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Media Preparation Using Tuna-Processing Wastes for Improved Lipase Production by Shrimp Gut Isolate *Staphylococcus epidermidis* CMST Pi 2

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Abstract Extracellular lipase production by *Staphylococcus epidermidis* CMST Pi 2 isolated from the intestine of shrimp *Penaeus indicus* has been investigated in shake-flask experiment using different preparations of tuna-processing waste such as raw fish meat, defatted fish meat, alkali hydrolysate, and acid hydrolysate as nitrogen source. Among the tested tuna preparations, defatted fish meat supported the maximum lipase production, and 2.5% concentration of the same was found to be optimum for maximizing the lipase production. The effect of carbon sources on lipase production revealed that glucose aided the higher lipase production than any other tested carbon source and a concentration of 2% glucose registered as optimum to enhance the lipase production. The halotolerancy of *S. epidermidis* CMST Pi 2 for lipase production indicated that 4% of sodium chloride was optimum to yield maximum lipase. Among the surfactants tested, lipase production was high in Tween 20 added medium when compared to other surfactants, and its optimum concentration recorded was 0.8%. Partial characterization of crude enzyme revealed that pH 7 and 55 °C temperature were optimum for maximum lipase activity.

Keywords Tuna waste · Lipase · Shrimp gut bacteria · *S. epidermidis* · Tween 20

Introduction

Lipases (triacylglycerol ester hydrolase, EC 3.1.1.3) catalyze the hydrolysis of fats to produce monoglycerides, diglycerides, free fatty acids, and glycerol. These reactions are reversible so that lipases also catalyze the formation of acylglycerols from glycerol and fatty acids. Lipases also possess characteristic properties like substrate specificity, stereospecificity, and the ability to catalyze heterogeneous reactions at the interface of water-soluble and water-insoluble systems [1].

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Lipases have been isolated from microorganisms especially from fungi, bacteria, and yeasts. The most productive fungal species belong to the genera *Geotrichum*, *Penicillium*, *Aspergillus*, *Rhizopus*, and *Ophiostoma*. The common lipase-producing bacterial strains are *Pseudomonas aeruginosa*, *P. fluorescens*, *P. fragi*, *B. coagulans*, *B. stearothermophilus*, *B. subtilis*, *Staphylococcus aureus*, *S. hyicus*, *S. epidermidis*, *S. warneri*, *Burkholderia glumae*, *B. cepacia*, *Streptomyces cinnamoneus*, etc. The yeasts include *Yarrowia lipolytica*, *Candida rugosam*, *C. valida*, *C. curvata*, *C. cylindracea*, *C. tropicalis*, *Saccharomyces lipolytica*, *S. crataegenesis*, *Rhodotorula glutinis*, *Rhodotorula*, *R. glutinis*, *Pichia bispora*, *P. maxicana*, and *P. sivicola* [2].

Lipase production by microorganisms is highly influenced by medium components like nitrogen sources, carbon sources such as fatty acids, triglycerides, and sugars or complex polysaccharides like glycogen and surfactants which can stimulate or repress lipase production. Nitrogen source is usually the most expensive component of microbial growth substrates for production of enzymes. At present, the important nitrogen source such as peptone is obtained from casein, soymeal, and gelatine [3]. The development of biotechnology has created an increasing demand for innovative, low-cost protein sources. In view of its high protein content of high quality, fish represents a potential source of industrial peptones for a wide range of applications.

Seafood is produced from a wide range of fishes, though only a part of those fishes are usually used as food. The rest of the by-products are simply thrown as waste and they are often rich in protein, which can be processed into useful products like fish protein concentrate, fish meal, fish silage, animal feed, etc. [4]. In view of the above, the present study was undertaken to investigate the lipase production by *Staphylococcus epidermidis* CMST Pi 2 using by-products obtained from tuna processing. Tuna is a fascinating fish resource, since total catch in the world is about 3.1 million metric tons per year. The meat is used by the canning industry, and this generates enormous amounts of waste material [5].

Materials and Methods

Bacterium and Lipase Activity

The potent lipolytic bacterium used in the present study was isolated from the gut of shrimp *Penaeus indicus* collected from Manakudy estuary of Kanyakumari District, Tamilnadu, India. The bacterium produced a clear zone (15 mm) when streaked on spirit blue agar after 48 h. The bacterium was identified as *Staphylococcus epidermidis* CMST Pi 2 at the Institute of Microbial Technology, Chandigarh, India (MTCC number 9040) (Table 1).

