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Lipase from Marine *Aeromonas hydrophila*

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ABSTRACT

A halotolerant strain of *Aeromonas hydrophila* was isolated from Vellar estuary, Parangipettai coast, Tamilnadu, India as a best enzyme producer using Tween 80 agar medium. Optimization studies revealed its maximum lipase production with optimized conditions such as 3% Tween80 as carbon source, 1% beef extract as nitrogen source, 0.3% fish waste as cheaper source, pH 9, temperature 37°C, 3% NaCl and 60 h incubation period. Optimized lipase partially purified with 60% Ammonium sulphate purification method results maximum enzyme production of 15 U/mL/L with 34 kDa molecular weight. The enzyme proved increased stability towards pH 7.5-9.5, 40-50°C and 15-30% of organic solvents for 1 h. The results concluded that the *Aeromonas hydrophila* is one of the potential alkaline lipase producing marine bacteria, with stability towards increased pH, temperature and organic solvents and preferable for further industrial applications.

Key words: Lipase, optimization, salinity, cheaper source, stability

INTRODUCTION

Lipases possess indigenous applications in recent technology. Lipases have emerged as key enzymes in swiftly growing biotechnology and are used in various industries like food, chemical, pharmaceutical, cosmetic, detergent production and leather processing (Jaeger and Eggert, 2002; Kulkarni and Gadre, 2002) and especially in biodiesel production (Nelson *et al.*, 1996). Lipases are hydrolytic enzymes which hydrolyse triglycerides to free fatty acids and glycerol (Sangeetha *et al.*, 2011).

In the last decade, lipases have gained importance to a certain extent over proteases and amylases, especially in the area of organic synthesis. Lipases shape a versatile group of enzymes, due to a big amount of catalyzed reactions, therefore a high potential of applications such as detergent, flavor development, paper recycle, chemical systems, racemic mixtures and so on (Jaeger *et al.*, 1999).

A variety of microbiological origin lipases with different properties and substrates specificity has been isolated and characterized so far. Microbial lipases have been a kind of favorable enzymes with their actual and potential applications. However, only a few microbial lipases are commercially exploited (Palekar *et al.*, 2000), for example the lipase produced by *Pseudomonas* sp. (Beissoon *et al.*, 2000). The widening application of microbial lipases in biotechnology has necessitated the continued research and development of novel lipases with broad substrate tolerance, high enantioselectivity and high stability (Sharma *et al.*, 2011).

Marine bacteria are highly resistance and have tremendous application in compared with terrestrial microbes and have valuable applications in biotechnology field. The characteristics of high pH and salinity tolerance of marine microbial lipases may greatly contribute to the related biotechnological field. Enzymes produced by halophilic microorganisms have developed particular trait. However, in spite of a growing interest in the use of halophilic enzymes for biotechnological applications, there are somewhat few reports in the literature about their production and characterization (Bhatnagar *et al.*, 2005).

Thus, the new findings of lipase producers and the optimized production strategy are very important especially with marine bacteria.

The genus *Aeromonas* belongs to the family Aeromonadaceae are primarily aquatic organisms. The organisms are straight or curved gram negative rods and are usually motile by singular polar flagella (Khardori and Fainstein, 1988).

The work was undertaken on lipase production, optimization, purification and characterization from the marine bacterium *Aeromonas hydrophila*.

MATERIALS AND METHODS

Screening for microorganisms with lipolytic activity: Estuarine sediment samples were collected for isolation of lipolytic bacteria from the Vellar estuary, Portonovo coast, Tamilnadu, India, 2009. The collected samples were stored immediately under aseptic condition. One gram of sample was serially diluted and inoculated on Tween 80 agar plates (Peptone-10 g, Beef extract -3 g, Calcium chloride-0.1 g, Tween 80-10 mL, Agar-20 g, 50% Aged Sea water -1000 mL, Rhodamine B indicator 0.01 g, pH-7±7.2) and incubated at 37°C for 48 h. All experimental steps have to be handled in strict aseptic condition. After incubation, distinct strains with zone of clearance were selectively isolated. The bacterial strain that possess higher values of lipolytic activity was identified up to species level following Bergey's manual of determinative bacteriology (Buchanan and Gibbons, 1974) and characterized for further optimization.

