J. Microbiol. Biotechnol. (2003), **13**(3), 451–456

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Overexpression of Thermoalkalophilic Lipase from *Bacillus stearothermophilus* L1 in *Saccharomyces cerevisiae*

AHN, JUNG OH[1], HYUNG WOOK JANG[2], HONG WEON LEE[3], EUI SUNG CHOI[4], SEUNG JOO HAAM[1]*, TAE KWANG OH[5], AND JOON KI JUNG[3]

[1]*Department of Chemical Engineering, Yonsei University, 134 Shinchon, Seoul 120-749, Korea*
[2]*Acebiotech Corporation, Chungwon, Chungbuk 363-942, Korea*
[3]*Bioventure Center, Bio-pilot Plant, Korea Research Institute of Bioscience and Biotechnology, P.O. Box 115, Yusong, Taejon 305-600, Korea*
[4]*Microbial Functions Laboratory, Korea Research Institute of Bioscience and Biotechnology, P.O. Box 115, Yusong, Taejon 305-600, Korea*
[5]*Microbial Genomics Applications Center, Korea Research Institute of Bioscience and Biotechnology, P.O. Box 115, Yusong, Taejon 305-600, Korea*

Received: January 16, 2003
Accepted: March 29, 2003

Abstract An expression vector system was developed for the secretory production of recombinant *Bacillus stearothermophilus* L1 lipase in *Saccharomyces cerevisiae*. The mature L1 lipase gene was fused to α-amylase signal sequence from *Aspergillus oryzae* for the effective secretion into the culture broth and the expression was controlled under *GAL10* (the gene coding UDP-galactose epimerase of *S. cerevisiae*) promoter. *S. cerevisiae* harboring the resulting plasmid successfully secreted L1 lipase into the culture broth. To examine an optimum condition for L1 lipase expression in the fed-batch culture, L1 lipase expression was induced at three different growth phases (early, mid, and late-exponential growth phases). Maximum production of L1 lipase (1,254,000 U/l, corresponding to 0.65 /l) was found when the culture was induced at an early growth phase. Secreted recombinant L1 lipase was purified only through CM-Sepharose chromatography, and the purified enzyme showed 1,963 U/mg of specific activity and thermoalkalophilic properties similar to those reported for the enzyme expressed in *Escherichia coli*.

Key words: *Bacillus stearothermophilus* L1, thermoalkalophilic lipase, induction time, *Saccharomyces cerevisiae*

Microbial lipases have a number of potential applications in oleochemistry, detergents, paper and food industry, and in organic synthesis [18]. In particular, thermoalkalophilic lipases isolated from thermophilic bacteria are playing increasingly important roles in industrial processes, because they are applicable in the enzymatic processing of lipids, even at high temperatures and alkaline condition [19]. The lipase isolated from *B. stearothermophilus* L1 is one of the well-characterized thermoalkalophilic lipases [6, 10, 12]. Kim *et al.* [10, 12] attempted to express L1 lipase in *E. coli* and investigated the characteristics of the purified enzyme. Recombinant L1 lipase produced by *E. coli* contained the thermoalkalophilic enzyme properties most active at 60–65°C and in alkaline pH 9–10 conditions [10]. These properties and its preference for long chain saturated fatty acids as substrates suggest that the L1 lipase can hydrolyze some solid lipids such as beef tallow and palm oil at high temperature [12]. As its industrial application increased, the secretion of this enzyme into the culture medium is required for easy separation and purification.

S. cerevisiae is an ideal host for the secretory production of recombinant proteins, because of its well-characterized secretion pathway and a small amount of its own secretory proteins [15]. Unlike *E. coli*, it does not produce endoxins and pyrogens, and has been considered as a safe organism for the production of food and health-care products [11, 16].

In this study, we attempted to express *B. stearothermophilus* L1 lipase as a secretory product in yeast. The physiological characteristics of transformants were investigated in the batch and fed-batch cultures. Effect of induction time on the production of L1 lipase was investigated during high cell density period in the fed-batch culture. Also, we purified secreted recombinant L1 lipase produced by *S. cerevisiae* and compared its characteristics to those of L1 lipase that was produced by *E. coli*.

