Expression of *LIP1* and *LIP2* Genes from *Geotrichum* Species in Baker's Yeast Strains and Their Application to the Bread-Making Process

Aurelia Monfort, Amalia Blasco, Pascual Sanz, and Jose A. Prieto*

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

Lipolytic baker's yeast strains able to produce extracellular active lipase have been constructed by transformation with plasmids containing the *LIP1* and *LIP2* genes from *Geotrichum* sp. under the control of the *Saccharomyces cerevisiae* actin promoter (*pACT1*). Lipase productivity differed between both constructs, YEpACT-LIP1-t and YEpACT-LIP2-t, being higher for the strain bearing the *LIP2* gene in all culture media tested. This result appeared not to be the consequence of a defect in the transcription of the *LIP1* gene as revealed by Northern blot analysis. Replacing the signal sequence of *LIP1* by that of *LIP2* in the YEpACT-LIP1-t plasmid enhanced significantly the secretion of lipase 1, but the levels of lipase activity were still lower than those found for the YEpACT-LIP2-t transformant. Recombinant lipase 2 protein produced by baker's yeast exhibited biochemical properties similar to those of the natural enzyme. Fermented dough prepared with YEpACT-LIP2-t-carrying cells rendered a bread with a higher loaf volume and a more uniform crumb structure than that prepared with control yeast. These effects were stronger by the addition in the bread dough formulas of a preferment enriched in recombinant lipase 2.

Keywords: *Lipase; bread; Geotrichum sp.; baking; baker's yeast; gene expression*

INTRODUCTION

The use of enzymes in the baking industry is widely extended. They modify the components of the flour and, as a result, the physical−chemical properties of the dough and/or the bread quality parameters (Poutanen et al., 1997). Regular application of enzymes in the dough system is carried out by exogenous addition of commercial preparations. Alternatively, recombinant baker's yeast strains producing a particular enzyme of interest can be employed to obtain the desired technological effect and to leaven efficiently the bread dough (Randez-Gil et al., 1995; Monfort et al., 1996). This strategy has several advantages with respect to the former because it avoids the presence of side activities and reduces the airborne allergen pollution at the workplace (Sanz and Prieto, 1997). Baker's yeast strains expressing the α-amylase from *Aspergillus oryzae* (Randez-Gil et al., 1995), the *Aspergillus nidulans* endoxylanase X22, X24, and X34, and the *Aspergillus niger* α-L-arabinofuranosidase (Monfort et al., 1997) have been constructed and successfully applied in the bread-making system. Combined secretion of α-amylase/xylanase activities from a baker's yeast has been also carried out (Monfort et al., 1996).

Lipases (glycerol ester hydrolases EC 3.1.1.3) catalyze the hydrolysis of triacylglycerols to render mono- and diacylglycerides and free fatty acids. Although the use of lipase in the bread-making process is quite recent as compared with the use of other enzymes such as protease and α-amylase, its industrial application is becoming important. It has been reported that the addition of lipase to the dough improves the bread volume and the crumb texture and retards the staling of bread (Olesen et al., 1994; Si and Hansen, 1994; Si, 1997). Like other enzymes, the effects of lipase depend on the type of flour and the baking formulation, being significant in dough systems without added shortening (Si, 1997). It is believed that lipase effects are specific for 1,3-triglyceride lipases, rendering an in situ emulsion by the production of mono- and diglycerides (Olesen et al., 1994). It has been also postulated that lipase could modify the interaction between lipids and gluten proteins, reducing the lipid−gluten overaggregation during the dough preparation (Weegels and Hamer, 1992). Nevertheless, the basis of the lipase benefits in bread-making is still unclear.

Lipases from the yeast *Geotrichum candidum* have been purified and extensively characterized (Sugihara et al., 1990; Sidebottom et al., 1991; Jacobsen and Poulsen, 1995). Two closely related lipase genes, *LIP1* and *LIP2*, have been cloned and sequenced (Shimada et al., 1989, 1990; Nagao et al., 1993), and the polymorphism in the lipase genes of *Geotrichum candidum* strains has been investigated (Bertolini et al., 1994). Furthermore, both genes have been expressed in *Saccharomyces cerevisiae* laboratory strains (Vernet et al., 1993; Bertolini et al., 1995). Enzymatic characterization of recombinant lipases from *G. candidum* showed that both enzymes hydrolyzed efficiently triacylglycerides with preference for substrates having long unsaturated fatty acid chains (Bertolini et al., 1995). Catalytic and biochemical properties of these enzymes make them suitable for potential applications in the baking industry.

In this paper, we describe the expression of the *LIP1* and *LIP2* genes from *Geotrichum* sp. in an industrial

* Author to whom correspondence should be addressed (telephone 34 6 3900022; fax 34 6 3636301; e-mail prieto@iata.csic.es).

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baker's yeast strain and the technological application of lipase-producing yeast in the bread-making process.