Lipase estimation: The supernatant of the fermented broth was recovered for the enzyme assay. Lipolytic activity was measured by titrimetric method of Beissin *et al.* (2000).

Optimization and mass scale culture for maximum lipase enzyme production: The culture conditions (carbon source, nitrogen source, cheaper source, pH, temperature, salinity and incubation period) were optimized for maximum enzyme production by the selected bacterium. The effect of different carbon sources on lipase production was investigated by using different carbon sources namely 3% of Tween 80, coconut oil, soyabean oil and olive oil. To test the effect of nitrogen sources on lipase production, four different organic nitrogen sources such as 1.0% of beef extract, yeast extract, peptone and soyabean meal were used to estimate the optimal lipase production. The resultant products such as 0.3% of fish waste, coconut cake, groundnut cake, wheat bran were tested individually by replacing beef extract present in the production medium and simultaneously, the beef extract containing production medium was taken as a control for testing the effect of cheaper source in all experiments. Optimum pH for lipase production was determined by measuring the optimum lipase production at different pH range from pH 4 to pH 12. Optimum temperature was determined by monitoring lipase production at different temperatures in the range of 20 to 60°C. With all other conditions kept constant as described, the effect of temperature upon lipase enzyme production was predicted. The effect of Sodium chloride (NaCl) at different (1, 1.5, 2, 2.5,

3 and 3.5%) concentration level was determined for optimum enzyme production. Lipase production was determined at different incubation period such as 0, 24, 36, 48, 60 and 72 h intervals for optimum lipase production. The 500 mL of optimized medium was prepared in Erlenmeyer flasks and inoculated with 10 mL broth culture preincubated with a stock culture and incubated.

Purification of lipase: The calculated amount of ammonium sulphate was added to cell free supernatant with constant stirring at 4°C to achieve 20, 40, 60 and 80% saturation. The precipitation thus obtained were harvested by centrifugation at 13,000x g for 30 min and resuspended in minimum volume of 25 mM sodium phosphate buffer (pH 7.2). This enzyme solution was subjected to dialysis for 24 h at 4°C against the same buffer with three intermittent changes of the buffer. Further, the filtrate was concentrated using lyophilization. Lipase activity was determined for both, the dialyzed and the concentrated filtrate sample (Ahmed *et al.*, 2010).

The enzyme concentrate obtained from dialysis was loaded onto a Sephadex G-100 gel filtration column (2.5×120 cm) at a flow rate of 0.5 mL min⁻¹. The column was equilibrated and eluted with Tris-HCl buffer (50 mM, pH 8.0) supplemented with CaCl₂ (1.0 mM). The eluted purified enzyme at different fractions were estimated to test the activity. The enzyme activity is defined as amount of enzyme which releases one milliequivalent/min/mg free fatty acid.

Effect of temperature, pH, organic solvents, on activity and stability of lipase: The stability of partially purified enzyme was estimated against various parameters. The pH stability of the enzyme was measured by incubating the enzyme in 50 mM Tris-HCl buffer at different pH (5, 6, 7, 8, 9, 10 and 11). The enzyme was incubated in 50 mM Tris-HCl buffer (pH 7.0) at the each temperature (20, 30, 40, 50, 60, 70 and 80°C) for 60 min and then the thermal stability was measured. Effect of organic solvents 50% (v/v), on lipase was analyzed by incubating at 10°C in 50 mM Tris-HCl buffer (pH 7.0) containing 0.1 or 1% (v/v) organic solvents (acetone, ethanol, hexane, methanol and butanol). Activity was measured at the beginning and at the end of incubation.

SDS-PAGE analysis: The relative molecular weight of the purified lipase was estimated by SDS-PAGE (Laemmli, 1970). The purified enzyme mixture was subjected to electrophoresis on 10% acrylamide gel and molecular weight was determined by comparing the relative mobility of the protein band with that of molecular weight markers. Lipase activity staining was performed using tributyrin agar according to the method of Singh *et al.* (2006).

