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Research Article

PRODUCTION AND PARTIAL CHARACTERIZATION OF LIPASE BY *BACILLUS SP* ISOLATED FROM VELLAR ESTUARY SEDIMENT

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ABSTRACT

Marine microorganisms have been attracting more and more attention as a resource for new enzymes, because the microbial enzymes are relatively more stable and active. In this study, a new lipolytic strain was isolated from estuary sediment sample. On measurement of biochemical test information, the strain was identified as Bacillus sp BM1. The maximum zone of hydrolysis (9.8 mm) and specific lipase activity (86.15 U/mg) was noticed for crude lipase production by the strain. For the lipase purification steps, 60% ammonium sulphate saturation was proved to be effective for maximum specific activity of 60.97U/mg with purification fold 14%. Among the different sources optimization conditions 4% NaCl, 45°C, pH7, xylose and yeast extract shows higher enzyme production. SDS-PAGE analysis showed a single band of partial purified lipase with a size of 54 kDa. From this study, it's evident that the enzyme has the property to tolerate a wide range of different sources which make it attractive towards industrial applications.

Keywords: Bacillus sp, estuary sediment, Lipase, Optimization, SDS-PAGE

INTRODUCTION

Lipase is a water-soluble enzyme that catalyzes the hydrolysis of ester bonds in water insoluble, lipid substrate. Most of the lipases exhibit a high activity on lipids with fatty acid residues of C8 to C18 chain length (Rakesh kumar *et al*., 2012). Lipases are ubiquitous enzymes of considerable physiological significance and industrial potential. The industrial applications of lipases have grown rapidly in recent years and are likely to markedly expand further in the coming years. Lipases may be used to produce fatty acids, biosurfactants, aroma and flavor compounds, lubricant and solvent esters, polyesters, and biomodified fats (Gandhi *et al*., 1995). Lipases are also widely used in the processing of fats and oils, detergents and degreasing formulations, food processing, the synthesis of fine chemicals and pharmaceuticals, paper manufacture and production of cosmetics. Most of the industrial microbiallipases are derived from fungi and bacteria (Tambekar *et al*., 2009).

Marine microorganisms have developed unique metabolic and physiological function but also offer a potential for the production of novel enzymes for wide exploitation. Lipase-producing microorganisms have been found in diverse habitats such as industrial wastes, vegetable oil processing factories, dairies, soil contaminated with oil, oil seeds, and decaying food, compost heaps, coal tips, and hot springs. Lipase-producing microorganisms include bacteria, fungi, yeasts, and *actinomycetes* (Gupta *et al*., 2004). Most of the bacterial lipases reported so far are constitutive and are nonspecific in their substrate specificity, and a few bacterial lipases are thermostable (Macrae and Hammond, 1985).

Lipase production is depends upon a number of factors including carbon, nitrogen sources, pH, temperature, aeration and inoculums size (Heravi *et al*., 2008). Marine *Vibrio sp.* VB-5 produces lipase that hydrolyzes n-3 polyunsaturated fatty acid (PUF A)-containing fish oil. (Chandrasekaran and Rajeev Kumar, (2002). As lipase producing microbes are widely distributed in nature, there is an immense need to explore natural habitats to isolate stable enzyme producing microbes. Hence, the present work was planned to isolate *Bacillus* from estuary sediment and partial characterization of lipase produced by the strain.

MATERIALS AND METHODS

Isolation and identification of bacterial strain:

The sediment sample was collected at Vellar estuary (Lat.11˚29’ N; Long.79˚46’E), Parangipettai, in Southeast coast of India. Samples were collected from 3 to 4 cm depth with the help of sterile spatula and transferred in to sterile plastic bags. They were brought to the Laboratory, inoculated in marine broth and incubated in 30°C for three days. After that they were transferred in to marine agar medium so that the

colonies appeared. After sub culturing each of the colonies, isolation was achieved. The morphological and biochemical characteristics of the selected isolate were performed according to the standard method (Baron and Finegold, 1990; Delost, 1997).