MATERIALS AND METHODS

Strains and Culture Media. *Geotrichum* sp. CECT1102 strain was obtained from the Spanish Type Culture Collection. The industrial baker's yeast strain CECT10837 (*trp1*) was used in transformation experiments (Randez-Gil and Sanz, 1994). A commercial baker's yeast strain was also used as control in the baking test. Yeast cells were grown at 30 °C in YP (1% yeast extract, 2% peptone) containing 2% glucose or 1% olive oil. In some experiments yeast cells were grown in MM minimal medium [0.67% YNB (Difco) plus the corresponding carbon source] supplemented with tryptophan, as described by Sherman et al. (1986). The oil−agar medium described by Baillargeon et al. (1989) was used to detect the production in the plate of recombinant lipase. Biomass from baker's yeast was prepared by growing cells in a liquid molasses medium as described by Randez-Gil et al. (1995).

Escherichia coli DH10B strain (Gibco BRL) was grown in Luria Bertani medium (1% peptone, 0.5% yeast extract, 0.5% NaCl).

Construction of the YEpACT-LIP1-t, YEpACT-LIP2-t, and YEpACT-ss2-LIP1-t Plasmids. The *LIP1* and *LIP2* coding regions containing their corresponding signal sequences were amplified by PCR from *Geotrichum* sp. genomic DNA and the oligonucleotides Lip1-1 5′CCTTTGATCTAGAACTGTTAATCATG3′ (*Xba*I site underlined), Lip1-2 5′CAAAGCATGCTAAATGGGATTAAC3′ (*Sph*I site underlined), Lip2-1 5′GTACCATCTAGAATTACTGTCAAAC3′ (*Xba*I site underlined), and Lip2-2 5′CGCATAGCATGCGGCTATAAAC3′ (*Sph*I site underlined), respectively. The amplified fragments were subcloned into the pUC18−*Sma*I plasmid, resulting in plasmids pUC-LIP1 and pUC-LIP2. The fragments containing the *Geotrichum* sp. *LIP1* and *LIP2* genes were released by digesting the plasmids with *Xba*I and *Sph*I and subcloned into plasmid YEpACT (Randez-Gil et al., 1995) previously digested with the same set of enzymes. In this way we obtained the plasmids YEpACT-LIP1 and YEpACT-LIP2. The expression cassettes were completed by insertion of the *FBP1* gene terminator. This was amplified by PCR using *S. cerevisiae* genomic DNA and the oligonucleotides Fbp-1 5′CTTTGTTCTGTACTTTAGCATGCG3′ (*Sph*I site underlined) and Fbp-2 5′CAATTCACCAGCTTTTGCAGCG3′. The amplified fragment was subcloned into pBlue-Script-*Eco*RV, resulting in plasmid pBS−FBPt. This vector was treated with *Sph*I and *Hind*III, and the fragment containing the *FBP1* terminator was inserted into plasmids YEpACT-LIP1 and YEpACT-LIP2 previously digested with these enzymes. As a result we obtained plasmids YEpACT-LIP1-t and YEpACT-LIP2-t containing the lipase DNAs between the *S. cerevisiae ACT1* promoter and the *FBP1* terminator.

For the construction of the plasmid YEpACT-ss2-LIP1-t, we treated the plasmid YEpACT-LIP2-t with *Eco*RI and *Sfi*I, releasing a fragment containing the *ACT1* promoter, the signal sequence of *LIP2*, and a piece of DNA corresponding to the N-terminal part of the mature Lip2 protein. This region is completely homologous to that found in the *LIP1* sequence. Thus, by interchanging the *Eco*RI/*Sfi*I fragments, we could replace the signal sequence of *LIP1* by that of *LIP2*. The resulting plasmid was named YEpACT-ss2-LIP1-t.

The plasmid YEplac112 (Gietz and Sugino, 1988) was used to construct a control transformant.

Yeast Transformation. Yeast transformation was carried out as described by Ito et al. (1983), using 3 μg of plasmid DNA. *E. coli* was transformed by electroporation following the manufacturer's instructions (Eppendorf).

DNA Sequencing. Inserts present in plasmids pUC-LIP1 and pUC-LIP2 were sequenced according to the dideoxy nucleotide chain termination procedure (Sanger et al., 1977).

Enzyme Determination. Lipase activity was determined by measuring the release of 4-methylumbelliferone by the action of the enzyme on 4-methylumbelliferyl oleate (MUF-oleate). The reaction mixture consisted of 1.35 mL of 0.1 M Tris buffer pH 8.5, 5 μL of 25 mM substrate solution (in ethylene glycol monomethyl ether), and 5−100 μL of enzyme extract. After vigorous shaking, the mixture was incubated at 1.000 rpm at 45 °C for 5 min, and the reaction was stopped by the addition of 250 μL of 1 M HCl. The fluorescence of the reaction was measured at λ_{ex} = 328 nm and λ_{em} = 470 nm using an RF-5000 spectrofluorophotometer (Shimadzu Corp., Kyoto). Quantification was done using 4-methylumbelliferone to generate a standard curve. One unit of enzyme activity was defined as the amount of enzyme necessary to produce 1 μmol of 4-methylumbelliferone per minute. The activity of lipase against other MUF derivatives of fatty acids (palmitic acid and butyric acid) was determined according to the standard procedure described above.