RESULTS AND DISCUSSION

The strain isolated with maximum lipase enzyme activity was identified as *Aeromonas hydrophila* (Table 1) is a gram negative bacilli, usually motile with single polar flagellum, Indole, Voges proskaur, Citrate, Oxidase, Catalase, Gelatin, Nitrate and Gas producing from glucose positive, Hydrogen sulfide positive bacteria. Screening of lipase producing microbial strains has been done using rhodamine B indicator as an orange halo around microbial colonies under UV light at 350 nm reported by Rajan *et al.* (2011).

Optimum enzyme production acquired by the bacterium with 3% Tween 80 as carbon source, 1% beef extract as nitrogen source, 0.3% fish waste as cheaper source, pH 9, temperature 37°C, 3% salt concentrations at 60th h incubation period.

Table 1: Identification of *Aeromonas hydrophila*

Bio chemical test	Results
Gram's stain	Negative
Shape	Bacilli
Motility	+
Indole	+
Methylred	-
Voges proskaur	+
Citrate	+
Oxidase	+
Catalase	+
Gelatin	+
Nitrate reduction	+
Gas from glucose	+
Lactose	V
Hydrogen sulfide	+

+: Positive, -: Negative, V: Variable

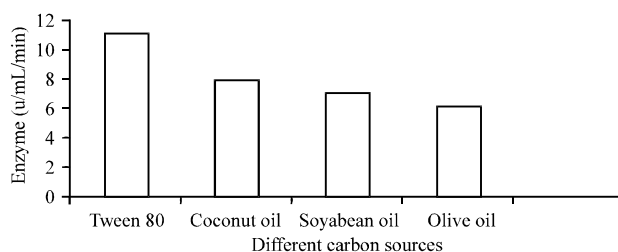


Fig. 1: Effect of different carbon sources on lipase production

In our present study 11.2 U/mL/min of maximum lipase activity was observed with Tween 80 as carbon source (Fig. 1) whereas coconut oil, soyabean oil and olive oil gave 08.01, 07.03 and 06.05 U/mL/min, respectively. Awan *et al.* (2003) found that Glucose, sucrose, starch, olive oil, mustard oil and Tween 80 gave lipase activity 32.56, 36.84, 27.54, 46.05, 31.09 and 62.84 UgG, respectively and Tween 80 was optimized as the best carbon source for lipase activity. The production of maximum extracellular lipase by addition of Tween 80 as best carbon source was observed by Handelsman and Shoham (1994). Variation in the concentration of Tween 80 was also effective for enhanced lipase production. Maximum lipase activity (71.65 UgG) was obtained at 0.5% concentration of Tween 80 as it provide optimum amount of carbon.

In our observation 9.3 U/mL/min of maximum lipase production was observed with 1% of beef extract as nitrogen source (Fig. 2) compared with yeast extract, peptone and soyabean meal. Joshi *et al.* (2006) and Zhang *et al.* (2009) suggested the beef extract as the best nitrogen source when compared with peptone and yeast extract. In our finding, an attempt has been made to reduce the production cost using a cheaper nitrogen source such as fish waste, coconut cake, groundnut cake, wheat bran. Among that, fish waste results 14.5 U/mL/min of maximum enzyme production compared with other cheaper source (Fig. 3).

Influence of pH on lipase production in the range 6-10 while keeping all the other conditions constant was determined. The optimum lipase production was observed at pH 9 which results in 10.01 U/mL/min lipase activity (Fig. 4). The purified lipase from *S. grimsii*, *S. marcescens*

