JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Efficient Display of Active *Geotrichum* sp. Lipase on *Pichia pastoris* Cell Wall and Its Application as a Whole-Cell Biocatalyst To Enrich EPA and DHA in Fish Oil

Xiao-Xing Pan, Li Xu,* Yan Zhang, Xiao Xiao, Xiao-Feng Wang, Yun Liu, Hou-jin Zhang, and Yun-Jun Yan*

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

Supporting Information

ABSTRACT: *Geotrichum* sp. lipase (GSL) was first displayed on the cell wall of *Pichia pastoris* on the basis of the a-agglutinin anchor system developed in *Saccharomyces cerevisiae*. Surface display levels were monitored using Western blotting, immunofluorescence miscroscopy, and fluorescence-activated cell sorting analysis. Lipase activity of the yeast whole cells reached a maximum at 273 ± 2.4 U/g of dry cells toward olive oil after 96 h of culture at 30 °C, with optimal pH and temperature at 7.5 and 45 °C, respectively. Displayed GSL exhibited relatively high stability between pH 6.0 and 8.0 and retained >70% of the maximum activity. The surface-displayed lipase retained 80% of its original activity after incubation at 45 °C for 4 h. Moreover, the GSL-displaying yeast whole cells were then used as a biocatalyst to enrich eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) from fish oil on the basis of selective hydrolysis. As a result, EPA and DHA increased from 1.53 and 24.1% in the original fish oil to 1.85 and 30.86%, which were increases of 1.21- and 1.29-fold, respectively. The total yield of EPA and DHA reached 46.62%.

KEYWORDS: *Geotrichum sp. lipase, Pichia pastoris, surface display, whole-cell biocatalyst, polyunsaturated fatty acid*

INTRODUCTION

It is well-known that long-chain polyunsaturated fatty acids (PUFAs) have beneficial effects on cardiovascular disease, pregnancy, and brain and nervous system development.[1−3] Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) have drawn increasing attention in recent years as the main active constituents in functional oils.[4,5] Therefore, it is important to enrich EPA and DHA from PUFA-containing oils. Thus far, there have been many reports regarding the concentration of PUFAs from fish oil, marine algae, and fishery byproducts using different chemical methods, such as urea inclusion complexation, molecular distillation, low-temperature fractional crystallization, liquid−liquid extraction−fraction, high-performance liquid chromatography, and salt solubility methods.[6,7] However, most of these chemical methods for concentrating PUFAs are not selective for fatty acids and consume large amounts of energy.[8] Methods catalyzed by lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) are promising alternative strategies that have been widely studied and have shown energy-saving and nutritionally desirable functionalities, which have mild operation conditions (temperature < 70 °C, normal atmospheric pressure) and narrow control of product distribution and are environmentally favorable.[8−13]

Lipase from the fungus *Geotrichum* sp. (GSL), a fatty acid-specific lipase, is highly selective toward substrates containing *cis*-Δ-9 unsaturated fatty acids, such as oleic, linoleic, and α-linolenic acid, whereas it discriminates against EPA (20:5-5*c*,8*c*,11*c*,14*c*,17*c*) and DHA (22:6-4*c*,7*c*,10*c*,13*c*,16*c*,19*c*).[13−17] Thus, GSL has an added advantage for the enrichment of PUFAs because non-PUFAs are partially released through selective hydrolysis, whereas the targeted fatty acids DHA and EPA can be retained in monoacylglycerol (MAG), diacylglycerol (DAG), and triacylglycerol (TAG).[13−16] Nevertheless, the high cost and relatively low operational stability of free enzyme hinders large scale industrialization of this technology. To overcome these limitations, GSL was immobilized and successfully applied to enrich EPA and DHA from fish oil in our previous work,[18] but the immobilization processes and purification of GSL from culture broth were still complex and tedious and did not markedly reduce production costs.

Surface-displayed lipase (self-immobilized on the surface of microorganisms), a type of whole-cell biocatalyst, can be used to avoid the laborious purification and immobilization and enable direct application of the lipase expression cells.[19] Among them, self-immobilized lipases on the surface of yeast are of great interest because of the mechanism of post-translational modification and easy culture.[20] Up to now, *Candida antarctica* lipase B,[21] *Rhizomucor miehei* lipase,[22] *Rhizopus oryzae* lipase,[23] and *Yarrowia lipolytica* lipases[24] have been shown to be displayed on the cell surface of *Pichia pastoris* and have been directly used in several applications. However, there have been few studies examining self-immobilized GSL on the surface of *P. pastoris* cells with high catalytic activity toward native macromolecular substrates as a whole-cell biocatalyst, although

Received: April 25, 2012
Revised: August 29, 2012
Accepted: August 30, 2012
Published: August 30, 2012

Table 1. Primers Used in This Study

PCR products	primer sequences[a]	templates
Aga1	5′-CAGTGGATCCATGACATTATCTTTCGCTCA-3′ 5′-CAGTGAATTCTTAACTGAAAATTACATTGCAAGCAACT-3′	EBY100 total genomic DNA
Aga2-Xa-HA-(G_4S)$_3$ (*AXHG*)	5′-TCATACAATTCTTGATATTCACAATGCAGTTACTTCGCTGTTTTTCAATA-3′ 5′-GTCTAAGGCTACAAACCTCGAG GCTAGCAGAACCACCACCACCAGAACC-3′	pCT-ΔGSL
FLD1	5′-GTTAGATCTGCATGCAGGAATCTCTG-3′ 5′-TATTGAAAAACAGCGAAGTAACTGCATTGTGAATATCAAGAATTGTATG-3′	pMD-FLD1
AOX1TT	5′-GGTTCTGGTGGTGGTGGTTCTGCTAGC CTCGAGGTTTGTAGCCTTAGAC-3′ 5′-GCTAGATCTGCACAAACGAAGGTCTCAC-3′	pPIC3.5k
FLD1-AXHG (*FA*)	5′--GTTAGATCTGCATGCAGGAATCTCTG-3′ 5′-GTCTAAGGCTACAAACCTCGAG GCTAGCAGAACCACCACCACCAGAACC-3′	*FLD1* gene *AXHG* gene
FA-AOX1TT (*FAA*)	5′-GTTAGATCTGCATGCAGGAATCTCTG-3′ 5′-GCTAGATCTGCACAAACGAAGGTCTCAC-3′	*FA* gene *AOX1TT* gene
ΔGSL	5′-CTTGCTAGCCAAGCTCCTACGGCTGTTCT-3′ 5′-CTTCTCGAGTTAACCGTAGAGATTAACGT-3′	pCT-ΔGSL

[a]All restriction sites are underlined.