*Corresponding author
Phone: 82-2-2123-2751; Fax: 82-2-312-6401;
E-mail: haam@yonsei.ac.kr

MATERIALS AND METHODS

Strains and Plasmids

Yeast host strain used in this study was *S. cerevisiae* 2805 (*MATa pep4::HIS3 prol-δ.6R can1 his3-200 ura3-52*). *E. coli* XL-1 Blue[*supE44 hsdR17 recA1 endA1 gyrA46 thi relA1 lac⁻F'(proAB⁺ lacI^q lacZ*M15 Tn10(tet^r)] was used for plasmid construction. For the construction of yeast expression plasmid, *E. coli*-yeast shuttle vector pGAL-1 containing the yeast *GAL10* promoter and the *GAL7* terminator [13] was used.

DNA Manipulation

For insertion of α-amylase signal peptide from *A. oryzae* to pGAL-1, the sense and antisense oligonucleotides were synthesized (Genotech, Korea) as follows: 5'-*AA TTC* ATG ATG GTC GCG TGG TGG TCT CTA TTT CTG TAC GGC CTT CAG GTC GCG GCA CCT GCT TTG GCT *CAT ATG* AAA GGT *AAG CTT G*-3' and 5'-*TC GAC AAG CTT* ACC TTT *CAT ATG* AGC CAA AGC AGG TGC CGC GAC CTG AAG GCC GTA CAG AAA TAG AGA CCA CCA CGC GAC CAT CAT *G*-3'. Characters in Italic and underlined indicate the restriction enzyme sites. A mixture of complementary strands were heated to 100°C, and cooled to room temperature for annealing. The resulting fragment included restriction enzyme sites of *Eco*RI and *Sal*I at the 5' end and 3' end, respectively. It was inserted into *Eco*RI/*Sal*I of digested pGAL-1. This plasmid was named pYEGA-AM. The L1 lipase gene encoding the open reading frame (ORF) was isolated by a treatment of *Nde*I and *Hin*dIII of pSLE2 [12]. This fragment containing the L1 lipase gene was inserted into *Nde*I/*Hin*dIII of digested pYEGA-AM. The resulting plasmid was designated as pYEGA-AM-Lip.

Batch and Fed-Batch Culture

Selection and maintenance of yeast transformants were performed by using YNBCAD medium (g/l): yeast nitrogen base without amino acids 6.7, casamino acid 5, and glucose 20. A single colony on YNBCAD agar plate was inoculated into 20 ml of YNBCAD medium and incubated overnight at 30°C. The culture was transferred into a 1,000-ml Erlenmeyer flask containing 200 ml of YNBCAD medium, and incubated as above. This culture was used as a seed for batch and fed-batch cultures. In a batch culture, the seed (200 ml) was inoculated to a 5-l fermentor (Kobiotech, Korea) containing 1,800 ml of complex medium (g/l): glucose 20, galactose 30, yeast extract 40, and Bacto-peptone 5. In fed-batch cultures, the seed (200 ml) was inoculated into a 5-l jar fermenter containing 1,800 ml of the initial media (g/l): glucose 5 (20 at early-induction), yeast extract 3, casamino acid 3, $MgSO_4 \cdot 7H_2O$ 1, K_2HPO_4 1.5, $(NH_4)_2SO_4$ 3, and 1 ml of the trace solution (μM): $(NH_4)_6MoO_{23}$ 3.0, H_3BO_3 400, $CuSO_4$ 10, $MnCl_2$ 80, and $ZnSO_4$ 10. When glucose was completely exhausted, growth medium was continuously fed to the fermentor and changed to the induction medium for the L1 lipase gene expression at optical cell densities of 10, 50, and 200. To avoid the catabolite repression, feeding rate was controlled manually to keep the glucose and ethanol concentrations below 1 g/l. The growth media contained the following composition (g/l): glucose 600, $MgSO_4 \cdot 7H_2O$ 8.5, yeast extract 37.5, casamino acid 30, K_2HPO_4 22.5, $(NH_4)_2SO_4$ 30, and 1 ml of the trace solution. Induction media consisted of the same components in the growth media, except for 300 g/l each of glucose and galactose as a carbon source.

