DOI:10.1021/jf1008555

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

Preparation of a Whole-Cell Biocatalyst of *Aspergillus niger* Lipase and Its Practical Properties

Wenshan Liu, Bin Jia, Heyun Zhao, Li Xu, and Yunjun Yan*

Key Laboratory of Molecular Biophysics, Ministry of Education, College of Life Science and Technology, Huazhong University of Science and Technology, Wuhan 430074, China

Aspergillus niger lipase (ANL), a widely used hydrolase, was displayed for the first time on the surface of *Saccharomyces cerevisiae* using a-agglutinin as an anchor protein. Localization of ANL on the cell surface was confirmed by immunofluorescence microscopy. The displayed ANL was confirmed to be active toward tributyrin and *p*-nitrophenyl caprylate (pNPC). The hydrolytic activity toward pNPC reached 43.8 U/g of dry cell weight after induction by galactose for 72 h. The ANL-displaying cells were characterized for their use as whole-cell biocatalysts. The optimum temperature was 45 °C, and the pH was 7.0. The cells had good thermostability, retaining almost 80% of the full activity after incubation at 60 °C for 1 h, and >80% of the full activity at 50 °C for 6 h. The displayed lipase showed a preference for medium-chain fatty acid *p*-nitrophenyl esters. Therefore, the produced whole-cell catalyst is likely to have a wide range of applications.

KEYWORDS: *Aspergillus niger* lipase; surface display; whole-cell catalysts; *Saccharomyces cerevisiae*

INTRODUCTION

Recently, owing to the safety and simplicity of genetic manipulation and the rigidity of the cell-wall structure in *Saccharomyces cerevisiae* (*1*), many heterologous proteins have been displayed on the cell surface of yeast. This method has a wide range of applications in biotechnology, such as whole-cell biocatalysis (*2*), adsorption of heavy metal ions (*3*), high-throughput screening of combinatorial protein libraries (*4*), production of live vaccines (*5*), and biosensors (*6*). In particular, enzyme-displayed yeast cells could be used as novel whole-cell biocatalysts because the enzymes are covalently linked to glucan in the cell wall, rendering them resistant to extraction (*7*). Furthermore, the whole cells could save multiple protein purification steps. Besides, it can also serve as an efficient, high-throughput screening method for protein engineering (*8*).

Lipases (EC 3.1.1.3) constitute an important group of biocatalysts in many fields, such as organic synthesis (*9*, *10*), detergents (*11*), and the food (*12*), pulp, and paper industries (*13*). *Aspergillus niger* lipase (ANL) is one of the most important industrial catalysts and is used in many applications (*14*). Moreover, *A. niger* is considered to be GRAS (generally regarded as safe) by the U.S. FDA (*15*). Therefore, ANL is a preferred choice in processes utilized in the pharmaceutical and food industries, such as organic synthesis (*16*), oil and fat modification (*17*), synthesis of structured lipids (*18*), and flavor enhancement (*19*). Some commercial ANLs can be obtained from Sigma-Aldrich (62301-1G-F, U.S. $35.30 per 1 g) and Amano Enzyme Group (Lipase A "Amano", U.S. $24.30 per 10 g). In all of these applications, ANL is used in its free form. It is deactivated easily during the operation processes and cannot be recycled. Hence, the immobilization of ANL has been explored in some studies (*20*, *21*). However, there have been no reports on the surface display of ANL since we first cloned and expressed the *ANL* gene from *A. niger* F044 (*22*). Surface display can endow ANL with broad application prospects and can also be used for protein engineering. Therefore, we want to explore surface display of ANL as whole-cell catalysts.

Until now, only a few lipases have been displayed on the surface of *S. cerevisiae*, and for this purpose mainly α-agglutinin Agα was used (*2*, *23*, *24*), but the activities of the displayed lipases were quite low. α-Agglutinin is a glycosylphosphatidylinositol (GPI) protein, which is covalently bound to the cell wall of *S. cerevisiae* through a GPI anchor attachment signal in its C-terminal. The activity of those lipases having active sites near the C terminus may be inhibited by fusion with the anchor proteins (*24*, *25*). Flo1 binds to the cell wall through its N-terminal portion and was used to display lipases (*25*). However, it is a noncovalent attachment (*26*). Therefore, cell wall proteins other than GPI proteins should be examined for surface display of lipases. Here, we chose a-agglutinin instead of the widely used α-agglutinin and Flo1 to display ANL on cell surface of *S. cerevisiae*. The a-agglutinin consists of two subunits, Aga1 and Aga2. Aga1 is attached covalently to *β*-glycan in the cell wall of *S. cerevisiae*. Aga2 binds to Aga1 through disulfide bridges (*4*).

