of the protein aggregates formed in the concentrated cell extracts is important to achieve the maximum activity of the cloned enzymes (Khalameyzer *et al*., 1999; Prim *et al*., 2000).

The molecular analysis of the cloned enzymes showed that both enzymes were small lipases of ca. 22 kDa and pI 9.5, confirming the MW and pI previously deduced for these lipases from their amino acid sequence, and reinforcing the hypothesis that the cloned lipases BMLipA and BP6LipA correspond to the unprocessed form of the secreted lipases of 19 kDa and pI 9.2 found in *B. megaterium* CECT 370 and *Bacillus* sp. BP6 supernatants.

4.5 FUTURE PERSPECTIVES

The small size and high stability of the cloned enzymes in a wide range of low-medium temperatures (they can be stored at room temperature for long-time periods, and show high activity up to 45 ºC), as well as their stability at acidic–basic pH, and the low inhibition caused by most of the cations (including calcium), detergents and other agents analyzed, make them of high interest in biotechnology. Moreover, BMLipA and BP6LipA do not require to be purified, and show a considerable activity in substrates of short-medium chain and in some long-chain substrates (MUF-oleate), which would favour even more their utilization in a wide range of biotechnological transformations. In fact, other lipases similar to the cloned enzymes have shown to be well suited for biotechnological applications: additives in the formulation of detergents (*B. pumilus* lipase; Jaeger *et al*., 1994), the enantioselective production of β-blocker compounds (*B. subtilis* LipA; Dröge *et al*., 2000), etc. Therefore, the cloned enzymes could be evaluated and used in certain manufacturing processes such as transformation

antibiotics and other drugs (Zock *et al*., 1994; Dröge *et al*., 2000), formulation of detergents for washing at low temperatures (Pandey *et al*., 1999), biodegradation of recalcitrant substances (Pohlenz *et al*., 1992) and conversion of low-cost fats into added value products (Schmidt-Dannert, 1999).

A second area of interest derived from this work is the isolation and characterization of the other lipolytic enzymes detected in the analysis of the strains *B. megaterium* CECT 370 and *Bacillus* sp. BP-6. In view of the high similarity found for BMLipA and BP6lipA to the secreted lipases of other *Bacillus* strains, a suitable strategy for isolating the putative cell-bound CEs detected on both strains and showing molecular properties similar to PnbA from *B. subtilis* (Zock *et al*., 1994), EstA from *Paenibacillus* sp. BP-23 (Prim *et al*., 2000), EstA1 from *Bacillus* sp. BP-7 (Prim *et al*., 2001) and other CEs from family VII of bacterial lipases, could be the design of consensus primers for the isolation of lipases from this family, since the mentioned esterases display a high similarity among them. On the contrary, isolation by amplification using consensus primers of the high-MW secreted lipase detected in *B. megaterium* CECT 370, and probably also in *Bacillus* sp. BP-6, seems to be more difficult task since no lipases showing this properties have been found for the moment in the genus *Bacillus*. Thus, other strategies such as the elaboration of a gene library, or N-terminal sequencing of the purified lipases would be required.

5 CONCLUSIONS

I. Strains *B. megaterium* CECT 370 and *Bacillus* sp. BP-6 were chosen from a collection of 26 Gram-positive isolates and *Bacillus* type culture strains due to showing high lipolytic activity and because of having a completely unknown lipolytic system.

II. The lipolytic system of *B. megaterium* involves at least two secreted lipases and probably two cell-bound lipases. One of the secreted lipases shows a MW of 57 kDa and pI of 5.7, whereas the second shows a MW of 19 kDa and a pI of 9.2, and probably corresponds to BMLipA cloned in this work. The non-secreted lipases show a MW of 43 and 37 kDa, respectively, and a pI of 5.5.

III. The lipolytic system of *Bacillus* sp. BP-6 involves one or two secreted lipases and from one to four cell-bound lipases. One of the secreted lipases shows a MW of 19 kDa and a pI of 9.2 and probably corresponds to BP6LipA cloned in this work, whereas the second shows a MW of 56 kDa and a pI of 9.2, although it could also correspond to a trimmer of the 19 kDa lipase. The non-secreted lipases show a MW range from 110 to 27 kDa and a pI of 5.2, showing one of them (52 kDa) the typical features of carboxylesterases from family VII of bacterial lipases.

IV. We have isolated the lipase-coding genes *B. megaterium* CECT 370 *lipA* (*BMlipA*) and *Bacillus* sp. BP-6 lipA (*BP6lipA*) by amplification using consensus primers for *B. subtilis* lipA-related lipases, followed by inverse PCR and subsequent amplification using specific primers for *B. subtilis lipB*.

V. The isolated lipases, showing respectively 99 and 100% identity to *B. subtilis* LipB, have been classified as members of a reduced cluster of secreted lipases belonging to subfamily I.4 of bacterial lipases.

VI. *BMlipA* and *BP6lipA* have been cloned in *E. coli* and the recombinant enzymes obtained (BMLipA and BP6LipA) have been characterized. Both enzymes

displayed the typical behaviour of carboxylesterases, showing maximum activity on short chain substrates, and displaying the typical kinetics behaviour of carboxylesterases, including absence of interfacial activation.

VII. The cloned enzymes displayed their maximum activity at 45 ºC and pH 7, although high activity was also found in a wide range of temperatures (from 20 to 50 ºC) and pH (from 5.5 to pH 8.5).

VIII. The cloned enzymes showed high stability in a wide range of temperatures (up to 45 ºC) and pH (from 4 to 12). Moreover, most of the cations, detergents and other agents analyzed caused low inhibition on these enzymes. Therefore, BMLipA and BP6LipA have a high biotechnological potential.

6 PUBLICATIONS

The work corresponding to this chapter has been published in the following scientific articles (see Annex I for the full text):

- **Ruiz, C., Blanco, A., Pastor, F.I.J. and Díaz, P.** (2002) Analysis of *Bacillus megaterium* lipolytic system and cloning of LipA, a novel subfamily I.4 bacterial lipase. *FEMS Microbiol. Lett.* 217: 263–267.

- **Prim, N., Sánchez M., Ruiz, C., Pastor, F.I.J. and Díaz, P.** (2003) Use of methylumbeliferyl-derivative substrates for lipase activity characterization. *J. Mol. Catal. B Enzym.* 22: 339–346.

- **Ruiz, C., Pastor, F.I.J. and Díaz, P.** (2003) Isolation and characterization of *Bacillus* sp. BP-6 LipA, a ubiquitous lipase among mesophilic *Bacillus* species. *Lett. Appl. Microbiol.* 37: 354–359.