For media optimization studies, lipase positive organism was initially cultivated in an enrichment medium containing beef extract (0.15%), peptone (0.5%), NaCl (0.5%), glucose (0.5%), and yeast extract (0.15%) at pH 7 for 24 h. Then 5 ml of enriched seed culture was inoculated in a 250-ml flask containing 50 ml basal medium. The composition of basal medium (g/l) was glucose 0.5 g; yeast extract 0.1 g; peptone 0.25 g; KH_2PO_4 0.05 g; $MgSO_4$ 0.01 g; NaCl 0.5 g; pH 7. The culture was then incubated for 48 h by shaking (150 rpm) at 30 °C. The cells were then harvested by centrifugation at 10,000 rpm for 15 min and the supernatant was used for lipase assay.

Table 1 Biochemical characteristics of *Staphylococcus epidermidis* CMST Pi 2.

No.	Biochemical tests	Characteristic
1	Gram staining	Positive
2	Shape	Cocci
3	Motility	Non-motile
4	Oxidase	Negative
5	Catalase	Positive
6	Spore production	Negative
7	Coagulase	Negative
8	Growth in 4% NaCl	Positive
9	Novobiocin	Susceptible
10	Polymixin B	Resistance
11	Nitrate reduction	Weakly positive
12	Alkaline phosphatase	Positive
13	Urease	Positive
14	Beta-galactosidase	Negative
15	Hemolysis	Negative
16	Tween 80 hydrolysis	Positive
Carbohydrate fermentation		
17	Xylose	Negative
18	Arabinose	Negative
19	Cellobiose	Negative
20	Raffinose	Negative
21	Mannitol	Negative
22	Lactose	Negative
23	Fructose	Positive
24	Mannose	positive
25	Sucrose	Positive

Lipase Assay

The lipase activity in the culture supernatant was determined by titrimetry method (olive oil emulsion method). One unit of enzyme activity is defined as the amount of enzyme required to liberate 1 μmol of equivalent fatty acid (ml/min) under the assay conditions followed [6]. The relative activity was calculated as 100% by relating the enzyme activity of the particular temperature or pH with that of the maximum activity of the related variable.

Media Optimization for Lipase Production

The lipase production by the selected bacterium was optimized through supplying different nutrients such as nitrogen sources, carbon sources, surfactants, and sodium chloride.

Preparation of Tuna Waste Products

In the present study, tuna waste collected from the processing industries was taken for its potentiality to yield lipase by the selected bacterium. Different preparations of waste such

as raw tuna waste, defatted tuna waste, alkali-hydrolyzed tuna waste, and acid-hydrolyzed tuna waste were tested for its lipase-yielding ability.

To obtain tuna waste powder, the tuna waste from the processing center was cooked until boiling. The bones were removed and the cooked tuna waste meat was pressed to remove water and fat. The resulting pressed product was minced in a meat grinder and dried at 80 °C for 24–48 h. The dried fish preparation was minced again to obtain a fine powder and then stored in a glass bottle at room temperature. The raw waste was defatted through extraction with chloroform:methanol (3:1). The acid hydrolysate of tuna waste was done according to the method described by Gao et al. [7]. In brief, the minced fine wastes were mixed with equal weight of water (1:1 ratio). After that, the initial pH of the waste slurry was adjusted to 1 with 6 M HCl. Finally, the slurry was hydrolyzed at 121 °C for 20 min and was further centrifuged at 5,000 rpm for 20 min. The supernatant was used as a nutrient source for lipase production.

The alkaline hydrolysate of tuna waste was performed according to the method described by Batista [8]. Briefly, the fish meat powder was mixed with 2 N NaOH at 3:8 (*w*/*v*) ratio and adjusted to pH 12. The slurry was mixed in a beaker using a magnetic stirrer and was allowed to hydrolyze at 100 °C for 30 min and was further centrifuged at 5,000 rpm for 20 min. The supernatant was used as a nutrient source for lipase production.

