Isolation of Lipase-producing Marine Yeast and Characterization of Lipase in Immobilized and Free State

Ying Xia Li[1,2], Bing Xue Dong[2], Guo Xia Zheng[3] and Feng Juan Zhao[4]

[1]Marine Science and Engineering College, Qingdao Agricultural University, Qingdao, China.
[2]School of Life Science and Technology, Nanyang Normal University, Nanyang, China.
[3]School of Environment and Chemical Engineering, DaLian University, Dalian China.
[4] School of Biological Sciences, BingZhou University, Binzhou China.

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To screen high lipase activity marine yeast, 39 samples from seawater and sea slug were collected. The marine yeasts were isolated through adopting Rhodamine preliminary screening plate and secondarily screening with shake flask loading fermentation medium. A higher lipase activity yeast was obtained and identified as *Pichia guilliermondii* based on the results of routine and molecular identification. Lipase produced by *P. guilliermondii* was partially purified by $(NH_4)_2SO_4$ precipitation and immobilized on silica. Lipolytic activities of the enzyme in free as well as immobilized crude enzyme were compared in different pH, temperature, metal ions and detergent and enzyme inhibitors. A sharp decrease in lipolytic activity was observed in case of free and immobilized crude enzyme at 50, 55 and 55 °C, respectively. The lipase activity of both free and immobilized crude extracts was inhibited when incubated with Na^+, K^+, Ag^+, Zn^{2+}, EDTA, PMSF, SDS and Tween80. Both the free and immobilized crude lipase forms were found to have a stimulatory effect in the presence of Mn^{2+}, Cu^{2+} , Mg^{2+}, Ca^{2+}, Ba^{2+}, Fe^{3+} and Triton X-100 showed an inhibitory effect on the activity of free as well as immobilized crude lipase.

Key words: Lipase; Lipolytic activities; *Pichia guilliermondii*.

Lipases (EC3.1.1.3.) is a group of enzymes with the ability to hydrolyze triglyceridesat lipid-water interfaces. Lipases are considered to be the third largest group according to the total sales volume, and widely used in industrial and medicinal applications due to their substrate specificity such as fatty acid, alcohol, regio- and stereo-specificity[1, 2].

Lipases occur in animals, plants and microorganisms. Microbial lipases have been attracted great attention because they are more stable compared with plant and animal lipases and they can be obtained cheaply[3,4]. Yeasts are one of the most important lipase sources for industrial application because yeast enzymes are usually excreted extracellularly, facilitating extraction from the fermentation media. Lipases from *Candia rugosa* and *C. Antarctica* have been extensively used in different fields, and a large numbers of yeast such as *Candida rugosa*, *C. tropicalis*, *C. antarctica*, *C. cylindracea*, *C. parapsilosis*, *Pichia maxicana*, *Pichia silvicola* have been studied for lipase production[5]. However, very few studies exist on the lipase produced by yeasts isolated from sea slug[6].

Lipase is relatively unstable on unusual conditions such as high pH and temperatures and various organic solvents. The high cost of the

* To whom all correspondence should be addressed.
E-mail: liyx108@163.com

enzymes often also makes the enzymatic processes economically unattractive. The immobilization of lipases on a propriate solid supports is one way to increase the applicability of lipases and to ensure their use and reuse for large-scale applications[7].

In this paper, the yeast has been isolated from seawater and sea sluge and identified based on the results of routine and molecular identification. The properties of lipases in free and immobilized status were compared on different pH, temperature, inhibitors, metal ions and organic solvents.

MATERIALS AND METHODS

Isolation of lipase-producing yeast

39 samples of seawater and maine sullage from Qingdao and Dalian. The lipase-producing yeasts were screening in Rhodomine agar plates according to the method of Ozcan *et al.*,[8]. Strains that formed a clear zone after 72 h incubation at 37°C were isolated. In order to select the best lipase producer for enzyme purification and characterization, yeastss with lipolytic activity on the plates were cultured in liquid medium (glucose 5, $(NH_4)_2S0_4$ 1, $MgSO_4.7H_2O$ 0. 5, K_2HP0_4 1, olive oil 10ml, seawater 1000 ml.), and lipase activity was determined spectrophotometrically with *p*-nitrophenyl-laurate as substrate. One yeast with higher lipase activity was selected for next test.

