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Screening of Halophilic Lipase-Producing Bacteria and Characterization of Enzyme for Fish Sauce Quality Improvement

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ABSTRACT

Twenty pure bacterial cultures isolated from Thai fish sauce were selected for lipase producing on Sehgal and Gibbons Complex (SGC)-Tween 80 agar containing 0-4 M NaCl. The results showed that isolate PB233 could produce the highest lipase activity. This isolate was identified based on morphological, physiological characteristics and compared 16S rRNA sequence to other bacteria. It was concluded that isolate PB233 belonged to *Staphylococcus warneri*. This strain could grow in 0-4 M NaCl but the best growth was observed at 0 M NaCl. Therefore, *Staphylococcus warneri* was classified as halotolerant bacteria. A halotolerant *Staphylococcus warneri* PB233 was cultured in mini jar fermenter using SGC medium containing 1% olive oil and 3 M NaCl. A typical pattern of lipase production showed that enzyme secretion was coupled to active cell multiplication and maximum activity was obtained at a stationary phase. The maximum production of lipase (90.12 U/ml) was observed at 48 hr. The lipase had optimum at pH 7.0 and at temperature of 40°C. It was stable between pH 7.0 and 9.0 and at temperatures between 30 and 40°C. This enzyme had marked halophilic enzyme properties and showed maximal activities in the presence of 2.5 M (15%) NaCl.

Key words: screening, halophilic lipase, halotolerant bacteria , *Staphylococcus warneri*, fish sauce

INTRODUCTION

As halophilic means "salt loving", halophilic enzymes or bacteria by definition require NaCl for activity or growth. In contrast, halotolerant forms do not require NaCl for growth (Garabito *et al*., 1998). Lipase (triacylglycerol acylhydrolase, EC 3.1.1.3) catalyzes the hydrolysis of ester bonds of triacylglycerols to glycerol and free fatty acids at oil-water interface and does not hydrolyze dissolved substrates in the bulk fluid (Sharma *et al*., 2001). The free fatty acids, especially low molecular weight volatile fatty acids (VFA) were shown to be associated with the aroma and flavor in food products, therefore, lipase has been used for development of flavor and aroma in cheese ripening, bakery products, sausages, yoghurt and beverages (Jaeger *et al*., 1994; Sharma *et al*., 2001). Fish sauce is a clear brown liquid, hydrolysis product of salted fish after a year of salting. It is commonly used as a condiment in Southeast Asia and an amino acid sources of certain social classes in the region (Sanceda *et al*., 2003). Fish sauce has a characteristic aroma which often serves as an indicator to measure the quality of fish sauce, since the very salty taste tends to overpower the other flavor constituents. Low molecular weight volatile fatty acids (VFA), in

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particular formic, acetic, propionic, n-butyric, isobutyric, n-valeric and isovaleric acids had been identified as a some of aroma in fish sauce (Saisithi, 1967; Dougan and Howard, 1975; McIver *et al*., 1982; Sanceda *et al*., 2003). Many research has been undertaken on fish sauce, mostly concerned with the use of enzymes to reduce the time taken in fish sauce production but aroma and flavor of fish sauce from these processes are often unsatisfactory. As far as we know, there has been no research related with the application of halophilic lipase to fish sauce production. It may be expected that volatile fatty acids produced from halophilic lipase will give a better aroma in fish sauce. Thus, the present work aims to screen halophilic lipase-producing bacteria and characterise enzyme, which might be helpful in fish sauce for improving of flavor and aroma.

MATERIALS AND METHODS

1. Bacteria preparation

Twenty pure bacterial cultures isolated from Thai fish sauce were stored at 4°C on agar slant of Sehgal and Gibbons complex medium (SGC medium) containing 7.5 g/l casamino acids, 10 g/l yeast extract, 2.0 g/l KCl, 3.0 g/l sodium citrate, 20.0 g/l $MgSO_4.7H_2O$ and 0.01 g/l $FeCl_2.nH_2O$ (Sehgal and Gibbons, 1960), supplemented with 0, 1, 2, 3 and 4 M NaCl. The medium was adjusted to pH 7.0 by 0.5 M NaOH.

