

Original Research Article

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Characterization and Optimization of Lipase Production from Soil Microorganism (*Serratia marcescens*)

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ABSTRACT

Lipase production by lipolytic bacteria (*Serratia marcescens*), isolated from soil in Ohuhu Community in Umuahia North Local Government Area of Abia State, was investigated using a basal medium containing KH_2PO_4 , Na_2SO_4 , $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, Yeast extract, K_2HPO_4 , Peptone, Glucose and Olive oil in a shake flask culture at 37°C for 48hours. The study of different carbon sources on lipase production revealed that glucose yielded higher lipase production of 7.6Uml^{-1} than sucrose (6.2Uml^{-1}), fructose (4.9Uml^{-1}), starch (3.6Uml^{-1}), lactose (2.4Uml^{-1}) and maltose (1.4Uml^{-1}). Subsequently, 2.5% concentration of glucose yielded higher lipase activity than the other concentrations (0.5%-4%) of glucose tested. Screening for the best nitrogen source revealed that peptone gave the highest activity of 28.5Uml^{-1} , others are tryptone (25.5Uml^{-1}), yeast extract (20.0Uml^{-1}) and meat extract (22.4Uml^{-1}), while NH_4Cl showed the least activity of 7.0Uml^{-1} . Also, 2.5% concentration of peptone gave the highest activity compared with other concentrations (0.5-4.5%) tested. Among the natural oils, olive oil gave the highest lipase activity of 25.2Uml^{-1} compared with other oils and 0.8% concentration of olive oil was found to increase lipase production by 36.2Uml^{-1} . Other lipid sources observed were palm oil (20.5Uml^{-1}), groundnut oil (19.5Uml^{-1}), soy oil (16.4Uml^{-1}) and crude oil (14.8Uml^{-1}). The most suitable source of metal ion was MgSO_4 (29.5Uml^{-1}) followed by MnSO_4 (27.9Uml^{-1}), KCl had the least activity of 13.9Uml^{-1} . Organic solvents on lipase activity revealed that methanol yielded higher lipase activity of 42.7Uml^{-1} than ethanol (30.5Uml^{-1}), isopropanol (15.6Uml^{-1}) and acetone (5.2Uml^{-1}). The results promises high production of lipase with an inexpensive and simple medium containing palm oil, peptone, and magnesium sulfate, at pH 6.0 and 150 rpm for 48hrs (2days). This could be employed in the enzyme industry for commercial purpose.

Keywords

Lipase, *Serratia marcescens*, Lipolytic bacteria, Characterization and Optimization.

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Introduction

Lipases are defined as glycerol ester hydrolases (EC3.1.1.3) hydrolyzing tri-, di- and mono-glycerides present at oil-water interface (Saxena *et al.*, 1999). Some lipases

are also able to catalyze esterification, trans-esterification and enantioselective hydrolysis reactions (Nini *et al.*, 2001; Shintre *et al.*, 2002; Nagayama *et al.*, 2002; Piao *et al.*,

2003; Kui *et al.*, 2003; Raku *et al.*, 2003). The interest in microbial lipase production has increased in the last decades, because of its large potential in a wide range of industrial applications as additives in food processing (flavor modification), fine chemicals (synthesis of esters), detergents (hydrolysis of fats), waste water treatment (decomposition and removal of oil substances), diagnostics, cosmetics (removal of lipids), pharmaceuticals (digestion of oil and fats in foods), leather (removal of lipids from animal skins) and medical (blood triglyceride assay) (Cardenas *et al.*, 2001; Elibol and Ozer, 2000).

Lipases are produced by many microorganisms and higher eukaryotes. Enzyme-producing microorganisms include bacteria (Kulkarni and Gadre, 2002), fungi (Fodiloglu and Erkmen, 1999), yeast (Corzo and Revah, 1999) and actinomycetes (Sommer *et al.*, 1997).

Lipases from microorganisms have drawn much attention for their potential use in biotechnology, mainly due to their availability and stability (Ghosh *et al.*, 1996). Lipase-producing bacteria have been found in diverse habitats such as soil contaminated with oil, dairies, industrial wastes, oilseeds and decaying food, compost heaps, coal tips and hot springs (Wang *et al.*, 1995).

A large number of beneficial thermophiles which produced lipases with good thermal stabilities have been found in diverse habitats (Wang *et al.*, 1995). Their thermal stabilities, particularly in the absence of appreciable amounts of water, increase their attractiveness industrially. Thermostable lipases have a lot of applications in the processing of food, domestic, industrial wastes, cosmetic, detergents and pharmaceutical industries (Saxena *et al.*, 1999)

Hence, this present work is centered on the isolation, characterization of lipase from lipase producing bacteria and the optimization of culture conditions required for maximum enzyme production by the isolate.

Materials and Methods

Sample collection

Soil sample was collected from 4-5cm depth from a palm oil processing environment located in Ohuhu Community in Umuahia North local Government Area of Abia State, Nigeria with sterile spatula in a sterile plastic bag and was transported to Microbiology Laboratory of Abia State Polytechnic, Aba.

Pre-enrichment

One gram of the soil sample was dissolved in 10ml sterile normal saline. After shaking, 5ml of the suspension was transferred into a 25ml enrichment medium with the following composition: 1% olive oil, 10g/l peptone, 3g/l beef extract and 5g/l NaCl at pH 7.0 (Pallavi *et al.*, 2010). The culture was incubated at 30°C for 24 hours. 1ml of the enriched culture suspension was serially diluted up to 10⁻⁷ dilutions and plated onto nutrient agar plates. Discrete colonies were picked and purified by sub-culturing onto NA, until their pure cultures were obtained and subjected to screening for extra cellular lipase production.

Lipase Assay

Lipase activity was determined by measuring the release of fatty acids from olive oil emulsion as described by Adinarayana *et al.*, (2004) using the titrimetric method. 0.2ml of the enzyme substrate mixture was incubated at 37°C for 30min. The reaction was terminated by adding 2ml acetone-ethanol mixture (1:1 v/v). The amount of fatty acid liberated was determined by titration with

0.01N NaOH using phenolphthalein as indicator. All experiments were run in triplicate sets and the mean values were presented. The values were analyzed using analysis of variance (ANOVA).

Effect of carbon sources on lipase activity

The effect of various carbon sources such as maltose, fructose, glucose, sucrose, lactose and starch on enzyme activity was determined by adding the carbon sources to the basal medium at 1% (w/v) as described by Gryzna *et al.*, (2007).

Effect of concentration of glucose on lipase activity

The effect of varying concentrations of the best carbon source (glucose) in the fermentation medium for the production of lipase was evaluated. 5mls of the broth culture was inoculated into 250ml conical flasks each containing 100ml sterile basal medium with ranging concentration (0.1-4.5% w/v) glucose.

Fermentation was carried out in an orbital shaker at 150rpm for 72hrs at 50°C thereafter, centrifuged. The cell free filtrate was used to assay for lipase activity as earlier described.

Effect of natural oil as additive on lipase activity

The effects of 1% v/v of various oils on lipase production were investigated by replacing olive oil used in the basal medium for preliminary study with; crude oil, palm oil, soy oil and groundnut oil as described by Pallavi *et al.*, (2010).

