IOSR Journal of Pharmacy and Biological Sciences (IOSR-JPBS)
e-ISSN: 2278-3008, p-ISSN:2319-7676. Volume 10, Issue 2 Ver. III (Mar -Apr. 2015), PP 70-78
www.iosrjournals.org

Optimization of Process Parameters for the Production of Lipase in Submerged Batch Fermentation by *Fusarium* specie.

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Abstract: *Production of lipase by Fusarium sp. isolated from soil in the University of Port Harcourt was studied in submerged batch fermentation. Lipase secretion by the fungus was detected in a Rhodamin B agar medium containing olive oil. Lipase production was investigated in shake-flask culture. Culture conditions and nutrient source modification studies involving carbon sources, nitrogen sources, medium pH as well as the time course were evaluated in this study. Lipase was detected in culture filtrate. Among the carbon and nitrogen sources; sucrose at 1.5% (7.7U/ml) and ammonium chloride at 2.5% (23.6 U/ml) was found to be the best carbon and nitrogen sources respectively. Manganese sulphate (0.1%) gave optimum lipase production among other metal ions studied. Among the different lipase inducers tested, crude oil (0.9%) and olive oil(1.0%) significantly ($p<0.05$) enhanced lipase production by the fungus. The optimum pH ant temperature for the production of lipase were found to be 2,5 at 30^0 C after 120h of fermentation with agitation rate of 150rpm. Lipase production by Fusarium sp. followed a logarithmic pattern with maximum lipase activity of 72.35U/ml obtained on the 5^{th} day of fermentation. Statistical analysis revealed that the culture conditions significantly ($p<0.05$) influenced lipase secretion by this fungus. Generally, results obtained in this study demonstrate that lipase from Fusarium sp. would be attractive for biotechnological and industrial explorations.*

Keywords: *Lipase, Fusarium sp., Optimization, Process, Parameters*

I. Introduction

Lipases (EC 3.1.1.3) are enzymes that catalyze the hydrolysis of triacylglycerides (Bora et al.,2007) into diacylglycerides monoacylglycerides, glycerol and fatty acids. They are widely distributed in animals, plants and microorganisms (Adinarayana et al., 2003).One important aspect of Lipase mediated catalysis is the unique physico-chemical character of the reactions i.e the catalysis at the lipid-water interface (Gupta et al. 2003; Ghosh et al.,1996).Microbial lipases have gained special industrial attention due to their stability, selectivity, and broad substrate specificity (Dutra et al., 2008;Griebeler et al.,2009) and occur widely in bacteria, yeasts and Moulds (Falony et al.,2010). Fungi are widely recognized as one of the best lipase sources and are widely used in the food industry. The search for new lipase showing diverse characteristics is a matter of industrial interest. From the industrial point of view, Fungi are the most preferred sources of lipases as they produce extracellular enzymes that facilitate downstream processing (Jesus et al.,1999). Most of the lipase focuses on the production of extracellular lipase from a variety of fungi; *Aspergillus niger.* (Falony et al., 2006; Rodriquez et al.,2006) *Rhizopus* sp., *Penicillum* sp., *Fusarium oxysporum* (Hala et al.,2010)

The interest in lipase has grown over the last few years due to their excellent catalytic properties (Falony et al., 2006) and their biodiverse industrial applications for example they have been used as additives in detergent, the elaborate of dietic foods for use in the food industry obtaining biomolecules in pharmaceuticals industry and production of pure optical compounds in chemical processes (Gupta, et al., 2007; Park et al.,2005; Grbavcic et al., 2007; Franken et al.,2009).

II. Materials And Methods

2.1. Pre-Enrichment And Isolation Of The Fungi

Soil sample was collected from the University of Port Harcourt, pre-enriched with olive oil (20% v/v) and incubated for 7days at 30^oC. one gram of the soil sample was serially diluted and plated onto Potato Dextrose Agar (PDA) incorporated with rifampicin (5µg/ml) for 4 days at 30^oC. Discrete colonies were picked and purified by sub-culturing onto PDA as described earlier until their pure cultures were obtained and subjected to screening for extracellular lipase production.