To determine the optimum pH, 0.1 M solutions consisting of sodium citrate (pH 3), sodium acetate (pH 4 and 5), sodium succinate (pH 6), and Tris-HCl (pH 7, 8, and 9) buffers were used in the standard assay. To test pH stability, a volume (30 μL) of the enzyme solution was mixed with 30 μL of the appropriate buffer and incubated at 45 °C for 1 h, and the remaining activity was assayed as before.

The effect of temperature on the relative activity of lipase at several temperatures (15−85 °C) was determined according to the standard procedure. Thermal stability experiments were conducted in 50 mM Tris-HCl buffer (pH 8.5) at the chosen temperature for 1 h. The enzyme samples were then cooled on ice, and the residual activity was measured as in the standard assay.

Lipase activity was also assayed by measuring the free fatty acids released by hydrolysis of olive oil (Lee and Rhee, 1993). Free fatty acids were extracted with isooctane (1.0 mL) and quantified according to the cupric acetate method (Kwon and Rhee, 1986). One unit of lipase activity was defined as micromoles of fatty acids equivalent released per minute under the assay conditions.

For lipase assay in bread dough, samples of fresh dough (200 mg) were transferred into a tube containing 0.3 mL of extraction buffer (50 mM Tris-HCl, pH 8.5) and 10 steel beads (1.2-mm diameter), and the mixture was shaken for 1 min in a Mini-bead beater apparatus (Biospec Products, Inc., Bartlesville, OK). Finally, the crude extract was centrifuged at 18000*g* (4 °C) for 10 min, and the supernatant was used for lipase analysis.

Electrophoretic Techniques. Protein samples from the culture supernatant of the different yeast strains assayed were concentrated 10 times by ultrafiltration through a membrane with a 10-kDa cutoff and loaded (100 mg of protein) onto a 10% SDS−PAGE gel. Electrophoresis was performed by using the buffer system described by Laemmli (1970). Transfer of proteins to a nitrocellulose membrane was carried out by Western blotting as described by Towbin et al. (1979). Blots were stained in 0.1% Amido Black-10B. Glycoproteins were detected by using the concanavalin A−peroxidase method (Hawkes, 1982; Millette et al., 1984). Total protein was determined according to the method of Lowry et al. (1951) using rabbit IgG as standard. Endo-H treatment of proteins was carried out as described previously (Prieto et al., 1995).

Bread-Making Procedure and Bread Volume Assessment. A commercial wheat bread flour [moisture, 14.9%; W, 8.6×10^{-3} J (Alveograph); P/L, 0.29, (Alveograph); protein, 14%; total lipid, 2.1%; falling number, 300 s] was used throughout this work. Bread dough was made according to a straight dough bread-making process (Barber et al., 1991). The dough formula was 100% flour, 2% yeast, 2% salt, 0.01% ascorbic acid, and 51.5% water (% flour basis). The baking test was carried out in duplicate for each yeast tested.

For the preparation of liquid preferment, flour was blended into a slurry with water (250%, flour basis) and salt (2%, flour basis). Duplicate samples were inoculated (2%, flour basis), with each yeast under study and incubated at 30 °C, with agitation (200 rpm). At 24 h, the flour slurry was centrifuged (12000 rpm, 10 min, 4 °C) and the clear supernatant poured off and used to prepare a bread dough. This was obtained by following the basic recipe cited above but using the liquid preferment instead of water.