Analytical Methods

The growth of yeast cells was monitored by measuring the optical density at 600 nm (OD_{600}) (UVICON930, Switzerland). The dry cell weight was estimated by a predetermined conversion factor of 0.27 g dry cell weight/l OD. The concentrations of glucose in the culture broth were measured by using a glucose analyzer (Yellow Springs Instrument 2700-D, U.S.A.), and the concentrations of galactose and ethanol were measured by HPLC (Gilson) analysis using an HPX 87H column and RI detector. Lipase activity was determined by the pH-stat analysis by using a 718 STAT Titrino pH-stat (Metrohm). Olive oil emulsion was prepared by emulsifying 5 ml of olive oil in 495 ml of the salt solution, containing 20 mM NaCl, 1 mM $CaCl_2$, and 0.5% (w/v) gum arabic, for 2 min at maximum speed in a Waring blender. After the pH of the substrate emulsion (40 ml) was adjusted to 8.0 by adding 10 mM NaOH at 50°C, an appropriate amount of enzyme solution was added and the rate of released fatty acid was measured [8]. For the extracellular lipase activity, the culture broth was centrifugated, and the supernatant was used for enzyme solution. For the intracellular lipase activity, cells were harvested by centrifugation, washed in 10 mM Tris-HCl buffer (pH 8.0), and suspended in the same buffer. This suspension was added to 425-600 nm of the glass bead (Sigma, U.S.A.) that was disrupted for 2 min by using the multi-power beater (Biogenia, Korea). The resulting supernatant was used for the enzyme source to carry out the intracellular lipase activity. One unit of enzyme activity was defined as the amount of enzyme required for releasing 1 μmol of fatty acid per min under assay conditions.

SDS-PAGE and Zymogram

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli [14]. For the zymogram analysis, after electrophoresis, the gel was kept in 50 mM phosphate buffer (pH 6.0) containing 1% Triton X-100. The gel was then washed twice in the same buffer without Triton X-100. The renatured gel was incubated on the agarose plate containing emulsion of 1% tributyrin.

Temperature and pH Effects on Lipase Activity

The optimum temperature and pH of the enzyme activity were measured by assaying the lipase activities at various temperatures and pHs as described above.

Purification of L1 Lipase

The culture supernatant was concentrated and dialyzed through the membrane of molecular weight cutoff of 10 kDa. The concentrated broth was placed onto CM-Sepharose column (2.5×20 cm) equilibrated with 10 mM phosphate buffer, pH 6.0, and the column was washed with the same buffer. The bound protein was eluted with an increasing gradient of KCl (0–0.5 M) in 400 ml of 10 mM phosphate buffer (pH 6.0).

RESULTS AND DISCUSSION

Construction of Vector for the Secretory Production of L1 Lipase in Yeast

An expression vector system was developed for the secretory production of *B. stearothermophilus* L1 lipase in *S. cerevisiae*. Figure 1 shows the scheme for the construction of the expression vector of L1 lipase (pYEGA-AM-Lip) in yeast. In this expression vector, the mature L1 lipase gene was fused to α-amylase signal sequence from *A. oryzae* and placed between the galactose-inducible *GAL10* promoter and the *GAL7* terminator (Fig. 1). The α-amylase signal sequence used in this system has already been successfully used for the effective secretion of several recombinant proteins such as over-glycosylated glucose oxidase and sweet potato peroxidase [3, 4, 9, 13]. Also, *GAL10* promoter is one of the most tightly regulatable promoters and has been routinely used for the production of recombinant proteins in *S. cerevisiae* [7, 16].

