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# CHARACTERISATION OF A BACTERIUM LIPASE (*Bacillus subtilis*) FROM VEGETABLE OIL POLLUTED SOIL

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## *ABSTRACT*

*Bacteria are very useful in protecting the environment. The lipolytic activities of physiologically diverse bacteria have great potential to degrade oil spills in the environment. There is a need for extensive characterisation of the bacterium lipase for the treatment of vegetable oil-polluted sites. This work was carried out to preliminarily characterise the lipase of Bacillus subtilis.*

*Bacillus subtilis was screened for lipase production using standard methods. Temperature, pH, ion concentration (NaNO and MgSO4), enzyme concentration, nitrogen concentration, substrate concentration, time course and agitation speed were optimized for the lipase activity as well as growth.*

*The Crude enzyme of Bacillus subtilis had the highest lipase activity and growth of 0.5 U/mL and 0.929mg/mL respectively at room temperature, but when production was optimised higher activity 0.6 U/mL was seen in the use of urea as nitrogen source. Agitation did not support lipase production. Glycerol as a substrate had highest growth of 1.637 mg/mL.*

*The enzyme has good potential for the hydrolysis of vegetable oils, which is an important factor in environmental cleanup of vegetable oil spill site.*

***KEYWORDS:*** *Characterisation, Vegetable Oil Spill, Lipase Production*

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## INTRODUCTION

Lipases (triacylglycerol acylhydrolases) are a group of enzymes, which have the ability to hydrolyze triacylglycerols at an oil-water interface to release free fatty acids and glycerol. Lipases are present in microorganisms, plants and animals (Jisheng *et al*., 2005).

Bacteria produce different classes of lipolytic enzymes including Carboxylesterases which hydrolyze water-soluble esters and lipases which hydrolyze long-chain triacylglycerol substrates (Rosenau and Jaeger, 2000). Many bacterial species produce lipases which hydrolyze esters of glycerol with long-chain fatty acids. They act at the interface generated by a hydrophobic lipid substrate in a hydrophilic aqueous medium. Lipases show interfacial activation, a sharp increase in lipase activity observed when the substrate starts to form an emulsion (Ece Yapasan, 2008). As a

consequence, the kinetics of a lipases reaction does not follow the classical Michaelis-Menten model with only a few exceptions, bacterial lipases are able to completely hydrolyze a triacylglycerol substrate although ester bonds are more favorable (Jaegar et al., 1994). Some important lipase - producing bacterial species are *Bacillus, Pseudomonas* and *Burkholderia* (Svendsen, 2000).

The synthesis and secretion of lipases by bacteria is influenced by a variety of environmental factors like ions, carbon sources or presence of non-metabolizable polysaccharides (Ece Yapasasn, 2008).

Bacterial lipases are mostly released outside of the cell that is called extracellular enzyme. They are influenced by nutritional and physic-chemical factors, such as temperature, pH, nitrogen and carbon sources, the presence of lipids, inorganic salts, stirring conditions, dissolved oxygen concentration (Rosenau and Jaeger, 2000).

The major factor for the expression of lipase enzyme is carbon source. Lipases generally are produced in the presence of lipid source such as oil, triacylglycerols, fatty acids, hydrolyzable esters, the tweens, and glycerols addition to the carbon source, the type of nitrogen source also influence the production of lipases. Generally, theorganic nitrogen source is preferred by bacteria, such as peptone and yeast extract (Gupta et al., 2004).

The initial pH of the growth medium is important for lipase production. Most bacteria prefer pH around 7.0 for their best growth and lipase production. The optimum temperature for lipase production is parallel with the growth temperature of the respective microorganism. It has been reported that lipases are produced in the temperature range from 20$^{o}$C to 45$^{o}$C (Jaeger et al., 1999) incubation periods change from few hours to many days until the maximum lipase production from bacteria is recorded.