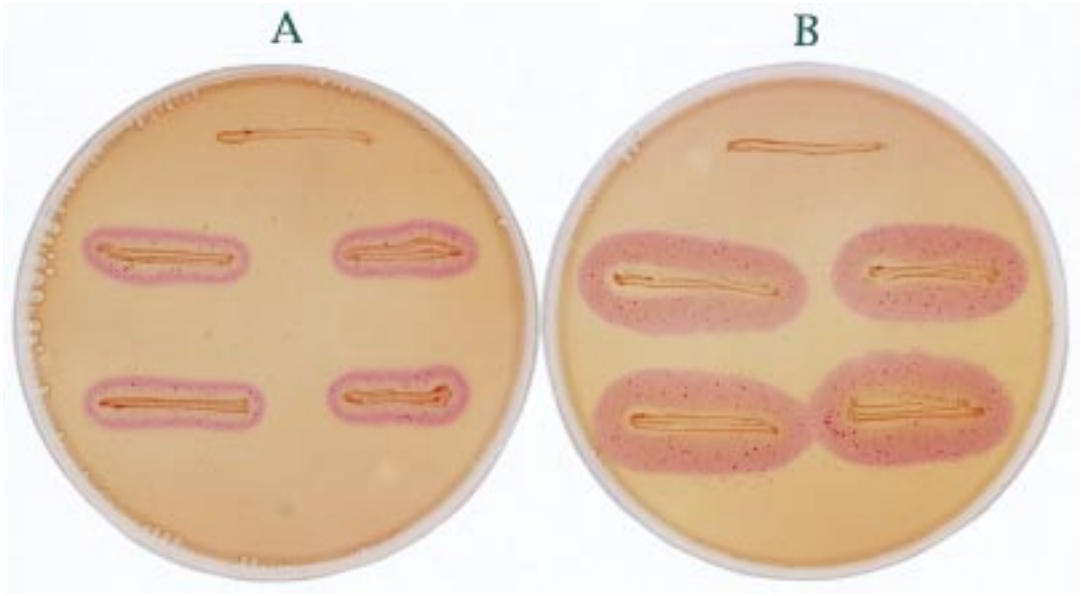

Figure 1. Production of recombinant lipase from four transformants of YEpACT-LIP1-t (A) and YEpACT-LIP2-t (B) grown on oil−agar plates containing Rhodamine B at 30 °C for 48 h. The presence of lipase activity was revealed by the presence of haloes around the colonies. The host yeast strain CECT10837 transformed with the plasmid YEplac112 was used as control (top of the plate).

Loaf volume measurements were performed as described previously (Monfort et al., 1996). At least six loaves of bread were assayed for each yeast sample.

RESULTS AND DISCUSSION

Expression of the *Geotrichum* Species *LIP1* and *LIP2* Genes in Baker's Yeast Cells and Production of Extracellular Lipase. The *LIP1* and *LIP2* genes from *Geotrichum* sp. were amplified using genomic DNA and a set of PCR primers designed from the sequence data of both genes previously reported in the *G. candidum* strain ATCC34614 (Nagao et al., 1993). The oligonucleotides contained the 5′ and 3′ flanking regions of the open reading frames (ORFs), where no significant homology between the *LIP1* and *LIP2* has been observed. The fragments were ligated into the pUC18 plasmid, and the nucleotide sequence in the 5′ and 3′ sides of the inserts were determined to confirm their identity. Partial DNA sequences for *LIP1* and *LIP2* genes from *Geotrichum* sp. were almost identical (data not shown) to those earlier reported for the lipase genes of the reference strain *G. candidum* ATCC34614 (Nagao et al., 1993), confirming that polymorphism of lipase genes into the *Geotrichum* genus is quite small (Bertolini et al., 1994).

To obtain the expression of the lipase genes in baker's yeast, plasmids YEpACT-LIP1-t and YEpACT-LIP2-t containing the corresponding expression cassettes were used to transform the CECT10837 baker's yeast strain (Randez-Gil and Sanz, 1994). Production of recombinant lipase from transformants was detected using oil−agar plates containing Rhodamine B. As shown in Figure 1, cells harboring YEpACT-LIP1-t and YEpACT-LIP2-t plasmids were able to produce active lipase as revealed by the appearance of fluorescent haloes around the colonies. The strain transformed with the plasmid containing the *LIP2* gene produced larger haloes, indicating a higher level of lipase secretion (Figure 1).

Lipase activities were also detected in the culture supernatant of both transformants grown in a YPD medium but not in the culture supernatant of control cells (Figure 2A). In agreement with previous studies showing the expression of heterologous protein in baker's yeast (Randez-Gil et al., 1995; Monfort et al., 1996), lipase activity was detected with the YEpACT-LIP2-t transformant at early exponential growth phase, reaching a maximal value (∼125 U/mL) when the cells entered the diauxic shift. Lipase production by the *LIP1*

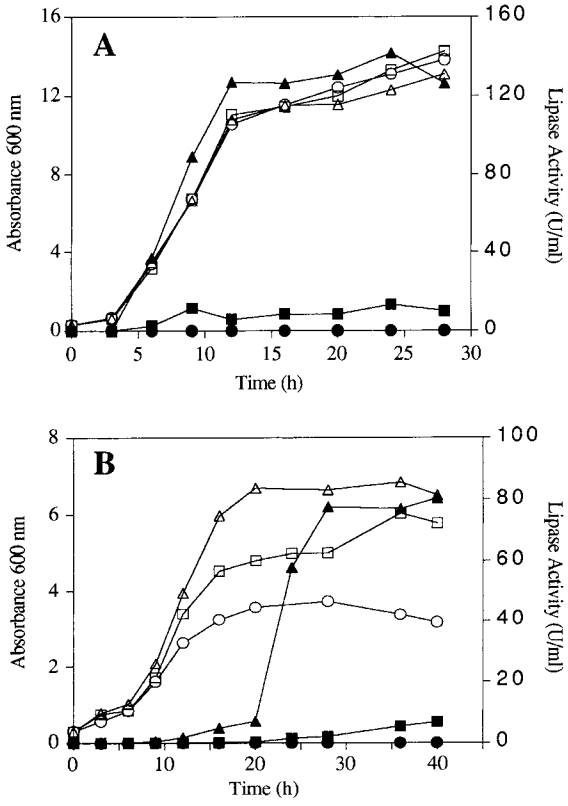

Figure 2. Growth (open symbols) and dynamics of lipase production (solid symbols) of YEpACT-LIP1-t (squares), YEpACT-LIP2-t (triangles), and YEplac112 control (circles) cells grown on YPD (A) or YP−oil (B) medium. Lipase activity was assayed in the supernatant of the culture medium using MUF-oleate as substrate.

