

Engineering of Baker's Yeasts, *E. coli* and *Bacillus* Hosts for the Production of *Bacillus subtilis* Lipase A

Marta Sánchez,¹ Núria Prim,¹ Francisca Rández-Gil,² F. I. Javier Pastor,¹ Pilar Díaz¹

¹Department of Microbiology, Faculty of Biology, University of Barcelona Av. Diagonal 645, 08028-Barcelona, Spain; telephone: +3493 4034627. fax: +3493 4034629; e-mail: pdiaz@bio.ub.es

²Departamento de Biotecnología, Instituto de Agroquímica y Tecnología de Alimentos (CSIC), P.O. Box 73, 46100-Burjassot Valencia, Spain

Received 26 July 2001; accepted 13 November 2001

DOI: 10.1002/bit.10201

Abstract: Lipases are versatile biocatalysts showing multiple applications in a wide range of biotechnological processes. The gene *lipA* coding for Lipase A from *Bacillus subtilis* was isolated by PCR amplification, cloned and expressed in *Escherichia coli*, *Saccharomyces cerevisiae* and *Bacillus subtilis* strains, using pBR322, YEplac112 and pUB110-derived vectors, respectively. Lipase activity analysis of the recombinant strains showed that the gene can be properly expressed in all hosts assayed, this being the first time a lipase from bacterial origin can be expressed in baker's *S. cerevisiae* strains. An important increase of lipase production was obtained in heterologous hosts with respect to that of parental strains, indicating that the described systems can represent a useful tool to enhance productivity of the enzyme for biotechnological applications, including the use of the lipase in bread making, or as a technological additive. © 2002 Wiley Periodicals, Inc. *Biotechnol Bioeng* 78: 339–345, 2002.

Keywords: lipase; triacylglycerol hydrolase; heterologous expression; baker's yeast; *Bacillus*

INTRODUCTION

Lipases (E.C. 3.1.1.3) are hydrolases acting on the carboxyl ester bonds of acylglycerols to liberate organic acids and glycerol. The catalytic mechanism of most known lipases depends upon the properties of the substrate and the conditions of the medium. Hydrolysis occurs in the presence of an oil-water interface, while at low water concentration, a synthesis reaction can take place (Jaeger et al., 1999). Lipase-catalyzed reactions show high selectivity and occur under mild conditions of pH and temperature, with no requirement for added cofactors. Accordingly, lipase-catalyzed processes usually require less energy and can be conducted in equipment of lower capital cost. All these properties have led to the recognition of lipases as very useful biocatalysts

because of their wide-ranging versatility in biotechnological applications (Jaeger and Reetz, 1998; Gunstone, 1999). Lipases have efficiently been used as additives in detergents and show multiple applications in textile or paper industry (Jaeger and Reetz, 1998). Lipases can also allow kinetic resolution of racemic mixtures and desymmetrization of prochiral substrates, used as pharmaceuticals or in the synthesis of added value organic compounds (Jaeger et al., 1999). In food industry, lipases have been widely used as ingredients or as additives in the transformation of oils and fats as well as in flavor development (Jaeger and Reetz, 1998). Additionally, lipases can effectively be used as additives in bread making, where they can improve both, the physical properties of the dough and the quality of bread by increasing the volume and retarding the process of staling (Si-Qi, 1997; Borch and Jensen, 1997). Nevertheless, the use of lipases as additives in baking industry must be very accurate to avoid the production of undesired fatty acids, occasionally released as side-products (Poutanen, 1997). Alternatively, recombinant baker's strains producing a particular lipase can be used to obtain the desired technological effect and to efficiently leaven the dough, without the presence of side-activities (Rández-Gil et al., 1995; Monfort et al., 1999).

Microorganisms are an important source of lipases for industry. Practical use of microbial lipases has determined a great interest concerning the improvement of both, the producing strains and the biochemical properties of lipolytic enzymes (Jaeger et al., 1999; Gunstone, 1999). A variety of lipases of microbial origin with different properties and specificities have been described and characterized (Arpigny and Jaeger, 1999). Among them, *Bacillus* lipases display properties that make them promising candidates for biotechnological applications (Dartois et al., 1994; Poutanen, 1997; Jaeger et al., 1999). Lipase A from *Bacillus sub-*

Correspondence to: Pilar Díaz

tilis was previously cloned and characterized, showing good hydrolytic activity on middle chain-length substrates (Kennedy and Lennarz, 1979; Dartois et al., 1992; Lesuisse et al., 1993). LipA is one of the smallest lipases known, well suited for biotechnological applications, including bread making or preparation of technological additives (Jaeger et al., 1999). Here we describe the isolation, by PCR techniques, of *lipA* gene from *Bacillus subtilis* and its cloning in *E. coli*, *S. cerevisiae* baker's yeast and *B. subtilis* strains in order to evaluate the performance of the different hosts in Lipase A activity production.