The raw fish tuna meat was used as such without any treatment. Crude protein content of the fish waste preparation was determined by multiplying total nitrogen content (Kjeldahl method) by a factor of 6.25. Total sugar content, dry matter, and ash analysis were estimated by AOAC methods [9]. Total lipids were estimated according to the method of Folch et al. [10].

Effect of Tuna Waste as Nitrogen Source on Lipase Production

The resultant products were tested individually by replacing peptone present in the basal medium, and simultaneously, the peptone-containing basal medium was taken as a control for commercial nitrogen source in all experiments. After screening the maximum lipase-yielding substrate, it was further optimized by varying concentrations (0.5%, 1.0%, 1.5%, 2.0%, 2.5%, 3.0%, 3.5%, and 4.0%). The time course of lipase production in the tested substrates was also studied during different time intervals of fermentation (24–120 h).

Effect of Carbon Sources on Lipase Production Combined with Substrate

For the identification of suitable carbon source for lipase production by this bacterium, 11 different carbon sources such as glucose, lactose, fructose, sucrose, xylose, galactose, maltose, arabinose, glycerol, corn starch, and starch solubles were tested individually at the level of 0.5%. Subsequently, the maximum lipase-producing carbon source was further optimized by varying its concentrations such as 0.5%, 1.0%, 1.5%, 2%, 2.5%, 3%, and 3.5% in the production medium.

Effect of Sodium Chloride on Lipase Production Combined with Substrate

The effect of NaCl on lipase production was tested by adding different concentrations of NaCl in the production medium. The NaCl concentrations tested were 1%, 2%, 3%, 4%, 5%, 6%, and 7%.

Selection and Optimization of Surfactants Combined with Substrate

To study the surfactant-induced production of lipase, five different surfactants were tested (Tween 20, Tween 40, Tween 60, Tween 80, polyethylene glycol (PEG), and Triton X 100). The selected surfactants were incorporated individually into the production medium at a concentration of 0.2% and the medium without surfactant was taken as control. Then, the surfactant showing the highest influence on lipase production was further optimized by varying concentrations such as 0.2%, 0.4%, 0.6%, 0.8%, 1%, 1.2%, and 1.4%.

Partial Characterization of Crude Lipase: Effect of pH and Temperature on Lipase Activity

Optimum pH for higher lipase activity was determined by using different pH buffers during the assay. The assay was carried out individually at various pH such as 3, 4, 5, 6, 7, 8, 9, and 10. The effect of temperature on lipase activity was studied by incubating the enzyme and substrate solution at various temperatures from 20 to 75 °C at an interval of 5 °C and the assay was carried out individually these tested temperatures.

Statistical Analysis

The results obtained in the present study were subjected to relevant statistical analysis using Microsoft Excel 2005. Tests for significant differences were analyzed using one-way and two-way analyses of variance (ANOVA).

Results and Discussion

One problem regarding enzyme production on a large scale is the production cost. A possible alternative for reduction in production cost will come about with the preparation of medium with low-cost nitrogen sources. Viewing this, in the present study, an attempt has been made to reduce the production cost using a cheap nitrogen source—fish wastes, i.e., tuna waste preparations. The present results on the effect of tuna fish waste preparations as nitrogen source revealed that defatted fish meat produces maximum amount of lipase (12.63±0.33 U/ml) when compared with commercial peptone (9.20±0.245 U/ml) and other fish preparations tested in this study. In all the tested substrates, 48 h was registered as the optimum fermentation duration for higher lipase production. The other preparations such as raw fish meat (8.17±0.287 U/ml), acid hydrolysate (8.03±0.287 U/ml), and alkali hydrolysate (8.13±0.287 U/ml) gave relatively low lipase production when compared to defatted fish meal (Fig. 1a). The variation on lipase production between different nitrogen tested sources was statistically insignificant (F (2) 1.1608; $P>0.05$), whereas the variation due to incubation periods was statistically significant (F (2) 4.2416; $P<0.05$). This variation in lipase production may be attributed to the variation in biochemical constituents of tuna waste preparations. Among the tested preparations, defatted fish meat contained a maximum amount of important energy and nutrient sources such as crude protein (78.90±1.82 g/100 g) and total sugar (12.42±0.26 g/100 g), while acid and alkali hydrolysates contain less amounts of biochemical constituents than defatted fish meat (Table 2). These results find support with the previous report of Ghorbel et al. [11] on lipase production by *Rhizopus oryzae*. They inferred that defatted protein hydrolysates of *Sardinella* wastes induce maximum lipase production than other raw fish meat hydrolysates. This study also supports the previous