Effect of concentration of olive oil on lipase activity

The effect of concentration (0.1-3.5% v/v) of the best natural oil source (olive oil) on lipase

production was evaluated. Five milliliters of the broth culture were inoculated into 250ml conical flasks each containing 100ml sterile production medium with ranging concentration (0.1-3.5% v/v) crude oil as described by Pallavi *et al.*, (2010).

Effect of nitrogen substrates on lipase activity

The following nitrogen substrates (2% w/v) were used to evaluate the influence of organic and inorganic nitrogen sources on lipase production by the bacteria; yeast extract, soy meal, peptone, groundnut meal, tryptone, meat extract, ammonium chloride (NH₄Cl) and potassium nitrate (KNO₃).

Each of the nitrogen sources was used as a sole source of nitrogen in place of yeast extract and peptone employed in the basal medium. Fermentation was carried out in an orbital shaking incubator at 130rpm for 48hrs at 37°C and cell free filtrate was obtained as had been described.

Effect of concentration of peptone on lipase activity

The effect of varying concentrations of the best Nitrogen source (peptone) in the fermentation medium for the production of lipase was evaluated. 5ml of the broth culture was inoculated into 250ml conical flasks each containing 100ml sterile basal medium with ranging concentration (0.1-4.5% w/v) peptone. Fermentation was carried out in an orbital shaker at 150rpm for 72hrs at 50°C thereafter, centrifuged. The cell free filtrate was used to assay for lipase activity as earlier described.

Effect of metal ions on lipase activity

The effect of different metal ions on lipase production was determined by pre-incubation of 1ml of the crude enzyme extract with 1.5%

concentration of the various salts of metals; FeSO₄, MgSO₄, NaCl, CaCl₂, ZnSO₄, KCL and MnSO₄) at 37°C for 1hour as described by Anjana *et al.*, (2009).

Effect of concentration of mgso₄ on lipase production

The effect of varying concentrations of the best metal ion (MgSO₄) was evaluated. Different concentration (0.5-3.5% w/v) was inoculated into 250ml conical flasks containing 1ml of the crude enzyme extract. Fermentation was carried out in an orbital shaker at 150rpm for 72hrs at 50⁰C. The residual activity was determined under the standard assay conditions.

Effect of pH on lipase activity

The effect of pH on the enzymatic activity of the lipase fraction was determined using a wide range of buffers, including 1M citrate phosphate (pH 3.0-7.0), 1M sodium phosphate (6.0-7.0), 1M Tris-HCL (pH 8.0) and 1M glycine-NaOH (pH 9-10) as described by Anjana *et al.*, (2009). One millilitre of the crude enzyme extract was incubated for 1hour at 37°C with respective buffers mentioned. The pH values were checked using a digital pH meter. The residual enzyme activity was estimated under standard assay conditions after the fermentation.

Effect of temperature on lipase activity

The effect of temperature on lipase activity was determined by assaying the enzyme activity at different temperatures ranging from 30°C-80⁰C at pH 9, as described by Anjana *et al.*, (2009). The thermostability was monitored by incubating 1ml of the crude enzyme extract at respective temperatures for 1hr and then enzyme activity measured under standard assay conditions.

Effect of organic solvents on lipase activity

The effect of different organic solvents on lipase production was determined by pre-incubation of 1ml of the crude enzyme extract with 1.5% concentration of the various solvents (acetone, isopropanol, ethanol and methanol) at 37°C for 1hour as described by Anjana *et al.*, (2009). The residual activity was determined after the fermentation.

Results and Discussion

Lipases are a group of enzymes, present in microorganisms, plants and animals, which have the ability to hydrolyse triacylglycerol at an oil-water interface to release free fatty acids and glycerol. Their biotechnological potential is relying on their ability to catalyze not only the hydrolysis of a given triacylglyceride, but also its synthesis from glycerol and fatty acids. However, the aim of this study was the isolation of lipolytic bacteria from the local soil sample.

Carbon source is an important substance for energy production in microorganisms especially in bacteria (Pogaku *et al.*, 2009). A range of different carbon sources has been reported to support both growth and lipase production (Dharmendra *et al.*, 2010). Carbon sources that were investigated showed the ability to stimulate lipase production. However, glucose showed the highest value for lipase stimulation (Fig. 1). The lipase yield was maximum at 2.5% concentration with activity of 19.2U/ml. The carbon concentrations at 1% and 2% results to the slight decline in lipase production to 15.0U/ml and 17.2U/ml respectively (Fig. 2). The decrease in the productivity at higher concentration could be due to the viscosity of the medium component. The influence of different carbon sources on the production of lipase was found to be statistically significant ($p < 0.05$) by the one-way ANOVA

All the natural oils; Groundnut oil, Crude oil, Soy oil, palm oil and Olive oil were shown to induce lipase production at varying degrees (Fig. 7). Various oils are reported to constitute different types of fatty acids some of which lipase have much higher affinity for than others. Interestingly this bacterium was able to produce similar high lipase activity with all studied oils. This is quite important since it means that the cheapest and most available oil could be used as a convenient carbon source for industrial scale production. This is in compliance with the report by Sirisha *et al.*, (2010). Among these, olive oil was found to be successful with respect to lipase production by *Serratia* sp. Olive oil was also previously reported as the best substrate for lipase production with *Bacillus* sp. strain 42 (Eltaweel *et al.*, 2005). The above finding was similar to that by Becker *et al.*, (1997) who found that olive oil was the best inducer of lipase production by the *Bacillus* sp.

This is also in agreement with the report by Sirisha *et al.*, (2010), Vishnupriya *et al.*, (2010), and Essakiraj *et al.*, (2010), who showed the ability of olive oil as best carbon source in lipase production when compared with other oils. Palm oil was selected as a potential substrate for the industrial production of lipase over olive oil based on economic reasons of availability and cost of purchase because olive oil is much more expensive and not readily available and this in turn could eventually affect the cost of production of lipase enzyme. One-way ANOVA showed that the influence of various lipid sources on total lipase production was statistically significant ($p < 0.05$).

Nitrogen sources play important role in the biosynthesis of lipase by microorganisms. Yeast extract, soy meal, peptone, ammonium chloride, malt extract, groundnut meal, potassium nitrate were investigated. Results

obtained on the effect of nitrogen sources shows the various effect of nitrogen sources with peptone as sole carbon source (Fig. 3 and 4). Peptone was preferred by the isolate for the production of lipase as a nitrogen source as it showed significant lipase activity of 28.5U/ml. The study by Breuil *et al.*, (1997) revealed peptone as the best source of nitrogen in *Cellotrichum glosporoides* using pongamia oil cake. Szatajer *et al.*, (1988) and Szatajer *et al.*, (1989) have also reported peptone as the best source of nitrogen for lipase production. Different concentration of peptone showed constant increment in activity and the highest was at 2.5%, then sudden decline at 3% concentration.

Ammonium chloride showed the least ability of the organism to produce lipase in huge amounts, this is in contrast with the work by Pogaku *et al.*, (2009) who reported the ability of Ammonium chloride to stimulate lipase activity when used for lipase production by *Staphylococcus* Sp LP 12.

