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EFFECTS OF NATURAL OILS, METAL IONS AND ORGANIC SOLVENTS ON LIPASE PRODUCTION BY *Serratia marcescens*

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ABSTRACT

Lipases are industrially important enzymes that are produced by a variety of microorganisms. Lipase production by lipolytic bacteria, isolated from soil in Ohuhu Community in Umuahia North Local Government Area of Abia State, was detected in a medium containing olive oil (3%) and rhodamine B. dye (1%). Among the several bacteria screened, a strain was selected for lipase production as it showed the largest orange fluorescence under UV light, indicating effective production of lipase. Lipase production was carried out using a basal medium containing KH_2PO_4, Na_2SO_4, $MgSO_4.7H_2O$, Yeast extract, K_2HPO_4, Peptone, Glucose and Olive oil. It was investigated in a shake flask culture at 37^0C for 48hours. Morphological, biochemical and molecular characterization of the isolate identified it as Serratia marcescens. 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}). Methanol showed significant high lipase activity at 1.5% concentrations. 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.

Key words: *Natural Oils, Metal Ions, Organic Solvents, Lipase, Serratia marcescens*

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). 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; Kamini *et al*., 2000). Lipases are produced by many microorganisms (Kamimura *et al*., 2001; Elibol and Ozer, 2000) and higher eukaryotes. Enzyme–producing microorganisms include bacteria (Kulkarni and Gadre, 2002), fungi (Fodiloglu and Erkmen, 1999; Shimada *et al*., 1992), yeast (Corzo and Revah, 1999) and actinomycetes (Sommer *et al*., 1997). Organic solvents are extremely harsh to living cells because they are able to bind to the cell membrane and affect its integrity as well as stability. These can disrupt the membrane and decrease the permeability of the barrier which leads to cellular metabolism damages, growth inhibition and finally to the cell death. Despite all these worse effects organic solvent-tolerant bacteria are capable of thriving in the presence of these toxic solvents .Currently bacterial lipases are of great demand because of potential industrial applications. Considering the ever increasing demand for the better lipases in the industry for the search for eco-friendly and economical sources of lipase producing bacteria the present study has been carried out for the optimization and the effect of natural oils, organic solvents and metal ions in extracellular lipase from *Serratia marcescens*.

MATERIALS AND METHODS

Pre-enrichment and Isolation of bacteria

Soil sample was collected from a palm oil processing environment located in Nkata Okpuala village, Okaiuga Nkwoegwu in Umuahia North local Government Area of Abia State with sterile spatula in a sterile plastic bag and was transported to Microbiology Laboratory of Abia State Polytechnic, Aba.

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 30^0C 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.

PRELIMINARY SCREENING FOR LIPASE ACTIVITY

The screening method of Savitha et *al.* (2007) was employed. Nutrient agar was incorporated with 1% rhodamine B dye and 3% olive oil. Each of the pure cultures was plated on the rhodamine B agar plates (NA with 1% rhodamine B and 3% olive oil) and Incubated at 37^{o}C for 48 days. To determine the production of extracellular lipase, plates were viewed under Ultra violet (UV) radiation.

IDENTIFICATION OF THE BACTERIA

Identification of the bacteria colony showing maximum activity was done by following Bergey's manual as described by Holt *et al.* (1994). The pure colonies were subjected to Gram staining, and further biochemical tests, to confirm the identity of the bacterium.

Preliminary Production of Lipase

A preliminary production of lipase was carried out using basal medium, of Saxena *et al.* (2011), which is composed of (g/L); KH_2PO_4 (1.0), Na_2SO4 (2.0), $MgSO_4$. $7H_2O$ (0.1), Yeast extract (5.0), K_2HPO_4 (3.0), Peptone (5.0), glucose (2.0) and olive oil (1% v/v). The initial pH of the medium was adjusted to pH 7.0. The enzyme preparation was obtained by inoculating 5 ml of broth culture from 48h culture into 250 conical flasks containing 50ml of sterile medium. Incubation was carried out in orbital-shaking incubator at 200rpm for 48 hrs. at 37^{o}C. The samples were centrifuged at 6000rpm for 15mins as described by Akanbi *et al.* (2010). The supernatant obtained were filtered through a Whatman No1 filter paper. The crude lipase solution was obtained by filtering through a Millipore 0.22um filter membrane. The extract was then assayed for lipase activity.

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. All experiments were run in triplicate sets and the mean values were presented. The values were analyzed using analysis of variance (ANOVA).