2. Screening of lipase producing isolate on plate agar

Lipase activity was tested on SGC agar medium containing 2 % of Tween 80 and supplemented with 1, 2, 3 and 4 M NaCl, while the control culture lacked NaCl. The bacterial isolates were spotted onto test agar and then incubated at 37°C for 10 days. A positive reaction for the lipase test was indicated by the opaque zone around the colony. The width of opaque zone was considered to be directly related to the amount of extracellular lipase produced. The ratio of enzyme production and growth were defined as A/B, where A was the diameter of opaque zone (mm) and B was the diameter of the colony size (mm). One isolate having the highest lipase activity was selected and subjected to the following experiments.

3. Identification of bacterial strain

The morphological and physiological characteristics of the selected isolate were performed according to the standard method (Cowan, 1974; Krieg and Holt, 1984; Washington, 1985; Baron and Finegold, 1990; Delost, 1997). Determination of 16S rRNA sequence was performed by direct sequencing of enzymatically amplified DNA with specific oligonucleotide primer (Arahal *et al*., 1996). The 16S rRNA sequence of the selected isolate was analysed by using an automatic DNA sequence and compared to 16S rRNA sequence with other bacteria from GenBank of National Center for Biotechnology Information (NCBI).

4. Effect of NaCl concentration on bacterial growth and lipase production

An inoculum was prepared by transferring one loop of the selected strain into 25 ml SGC medium (pH 7.0) in 125 ml Erlenmeyer flask. This was then incubated on a rotary shaker at 37°C, 250 rpm for 18 hr. Later, 5 ml of this seed culture was inoculated into 50 ml SGC medium (pH 7.0), containing 1% of olive oil and various concentrations of NaCl (0-4 M), in 250 ml Erlenmeyer flask. Cultivation conditions were the same as for the seed culture. About 3 ml of culture broth was taken out every day and optical density was measured at wavelength 660 nm by using a spectrophotometer (UV-1201 Shimadzu, Japan). After measurement, the culture broth was centrifuged at 8,000 ×g, 5°C for 20 min and the supernatant was used for determination of lipase activity.

5. Growth and lipase production in mini jar fermenter

The SGC medium (pH 7.0) containing 1% of olive oil and supplemented with optimal concentration of NaCl was used for enzyme production. Inocula (150 ml) of an 18 hr old culture of selected strain grown in SGC medium were added to a 2-liters mini jar fermenter (New Brunswick Scientific, USA) containing 1,350 ml of SGC production medium. Fermentation conditions were maintained at 37°C, 600 rpm agitation and air supply at 1.0 vvm for 72 hr. The pH of the broth was not controlled during this process. About 10 ml of culture broth was taken out every 4 hr for measurement of the optical density at 660 nm and pH. After measurement, the culture broth was centrifuged at 8,000 ×g, 5°C for 20 min and the supernatant was used for determination of lipase activity.

6. Characterization of crude lipase

The pH optimum for the crude lipase was measured at 37°C for 1 hr in 0.2 M buffers of various pH values (pH 4.0-12.0).

To determine the pH stability, the crude lipase (pH 7.0) was mixed with buffer at a ratio of 1:1. The mixture was kept at 4°C for 24 hr in 0.2 M buffers of various pH values (pH 4.0-12.0). Subsequently, pH was adjusted to 7.0 by adding 1 M phosphate buffer, pH 7.0, and residual lipase activity was measured.

The pH optimum and stability were determined using the following buffers: 0.2 M citrate buffer (pH 4.0-5.0), 0.2 M phosphate buffer (pH 6.0-7.0), 0.2 M Tris-HCl buffer (pH 8.0-9.0), 0.2 M glycine-NaOH buffer (pH 10.0-12.0).

The temperature optimum for the crude lipase was determined at various temperatures (30-70°C) for 1 hr in 0.2 M phosphate buffer, pH 7.0.

To determine temperature stability, the crude lipase solutions prepared in 0.2 M phosphate buffer, pH 7.0 were kept for 30 min at various temperatures (30-70°C). The mixture was then cooled to 4°C and residual lipase activity was measured.