The results obtained showed that ammonium chloride slightly stimulated lipase production when compared with other nitrogen sources. Malt extract showed the ability to stimulate lipase synthesis but showed highest increase when crude oil was used as a sole carbon source. Sekhon *et al.*, (2006) has reported on the use of malt extract as a source of nitrogen for lipase production. There exist reports on the use of soy meal as a source of nitrogen but more reported that it was found not to be the best source with any organism (Essakiraj *et al.*, 2010; Vargas *et al.*, 2008; Kempka *et al.*, 2008). This could mean that soymeal contain certain cations which repress or inhibit lipase activity and can only be employed when these cations had been removed by an ion-exchanger; which when considered from the economic standpoint may not be ideal or good for lipase production or may be cost-intensive.

Therefore soybean may have the tendency to stimulate lipase production, but cannot be employed in the industrial scale production of the enzyme. Reports have shown that yeast extract can be dependable for large scale production of lipase enzyme (Dheeman *et al.*,

2010). Yeast extract stimulated lipase production in the present study but was not generally found to be the best source This is in contrast with the observation of Dheeman *et al.*, (2010); who reported the ability of yeast to stimulate maximum activity.

Plate.1 Showing orange fluorescence colonies of isolate on Rhodamine B Agar under UV irradiation



Plate.2 Showing no visible orange fluorescence colonies under UV irradiation



Fig.1 Effect of carbon sources on lipase activity

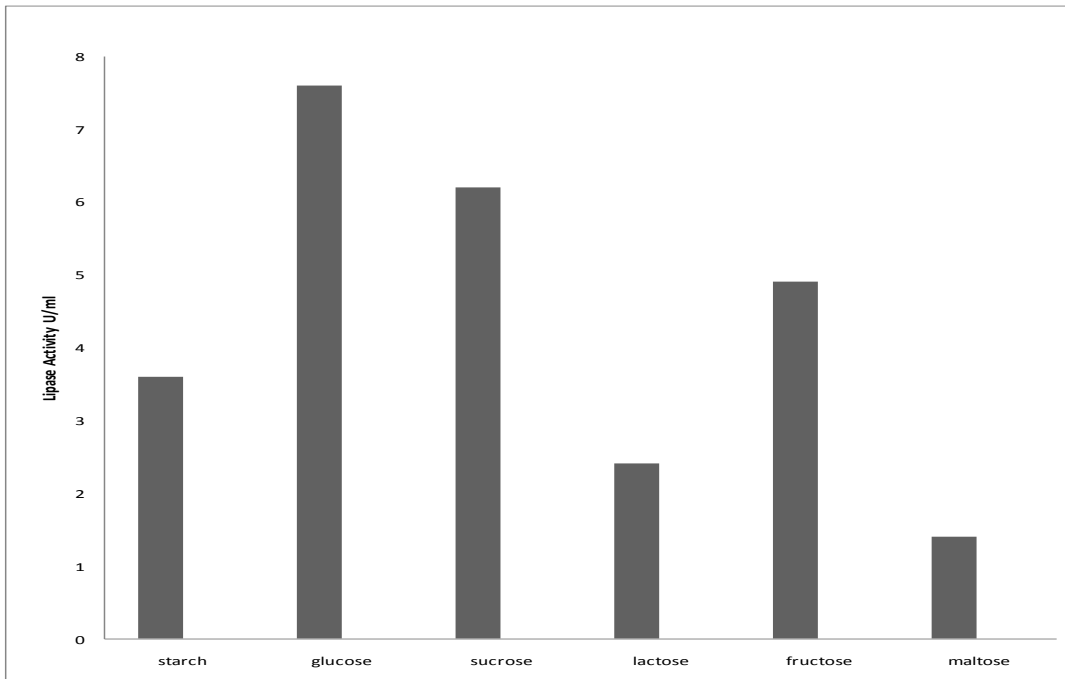


Fig.2 Effect of concentrations of carbon source (glucose) on lipase production by *Serratia marsecens*