MATERIALS AND METHODS

Strains, Plasmids, and Media

Bacillus subtilis MB216 (Lampen et al., 1986) was used as donor strain for the isolation of *lipA* gene. Strains *Escherichia coli* 5K (Juárez et al., 1984), *Saccharomyces cerevisiae* baker's strains 13bxV4 (CECT10837, [*trpI*]) and CENPK 113-11A (*trpI*-289, *his3*) (Monfort et al., 1996, Monfort et al., 1999), and *Bacillus subtilis* strains MB216 and BCL1050 (Dartois et al., 1994) were used as recipient hosts. Plasmids pBR322 (Sambrook et al., 1989), YEplac112 (Rández-Gil et al., 1995), and the pUB110-derivative plasmid pRB473 (Brückner, 1992; Zyprian and Matzura, 1986) were used as cloning and expression vectors.

E. coli was grown in LB medium at 37°C. *B. subtilis* cells were grown in nutrient broth at 30°C. *S. cerevisiae* strains were routinely grown at 30°C in YPD (Monfort et al., 1999). Occasionally, yeast cells were grown in Difco Minimal Medium (0.67% YNB w/o amino acids) plus glucose (2%) or olive oil (1%), supplemented in some cases with histidine (Sherman et al., 1986). Detection of lipase-producing recombinant hosts was performed either on CeNAN (ADSA-Micro) or YNB (DIFCO) agar plates, supplemented with tributyrin (1%) or olive oil (1%) and Rhodamine B (0.0002%), as previously described (Kouker and Jaeger, 1987; Prim et al., 2000), or by fluorescence emission of cell suspensions (Diaz et al., 1999).

Nucleic Acid Manipulation

Plasmid and genomic DNA were purified essentially as described (Sambrook et al., 1989). Restriction nucleases and DNA-modifying enzymes were obtained from BioLabs and used according to the manufacturer's specifications. Northern hybridization analysis and DNA manipulations were performed as described (Sambrook et al., 1989). Primer oligonucleotides were purchased at Gifco BRL, and *pfu* polymerase was from Stratagene. DNA was sequenced as described (Blanco et al., 1998), homology analyzed through BLAST (Altschul et al.,

1997), and alignments were performed using ClustalW (1.74) Multalign software.

PCR Strategies

Two sets of primers were designed for *lipA* amplification, using the nucleotide sequence of *B. subtilis* 168 as a template (Dartois et al., 1992). *XbaI* (FW) and *SphI* (BK) restriction sites were included in the oligonucleotide sequences of the primers: FWBSLA 5'-TCT **AGA** **GGA GGA TAT TAT GAA ATT TG** (*XbaI* site underlined, Shine-Dalgarno region in bold). BKBSLA 5'-GCA TGC **CAT TAA TTC GTA TTC TGG CC** (*SphI* site underlined, Stop codon in bold). A single colony of *B. subtilis* MB216 suspended in 50 µl H₂O was used as template for *lipA* amplification by-PCR, using *pfu* polymerase and 29 cycling periods of 30" at 94°C, 40" at 53°C, and 4' at 72°C. A single band of 650bp was obtained, sequenced, and aligned to *B. subtilis* 168 *lipA* gene for confirmation.

The expression cassette YE[pACT1-T] for LipA production in baker's yeast strains was kindly provided by Dr. J. A. Prieto (Monfort et al., 1996). Replacement of LipA signal peptide by the *Geotrichum* sp. Lip2 (Monfort et al., 1999) or by the *S. cerevisiae* invertase gene (Sarokin and Carlson, 1985) signal sequence was performed by PCR amplification of *lipA*, using as primers the *in frame* fusion sequences of each signal peptide and the 5' region of *lipA* ORF devoid of the signal sequence. The primers used were: FWssGeo-LipA 5'-TCT **AGA** **ATG GTT TCC AAA ACC TTT TTT TTG GCT GCG GCG CTC AAC GTA GTG GGC ACC TTG GCC GCT GAA CAC AAT CCA GTC GTT ATG** (*XbaI* site underlined; *Geotrichum* Lip2 signal sequence in bold); FwssInv-LipA 5'-TCT **AGA** **ATG CTT TTG CAA GCT TTC CTT TTC CTT TTG GCT GGT TTT GCA GCC AAA ATA TCT GCA TCA ATG ACA AAC GCT GAA CAC AAT CCA GTC GTT ATG** (*XbaI* site underlined; *S. cerevisiae* invertase signal sequence in bold). The isolated fragments were sequenced for confirmation of the *in frame* fusions, and ligated to *EcoRV*-digested pBR322, resulting in plasmids pBRssGeo-LipA or pBRssInv-LipA, respectively. *XbaI-SphI* digestion of both recombinant plasmids rendered the fusion sequences ssGeo-LipA or ssInv-LipA that were then ligated to YE[pACT1-T], producing plasmids YE[pACT1-ssGeo-LipA-T] or YE[pACT1-ssInv-LipA-T], used to transform *E. coli* 5K and baker's yeast strains 13bxV4 and CENPK.