Enzyme assay for lipase:

Lipase activity was determined as described by Gopinath *et al*. (2005), by using olive oil as a substrate. The substrate emulsion was prepared with 50 ml olive oil and 100ml phosphate buffer. The reaction mixture contained 1 ml enzyme, 5 ml substrate and 2 ml of 50 mM phosphate buffer, pH 6.8 and incubated for 1 hour at 37ºC with shaking. The reaction was stopped by adding 4 ml of acetone: ethanol (1:1) containing 0.09% phenolphthalein as an indicator. Enzyme activity was determined by titration of the fatty acid released with 50mM sodium hydroxide solution. One international unit of enzyme is defined as enzyme activity that produced 1µmole of fatty acid per min.

Lipase production:

The selected bacterium was initially enriched by: beef extract (0.15%), peptone (0.5%), sodium chloride (1.0%) and glucose (0.5%), pH 7, at 32°C for 24 h. Then, 5% of enriched seed culture was inoculated into a 50 ml medium (w/v) containing potassium peptone 0.5%, dihydrogen orthophosphate 0.1%; sodium chloride 1% and magnesium sulphate 0.01%. It was then incubated at 32°C at 150 rpm. After incubation it was centrifuged at 10000 rpm and the supernatant was used as lipase enzyme source (Kanlayarki and Boonpan, 2007).

Partial purification of lipase:

The crude lipase was fractionated by ammonium sulphate precipitation at various concentration *i.e*. 20%, 40%, 60%, 80% and 100%. After addition of ammonium sulphate, the flasks were stirred on magnetic stirrer. Then it was placed in refrigerator for overnight. Precipitated fractions were collected by centrifugation at 10,000 rpm at 4˚C for 10 min. The fractions were dissolved in little amount of phosphate buffer and dialyzed by dialysis membrane (Himedia) at 4˚C for overnight (Ramadas and Lavanya, 2012).

Optimization of lipase production:

Effect of Sodium chloride:

Sodium chloride at varying concentration was added to all the enzyme production broth *viz.* 1%, 2%, 3%, 4% and 5%. After incubation of the strain, the flasks were maintained at the specific temperature indentified for the individual strains for seven days. Simultaneously a control was maintained without sodium

chloride. Cultures were maintained at previously standardized parameters (Rajkumar, 2010).

Effect of temperature and Ph:

Effect of different temperature values on growth and enzyme production, five different temperature (˚C) the reaction mixture containing 2.0ml of 0.5% casin solution in 0.1m carbonate buffer (pH 0.3) and 0.1ml of enzyme solution in the total volume of 2.1ml after incubation at 25˚C, 30˚C, 35˚C, 40˚C and 45˚C For 5mins, the reaction was stopped by adding of 3.0 ml of 10% ice cold and centrifuged at 10000 rpm for 5mins.and measured the enzyme production.

Effect of different pH grown in nutrient broth containing 1% casein and 3% NaCl. Effect of pH on enzyme production was studied by adjusting the culture media pH (4.0, 5.0, 6.0, 7.0, 8.0, and 9.0) prior to incubate at 37°C for 48 hr in an orbital shaker at 150 rpm. The contents were centrifuged at 10,000 rpm at 4°C for 10 min and enzyme production was checked in the cell free extract.

Effect of carbon and nitrogen sources:

To find the optimum carbon source for enzyme production, five carbon sources (1%) (starch, glucose, sucrose, lactose and xylose) were selected and added to nutrient broth. The organism was inoculated and incubated for 48 hrs at 37°C and the enzyme activity was assayed in the culture supernatant (Lesuisse *et al*., 1993). To optimize the nitrogen source for enzyme production, five different nitrogen sources (1%) (yeast extract, gelatin, casein, urea and ammonium chloride) were added to nutrient broth and the organism was inoculated and incubated for 48hrs at 37˚C The enzyme activity was assayed in the culture supernatant (Lesuisse *et al*., 1993).