2.3. Preliminary Screening For Lipase Activity

The screening method of Savitha et al., (2007) was employed. PDA was incorporated with 1% rhodamine B dye and 3% olive oil. Each of the pure cultures was plated on the rhodamine B agar plates (PDA

with 1% rhodamine B and 3% olive oil) and Incubated at 30^{o}C for 5 days. To determine the production of extracellular lipase, plates were viewed under Ultra violet (UV) illumination.

2.4. Enzyme Assay

Lipase activity was determined using olive oil emulsion method. The assay mixture consisted of 1.0ml of the substrate emulsion (7ml emulsifying reagent with 30ml olive oil homogenized for 5 minutes using a vortex mixer (VMG 701). The emulsification reagent (NaCl 17.9g/L KH_2PO_4 0.41g/L, glycerol 540ml/L, gum Arabic 10.0g/L and distilled water to a volume of 1litre). 0.8ml of 0.2M potassium phosphate buffer (PH 7.0) and 0.2ml of the enzyme were incubated 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. One unit of lipase activity was defined as the amount of enzyme required to release 1µmoL of fatty acid per ml per min under above assay conditions. Assay was carried out in duplicates and the mean values were presented. (Adinarayana et al., 2003)

2.6. Production Studies Of Lipase

Preliminary Production of Lipase

A preliminary study of lipase in submerged fermentation was carried out using basal medium, of Kamimura et al., (1999), which is composed of (g/L); KH_2PO_4 (1.5), NH_4Cl (1.0), $MgSO_4$. $7H_2O$ (1.2), Yeast extract (2.0), $MnSO_4$ (17mg), $ZnSO_4$ (17mg), $FeSO_4$ (17mg) and olive oil (1% v/v). The enzyme preparation was obtained by inoculating 5 ml of dislodged spores from a 96h, culture into 250 conical flask containing 100ml of sterile fermented medium. Fermentation was carried out in orbital-shaking incubator at 130rpm for 3 days at 30^{o}C. After fermentation, mycelia were separated using Whatman no. 1 filter paper at room temperature, (Rajesh et al., 2010;Vishnupriya et al., 2010). The cell-free filtrate was used to assay for lipase activity. All experiments in this study were carried out in duplicates.

2.6.1 Effect of Carbon Substrates

The effects of various carbon sources on lipase production were investigated by replacing olive oil (control) used in the basal medium for preliminary study with; sucrose, crude oil, palm oil, soy oil and groundnut oil. A hundred milliliter of each media was dispensed as described earlier and sterilized at 15psi for 15 min after which 5ml of dislodged spores from the fungus was used to inoculate the various media. Fermentation was carried out in an orbital shaking incubation at 130rpm for 3 days (72h) at 30^{o}C following fermentation, the culture broth was filtered through Whatman no 1, filter paper at room temperature and filtrate covered. The cell free filtrate was used to assay for lipase. (Kamimura et al.,1999)

2.6.2 Effect of Concentration of Sucrose

The effect of concentration (0.1-3.5% w/v) of the sucrose in lipase, production was evaluated. Five millilitre of dislodged spores of *Fusarium* culture cultivated on PDA for 96h at 30^{o}c were inoculated into 250ml conical flasks each containing 100ml sterile basal medium with ranging concentration (0.1-3.5% w/v) of sucrose. Fermentation was carried out in an orbital shaking incubator at 130rpm for 72hrs at 30^{o}C. Thereafter, the culture broth was filtered through Whatman No 1 filter paper at room temperature and filtrate recovered. The cell free filtrate was used to assay for lipase activity as earlier described.

2.6.3 Effect of nitrogen Substrate

The following nitrogen substrates (2% w/v) were used to evaluate the influence of organic and inorganic nitrogen sources on lipase production by the fungus: yeast extract, soymeal, peptone, groundnut meal, malt extract, ammonium chloride (NH_4Cl) and potassium nitrate (KNO_3). Each of the nitrogen sources was used as a sole source of nitrogen in place of yeast extract and ammonium chloride employed in the basal medium. Fermentation was carried out in an orbital shaking incubator at 130rpm for 72hrs at 30^{o}C and cell free filtrate was obtained as had been described. Groundnut meal and Soybean meal were prepared in the laboratory using standard procedure and all inorganic nitrogen compounds used in this study were of analytical grade.