In this study, for the first time, ANL was displayed on the surface of *S. cerevisiae* using a-agglutinin as an anchor protein. The practical properties of the displayed lipase, such as optimal temperature and pH, thermostability, and its optimal substrate, were further characterized.

MATERIALS AND METHODS

Strains and Media. *Escherichia coli* DH5α [F^- *endA1 hsdR17* (r_K^-/m_K^+) *supE44 thi-l* λ^- *recAl gyrA96* Δ*lacU169* (Φ80*lacZD*M15)]

*Corresponding author (phone 86-027-87792213; fax 86-027-87792213; e-mail yanyunjun@tom.com).

 Published on Web 09/09/2010

was used as a host for DNA manipulation and was grown in LB medium [1% (w/v) tryptone, 0.5% (w/v) yeast extract, and 1% (w/v) sodium chloride] containing 50 μg/mL ampicillin. *S. cerevisiae* strain EBY100 (*MATa GAL1-AGA1::URA3 ura3−52 trp1 leu2Δ1 his3Δ200 pep4::HIS2 prb1Δ1.6R can1 GAL*) (a gift of Dr. Eric T. Boder) was used for the production of Aga2−ANL fusion proteins. The EBY100 or recombinant EBY100 was grown in YPD [1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) dextrose], SD medium [0.67% (w/v) YNB, 2% (w/v) dextrose, 0.01% (w/v) leucine], SD+CAA medium [0.67% (w/v) YNB, 0.54% (w/v) Na_2HPO_4, 0.86 (w/v) NaH_2PO_4, 2% (w/v) dextrose, 0.5% (w/v) casamino acids], or SG+CAA medium (same as SD+CAA, except that dextrose is replaced by galactose). For the plate medium, 2% (w/v) agar was added.

Construction of the Plasmid. In a previous study, *A. niger* F044 strain was isolated from the soil around the oil refinery in Wuhan, China (*27*). For the expression of the *AGA2−ANL* fusion gene, the mature ANL-encoding gene was amplified from pET28a-*lipanl* (*27*), using the forward primer 5′-CT*GCTAGC*AGTGTCTCGACTTCCACGTTGG-3′ (*Nhe* I site in italics) and the reverse primer 5′-CTT*AGATCT*TTATAGCAGG-CACTCGGAAATC-3′ (*Bgl*II site in italics). The amplified fragments were digested by *Nhe*I and *Bgl*II and introduced into the *Nhe*I and *Bgl*II sites of plasmid pCT302 (provided by Dr. Eric T. Boder). The resulting plasmid was named pCTANL and was used to transform the yeast *S. cerevisiae* EBY100 by the lithium acetate method (*28*). The transformants were selected by plating and incubating for 3 days on a plate with SD medium.

Preparation of the Lipase. An EBY100/pCTANL single yeast colony was inoculated into SD+CAA medium and cultivated overnight at 30 °C. Cells were harvested by centrifugation for 5 min at 4 °C and 4000 rpm. Cell pellets were washed twice, resuspended in SG+CAA medium, and cultivated at 20 °C. Yeast cells harvested from the culture broth were washed, resuspended in 50 mM potassium phosphate buffer (pH 7.0), and then used as whole-cell catalysts.

Immunofluorescence Microscopy Assays. Immunostaining was performed as follows: The mouse polyclonal antibody against HA tag (Tiangen Biotech, China) was used as the primary antibody at a dilution rate of 1:100. Cells and the antibody were incubated on ice for 30 min. After the cells had been washed with PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4) (*29*), the second antibody, fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG diluted 1:200, was reacted with the cells on ice for 30 min. After washing with PBS, the cells were observed with a fluorescence microscope.

Halo Assay. Transformants were inoculated on tributyrin agar plates [0.5% (v/v) tributyrin was added into SG+CAA medium] for 3−5 days. The activity of the lipase was examined by the halo formed around the colony.