Molecular weight determination:

Molecular weight of lipase was determined using SDS PAGE by following the procedure of Rakesh kumar *et al*., (2012). Glass plates were assembled and 20ml of 15% resolving gel was prepared and poured immediately to the notch plate. It was over laid with butanol, after polymerization was completed, over lay was poured off and washed the top layer with deionized water. Then 8ml of stacking gel was over laid. 10µl gel loading buffer and sample was heated at 100 ºC for 3 min. Assembly was fixed in electrophoresis apparatus then 15µ1 of sample and marker (14-78 kDa) was loaded in adjacent wells, run the gel and strain with coomassie brilliant blue.

RESULTS

Isolation and identification of bacterial strain:

Six bacterial isolates were grown in tributyrin agar medium, pH 7.0 at 37°C to get bacteria for potent

lipase producing ability. A screening test of lipolytic activity of all bacterial isolates resulted in the fact that only one bacterial isolates were found to be very good result. The most potent one bacterial isolate was selected for further study. The gram staining result showed the bacterium was gram positive Bacillus (Figure.1). Based on the morphological and biochemical features the bacteria was identified as *Bacillus* sp BM1 (Table.1).

Biochemical test	**Results**
Gram staining	+
Morphology	Rod
Motility	+
Oxidase	+
Catalase	+
Methyl rad	+
Indole	-
Voges-Proskauer	+
Urea	-
Citrate utilization	-

Table 1: Biochemical test for isolated bacteria

+ (positive) – (negative)

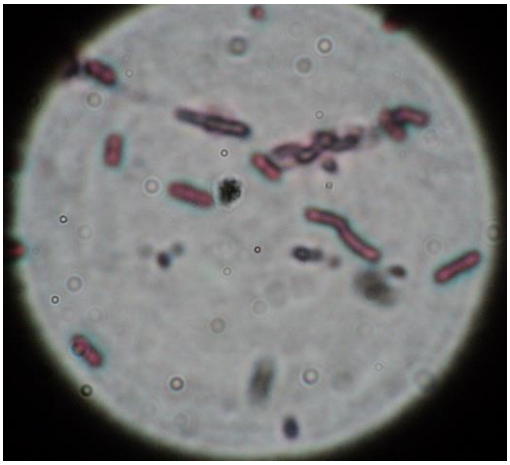

Figure 1: Gram positive *Bacillus* bacteria

Enzyme assay for lipase:

The maximum zone of hydrolysis (9.8 mm) and specific lipase activity (86.15 U/mg) was noticed for crude enzyme from BM1 strain while minimum zone of clearance (3.1mm) and specific lipase activity (0.58

U/mg) was observed in BM1 strain (Figure.2).

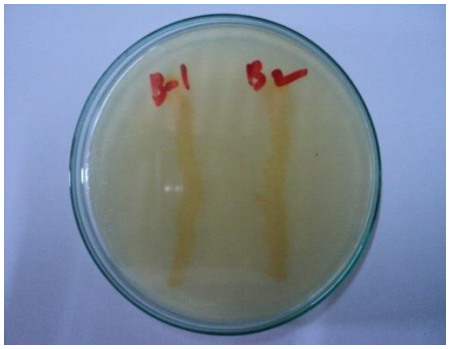

Figure 2: The plate showing the lipase activity by *Bacillus sp*

Partial purification of lipase:

To precipitate lipase by ammonium sulphate, experiment was conducted at 20%, 40%, 60%, 80% and 90% saturation of ammonium sulphate salt. Results revealed that 60% saturation was proved to be effective for maximum specific activity of 60.97 U/mg with purification fold of 14%.

Effect of Sodium chloride:

The bacteria were isolated from the sediment and the effect of NaCl on lipase production was tested. The effect of salinity (1%, 2%, 3%, 4% and 5%) on lipase production was as given in Fig. 3. Among the five different salinity conditions 4% NaCl shows higher (95.18 U/ml) enzyme production.