2.6.5. Effect of concentration of ammonium chloride

The effect of varying concentration of ammonium chloride in the fermentation medium for the production of lipase was evaluated. A 5ml of dislodge spores from a 96hours old *Fusarium* culture were inoculated into 250ml conical flasks each containing .A Hundred milliliter of sterile fermentation medium with varied concentrations (0.1 - 4.5w/v) of ammonium chloride (NH_4Cl). Fermentation was carried out in an orbital shaking flask at 130rpm for 72hrs at 30^{o}C, this was followed by filtration to obtain a cell-free filtrate which was to assay for lipase activity as earlier described.

2.7.7. Effect of Metals ions on Lipase Production.

The effect of different metal ions on lipase production was determined by substituting (g/l) KH_2PO_4 (1.5), NH_4Cl (1.0), $MgSO_4$. $7H_2O$ (1.2), Yeast extract (2.0), $MnSO_4$ (17mg), $ZnSO_4$ (17mg), $FeSO_4$ (17mg) in the basal medium with each of these various salts of metals at 1.5% concentration: KH_2PO_4, $MgSO4.7H_2O$, NaCl, CoCl, KNO_3, CaCl, $CaCO_3$, $ZnSO_4$ H_2O, $FeSO_4.H_2O$, and $MnSO_4$ H_2O. The medium was constituted followed by their sterilization. Five milliliter of dislodged spores were inoculated into a 250ml conical flasks containing 100ml of each of the medium with a particular metallic salts. Fermentation was carried out just as had been described for other treatment, followed by fermentation and subsequent filtration using Whatman no. 1 filter paper at room temperature to obtain a cell free filtrate which was used to assay for lipase activity.

2.7.8. Effect of Concentration of $MnSO_4.H_2O$ on Lipase Production.

Changes in lipase production in response to varied concentration of $MnSO_4.H_2O$ were evaluated. Five milliliter (5ml) of dislodged spores from 96h old *Fusarium* culture used to inoculate 100ml each of fermentation broth containing varying concentrations (0.1 - 3.0% w/v) of $MnSO_4$. Fermentation was carried out as had been described, followed by filtration using whatman no 1 filter paper. The cell-free filtrate was employed for enzyme essay as had been described.

2.7.9. Effect of additives on lipase production

Sources of nourishment (additives) (1% v/v) were employed to investigate the effect of combinations of sucrose with various oils and surfactants on lipase production. The following additives; palm oil, olive oil, crude oil, glycerol and Tween 80 were employed. Fermentation was carried out under conditions as earlier described. assay for lipase activity and biomass was carried out as earlier described.

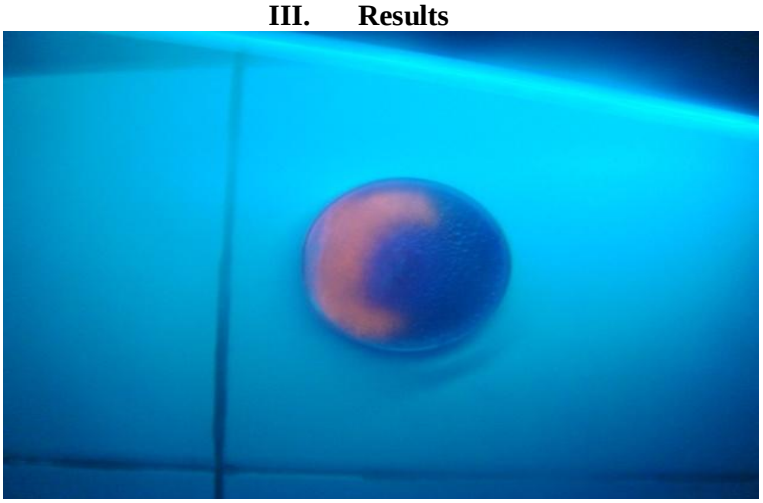
2.7.10. Effect of Concentrations of crude oil on Lipase Production

Varied concentrations of (0.1-1% v/v) of crude oil on lipase production were employed in fermentation broth were investigated. Same volume of dislodged spores of the fungus was employed in 250ml conical flask containing 95ml of fermentation broth and each concentration (0.1-1%) of crude oil. Fermentation in orbital shaker at 130rpm for 72hrs at 30% ensures followed by termination of fermentation and subsequent filtration using whatman no 1 filter paper to obtain a cell-free filtrate, which was used to carry out enzyme activity assay as earlier described.