GSL was successfully displayed on the cell surface of *Saccharomyces cerevisiae* in our previous study.[25]

In this context, we attempted to construct a novel expression system for displaying GSL on the cell wall of *P. pastoris*, a cellular host with high cell densities.[26] Through homology modeling using Swiss-MODEL, the catalytic triad active center of GSL (Ser^{236}-Glu^{373}-His^{482}) was found close to the C-terminus. Therefore, the N-terminal anchor protein was selected for self-immobilization of GSL to avoid conformation variations at the active center.[27,28] In previous studies, the anchor protein a-agglutinin derived from *S. cerevisiae* was shown to be capable of immobilizing proteins in the cell wall of *P. pastoris* with high efficiency,[29] whereas there have been no reports related to displaying the enzyme using the anchor protein a-agglutinin for biocatalysis.

In this study, a display system based on a-agglutinin was successfully constructed. GSL was first self-immobilized on the cell wall of *P. pastoris*, and the characteristics of the displayed lipase including optimum temperature, optimum pH, and thermostability were further investigated. Importantly, the derivative lipase was successfully applied in the enrichment of DHA and EPA from fish oil so as to assess its potential in industrial application as whole-cell catalysts.

MATERIALS AND METHODS

Strains, Vectors, and Culture Media. *S. cerevisiae* strain EBY100 (*MATa GAL1-AGA1::URA3 ura3-52 trp1 leu2Δ1 his3Δ200 pep4::HIS2 prb1Δ1.6R can1 GAL*) (a gift from Dr. Eric T. Boder, Department of Chemical and Biomolecular Engineering, University of Tennessee, Knoxville, TN, USA), used to amplify the *Aga1* gene, was grown in YPD medium (2% (w/v) tryptone, 1% (w/v) yeast extract, 1% (w/v) dextrose). The plasmids pMD-FLD1 and pCT-ΔGSL, which contained the *FLD1* and *Aga2-Xa-HA-(G_4S)$_3$* genes, respectively, were constructed in our laboratory. *Escherichia coli* DH5α (*F$^-$ endA1 hsdR17* (r_K^-/m_K^+) *supE44 thi-l λ$^-$ recAl gyrA96 ΔlacU169* (Φ80*lacZD*M15)) was used as the host for DNA manipulation. *P. pastoris* strain X-33 and recombinant X-33 were grown in YPD medium (2% (w/v) tryptone, 1% (w/v) yeast extract, 1% (w/v) dextrose), YPDS medium (2% (w/v) tryptone, 1% (w/v) yeast extract, 1% (w/v) dextrose, 1 M sorbitol, 2% agar) containing 100 μg/mL Zeocin, BMGY medium (2% (w/v) tryptone, 1% (w/v) yeast extract, 50 mM potassium phosphate, pH 7.0, 1.34% yeast nitrogen base (YNB), 4×10^{-5}% biotin, 1% glycerol), or BMMY medium (2% (w/v) tryptone, 1% (w/v) yeast extract, 50 mM potassium phosphate, pH 7.0, 1.34% YNB, 4×10^{-5}% biotin, 1% methanol). *P. pastoris* strain X-33, the vector pPICZαA, and pPIC3.5k were purchased from Invitrogen (Carlsbad, CA, USA). PrimeSTAR HS DNA Polymerase, restriction enzymes, and DNA ligation kit ver. 2.0 were purchased from TaKaRa Biotechnology Co., Ltd. (Shiga, Japan). Factor Xa protease was purchased from New England BioLabs (Ipswich, MA, USA). Fish oil was purchased from Yuwang Pharmaceutical Co., Ltd. (Shandong, China). Its main properties include a saponification value of 105.068 mg KOH/g and an acid value of 0.231 mg KOH/g. Its main fatty acid composition was 8.51% myristic acid, 22.11% palmitic acid, 1.74% stearic acid, 2.69% tetracosanoic acid, 3.03% palmitoleic acid, 8.95% oleic acid, 2.14% linoleic acid, 3.61% arachidonic acid, 10.17% docosapentenoic acid, 1.53% EPA, and 24.1% DHA.

Construction of Plasmids. The *Aga1* gene was amplified using EBY100 total DNA as a template. The amplified products were ligated into the vector pPIC3.5k at the *Bam*HI and *Eco*RI sites, resulting in recombinant plasmid designated pPIC3.5k-Aga1. The fragment of *Sac*I−*Not*I with gene *Aga1* from the plasmid pPIC3.5k-Aga1 was inserted into the vector pPICZαA, yielding pPICZX-Aga1. The *Aga2-Xa-HA-(G_4S)$_3$* (*AXHG*) gene was amplified using the plasmid pCT-ΔGSL as a template and then fused with the *FLD1* gene from pMD-FLD1 at the N-terminus and the *AOX1TT* gene at the C-terminus using overlap extension PCR. The resulting fragment *FLD1-AXHG-AOX1TT* (*FAA*) was digested with *Bgl*II and inserted into the retroplasmid pPICZX-Aga1 to avoid an interaction between *pAOX1* and *pFLD1*. The resulting plasmid construct was named pPICZXFa. The *ΔGSL* gene (*GSL* gene without signal peptide) was cloned from the plasmid pCT-ΔGSL. Next, it was cut using *Nhe*I and *Xho*I and introduced into the plasmid pPICZXFa, resulting in pPICZXFa-ΔGSL. All PCRs were carried out using PrimeSTAR HS DNA Polymerase. All recombinant plasmids constructed in this study were transformed into *E. coli* DH5α, and the transformants were screened in LB medium containing 50 μg ampicillin/mL. All plasmid sequences were confirmed through DNA sequencing by Sangon (Shanghai, China), and all primers used in this study are shown in Table 1.