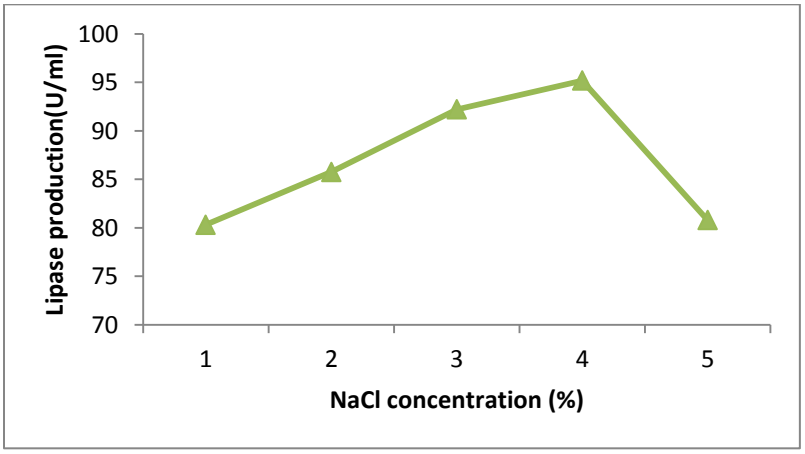

Figure 3: Production of lipase at different NaCl concentration

Effect of temperature and pH:

The effect of temperature (25, 30, 35, 40 and 45°C) on lipase production is given in Figure. 4. Among

the five different temperature conditions 45°C shows higher (87.25 U/ml) enzyme production in the liquid media. The effect of pH on lipase production was studied by growing the bacteria in fermentation media set at different pH (4, 5, 6, 7, 8 and 9). Among the seven pH ranges, pH7 shows high (140.29 U/ml) lipase production (Figure. 5).

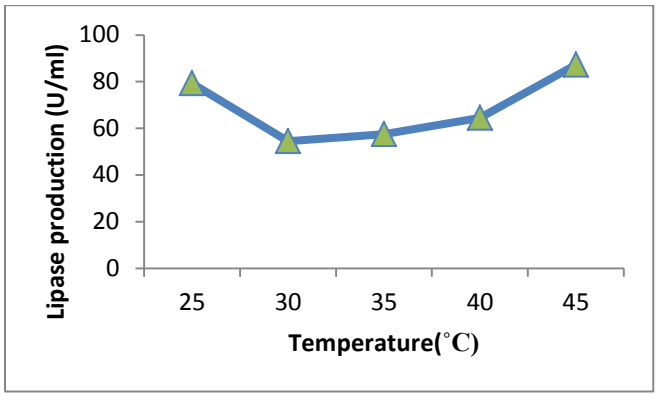

Figure 4: Production of lipase at different temperature

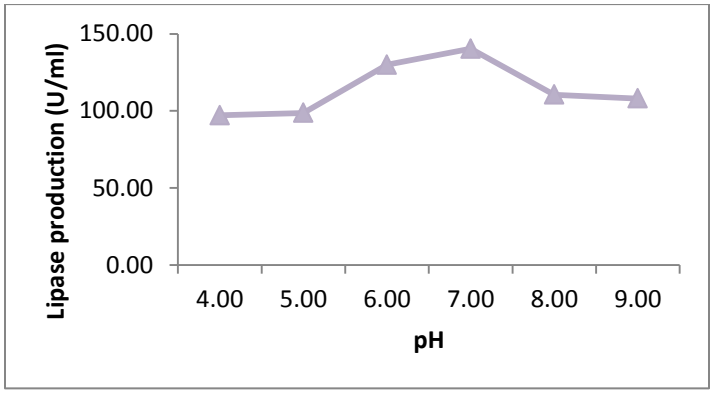

Figure 5: Production of lipase at different pH

Effect of carbon and nitrogen sources on lipase production:

The effect of carbon sources on lipase production was tested by using five different carbon sources. Among the five difference carbon sources, xylose shows high (102.12 U/ml) enzyme production (Figure. 6).

