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Characterisation of a Bacterium Lipase (Pseudomonas Fluoresens) 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 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 Pseudomonas fluoresens.*
Pseudomonas fluoresens was screened for lipase production using standard methods. Temperature, pH, ion concentration ($NaNO_3$ and $MgSO_4$), enzyme concentration, nitrogen concentration, substrate concentration, time course and agitation speed were optimised for the lipase activity as well as growth.
Crude enzyme of Pseudomonas fluoresens had the highest lipase activity and growth of 0.8 U/mL and 1.418 mg/mL respectively at room temperature, but when production was optimised higher activity 0.9 U/mL was seen in the use of glucose as substrate. Agitation with the speed used did not support lipase production but supported growth (1.998 mg/mL) at agitation speed of 100 rpm. Different substrate concentration which had optimum enzyme activity for Pseudomonas fluoresens had activity of (1.1 U/mL), was at 1.0 %. At 30^oC Pseudomonas fluoresens had activity of 1.0 u/mL. Substrate concentration had optimum growth (1.778 mg/mL) at 0.5 %.
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: *Vegetable oil spill, Lipase production, characterisation.*

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I. Introduction

Lipases (triacylglycerol acylhydrolases) are a group of enzymes, which have the ability to hydrolyse 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).

Lipases catalyse a wide range of reactions, including hydrolysis, inter-esterification, alcoholysis, acidolysis, esterification and aminolysis (Joseph *et al*., 2008). This hydrolytic reaction is reversible. In the presence of organic solvents, the enzymes are effective catalysts for various inter-esterification and trans-esterification reactions.

Further, microbial lipases show regiospecificity and chiral selectivity (Gupta *et al*., 2003). Especially microbial lipases have different enzymological properties and substrate specificities. Many species of bacteria, yeast and molds are found to produce lipases (Liu *et al*., 2008).

Their biotechnological potential is relying on their ability to catalyse not only the hydrolysis of a given triglyceride, but also its synthesis from glycerol and fatty acids. Therefore, microbial lipases have many industrial applications (Jaeger *et al*., 1999). The temperature stability of lipases is the most important characteristic for industrial use (Choo *et al*., 1997). Lipase catalysed reactions are widely used in the manufacturing of fats and oils, detergents and degreasing formulations, food processing, the synthesis of fire chemical and pharmaceuticals, paper manufacture, and production of cosmetics.

Lipases are also used to accelerate the degradation of fatty wastes and polyurethane (Jisheng *et al*., 2005). Among microbial lipases extensive reviews have been written on bacterial lipases (Jaeger *et al*. 1999; Arpigny and Jaeger., 1999).

In view of other numerous potential applications of bacterial lipases, their availability with specific characteristics is still a limiting factor as well as high cost of the enzyme. Therefore, in this study, an attempt was made to access the bio-potentials of the bacterium enzyme *Pseudomonas fluoresens* under laboratory conditions, by studying the effect of the environment on the growth and lipolytic enzyme production by the isolate and also characterizing the lipolytic enzyme of the bacterium isolate. The characteristics of this lipase were checked in order to understand enzyme functions better and enhance enzyme production by applying suitable substrate as well as process parameters optimization.

II. Materials And Methods

2.1 Growth studies and Production of the Enzymes

2.1.1 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 sterilisation. Sterile olive oil (Goya) was added as carbon source.

2.1.2Lipase Production

2.1.2.1 Preparation of Inoculum

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

2.1.2.2Production 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°C $\pm$ 2°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.

2.1.3 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 blank (Gojkovic, 2009).

2.1.4 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 describe 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 as the release of one micromole of free fatty acid (FFA)/min at room temperature.

2.2 Optimisation of Production Conditions

2.2.1 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 appropriate procedure.

2.2.2 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°C, 27°C, 40°C, 50°C, 60°C to 70°C for 24hours. The lipase activity and growth in the culture supernatant were determined.