Assay of Lipase Activity. The hydrolytic activity was measured by a spectrophotometric method, using *p*-nitrophenyl caprylate (pNPC) as substrate, according to a previously described method (*30*). The pNPC dissolved in acetonitrile at a concentration of 10 mM ethanol and 50 mM potassium phosphate buffer (pH 7.0) were subsequently added to a final ratio of 1:4:95 (v/v/v). Resuspended cells were added to 3 mL of the substrate solution for determination of lipase activity. The OD_{410} was measured after the mixture had been incubated at 45 °C for 10 min. One unit of enzyme activity was defined as the amount of enzyme releasing 1 μmol of *p*-nitrophenol/min.

Characterization of the Lipase. The optimum temperature of lipase under our reaction conditions was examined in the same substrate solution described above, at controlled temperatures from 25 to 60 °C. The optimum pH was also examined in the same substrate solution, except that the potassium phosphate buffer (pH 7.0) was replaced with 50 mM acetate buffer, 50 mM Tris-HCl buffer, or 50 mM carbonate−bicarbonate buffer at various pH values ranging from 3 to 10. The reaction was carried out at 45 °C for 10 min. The effect of Ca^{2+} was evaluated by detecting lipase activity in the same solution containing 2 mM Ca^{2+} at 45 °C.

To examine the thermostability of the lipase, the resuspended cells were incubated at controlled temperature from 30 to 60 °C for up to 1 h and incubated for 1−6 h at 50 °C in 50 mM potassium phosphate buffer (pH 7.0). Then the residual activity was measured at 45 °C by the spectrophotometric method described under "Assay of Lipase Activity" section.

The effect of the substrate chain length was determined by adding a 10 mM solution of *p*-nitrophenol butyrate (C4), *p*-nitrophenol decanoate (C10), *p*-nitrophenol laurate (C12), or *p*-nitrophenol palmitate (C16) instead of pNPC (C8) into the substrate solution for activity measurements according to the spectrophotometric method described under "Assay of Lipase Activity" section.

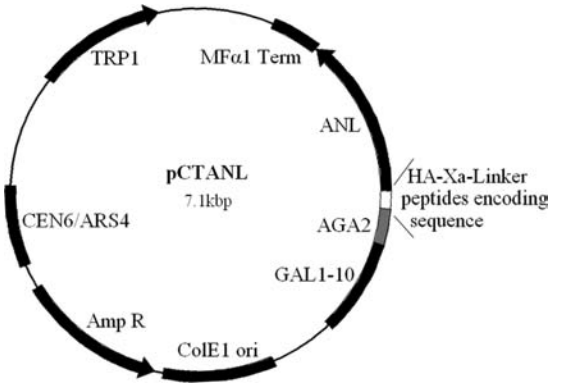

Figure 1. Construction of the plasmid pCTANL for surface display of *Aspergillus niger* lipase (ANL).

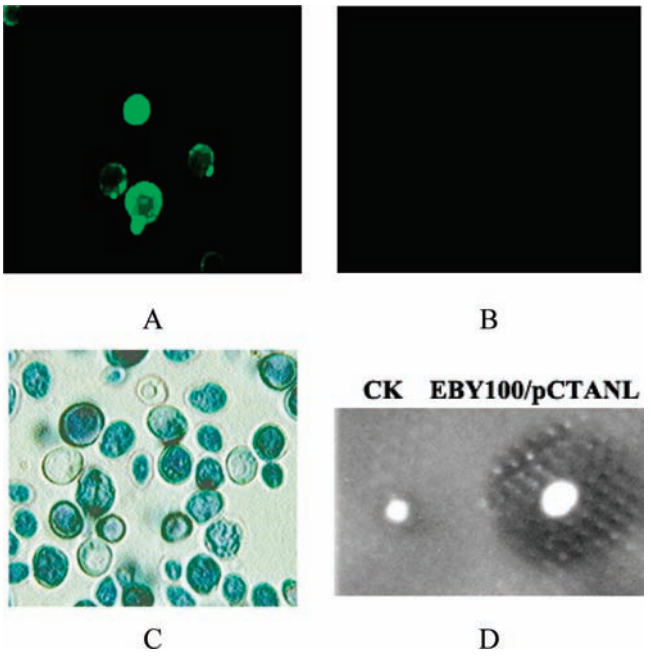

Figure 2. Confirmation of successful surface display of ANL. (**A**) Through fluorescence microscope assay, cells harboring pCTANL were immunologically labeled with the anti-HA antibody as the first antibody and FITC-conjugated anti-IgG as the second antibody. (**B**) EBY100 cells as the control for fluorescence microscope assay. (**C**) The cells (most dead cells were stained blue by methylene blue trihydrate, whereas a few live cells were not stained) observed in optical microscope after the cell suspension was conserved at 4 °C for half a month. (**D**) Halo formation of ANL-displaying yeast on tributyrin agar plate. CK, EBY100 harboring pCT302 as the control.

