

Full Length Research Paper

Characterization of a novel extracellular lipase from a halophilic isolate, *Chromohalobacter* sp. LY7-8

Xin Li* and Hui-Ying Yu

Life Science College, Yuncheng University, Yuncheng, 044000, China.

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A newly moderate halophilic strain LY7-8 with high lipolytic activity was isolated from salt lake of Yuncheng, China. Biochemical and physiological characterization, along with 16S rRNA sequence analysis placed the isolate in the genus *Chromohalobacter*. Lipase production started from the early-exponential phase of bacterial growth and reached a maximum level during the mid-stationary phase. The enzyme was highly active against *p*-nitrophenyl esters with acyl chain lengths of C8 to C18, indicating a lipase activity. Enzyme purification was carried out by ammonium sulphate precipitation and Sephacryl S-100 gel filtration chromatography. Molecular weight of the lipase was determined to be 44 kDa on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The purified enzyme was highly active over broad temperature (30 to 90°C), pH (6.0-12.0) and NaCl concentration (0 to 20%) ranges, showing optimal activity at 60°C, pH 9.0 and 12.5% NaCl. Significant inhibition of the lipase was shown by ethylenediaminetetraacetic acid (EDTA), phenylmethylsulfonyl fluoride (PMSF) and diethyl pyrocarbonate (DEPC), indicating it was a metalloenzyme with serine and histidine residues essential for its catalytic function. Moreover, it exhibited high activity in the presence of surfactants, such as SDS and Triton X-100. Results from the present study indicated that the extracellular lipase from LY7-8 may have considerable potential for industrial application from the perspectives of its properties.

Key words: Moderately halophile, lipase, purification, *Chromohalobacter*.

INTRODUCTION

Carboxyl ester hydrolases, like lipases and esterases, constitute an important group of biocatalysts. These enzymes are essentially distinguished on the basis of their substrate specificity. Lipases preferentially hydrolyze triglycerides made up of long chain fatty acids, and esterases hydrolyze only water soluble esters, for example, short-chain fatty acid triglycerides (Fojan et al., 2000). Microbial lipases are currently receiving more and more attention because of their potential applications in synthetic chemistry, food processing and environmental monitoring, such as biodegradation of industrial wastewater and agricultural pesticides (Jaeger et al., 1994; Jaeger and Reetz, 1998). However, most industrial processes are carried out under harsh physicochemical

conditions which may not be definitively adjusted to the optimal points required for the activity of the available enzymes. Therefore, it would be of great importance to develop new enzymes that exhibited optimal activities at various ranges of salt concentration, pH and temperature.

Moderately halophiles are a group of halophilic microorganisms able to grow optimally in media containing 3 to 15% NaCl (Ventosa et al., 1998). Enzymes from these prokaryotes usually showed high stability under extreme conditions, making them robust biocatalysts with potential applications in harsh industrial processes (Oren, 2002). There are numerous reports about lipase production from halophiles (Amoozegar et al., 2008; Boutaiba et al., 2006; Ozcan et al., 2009; Rohban et al., 2009); however, scarce studies on their purification and characterization were reported. Enzymatic properties of these lipases were mostly obtained from their crude extracts. Recently, a screening of lipase activity was carried out on halophilic bacteria from salt lake of Yuncheng, China. In this work,

*Corresponding author. E-mail: lixin-eva@163.com. Tel/Fax: +86-(0)359-2090324.

we reported a moderately halophilic strain LY7-8 with high esterase activity, and characterization of a novel extracellular lipase was performed.

MATERIALS AND METHODS

Bacterial strain isolation, identification and lipase production

The strain LY7-8 was isolated from the saline soil in Yuncheng, China, and cultivated aerobically at 37°C in the complex medium (CM) with the following composition (g/l): casein peptone 7.5; yeast extract 10.0; sodium citrate 3.0; MgSO₄·7H₂O 20.0; KCl 2.0; FeSO₄·7H₂O 0.01; NaCl 120.0 and pH 7.0.

Morphological, physiological and biochemical characteristics of LY7-8 were studied either on CM agar plate (2% agar, w/v) or in CM broth plus 12% NaCl, according to Sánchez-Porro et al. (2007). 16S rRNA gene was amplified using the general bacterial primers 8F and 1492R. The polymerase chain reaction (PCR) product was purified and sequenced in both directions using an automated sequencer by Seq Lab laboratory (Germany). The obtained 16S rRNA gene sequence was aligned with its closely related neighbor sequences retrieved from GenBank and a phylogenetic tree was constructed as described previously (Sánchez-Porro et al., 2007).