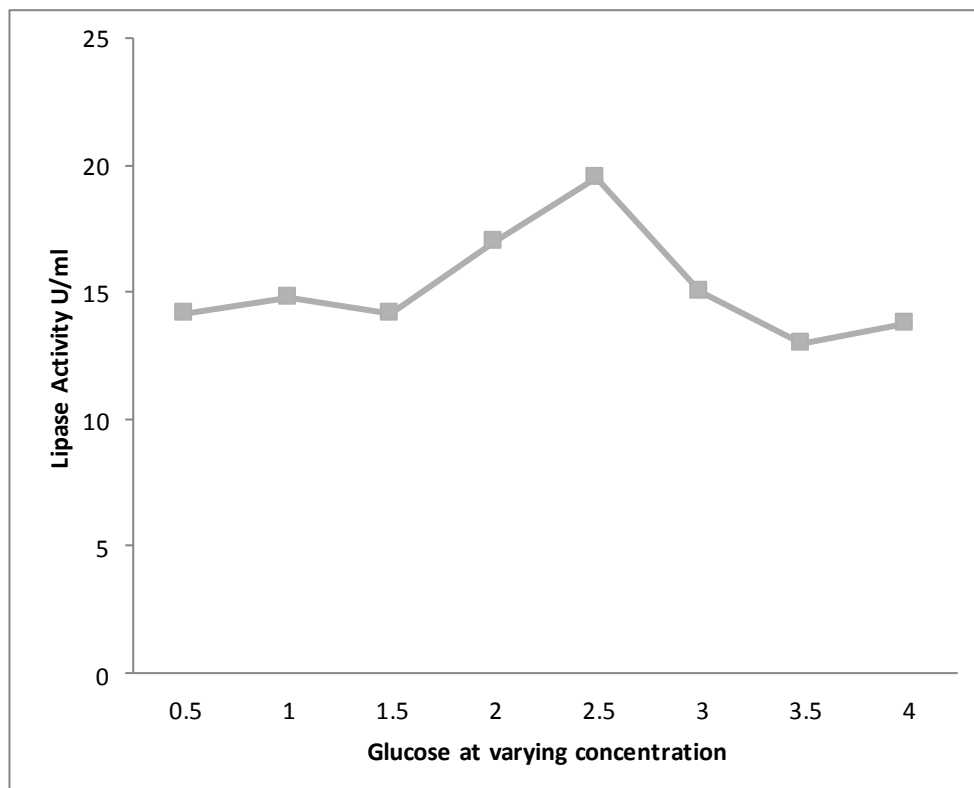


Fig.3 Effect of nitrogen sources on lipase activity

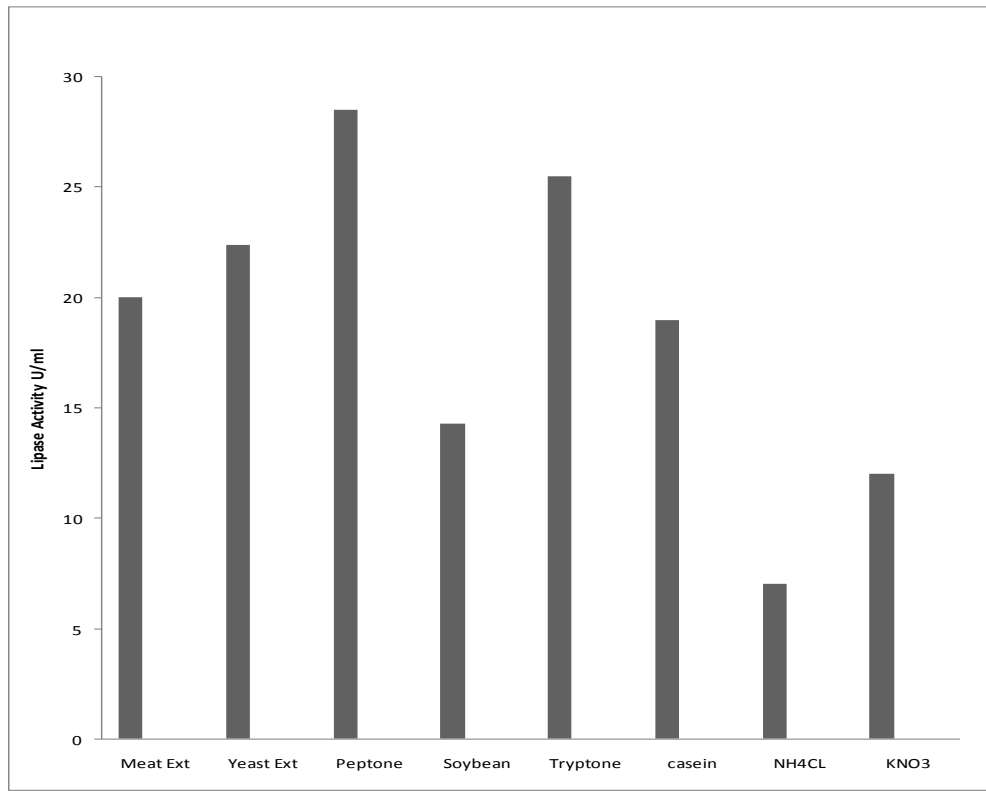


Fig.4 Effect of concentration of nitrogen source (peptone) on lipase production by *Serratia marsecens*