Molecular Identification of endophytic bacteria

ITS sequence was amplificated using genomic DNA as the template and ITS generic primers: upstream primer: 5' -TCC GTA GGT GAACCT GCG G 3' primers: 5' -TCC TCC GCT TA TTGA TA T GC-3' The thermal profile involves 30 cycles of denaturing at 94 °C for 30 s, primer annealing at 54°C for 45s, and extension at 72 °C for 30 s (10 min in the last cycle). PCR products were send to Shanghai Sangni Biotechnology Co., Ltd. to sequence.

Cell culture

For the production of lipase, inoculum was prepared in medium having the following composition (g/l): glucose 5, $(NH_4)_2S0_4$ 1, $MgSO_4.7H_2O$ 0.5, K_2HPO_4 1, olive oil 10ml, seawater 1000ml. After 3 days of incubation at 30 °C, yeasts were separated by centrifugation at 6000 g for 10 min. The supernatant was used for pufification of lipse.

Purification of lipase

The optimum amount of $(NH_4)_2S0_4$ to precipitate lipase was determined by adding a varying amount $(NH_4)_2S0_4$ (20-80 %) of ice cold solvent. To 900 ml of the culture supernatant, $(NH_4)_2S0_4$ was added gradually at 4 °C with constant stirring. The precipitate was collected by centrifugation at 10,000 g at 4 °C for 20 min, and dissolved in 20mMTris-HCl buffer, pH 8.0. Lipase activity was determined after each batch of precipitate obtained.

Determination of protein concentration

The protein concentration was determined by the Bio-Rad Protein Assay Kit according to the manufacturer's instructions with bovine serum albumin (sigma).

Enzyme assay for the crude lipase

The lipase activity was evaluated by measuring the free fatty acid sproduced by nitrophenol produced from p-nitrophenyl stearate and 4-nitrophenyl palmitate. The amount of p-nitrophenol produced was quantified spectrophotometrically at 420 nm. 20 µl lipase was amixed with 880 µl reaction buffer and incubated for 5 min at 37 °C. The reaction was initiated by adding 100 µl of 8 mM substrate solubilized in isopropanol. Then, 0.5 ml of 3 M HCl was added to stop the reaction. After10 min centrifugation, 333 µl supernatant was mixed with 1ml of 2 M NaOH and absorbance was measured at 420 nm. Standard curve was acquired with known concentrations of p-nitrophenol. The unit of activity is defined as the amount of enzyme that hydrolyzes 1 µmol substrate in 1min.

Immobilization of lipase

The immobilization of lipase was according the method of Li *et al.*,[9]. Five grams of silica was mixed with 3 % methanesulfonic acid and washed with distilled water, then dried with vacuum drier. The treated silica was mixed with 3-chloropropyltrimethoxysilane and acetone at 80 °C for 6 h. White precipitate was collected by filtration, washed with water and dried at room temperature in air. Finally, the product was calcined inair at 500 °C for 5 h in a tube furnace to remove the organic templates. The treated silica was then suspended in 20 ml of 1mM phosphate buffer solution (pH= 7). 2 ml of glutataldehyde (25 % v/v) was added to this solution followed by incubation at room temperature for 2h to activate the silica which was

then washed with distilled water and dried at 60 °C for 2h. 50 mg activated silica and 50mg lipase were added to 25 ml phosphate buffer (pH = 7.0) and stirred by a magnetic stirrer at 4 °C for 6h. The supernatant was separated from solid material by centrifugation, and the solid material was washed with phosphate buffer, and then dried overnight at room temperature.