Effect of Natural Oil as additive

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 Natural Oil

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). Fermentation was carried out in an orbital shaker at 150rpm for 72hrs at 50^0C thereafter, centrifuged. The cell free filtrate was used to assay for lipase activity as earlier described.

Effect of Organic Solvents on Lipase ctivity

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 37^0C for 1hour as described by Anjana *et al*. (2009). The residual activity was determined after the fermentation.

Effect of Concentration of Methanol on Lipase Production.

The effect of varying concentrations of the best organic solvent (Methanol) 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 50^0C.The residual activity was determined under the standard assay conditions.

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; $FeSO_4$, $MgSO_4$, NaCl, $CaCl_2$, $ZnSO_4$, KCL and $MnSO_4$) at 37^0C for 1hour as described by Anjana *et al*. (2009). The residual activity was determined after the fermentation.

Effect of Concentration of Metal ion on Lipase Production

The effect of varying concentrations of the best metal ion ($MgSO_4$) 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 50^0C.The residual activity was determined under the standard

assay conditions.

RESULTS

Lipase production by the bacteria was identified as orange fluorescence under U.V. light. The bacterium showed brilliant orange fluorescence upon UV irradiation of the rhodamine B agar plates.

The isolate studied was Gram negative, short rods. The organisms were motile. On solid media, the colonies were red pigmented. The bacterial isolates showed positive for Coagulase test, Catalase test, Citrate, lipase, Voges proskauer's test, glucose, and sucrose fermentation. The following characteristics were negative for the strain – Indole test, methyl red, lactose fermentation and H_2S production. The isolate was also taken to Nigerian Institute for Medical Research (NIMR), Lagos where further tests were carried out as seen in the appendix. These observations were in accordance with the reported biochemical characters of *Serratia* according to Aparna and Sarada (2012). Thus based on biochemical, cultural, morphological and genetic characteristics the isolate was identified as *Serratia marcescens*. (Table 1)

Lipase production was investigated with soya oil, groundnut oil, palm oil, crude oil and olive oil. Results obtained are shown in Table 5. Results show that the best additive is shown to be Olive oil (25.2 U/ml). Followed by Palm oil (20.5 U/ml), groundnut oil (19.5 U/ml). The least activity was observed with Crude oil (14.8 U/ml).

Varied concentrations of natural oil (Olive oil) were employed in the study to investigate its effect on lipase production. Result in Figure 4 shows that concentration 0.8% gave the highest lipase production which decreased upon increased in concentration higher than 0.8%. The least activity was shown with 0.5% concentration (21.8 U/ml) whereas the highest was found with 0.8% concentration (36.2 U/ml).

Organic solvents were found to influence lipase production. The addition of methanol and ethanol gave the highest lipase production of 42.7 U/ml and 30.5 U/ml (Table 9) respectively. Isopropanol gave an activity 15.6U/ml. Acetone gave the least activity of 5.2U/ml

The result on the effects of various concentrations of the organic solvent (Methanol) is shown in Figure 5. The highest activity occurred at 1.5% concentration with 60.4 U/ml, further increase in concentration showed decreased enzyme synthesis as 2.0% concentration showed an activity of 20.4 U/ml. At 2.5% concentration, it slightly increased with an activity of 32.7 U/ml. Subsequent increase in concentrations declined the activity.

Metal ions are essential for the production of lipase. Result on the effect of metal ions is shown in Table 4. $MgSO_4$ gave the highest lipase activity (29.5 U/ml). $MnSO_4$ gave an activity of (27.9 U/ml), $FeSO_4$ gave an activity of 25.7 U/ml. NaCl gave an activity of 15.2 U/ml; $CaCl_2$ gave an activity of 22.6 U/ml. The least activity was found with KCL (13.9 U/ml).

The result on the effects of various concentrations of the best source of metal ion ($MgSO_4$) is shown in Figure.3. The highest activity occurred at 0.5% concentration with 48.5 U/ml, further increase in concentration showed decreased enzyme activity as 1.0% concentration showed an activity of 30.2 U/ml, 2.0 0% concentrations gave an activity of 20.2 U/ml.