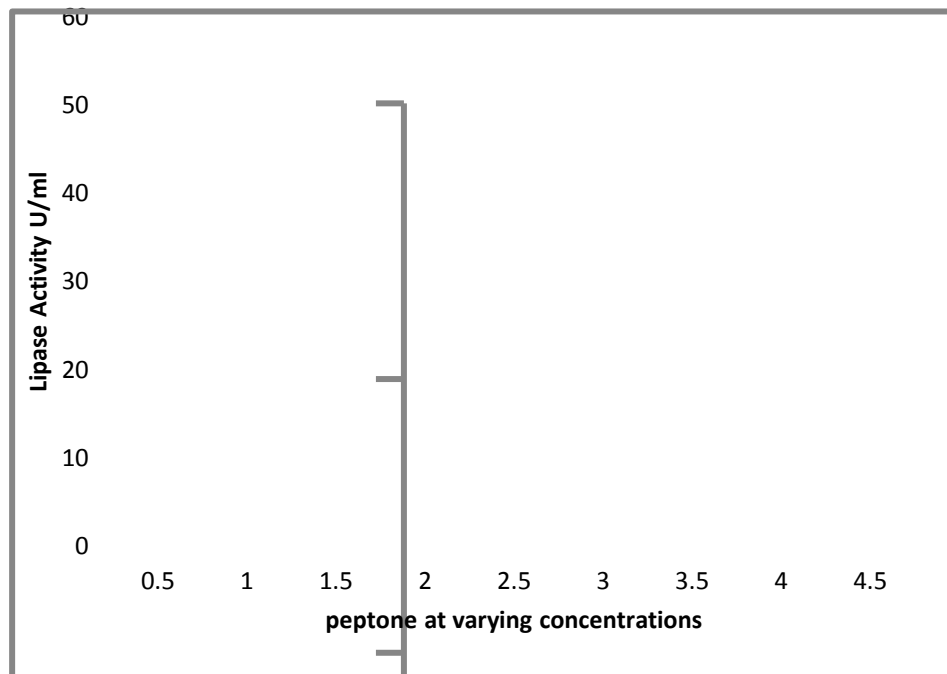


Fig.5 Effect of metal ions on lipase activity

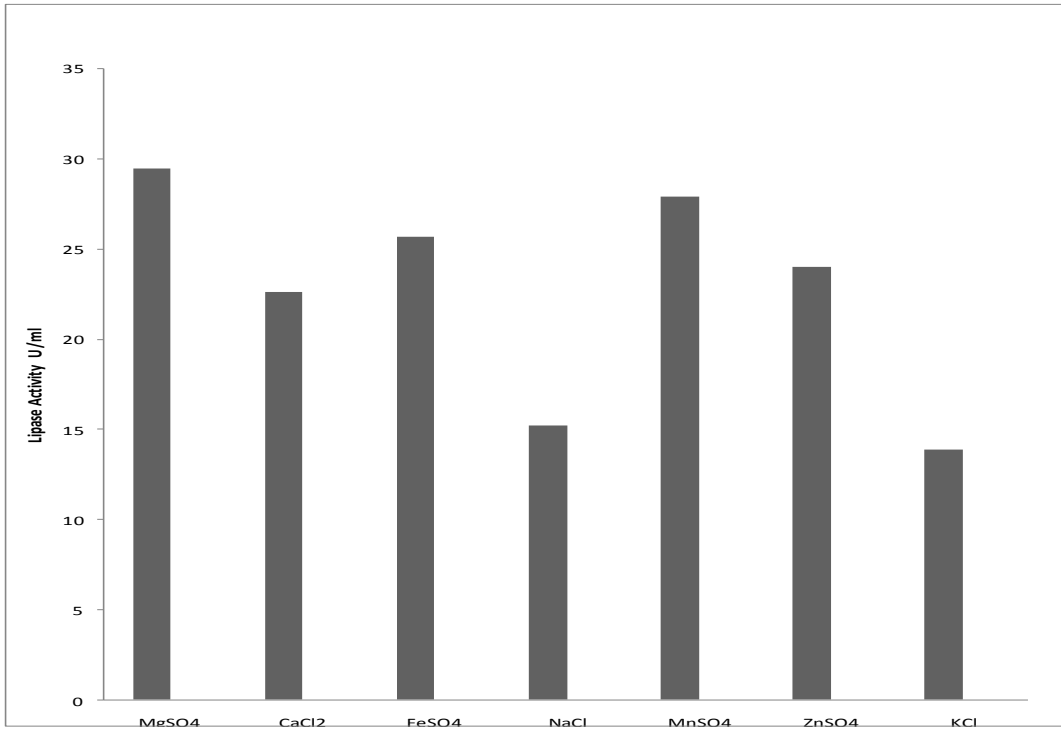


Fig.6 Effect of metal ion concentrations ($MgSO_4$) on lipase production

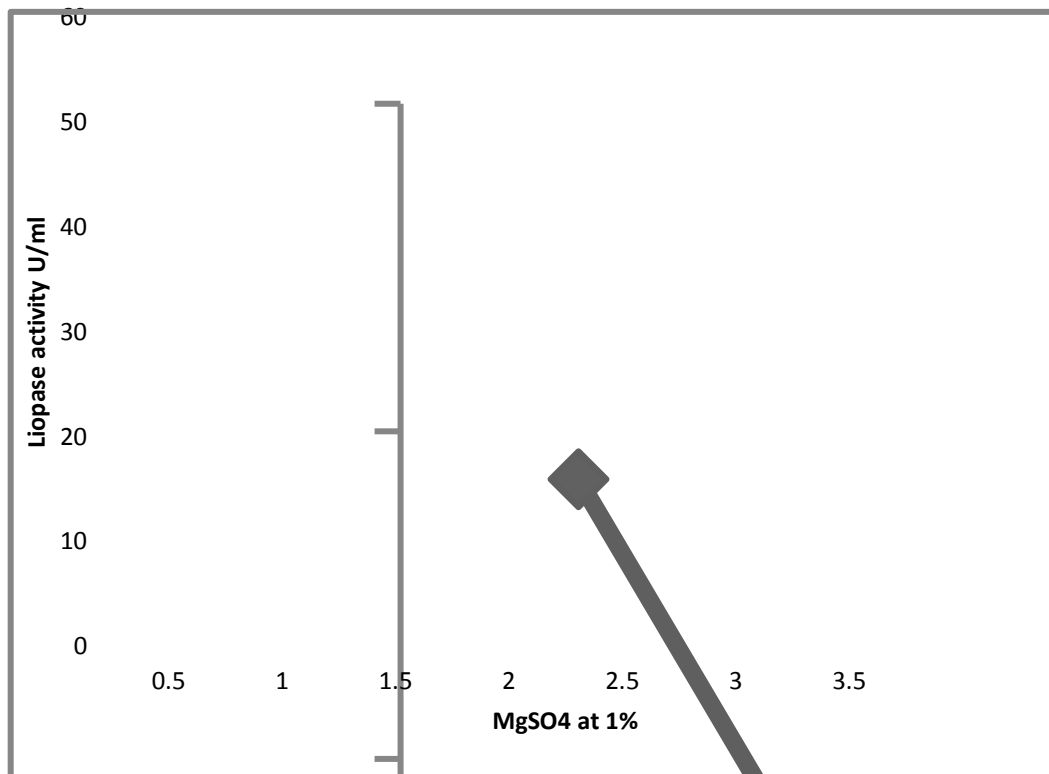


Fig.7 Effect of natural oil on lipase activity

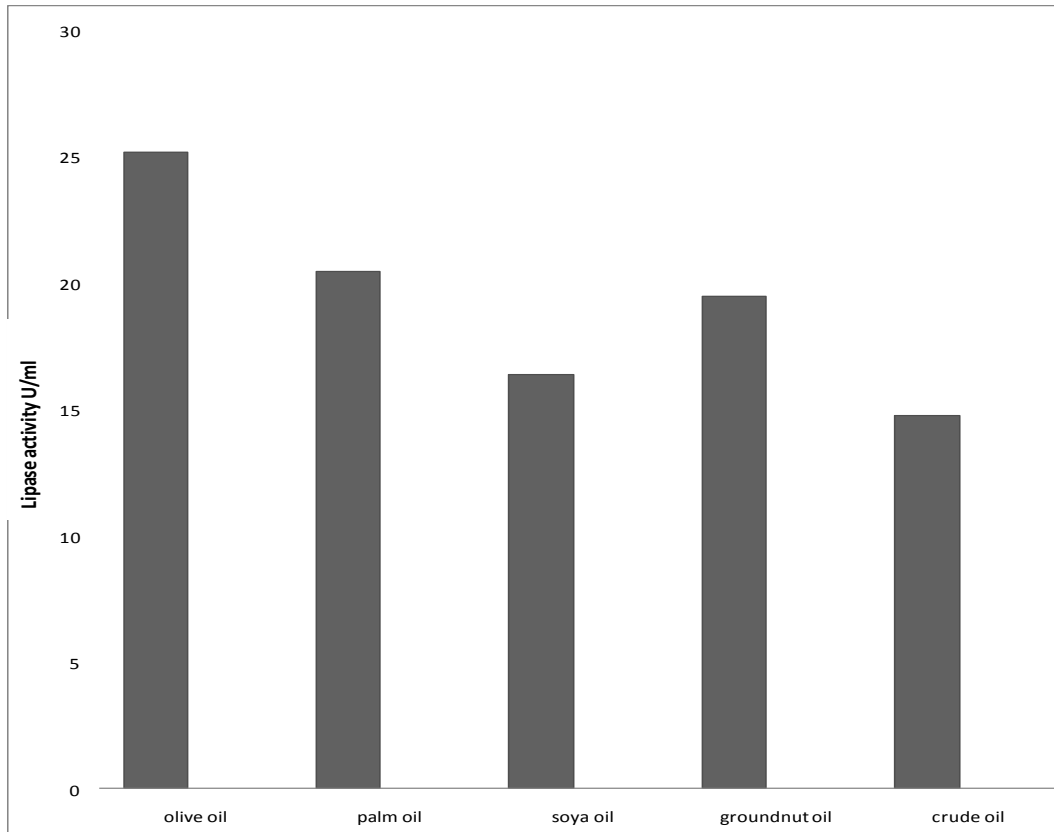


Fig.8 Effect of olive oil concentrations on lipase production by *Serratia marsecens*