Secretion of L1 Lipase into the Culture Broth

pYEGA-AM-Lip was transformed into *S. cerevisiae* 2805. For preliminary detection of the secretory production of L1 lipase, the transformants were cultivated on YPG agar plates containing an emulsion of tributyrin. Lipolytic activities were observed around transformants by clearing the opaque triglyceride, but no activity was observed around *S. cerevisiae*/pYEGA-AM as a control strain (data not shown). To investigate the expression and secretion level of L1 lipase in yeast, batch cultivation was carried out in 5-l fermentor. Figure 2 shows the time profiles of the batch culture. Cell growth in batch culture was divided into two distinct growth phases (Fig. 2). First, the exponential growth phase on glucose was observed, and during this

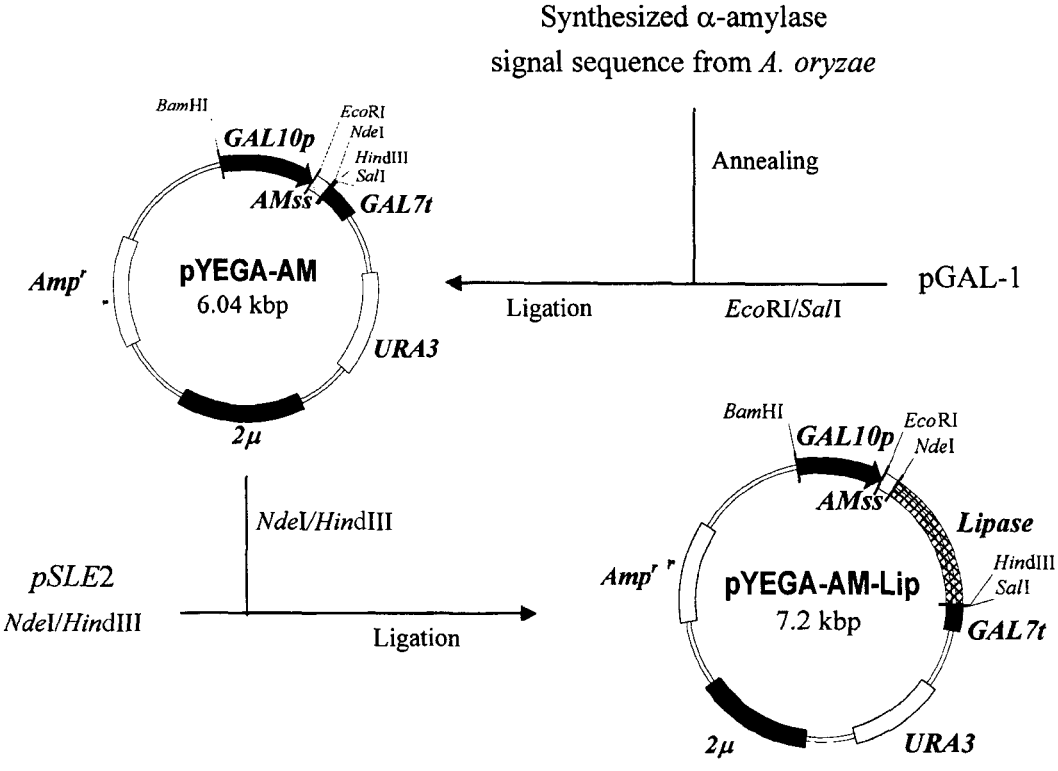

Fig. 1. Construction of vector for expression and secretion of L1 lipase in *S. cerevisiae*.
pYEGA-AM-Lip is a multicopy-episomal expression vector that contains the structural gene of L1 lipase fused to the α-amylase signal sequence from *Aspergilus oryzae* (*Amss*) that was placed between *GAL10* promoter (*GAL10p*) and *GAL7* terminator (*GAL7t*).