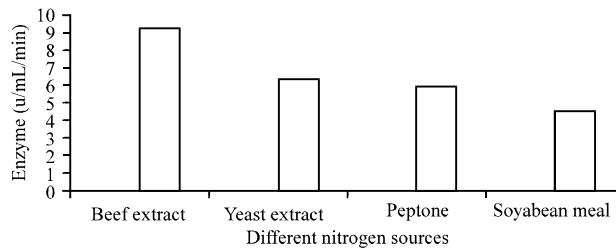


Fig. 2: Effect of different nitrogen source for enzyme production

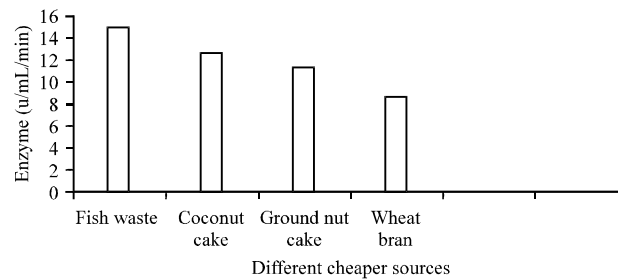


Fig. 3: Effect of cheaper source for enzyme production

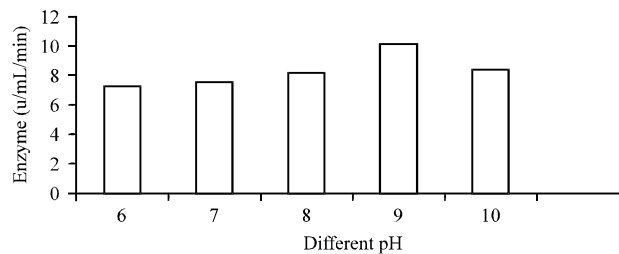


Fig. 4: Effect of pH for enzyme production

Sr418000 (Abdou, 2003; Matsumae and Shibatani, 1994), *Pseudomonas* (Landass and Solberg, 1978), *P. fluorescens* (Fox and Stepaniak, 1983) observed optimum lipase activity in the range of 8-9 pH. The activity of the enzyme was compared with the works done by Pabai (1997). Most *Pseudomonas* species have maximum lipase activity in the pH range of 7.0 to 9.0, including enzymes from *P. putida*. The highest activity was found at pH 9.5 to 10.0. However, low enzymatic activity was determined at pH values less than 7.0 (Macrae, 1983; Gilbert *et al.*, 1991; Baral and Fox, 1997; Schuepp *et al.*, 1997). Mostly higher pH range supported the maximum enzyme production. Lower pH range supported minimum enzyme production. This low activity could be due to the incomplete ionization of the free fatty acids rather than to the inactivity of the lipase ions.

In our observation, influence of temperature on lipase production in the range 30-50°C while keeping all the other conditions constant was determined. The lipase activity was optimum at 37°C results in 12.03 Unit/mL/min (Fig. 5). Kiran *et al.* (2008) observed that 37°C was optimum for lipase production. Sarkar *et al.* (1998) also reported that optimal temperature determined for lipase production by *Pseudomonas* MSI057 was 37°C. Schuepp *et al.* (1997) reported the optimum temperature of 35°C for the partially purified extracellular lipase from *Pseudomonas* sp.

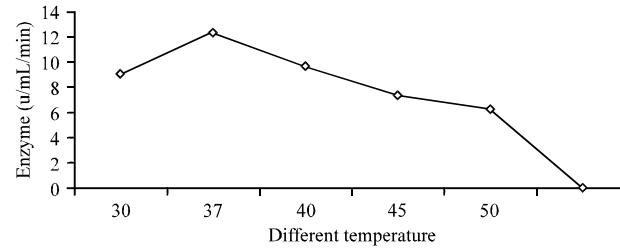


Fig. 5: Effect of temperature for enzyme production

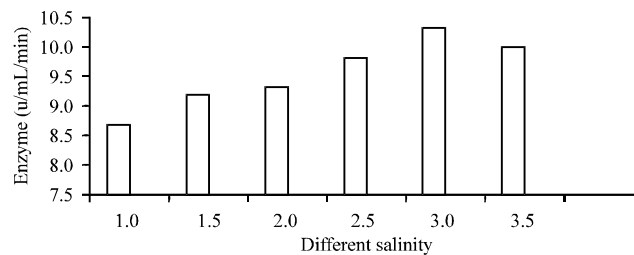


Fig. 6: Effect of salt concentration for enzyme production

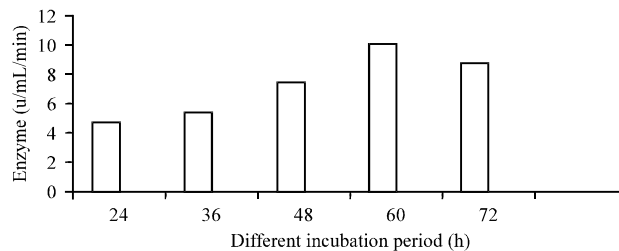


Fig. 7: Effect of incubation time for enzyme production

Kosugi and Kamibayashi (1971) reported reduced lipase activity at temperature ranging from 60 to 80°C. Shukla *et al.* (2007) founded the optimum lipase enzyme activity by *Rhizopus oryzae* KG-10 at 37°C.