RESULTS AND DISCUSSION

Construction of the Plasmid. The plasmid pCTANL was constructed as described above. The mature ANL encoding gene was inserted into the yeast vector pCT302 as a 3′-terminal fusion to the *AGA2* gene through a linker sequence and was expressed under the control of the GAL1 galactose-inducible promoter (**Figure 1**). The fusion proteins were bound to Aga1 via disulfide bridges, and Aga1 was covalently anchored to the cell wall of *S. cerevisiae* through a GPI anchor. Between the lipase and Aga2, the linker peptide included an HA epitope (nine amino acids) and

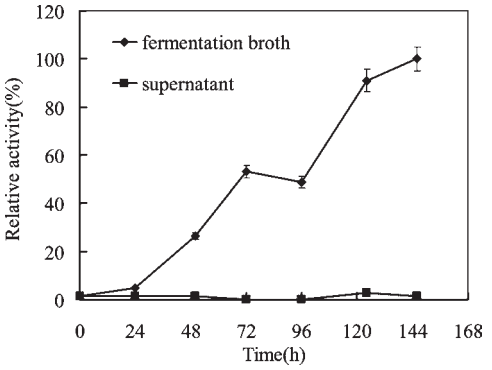

Figure 3. Lipase activity of culture medium and supernatant in different induction times. The activities were detected by adding fermentation broth or supernatant into the buffer [the volume ratio of 10 mM pNPC/ethanol/50 mM potassium phosphate buffer (pH 7.0) was 1:4:95] for 10 min at 45 °C. Then, the OD_{410} was detected. Relative activity was equal to the relative OD_{410}, which was calculated by assuming the OD_{410} obtained at 144 h to be 100% (OD_{410} = 0.63).

a $(G_4S)_3$ spacer. The HA tag was used for immunofluorescent detection. The constructed plasmids were introduced into *S. cerevisiae* EBY100, in which the expression of Aga1 was also inducible by galactose from a single *GAL1*−*AGA1* expression cassette integrated in the genome of EBY100.

Confirmation of Lipase Display on the Cell Surface. To examine whether the lipases were indeed displayed on the surface of *S. cerevisiae*, the cells were examined by immunofluorescence microscopy. As shown in **Figure 2A**, *S. cerevisiae* EBY100 cells anchoring the Aga2−HA-XA-$(G_4S)_3$−ANL fusion protein became fluorescent as a result of the binding of anti-HA tag antibody, followed by binding of FITC-conjugated secondary antibody, whereas the control cells EBY100 were not fluorescent (**Figure 2B**). This indicates that the displayed lipase was not hindered by the structure of the cell surface and could easily bind to the antibody. Thus, the ANL was successfully displayed on the cell surface of EBY100 cells. Meanwhile, methylene blue trihydrate was used to check whether the dead yeast cells maintained their shape. As shown in **Figure 2C**, blue dead cells and live cells both had the expected shape after being kept at 4 °C for 15 days. Furthermore, as shown in **Figure 2D**, the ANL-displaying EBY100 cells produced a clear halo around the colony on tributyrin agar plates. This observation indicates that the surface-displayed ANL could hydrolyze tributyrin, further indicating the accessibility of surface-displayed ANL.

Assay of Lipase Activity. After confirming the successful display of ANL on the surface of *S. cerevisiae*, we further examined quantitatively its hydrolytic activity, using pNPC as the substrate. Hydrolytic activity of the whole-cell EBY100/pCTANL reached 43.8 U/g of dry cell weight, following induction by galactose for 72 h at 20 °C. We also examined the activity of the fermentation broth and the supernatant after induction by galactose for 0−146 h at 20 °C. As shown in **Figure 3**, when the cultures were used to catalyze the hydrolysis of pNPC, enzyme activity increased markedly with time, whereas that of the supernatant was negligible. This indicates that the fusion protein was all anchored on the cell surface. In addition, when compared with surface-displayed lipases using a C-terminal anchor system, the activity of the displayed ANL here was higher than that of the displayed *Rhizopus oryzae* lipase (4.1 U/g of dry cell weight) (*24*), *Yarrowia lipolytica* lipase Lip2 (7.6 U/g of dry cell weight) (*31*), and *Candida antarctica* lipase B (20.4 U/g of dry cell weight) (*2*). When compared with surface-displayed lipases using an N-terminal anchor system, the activity of the displayed ANL was slightly lower than that of *R. oryzae* lipase (61.3 U/g of dry cell weight) (*25*).