The effect of NaCl on the crude lipase activity was determined by measuring the activity at various NaCl concentrations (0-4 M NaCl) for 1 hr at 37°C in 0.2 M phosphate buffer, pH 7.0.

7. Lipase activity assay

Lipase activity was measured by a modification of the method used by Yamada *et al.* (1962). Olive oil (10%) was used as a substrate and treated with 45% of 2% polyvinyl alcohol and 45% of distilled water. The olive oil emulsion (5.0 ml) was hydrolyzed with 2.0 ml enzyme solution in 3.0 ml of 0.2 M phosphate buffer, pH 7.0 at 37°C, 250 rpm for 1 hr and the reaction was stopped by addition of 20 ml of a mixture solution containing acetone and 95% ethanol in the ratio of 1 : 1. The liberated oleic acid was calculated by titration with 0.05 N NaOH. One unit of lipase activity was defined as the amount of enzyme required to release 1 μmol of oleic acid per ml per hour.

RESULTS AND DISCUSSION

1. Screening of lipase producing isolate on plate agar

The screening for lipase producing isolate was carried out using SGC agar plates with Tween 80 as a substrate at several concentrations of NaCl as shown in Table 1. Among twenty isolates, the isolate PB233 had the highest value for the ratio of colony size to opaque zone size, indicating that it exhibited the highest lipase producing ability. This isolate also had a strongly positive activity on the lipase test and had a positive ratio of lipase activity of 4.95 at 3 M NaCl for 10 days. In addition, it produced lipase between 1 and 4 M NaCl but the activity at 3 M NaCl was higher than these at 1, 2 and 4 M. On the basis of this result, isolate PB233 was selected for further studies since it appeared to be the best lipase producer.

Table 1 Screening of lipase producing-bacterial isolates on SGC- Tween 80 Agar.

Isolate No.	Ratio of opaque zone diameter (mm) and colony diameter (mm)				
	0 M NaCl	1 M NaCl	2 M NaCl	3 M NaCl	4 M NaCl
PB 213	-	1.80	4.10	1.50	1.00
PB 214	-	-	-	2.63	1.10
PB 221	-	-	-	3.17	-
PB 222	-	-	-	-	-
PB 232	-	-	-	2.28	-
PB 233	-	2.80	3.50	4.95	1.80
PB 241	-	-	-	1.75	1.70
PB 251	-	1.30	1.42	1.50	1.20
PB 262	-	-	1.35	3.14	1.90
PB 264	-	-	-	2.40	2.10
PB 311	-	-	-	2.00	-
PB 314	-	2.57	2.88	3.25	1.90
PB 315	-	1.57	2.83	2.00	-
PB 322	-	1.63	1.20	2.57	-
PB 361	-	-	1.33	1.28	-
PB 372	-	-	3.66	4.13	-
PB 373	-	1.62	2.83	3.43	1.50
PB 374	-	-	4.00	2.50	-
PB 375	-	2.16	2.83	1.55	1.15
PB 376	-	1.78	3.00	2.50	1.10

2. Identification of bacterial strain

The morphological and physiological characteristics of isolate PB233, determined by a standard method as shown at Table 2 (Cowan, 1974; Krieg and Holt, 1984; Washington, 1985; Baron and Finegold, 1990; Delost, 1997). At the first stage of identification, the organism was characterized as gram-positive cocci and exhibited positive urease and catalase reactions, and gelatin liquefaction. Selected isolate could aerobically and anaerobically grow in liquid SGC medium containing 0-4 M NaCl at 25-45°C. According to its morphological and physiological characteristics, the isolate PB233 was closely related to *Staphylococcus warneri* (Krieg and Holt,1984). Further identification was carried out by comparative sequence analyses of the 16S rRNA of selected isolate and other bacteria in the database. The 16S rRNA sequence of isolate PB233 showed a 99% similarity to *Staphylococcus warneri*, *Staphylococcus* sp. LMG 19417, *Staphylococcus* sp. ARCTIC-P62 and *Staphylococcus pasteuri* (Table 3). On the basis of morphological and physiological characteristics (Table 2) and 16S rRNA sequence comparative data (Table 3), it was concluded that the bacterial isolate PB233 belonged to *Staphylococcus warneri*.