## MATERIALS AND METHODS

### Growth studies and Production of the Enzymes

### Growth Media

Isolates were grown in a complex basal medium whose composition was a modification of the medium of Tsujisaka *et al.* (1973) with glucose omitted. This medium contained 5% peptone, 0.1% $NaNO_3$ and 0.1% $MgSO_4$, adjusted to the desired pH, before sterilization. Sterile olive oil (Goya) was added as the carbon source.

### Lipase Production

### Preparation of Inoculum

A loopfull of the pure culture of the bacteria were grown overnight in nutrientsbroth.

### Production Procedure

One milliliter from the above preparation was inoculated into 65.0ml of sterile medium in 250ml Erlenmeyer flasks and incubated at room temperature (27$^{o}$C $^{\pm}$2$^{o}$C) from 24hrs to many days until the maximum lipase production was recorded. The medium was centrifuged at 30,000 g for 15min using Himac High-speed Refrigerated Centrifuge (Hitachi model CR21GII).The supernatant of the centrifuged culture broth was then decanted leaving the cells at the bottom. The cell-free extract acted as the crude lipase enzyme.

## Growth of the Isolates

Growth of the Isolates in the growth medium was examined spectrophotometrically using a Jenway 640 UV/VIS spectrophotometer at 540nm, absorbances were measured against a blank (Gojkovic, 2009).

## Lipase Assay

Lipase activity was measured by a modification of the assay of Parry *et al.* (1966) using as substrate a 10% Olive oil-gum arabic solution emulsified by sonication for 2mins at 25watts output according to Linfield *et al.* (1985). One milliliter of cell-free fermentation broth prepared by centrifugation as described above was added to 5ml of emulsion and incubated at room temperature for 1h with rapid stirring. Ethanol was added to stop the reaction and the free fatty acids produced were quantified by titration to pH 9.5 against 0.1N NaOH using a radiometer titration system. Blanks with 1ml of fermentation broth were employed in each experiment. Blanks ran with sterile or actual uninoculated broths were the same within experimental error. Samples were run in duplicate.

A unit of lipase activity was defined as the amount of sodium hydroxide (NaOH) used in the titration to bring the reaction mixture to a pH of 9.5 per min under the defined assay conditions. Alternatively, it is considered the release of one micromole of free fatty acid (FFA)/min at room temperature.

## Optimization of Production Conditions

### Effect of pH on Lipase Production

This was carried out using a modified method of Tsujisaka *et al.* (1973). Growth medium was prepared in 0.2M phosphate buffer and (0.1M citric acid mixed with 0.2M $Na_2HPO_4$ ) citrate phosphate buffer of varying pH (5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5). The lipase activity and the growth in the culture supernatant were determined using the appropriate procedure.

### Effect of Temperature on Lipase Production

The effect of temperature on lipase activity was determined using the above method. The organism was cultivated in the growth medium at different temperatures, which ranged from 20$^{o}$C, 27$^{o}$C, 40$^{o}$C, 50$^{o}$C, 60$^{o}$C to 70$^{o}$C for 24hours. The lipase activity and growth in the culture supernatant was determined.

### Effect of Aeration on Lipase Production

After inoculating the organisms into the growth medium, the flasks were continuously shaken at 27$^{o}$C for 24hours at varied revolutions per minute (80, 100, 120 and 140) using orbital shaker Stuart SSLI. The lipase activity and growth in the culture supernatant were then determined.

### Time Course of Lipase Production

The organisms were cultivated in the growth medium for different periods that ranged from 24hours to 72hours. Samples were removed periodically and growth and lipase activity in the culture supernatant were determined.

### Effect of Different Nitrogen Sources on Lipase Production

The main nitrogen source in the growth medium was replaced by other nitrogen sources such as casein, urea and yeast extract at the same concentration (5 %). The lipase activity and growth in the culture supernatant were then determined.

### Effect of Different Substrates on Lipase Production

To determine the suitable substrate (carbon source) for the production of lipase by the organisms, substrates such as; glycerol, soy oil, olive oil and a simple sugar (glucose) were used. They were individually tested by replacing the substrate present in the growth medium at the concentration of 2%. Thereafter, the lipase activities ,as well as growth in the culture supernatant, were determined.