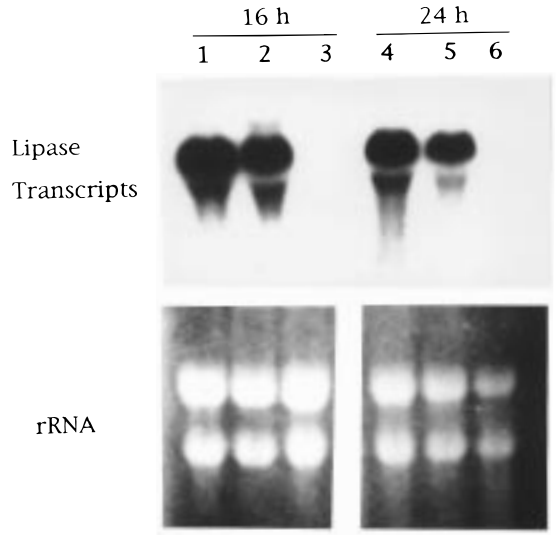

Figure 3. Northern blot analysis of the expression of the *LIP1* and *LIP2* genes in transformants of baker's yeast. Total RNA was prepared from the 10a12-13x28b4 yeast strain transformed with plasmid YEpACT-LIP1-t (lanes 1 and 4), YEpACT-LIP2-t (lanes 2 and 5), or YEplac112 (control, lanes 3 and 6) grown at the indicated times on YPD medium. RNA ethidium bromide staining was performed as a control of loading and transfer.

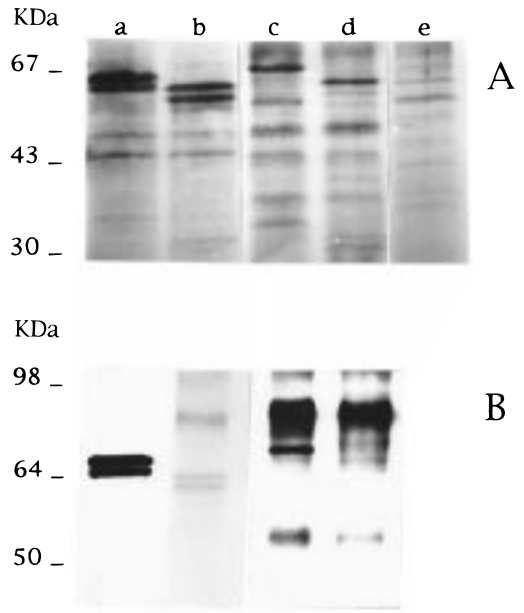

Figure 4. SDS−PAGE analysis of concentrated protein extracts from the culture supernatant of *Geotrichum* sp. (lanes a and b), YEpACT-LIP2-t (lanes c and d), and YEpACT-LIP1-t (lane e) transformant cells. Proteins were electroblotted to nitrocellulose paper and stained with Amido Black-10B (A) or concanavalin A (B). Samples were analyzed before (lanes a, c, and e) and after (lanes b and d) deglycosylation with Endo-H. For more details, see Materials and Methods.

transformant became significant only at the middle of the logarithmic phase, achieving a level of ~10% of that found for the YEpACT-LIP2-t construct (Figure 2A).

Cell growth and secretion of lipase were also followed in a YP medium containing 1% olive oil as sole carbon source. As can be seen (Figure 2B), the lipase production pattern was different from that observed in YPD medium. Thus, the expression levels were lower and production started to be detected later. Lipase production affected the rate and extent of growth of the different yeast strains assayed (Figure 2B). Thus, the lipase-producing yeast grew faster and reached a larger cell biomass than control cells, especially when nutrients became limiting (12−18 h). The construct YEpACT-LIP1-t showed a growing pattern intermediate between that of the control and the *LIP2* transformant strain, in accordance with the low level of lipase activity detected. Only at the end of the growth, when the amount of lipase 1 increased, were the cell biomasses of the cultures of both transformants similar (Figure 2B).

Transcription of the Lipase Genes and Secretion of Lipase 1 by Using the Signal Sequence of Lipase 2. Previous work has shown that in *G. candidum* the amount of *LIP2* mRNA is greater than that of *LIP1* mRNA (Shimada et al., 1992). This fact was ascribed to the existence in the promoter region of the *LIP2* gene of a specific structure responsible for a putative higher transcriptional efficiency (Nagao et al., 1993). The lipases cloned in our work contained an entire ORF, under the control of a *S. cerevisiae* constitutive promoter (*pACT1*). Nevertheless, lipase-specific transcripts were analyzed to determine if the mRNA level in the *LIP1* and *LIP2* transformants could account for the differences observed in the lipase production reported above (see Figures 1 and 2). Northern blot analysis of total RNA from cells grown in YPD medium showed a single band of hybridization that was absent in samples of the control strain transformed with an empty plasmid (Figure 3, lanes 3 and 6). Difference between the expression levels of *LIP1* and *LIP2* were insignificant for cells either growing actively (Figure 3, lanes 1 and 2) or in stationary phase (Figure 3, lanes 4 and 5). Neither the YEpACT-LIP1-t nor the YEpACT-LIP2-t transformant cells exhibited abnormally sized lipase messengers. These results rule out a defect in the transcription of the *LIP1* gene as the origin of the poor lipase-productivity of the YEpACT-LIP1-t construct.