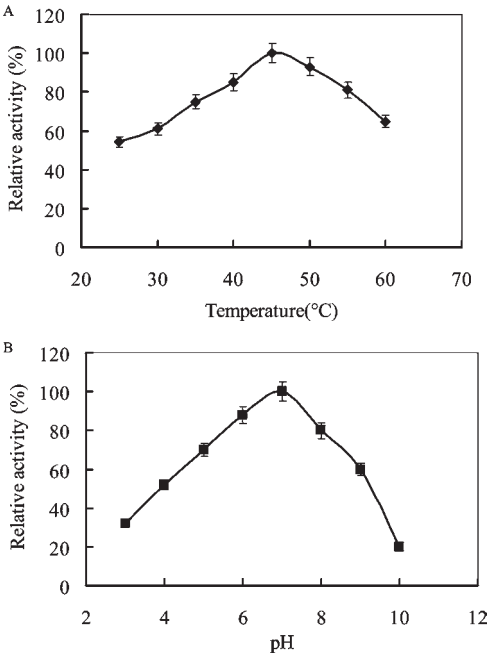

Figure 4. Effect of temperature (**A**) and pH (**B**) on the activity of surface-displayed lipase. The optimal temperature was determined by incubating the displayed ANL in 50 mM potassium phosphate buffer (pH 7.0) at different temperatures from 20 to 70 °C. The optimal pH was determined by incubating the displayed ANL at different pH values from 3 to 10 at 45 °C. Relative activity was calculated by assuming the activity obtained at 45 °C (**A**) and pH 7.0 (**B**) to be 100% (43.8 U/g of dry cell weight).

Characterization of the Displayed Lipase. To evaluate the potential of the displayed lipase as a whole-cell catalyst, its optimal temperature and pH, and its thermostability, the effects of Ca^{2+} and substrate specificity were characterized. The optimal temperature and pH of the displayed lipase were tested at various temperatures, ranging from 20 to 70 °C, and pH, from 3.0 to 10.0. The highest activity of the displayed ANL was observed at 45 °C and pH 7.0 (**Figure 4**). These values were consistent with the results of Shu et al. (*22*). The displayed ANL had > 80% of the maximal activity in the temperature range from 40 to 55 °C and in the pH range from pH 6 to 8. As shown in **Figure 4A**, the displayed ANL could tolerate rather high temperature; it retained 54% of its maximal activity at 25 °C and 65% of its maximal activity at 60 °C. The pH influenced remarkably the activity of the displayed ANL (**Figure 4B**), which retained only 32 and 20% of its maximal activity at pH 3.0 and 10.0, respectively. Therefore, the displayed ANL is more easily inactivated at alkaline pH.

The thermostability of the lipase was also evaluated by detecting the remaining lipase activity after incubation for 1 h at temperatures ranging from 30 to 60 °C. As shown in **Figure 5A**, the surface-displayed ANL had good thermal stability. It lost < 1% of its original activity after incubation at 30 °C, did not lose any detectable activity at 40 °C for 1 h, and retained 78.4% of its original activity after incubation at 60 °C for 1 h. The thermostability was further examined by incubating the displayed ANL at the relatively high temperature of 50 °C for 1−6 h. As shown in **Figure 5B**, the displayed ANL retained > 95% of its activity after incubation for 4 h and > 80% of its activity after 6 h. These results coincided with the purified free form of the enzyme from the wild strain of *A. niger* F044 (*22*). They were better than the free lipases