The expression cassette for *Bacillus* strains (Brückner, 1992), was obtained by insertion of SPO2 (Schoner et al., 1983) *Bacillus* promoter in plasmid pRB473. The promoter was isolated by PCR amplification using plasmid pPL608 (Schoner et al., 1983) as template. The following oligonucleotides were used: FWSP0 5'-TCT **AGA** **CAC TGG CCT TGG TTA AGG** (*XbaI* site underlined);

BKSPO 5'-GGA TCC TCT TCT TCA TGA ACT TC (*Bam*HI site underlined). The amplified fragment was sequenced for confirmation and cloned into pBR322-*Eco*RV, resulting in plasmid pBRSP0. This was then treated with *Xba*I and *Bam*HI, and the fragment containing the promoter was inserted into plasmid pRB473, previously digested with the same set of enzymes. As a result, plasmid pRB473[pSPO2-T] was obtained and used for expression of LipA in *Bacillus* hosts.

Cloning Procedures

The amplified *lipA* gene was ligated to *Eco*RV digested pBR322, resulting in plasmid pBRLipA, and cloned in *E. coli* 5K by transformation (Sambrook et al., 1989). Ampicillin-resistant, tetracycline-sensitive transformants were isolated, and those showing activity (Prim et al., 2000, Prim et al., 2001) were selected. Plasmid DNA sequencing showed that the inserted *lipA* gene was downstream the pBR322 tetracycline promoter. *lipA* gene was released from pBRLipA by *Xba*I and *Sph*I digestion, and ligated to YE[pACT1-T], previously digested with the same set of enzymes. As a result, plasmid YE[pACT1-LipA-T] was obtained, containing the *lipA* gene between the *S. cerevisiae* ACT1 promoter and the FBP1 (T) terminator (Monfort et al., 1999). The new recombinant vector was used for transformation of both, *E. coli* 5K and *S. cerevisiae* strains (Ito et al., 1983). Recombinant baker's yeast clones were selected on the basis of complementation of the auxotrophic *trp1* and *his3* mutations, and presence of activity on olive-oil/Rhodamine plates. The amplified *lipA* gene was blunt-end ligated to pRB473[pSPO2-T]-*Sma*I, rendering plasmid pRB473[pSPO2-LipA-T]. The correct orientation of *lipA* was confirmed by DNA sequencing, and the construction used for transformation of *B. subtilis* strains (Contente and Dubnau, 1979). *Bacillus* recombinants were selected on the basis of chloramphenicol (50 $\mu\text{g} \cdot \text{ml}^{-1}$) resistance, restriction analysis, and detection of activity on MUF-butyrate (Diaz et al., 1999).

Activity Assays

Lipase activity was detected on agar plates (Kouker and Jaeger, 1987) or from cell suspensions, as previously described (Diaz et al., 1999). Determination of lipolytic activity was routinely performed from crude cell extracts or concentrated culture media. Assays were carried out by measuring the release of *para*-nitrophenol (*p*NP) or 4-methylumbelliferone (MUF) from *p*NP or MUF-derivative substrates (Sigma), as previously described (Prim et al., 2000). Specific activity was calculated using a calibration curve for each reaction

product (*p*NP or MUF). One unit of activity was defined as the amount of enzyme necessary to release 1 μmol of MUF or *p*NP per minute under the assay conditions described.

RESULTS AND DISCUSSION

Isolation of *lipA* Gene

Genomic DNA from *B. subtilis* MB216 was used for PCR isolation of *lipA* gene. A single band of ca. 650 base pairs could be amplified using the oligonucleotides described in Materials and Methods. The amplified DNA fragment was then purified and its nucleotide sequence determined, showing to be identical to that reported for the ORF of *B. subtilis* 168 *lipA* gene (Dartois et al., 1992).

Expression of LipA in *E. coli*

Plasmid pBRLipA containing the isolated *lipA* gene (see Materials and Methods) was used to transform *E. coli* 5K cells. Several recombinant *E. coli* 5K clones were selected and analyzed. No activity on olive oil/Rhodamine-supplemented agar plates was detected for colonies lacking the *lipA* insert. On the contrary, all recombinant clones produced haloes and fluorescence (Kouker and Jaeger, 1987) when grown on tributyrin or olive oil/Rhodamine plates, showing also activity on MUF-butyrate and MUF-oleate (Fig. 1A) (Diaz et al., 1999). Our results indicate that in contrast to what had