Yeast Transformation and Inducible Expression of Displayed GSL. Plasmids pPICZXFa-ΔGSL and pPICZXFa were

linearized using *Bst*XI and transformed into *P. pastoris* X-33 using electroporation with a Gene Pulser apparatus (Bio-Rad, Hercules, CA, USA) (1500 V, 25 μF, and 200 Ω, using a 0.2 cm cuvette) following the manufacturer's instructions (Invitrogen). Transformants were screened on YPDS plates containing 100 μg Zeocin/mL, which had been incubated at 30 °C for 2−3 days.

Individual colonies were inoculated into YPD medium and incubated overnight at 30 °C and 200 rpm. Next, the culture was transferred into BMGY medium and cultured under the same conditions until reaching an OD_{600} of 2.0−6.0. Cells were harvested by centrifugation at 4 °C and resuspended into BMMY medium for inducible expression. Fresh methanol was added to a final concentration of 1% (v/v) every 24 h. The recombinant yeasts were harvested, washed, and resuspended in sterile water for activity assay. Each sample contained 150 mg of dried weight cells of the displayed GSL per milliliter.

Immunofluorescence Assay. An immunofluorescence assay was performed using a mouse monoclonal anti-hemagglutinin (HA) tag and a fluorescein isothiocyante (FITC)-conjugated goat anti-mouse IgG. The mouse monoclonal anti-HA tag was used as the primary antibody against the displayed GSL. The harvested yeast cells were washed with PBS (pH 7.4) containing 1 mg/mL bovine serum albumin (BSA) and then incubated with the primary antisera in a dilution of 1:100 in phosphate-buffered saline (PBS) for 30 min on ice, washed three times with PBS (pH 7.4) containing 1 mg/mL BSA, and then incubated with FITC-conjugated goat anti-mouse IgG diluted 1:200 on ice for 30 min in the dark. The cells were then washed with PBS, and the signal was detected using fluorescence microscopy (Nikon) or the Cell Lab Quanta SC flow cytometer (Beckman Coulter, Fullerton, CA, USA). For the latter, the data were analyzed using CXP software (Beckman Coulter). Cells transformed with pPICZXFa were also processed in the same manner to serve as the negative controls.

Western Blot Analysis. GSL with an HA epitope tag was cleaved from the cell wall using factor Xa protease (New England Biolabs) as follows.[30,31] Induced cells were harvested, washed twice with ice-cold water, resuspended in 50 μL of 20 mM Tris-HCl containing 100 mM NaCl and 2 mM $CaCl_2$ (pH 8.0), and treated with 1 U of facter Xa protease at 23 °C for 6 h. The collected lipase conjugated with the HA epitope tag in the supernatant was boiled in Laemmli buffer for 5 min after removal of cell debris by centrifugation at 12000*g* for 3 min at 4 °C. The resulting proteins were separated by 12% SDS-PAGE using the method of Laemmli[31] and transferred to a polyvinylidene fluoride (PVDF) membrane (EMD Millipore, Billerica, MA, USA) in 25 mM Tris, 192 mM glycine, and 20% (v/v) methanol using a Transblot apparatus (Beijing Liuyi Instrument Factory, Beijing, China) for 1 h at 200 mA according to the method of Towbin et al.[32] The membranes were blocked with 5% (w/v) nonfat dried milk in Tris-buffered saline (pH 8.0) and Tween 20 (TBST, 20 mM Tris, 137 mM NaCl, 0.1% (v/v) Tween 20), incubated with anti-HA mouse monoclonal antibody (Tiangen Biotech (Beijing) Co., Ltd., Beijing, China) in a 1/1000 dilution in TBST for 2 h at room temperature, washed with TBST, and then incubated for 2 h with horseradish peroxidase-conjugated goat anti-mouse IgG (Tiangen Biotech) in a 1/2000 dilution in TBST at room temperature. The blots were developed in diaminobenzidine (DAB, Pierce Biotechnology) and 0.01% H_2O_2 and then detected with enhanced chemiluminescence (ECL, Bio-Rad).

Plate Assay. Transformants were inoculated on BMMY plates supplemented with 1% (v/v) olive oil and 0.002% (v/v) rhodamine B for 3 days. Next, 200 μL of methanol was added every 24 h to induce expression of the AGA1− and AGA2−lipase fusion proteins. Lipase activity was examined on the basis of the fluorescent halo that had formed around the colony.

Activity Assay of the Displayed GSL. The activity of the displayed GSL was determined using the acid−base titration method.[33] The reaction mixture containing 4 mL of substrate (25% (v/v) olive oil emulsified with 2% (w/v) polyvinyl alcohol solution), 5 mL of 50 mM phosphate buffer (pH 7.5), and 1 mL of resuspended cells was incubated at 45 °C for 10 min with 150 rpm of orbital shaking. The reaction was immediately terminated by the addition of 15 mL of a cold acetone/ethanol mixture (1:1, v/v). Liberated free fatty acid amount was measured by titration with 50 mM NaOH using phenolphthalein as an indicator. One enzyme unit (U) was defined as the amount of dry cells that liberated a 1 μmol equivalent of fatty acid per minute under the assay conditions. The dry weight of cells was determined as follows. The cells were harvested from culture broth by centrifuging in a preweighed centrifuge tube at 8000 × *g* for 15 min, washed, resuspended in distilled water, and divided into two equal parts. Finally, one equivalent was dried at 105 °C to a constant weight, and the other equivalent was used to estimate lipase activity.

Characterization of the Displayed GSL. To characterize the displayed GSL, each sample contained 150 mg of dried weight of cells of the displayed GSL. The effects of temperature and pH on lipase activity were measured as described below.