3. Effect of NaCl concentrations on bacterial growth and lipase production

The effect of NaCl concentration on bacterial growth and lipase production was studied by cultivation of *Staphylococcus warneri* PB233 in SGC liquid medium (pH 7.0), containing 1% of olive oil and various concentrations of NaCl (0-4 M). From Figure 1, it was observed that *Staphylococcus warneri* PB233 could grow in

Table 2 Morphological and physiological properties of isolate PB233.

Characteristics	PB233
Gram strain	Positive
Morphology	Cocci
Pigment	Yellowish
Oxidase test	-
Urease test	+
Catalase test	+
Nitrate reduction	-
Oxidation-Fermentation (O-F test)	Oxidative
Gelatin hydrolysis	+
Anaerobic growth	+
Carbohydrate fermentation	
Galactose	+
Fructose	-
Lactose	+
Sucrose	+
Growth in : 0 M NaCl	+
1 M NaCl	+
2 M NaCl	+
3 M NaCl	+
4 M NaCl	+
Growth at : 25°C	+
30°C	+
37°C	+
45°C	+

Symbols: +, positive reaction; -, negative reaction

medium containing 0-4 M NaCl but the best growth was observed in medium without NaCl. This showed that *Staphylococcus warneri* PB233 should be classified as halotolerant bacteria, as the growth at 0 M NaCl was higer than at 1, 2, 3 and 4 M NaCl (Garabito *et al*., 1998). *Staphylococcus warneri* PB233 could produce lipase both in the presence or absence of NaCl. However, lipase production was the higest in medium containing 3 M NaCl (Figure 2). There was no correlation between the optimal NaCl concentration for growth (0 M NaCl) and for lipase production (3 M NaCl).

4. Growth and lipase production in mini jar fermenter

Production of lipase during growth of *Staphylococcus warneri* PB233 was studied in batch fermentation in a 2-liters mini jar fermenter using SGC medium containing 1% olive oil and 3 M NaCl. A time course of lipase production from this strain showed that the maximum lipase activity (90.12 U/ml) was detected at 48 hr of incubation at the stationary phase (Figure 3). The pattern of lipase production from *Staphylococcus warneri* PB233 was shown to be growth-associated (Lee *et al*., 1999). In the last phase, growth was still stationary, but enzyme activity was decreased. It was possible that cells lost activity due to a high concentration of enzyme (production inhibition) (Kanlayakrit *et al*., 2001).

Table 3 16S rRNA similarity values for isolating PB233 and members of *Staphylococcus* sp.

Organisms	% Similarity
	PB233
1. *Staphylococcus warneri*	99
2. *Staphylococcus* sp. LMG 19417	99
3. *Staphylococcus* sp. ARCTIC-P62	99
4. *Staphylococcus pasteuri*	99
5. *Staphylococcus epidermidis* ATCC 12228	98
6. *Staphylococcus* sp. MO28	98
7. *Staphylococcus aureus* MO50	98
8. *Staphylococcus succinus*	97
9. *Staphylococcus hominis*	97
10. *Staphylococcus saprophyticus*	97
11. *Staphylococcus haemolyticus*	96
12. *Staphylococcus xylosus*	96
13. *Staphylococcus capitis*	96
14. *Staphylococcus cohnii*	95
15. *Staphylococcus capprae*	95