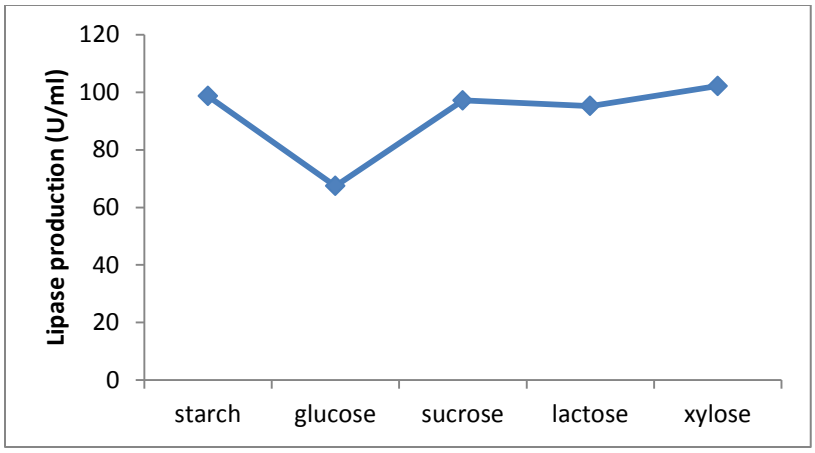

Figure 6: Production of lipase enzyme at different carbon source

The effect of nitrogen sources on lipase production was tested by using six different nitrogen sources. Among these five different nitrogen sources, yeast extract shows high (72.32 U/ml) enzyme production and the result is given in Figure. 7.

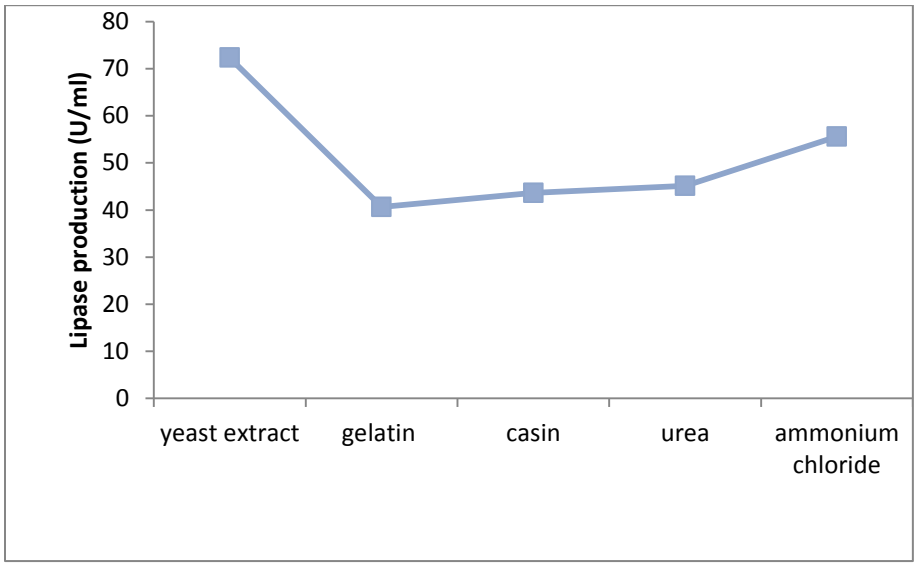

Figure 7: Production of lipase at different nitrogen source

Molecular weight of lipase:

SDS-PAGE analysis showed a single band of partial purified lipase after ammonium sulphate precipitated. Partial purified lipase yields one well defined band with as size of 54 kDa when comparing with standard molecular weight markers (14 to 78 kDa) (Figure. 8).