2.2.3 Effect of Aeration on Lipase Production

After inoculating the organisms into the growth medium, the flasks were continuously shaken at 27°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.

2.2.4 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.

2.2.5 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.

2.2.6 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.

2.2.7 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.

III. Result

Figure I shows the effect of pH on growth and lipase production of *Pseudomonas Fluorescens,* maximum growth and lipase production was recorded at pH 7.0. In the case of the effect on temperature as shown in figure II, from 27°C as temperature increase, growth decreases and no lipase production at all. Agitation, within the agitation speed tested did not support lipase production but growth as shown in figure III. At 48hours of incubation maximum growth was recorded (1.498mg/ml) for *pseudomonas fluorescens* but lipase production decreased with increase in incubation time as shown in figure IV.

Figure V shows the effect of the different carbon sources on growth and lipase production of *Pseudomonas Fluorescens* glucose enhanced highest lipase production (0.9 U/mL). Figure VI shows the effect of the different nitrogen sources on growth and lipase production by *Pseudomonas Fluorescens,* Urea least supported both growth and lipase production. Figures VII and VIII shows the effect of metal ions on growth and lipase production of *Pseudomonas Fluorescens,* in the case of effect of cations on growth and lipase production of *Pseudomonas Fluorescens* $MgSO_4$ supported both growth and lipase production, KNO_3 supported growth but not lipase production, amongst the anions tested Na_2CO_3 supported growth most but least supported lipase production as shown in figure VIII.

Temperature 25°C had the maximum growth, while temperature 27°C had the maximum lipase production as shown in Table I. Table II, shows the effect of different concentration of substrate (olive oil) on growth and lipolytic activity of *Pseudomonas Fluorescens*. At 1.0 % lipase production was maximum while growth was at 0.5 %. Table III shows the effect of different concentration of anion and cation on growth and lipolytic activity of *Pseudomonas fluoresens* with maximum growth and lipase production at 0.1 %. Effect of different concentration of crude enzyme on growth and lipolytic activity of *Pseudomonas Fluorescens* as shown in table IV, at concentration 1.0 mL growth and lipolytic activity was maximum.

Table V shows the effect of different concentration of nitrogen source (peptone) on growth and lipolytic activity of *Pseudomonas Fluorescens*, maximum growth as well as lipase production was at 5.0 g.