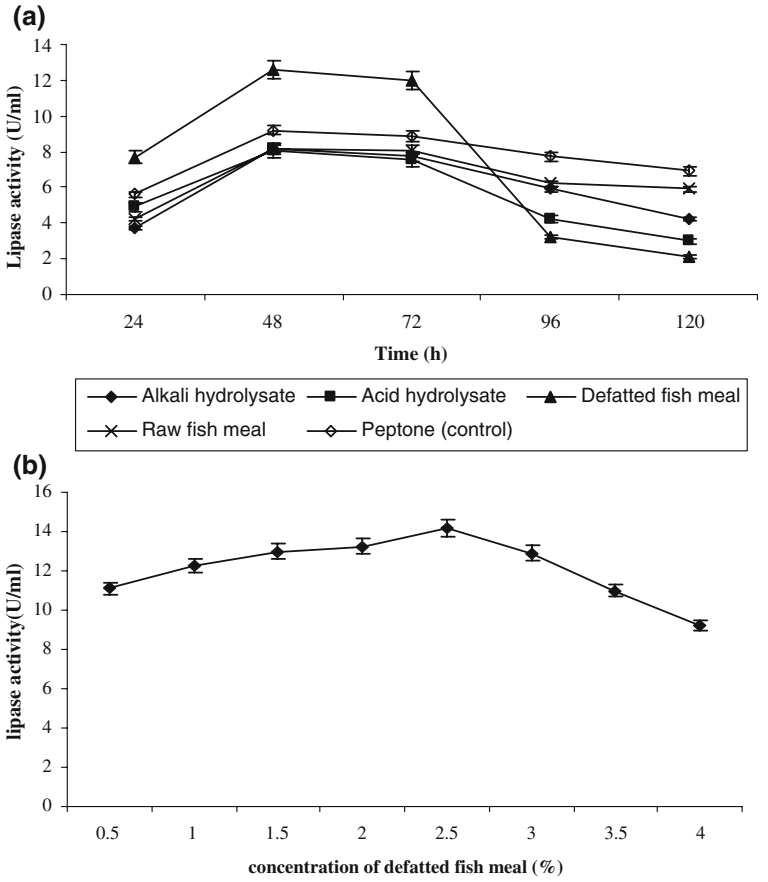

Fig. 1 Effect of different preparations of tuna-processing wastes on lipase production (**a**) and effect of various concentrations of defatted tuna meat on lipase production (**b**)

findings of Ellouz et al. [3, 12] and Souissi et al. [13] on the possible use of fish wastes as nitrogen source for bacterial growth and protease production.

Because of more lipase production in the defatted tuna waste meat-supplemented media, its different concentrations were tested for lipase production. The variation on lipase production between different concentrations of defatted tuna waste was statistically more

Table 2 Biochemical composition of different tuna waste preparations.

Tuna waste preparations	Crude protein	Total sugars	Total lipids	Dry matter	Ash content
Raw fish meat (g/100 g powder)	63.50±1.27	11.5±0.23	7.94±0.32	55.80±1.42	21.20±0.41
Defatted fish meat (g/100 g powder)	72.90±1.18	12.42±0.26	2.82±0.44	60.50±1.00	22.50±0.36
Acid hydrolysate (g/100 ml product)	6.72±0.04	0.58±0.001	0.21±0.004	11.20±0.48	5.42±0.08
Alkali hydrolysate (g/100 ml product)	6.31±0.03	0.53±0.002	0.23±0.003	12.0±0.61	5.94±0.12

significant (F (1) 25.1491; $P<0.001$). Among the concentrations tested, 2.5% was found to be the optimum to produce maximum lipase (14.20±0.287 U/ml), and above this concentration, the enzyme production declined (Fig. 1b).