Salinity was found to be a critical factor on the production of lipase. In the absence of sodium chloride, only traces of enzyme production and growth were observed. Since the strain was a marine isolate, it showed maximum lipase production in a medium supplemented with NaCl. The lipase production reached the highest when the production medium contained 3% of NaCl (Fig. 6). Likewise at 3% of salt concentration, 10.02 Unit/mL/min was observed. Whereas Kiran *et al.* (2008) reported that 1.5% NaCl was optimum for lipase production.

Maximum production of enzyme resulted at 60th h of incubation in all the optimizing conditions of various parameters (Fig. 7). However, in the mass scale production in a fermentor these optimum conditions are needed to assess the high yield and activity of lipase enzyme. The incubation period for optimum lipase production was 60 h. Kumari *et al.* (2009) also observed that the optimum incubation time for *E. aerogenes*, for lipase production was fixed as 60 h. Lipase production is influenced by the type and concentration of carbon and nitrogen sources, the culture pH, the growth temperature and the dissolved oxygen concentration (Elibol and Ozer, 2000).

Optimized lipase enzyme shows increased stability towards pH 8-10 (Fig. 8), temperature range of 40-50°C (Fig. 9) and organic solvent (Fig. 10) acetone. Lee *et al.* (2004) reported on cell surface displayed lipase stability in *E. coli* shows higher stability at 50°C and between pH 9-10. Chouhan and Dawande (2010) demonstrated the lipase obtained from *P. aeruginosa* (MTCC 2488) found to be thermostable at optimum temperature from 50-60°C when Tween-20 was used as the substrate.

The 20% of ammonium sulphate saturation resulted in the yield of 0.16 g of enzyme powder where as at 40, 60 and 80% of, respectively 0.19, 0.38 and 0.22 g were obtained, respectively. The enzyme purified from the 60% of ammonium sulphate saturation gave the maximum enzyme activity on tween 80 agar plate assay with Rhodamine B indicator and it was comparable to the commercially available lipase. Optimized lipase enzyme shows stability towards pH 7.5-9.5,