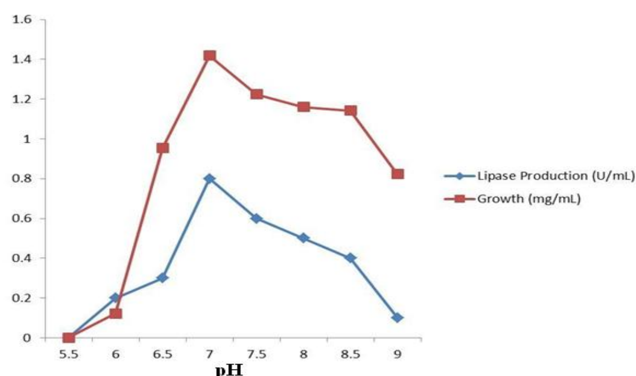

Fig I: Effect of pH on growth and lipase production of Pseudomonas fluoresens

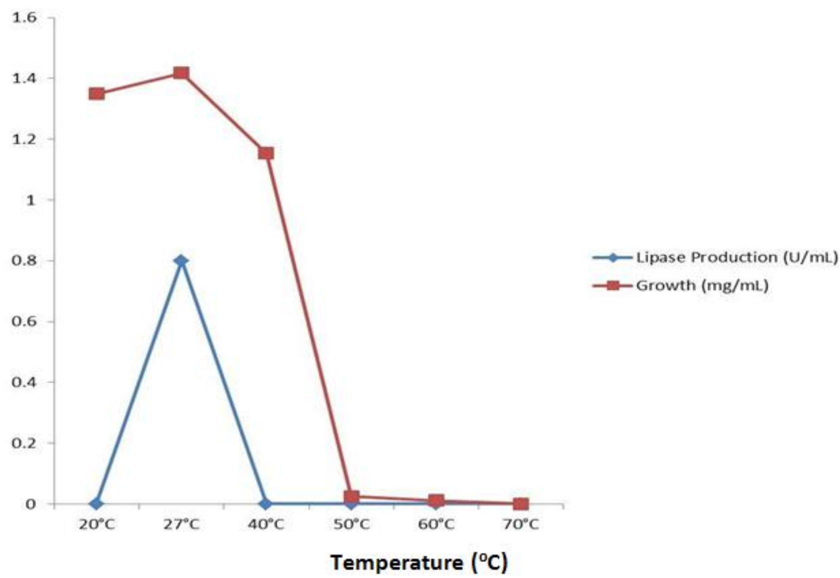

Fig II: Effect of temperature on growth and lipase production by Pseudomonas fluoresens

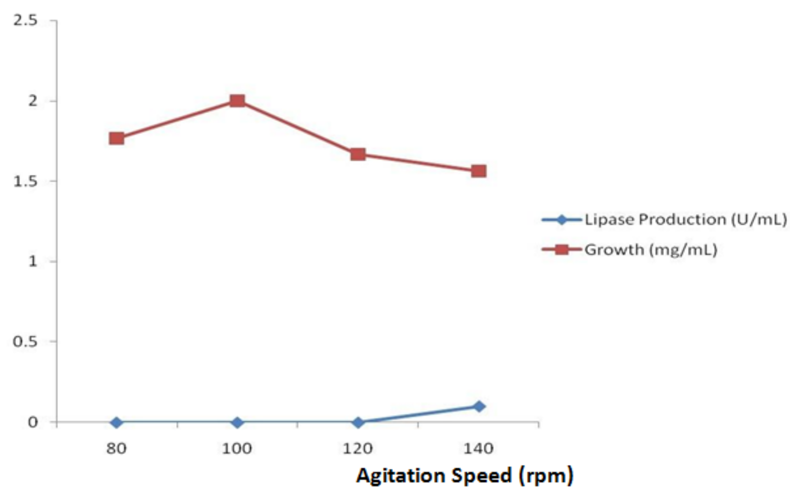

Fig III: Effect of agitation on growth and lipase production by Pseudomonas fluoresens

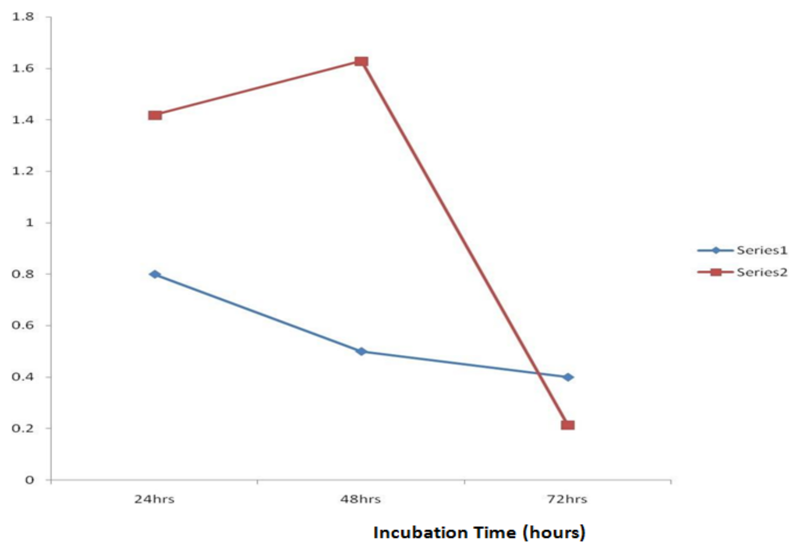

Fig IV: Effect of Incubation time on growth and lipase production by Pseudomonas fluoresens