The selection and optimization of suitable carbon source for lipase production revealed that glucose in the basal medium was found to be the best source for maximum lipase production (15.07±0.249 U/ml). Fructose (14.82±0.247 U/ml) and sucrose (14.04±0.251 U/ml) added medium also gave maximum lipase production next to glucose (Fig. 2a). The variation on lipase production between tested carbon sources was statistically more significant (F (1) 31.7318; $P<0.001$). In correlation with the present study, lipase production by *Penicillium chrysogenum* 9′ was higher in sucrose added medium [14]. They also observed a higher biomass production in glucose supplied medium, which is next to sucrose added medium. This result also supports the previous study by Dharmsthiti and Kuhasuntisuk [15] on lipase production by *Pseudomonas aeruginosa* (LP602); here, they reported an increase in lipase production in glucose and soybean oil supplied medium. Lin

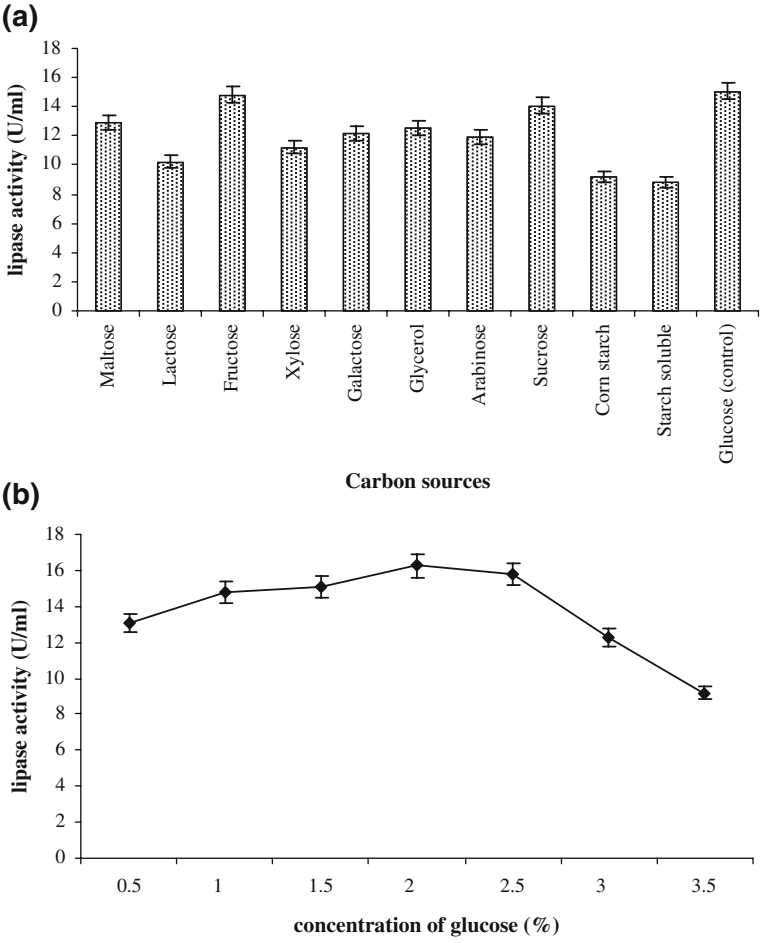

Fig. 2 Effect of different carbon sources on lipase production (**a**) and effect of various concentrations of glucose on lipase production (**b**)

and Ko [16] reported the importance of glucose in lipase production by Basidiomycete *Antrodia cinnamomea*. In contrast to the present study, Abdul Rahman et al. [17] reported that about 46% of initial lipase production was inhibited in glucose supplied medium. Considering the support of glucose for lipase production, different concentrations were then tested, and 2% was found to be an optimum, which maximized lipase production (16.28± 0.28 U/ml) (Fig. 2b), and on both sides of this optimum level the lipase production decreased. The variation in lipase production between glucose concentrations was statistically more significant (F (1) 10.2536; $P<0.001$).