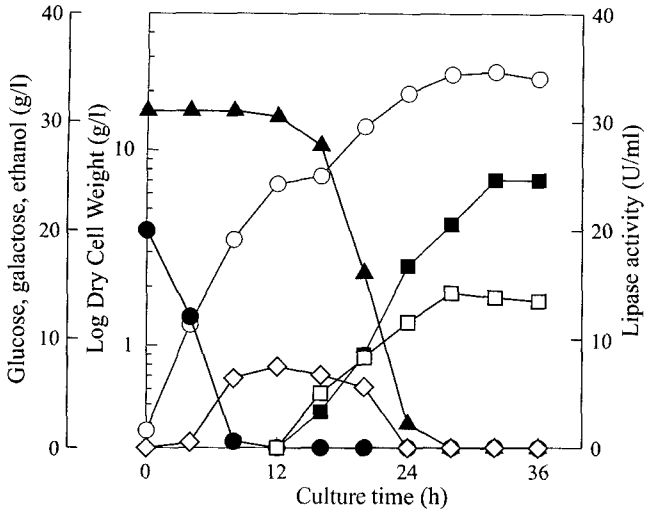

Fig. 2. Time profiles for batch culture of *S. cerevisiae* harboring pYEGA-AM-Lip.
Symbols: ○, Dry cell weight; ●, Glucose concentration; ▲, Galactose concentration; ◇, Ethanol concentration; ■, Extracellular lipase activity; ❑, Intracellular lipase activity.

phase, ethanol was accumulated to about 10 g/l due to the Crabtree effect [2]. As glucose was depleted, another exponential growth phase on accumulated ethanol and galactose was preceded by the lag phase (Fig. 2). The entire carbon sources were depleted in 28 h, and the stationary phase began. L1 lipase gene began to be expressed after the uptake of galactose took place. L1 lipase activity was observed both in the culture broth and within the cell. The intracellular lipase activity remained unchanged in the stationary phase, which resulted from the decrease of expression due to energy limitation caused by depletion of carbon source. At the end of the batch cultivation, extracellular and intracellular activities of L1 lipase were 24,000 and 13,000 U/l, respectively, and secretion level was approximately 65%. Thus, L1 lipase was successfully expressed and its product was effectively secreted into the culture broth by *S. cereivisae*/pYEGA-AM-Lip.

Effect of Induction Time in Fed-Batch Culture

To develop a strong expression process by using an inducible promoter, selection of induction time is an important factor to be taken into consideration [1, 5]. Therefore, effect of induction time on L1 lipase expression