Because the constructions employed here, YEpACT-LIP1-t and YEpACT-LIP2-t, contained the signal sequence corresponding to the *LIP1* and *LIP2* genes, respectively, we replaced the signal sequence of *LIP1* by that of *LIP2* in the YEpACT-LIP1-t plasmid. This change enhanced significantly the production of lipase 1 of the YEpACT-ss2-LIP1-t transformant. After 24 h of culture in a YPD medium, the level of lipase activity reached values of ~45 U/mL, ~4-fold higher than that found for the secretion of lipase 1 with its own signal sequence (see Figure 2A). This result stresses the importance of the signal sequence in the secretion yield of heterologous protein, although lipase 1 production by the YEpACT-ss2-LIP1-t construct was still far from that found for the transformant harboring the *LIP2* gene (see Figure 2A). Other factors could be thus responsible for the reduced lipase 1 production.

Characterization of the Enzyme Lipase 2 Produced by Baker's Yeast. The recombinant protein produced by the *LIP2* transformant was biochemically and enzymatically characterized to compare it with the wild-type enzyme. Figure 4A shows the SDS−PAGE pattern of enzyme preparations from the culture supernatant of *Geotrichum* sp., YEpACT-LIP1-t, and YEpACT-LIP2-t transformant cells. The protein profile from *Geotrichum* sp. (lane a) revealed two main Amido-Black-stained bands corresponding to proteins with apparent sizes of ~62 and ~66 kDa. These values agreed with the previously reported molecular masses of lipases 1

and 2 from *G. candidum* (Vernet et al., 1993; Bertolini et al., 1995). Proteins produced by the lipase 2-producing baker's yeast contained a main species (lane c) moving to a similar position to that of wild-type lipase 2 (lane a). These species were absent in samples of control (data not shown) and *LIP1* transformant (lane e) cells, indicating that they corresponded to the recombinant lipase 2. In consonance with the results of lipase activity described above, the SDS−PAGE pattern of proteins samples of the YEpACT-LIP1-t construct (lane e) showed no differential bands corresponding to the relative mobility of natural lipase 1 (lane a), confirming that the amount of lipase 1 secreted into the culture medium was not significant.

Deglycosylation treatment of protein samples and later staining with concanavalin A revealed that recombinant lipase 2 was glycosylated in a manner similar to that of the wild type (Figure 4A,B). After digestion with Endo-H, only a slight decrease in the apparent M_r of the band was observed (Figure 4A, lanes a−d), whereas the concanavalin A staining of these forms almost disappeared (Figure 4B, lanes a−d). Similar results were also found after *N*-glycosidase F treatment (data not shown).

Recombinant lipase 2 showed preference for substrates with a long *cis*-9 unsaturated fatty acid chain, the activity for the hydrolysis of other substrates with palmitic and butyric acid chain being 4 and 1%, respectively, of that observed for the 18:1 derivative. Maximal recombinant lipase 2 activity was found at pH 8.5 and at 45 °C, although the enzyme was active from pH 5.0 to 9.0 (20−40%) and from 15 to 70 °C (15−15%). The pH and temperature stability profile revealed that the activity was highly conserved in a wide range of pH (4.0−9.0) and temperature (30−50 °C) over a 1-h period. Complete loss of activity by thermal inactivation of the enzyme occurred at 60 °C under the incubation conditions. These results were similar to those previously reported for the wild-type enzyme of *G. candidum*, except that the optimal pH was 6.0 for natural lipase 2 (Sugihara et al., 1990). These differences could originate from variations in the ratio of isoforms of lipase 2 present in the preparations analyzed by other authors. For this reason, characterization of lipases from *G. candidum* strains have resulted in several conflicting reports regarding enzymatic characteristics (Sugihara et al., 1990; Sidebottom et al., 1991; Charton and Macrae, 1992; Jacobsen and Poulsen, 1995).

Application of Lipase-Producing Yeast in Bread-Making. Because the construct YEpACT-LIP2-t produced the highest level of extracellular lipase activity in all media tested, we used this transformant in the baking test. Dough produced with the recombinant yeast gave a bread with a more uniform crumb structure and a higher volume (4.24 ± 0.04 cm^3/g of bread) than that prepared with commercial baker's yeast (3.97 ± 0.08 cm^3/g of bread) or control yeast transformed with an empty plasmid (3.90 ± 0.06 cm^3/g of bread).