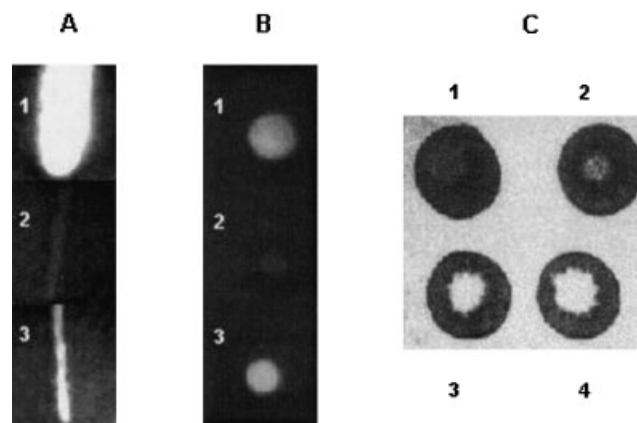


Figure 1. Lipase activity of recombinant *E. coli* 5K (A), *S. cerevisiae* (B), and *B. subtilis* (C) recombinant strains carrying *lipA* gene. (A) Release of fluorescence on olive-oil/Rhodamine-plates produced by recombinant *E. coli* 5K bearing plasmid pBRLipA (1) or YE[pACT1-LipA-T] (3). *E. coli* 5K/pBR322 (2) is shown as negative control. (B) Fluorescence emission on olive-oil/Rhodamine-plates produced by recombinant baker's yeast strains 13bxV4 (1) and CENPK (3) carrying plasmid YE[pACT1-LipA-T]. Strain *S. cerevisiae* 13bxV4/Yeplac112 (2) was used as negative control. (C) Release of fluorescence from MUF-butyrate by cell suspensions of *B. subtilis* strains MB216 (2, 4) and BCL1050 (1, 3) bearing plasmid pRB473[pSPO2-LipA-T] (3, 4). Parental strains are shown as controls (1, 2).

previously been suggested (Dartois et al., 1992), expression of the *B. subtilis* lipase gene caused no growth inhibition of *E. coli* cells, which in turn, could properly express the cloned gene, even in the absence of its own promoter region. Expression of *lipA* in the transformants was directed by the pBR322 tetracycline promoter, responsible for a rate of expression sufficiently low to avoid growth inhibition.

Lipase activity of late exponential-phase cultures of parental *B. subtilis* MB216 and recombinant *E. coli* 5K/pBRLipA was assayed using MUF-butyrate and MUF-oleate as substrates. Crude cell extracts of *E. coli* 5K/pBRLipA displayed 0.13 (± 0.01) and 0.02 (± 0.004) activity units mg prot⁻¹ on MUF-butyrate and MUF-oleate, respectively, while activity at the culture supernatants was negligible. Under the same conditions, *B. subtilis* MB216 showed an overall lipolytic activity (cell extract plus supernatant) on MUF butyrate of 7.1×10^{-4} units mg prot⁻¹. According to the results obtained, lipase activity was 183-fold greater in recombinant *E. coli* 5K/pBRLipA than in the parental strain. The observed increase of lipase activity in recombinant *E. coli* 5K/pBRLipA could be justified by the higher copy number of the gene in the recombinant host. This enhanced production of LipA represents an important source of enzyme for biotechnological applications as those of paper or textile industries, where enzyme purification and GRAS microorganisms are not required (Jaeger and Reetz, 1998).

Lipases exhibit different kinetic behaviors depending on the properties and concentration of the substrate they hydrolyze (Jaeger et al., 1999). An interfacial activation of true lipases occurs at high substrate concentration or when long chain-length substrates are used. On the contrary, esterases and other lipolytic enzymes display a Michaelis-Menten behavior, without interfacial activation (Jaeger et al., 1994). Therefore, we assayed the kinetic parameters of the cloned enzyme using MUF-butyrate and MUF-oleate as different chain-length substrates. Crude cell extracts of *E. coli* 5K/pBRLipA showed a Michaelis-Menten hyperbola on both MUF-butyrate and MUF-oleate substrates (not shown), with a calculated Km of 0.031 mM and 0.044 mM, respectively. This indicates that no interfacial activation is required for LipA activity, a result that agrees with previous reports indicating that although LipA is considered a true lipase, it lacks the structural α -helix fold that acts to prevent true lipases from displaying activity in the absence of an interface (Ransac et al., 1994, Jaeger et al., 1999).

Expression of LipA in Baker's Yeast Strains

In order to get the expression of *B. subtilis* LipA in baker's yeast strains, plasmid YE[pACT1-LipA-T] containing the adequate expression cassette, in which

lipA structural gene was controlled by the *S. cerevisiae* constitutive promoter pACT1, was used to transform *E. coli* 5K, *S. cerevisiae* 13bxV4 and CENPK strains (Monfort et al., 1999). Production of Lipase A from recombinant clones was detected using olive oil/Rhodamine YNB-agar plates (Fig. 1) (Kouker and Jaeger, 1987). As shown in Figure 1B, yeast cells harboring YE[pACT1-LipA-T] were able to produce active lipase, as revealed by the presence of hydrolysis haloes and fluorescence. No interference caused by glycosilation was detected for LipA activity. To our knowledge, this is the first time a bacterial lipase is reported to be produced in baker's yeast strains, a result that contributes to increase the knowledge for expression of bacterial genes in yeasts and that is in agreement with previous reports showing that fungal lipases can be properly produced in baker's *S. cerevisiae* strains (Rández-Gil et al., 1995; Monfort et al., 1996, Monfort et al., 1999).