The kinetics of bacterial growth and esterase production were determined at different time intervals. Bacterial growth, along with enzyme activity, was measured by spectrometric method (Shimadzu model UV-160A).

Lipase activity assay

The lipase activity was determined by using *p*-NPM (*p*-nitrophenyl myristate) as substrate. The substrate was dissolved in 2-propanol to give a final concentration of 1 mM in the reaction mixture. After pre-incubation at 30°C for 10 min, the enzymatic assay was initiated by adding 0.2 ml of the purified enzyme solution to the reaction mixture (pH 9.0) and incubated at 60°C for 20 min. Following the addition of 2.0 ml of Na₂CO₃ solution (0.25 M) to stop the reaction, the amount of *p*-NP (*p*-nitrophenol) released was measured at 410 nm against a blank. One unit (U) was defined as the amount of enzyme liberating 1 μmol of *p*-NP per minute. The specific activity was expressed in the units of enzyme activity per milligram of protein.

Lipase purification

Culture supernatant obtained by centrifugation was treated with solid ammonium sulphate to 80% saturation and stirred overnight at 4°C. The precipitate collected by centrifugation was dissolved in 20 mM Tris-HCl buffer containing 12.5% NaCl (pH 9.0). After dialysis against the same buffer overnight, the sample was applied to a Sephacryl S-100 gel filtration column (1.6 cm×60 cm). The column was then washed with Tris-HCl buffer and bound proteins were eluted with a linear gradient of 0.1 to 0.8 M NaCl in the same buffer at a flow rate of 0.5 ml/min. Active fractions were pooled and concentrated by freeze-drying. Protein content was determined by the method of Lowry et al. (1951), using bovine serum albumin (BSA) as standard.

Molecular weight determination

SDS-PAGE (12% acrylamide) was performed to determine the molecular weight of the purified esterase, according to the method

of Laemmli (1970). Next after running the gel, the proteins were stained with Coomassie Brilliant Blue R-250.

Effects of temperature, pH and salt on lipase activity

The temperature optimum for the purified lipase was determined at various temperatures of 30 to 90°C. Effect of pH on enzyme activity was studied in the range of 6.0 to 12.0, using appropriate buffers at a concentration of 0.05 M (6.0 to 7.0, sodium phosphate; 8.0 to 9.0, Tris-HCl; 10.0 to 12.0, glycine-NaOH). Moreover, lipase activity was measured with different NaCl concentrations (0 to 20%) in the reaction mixtures.

Effects of metal ions and chemical reagents

The effects of metal ions and chemical reagents EDTA, PMSF, phenylarsine oxide (PAO), DEPC, β-mercaptoethanol, SDS and Triton X-100 on the lipase activity were examined after the enzyme had been pre-incubated with them for 30 min at 60°C, respectively, and then, residual activity was determined using the standard assay method as described previously. Activity in the absence of any additives was taken as 100%.

Substrate specificity

The substrate specificity of the purified lipase was studied by a spectrophotometric assay with various *p*-nitrophenyl (*p*-NP) esters (C2-C18, Sigma) as described previously (Lv et al., 2010). 0.1% Triton X-100 was added in the presence of *p*-NPO, while 2% 2-propanol was included when other compounds was used as substrate in order to solubilize the substrate. Data were expressed as the percentage of the observed maximal activity obtained with *p*-NPM.

RESULTS

Identification of strain LY7-8

Based on morphological, physiological and biochemical characterization, the isolate LY7-8 were Gram-negative, non-spore-forming, rod-shaped and motile bacterium. Colonies are cream, opaque and circular on CM agar plate. It grows well over a wide range of salt concentrations (0.9 to 20%). Optimal bacterial growth was observed at pH 8.0, 28 to 37°C and 10% NaCl. H₂S production, methyl red and Voges-Proskauer tests were negative, while nitrate reduction, oxidase, hydrolysis of tween 80 and gelatin were positive. Acid was produced from maltose, sucrose and glucose. Phylogenetic analysis based on 16S rRNA gene sequence comparisons revealed that the isolate LY7-8 (accession number in GenBank: HQ683732) fall within the branch encompassing the members of the genus *Chromohalobacter* (Figure 1). Thus, it was tentatively named as *Chromohalobacter* sp. LY7-8.