Bread quality parameters analyzed in this study were thus affected positively by the application of the lipase-producing yeast. Nevertheless, the observed effects were lower than those reported by the addition of exogenous lipase. Si (1997) reports ∼20-30% of increase in loaf volume at the dosages recommended for commercial preparations, 1000−2000 units of lipase activity/kg of flour. In fermented bread dough, the production of lipase by the transformant strain, as assayed using olive oil as substrate, was found to reach values ∼44 U/kg of flour. The level of lipase activity produced by the transformant yeast is low in comparison to the recommended dosage for commercial lipase. This could be the reason for the observed effect on bread volume that was lower than expected.

We decided to test if the increase in the amount of recombinant lipase could further improve the bread quality parameters. YEpACT-LIP2-t construct cells were cultivated for 24 h in a flour slurry to obtain a preferment enriched in lipase activity. The slurry was centrifuged, and the supernatant containing lipase was used instead of water in the bread dough formula yeasted with the YEpACT-LIP2-t transformant. Thus, an increase of 2-fold in the initial level of lipase activity of the unfermented dough (90 U/kg of flour, using olive oil as substrate) was obtained. Similarly, a preferment of commercial baker's yeast cells cultivated in a wheat flour slurry was employed to prepare the corresponding control bread dough proofed with the same reference strain. Quality parameters of control bread were comparable to those of the product elaborated without the addition of a wheat flour preferment, indicating that the liquid ferment itself had no effect in the bread characteristics. Greater levels of lipase in the bread dough elaborated with the lipase-containing preferment provided a larger increase of loaf volume (∼ +11%) than that observed above. Therefore, the effects observed were specifically related to the presence of more recombinant lipase activity. The results obtained support the idea that the performance of a particular enzyme in baking may be achieved by the yeast producing and secreting the enzymes required.

The strategy followed in this work, making use of recombinant baker's yeast, appears thus to fulfill the requirements of the bread-making process. However, further research is needed to construct yeast strains with optimized enzyme-producing capabilities. Efforts to achieve this by the use of different promoters exhibiting a high activity under the bread dough conditions are now in progress.

LITERATURE CITED

Baillargeon, M. W.; Bistline, R. G.; Sonnet, P. E. Evaluation of strains of *Geotrichum candidum* for lipase production and fatty acid specificity. *Appl. Microbiol. Biotechnol.* **1989**, *30*, 92−96.

Barber, B.; Martínez, J.; Cornejo, L.; Benedito, C. Efectos de distintas masas madre sobre las características reológicas y fermentatívas de la masa panaria. *Rev. Agroquim. Tecnol. Aliment.* **1991**, *31*, 512−522.

Bertolini, M. C.; Laramée, L.; Thomas, D. Y.; Cygler, M.; Schrag, J. D.; Vernet, T. Polymorphism in the lipase genes of *Geotrichum candidum* strains. *Eur. J. Biochem.* **1994**, *219*, 119−125.

Bertolini, M. C.; Schrag, J. D.; Cygler, M.; Ziomek, E.; Thomas, D. Y.; Vernet, T. Expression and characterization of *Geotrichum candidum* lipase I gene. Comparison of specificity profile with lipase II. *Eur. J. Biochem.* **1995**, *228*, 863−869.

Charton, E.; Macrae, A. R.Substrate specificities of lipases A and B from *Geotrichum candidum* CMICC 335426. *Biochim. Biophys. Acta* **1992**, *1123*, 59−64.

Gietz, R. D.; Sugino, A. New yeast-*Escherichia coli* shuttle vectors constructed with in vitro mutagenized yeast genes lacking six-base pair restriction sites. *Gene* **1988**, *74*, 527−534.

Hawkes, R. Identification of concanavalin A-binding proteins after sodium dodecyl sulfate-gel electrophoresis and protein blotting. *Anal. Biochem.* **1982**, *123*, 143−146.

Ito, H.; Jukuda, K.; Murata, K.; Kimura, A. Transformation of intact yeast cells treated with alkali cations. *J. Bacteriol.* **1983**, *153*, 163−168.

Jacobsen, T.; Poulsen, O. M. Comparison of lipases from different strains of the fungus *Geotrichum candidum*. *Biochim. Biophys. Acta* **1995**, *1257*, 96−102.

Kwon, D. Y.; Rhee, J. S. A simple and rapid colorimetric method for determination of free fatty acids for lipase assay. *J. Am. Oil Chem. Soc.* **1986**, *63*, 89−92.

Laemmli, V. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* **1970**, *227*, 680−685.

Lee, S. Y.; Rhee, J. S. Production and partial purification of a lipase from *Pseudomonas putida* 3SK. *Enzyme Microb. Technol.* **1993**, *15*, 617−623.

Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. Protein measurement with the Folin-phenol reagent. *J. Biol. Chem.* **1951**, *193*, 265−275.