Lipase activity of overnight cultures of 13bxV4/YE[pACT1-LipA-T] and CENPK/YE[pACT1-LipA-T] were assayed using MUF-butyrate as a substrate. Activity was determined in both, cell extracts and culture supernatants. Unexpectedly, no activity was detected in the culture supernatants, suggesting that Lipase A signal peptide was not being processed by *S. cerevisiae* signal-peptidase. On the contrary, activity at the cell extracts of strains 13bxV4/YE[pACT1-LipA-T] and CENPK/YE[pACT1-LipA-T] was 6.2×10^{-3} and 1.5×10^{-2} units mg prot⁻¹, respectively, while control 13bxV4 and CENPK strains containing the same plasmid but lacking the *lipA* gene showed 2.5×10^{-3} and 2.1×10^{-3} activity units mg prot⁻¹, respectively, indicating that some esterase activity background was present in the recipient strains (Jaeger et al., 1999; Prim et al., 2000).

Transcription of *lipA* gene in recombinant strains 13bxV4/YE[pACT1-LipA-T] and CENPK/YE[pACT1-LipA-T] was analyzed by Northern hybridization using the isolated *lipA* gene as a probe. Yeast strains containing the same plasmid but lacking the *lipA* gene were also assayed as negative controls. Total RNA from cells grown overnight in YNB medium showed a specific hybridization band of the expected size in strains bearing plasmid YE[pACT1-LipA-T], that was absent in samples of control strains (Fig. 2). In agreement with the activity results obtained, expression of *lipA* gene was more efficient in CENPK than in 13bxV4 transformants.

The results obtained indicate that *lipA* gene can properly be expressed in *S. cerevisiae* baker's strains, providing activity to the cell extracts. The recombinant lipase produced by these strains exhibited biochemical properties similar to those of the original enzyme (not shown), suggesting that it could be used in bread making during dough fermentation, to render bread with a higher volume and a more uniform crumb structure (Si-Qi, 1997). Nevertheless, absence of activity in the culture supernatants indicates that the enzyme cannot be secreted to the culture media, where it could be more ef-

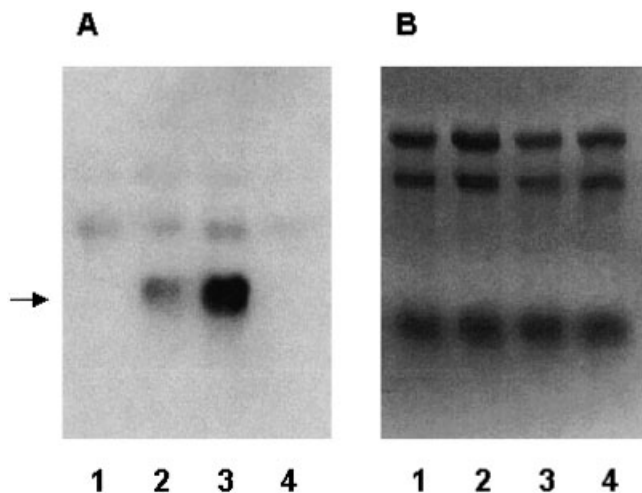


Figure 2. Northern blot analysis of *lipA* gene expression in baker's yeast strains 13bxV4 (1, 2) and CENPK (3, 4). (A) Total RNA was prepared from transformants bearing plasmid YE[pACT1-LipA-T] (2, 3) or YEplac112 (controls, 1, 4). A band of hybridization appeared for recombinant strains carrying the *lipA* gene. (B) RNA ethidium bromide staining of the same samples was performed as a control of loading and transfer.

fectively used for bread making (Si-Qi, 1997; Monfort et al., 1999). In order to get the enzyme secreted into the medium, the signal peptide of LipA was replaced by the signal sequence of *Geotrichum* sp. Lip2 gene (Monfort et al., 1999), by isolation of the *in frame* fusion fragment of both, the signal sequence of *Geotrichum* sp. Lip2 gene and the signal sequence-depleted *Bacillus lipA* gene (see Materials and Methods). Plasmids pBRssGeo-LipA and YE[pACT1-ssGeo-LipA-T] were obtained and used to transform *E. coli* 5K and *S. cerevisiae* strains 13bxV4 and CENPK. Lipase activity of the newly transformed yeast strains containing the *Geotrichum* sp. Lip2 signal peptide was determined using MUF-butyrate as a substrate. As for the previous constructions, no activity was detected in the culture media, suggesting that the *Geotrichum* sp. Lip2 signal peptide was not efficient in secreting the lipase. Surprisingly, activity could neither be found at the cell extract fraction of recombinant baker's yeast strains (5.9×10^{-4} units mg prot⁻¹), indicating that some kind of enzyme inactivation had occurred. A deficiency in the folding of the enzyme was suspected, as we had previously observed that *lipA* gene could properly be transcribed in *S. cerevisiae* (Fig. 2). This hypothesis was confirmed when lipase activity was determined in *E. coli* 5K/pBRssGeo-LipA cell extracts. Again, no significant activity was found in this strain (7.4×10^{-4} units mg prot⁻¹), suggesting that the presence of an exogenous signal peptide was responsible for a wrong folding of the protein, which in turn, resulted inactive.