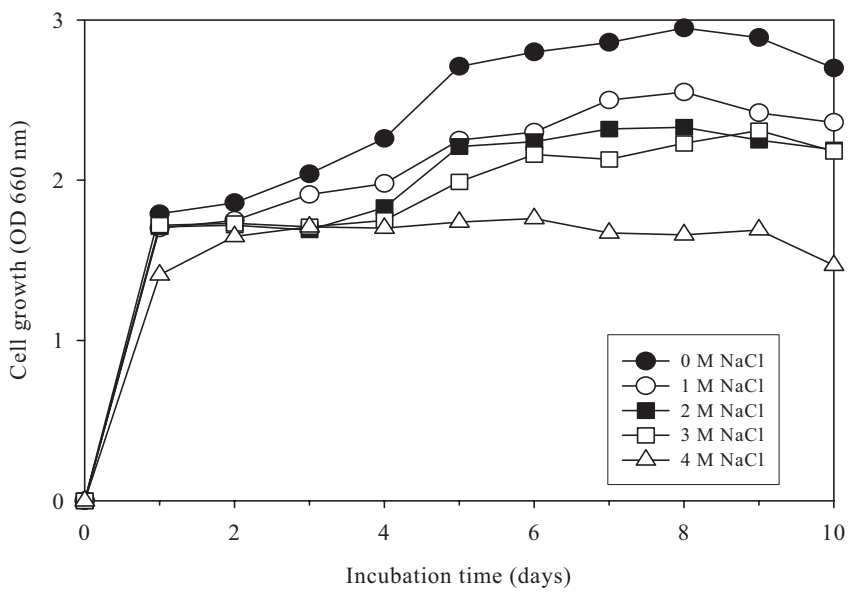

Figure 1 Effect of NaCl concentrations on growth of *Staphylococcus warneri* PB233 in liquid SGC medium.

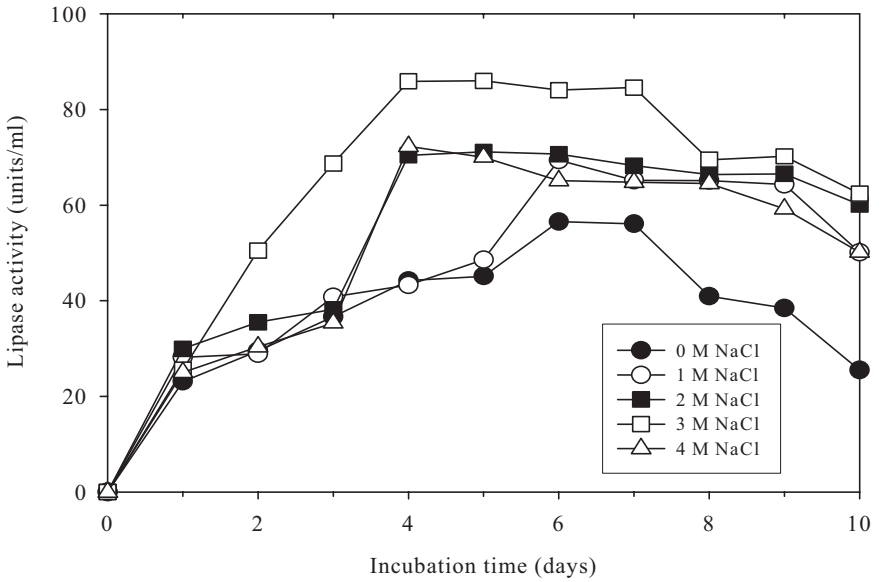

Figure 2 Effect of NaCl concentrations on lipase production of *Staphylococcus warneri* PB233 in liquid SGC medium.