### Effect of Metal ion on Lipase Production

The growth medium in which the organisms were cultivated had their metal ions varied. $ZnSO_4$, $FeSO_4$, $(NH_4)_2SO_4$, $KNO_3$, $AgNO_3$, $CaNO_3$, $Na_2CO_3$ and NaCl were used in equimolar concentration (0.1mM) instead of $MgSO_4$ and $NaNO_3$. The lipase activity and growth in the culture supernatant were then determined.

Furthermore, the effect of different concentrations of the substrate, nitrogen, anion and cation, crude enzyme and varied temperature on growth and lipolytic activity of *Pseudomonas fluoresens*were also studied according to the assay method described above.

## RESULTS

Figure 3.1 shows the effect of pH on growth and lipase production of *Bacillus subtilis,* maximum growth, and lipase production was recorded at pH 7.5, while maximum lipase production was recorded at pH 7.0.Efffect of temperature on growth and lipase production on the bacterium as shown in Figure 3.2, maximum growth and lipase production was recorded at 27$^{o}$C. Increase in agitation speed increased growth but agitation did not support lipase production at all as shown in figure 3.3. At 48hours of incubation maximum growth was recorded (1.218mg/ml) for *Bacillus subtilis* but lipase production decreased with an increase in incubation time as shown in figure 3.4.

Figure 3.5 shows the effect of the different carbon sources on growth and lipase production of *Bacillus subtilis* glycerol enhanced highest growth and lipase production, while Olive oil enhanced highest lipase production. Yeast extract supported growth but not lipase production while Urea least supported growth but recorded highest lipase production as shown in Figure 3.6. Figures 3.7 and 3.8 shows the effect of metal ions on growth and lipase production of *Bacillus subtilis,* in the case of effect of cations on growth and lipase production of *Bacillus subtilis* $KNO_3$ supported growth but not lipase production, amongst the anions, tested NaCl had the least support for growth and Lipase Production as shown in Figure 3.8.