The optimum temperature was determined in the same substrate solution at different temperatures ranging from 30 to 60 °C for 10 min under pH 7.5. To study the thermostability of the displayed GSL, 1 mL samples containing 150 mg of dried weight cells, were incubated at 45 °C for up to 6 h in 50 mM potassium phosphate buffer (pH 7.5). Residual activity was measured each hour using titrimetry with olive oil emulsion as the substrate. Each test condition was measured in triplicate.

To determine the optimum pH of the displayed GSL, different pH buffers including 50 mM potassium phosphate buffer (pH 6.0−7.5), 50 mM Tris-HCl buffer (pH 8.0−9.0), and 50 mM carbonate−bicarbonate buffer (pH 9.5−10.0) were used. The effect on the displayed GSL was dertermined by measuring the remaining activity after incubation for 10 min at 45 °C in the different pH buffers. Each condition was measured in triplicate.

Hydrolysis of Fish Oil for Enrichment of EPA and DHA with the Displayed GSL. The reaction mixture was similar to that described previously except that substrate was replaced by fish oil emulsion.[18] The initial reaction system containing 1 mL of fish oil and 3 mL of polyvinyl alcohol solution (PVA, 2%, w/v) was emulsified at 4000 rpm for 10 min by using a homogenizer and placed in a 50 mL stoppered conical flask. Next, the 4 mL of emulsified fish oil was mixed with 5 mL of 50 mM K_2HPO_4/KH_2PO_4 buffer (pH 7.5) and preincubated at 45 °C and 200 rpm for 10 min in a water bath shaker. Next, 1 mL of cell suspension containing 150 mg dried weight cells of the displayed GSL was added to these emulsified substrates to start the hydrolysis reaction, which was carried out in a rocking incubator at 150 rpm and 45 °C. The initial reaction rate was measured during the first 2 h. Samples were removed from the reaction mixture every 2 h to assay the degree of hydrolysis and fatty acid compositions in the glyceride fraction. Fractionation of glycerides in reaction mixture was extracted with 100 mL of *n*-hexane after the addition of 50 mL of ethanolic KOH (500 mM) to the hydrolysis reaction mixture. EPA and DHA in the glyceride fraction were identified and quantified using Agilent 7890A/5975C gas chromatography (GC) and mass spectrometry (GC-MS) equipped with a capillary column (DB-WAX, 30 m × 250 μm × 0.25 μm).

The GC-MS program was performed as follows. The oven temperature was increased from 150 to 250 °C at a rate of 10 °C/min, which was maintained for 10 min. The temperatures of the injector and connector were set at 250 and 260 °C, respectively. Split sampling was employed with an injection volume of 1 μL and a split ratio of 50:1. The MS program was performed as follows. The time of solvent delay was 3 min, with an He flow rate of 1 mL/min. The temperatures of the MS source and MS quad were 230 and 150 °C, respectively. The detectable molecular weight was in the range from 30 to 500.

The hydrolysis degree is described by the equation

$$\text{hydrolysis degree} = \frac{AV_t - AV}{SV - AV} \times 100\%$$

in which SV and AV indicate the saponification value and acid value of the original fish oil, respectively, and AV_t indicates the acid value of the reaction mixture at a designated time.[18] Each condition was evaluated in triplicate, and each experiment was performed three times. Results are shown as the mean ± standard deviation (SD) of triplicate

measurements for three separate experiments. Group comparisons were performed using one-factor analysis of variance and a paired *t* test when appropriate. Calculated *P* values of <0.05 were considered to be significant.

Recycling of the Displayed GSL. Hydrolysis was repeated for the fish oil using the *P. pastoris* X-33/pPICZXFa-ΔGSL cells. After one cycle of hydrolysis, self-immobilized cells were recovered from the reaction mixture and washed with 50 mM K_2HPO_4/KH_2PO_4 buffer (pH 7.5). The recovered yeast cells were repeatedly applied to the hydrolysis of fish oil as the catalyst for the next cycle. Recyclability was characterized by residual hydrolysis degree in subsequent reactions relative to that of the first reaction.

RESULTS AND DISCUSSION

Construction of a *P. pastoris* Surface Display System. To self-immobilize active lipases for practical application as important whole-cell biocatalysts, a novel *P. pastoris* surface display system was constructed. As shown in Figure 1, the *Aga1*

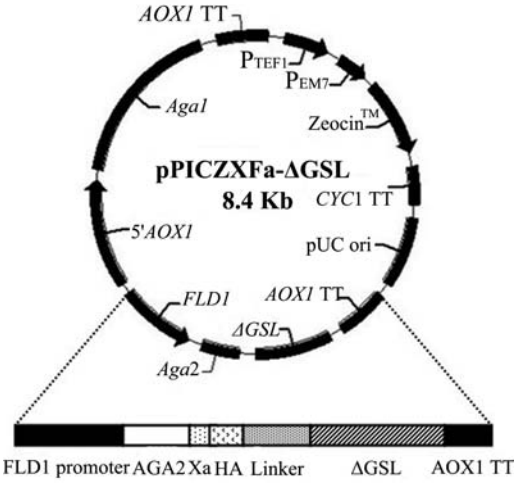

Figure 1. Schematic diagram of plasmid pPICZXFa-ΔGSL encoding the AGA1 protein and AGA2−ΔGSL fusion protein.

gene was placed under the direction of the alcohol oxidase 1 promoter (*pAOX1*), whereas the *Aga2-ΔGSL* fusion gene was under the control of the formaldehyde dehydrogenase 1 promoter (*pFLD1*). The resulting recombinant plasmid was named pPICZXFa-ΔGSL. Both *pAOX1* and *pFLD1* could be strongly induced by methanol, and their coexistence on one vector did not affect the efficiency of protein expression.[34] Xa, downstream of the *Aga2* gene, was designed by cleaving displayed proteins from the cell wall. The HA tag served as an epitope tag for immunofluorescent localization. The linker sequence $(G_4S)_3$ was used to prevent possible functional interference between AGA2 and lipase. The *ΔGSL* gene was inserted at the C-terminus fused to the *Aga2* gene. When induced with methanol, transformants harboring pPICZXFa-ΔGSL expressed the AGA2−ΔGSL fusion protein and AGA1. The fusion proteins tethered the AGA1 protein via disulfide bridges, and the AGA1 covalently bonded to the cell wall through a glycosylphosphatidylinositol (GPI) anchor attachment signal sequence.