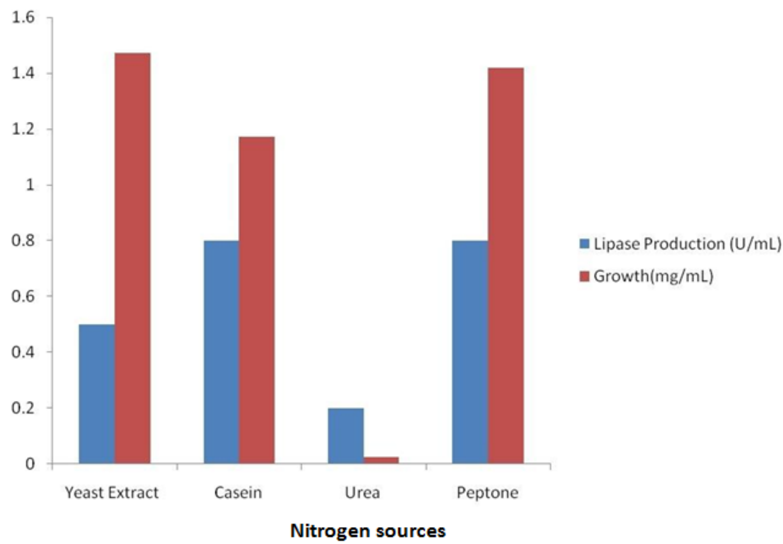

Fig V: Effect of 5% different nitrogen sources on growth and lipase production by Pseudomonas fluoresens

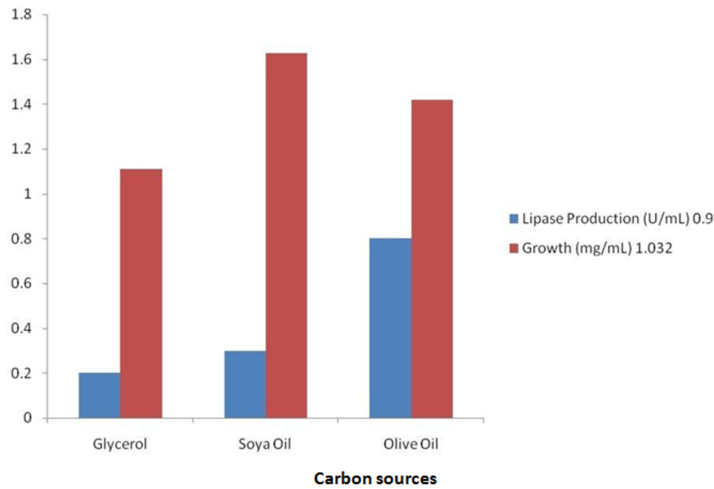

Fig VI: Effect of 2% different carbon sources on growth and lipase production by Pseudomonas fluoresens

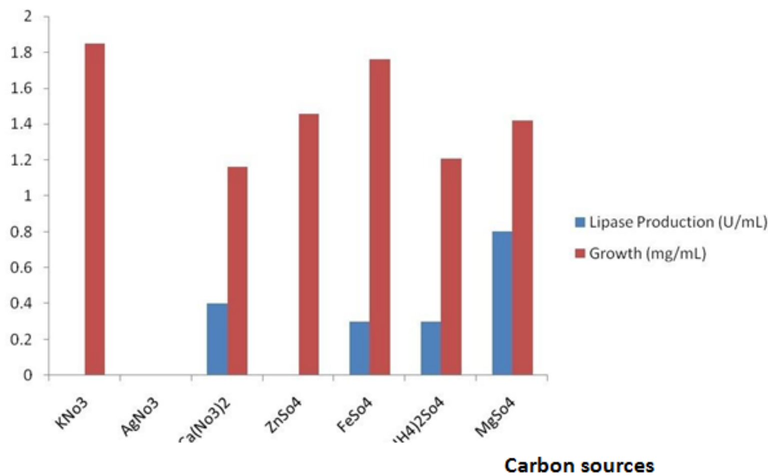

Fig VII: Effect of 2% different carbon sources on growth and lipase production by Pseudomonas fluoresens