Kinetics of bacterial growth and enzymes production

As shown in Figure 2, the Lag phase of bacterial growth

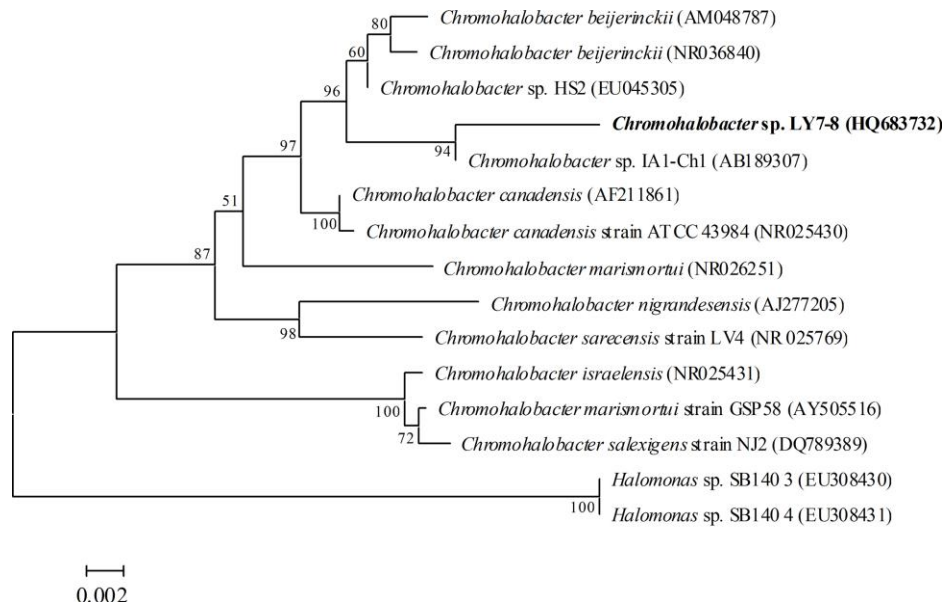


Figure 1. Phylogenetic tree based on 16S rRNA sequences, showing the relationship of the isolate LY7-8 to other members of the genus *Chromohalobacter*. Accession numbers of the sequences used in this study are shown in parentheses after the strain designation. Numbers at nodes are percentage bootstrap values based on 1,000 replications; only values greater than 50% are shown. Bar 0.002 substitutions per nucleotide position.