Expression and Localization of GSL in *P. pastoris*. As shown in Figure 2A, clear halos were observed around the *P. pastoris* X-33/pPICZXFa-ΔGSL cells, whereas no halo was observed around cells harboring the control plasmid pPICZXFa. This suggests that the displayed lipase was

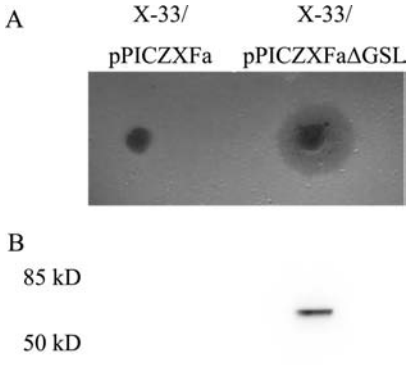

Figure 2. (A) Halo formation of lipase-displaying yeast on BMMY agar plate containing olive oil and rhodamine B. Clear halos were observed around *P. pastoris* X-33/pPICZXFa-ΔGSL, whereas there was no halo around the control *P. pastoris* X-33/pPICZXFa colony. (B) Western blot analysis of GSL with an HA tag cleaved from *P. pastoris* X-33/pPICZXFa-ΔGSL by factor Xa protease detected using mouse anti-HA monoclonal antibodies. The position of molecular weight markers is indicated.

functionally active. To further explore the molecular weight and expression pattern of the recombinant GSL, Western blot analysis of GSL with an HA tag cut from the cell wall of recombinant *P. pastoris* X-33/pPICZXFa-ΔGSL by factor Xa protease was performed utilizing mouse anti-HA monoclonal antibodies. As shown in Figure 2B, a specific band of approximately 60 kDa for GSL with an HA tag was detected as expected. In contrast, no specific bands were observed for the control transfectants. These results suggest that GSL and HA molecules were coexpressed on the cell surface of *P. pastoris* X-33/pPICZXFa-ΔGSL transfectants.

Localization of GSL in the *P. pastoris* X-33 transformants was analyzed by indirect immunofluorescence labeling using the mouse anti-HA tag antisera and FITC-conjugated goat anti-mouse IgG and flow cytometry. As shown in Figure 3, *P.*

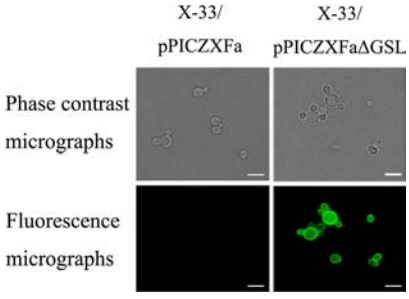

Figure 3. Immunofluorescent labeling of GSL on the cell surface of *P. pastoris*. Cells harboring the plasmid pPICZXFa-ΔGSL were immunologically labeled with the anti-HA antibody as the first antibody and FITC-conjugated anti-IgG as the second antibody. Control cells (X-33/pPICZXFa) showed no fluorescence. Scale bar = 10 μm.

pastoris X-33 cells harboring the plasmid pPICZXFa-ΔGSL exhibited clear green fluorescence localizing on the surface due to the binding of anti-HA tag antibody followed by binding of the FITC-conjugated secondary antibody. However, the cell surface of *P. pastoris* X-33 harboring the control plasmid pPICZXFa showed no fluorecence.

Additionally, flow cytometry analysis demonstrated that >99.3% of *P. pastoris* X-33/pPICZXFa-ΔGSL cells expressed

the fusion protein on their cell surface, whereas the intensities of the fluorescence signals with the *P. pastoris* X-33/pPICZXFa cells could be ignored as background (Figure 4). All of these results confirmed that the *Geotrichum* sp. lipase was successfully displayed on the yeast cell surface via the small cell wall anchor domain AGA2.

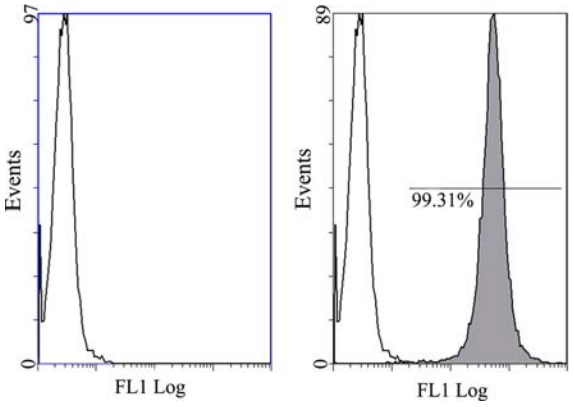

Figure 4. FACS analysis of surface-displayed GSL. Expression levels on the surfaces of X-33 were determined using flow cytometry. Approximately 99.31% of the cells expressed the fusion protein on their cell walls. Unlabeled cells (negative control) are shown as unfilled histograms.

Functional Activity of Surface-Displayed GSL. After confirming the successful display of GSL on the surface of *P. pastoris*, we examined its functional activity to estimate its potential application value. Notably, the *Pichia* displaying GSL could efficiently hydrolyze the macromolecular substrate olive oil, and its maximal activity reached >273 ± 2.4 U/g of dry cell. However, GSL displayed on the cell surface of *S. cerevisiae* exhibited activity toward only the small molecular substances tributyrin and *p*-nitrophenyl ester, which may have been caused by hyperglycosylation and agreed with the results of previous studies.[25,27,35] These results indicate that self-immobilized GSL on the surface of *P. pastoris* has the correct spatial configuration and could catalyze macromolecular substrates with high efficiency due to more convenient accessibility of the substrates.