The immobilized yield was calculated using the following formula:

Immobilized yield= (total immobilized specific activity/ total initial soluble lipase activity)×100

Effect of temperature on lipase stability

The optimum temperature of lipase activity was measured at different temperature under standard assay conditions. The thermal stability was studied by incubating lipase at 20, 25, 30, 35, 40, 45, 50, 55, 60, 65 and 70 °C and measuring the residual activity with time under standard assay conditions. The relative lipase activity was calculated by taking the non-heated lipase activity as 100 %.

Effect of pH on lipase stability

The effect of pH on the activity of free and immobilized lipase was measured by incubating the enzyme at 37 °C for 1 h at various pH (4–11). Buffer solutions (20 mM) of different pH values were used: acetate buffer (pH 4, 5, 6), phosphate buffer (pH 7, 8, 9) and glycine-NaOH buffer (pH 10, 11). The activity was observed under the standard conditions.

Effect of detergent and enzyme inhibitors on lipase activity

The effect of inhibitors including ethylenediaminetetraacetic acid (EDTA), sodium dodecyl sulphate (SDS), phenylmethyl sulphonyl fluoride (PMSF), Triton X-100, β-Mercaptoethanol and Tween80 on the activity of lipase in free and immobilized state was determined. Lipase activity was observed after incubating the enzyme with 1 mM of the given inhibitors at 37 °C for 1 h.

Effect of the metal ions

The effect of various metal ions on lipolytic activity of the free and immobilized enzyme was examined by incubating the enzyme with 1 mM metal ions at 37 °C for 1 h. The metal ions tested were: Na+, K^+, Ag^+, Ca^{2+}, Ba^{2+}, Mg^{2+}, Mn^+, $Cu^{2+,}$ Zn^{2+}, Ba^{2+} and Fe^{3+}.

RESULTS AND DISCUSSION

The screening and identified yeast

To screen high lipase activity marine yeast, 39 samples from seawater and sea slug were collected. The marine yeasts were isolated through adopting Rhodamine preliminary screening plate and secondarily screening with shake flask loading fermentation medium. The yeast with higher lipase activity was obtained. The ITS sequencing of yeast showed 99 % homology with *Pichia guilliermondii* (Fig. 1). Further results based on fermentation and assimilation of different carbohydrates (Table 1) identify the yeast was *P. guilliermondii*.

Production and partial purification of lipase from yeast

Lipolytic activities were determined at different time intervals. The maximum activity was observed after 60 h of incubation (7.04 U/ml) in cell-free supernatant crude enzyme extract. Different concentrations of $(NH_4)_2SO_4$ (20–80 %) were added to the cell-free supernatant. Maximum percentage of lipase was obtained at 40 % $(NH_4)_2SO_4$ concentration. The lipolytic activity partially purified lipase after $(NH_4)_2SO_4$ precipitation was lower than of crude lipase (5.63 U/ml).

Effect of temperature on the free and immobilized lipase

Immobilization provides operational flexibility and improves enzymes' thermal and chemical stability[10]. Both of the lipases in free and immobilized were stable at the low temperature. Free and immobilized lipase retained similar activity after being incubated at 30 °Cfor 60 min. However,

Table 1. Fermentation and assimilation of different carbohydrates by the marine yeast strains

Fermentation	Result	Assimilation	Result
Glucose	+	Glucose	+
Maltose	-	Maltose	+
Galactose	-	Galactose	+
Sucrose	-	Sucrose	+
Lactose	-	Lactose	-
Raffinose	-	Raffinose	-
Melibiose	-	Melibiose	-

at 40 °C, the residual activity in case of free and immobilized state was recorded as 82.6 and 68.5 %, respectively (Fig. 2).