Lipase production at various concentrations of NaCl indicated that it was maximum at 4% concentration (17.04±0.205 U/ml) (Fig. 3). Beyond 4% NaCl, lipase production decreased sharply and at 7% only 44% production was retained. At NaCl added medium, the variation in lipase production between concentrations was statistically more significant (F (1) 13.1191; $P<0.001$). This result revealed that this bacterium is moderately halophilic, because this bacterium was isolated from the gut of shrimp *P. indicus* which inhabits estuaries and its environment is often known to change due to inflow of freshwater and seawater. At present, the halophilic enzymes receive due consideration because of its multiple uses particularly in detergent industry. This result agrees with the previous report by Joseph et al. [18], and they inferred that the extracellular lipase production by *Staphylococcus epidermidis* was more in medium added with NaCl. Boutaiba et al. [19] found out that the lipase production by halophilic bacterium *Natronococcus* sp. was high at the optimized concentration of 3.5–4 M NaCl (14–16%). Martin et al. [20] reported that *Marinobacter lipolyticus* from hypersaline environment of Spain produced lipase and grown well at an optimum concentration of 7.5% NaCl. Similarly, Joshi et al. [21] reported that lipolytic bacterium *Halomonas campisalis* (MCM B-365) from Lonar Lake of India was found to grow up to 16% NaCl.

The effect of surfactants on lipase production by *S. epidermidis* CMST Pi 2 revealed that Tween 20 supplied medium supported for higher lipase production than others (21.1± 0.327 U/ml) (Fig. 4a). In polyethylene glycol (PEG) supplied medium, lipase production decreased drastically when compared with control devoid of surfactant (14.03±0.287 U/ml). The variation in lipase production between different surfactants was statistically highly significant (F (1) 143.844; $P<0.0001$). In contrast to the present study, lipase production by *Yarrowia lipolytica* was maximum in medium containing PEG with sunflower oil [22]. Earlier studies reported the influence of surfactants on enzyme production including lipase. Johri et al. [23] observed higher lipase production by *Rhizopus oligosorus* in medium added with Tween 20. Dharmsthiti et al. [24] have reported higher lipase production by *Acinetobacter*

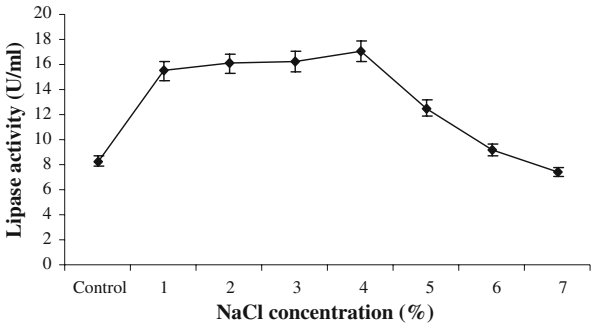

Fig. 3 Effect of different concentrations of NaCl on lipase production

calcoaceticus (LP009), in Tween 80 added medium followed by Tween 20. Abdul Rahman et al. [17] reported that lipase production by *Pseudomonas* sp. was highly induced in Tween 80 and Tween 60 added medium. Bancerz and Ginalska [25] reported the highest lipase activity by Basidiomycete *Bjerkandera adusta* R59 in Triton X 100 followed by Tween 20. Considering maximum lipase production in Tween 20, further optimization was carried out and indicated that 0.8% of Tween 20 was the optimum concentration for better lipase production (21.94±0.331 U/ml) (Fig. 4b). The variation in lipase production between different concentrations of Tween 20 was statistically more significant (F (1) 24.3642; $P<0.001$).

Partial characterization of crude lipase was assessed through the effect of pH and temperature on lipase activity. The effect of pH showed that the lipase is active over a narrow pH range of 6–8. Maximum activity was observed at pH 7 and the activity fell down rapidly at above pH 7, and at pH 10 only 65% activity was restored (Fig. 5). The variation in lipase activity between different pH was statistically more significant (F (1) 234.281; $P<0.0001$). Studies on the characterization of extracellular enzymes from the fish intestinal isolates are very scarce. Hoshino et al. [26] inferred that the protease activity by fish intestinal isolate *Pseudomonas* sp. was optimum at pH 7. Dharmsthiti and Kuhasuntisuk [15] reported the effect of pH on lipase activity by *Pseudomonas* sp. and they observed that pH 7–8 was optimum for increasing lipase activity. The study of Ginalska et al. [27] on *Geotrichum* sp. indicated that lipase activity by these fungi was high