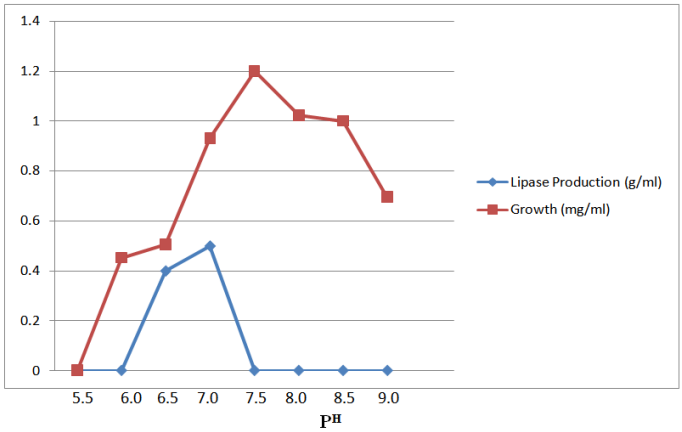


**Figure 3.1: Effect of PH on Growth and Lipase Production by *Bacillus Subtilis***

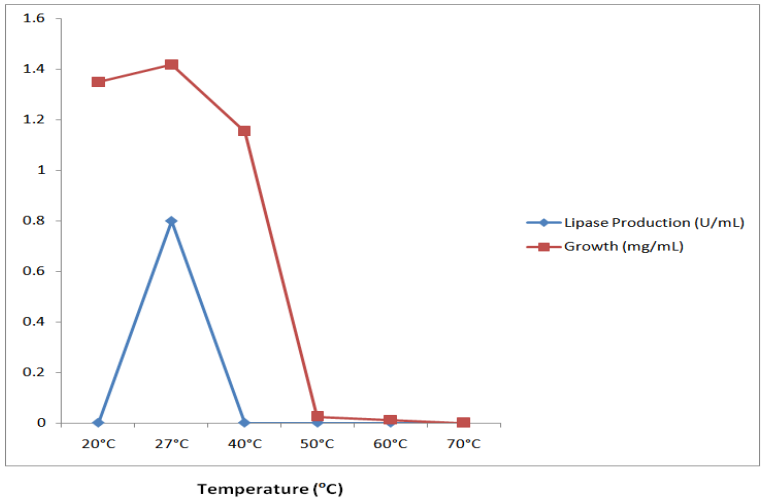


**Figure 3.2: Effect of Temperature on Growth and Lipase Production by Bacillus Subtilis**

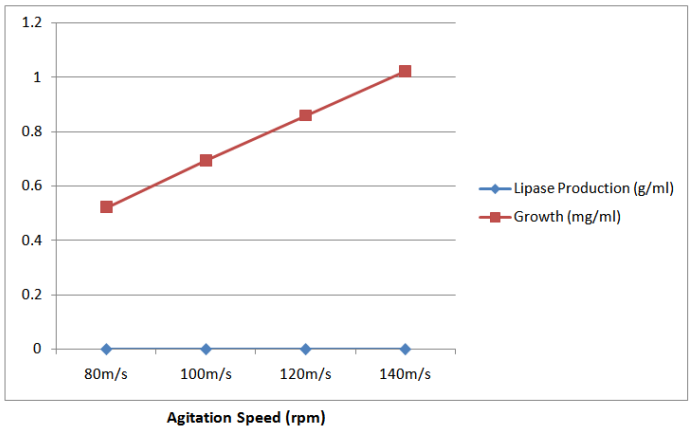


**Figure 3.3: Effect of Agitation on Growth and Lipase Production by *Bacillus Subtilis***

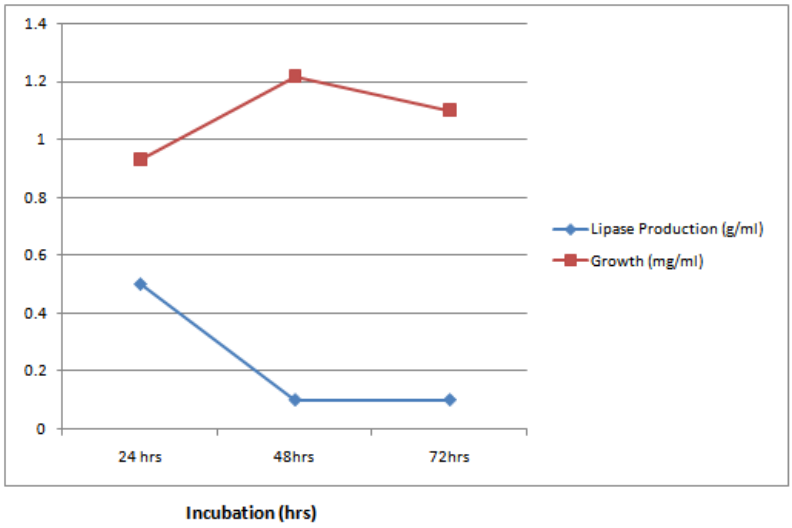


**Figure 3.4: Effect of Incubation Time on Growth and Lipase Production by *Bacillus Subtilis***

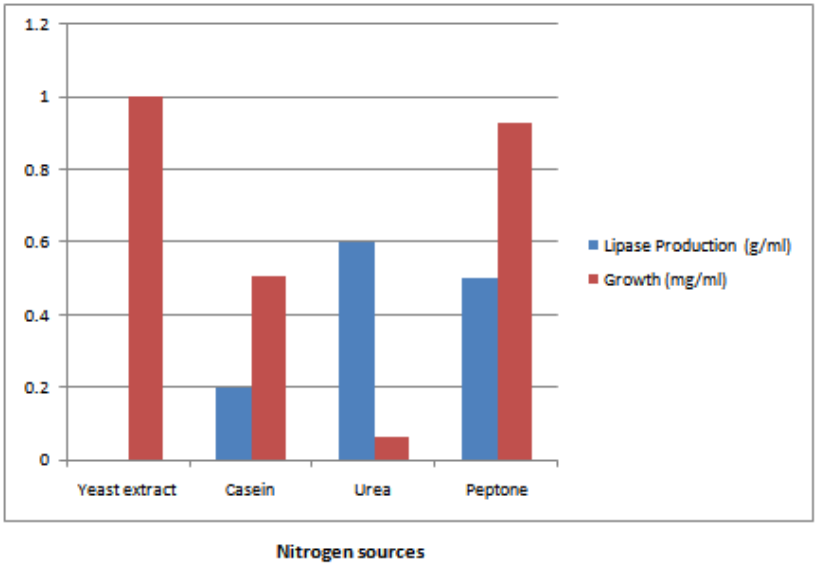


**Figure 3.6: Effect of (5%) Different Nitrogen Sources on Growth and Lipase Production by *Bacillus Subtilis***

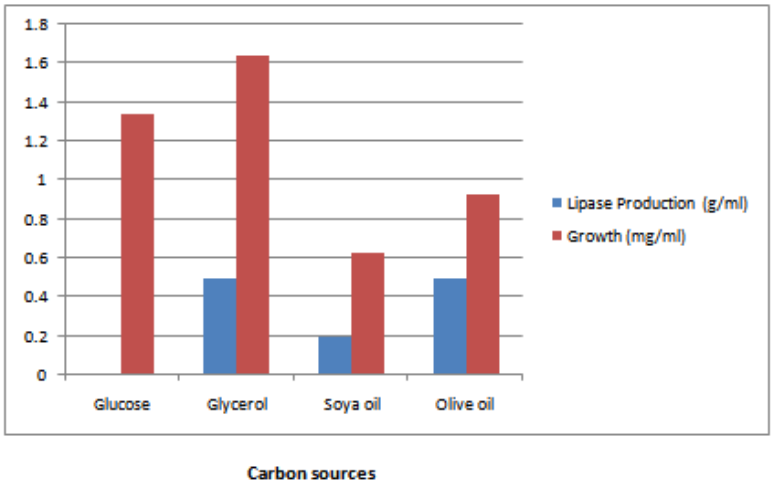


**Figure 3.6: Effect of (2%) Different Carbon Sources on Growth and Lipase Production by *Bacillus Subtilis***

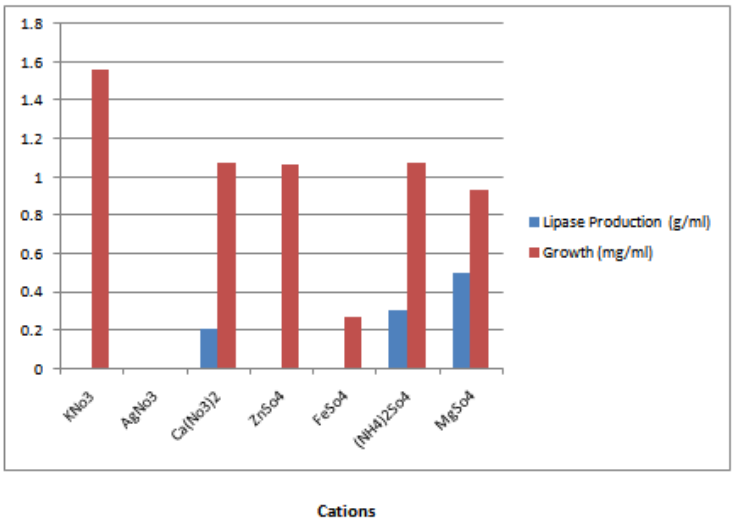


**Figure 3.7:Effect of (0.1%) Different Cations on Growth and Lipase Production by *Bacillus Subtilis***

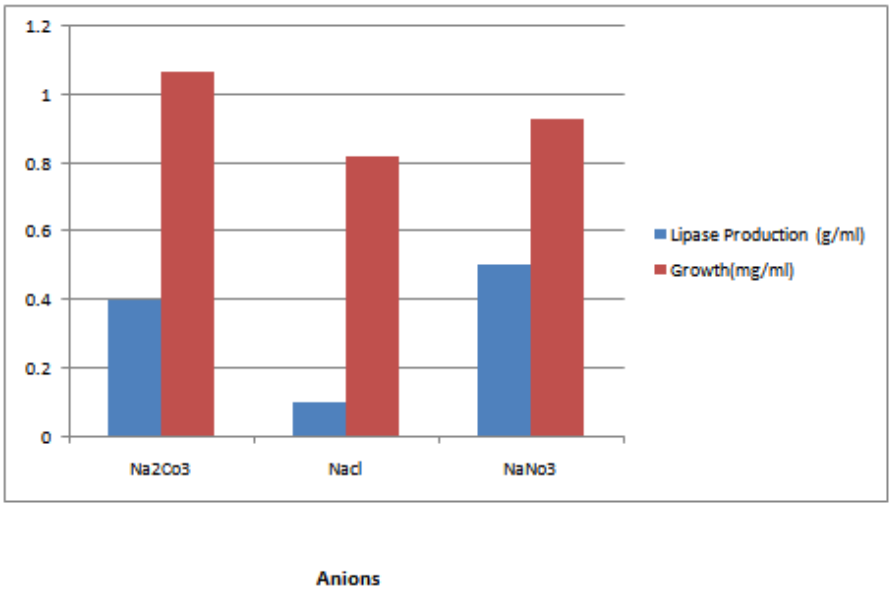