Characterization of Surface-Displayed GSL. To further estimate the potential application of display GSL as whole-cell catalysts, the optimal pH, optimal temperature, and thermostability of cell surface displayed lipase was investigated using olive oil emulsion as substrate. As shown in Figure 5A, the optimum pH was 7.5, which was similar to that experimentally observed for recombinant lipases from different *Geotrichum* having optimum pH in the range of pH 7.0−8.0.[36−39] More importantly, the displayed GSL showed relatively high stability in acidic pH values and remained >70% of the maximal activity when pH values ranged from 6.0 to 8.0. As shown in Figure 5B, the optimum temperature of the displayed lipase was 45 °C. Most importantly, the displayed GSL on the *P. pastoris* showed excellent thermostability and remained >80% of the original activity after incubation at 45 °C for 4 h at pH 7.5 using olive oil as a substrate. The enhancement of thermal stability coincided with the previous results due to the protection of the cell wall structure.[23] All results described above were promising for practical application in industry.

Application of the GSL Whole-Cell Biocatalysts for Enrichment of EPA and DHA. Recombinant X-33/pPICZXFa-ΔGSL yeast cells were harvested after 96 h of

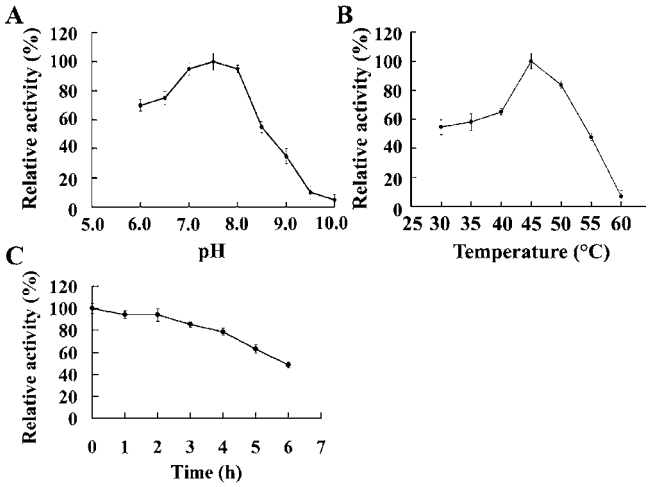

Figure 5. Effects of pH (A) and temperature (B) on the activities of the surface-displayed GSL. (A) The effect of pH on the activities of surface-displayed GSL was calculated by assuming the activity obtained at 45 °C using olive oil as a substrate at different pH values ranging from 6.0 to 10.0 after 10 min of incubation at the optimal temperature of 45 °C. Relative activity was calculated by setting to 100% the activity obtained at pH 7.5. All values are represented as the mean ± standard deviation (SD) of three independent experiments. (B) The effect of temperature on the activity of surface-displayed GSL was calculated by comparing the activity obtained at pH 7.5. (C) The thermal stability of the displayed GSL was measured using titration with olive oil as the substrate at every 1 h of incubation at 45 °C in 50 mM potassium phosphate buffer (pH 7.5). Relative activity was calculated by setting the original activity at 100%. All values are represented as the mean ± standard deviation (SD) of three independent experiments.

culture and used to catalyze the hydrolysis of fish oil for enriching the DHA and EPA as whole-cell biocatalysts. Similarly to free GSL,[36] self-immobilized lipase showed specificity toward its natural substrate, fish oil, and the contents of EPA and DHA in glyceride fraction were increased along with hydrolysis degree. After 14 h of treatment, maximum hydrolysis reached 28.6%. The contents of EPA and DHA increased from 1.53 ± 0.145 and 24.1 ± 1.002% in original fish oil to 1.85 ± 0.148 and 30.86 ± 1.11%, which were 1.21- and 1.29-fold increases, respectively. The total yield of EPA and DHA reached approximately 46.62% (Figure 6A). As shown in Figure 6B, the contents of DHA in fish oil before hydrolysis showed a significant difference when compared with that of the glyceride fraction after hydrolysis by displayed lipase ($P < 0.05$). The contents of EPA in fish oil before hydrolysis also showed a significant difference when compared with that of the glyceride fraction after hydrolysis ($P < 0.05$).

To evaluate the operational stability of the whole-cell catalyst, repeated hydrolysis reactions were preliminarily examined. The result showed that >50% hydrolysis activity was retained after five subsequent reactions, suggesting that the self-immobilized lipase exhibits a relatively good operational stability.

Thus, this method is not only a convenient method for preparing a stable whole-cell biocatalyst GSL with native activity but also a useful strategy for immobilizing industrial lipases, particularly for hyperglycosylated lipases in *S. cerevisiae*, with high stability and correct configuration as whole-cell biocatalysts in a cost-effective manner. Interestingly, displayed GSL has great potential applications as whole-cell catalysts for

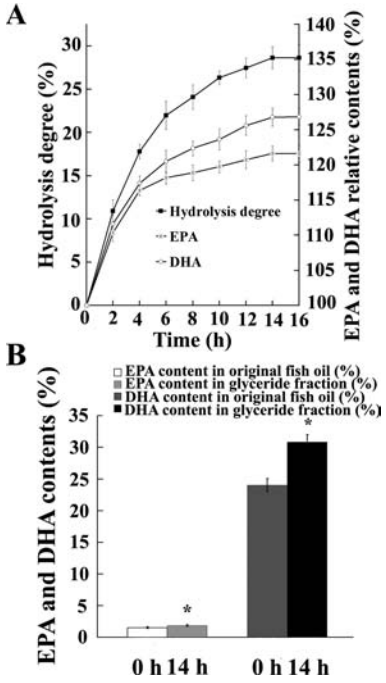

Figure 6. (A) Relationship between degree of hydrolysis and the relative contents of EPA and DHA compared with their own initial contents in original fish oil before hydrolysis, respectively. Relative contents of EPA and DHA were calculated by assuming that the original content in fish oil before hydrolysis was 100%. All values are represented as the mean ± standard deviation (SD) of three independent experiments. (B) The contents of DHA and EPA in the glyceride fraction after 14 h of hydrolysis by displayed lipase were compared with those of fish oil before hydrolysis. Group comparisons were performed with the one-factor analysis of variance and a paired *t* test when appropriate. Calculated *P* values of <0.05 were considered to be significant (*).

enriching PUFAs from natural oils in the food and pharmaceutical industries.