As the signal peptide used in the previous non-active constructions came from a different fungal species (*Geotrichum* sp.), we tried to get the lipase secreted by

using the signal sequence of *S. cerevisiae* own's invertase gene (Sarokin and Carlson, 1985). For this purpose, the *in frame* fusion fragment of both, the signal sequence of *S. cerevisiae* invertase gene and the signal sequence-depleted *Bacillus lipA* was isolated (see Materials and Methods), and used to obtain plasmids pBRssInv-LipA and YE[pACT1-ssInv-LipA-T]. *E. coli* 5K and *S. cerevisiae* strains 13bxV4 and CENPK were transformed with these plasmids, and their activity on MUF-butyrate and MUF-oleate measured. The overall activity of recombinant *E. coli* 5K transformants rendered values similar to those obtained for *E. coli* 5K/pBRssLipA, indicating that folding of the protein was not being affected by the use of *S. cerevisiae* invertase signal sequence.

Although high levels of activity were detected at the cell extract fractions of recombinant 13bxV4 and CENPK/YE[pACT1-ssInv-LipA-T] strains, neither in this occasion could activity be found at the supernatant fractions, suggesting that the cell envelope of *S. cerevisiae* was acting as a barrier for the secretion of

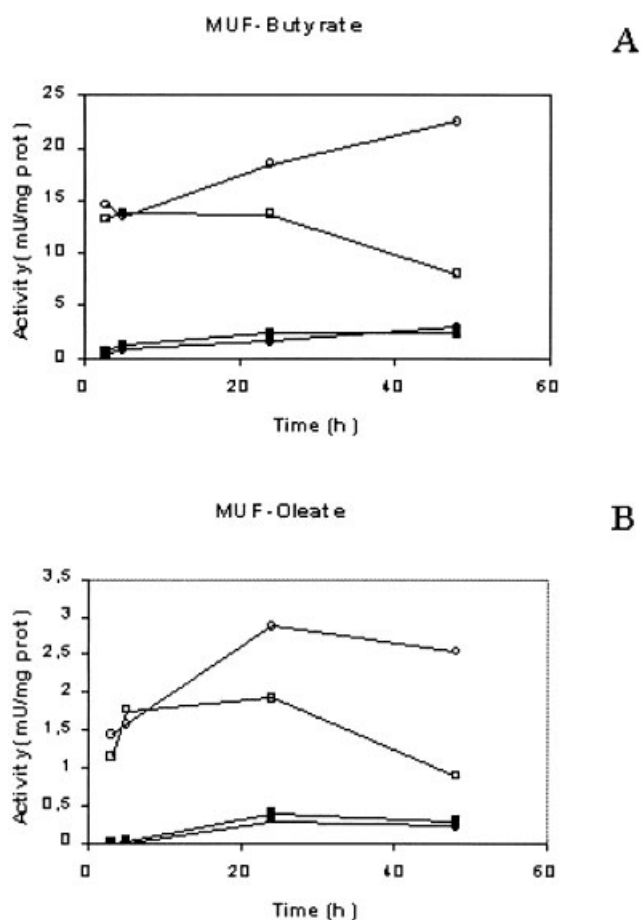


Figure 3. Cell Extract lipase activity found on MUF-Butyrate (A) or MUF-Oleate (B) for strains 13bxV4 (squares) and CENPK (circles) transformed with plasmid YE[pACT1-ssInv-LipA-T] (empty symbols) or YEplac112 (control samples, solid symbols), grown in YPD medium.

Lipase A. Activity at the cell extract fractions of both parental and recombinant strains was determined along growth using MUF-butyrate or MUF-oleate as substrates (Fig. 3). Recombinant 13bxV4 and CENPK/YE[pACT1-ssInv-LipA-T] strains showed a significant increase of lipase activity with respect to the same *S. cerevisiae* host strains. A 7-fold and 9.3-fold increase of activity on MUF-butyrate, respectively, was found for recombinant 13bxV4 and CENPK/YE[pACT1-ssInv-LipA-T] strains grown overnight in YNB medium. The results obtained indicate that the activity produced in the presence of *S. cerevisiae* invertase gene signal sequence was even higher than that found for the recombinant strains bearing Lipase A own's signal peptide. Moreover, activity of cell extract fractions of recombinant 13bxV4 and CENPK/YE[pACT1-ssInv-LipA-T] revealed an overall increase in lipase activity of 24.5-fold and 27-fold, respectively if compared to that of the *B. subtilis* parental strain. Although comparison of lipase production in yeast cell extracts and *Bacillus* is only approximative in terms of protein expression, our results are in agreement with the naturally occurring higher production of enzymes in yeast hosts, being of great utility for large scale production of the enzyme.