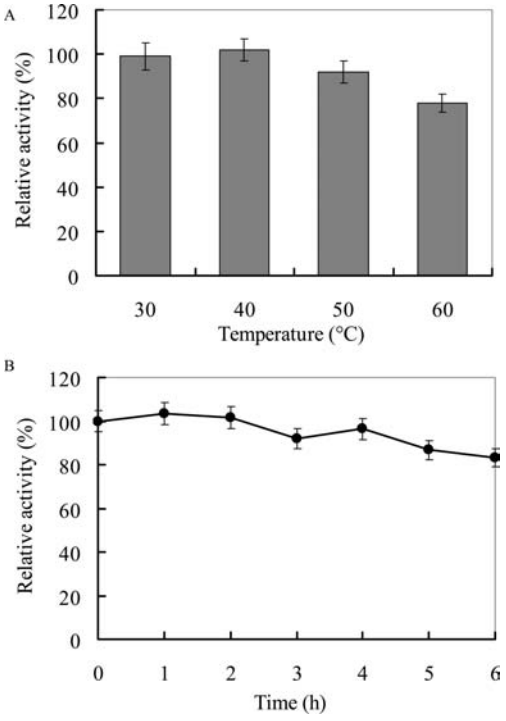

Figure 5. Thermostability of displayed lipase. (**A**) The displayed ANL was incubated at controlled temperature from 30 to 60 °C for up to 1 h in 50 mM potassium phosphate buffer (pH 7.0). Then the residual lipase activity was measured by spectrophotometric method. (**B**) The displayed ANL was incubated for 1−6 h at 50 °C in 50 mM potassium phosphate buffer (pH 7.0). Then the residual lipase activity was measured by spectrophotometric method. Relative activity was calculated by assuming the original activity to be 100% (43.8 U/g of dry cell weight).

from the wild type strain BTL of *A. niger* (that retained about 50% of the original activity after incubation at 50 °C for 0.5 h) (*32*) and the *A. niger* strain from Hindustan Lever Research Centre (Bombay, India) (that retained about 50% of the original activity after incubation at 60 °C for 1 h) (*33*).

The effect of Ca^{2+} was also examined. The surface-displayed ANL was sensitive to the presence of Ca^{2+}. The enzymatic activity with 2 mmol/L Ca^{2+} was 2.3-fold higher than that without Ca^{2+}; this result is similar to that obtained with the purified free ANL from *A. niger* F044 (*22*) and the free ANL reported by Sugihara et al. (*34*).

The substrate specificity of the cell surface lipase was also explored. The displayed ANL showed a marked preference for medium-chain fatty acid *p*-nitrophenyl esters. The highest activity of the displayed ANL was detected when *p*-nitrophenol decanoate (C10) was used as a substrate (**Figure 6**). The lipase activity toward *p*-nitrophenol caprylate (C8) and *p*-nitrophenol laurate (C12) was also relatively high, reaching 84.0 and 62.5% that of C10, respectively. However, the activity of displayed ANL toward *p*-nitrophenol palmitate (C16) was relatively low, only 23.5% of that of C10.

In conclusion, ANL was successfully displayed on the surface of *S. cerevisiae* cells for the first time using the a-agglutinin anchor system. The displayed ANL showed high activity (43.8 U/g of dry cell weight) and excellent thermostability. It retained almost 80% activity after incubation at 60 °C for 1 h and did not lose any significant activity after incubation at 50 °C for 4 h. In addition, the displayed ANL could be recycled simply and saved time spent on the purification of immobilized enzymes. Therefore, whole-cell catalysts with good catalytic properties can be prepared simply and inexpensively and will have many

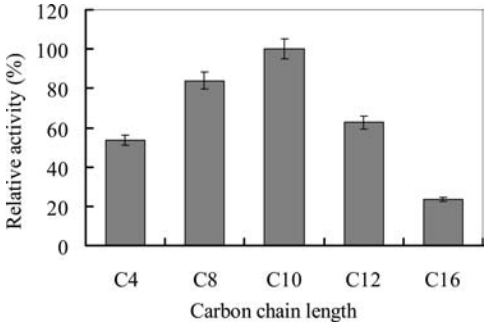

Figure 6. Substrate specificity of displayed lipase. The enzyme activity was determined at 45 °C using different *p*-nitrophenyl esters (C4−C16) as substrates in 50 mM potassium phosphate buffer (pH 7.0) for 10 min. Relative activity was calculated by assuming the activity obtained using C10 as substrates to be 100% (53.2 U/g of dry cell weight).

applications in biotechnology including organic synthesis and food processing.

ABBREVIATIONS USED

ANL, *Aspergillus niger* lipase; pNPC, *p*-nitrophenyl caprylate; GRAS, generally regarded as safe; GPI, glycosylphosphatidylinositol; FITC, fluorescein isothiocyanate.

ACKNOWLEDGMENT

We are grateful for the kind present of plasmid and strain from Dr. Eric T. Boder.