2.7.11 Effect of Initial pH on Lipase Production

The influence of different initial pH values (PH 2.5-13.0) of fermentation media on lipase production was investigated by varied pH values. Buffers used were lM citrate buffer (3-5) and lM phosphate buffer (5-8) and the pH of the medium was adjusted prior to autoclaving with lM HCl and lM NaOH. Fermentation and enzyme assay were carried out as previously described.

III. Results



In the present study, the isolate tested showed detectable level of fluorescence on rhodamine B agar indicating the liberation of fatty acids from the incorporated triacylglycerol (olive oil) which can be seen as orange halos in the culture medium on which the organism is growing due to the action of secreted enzyme in the medium (Savitha et al., 2007).

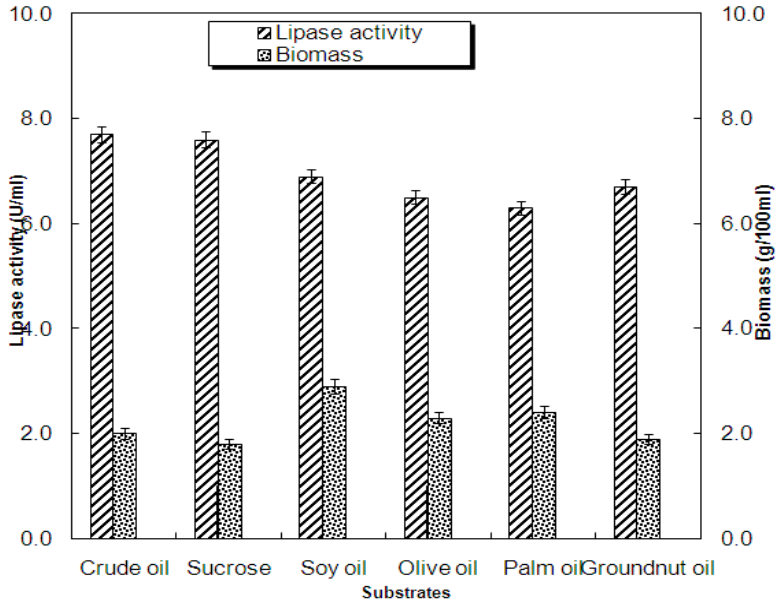


Figure 1: Effect of carbon sources on Growth and Lipase production by *Fusarium* sp.

Carbon source is an important substance for energy production in microorganism especially Fungi (Pogaku et al., 2009). A range of different carbon sources (carbohydrates, alcohol, acids, lipids.) has been reported to support both growth and lipase production (Dheeman et al., 2010),. Figure 1 shows the effect of various carbon sources on the production of lipase by *Fusarium* sp. Sucrose was found to greatly stimulate lipase production in *Fusarium* sp, This result is in agreement with the report by other workers whose investigations revealed sucrose as the best carbon source for lipase production by different organism. (Gunalashimi et al.,2008; Jagtap et al., 2010), Ginalska et al., (2006) reported the ability of sucrose to stimulate lipase production by *Geotrichum* sp strain R-59 (Basidiomycetes) . Sucrose was selected as the best potential substrate for the industrial production of lipase over crude oil based on economic reasons of availability and cost of purchase because crude oil is much more expensive and not readily available and this in turn could eventually affect the cost of production of lipase enzyme. No linear relationship exists between the ability of the carbon substrates to stimulate lipase production and ability to enhance the growth of the organism. Result obtained on the effect of carbon sources on biomass shows that the best stimulating substrate is not necessarily the best with the highest biomass, instead the increase in growth and lipase production depends entirely on the ability of the substrate to stimulate lipase production and is at the same time easily metabolized by the organism..