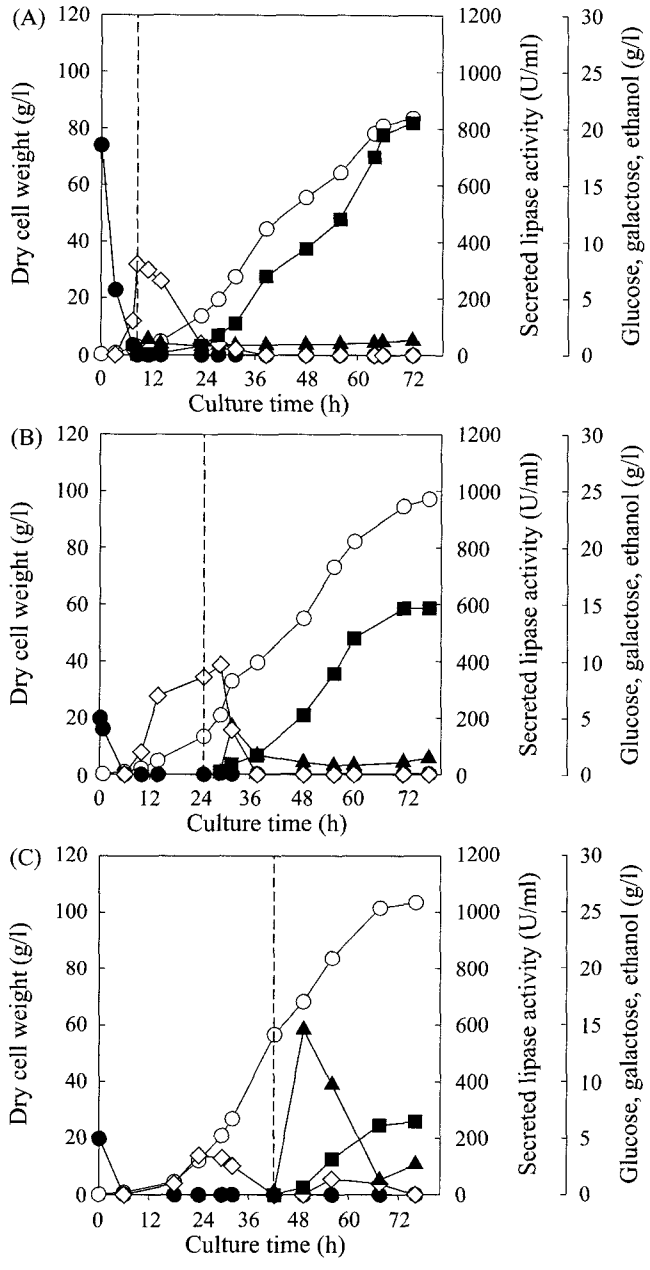

Fig. 3. Time profiles for fed-batch cultures in which the expression of L1 lipase was induced at early (A), mid (B), and late (C) growth phases, respectively.
Symbols: ○, Dry cell weight; ●, Glucose concentration; ▲, Galactose concentration; ◇, Ethanol concentration; ■, Extracellular L1 lipase. The dashed lines indicate the induction time and feeding point of induction media.

Table 1. Comparison of the main results obtained at the end of fed-batch cultures with different induction times.

Induction time	Culture time (h)	Dry cell weight (g/l)	Lipase activity (U/ml)		Total activity (U/ml)	Secretion level* (%)	Total activity/Dry cell weight (U/g cell)
			Extra.	Intra.			
Early phase	73	83.7	820	434	1,254	65	14,982
Mid phase	79	97.2	586	386	972	60	10,000
Late phase	76	103.4	259	158	417	62	4,033

*Secretion level was identified as extracellular lipase activity versus total activity.

Table 2. Purification of secreted recombinant L1 lipase produced by *S. cerevisiae*.

Purification step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Ultrafiltration (10 K)	67,342	74.89	911	100	1.00
CM-Sepharose	30,624	15.67	1,953	46	2.14

was examined. The term "induction time" here means feeding point of induction medium containing galactose, because the expression of L1 lipase in the vector pYEGA-AM-Lip was induced by galactose. Experiment was performed in the fed-batch culture, and L1 lipase expression was induced at different cell growth phases; early-, mid-, and late-stage of the culture.

Figures 3A, 3B, and 3C show the time profiles for three types of fed-batch cultures with different starting points of the induction media feeding at a cell growth phase. For the induction at an early stage of the culture (at dry cell weight, 2.7 g/l), induction medium began to be fed into the fermentor until the end of culture, when initial glucose (20 g/l) was depleted completely. For mid- and late- stages of cultures (at dry cell weight 14 and 54 g/l, respectively), growth medium containing glucose as a sole carbon source was first fed to the fermentor until dry cell weight reached 14 and 54 g/l, respectively, and then changed to the induction medium. To avoid catabolite repression, the feeding rate was manually controlled by using a stepwise feeding method, and glucose concentration was maintained below 1 g/l after feeding both growth and induction media in all cultures (Figs. 3A, 3B, and 3C). As shown in Figs. 3A, 3B, and 3C, the accumulation of galactose was observed for a few hours after induction medium was fed, which was the time required to synthesize galactose-utilizing enzymes [1]. After this period, the galactose was consumed and maintained between 1 and 2 g/l in all cultures. It should be noted that the glucose and galactose were consumed simultaneously: Although the glucose and galactose in a batch culture were not consumed simultaneously due to catabolite repression by glucose, they were consumed simultaneously in the fed-batch cultures due to glucose-limited condition. L1 lipase began to be expressed after the uptake of galactose in all cultures, and its production was related with cell growth (Figs. 3A, 3B, and 3C).