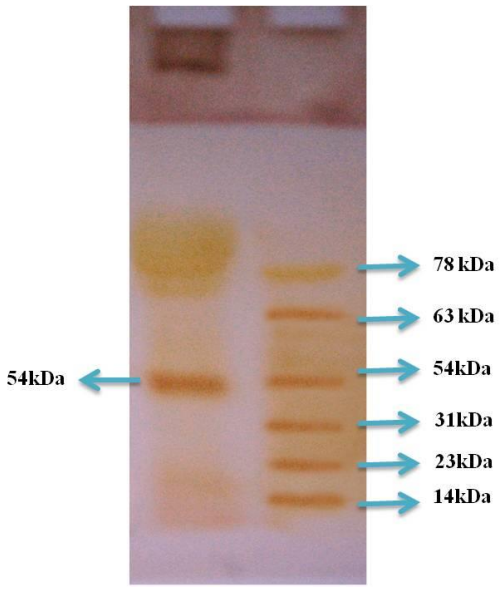

Figure 8: SDS-PAGE zymogram of lipase produced by *Bacillus sp*

DISCUSSION

Microorganisms play major roles in energy transformations and biochemical processes in exceedingly diverse habitats especially, in marine environments. Knowledge of both microbial diversity and microbial activity in the coastal area could be important for business and industry as well as in maintaining the health of near-shore ecosystems (Leow *et al*., 2007). Studies in the past two decades show that heterotrophic bacteria not only function as decomposers (such as lipase producing bacteria), but also as a channel dissolved organic substances and inorganic nutrients to higher atrophic levels through the microbial food web (Seinthikumar and Selvakumar, 2008).

Microbial lipases are currently receiving much attention with the rapid development of enzyme technology. In the present study, six bacterial isolates were grown in tributyrin agar medium, pH 7.0 at 37°C in order to isolate bacteria for potent lipase producing ability. A screening test of lipolytic activity of all bacterial isolates was made and only one bacterial isolates was found to as good lipase producer. The gram staining result showed the bacterium was gram positive Bacillus. Although biochemical characteristics suggested the bacterial isolate as *Bacillus sp* and a common inhabitants of estuary environments.

The plates contained a lipid substrate and lipolysis was observed as clear halos or opaque zones around the culture. Nair and Kumar (2007) recommended these methods of lipase detection on agar plates. In the present study, the maximum zone of hydrolysis (9.8 mm) and specific lipase activity (86.15 U/mg) was noticed for crude lipase from BM1 while minimum zone of clearance (3.1mm) and specific lipase activity

(0.58 U/mg) was observed for BM3. Presence of extra cellular lipase in cell free extract is possibly responsible for the lipolytic activity of the isolates (Kalogridou-Vassiliadou, 1984). In titrimetric method lipase activity was measured as a release of free fatty acids during specific time duration by using phenolphthalein as end point indicator. Action of lipase hydrolyzes the lipid into fatty acids and glycerol. Hydrolysis of tributyrin results increase in acidity of reaction mixture which can be measured titrimetrically (Deeth and Touch, 2000). Two bacterial strains were isolated and identified as Bacillus sp according to the morphological and biochemical assays. Tween 80, Rhodamine B-Olive oil plates were used and screened for production of lipase (Seinthikumar and Selvakumar, 2008). Sebdani *et al*. (2011) estimated that the maximum lipase activity showed in the 3rd day (72 hours) and the production of lipase enzyme on the first day was 25.25 U/ml for BB-1 and 25.50 U/ml for BB-2.

Precipitation of enzymes was carried out by ammonium sulphate since it was highly soluble in water, cheap and had no deleterious effect on structure of protein. In the present study, the precipitate lipase by ammonium sulphate, experiment was conducted at 20%, 40%, 60%, 80% and 90% saturation of ammonium sulphate salt. Results revealed that 60% saturation was proved to be effective for maximum specific activity of 60.97U/mg with purification fold of 14%. These results matches with the purification strategies followed by Kanwar *et al*. (2002) for the precipitation of lipase from *Pseudomonas sp*. G6. Rakesh Kumar *et al*. (2012) reported that 60% saturation was effective concentration with 6.53 fold specific activities, among tested different saturations. Lipase from *Pseudomonas sp.* Yo103 was purified using ammonium sulphate fractionation, Sphadex G200 to 62 fold increase and 3.7% yield by Kim *et al.* (2000). In this study the results are encouraging in the sense that only partial purification steps are needed to achieve high fold purification. At industrial point of view the minimum steps are prerequisite to purify the enzyme at homogeneity so that the production cost can be controlled.