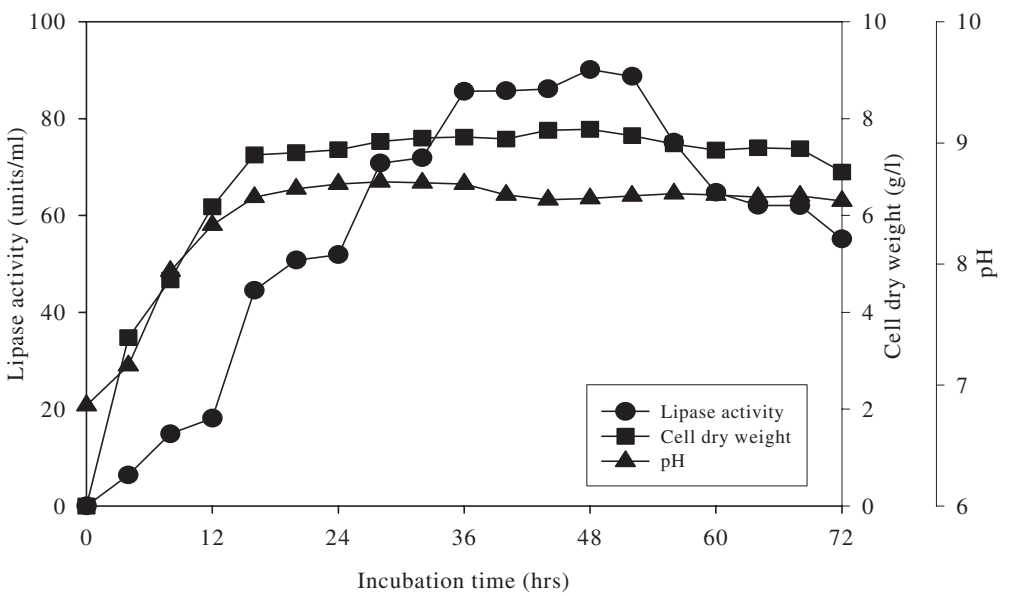

Figure 3 Time courses of growth, lipase production and pH of halotolerant *Staphylococcus warneri* PB233 in a 2-liters mini jar fermenter.

5. Characterization of crude lipase

Characteristics of crude lipase obtained from batch fermentation of *Staphylococcus warneri* PB233 in a 2-liters mini jar fermenter using SGC medium containing 1% olive oil and 3 M NaCl were studied. The crude lipase activity had an optimal pH and temperature of 7.0 and 40°C, respectively (Figure 4 A, B). The crude lipase was stable between pH 7.0 and 9.0 and at temperature between 30 and 40°C (Figure 5 A, B). The effect of NaCl on activity of the crude lipase is presented in Figure 6. This enzyme had marked halophilic properties, which required an optimal NaCl level of 2.5 M (15%). From these properties, it was concluded that lipase from halotolerant *Staphylococcus warneri* PB233 was a form of halophilic lipase. There were reports on halophilic enzyme, in particular nuclease, ribonuclease and protease production by halophilic and halotolerant bacteria (Kamekura and Onishi, 1974; Qua *et.al*., 1981, Onishi *et al*., 1983; Kanlayakrit *et al*., 2001) but there was no report on halophilic lipase, therefore, this was the first research report on halophilic lipase from halotolerant *Staphylococcus warneri* PB233 isolated from fish sauce.

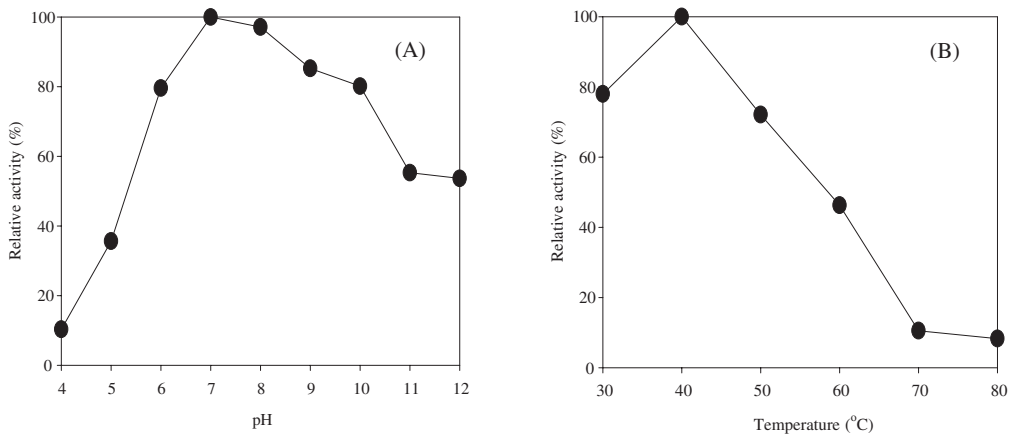

Figure 4 Effect of pH (A) and temperature (B) on activity of the crude lipase from halotolerant *Staphylococcus warneri* PB233.