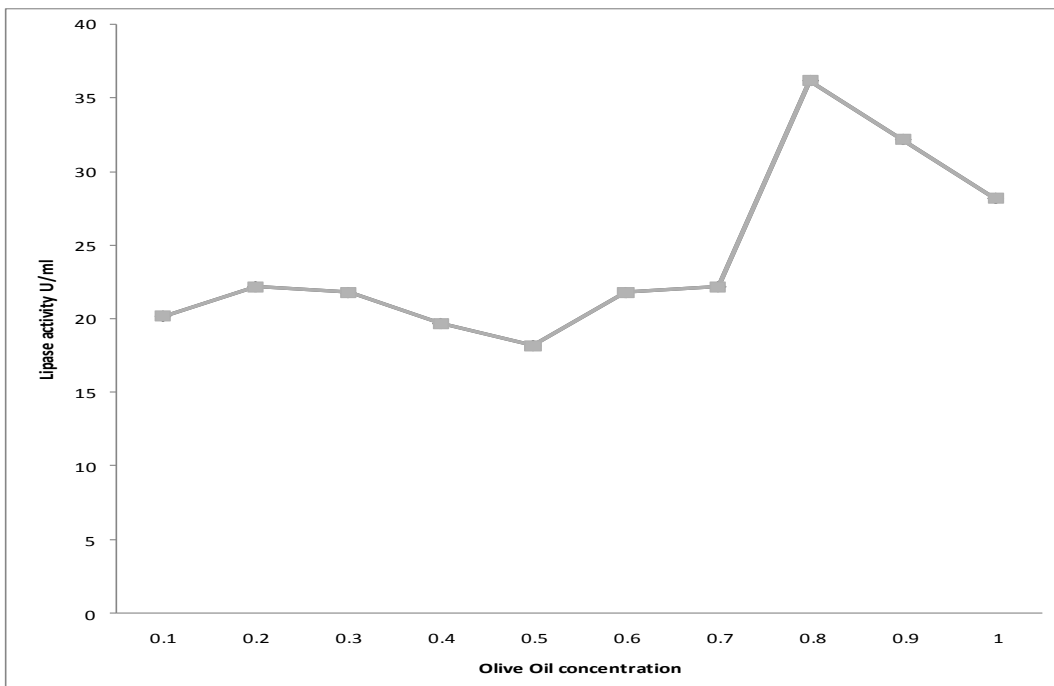


Fig.9 Effect of agitation rates on lipase activity

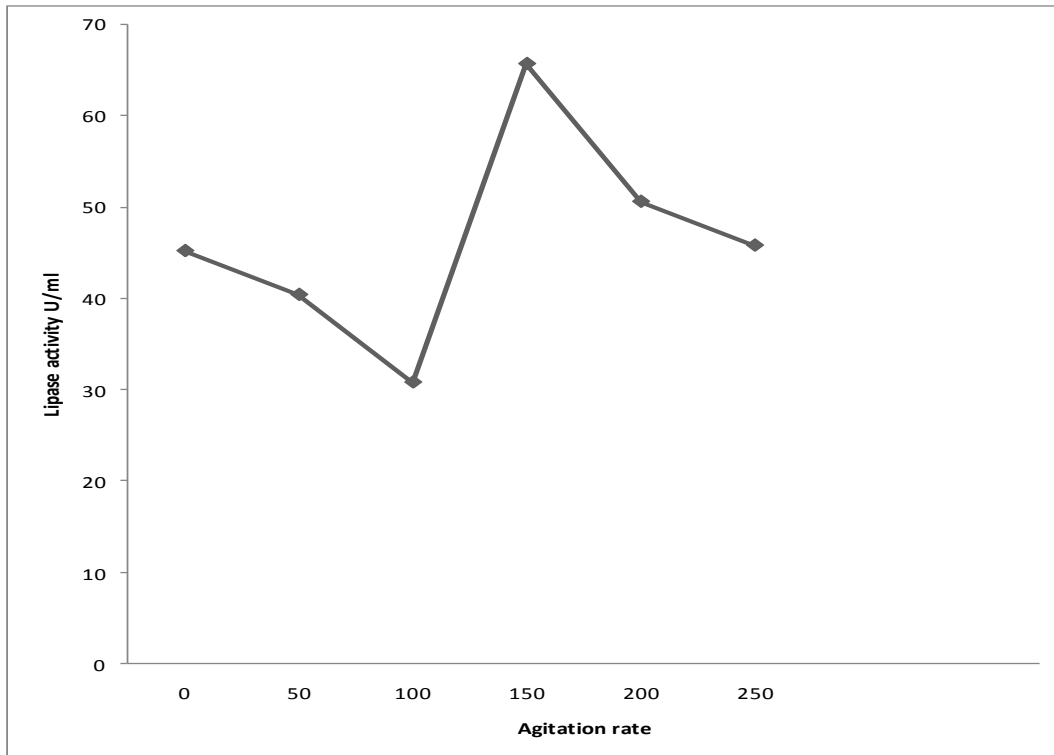


Fig.10 Effect of temperature ranges on lipase activity

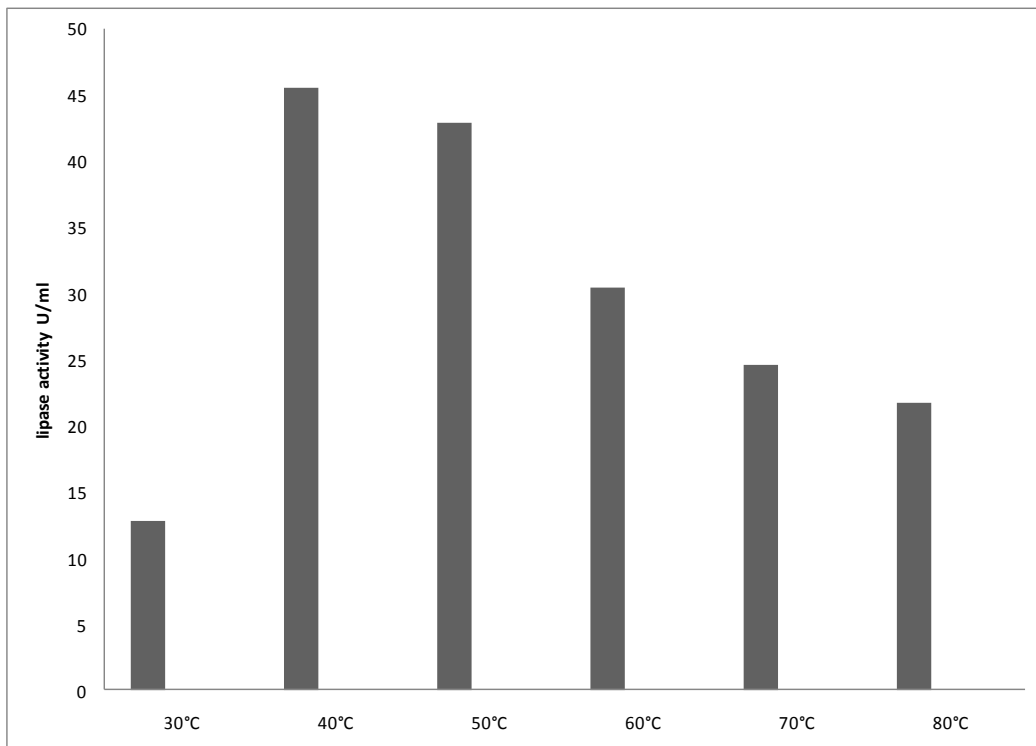


Fig.11 Effect of pH on lipase activity