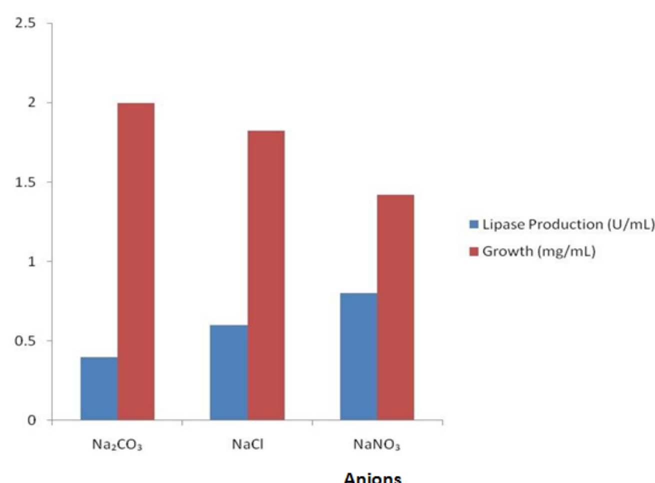

Fig VIII: Effect of 0.1% different anions on growth and lipase production by Pseudomonas fluoresens

TABLE I: EFFECT OF TEMPERATURE ON GROWTH AND LIPOLYTIC ACTIVITY OF *Pseudomonas fluoresens*

PARAMETERS	Different Temperature Investigation						
	20°C	25°C	27°C	30°C	35°C	37°C	40°C
Growth (mg/mL)	1.349±0.0005	1.595±0.0005	1.418±0.00025	1.386±0.0025	1.319±0.00025	1.293±0.00005	1.154±0.0001
Lipolytic activity (u/mL)	0.000±0.000	0.60±0.005	0.8±0.005	1.00±0.005	0.70±0.005	0.000±0.000	0.000±0.000

Each value is a mean of duplicate determinations ± standard error

TABLE II: EFFECT OF DIFFERENT CONCENTRATION OF SUBSTRATE (OLIVE OIL) ON GROWTH AND LIPOLYTIC ACTIVITY OF *Pseudomonas fluoresens*

PARAMETERS	Different Concentration of Olive Oil				
	0.5%	1.0%	1.5%	2.0%	2.5%
Growth (mg/mL)	1.778±0.00005	1.436±0.00005	1.422±0.00015	1.418±0.001	1.122±0.00005
Lipolytic activity (u/mL)	0.000±0.000	1.10±0.0050	0.90±0.0005	0.80±0.005	0.20±0.0005

Each value is a mean of duplicate determinations ± standard error

TABLE III: EFFECT OF DIFFERENT CONCENTRATION OF ANION AND CATION ON GROWTH AND LIPOLYTIC ACTIVITY OF *Pseudomonas fluoresens*

PARAMETERS	Different Concentration of Mg SO_4 and $NaNO_3$ Tested			
	0.05%	0.1%	0.15%	0.2%
Growth (mg/mL)	1.046±0.00005	1.418±0.00005	1.351±0.00005	1.344±0.00005
Lipolytic activity (u/mL)	0.00±0.00	0.800±0.0005	0.700±0.0005	0.00±0.000

Each value is a mean of duplicate determinations ± standard error

TABLE IV: EFFECT OF DIFFERENT CONCENTRATION OF CRUDE ENZYME ON GROWTH AND LIPOLYTIC ACTIVITY OF *Pseudomonas fluoresens*

PARAMETERS	Different Concentration of Crude Enzyme			
	0.5ml	1.0ml	1.5ml	2.0ml
Growth (mg/mL)	1.410±0.0005	1.418±0.00005	1.372±0.00005	1.370±0.0005
Lipolytic activity (u/mL)	0.00±0.00	0.800±0.0005	0.200±0.0005	0.100±0.0005