Effect of pH on free and immobilized lipase

Residual lipolytic activity was the maximum in the case of free crude enzyme at pH 6 state and in the case of immobilized enzyme at pH 7. The residual activity of free crude enzyme was 65.4, 89.1, and 75.3 % at pH 5, 7, and 8, respectively. The residual activity of the immobilized crude

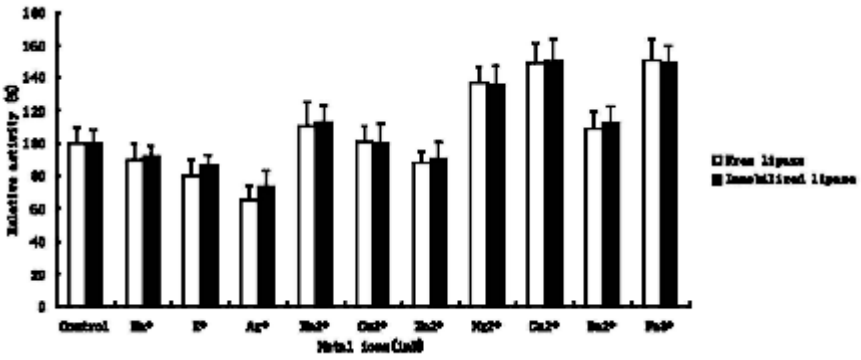

Fig. 1. The phylogenetic tree of lipase-producing yeast

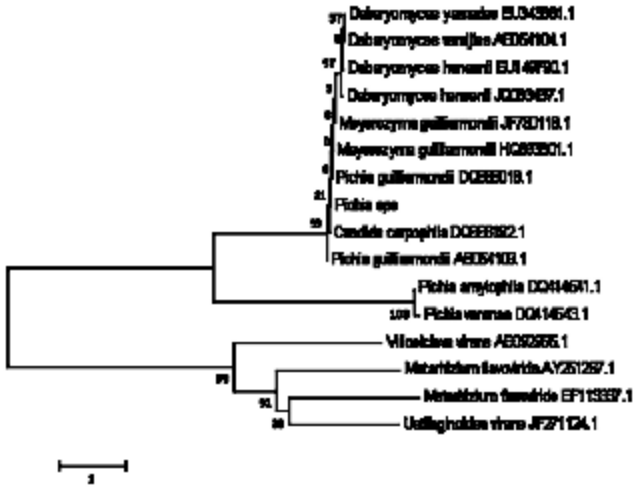

Fig. 2. Effect of temperature on free and immobilized crude lipase

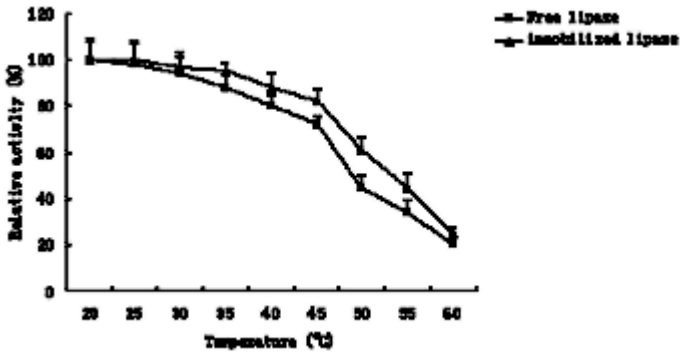

Fig. 3. Effect pH on free and immobilized crude lipase

enzyme was 87.16, 80.3, and 62.4% at pH 6, 8, and 9, respectively (Fig. 3). Free and immobilized showed different behaviors of pH dependence, similar result was also concluded in Bacillus sp. FH5 [11]. However, The native lipase and lipases immobilized on celite showed the same behaviors of pH dependence[12]. The discrepancy might be due to the difference of supports for immobilization.

Effect of detergent and enzyme inhibitors on lipase activity

Residual lipolytic activities of about 81.5 and 85.4% were observed in the presence of SDS in the case of free and immobilized lipase, respectively. In the presence of EDTA, PMSF and Tween80, the lipase activity was also inhibited in the case of free and immobilized lipase (Fig.4). The enzyme was sensitive to EDTA indicating that it was a metalloenzyme[13]. Microbial lipases were initially classified as serine hydrolyases according to the inhibition of their activity by chemical modification. In this study, PMSF inhibited the lipase activity, suggesting that Ser residues were essential for the enzyme active sites[14]. In contrast, Triton X-100 could promote the activities.