3.3.2. Effect Of Concentrations Of Sucrose

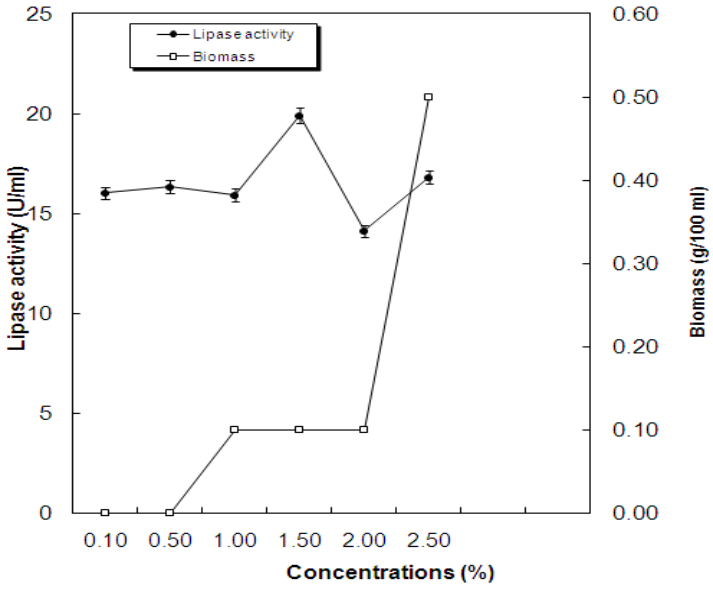


Figure 2: Effect of concentrations of carbon source (sucrose) on Growth and Lipase production by *Fusarium sp.*

The effect of various concentrations of sucrose is depicted in Figure 2.

Further study on the effect of various concentrations of sucrose reveals that lipase production was at its peak at concentration 1.5% but declined at concentration higher than 1.5%. Result showed that it was less significant ($p>0.05$).This is in contrast with the report of Ginalska et al (2006) who reported that Geotrichum like R59 strain was able to show maximum production at 0.5% concentration which declined when the concentration of the substrate increased to 1.5%. Sucrose is a good option when lipase production is thought among other substrates when an array of degradable substrate are available as options to be chosen for production using this organism.Armas et al., (2008) have reported slight superior lipase activity when sucrose was used in combination with coconut oil than when coconut was used alone.

Nitrogen sources play important role in the biosynthesis of lipase by microorganisms (Dheeman et al., 2010). Results obtained on the effect of nitrogen sources showed that treatment was significant($p<0.05$). Ammonium chloride was found to give the best yield. Ammonium chloride was found to be the best source of nitrogen with sucrose. The use of ammonium chloride as the best nitrogen source had been reported by D'annibale et al., (2006) with *Penicillium citrinum* on the contrary, Moataza et al.,(2008) reported the ability of ammonium chloride to stimulate lipase production but was not found to be the best. This in other words, points out that source of nitrogen from ammonium or its salts are highly dependable for lipase production than any other source (Essakkiraj et al., 2010, Rodriquez et al., 2006).