Table 1 shows the final dry cell weight, extracellular lipase activity, intracellular lipase activity, total lipase activity/final dry cell weight, and secretion level at the end of the fed-batch culture. High cell concentration (above 80 g/l) was obtained in all cultures, and maximum final cell concentration (103.4 g/l) was obtained at late-induction. Extracellular lipase activity at an early induction (820,000 U/l) was 1.3-fold higher than that at mid-induction, and 3.2-fold higher than that at the late-induction. Also, total lipase activity/final dry cell weight was 1.5- and 4-fold higher than those at mid- and late-induction, respectively. Secretion levels (around 62%) were similar in all cultures.

Purification and Characteristics of L1 Lipase

Recombinant L1 lipase was purified from the culture broth obtained from a fed-batch culture and this is shown in Fig. 3A. The culture broth was first concentrated and dialyzed by ultrafiltration through a membrane of 10 kDa molecular weight cutoff. Then, L1 lipase was purified through CM-Sepharose column chromatography with linear gradient of KCl from 0–0.5 M, and the lipase dialyzed. CM-Sepharose chromatography yielded a relatively sharp single protein peak at KCl concentration, somewhere between 0.15–0.25 M. The purification results are summarized in Table 2. L1 lipase purified by the single procedure showed 1,953 U/mg of specific activity that was comparable to the purified enzyme produced by *E. coli*, and its recovery was 45.5% [12]. As shown by SDS-PAGE and zymogram analyses of the purified enzyme (Fig. 4), the secreted recombinant L1 lipase produced by *S. cerevisiae* was 43 kDa, equal to the size of L1 lipase produced by *E. coli* [10]. In order to determine optimal temperature and pH, the lipase activities of both L1 lipases in *S. cerevisiae* and *E. coli* were measured at different temperatures at pH 8.0 and different pHs at 50°C. Similar to recombinant L1 lipase expressed in *E. coli* [12], the secreted recombinant

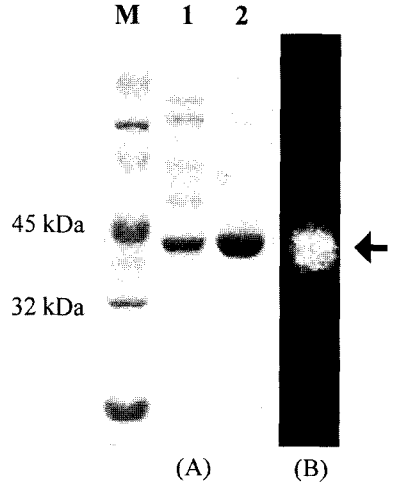

Fig. 4. SDS-PAGE (A) and zymogram (B) of purified recombinant L1 lipase produced by *S. cerevisae*.
Lane M, molecular weight marker; lane 1, crude sample (5 μg) produced by *S. cerevisiae*; lane 2, purified sample (5 μg) through CM-Sepharose. Arrow indicates the L1 lipase.

L1 lipase produced by *S. cerevisiae* was active at 60-65°C and pH 9.0-10 (data not shown).