**Figure 3.8: Effect of 0.1% Different Anions on Growth and Lipase Production of *Bacillus Subtilis***

## DISCUSSIONS AND CONCLUSIONS

Increase in microbial growth does not necessarily mean the increase in lipolytic activity as generally observed from this study. On the contrary, Becker *et al*., (1999), Keenan and Sabelnikov (2000) used microbial growth parameters to measure lipids degradation. However, according to Kramer (1971) as seen in this study ( a physiological study of *Bacillus subtilis)* an increase on biomass concentration may not produce an increase in lipid matter hydrolysis, because lipase production is not a function of cell growth or concentration.

The obtained bacterium lipase was generally observed to work best in alkaline neutral pH (7.0) (Sugihara *et al.*1991. In low and high medium pH tested the lipase activity was less. This result is consistent with the report of Mohan *et al.* (2008). They stated that the lipase activity of *Bacillus sp* was optimal at pH 7 during the 24h culture period.

In this study, the bacterium lipase had its optimum growth temperature and lipase activity at 27$^{o}$C. Ece Yapasan (2008) reported the optimum bacterial growth temperature to be 25$^{o}$C for *Pseudomonas sp* with lipase enzyme showing activity above and below this temperature. Temperature changes give rise to cleavage of hydrogen bonds between substrate and enzyme active sites. Optimum temperature value promotes the binding potential of enzymes and substrate. However, in this study, increase in temperature above known optimum tends most likely to denature the enzymes thereby reducing the enzyme activity.

In this study, agitation supported the growth of the experimental organism, signifying that it is aerobic in nature and required large quantities of dissolved oxygen for its growth and multiplication. This is also affirmed by Chander *et al.* (1980). But on the contrary, Chander *et al.* (1980) noted that agitation improved lipase production whereas stationary cultures produced better lipolytic activity than agitated cultures in this experiment. Further, Ebrahimpour *et al.* (2008) showed that shallow layer (static culture) where aeration is moderate produced much more lipase than shake cultures (high aeration). Increase in lipase production on increasing agitation could be due to increased oxygen transfer rate, increased surface area contact with the media component or better dispersibility of the carbon sources.