Production of LipA in *B. Subtilis* Strains

The difficulty to solve the problem of secreting the protein in baker's yeast strains led us to develop other strategies to secrete LipA in a different host, *Bacillus*, so that it could be used as a technological additive for food industry (Jaeger and Reetz, 1998). *B. subtilis* strains MB216 and BCL1050 were transformed with plasmid pRB473[pSPO2-LipA-T] (see Materials and Methods). Cell suspensions of the recombinant clones containing the structural *lipA* gene downstream the SPO2 *Bacillus* promoter, released fluorescence when analyzed in the presence of MUF-butyrate (Fig. 1C) (Diaz et al., 1999), indicating that the enzyme was being produced. Lipase activity on MUF-butyrate of cell extracts and culture supernatants of recombinant *Bacillus* clones was determined along growth, using the parental strains as control samples. Cell extract fractions showed maximum activity after 24 h growth at 30°C (not shown). Activity at the culture supernatants appeared after 48h growth in all, parental and recombinant strains (Fig. 4). As expected, lipase activity was much lower (10-fold) at the cell extract fractions than that secreted to the culture medium. Both strains, MB216 and BCL1050 carrying plasmid pRB473[pSPO2-LipA-T] showed a significant increase of lipase activity over the lipolytic background of the parental strains. After 48h growth, the lipase-disrupted mutant, protease-deficient strain BCL1050 (Dartois et al., 1994) bearing plasmid pRB473[pSPO2-LipA-T] displayed the best performance in LipA production (Fig. 4): a 9.2-fold increase in production with

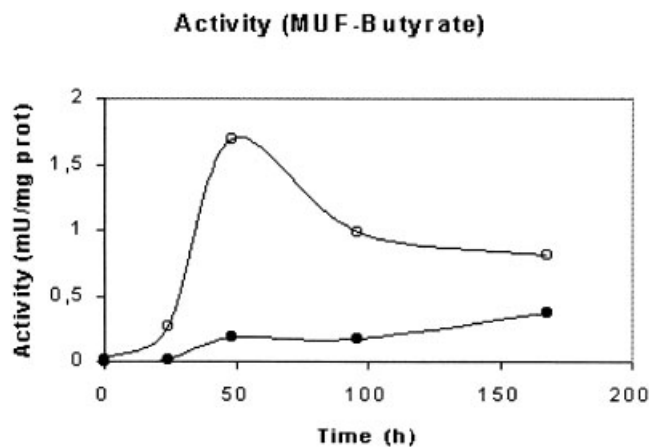


Figure 4. Lipase activity profile on MUF-butyrate of culture supernatants from *B. subtilis* BCL1050 transformed with plasmid pRB347[pSPO2-LipA-T] (open circles). The lipolytic activity of the parental strain is shown in solid circles, and used as a control.

respect to the parental strain. When strain MB216 was used as recipient host, a 2-fold increase of activity production was detected (not shown), due to the lipolytic background of this strain. In general, a remarkable increase of overall enzyme production was obtained from the recombinant strains. In agreement with previously reported data (Dartois et al., 1994), our results indicate that LipA can properly be expressed and secreted when cloned in different *Bacillus* strains, suggesting that the described system may constitute an efficient tool to enhance the productivity of the enzyme. Experiments are now in progress to produce and purify LipA in order to evaluate its performance as a technological additive in food processing and baking industry.

We thank V. Dartois, and G. Pérez-Martínez for generously providing strains and plasmids. Our acknowledgement is also for J. A. Prieto for advisory and technical support. J. L. López is acknowledged for his aid in vector preparation. We thank Serveis Científico-Tècnics of the University of Barcelona for sequencing. This work was partially financed by the Scientific and Technological Research Council (CICYT, Spain), grant ALI97-0356-C02-02, and by the II Pla de Recerca de Catalunya (Generalitat de Catalunya), grant 1999SGR-00024. M. Sánchez is a recipient of a fellowship from the Spanish Ministry of Education and Science.

References

- Altschul S, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ. 1997. Gapped-BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* 25:3389-3402.
- Arpigny JL, Jaeger KE. 1999. Bacterial lipolytic enzymes: classification and properties. *Biochem J* 134:177-183.
- Blanco A, Diaz P, Martínez J, Vidal T, Torres AL, Pastor FIJ. 1998. Cloning of a new endoglucanase from *Bacillus* sp. BP-23 and characterization of the enzyme. Performance in paper manufacture from cereal straw. *Appl Microbiol Biotechnol* 50:48-54.