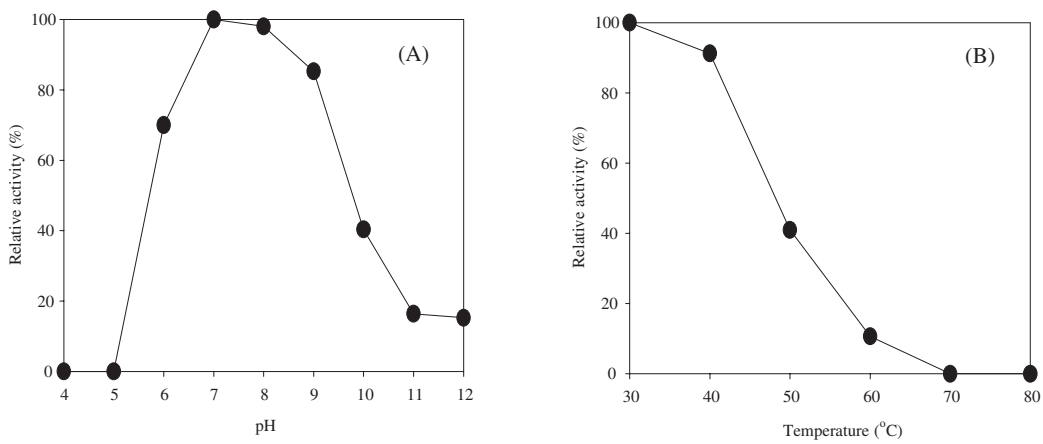

Figure 5 pH (A) and temperature (B) stability of the crude lipase from halotolerant *Staphylococcus warneri* PB233.

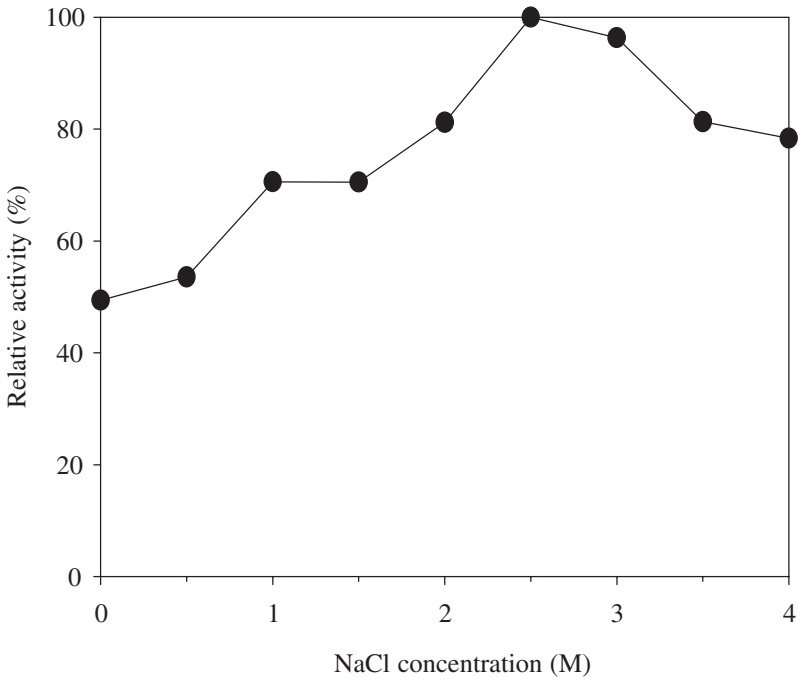

Figure 6 Effect of NaCl concentrations on activity of the crude lipase from halotolerant *Staphylococcus warneri* PB233.

CONCLUSION

The properties of halophilic lipase from a halotolerant *Staphylococcus warneri* PB233 showed several advantagenous features for industrial applications, which may be helpful for possible application in the production of volatile fatty acid by enzymatic hydrolysis in food products. In addition, studies on production and characterization of halophilic lipase from a halotolerant *Staphylococcus warneri* PB233 may lead to further apply this enzyme to fish sauce production for flavor and aroma improvement since optimum values of enzyme characteristics are the same as the actual fermentation conditions of fish sauce production.

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