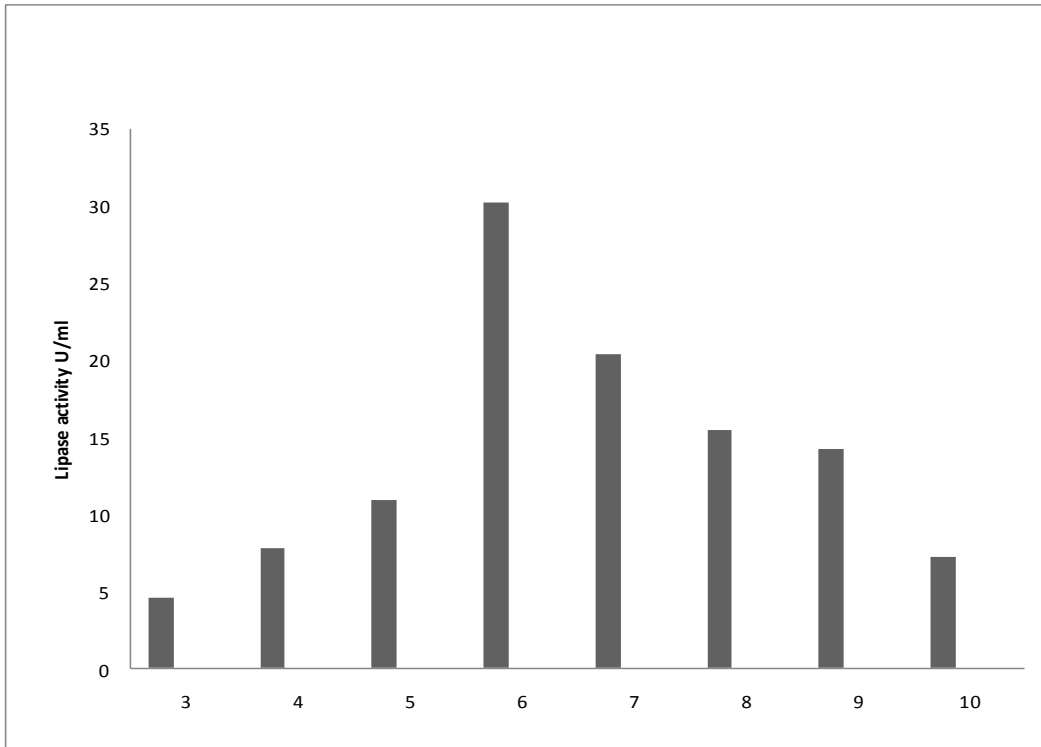


Fig.12 Effect of organic solvents on lipase activity

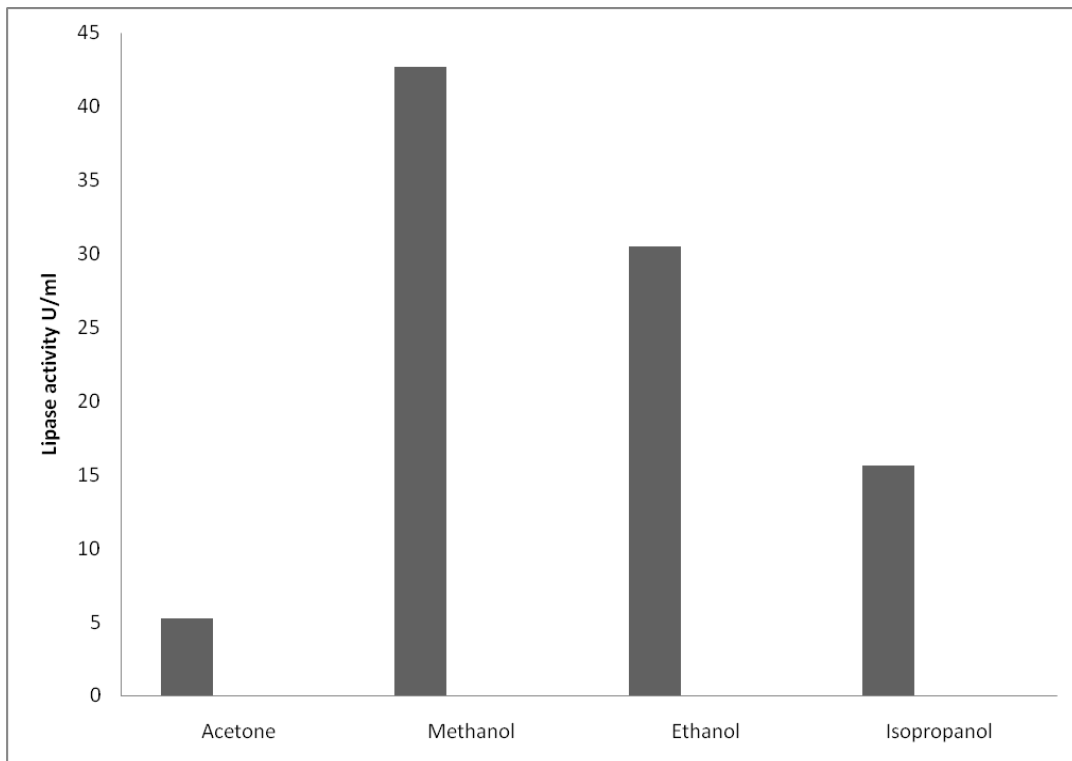


Fig.13 Effect of different concentrations of methanol on lipase production

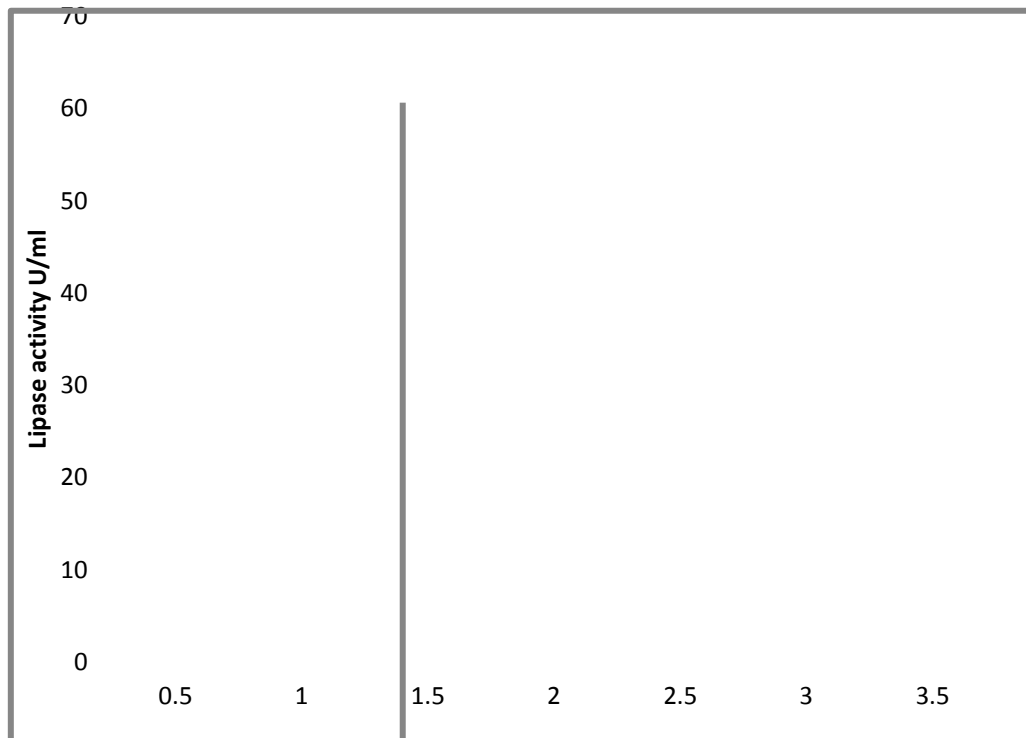
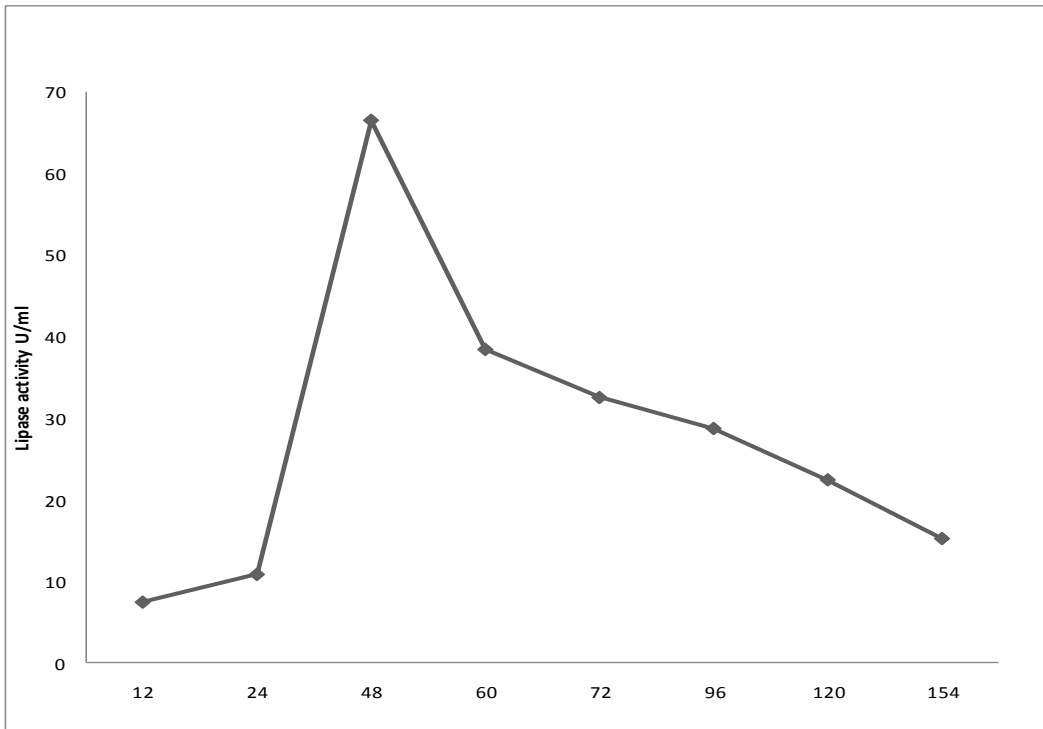