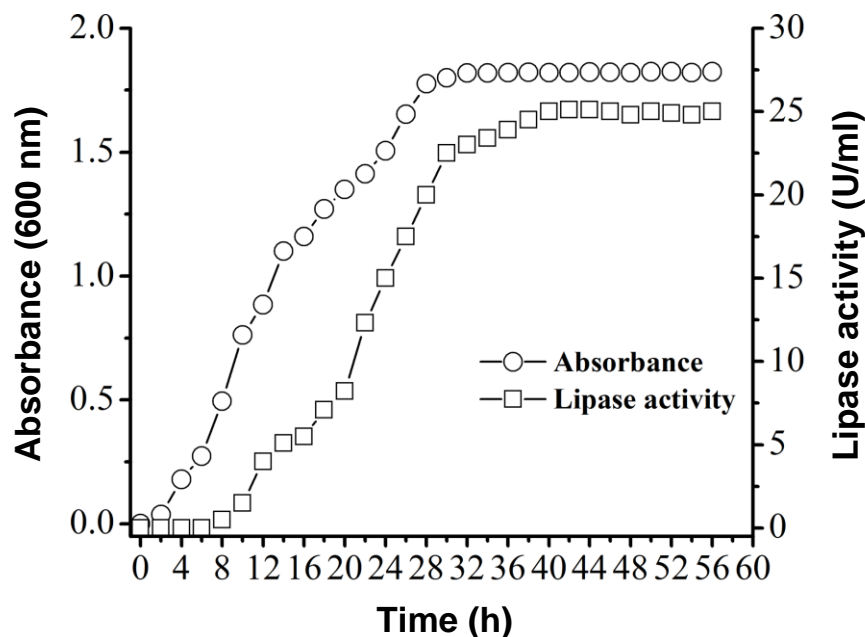


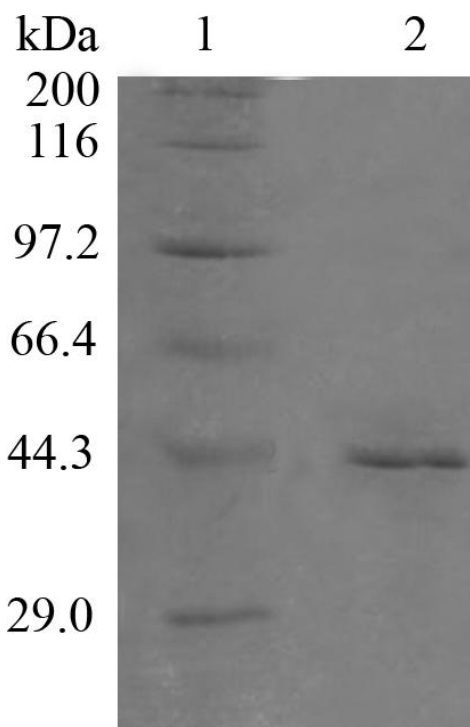
Figure 2. Kinetics of bacterial growth and extracellular lipase production of strain LY7-8 in CM broth at 37°C. Results represent the means of three independent experiments.

was short (2 h), and after 32 h the bacterial growth reached to the stationary phase. No lipase activity was detected during the early-exponential growth phase.

Lipase production started from the early-exponential phase (6 h) and reached a maximum level during the mid-stationary phase (40 h).

Table 1. Summary of the purification of the lipase from strain LY7-8.

Purification steps	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Crude enzyme	1220	47.5	25.6	1.0	100
80% (NH ₄) ₂ SO ₄	895	19.3	46.4	1.8	73.4
Sephacryl S-100	157	0.6	261.7	10.2	12.9

**Figure 3.** SDS-PAGE analysis of the purified lipase. Lane 1, molecular mass markers; lane 2, purified lipase.

Enzymes purification

The lipase was well purified by combination of 80% ammonium sulphate precipitation and Sephacryl S-100 gel filtration chromatography. The results of each purification procedure are summarized in Table 1. The enzyme was purified 10.2-fold with a recovery of 12.9% and a specific activity of 261.7 U/mg (protein). The purified enzyme showed a single band corresponding to an apparent molecular weight of 44 kDa on SDS-PAGE (Figure 3, lane 2).

Effect of temperature, pH and salt on lipase activity

The lipase was highly active over a broad temperature range with an optimum at 60°C. More than 50% of the activity remained at 90°C (Figure 4a). Moreover, it

showed activity over a wide pH range (6.0 to 12.0). Optimal pH was found to be 9.0. It retained 40% of activity at pH 12.0 (Figure 4b).

Enzyme activity was also determined with different NaCl concentrations at 60°C and pH 9.0 (Figure 4c). The highest activity was shown at 12.5% NaCl. Even under higher (20%) salinity, about 60% of the lipase activity was retained. Furthermore, in the absence of NaCl, the enzyme still retained more than 60% of its activity.

Effect of metal ions and chemical reagents

As shown in Table 2, none of the metal ions tested was found to stimulate the lipase activity. Effects of some known enzyme inhibitors revealed EDTA inhibited the enzyme greatly, as more than 80% of the activity was lost. Meanwhile, complete inactivation of the lipase was shown by PMSF and DEPC. Other enzyme inhibitors did not affect the enzyme, such as, β -mercaptoethanol and PAO. In addition, the enzyme was highly active towards surfactants, such as, SDS and Triton X-100.

Substrate specificity of the purified enzyme

The enzyme was highly active against a wide range of *p*-NP esters with acyl chain lengths of C8 to C18 (Figure 5). Maximal activity was obtained against *p*-NPM (C14). Lipase activity declines along with shorter chain-length, reaching 60% activity with *p*-NPH (C6), 30% with *p*-NPB (C4) and 12.1% with *p*-NPA (C2), respectively. Little activity was detected with *p*-NP esters with acyl chain lengths longer than C12.