LITERATURE CITED

(1) Lipke, P. N.; Ovalle, R. Cell wall architecture in yeast: new structure and new challenges. *J. Bacteriol.* **1998**, *180*, 3735−3740.
(2) Tanino, T.; Ohno, T.; Aoki, T.; Fukuda, H.; Kondo, A. Development of yeast cells displaying *Candida Antarctica* lipase B and their application to ester synthesis reaction. *Appl. Microbiol. Biotechnol.* **2007**, *75*, 1319−1325.
(3) Vinopal, S.; Ruml, T.; Kotrba, P. Biosorption of Cd^{2+} and Zn^{2+} by cell surface-engineered *Saccharomyces cerevisiae*. *Int. Biodeter. Biodegr.* **2007**, *60*, 96−102.
(4) Boder, E. T.; Wittrup, K. D. Yeast surface display for screening combinatorial polypeptide libraries. *Nat. Biotechnol.* **1997**, *15*, 553−557.
(5) Zhu, K. L.; Chi, Z. M.; Li, J.; Zhang, F. L.; Li, M. J.; Yasoda, H. N.; Wu, L. F. The surface display of haemolysin from *Vibrio harveyi* on yeast cells and their potential applications as live vaccine in marine fish. *Vaccine* **2006**, *24*, 6046−6052.
(6) Shibasaki, S.; Ueda, M.; Ye, K.; Shimizu, K.; Kamasawa, N.; Osumi, M.; Tanaka, A. Creation of cell surface engineered yeast which can emit different fluorescence in response to the glucose concentration. *Appl. Microbiol. Biotechnol.* **2001**, *57*, 528−533.
(7) Ueda, M.; Tanaka, A. Genetic immobilization of proteins on the yeast cell surface. *Biotechnol. Adv.* **2000**, *18*, 121−140.
(8) Shibamoto, H.; Matsumoto, T.; Fukuda, H.; Kondo, A. Molecular engineering of *Rhizopus oryzae* lipase using a combinatorial protein library constructed on the yeast cell surface. *J. Mol. Catal. B: Enzym.* **2004**, *28*, 235−239.
(9) Jaeger, K. E.; Reetz, T. M. Microbial lipases from versatile tools for biotechnology. *Trends Biotechnol.* **1998**, *16*, 396−403.
(10) Muralidhar, R. V.; Chirumamilla, R. R.; Marchant, R.; Ramachandran, V. N.; Ward, O. P.; Nigam, P. Understanding lipase stereoselectivity. *World J. Microbiol. Biotechnol.* **2002**, *18*, 81−97.
(11) Cardenas, F.; Alvarez, E.; de Castro-Alvarez, M.-S.; Sanchez-Montero, J.-M.; Valmaseda, M.; Elson, S. W.; Sinisterra, J.-V. Screening and catalytic activity in organic synthesis of novel fungal and yeast lipases. *J. Mol. Catal. B: Enzym.* **2001**, *14*, 111−123.
(12) Undurraga, D.; Markovits, A.; Erazo, S. Cocoa butter equivalent through enzymic interesterification of palm oil midfraction. *Process Biochem.* **2001**, *36*, 933−939.