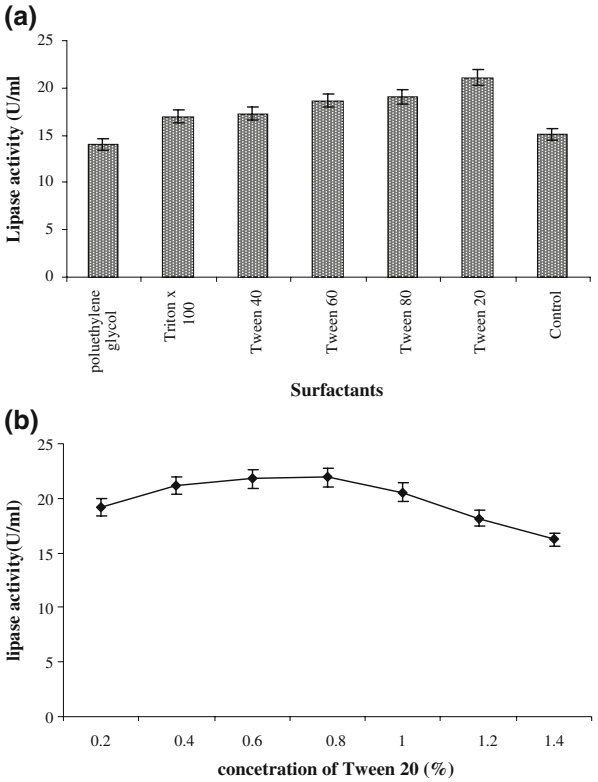

Fig. 4 Effect of different surfactants on lipase production (**a**) and effect of various concentrations of Tween 80 on lipase production (**b**)

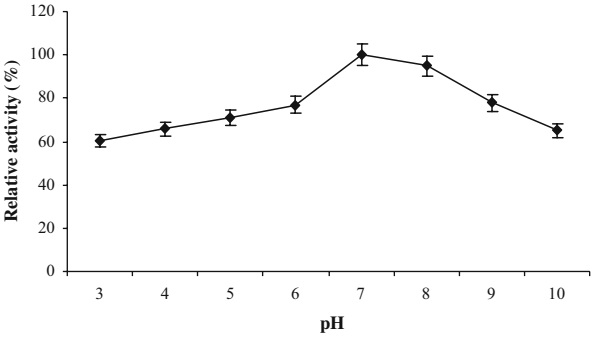

Fig. 5 Effect of different pH on lipase activity

at pH 7 and at higher pH the enzyme activity decreased. Falony et al. [28] also reported that the lipase activity by *Aspergillus niger* was also high in a pH range of 6–7.

The experiment on the effect of temperature revealed that lipase activity was stable over the temperature range of 45–60 °C and activity was specifically high at 55 °C, and then started to decline over 55 °C. At 75 °C, only 44% activity was retained (Fig. 6). The variation in lipase activity between different temperatures was statistically more significant (F (1) 250.864; $P<$ 0.0001). Dharmsthiti and Kuhasuntisuk [15] reported the effect of temperature on lipase activity by *Pseudomonas* sp.; they observed maximum activity at 55 °C. Prazeres et al. [29] also reported a higher lipase activity by *Fusarium oxysporum* at 50 °C.

Conclusion

Lipases have many industrial applications especially in food and detergent industry, and one of the main criteria in bioprocessing of enzymes including lipase is production cost. The present study reports the production of novel halophilic thermostable lipase by *Staphylococcus epidermidis* isolated from the gut of shrimp *P. indicus* using by-products from tuna fish processing. Owing the potential of fish waste proteins for maximizing lipase production, it can be used for the media formulation for

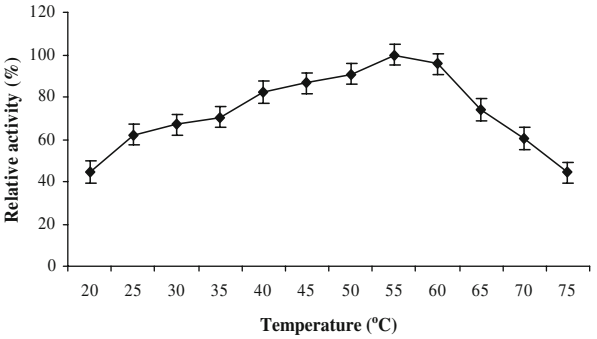

Fig. 6 Effect of different temperature on lipase activity

microbiological growth, and subsequently it adds value for waste outputs from processing. Besides this, the thermostability and halotolerance of lipase can be used for many industrial purposes.

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