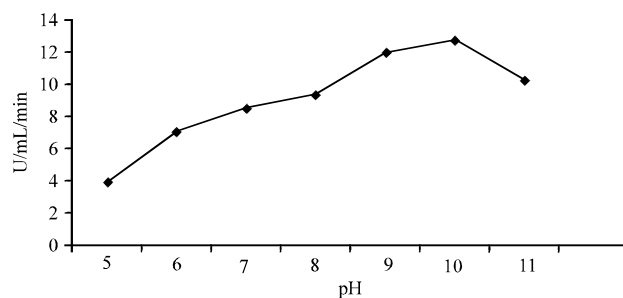


Fig. 8: Effect of pH on lipase stability

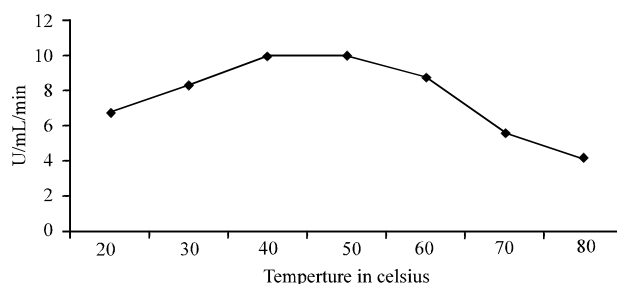


Fig. 9: Effect of temperature on lipase stability

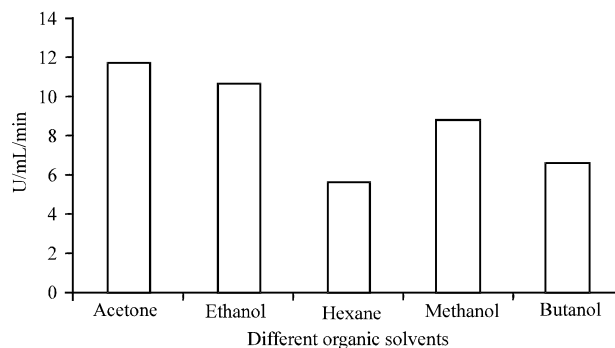


Fig. 10: Effect of organic solvents on lipase activity

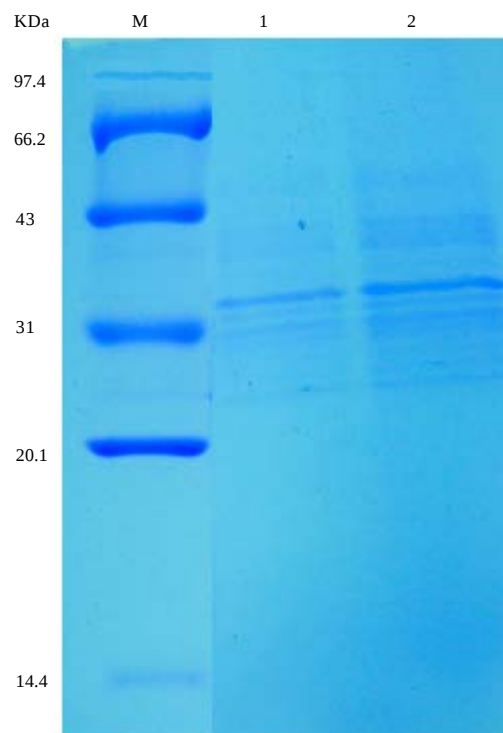


Fig. 11: SDS-PAGE profile of lipase (34 kDa) enzyme protein

temperature range of 40-50°C and organic solvent acetone for 1 h and molecular weight was determined as 34 kDa (Fig. 11).

The molecular mass of lipase was determined by SDS-PAGE to be 34 kDa (Fig. 11). The molecular weight was determined as 31 kDa stated by Kumar *et al.* (2005). The molecular weight of protease and lipase enzymes determined as 40 kDa reported by Sangeetha *et al.* (2010). Thermostable lipases from many *Bacillus* species have been found to possess a molecular mass of 43-45 kDa (Nawani and Kaur, 2000; Lee *et al.*, 2001). Recently, a 62-kDa lipase from *Geotrichum marinum* has been reported by Huang *et al.* (2004). Dharmsthiti and Luchai (1999) reported on thermophilic lipase from *Bacillus* sp. THL027 to be the molecular weight of 69 kDa. In contrast, lipase with very high molecular weight (112 kDa) has been reported from a mesophilic *Bacillus* sp. (Dosanjh and Kaur, 2002; Castro-Ochoa *et al.*, 2005). The lipase was confirmed to be homogeneous by a single band on SDS-PAGE with a relative molecular mass of 11 kDa. Reported molecular weights of microbial lipases are variable, ranging from 12 to 76 kDa (Jurgens *et al.*, 1981). In particular, molecular sizes of lipases from thermophilic bacteria range from 16 kDa in *Bacillus thermocatenulatus* (Schmidt-Dannert *et al.*, 1994) to 69 kDa in *Bacillus* spTHL027 (Dharmsthiti and Luchai, 1999).

CONCLUSION

Selection of medium composition and determination of some important environmental parameters on lipase production by *Aeromonas hydrophila* were performed. This isolated strain utilizes tween 80 as a sole carbon source, beef extract as nitrogen source, fish waste as cheaper

source, pH 9, temperature 37°C and 3% salinity at 60th incubation period. The study proved that the optimization of growth parameters has significant effect on improved production. This is one of the prime objectives of industrial microbiology for large-scale production of valuable metabolites which can be achieved with balanced nutrient supply.

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