Millette, C. F.; Scott, B. K. Identification of spermatogenic all plasma membrane glycoproteins by two-dimensional electrophoresis and lectin blotting. *J. Cell. Sci.* **1984**, *65*, 233−248.

Monfort, A.; Blasco, A.; Prieto, J. A.; Sanz, P. Combined expression of *Aspergillus nidulans* endoxylanase ×24 and *Aspergillus oryzae* α-amylase in industrial baker's yeasts and their use in bread making. *Appl. Environ. Microbiol.* **1996**, *62*, 3712−3715.

Monfort, A.; Blasco, A.; Prieto, J. A.; Sanz, P. Construction of baker's yeast strains that secrete different xylanolytic enzymes and their use in bread making. *J. Cereal Sci.* **1997**, *26*, 195−199.

Nagao, T.; Shimada, Y.; Sugihara, A.; Tominaga, Y. Cloning and sequencing of two chromosomal lipase genes from *Geotrichum candidum*. *J. Biochem.* **1993**, *113*, 776−780.

Olesen, T.; Qi Si, J.; Donelyan, V. Use of lipase in baking. Patent PCT/DK93/00274, 1994.

Poutanen, K.; Luonteri, E.; Tenkanen, M.; Autio, K.; Forssell, P. Enzymes as baking aids. In *Biotechnology in the Food Chain. New Tools and Applications for Future Foods*; Proceedings of the VTT Symposium 177; Espoo, Finland, 1997; pp 67−73.

Prieto, J. A.; Bort, B. R.; Martinez, J.; Randez-Gil, F.; Buesa, C.; Sanz, P. Purification and characterization of a new α-amylase of intermediate thermal stability from the yeast *Lipomyces kononenkoae*. *Biochem. Cell Biol.* **1995**, *73*, 41−49.

Randez-Gil, F.; Sanz, P. Construction of industrial baker's yeast strains able to assimilate maltose under catabolite repression conditions. *Appl. Microbiol. Biotechnol.* **1994**, *42*, 581−586.

Randez-Gil, F.; Prieto, J. A.; Murcia, A.; Sanz, P. Construction of baker's yeast strains that secrete *Aspergillus oryzae* α-amylase and their use in bread making. *J. Cereal Sci.* **1995**, *21*, 185−193.

Sanger, F.; Nicklen, S.; Coulson, A. R. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. U.S.A.* **1977**, *74*, 5463−5467.

Sanz, P.; Prieto, J. A. Construction and benefits of enzyme supplemented baker's yeasts. In *Biotechnology in the Food Chain. New Tools and Applications for Future Foods*; Proceedings of the VTT Symposium 177; Espoo, Finland, 1997; pp 75−81.

Sherman, F.; Fink, G. R.; Hicks, J. B. *Methods in Yeast Genetics*; Cold Spring Harbor Laboratory: Cold Spring Harbor, NY, 1986.

Shimada, Y.; Sugihara, A.; Tominaga, Y.; Iizumi, T.; Tsunasawa, S. cDNA molecular cloning of *Geotrichum candidum* lipase. *J. Biochem.* **1989**, *106*, 383−388.

Shimada, Y.; Sugihara, A.; Iizumi, T.; Tominaga, Y. cDNA cloning and characterization of *Geotrichum candidum* lipase II. *J. Biochem.* **1990**, *107*, 703−707.

Shimada, Y.; Sugihara, A.; Nagao, T.; Tominaga, Y. Induction of *Geotrichum candidum* lipase by long-chain fatty acids. *J. Ferment. Bioeng.* **1992**, *74*, 77−78.

Si, J. Q.; Hansen, T. T. Effect of lipase on bread-making in correlation with their effects on dough rheology and wheat lipids. In *Proceedings of the International Symposium of the AACC/ICC/CCOA*; Beijing, 1994.

Si, J. Q. Synergistic effect of enzymes for breadbaking. *Cereal Foods World* **1997**, *42*, 802−807.

Sidebottom, C. M.; Charton, E.; Dunn, P. P. J.; Mycock, G.; Davies, Ch. J.; Sutton, L.; MacRae, A. R.; Slabas, A. R. *Geotrichum candidum* produces several lipases with markedly different substrate specificities. *Eur. J. Biochem.* **1991**, *202*, 485−491.

Sugihara, A.; Shimada, Y.; Tominaga, Y. Separation and characterization of two molecular forms of *Geotrichum candidum* lipase. *J. Biochem.* **1990**, *107*, 426−430.

Towbin, H.; Staehelin, T.; Gordon, J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. U.S.A.* **1979**, *76*, 4350−4354.

Vernet, T.; Ziomek, E.; Recktenwald, A.; Schrag, J. D.; De Montigny, Ch.; Tessier, D. C.; Thomas, D. Y.; Cygler, M. Cloning and expression of *Geotrichum candidum* lipase II gene in yeast. *J. Biol. Chem.* **1993**, *268*, 26212−26219.

Weegels, P. L.; Hamer, R. J. Improving the bread-making quality of gluten. *Cereal Foods World* **1992**, *37*, 379−380, 382−385.

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