- Borch J, Jensen B. 1997. Effect and functionality of lipases in dough and bread. DANISCO Ingredients, Denmark. VII Meeting on Industrial Applications of Enzymes, Barcelona, Spain.
- Brückner R. 1992. A series of shuttle vectors for *Bacillus subtilis* and *Escherichia coli*. *Gene* 122:187–192.
- Contente S, Dubnau D. 1979. Characterization of plasmid transformation in *Bacillus subtilis*: kinetic properties and the effect of DNA conformation. *Mol Gen Genet* 167:251–258.
- Dartois V, Baulard A, Schanck K, Colson C. 1992. Cloning, nucleotide sequence and expression in *Escherichia coli* of a lipase gene from *Bacillus subtilis* 168. *Biochim Biophys Acta* 1131:253–260.
- Dartois V, Coppée JY, Colson C, Baulard A. 1994. Genetic analysis and overexpression of lipolytic activity in *Bacillus subtilis*. *Appl Environ Microbiol* 60:1670–1673.
- Diaz P, Prim N, Pastor FIJ. 1999. Direct fluorescence-based lipase activity assay. *BioTechniques* 27:696–700.
- Gunstone FD. 1999. Enzymes as biocatalysts in the modification of natural lipids. *J Sci Food Agric* 79:1535–1549.
- Ito H, Jukuda K, Murata K, Kimura A. 1983. Transformation of intact yeast cells treated with alkali cations. *J Bacteriol* 153:163–168.
- Jaeger KE, Reetz MT. 1998. Microbial lipases form versatile tools for biotechnology. *Trends Biochem Biotech* 16:396–403.
- Jaeger KE, Dijkstra BW, Reetz MT. 1999. Bacterial biocatalysts: molecular biology, three-dimensional structures, and biotechnological applications of lipases. *Annu Rev Microbiol* 53:315–351.
- Jaeger KE, Ransac S, Dijkstra BW, Colson C, Heuvel M, Misset O. 1994. Bacterial lipases. *FEMS Microbiol Rev* 15:29–63.
- Juárez A, Härtlein M, Goebel W. 1984. Study of regulation and transport of hemolysin by using fusion of the β -galactosidase gene (*lacZ*) to hemolysin genes. *J Bacteriol* 160:161–168.
- Kennedy MB, Lennarz WJ. 1979. Characterization of the extracellular lipase from *Bacillus subtilis* and its relationship to a membrane-bound lipase found in a mutant strain. *J Biol Chem* 254:1080–1089.
- Kouker G, Jaeger KE. 1987. Specific and sensitive plate assay for bacterial lipases. *Appl Environ Microbiol* 53:211–213.
- Lampen JO, Pastor FIJ, Hussain M. 1986. Processing of secreted proteins and the signal peptidases of Bacilli. In: Leive L, Bonventre PF, Morello JA, Silver SD, Wu HC, editors. *Microbiology*-1986. Washington: American Society for Microbiology p 279–282.
- Lesuisse E, Schank K, Colson C. 1993. Purification and primary characterization of the extracellular lipase of *Bacillus subtilis* 168, an extreme basic pH tolerant enzyme. *Eur J Biochem* 216:155–160.
- Monfort A, Blasco A, Prieto JA, Sanz P. 1996. Construction of baker's yeast strains that secrete different xylanolytic enzymes and their use in bread making. *Appl Environ Microbiol* 62:3712–3715.
- Monfort A, Blasco A, Sanz P, Prieto JA. 1999. Expression of *LIP1* and *LIP2* genes from *Geotrichum* species in baker's yeast strains and their application to the bread-making. *J Agric Food Chem* 47:803–808.
- Poutanen K. 1997. Enzymes: an important tool in the improvement of the quality of cereal foods. *Trends Food Sci Technol* 8:300–306.
- Prim N, Blanco A, Martínez J, Pastor FIJ, Diaz P. 2000. *estA*, a gene coding for a cell-bound esterase from *Paenibacillus* sp. BP-23, is a new member of the bacterial subclass of type B carboxylesterases. *Res Microbiol* 151:303–312.
- Prim N, Pastor FIJ, Diaz P. 2001. Cloning and characterization of a bacterial cell-bound type B carboxylesterase from *Bacillus* sp. BP-7. *Curr Microbiol* 42:237–240.
- Rández-Gil F, Prieto JA, Murcia A, Sanz P. 1995. Construction of baker's yeast strains that secrete *Aspergillus oryzae* alpha-amylase and their use in bread making. *J Cereal Sci* 21:185–193.
- Ransac S, Blaaup M, Lesuisse E, Schank K, Colson C. 1994. Crystallization and preliminary X-ray analysis of lipase from *Bacillus subtilis*. *J Mol Biol* 238:857–859.
- Sambrook J, Fritsch EF, Maniatis T. 1989. *Molecular cloning: a laboratory manual*. 2nd Ed. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.
- Sarokin L, Carlson M. 1985. Comparison of two yeast invertase genes: conservation of the upstream regulatory region. *Nucleic Acids Res* 13:6089–6103.
- Schoner RG, Williams DM, Lovett PS. 1983. Enhanced expression of mouse dihydrofolate reductase in *Bacillus subtilis*. *Gene* 22:47–57.
- Sherman F, Fink GR, Hicks JB. 1986. *Methods in yeast genetics*. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.
- Si-Qi J. 1997. Synergistic effect of enzymes for bread-baking. *Cereal Foods World* 42:802–807.
- Zyprian E, Matzura H. 1986. Characterization of signals promoting gene expression on the *Staphylococcus aureus* plasmid pUB110 and development of a gram-positive expression vector system. *DNA* 5:219–225.