DISCUSSION

In recent years, the ability of the moderate halophiles to grow and produce enzymes over a very wide range of salinities make them very attractive for research and for screening of novel enzymes with unusual properties. In this investigation, some halophilic bacteria were isolated from salt lake of Yuncheng, China. Among these, the isolate LY7-8 was selected for further studies because it appeared to be the best producer of extracellular lipase. As determined by 16S rRNA sequence analysis, the isolate was closely related to the species of the genus

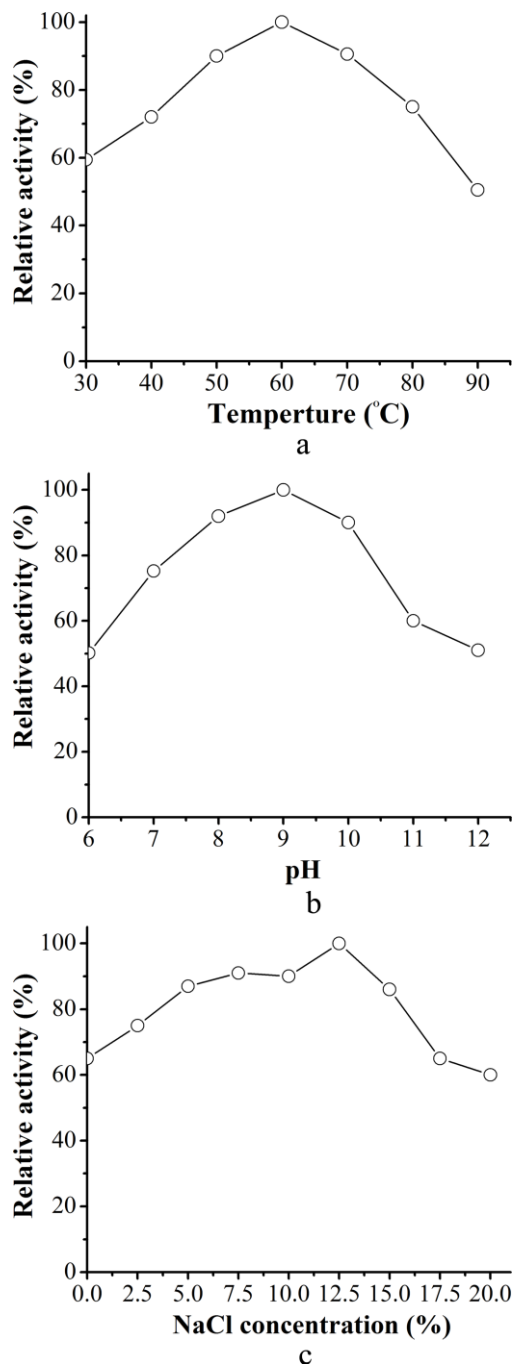


Figure 4. (a) Effect of temperature, (b) pH and (c) NaCl concentration on the activity of the purified lipase. The relative activity was defined as the percentage of activity detected with respect to the maximum enzyme activity. Values are averages of three independent experiments. See "Materials and Methods" for further details.

Chromohalobacter (Figure 1).

Lipase production of the strain LY7-8 started from the early-exponential phase of bacterial growth and reached

its maximum level during the mid-stationary phase. In contrast to other moderate halophiles, such as, *Marinobacter lipolyticus*, *Salinivibrio* sp. strain SA-2 and *Thalassobacillus* sp. strain DF-E4, lipolytic activity was detected at the end of the exponential growth phase (Martin et al., 2003; Amoozegar et al., 2008; Lv et al., 2010). Substrate specificity test revealed that the enzyme was highly active against *p*-NP esters with acyl chain lengths of C8 to C18 (Figure 5). Meanwhile, lipolytic activity on Rhodamine B agar plates (Kouker and Jaeger, 1987) indicated it could hydrolyze olive oil (data not shown). These results confirmed the lipase nature of this enzyme, with it being more active on longer-chain fatty acid esters.

The extracellular lipase can be classified as moderately thermoactive enzyme because of its optimal activity at 60°C. However, it was worth noting that the enzyme showed relatively high activity (50%) at 90°C. This characteristic made it obviously different from other lipases described previously, which were usually inactive under temperatures higher than 80°C (Ozcan et al., 2009; Lv et al., 2010). Optimal pH for the enzyme was found to be 9.0, indicating its alkali-stable nature. Similarly, Boutaiba et al. (2006) reported that lipase from *Natronococcus* sp. exhibited an optimum activity at pH 7.0, while Ozcan et al. (2009) reported a range of pH of 8.0 to 8.5 for optimal esterase activities of five halophilic archaeal strains. The lipase showed activity under high salinity (20%), which made it an interesting candidate for future investigation as biocatalysis in non-aqueous solvents, since it could be considered as active and robust biocatalyst at low water activity. Similar extreme halotolerance was also reported in other lipases from *Haloarcula marismortui* (Camacho et al., 2009) and *Halobacterium* sp. NRC-1 (Camacho et al., 2010). As we all know, the catalytic activity of most enzymes from halophilic microorganisms that normally live in a saline environment falls off dramatically and irreversibly when the enzyme is exposed to lower salt concentration. However, the purified lipase from LY7-8 was found to be highly active in the absence of NaCl. Similarly, other halophilic enzymes were also reported to retain their activity at low salt concentrations or even in its absence (Johnsen and Schonheit, 2004; Lv et al., 2010).