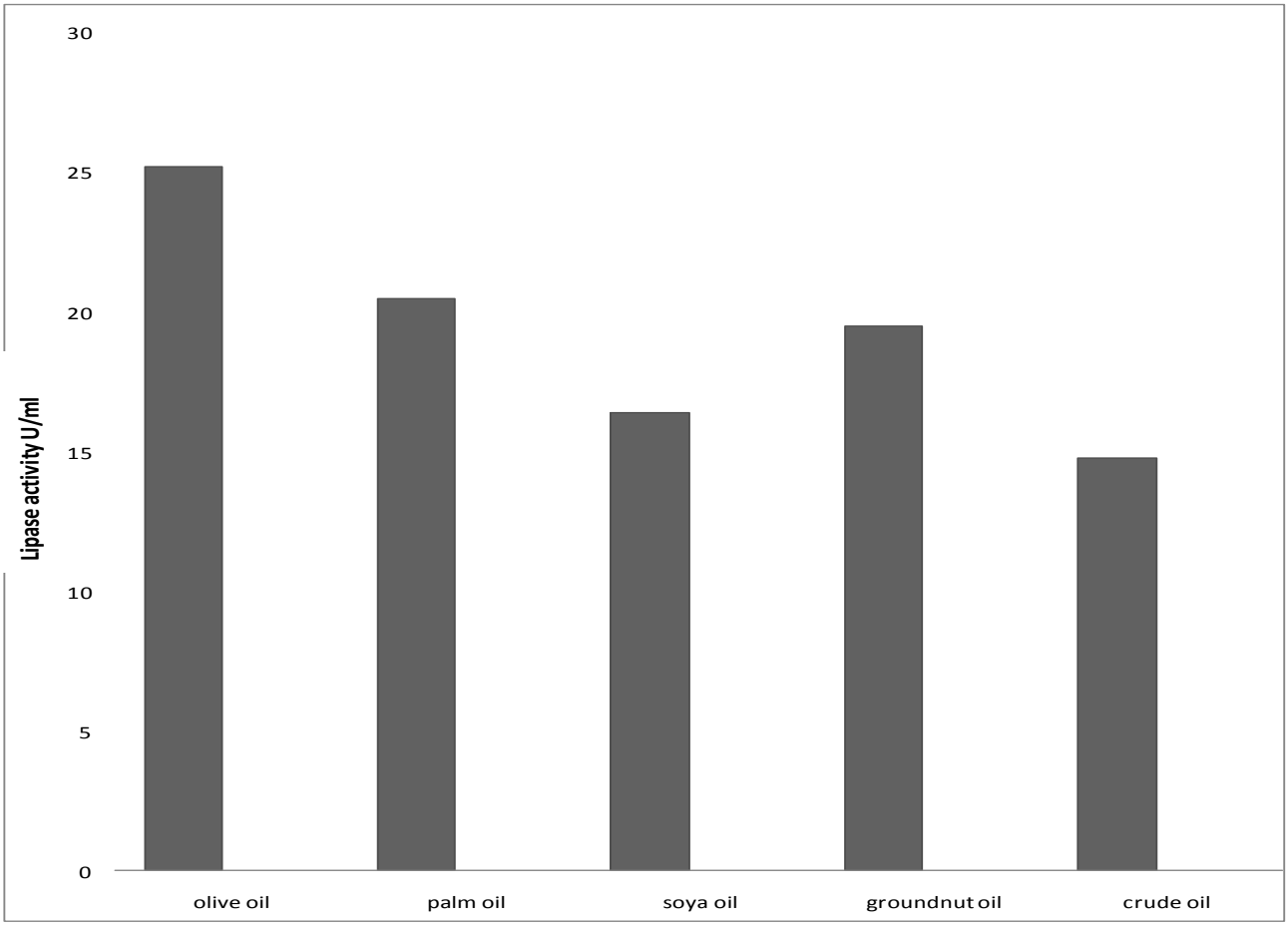

Fig 1: Effect of Natural Oil on Lipase Activity

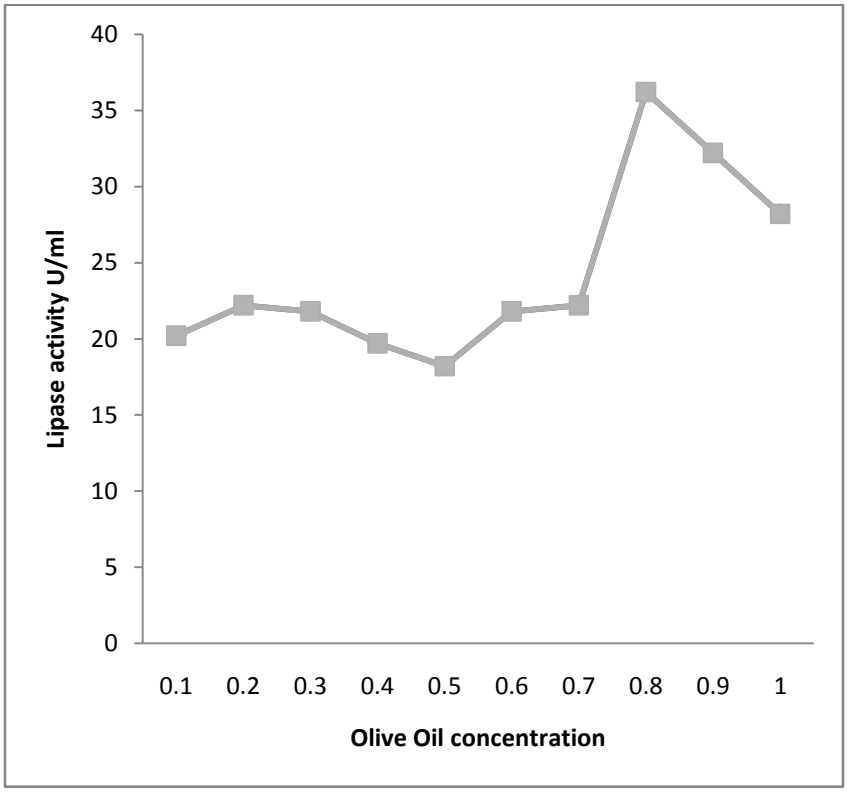

Fig 2: Effect of Olive oil concentrations on lipase production by *Serratia marsecens*