Fig.14 Effect of incubation time (hours) on lipase activity



Considering the effects of the various treatments involving nitrogen sources, the results showed that organic sources of nitrogen is a very important component for the growth of organism as appreciable growth values were observed with organic sources but not with inorganic sources. This could be because inorganic nitrogen sources are used up quickly, while organic nitrogen source can supply cell growth factors and amino acids needed for cell metabolism and enzyme synthesis.

Nitrogen sources are highly important for both growth of the bacterium and lipase production. Their tendency to stimulate lipase production more than carbon sources, make them the most important nutrient for the maximum production of lipase. The statistical analysis by one-way ANOVA showed that the influence of nitrogen sources on total lipase production was statistically significant ($p < 0.05$). An increase in lipase activity (synthesis and hydrolysis) after incubation in organic solvents (Fig. 12 and 13) showed that methanol and ethanol gave the highest lipase production of 42.7 U/ml and 30.5 U/ml respectively while Isopropanol gave an activity 15.6U/ml. Acetone gave the least activity of 5.2U/ml. As suggested by Chen and Wu (2003), the increase in enzymatic activity could be explained by the structural transition from the inactive “closed” form of lipase to the active “open” form, as a result of the treatment with organic solvents. Methanol is widely used as the acyl acceptor and is often favoured over other alcohols because of its low cost.

All the trace elements employed in the original medium used in the initial production were substituted with various salts of both macro elements and micro elements alone without any other salt. Trace elements such as Fe^{2+} and Mn^{2+} among others are found in almost all the media used for lipase

production. These salts include ferrous sulphate, manganese sulphate, magnesium sulphate, sodium chloride, potassium chloride, calcium chloride and zinc sulphate were all employed.

Potassium chloride and sodium chloride found to yield almost equal synthesis of lipase production were also found to be the least among the rest in its ability to stimulate lipase production. This shows that potassium ions along side with sodium are not good lipase stimulators. The ability of the rest of the salts to stimulate lipase production is not new; manganese and ferrous sulfate had been shown by to have stimulated lipase production. Dheeman *et al.*, (2010) has reported the use of Mn^{2+} , Fe^{2+} , Mg^{2+} and NH_4^+ to enhance lipase production. Increase lipase production was observed when Magnesium sulphate was used followed by manganese sulfate (Fig. 5 and 6). This is in agreement with the work of Jagtap *et al.*, (2010) who reported that the best source of metal ion was found to be magnesium sulphate, followed by ferrous sulphate. Increased lipase activity has been found to increase when combinations of glucose and magnesium salts are employed as carbon source and metallic salts respectively (Jagtap *et al.*, 2010). Concentration of 0.5% gave the maximum lipase activity and gradually decreased upon increasing concentration; this shows that the effect of magnesium sulphate required minimal concentration even though their effects at increase concentration are not negligible. Magnesium sulphate was found to give the best yield.

In the present study, lipase activity showed gradual increase in temperature from 30°C-40°C and further increase of temperature beyond 40°C, the production decreased (Fig 10). Thus the action of protease may be responsible for the remarkable thermal stability of the crude enzyme. This may be

ascribed to the poor stability of extracellular lipase, the release of more protease from dead cells, and higher protease reaction rate at higher temperature (Hong *et al.*, 2002). Statistical analysis by one-way ANOVA test for the data on lipase activity as a function of medium temperature change indicated a highly significant variation ($p < 0.05$). The pH of the medium is important for lipase production. Lipase activity and growth is affected by the pH of the medium. Molecular charges and consequently molecular interactions and functions are directly related to medium pH (Dheeman *et al.*, 2010); thus any change in medium affects any biological functions (Moon and Parulekar, 1991). The results of lipase production at increasing pH showed decreasing lipase across the pH gradient with the highest production found at pH 6.0; followed by pH 7.0 and the least at pH 3.0 (Fig. 11). This shows that the lipase produced by this organism is an acidic lipase. The ability of organisms to produce slightly acidic lipase has been reported by Pogaku *et al.*, (2010) and Rajesh *et al.*, (2010). There is an indication that proteases which are known to be secreted from dead cells may be more active at pH greater than 7.00 according to Hong *et al.*, (2002). One-way ANOVA test conducted to obtain the data on lipase production as a function of medium pH revealed a highly significant variation ($p < 0.05$).

Determination of the effect of agitation of production media, were kept in static and different shaking conditions. At static conditions lipase activity was much more lower than that of subsequent 50rpm - 100rpm agitation. Optimum shaking conditions required for lipase production was found to be 150rpm (Fig. 9). At 200rpm shaking condition, lipase production was slightly decreased this maybe due to cell disruption and resulting in the release of intracellular enzymes such as protease and esterase that

can digest lipase enzymes (Rajesh *et al.*, 2010). Shaking speeds, 100 and 50rpm seemed to disrupt the microaerophilic or near aerobic conditions required for maximum lipase production. This reveals the ability of the organism to produce lipase enzyme maximally at high increase in the availability of oxygen.

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