■ ASSOCIATED CONTENT

Supporting Information

The quality of fish oil before hydrolysis or after hydrolysis by displayed GSL was further determined by using Agilent 7890A/5975C gas chromatography (GS) and mass spectrometry (GC-MS), respectively. The quantitative and qualitative analysis of fatty acid in glyceride fraction is shown in supplementary Figures 1 and 2. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*(L.X.) Phone: +86-027-87792214-12. Fax: +86-027-87792213. E-mail: xuli1881@yahoo.cn. (Y.-J.Y.) Phone: +86-027-87792213. Fax: +86-027-87792213. E-mail: yanyunjun@mail.hust.edu.cn.

Funding

We acknowledge financial support by the National Natural Science Foundation of People's Republic of China (NSFC) (No. 31070089 and 31170078), the National High Technology Research Development Program of People's Republic of China (863 Program) (No. 2011AA02A204), and the Innovation Foundation of HUST (No. 2011TS100).

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

Many thanks are given to Hong Chen (Analytical and Testing Center of Huazhong University of Science and Technology) for her valuable assistance in testing the samples by GC-MS and Fenfen Fu for aiding with flow cytometry analysis.

■ REFERENCES

(1) Holub, D. J.; Holub, B. J. Omega-3 fatty acids from fish oils and cardiovascular disease. *Mol. Cell. Biochem.* **2004**, *263*, 217−225.

(2) Thies, F.; Garry, J. M.; Yaqoob, P.; Rerkasem, K.; Williams, J.; Shearman, C. P.; Gallagher, P. J.; Calder, P. C.; Grimble, R. F. Association of n-3 polyunsaturated fatty acids with stability of atherosclerotic plaques: a randomized controlled trial. *Lancet* **2003**, *361*, 477−485.

(3) von Schacky, C. Omega-3 fatty acids and cardiovascular disease. *Curr. Opin. Clin. Nutr. Metab. Care* **2007**, *10*, 129−135.

(4) Belayev, L.; Khoutorova, L.; Atkins, K. D.; Eady, T. N.; Hong, S.; Lu, Y.; Obenaus, A.; Bazan, N. G. Docosahexaenoic acid therapy of experimental ischemic stroke. *Transl. Stroke Res.* **2011**, *2*, 33−41.

(5) Holub, B. J. Docosahexaenoic acid (DHA) and cardiovascular disease risk factors. *Prostaglandins, Leukotrienes Essent. Fatty Acids* **2009**, *81*, 199−204.

(6) Chakraborty, K.; Paulraj, R. Eicosapentaenoic acid enrichment from sardine oil by argentation chromatography. *J. Agric. Food Chem.* **2007**, *55*, 7586−7595.

(7) Guil-Guerrero, J. L.; López-Martínez, J. C.; Rincón-Cervera, M. A.; Campra-Madrid, P. One-step extrction and concentration of polyunsaturated fatty acids from fish liver. *J. Am. Oil Chem. Soc.* **2007**, *84*, 357−361.

(8) Chakraborty, K.; Vijayagopal, P.; Chakraborty, R. D.; Vijayan, K. K. Preparation of eicosapentaenoic acid concentrates from sardine oil by *Bacillus circulans* lipase. *Food Chem.* **2010**, *120*, 433−442.

(9) Fajardo, A. R.; Cerdán, L. E.; Medina, A. R.; Martínez, M. M.; Peña, E. H.; Grima, E. M. Concentration of eicosapentaenoic acid by selective esterification using lipases. *J. Am. Oil Chem. Soc.* **2006**, *83*, 215−221.

(10) Hayes, D. Enzyme-catalyzed modification of oilseed materials to produce eco-friendly products. *J. Am. Oil Chem. Soc.* **2004**, *81*, 1077−1103.

(11) Shimada, Y.; Sugihara, A.; Tominaga, Y. Enzymatic purification of polyunsaturated fatty acids. *J. Biosci. Bioeng.* **2001**, *91*, 529−538.

(12) Wanasundara, U. N.; Shahidi, F. Lipase-assisted concentration of n-3 polyunsaturated fatty acids in acylglycerols from marine oils. *J. Am. Oil Chem. Soc.* **1998**, *75*, 945−951.

(13) Holmquist, M. Insights into the molecular basis for fatty acyl specificities of lipases from *Geotrichum candidum* and *Candida rugosa*. *Chem. Phys. Lipids* **1998**, *93*, 57−65.

(14) Jensen, R. G.; Galluzzo, D. R.; Bush, V. J. Selectivity is an important characteristic of lipases (acylglycerol hydrolases). *Biocatalysis* **1990**, *3*, 307−317.

(15) Loo, J. L.; Lai, O. M.; Long, K.; Ghazali, H. M. Fatty acid preference of mycelium-bound lipase from a locally isolated strain of *Geotrichum candidum*. *World J. Microbiol. Biotechnol.* **2007**, *23*, 1771−1778.

(16) Shimada, Y.; Maruyama, K; Nakamura, M.; Nakayama, S.; Sugihara, A.; Tominaga, Y. Selective hydrolysis of polyunsaturated fatty acid-containing oil with *Geotrichum candidum* lipase. *J. Am. Oil Chem. Soc.* **1995**, *72*, 1577−1581.

(17) Treichel, H.; Oliveira, D.; Mazutti, M. A.; Luccio, M. D.; Oliveira, J. V. A review on microbial lipases production. *Food Bioprocess. Technol.* **2010**, *3*, 182−196.