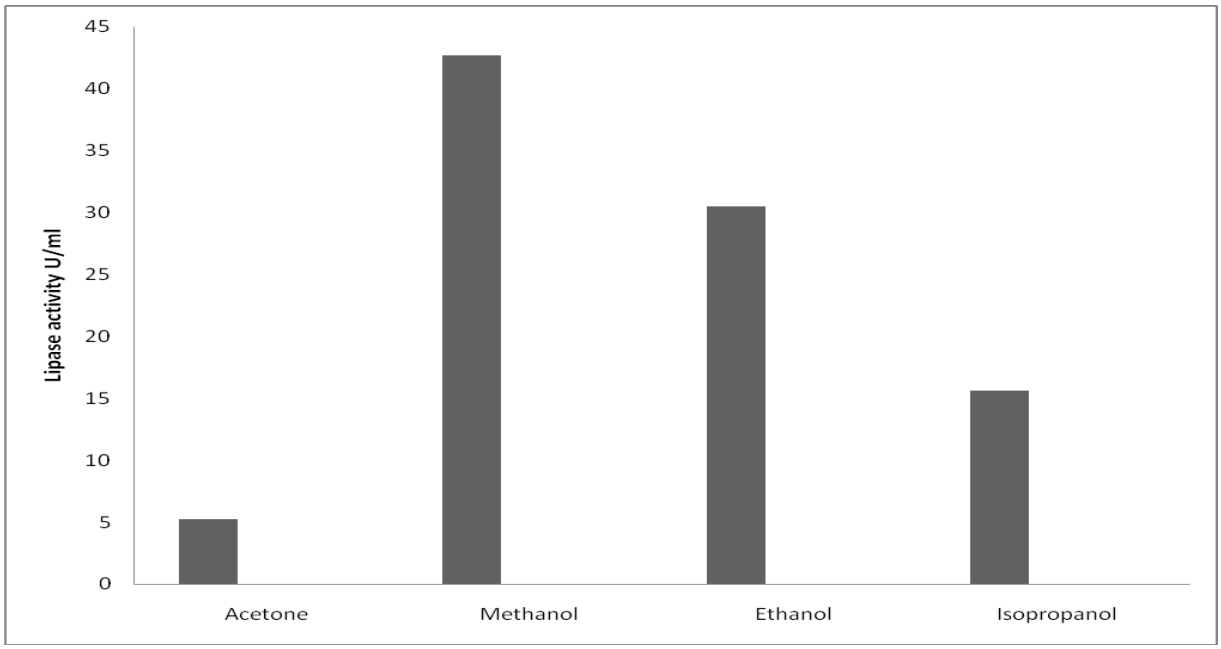

Fig 3: Effect of Organic Solvents on Lipase Activity

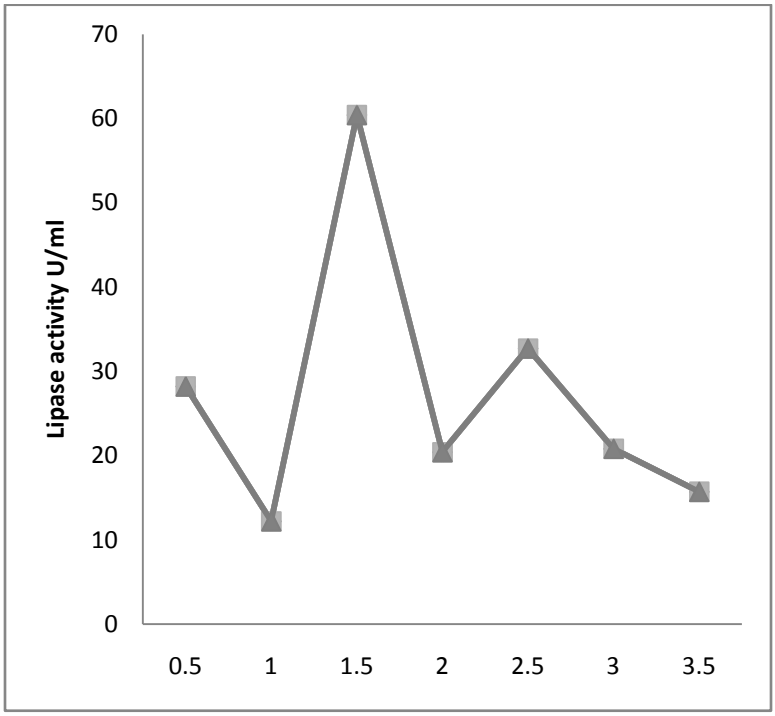

Fig 4: Effect of different concentrations of methanol on lipase production

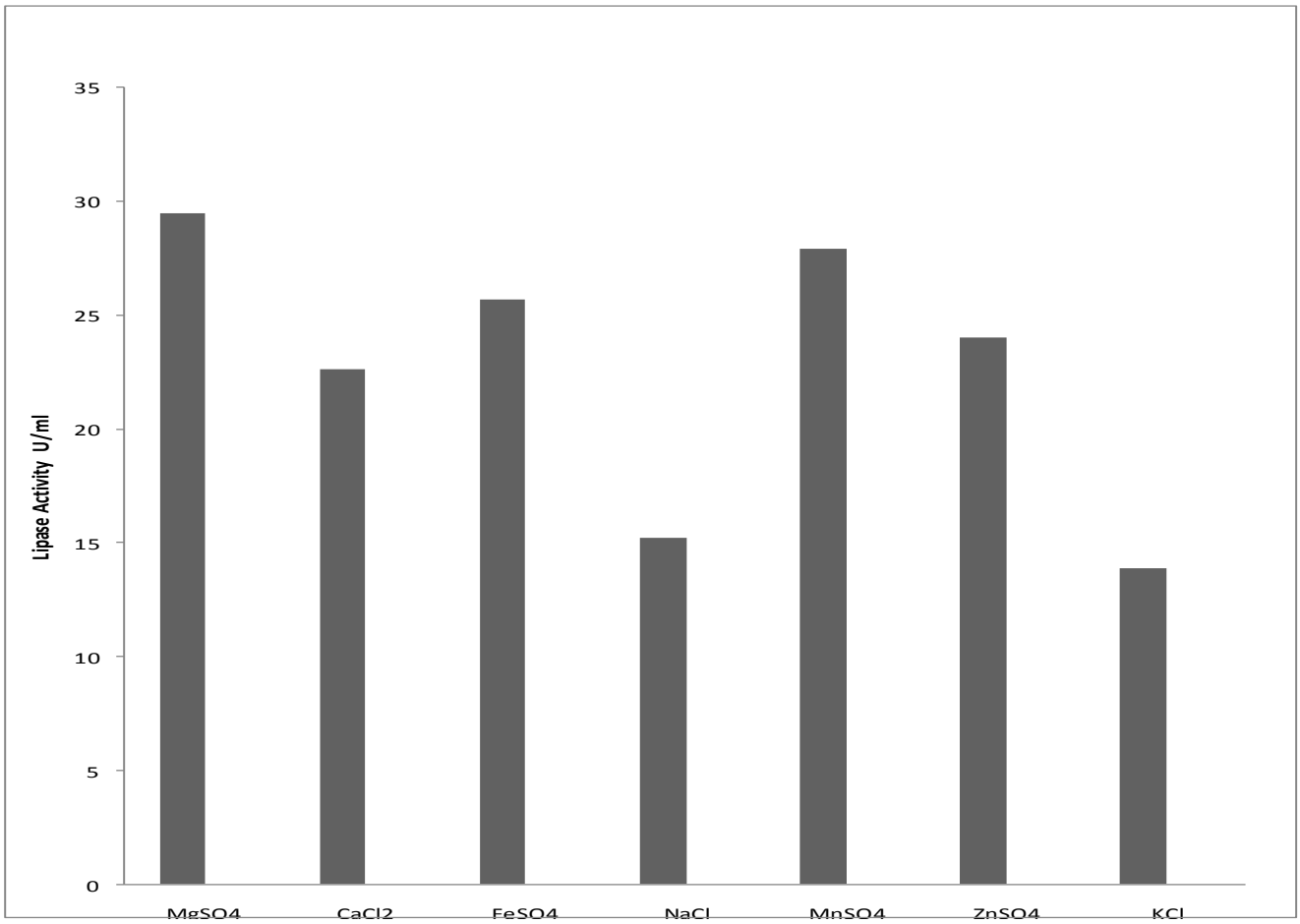

Fig 6: Effect of Metal Ions on Lipase Activity

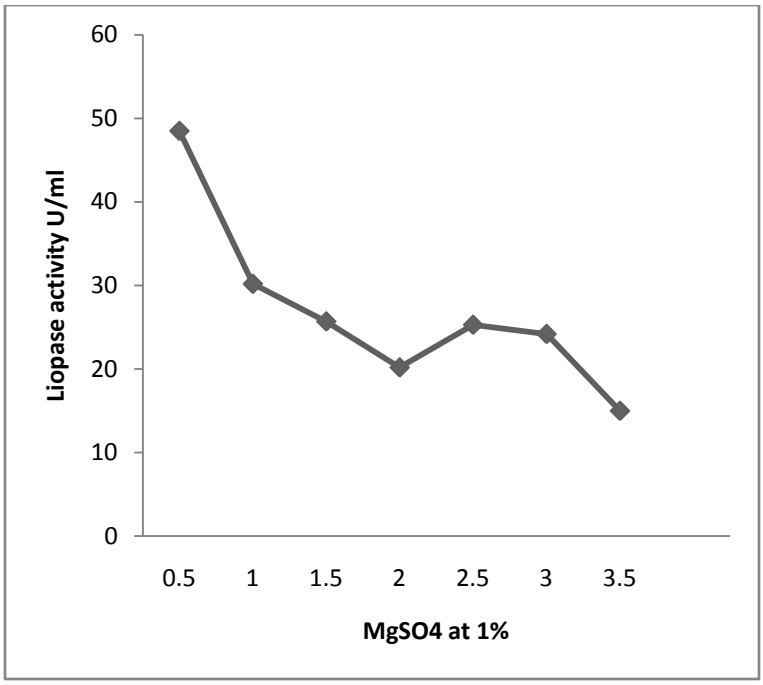

Fig 7: Effect of metal ion concentrations ($MgSO_4$) on lipase production

DISCUSSION

The various oils; Groundnut oil, Crude oil, Soy oil, palm oil and Olive oil were shown to induce lipase production at varying degrees. 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 *Serretia* 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 ($p < 0.05$). An increase in lipase activity (synthesis and hydrolysis) after incubation in organic solvents was observed as follows: 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.

Potassium chloride and sodium chloride found to yield almost equal synthesis of lipase production and 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 manganesse sulfate. 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 magnessium sulphate, followed by ferrous sulphate. Increased lipase activity has been found to increase when combinations of glucose and magnessium 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 magnessium sulphate required minimal concentration even though their effects at increase concentration are not negligible. Magnessium sulphate was found to give the best yield.

CONCLUSION

The growing demand for lipases has shifted the trend towards prospecting for novel lipases, improving the properties of existing lipases for established technical applications and producing new enzymes tailor-made for entirely new areas of application. This has largely been possible due to outstanding events in the field of molecular enzymology. The number of novel microbial lipases being cloned and biochemically characterized is on the rise.

These results are promising as a cheap and effective medium can decrease the production cost, so it is crucial to the industrialization of the enzyme method. However, it would be recommended that the research involved in this enzyme's characterization should be continued in order to get a full image of its potential and its use in application. Further study must be performed in order to fully characterize the new lipase and design its production to serve environmental friendly purposes.

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