As observed in this research, maximum lipase activity for the *Bacillus subtilis* enzyme studied was studied was obtained after 24hrs of incubation, indicating that lipase was necessary for the first stages of growth, while growth was decreased after 48hrs, an observation in agreement with Ginalska *et al.* (2007). Further, at longer incubation periods, the lipase activity decreased which might be due to the depletion of nutrients, accumulation of toxic end-products, the change in pH of the medium, or loss of moisture.

Generally, peptone stimulated lipase production better than most of the nitrogen sources experimented with in this study. This is in agreement with the work of Tembhurkar *et al.* (2012).It is also in agreement with the finding of Sirisha *et al.* (2010) who recorded better lipase production by *Staphylococcus* when peptone was used in place of yeast extract and tryptone as a nitrogen source. Gupta *et al.* (2004) generally observed that organic nitrogen source such as peptone and yeast extract is preferred by bacteria. Also, peptone and yeast extract has been used as a nitrogen source for lipase production by various *Bacillus* spp., various *Pseudomonads* and *Staphylococcus haemolyticus,* respectively (Wang *et al.,* 1995; Khyami-Horani, 1996; Pabai *et al.,* 1995; Oh *et al.,* 1999; Ghanem *et al.*,2000; Lanser *et al.,* 2002).Inorganic nitrogen sources such as ammonium chloride and di-ammonium hydrogen phosphate have also been reported to be effective in some microbes (Gilbert *et al.,* 1991; Bradoo *et al.,* 1999; Dong *et al.,* 1990; and Rathi *et al.,* 2001).

In the case of substrate hydrolysis, almost all the substrates tested were hydrolyzed by the tested organism. Most of the substrates have long carbon chains (Olive oil has C18:1) which may take time to dissolve. Since lipases hydrolyze esters in the emulsion and usually water - insoluble substrates, the organisms take up the substrate at the different concentration, form and time. Typically, triglycerides composed of long-chain fatty acids, whereas esterases preferentially hydrolyze “simple” esters and usually only triglycerides bearing fatty acids shorter than six carbon chains (C6). Thus, these results strongly suggest that the enzyme used in this study show lipase activity. The findings of Pogaku *et al.* (2010), olive oil supported good growth and increased lipase activity significantly. Olive oil in this case, acted as an inducer of lipase production; hence lipase production has been shown to be induced remarkably in the presence of lipidic carbon sources like oils and fatty acids. Glucose was found effective in stimulating lipase production. Prabhakar *et al.* (2002) obtained maximum production of the enzyme with glucose among various carbon sources studied.

The catalytic activities of the lipases in the current study were enhanced in the presence of $Ca^{2+}$,$Mg^{2+}$and $NH_3^+$, but inhibited by $Zn^{2+}$ and $Hg^{2+}$ . These results are in agreement with Chakraborty and Raj (2008).$Ca^{2+}$ ions have been known to stimulate lipase activity in varying concentrations. It has been reported that in the presence of $Ca^{2+}$, lipase activity of *Bacillus licheniformis* strain H1 increased up to 120% (Khyami-Horani, 1996), (Sharma *et al.*2002) also reported stimulation of lipase production from *Bacillus sp.*in the presence of $Ca^{2+}$ and $Mg^{2+.}$ However, most other metal ion salts were inhibitory to lipase production. The enzyme was inhibited by $Al^{3+}$, $Co^{2+}$, $Mn^{2+}$ and $Zn^{2+}$ ions while $K^+$, $Fe^{3+}$, $Ag^{2+}$ and $Mg^{2+}$ ions enhance the enzyme activity, $Na^+$had no effect on enzyme activity (Kumar *et. al*, 2005). Mukeshkumar *et. al,* (2012) reported that $Fe^{2+}$, $Mg^{2+}$, Triton X-100 and Tween esters enhance lipase production in *Bacillus sp.*MPTK912.

Hence, lipase production is generally 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, 2001). The enzyme has good potential for hydrolysis of vegetable oils which is an important factor in environmental cleanup of vegetable oil spill site.

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