Each value is a mean of duplicate determinations ± standard error

TABLE V: EFFECT OF DIFFERENT CONCENTRATION OF NITROGEN SOURCE (PEPTONE) ON GROWTH AND LIPOLYTIC ACTIVITY OF *Pseudomonas fluoresens*

PARAMETERS	Different Temperature Investigation					
	1.0g	2.0g	3.0g	4.0g	5.0g	6.0g
Growth (mg/mL)	0.916±0.00025	1.158±0.0005	1.195±0.00005	1.202±0.00005	1.418±0.00005	0.696 ±0.00005
Lipolytic activity (u/mL)	0.00±0.00	0.30±0.005	0.600±0.0045	0.700±0.0040	0.80±0.0005	0.20±0.0005

Each value is a mean of duplicate determinations ± standard error

IV. Discussion And Conclusion

Increase in microbial growth does not necessarily mean 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 (physiological study of *Pseudomonas fluorescens)* 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) (Ece Yapasan, 2008). 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°C. Ece Yapasan (2008) reported the optimum bacterial growth temperature to be 25°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 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 dispersability of the carbon sources.

As observed in this research, maximum lipase activity for the *Pseudomonas Fluorescens* enzyme studied was studied was obtained after 24hrs of incubation, indicating that lipase was necessary for the first stages of growth, while minimum growth was detected 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 other 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 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 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 hydrolysed 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 hydrolyse esters in emulsion and usually water - insolude substrates, the organisms take up the substrate at different concentration, form and time. Typically, triglycerides composed of long-chain fatty acids, whereas esterases preferentially hydrolyse "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 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+}, $Mg2^{+}$ 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) while the activity of lipase from a *Pseudomonas* sp has been reported to be increased by 250% (Dong *et al.,* 1990). Ba^{2+} is also known to enhance lipase activity of lipase isolated from *Burkholderia* sp (Rathi *et al.,* 2001). Metal ions like Hg^{2+}, Zn^{2+} and Cu^{2+} have been reported to have inhibitory effect on *Pseudomonas* lipases

(Iizumi *et al.,* 1990). *Pseudomonas* sp lipase has also been reported to be inhibited in the presence of Al^{3+}, Mn^{2+}, Ni^{2+} and Fe^{3+} (Dong *et al.,* 1990).

As also observed in this study, 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, 2001).

The activity of lipase in synchrony with growth was found to be best at 0.1% concentration of $MgS0_4$ and $NaN0_3$ for *Pseudomonas fluorescens*. Islam *et al.* (2009) found that higher concentration of Ca^{2+} decreased lipase activities. Similar results have been reported for *Pseudomonas* lipase by Ece Yapasan (2008). These metal ions may increase the binding affinity of enzyme to the substrate (Ece Yapasan, 2008).

The enzyme had optimum lipase activity at substrate concentration of 1%. Similar results have been observed by Sugihara *et al.* (1991) who reported lipase production from *Bacillus* sp in the presence of 1% olive oil in the culture medium. Several other scholars have observed different percentages of olive oil in culture medium for lipase production by different enzymes. An example is Rajesh *et al.* (2010) who note lipase production by *Trichoderma reesei* at 2% concentration.

The inhibition of the synthesis of lipases at higher olive oil concentration could be due to poor oxygen transfer into the medium, low oxygen supplies can alter microbial metabolism and consequently, the production of lipase.

Temperature effect in this study showed that optimum *Pseudomonas* growth temperature was 25°C, whereas enzymatic activity was best at 30°C. This is in agreement with the report of Ece Yapasan (2008). Alteration of the optimum temperature resulted in gradual decrease in growth. This could be interpreted to mean increase in temperature alters the cell membrane composition and stimulates protein catabolism, thus causing cell death (Craig, 1985). In the case of lipase activity, temperature changes gave rise to cleavage of hydrogen bonds between substrates and enzyme active sides (Ece Yapasan, 2008). Optimum temperature value promotes binding potential of enzyme and substrates (Ece Yapasan, 2008).

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 clean up of vegetable oil spill site.

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