REFERENCES

1. Chung, B. H., B. M. Kim, S. K. Rhee, Y. H. Park, and S. W. Nam. 1995. Effect of galactose feeding strategy on heterologous human lipocortin-I production in the fed-batch culture of *Saccharomyces cerevisiae* controlled by the *GAL10* promoter. *J. Microbiol. Biotechnol.* **5:** 224-228.
2. De Deken, R. H. 1966. The Crabtree effect: A regulatory system in yeast. *J. Gen. Microbiol.* **44:** 149-156.
3. El-enshasy, H., K. Hellmuth, and U. Rinas. 1999. GpdA-promoter-controlled production of glucose oxidase by recombinant *Aspergillus niger* nonglucose carbon sources. *Appl. Biochem. Biotechnol.* **90:** 57-66.
4. Hellmuth, K., S. Pluschkell, J. K. Jung, E. Ruttkowski, and U. Rinos. 1995. Optimization of glucose oxidase production by *Aspergillus niger* using genetic- and process-engineering techniques. *Appl. Microbiol. Biotechnol.* **43:** 978-984.
5. Jeong, K. J. and S. Y. Lee. 1999. High-level production of human leptin by fed-batch cultivation of recombinant *Escherichia coli* and its purification. *Appl. Environ. Microbiol.* **65:** 3027-3032.
6. Jeong, S. T., H. K. Kim, S. J. Kim, J. G. Pan, T. K. Oh, and S. G. Ryu. 2001. Crystallization and preliminary X-ray analysis of thermoalkalophilic lipase from *Bacillus stearothermophilus* L1. *Biologic. Crystal.* **D57:** 1300-1302.
7. Johnston, M. 1987. A model fungal gene regulatory mechanism: The *GAL* genes of *Saccharomyces cerevisiae*. *Microbial Rev.* **51:** 458-476.
8. Jung, W. H., H. K. Kim, C. Y. Lee, and T. K. Oh. 2002. Biochemical properties and substrate specificity of lipase from *Staphylococcus aureus* B56. *J. Microbiol. Biotechnol.* **12:** 25-30.
9. Kapat, A., J. K. Jung, and Y. H. Park. 2000. Effect of continuous feeding of galactose on the production of recombinant glucose oxidase using *Saccharomyces cerevisiae*. *Bioproc. Eng.* **23:** 37-40.
10. Kim, H. K., S. Y. Park, J. K. Lee, and T. K. Oh. 1998. Gene cloning and characterization of thermostable lipase from *Bacillus stearothermophilus* L1. *Biosci. Biotech. Biochem.* **62:** 66-71.
11. Kim, K. Y., M. D. Kim, S. H. Nam, and J. H. Seo. 2002. Display of *Bacillus macerans* cyclodextrin glucanotransferase on cell surface of *Saccharomyces cerevisiae*. *J. Microbiol. Biotechnol.* **12:** 411-416.
12. Kim, M. H., H. K. Kim, J. K. Lee, and T. K. Oh. 2000. Thermostable lipase of *Bacillus stearothermophilus*: High-level production, purification, and calcium-dependent thermostability. *Biosci. Biotech. Biochem.* **64:** 280-286.
13. Kim, T. H., J. K. Jung, S. S. Kwak, S. W. Nam, M. J. Chun, and Y. H. Park. 2002. Heterologous expression and secretion of sweet potato peroxidase isoenzyme A1 in recombinant *Saccharomyces cerevisiae*. *Biotechnol. Lett.* **24:** 279-286.
14. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227:** 680-685.
15. Novick, P., S. Ferro, and R. Scheckman. 1985. Order events in the yeast secretory form yeast. *Science* **25:** 461-469.
16. Romanos, M. A., C. A. Scorer, and J. J. Clare. 1992. Foreign gene expression in yeast: a review. *Yeast* **8:** 423-488.
17. Rua, M. L., H. Atomi, C. Schmidt-Dannert, and R. D. Schmid. 1998. High-level expression of the thermoalkalophilic lipase from *Bacillus thermocatenulatus* in *Escherichia coli*. *Appl. Microbiol. Biotechnol.* **49:** 405-410.
18. Schmidt-Dannert, C. 1999. Recombinant microbial lipase for biotechnological applications. *Biol. Med. Chem.* **7:** 2123-2130.
19. Schmidt-Dannert, C., M. L. Rua, H. Atomi, and R. D. Schmid. 1996. Thermoalkalophilic lipase of *Bacillus thermocatenulatus* l. Molecular cloning, nucleotide sequence, purification and some properties. *Biochim. Biophys. Acta* **1301:** 105-114.