Significant inhibition by EDTA and PMSF indicated that the purified lipase was a serine metalloenzyme. Moreover, it was completely inhibited by DEPC, a histidine modifier (Wragg et al., 1997), suggesting histidine residue was essential for its catalytic function. However, no significant effect was detected with β -mercaptoethanol, indicating disulfide bonds did not have important role for its catalysis. These structural characteristics have not been observed in other lipases and esterases (Lv et al., 2010). In comparison with other halophilic lipases (Sana et al., 2007; Rao et al., 2009; Lv et al., 2010), the enzyme from LY7-8 had similar molecular weight, which was determined to be 44 kDa (Figure 3). In addition, it

Table 2. Effects of different metal ions and chemical reagents on lipase activity.

Substances	Concentration (mM)	Residual activity (%)
Control	-	100
Ca ²⁺	5	98.1±1.1
Zn ²⁺	5	95.6±0.5
Fe ²⁺	5	91.7±0.7
Fe ³⁺	5	90.9±0.5
Cu ²⁺	5	80.8±1.5
Mn ²⁺	5	97.5±1.5
Hg ²⁺	5	78.4±1.2
Mg ²⁺	5	99.7±1.5
EDTA	2	18.2±0.1
SDS	2	88.1±1.3
β-mercaptoethanol	2	99.3±1.1
Triton X-100	2	90.1±1.3
PMSF	2	0
DEPC	2	0
PAO	2	98.4±1.2

Residual activity was determined as described in "Materials and Methods" and expressed as the percentage of the control value (without any additives). Values are expressed as the averages of three independent experiments ± standard deviations.

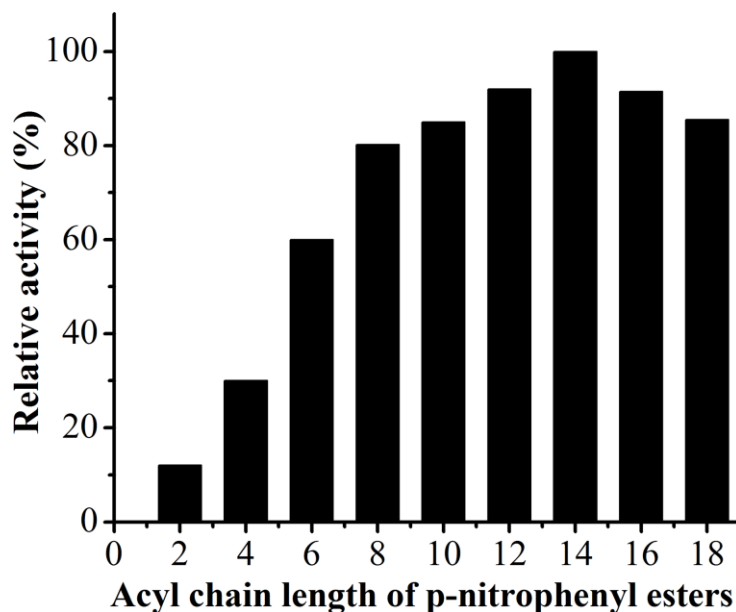


Figure 5. Substrate specificity test of the purified lipases towards the *p*-NP esters with different acyl chain lengths. Assays were done with 12.5% of NaCl at 60°C and pH 9.0. Data represented the means of three independent experiments.

exhibited remarkable stability towards surfactants, such as, SDS and Triton X-100, and may be useful in surfactant industries (Arikan, 2008).

In the present investigation, we described a novel moderately thermoactive, surfactant stable, alkali-stable

and halotolerant lipase from *Chromohalobacter* sp. LY7-8, with serine and histidine residues located in its active site. All of these results led us to conclude that the enzyme may have considerable potential for industrial application from the perspectives of its properties.

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