(18) Yan, J. Y.; Liu, S. X.; Hu, J.; Gui, X. H.; Wang, G. L.; Yan, Y. J. Enzymatic enrichment of polyunsaturated fatty acids using novel lipase preparations modified by combination of immobilization and fish oil treatment. *Bioresour. Technol.* **2011**, *102*, 7154−7158.

(19) Kondo, A.; Ueda, M. Yeast cell-surface display − applications of molecular display. *Appl. Microbiol. Biotechnol.* **2004**, *64*, 28−40.
(20) Cregg, J. M.; Cereghino, J. L.; Shi, J.; Hiqqins, D. R. Recombinant protein expression in *Pichia pastoris*. *Mol. Biotechnol.* **2000**, *16*, 23−52.
(21) Tanino, T.; Aoki, T.; Chung, W. Y.; Watanabe, Y.; Ogino, C.; Fukuda, H.; Kondo, A. Improvement of a *Candida antarctica* lipase B-displaying yeast whole-cell biocatalyst and its application to the polyester synthesis reaction. *Appl. Microbiol. Biotechnol.* **2009**, *82*, 59−66.
(22) Han, Z. L.; Han, S. Y.; Zheng, S. P.; Lin, Y. Enhancing thermostability of a *Rhizomucor miehei* lipase by engineering a disulfide bond and displaying on the yeast cell surface. *Appl. Microbiol. Biotechnol.* **2009**, *85*, 117−126.
(23) Shiraga, S.; Kawakami, M.; Ishiguro, M.; Ueda, M. Enhanced reactivity of *Rhizopus oryzae* lipase displayed on yeast cell surfaces in organic solvents: potential as a whole-cell biocatalyst in organic solvents. *Appl. Environ. Microbiol.* **2005**, *71*, 4335−4338.
(24) Jiang, Z. B.; Song, H. T.; Gupta, N.; Ma, L. X.; Wu, Z. B. Cell surface display of functionally active lipases from *Yarrowia lipolytica* in *Pichia pastoris*. *Protein Expres. Purif.* **2007**, *56*, 35−39.
(25) Xu, L.; Pan, X. X.; Liu, W. S.; Shen, X. G.; Wang, X. F.; Liu, Y.; Yan, Y. J. Preparation of a promising whole cell biocatalyst of *Geotrichum* sp. lipase and its properties. *J. Chem. Technol. Biotechnol.* **2012**, *87*, 498−504.
(26) Daly, R.; Hearn, M. T. Expression of heterologous proteins in *Pichia pastoris*: a useful experimental tool in protein engineering and production. *J. Mol. Recognit.* **2005**, *18*, 119−138.
(27) Liu, W. S.; Jia, B.; Zhao, H. Y.; Xu, L.; Yan, Y. J. Preparation of a whole-cell biocatalyst of *Aspergillus niger* lipase and its practical properties. *J. Agric. Food Chem.* **2010**, *58*, 10426−10430.
(28) Van der Vaart, J. M.; Biesebeke, R.; Chapman, J. W.; Toshka, H. Y.; Klis, F. M.; Verrips, C. T. Comparison of cell wall proteins of *Saccharomyces cerevisiae* as anchors for cell surface expression of heterologous proteins. *Appl. Environ. Microbiol.* **1997**, *63*, 615−620.
(29) Jacobs, P. P.; Ryckaert, S.; Geysens, S.; Vusser, K. D.; Callewaert, N.; Contreras, R. *Pichia* surface display: display of proteins on the surface of glycoengineered *Pichia pastoris* strains. *Biotechnol. Lett.* **2008**, *30*, 2173−2181.
(30) Eaton, D.; Rodriguez, H.; Vehar, G. A. Proteolytic processing of human factor VIII. Correlation of specific cleavages by thrombin, factor Xa, and activated protein C with activation and inactivation of factor VIII coagulant activity. *Biochemistry* **1986**, *25*, 505−512.
(31) Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **1970**, *227*, 680−685.
(32) Towbin, H.; Staehlin, T.; Gordon, J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedures and some applications. *Proc. Natl. Acad. Sci. U.S.A.* **1979**, *76*, 4350−4354.
(33) Kojima, Y.; Shimizu, S. Purification and characterization of the lipase from *Pseudomonas fluorescens* HU380. *J. Biosci. Bioeng.* **2003**, *96*, 219−226.
(34) Duan, H. M.; Umar, S.; Hu, Y. P.; Chen, J. C. Both the *AOX1* promoter and the *FLD1* promoter work together in a *Pichia pastoris* expression vector. *World J. Microbiol. Biotechnol.* **2009**, *25*, 1779−1783.
(35) Gemmill, T. R.; Trimble, R. B. Overview of N- and O-linked oligosaccharide structures found in various yeast species. *Biochim. Biophys. Acta* **1999**, *1426*, 227−237.
(36) Yan, J. Y.; Yang, J. K.; Xu, L.; Yan, Y. J. Gene cloning, overexpression and characterization of a novel organic solvent tolerant and thermostable lipase from *Galactomyces geotrichum* Y05. *J. Mol. Catal. B: Enzym.* **2007**, *49*, 28−35.
(37) Holmquist, M.; Tessier, D. C.; Cygler, M. High-level production of recombinant *Geotrichum candidum* lipases in yeast *Pichia pastoris*. *Protein Expression Purif.* **1997**, *11*, 35−40.
(38) Catoni, E.; Schmidt-Dannert, C.; Brocca, S.; Schmid, R. P. Overexpression of lipase A and B of *Geotrichum candidum* in *Pichia pastoris*: high-level production and some properties of functional expressed lipase B. *Biotechnol. Lett.* **1997**, *11*, 689−695.
(39) Fernández, L.; Pérez-Victoria, I.; Zafra, A.; Benítez, P. L.; Morales, J. C.; Velasco, J.; Adrio, J. L. High-level expression and characterization of *Galactomyces geotrichum* (BT107) lipase I in *Pichia pastoris*. *Protein Expression Purif.* **2006**, *49*, 256−264.