(13) Farrell, R. L.; Hata, K.; Wall, M. B. Solving pitch problems in pulp and paper processes by the use of enzymes or fungi. *Adv. Biochem. Eng. Biotechnol.* **1997**, *57*, 197−212.
(14) Sariyar, L.; Heperkan, D. The role of *Aspergillus flavus* and *Aspergillus niger* in the hydrolysis of hazelnut fat. *Int. J. Food Sci. Technol.* **2003**, *38*, 487−492.
(15) Schuster, E.; Dunn-Coleman, N.; Frisvad, J. C.; van Dijck, P. W. M. On the safety of *Aspergillus niger* − a review. *Appl. Microbiol. Biotechnol.* **2002**, *59*, 426−435.
(16) Contesini, F. J.; Carvalho, P. O. Esterification of (RS)-Ibuprofen by native and commercial lipases in a two-phase system containing ionic liquids. *Tetrahedron: Asymmetry* **2006**, *17*, 2069−2073.
(17) Chen, C. A.; Lu, W.; Shi, C. J. Synthesis of 9*Z*,11*E*-octadecadienoic and 10*E*,12*Z*-octadecadienoic acids, the major components of conjugated linoleic acid. *Lipids* **1999**, *34*, 879−884.
(18) Hamam, F.; Shahidi, F. Synthesis of structured lipids containing medium-chain and omega-3 fatty acids. *J. Agric. Food Chem.* **2006**, *54*, 4390−4396.
(19) Kwon, D. Y.; Hong, Y. J.; Yoon, S. H. Enantiomeric synthesis of (*S*)-2- methylbutanoic acid methyl ester, apple flavor, using lipases in organic solvent. *J. Agric. Food Chem.* **2000**, *48*, 524−530.
(20) Da Silva, V. C. F.; Contesini, F. J.; Carvalho, P. O. Characterization and catalytic activity of free and immobilized lipase from *Aspergillus niger*: a comparative study. *J. Brazil. Chem. Soc.* **2008**, *19*, 1468−1474.
(21) Da Silva, V. C. F.; Contesini, F. J.; Carvalho, P. O. Enantioselective behavior of lipases from *Aspergillus niger* immobilized in different supports. *J. Ind. Microbiol. Biot.* **2009**, *36*, 949−954.
(22) Shu, Z. Y.; Yang, J. K.; Yan, Y. J. Purification and characterization of a lipase from *Aspergillus niger* F044. *Chin. J. Biotechnol.* **2007**, *23*, 96−100.
(23) Schreuder, M. P.; Mooren, A. T. A.; Toschka, H. Y.; Verrips, C. T.; Klis, F. M. Immobilizing proteins on the yeast cells. *Trends Biotechnol.* **1996**, *14*, 115−120.
(24) Washida, M.; Takahashi, S.; Ueda, M.; Tanaka, A. Spacer-mediated display of active lipase on the yeast cell surface. *Appl. Microbiol. Biotechnol.* **2001**, *56*, 681−686.
(25) Matsumoto, T.; Fukuda, H.; Ueda, M.; Tanaka, A.; Kondo, A. Construction of yeast strains with high cell surface lipase activity by using novel display systems based on the Flo1p flocculation functional domain. *Appl. Environ. Microbiol.* **2002**, *68*, 4517−4522.
(26) Bony, M.; Thines-Sempoux, D.; Barre, P.; Blondin, B. Localization and cell surface anchoring of the *Saccharomyces cerevisiae* flocculation protein Flo1p. *J. Bacteriol.* **1997**, *179*, 4929−4936.
(27) Shu, Z. Y.; Yan, Y. J.; Yang, J. K.; Xu, L. *Aspergillus niger* lipase: gene cloning, over-expression in *Escherichia coli* and in vitro refolding. *Biotechnol. Lett.* **2007**, *29*, 1875−1879.
(28) Ito, H.; Fukuda, Y.; Murata, K.; Kimura, A. Transformation of intact yeast cells treated with alkali cations. *J. Bacteriol.* **1983**, *153*, 163−168.
(29) Dulbecco, R.; Vogt, M. Plaque formation and isolation of pure lines with poliomyelitis viruses. *J. Exp. Med.* **1954**, *99*, 167−182.
(30) Lee, S. H.; Choi, J. I.; Park, S. J.; Lee, S. Y.; Park, B. C. Display of bacterial lipase on the *Escherichia coli* cell surface by using FadL as an anchoring motif and use of the enzyme in enantioselective biocatalysis. *Appl. Environ. Microbiol.* **2004**, *70*, 5074−5080.
(31) Liu, W. S.; Zhao, H. Y.; Jia, B.; Xu, L.; Yan, Y. J. Surface display of active lipase in *Saccharomyces cerevisiae* using Cwp2 as an anchor protein. *Biotechnol. Lett.* **2010**, *32*, 255−260.
(32) Hatzinikolaou, D. G.; Macris, J. B.; Christakopoulos, P.; Kekos, D.; Kolisis, F. N.; Fountoukidis, G. Production and partial characterisation of extracellular lipase from *Aspergillus niger*. *Biotechnol. Lett.* **1996**, *18*, 547−552.
(33) Namboodiri, V. M. H.; Chattopadhyaya, R. Purification and biochemical characterization of a novel thermostable lipase from *Aspergillus niger*. *Lipids* **2000**, *35*, 495−502.
(34) Sugihara, A.; Shimada, Y.; Tominaga, Y. Purification and characterization of *Aspergillus niger* lipase. *Agric. Biol. Chem.* **1998**, *52*, 1591−1592.

Received for review March 2, 2010. Revised manuscript received August 26, 2010. Accepted August 27, 2010. This work was funded by the National High Technology Research and Development Program of China (863 Program) (No. 2006AA020203, 2007AA05Z417, 2007AA100703, 2009AA03Z232, and 2010AA101501), the Program for New Century Excellent Talents in University (NCET-07-0336), and the Natural Science Foundation of Hubei Province (2009CDA046).