In the present study, among the five different salinity conditions, 4% NaCl shows higher (95.18 U/ml) enzyme production. Previously, Sharma and Rathore (2010) reported that the enzyme activity of certain isolates could be observed even at 7.5 % NaCl concentration but optimum activity was observed between 2.5 – 4.5%. Addition of NaCl or change in osmolarity altered water activity of media which in turn modulate the permeability of bacterial cell wall hence eased the release of lipase enzyme in external environment (Takahashi and Gibbons, 1957). According to MacLeod (1965) Na+ concentrations are needed for the membrane active transport hence enhanced the secretion of lipase. Whereas for some isolates, increased osmolarity or salinity due to the presence of NaCl create hypertonic environment condition which adversely affects the growth of bacteria by shrinkage effect and it directly affects the secretion of lipase from bacterial isolates.

Various industrial applications require lipase that remain thermo stable (Leow *et al*., 2007). Among the five different temperature conditions, 45°C shows higher (87.25 U/ml) enzyme production in the liquid

media. Previously, lipase from all the bacterial isolates retained stability at all provided temperature ranges *i.e.* 15°C to 45°C (Sharma and Rathore, 2010). Earlier studies regarding lipase production by *Serratia* sp. isolated from raw milk samples stated that optimum temperature for lipase production was 30–40°C (Abdou, 1997). But in the present study the production gradually increases with the increase of temperature from 10 to 40°C and further increase of temperature, beyond 40°C, decreased the production of lipase. Among the seven pH ranges, pH7 shows high (140.29 U/ml) lipase production. Invariably, the lipase production by *Serratia marcescens* confirms the same pH condition (Gao *et al*., 2004)

In the present study, the effect of carbon sources on lipase production was tested by using five different carbon sources. Among the five difference carbon sources, xylose shows high (102.12 U/ml) enzyme production. This result supports the earlier observations of lipase higher production by *Bacillus circulans* induced with starch (Sztajer and Malszewska.1988). The lipase production by *Streptomyces sp*. and *P. fluorescens* was increased when these organisms were cultured in the medium containing soluble starch (Sztajer and Malszewska, 1988). Immanuel *et al*. (2008) reported that the effect of carbon source on lipase production indicated that starch was suitable substrate to maximize lipase production (15.60 ± 0.20 U/ml) and the optimum concentration registered was 4 g/L/ U/mL). Rathi *et al*. (2002) observed that olive oil is the most used lipid substrate to induce lipase production by bacteria however other carbon sources like molasses have been found to completely inhibit the lipase production by *Bacillus* sp. Eltaweel *et al*. (2005). reported that bacterial lipases are mostly extracellular; where the biggest factor in the expression of lipolytic activity has always been the requirement of an adequate carbon source.

Among the five tested nitrogen sources, yeast extract shows high (72.32 U/ml) enzyme production. Whereas the lowest lipase production was registered in yeast extract-supplied medium for Immanuel *et al*. (2008). The quantities of lipase measured were generally lower than that detected for fungi like *Geotricum* that showed maximum lipase production (146 U/ml) when urea was used as nitrogen source Ginalska *et al*., (2007). A possible mechanism may be that yeast extract is a complex nitrogen source and thus requires the cells to secrete more protease for its enzymatic degradation before utilization.

Partial purified lipase showed single band with the size of 54 kDa in SDS-PAGE. Previously, Abdou (2003) also reported that the molecular mass of purified lipase of *S. marcescens* was 52kDa. The observation of Arpigny and Jaeger (1999) inferred that the molecular mass of lipases from *P. fluorescens* and *S. marcescens* was 50 and 65 kDa, respectively. Lower molecular weight lipase (50 kDa) was purified from the culture filtrate of *Streptomyces cinnamomeus* (Sommer *et al*., 1997). From this study it is evident that the isolated *Bacillus sp*. from estuary sediment has the potential to produce lipase enzyme. The enzyme had property to tolerate a wide range of different sources which make it attractive towards industrial applications.

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