Effect of metal ions on lipase activity

It was found that Mn^{2+}, Cu^{2+}, Ca^{2+}, Ba^{2+}, Mg^{2+} and Fe^{3+} acted as inhibitors in decreasing activity of the free and immobilized lipase, with K^{+}, Na^{+}, Ag^{+}, Zn^{2+} having the strongest inhibitory effect on lipase activity (Fig.5). Among the various metal ions used, the lipase activity was stimulated in the following order: Fe^{3+}> Ca^{2+}> Mg^{2+}> Ba^{2+}> Mn^{2+} >Cu^{2+}, and inhibited in the following order: Ag^{+}> Na^{+}> Zn^{2+}> K^{+}. Metal cations play important roles in influencing the structure and function of enzyme[15], and various metal ions showed different effect on lipase from different sources. Activity of a lipase from *Geotrichum marinum, Yarrowia*

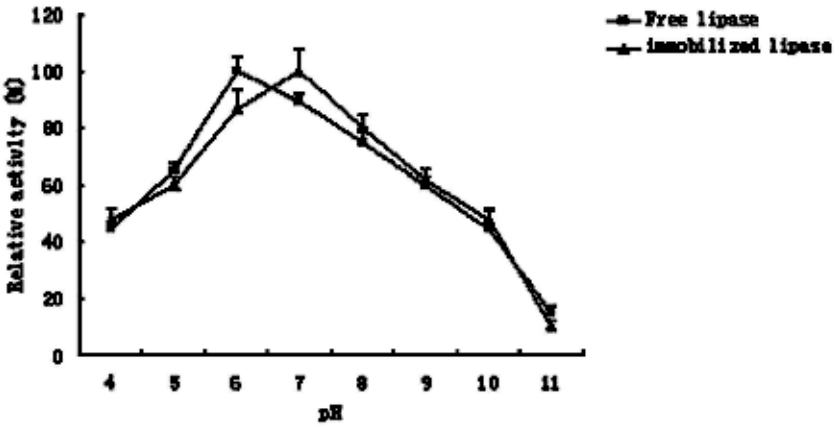

Fig.4. Effect detergent and enzyme inhibitors on free and immobilized crude lipase

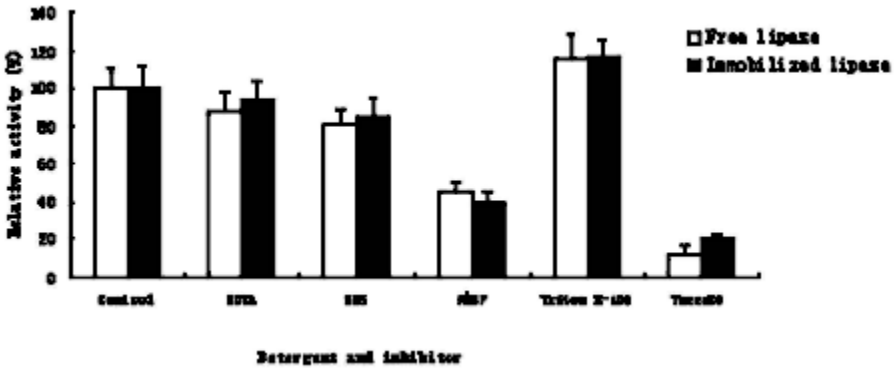

Fig. 5. Effect metal ions on free and immobilized crude lipase

lipolytica and *Aspergillus carneus* was enhanced by and Mg^{2+} ions[16-18]. Ca^{2+} can inhibite the activity of the lipase from *Pseudomonas fluorescens*, *Bacillus thermoleovorans*[19, 20]. However, reverse results also concluded. Fe^{3+} reduced the activity of lipase from *Pseudomonas fluorescens*[19] . Na^{+} promoted the activity of lipase from *Aspergillus carneus*[17].

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