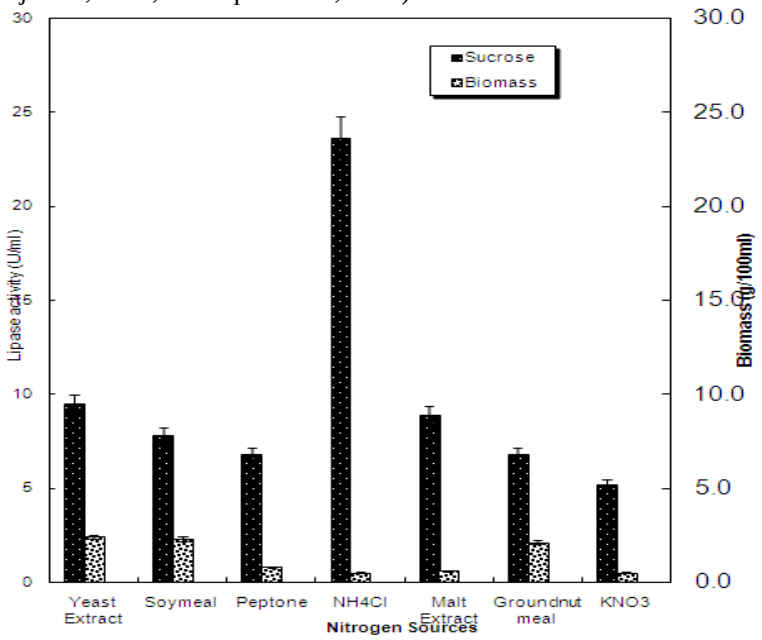


Figure 3: Effect of various nitrogen sources with sucrose on growth and Lipase production by *Fusarium sp.*

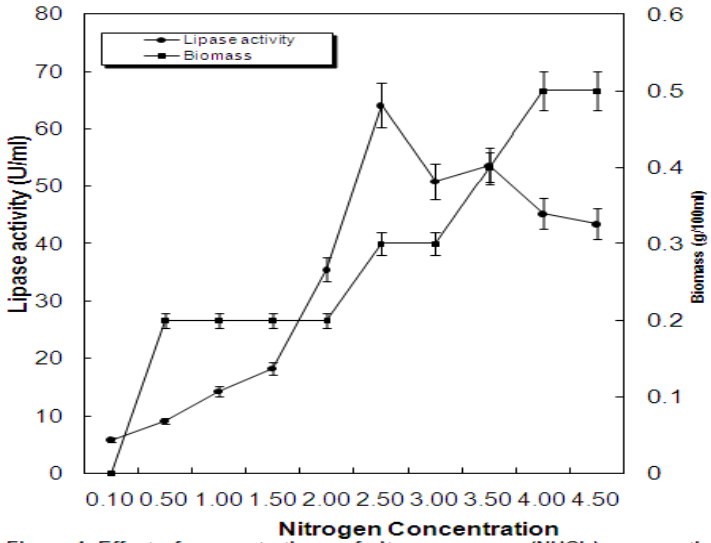


Figure 4: Effect of concentrations of nitrogen source ($NHCl_4$) on growth and Lipase production *Fusarium sp.*

Considering the effects of the various treatments involving nitrogen sources and 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 (Tan et al.,2004; Dheeman et al.,2010)
. A linear increase in their ability to increase lipase production by different concentrations was observed until a peak was reached at 2.5% concentration which then followed by a sharp decline at 3% concentration, this is in line with that which was reported by Essakkiraj et al., (2010). More so, increase in the concentration of ammonium chloride did not affect the growth of the organism quantitatively but that at concentration higher than 0.1% substantial growth must occur.

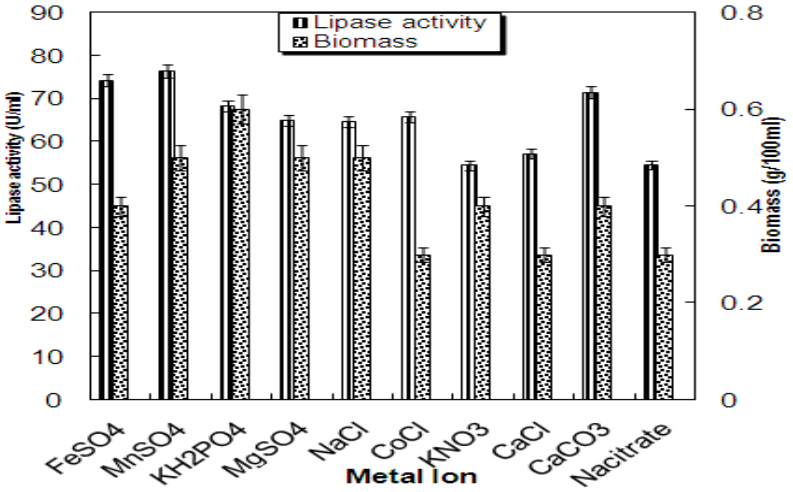


Figure 5: Effect of metal salts on Growth and Lipase production by *Fusarium sp.*

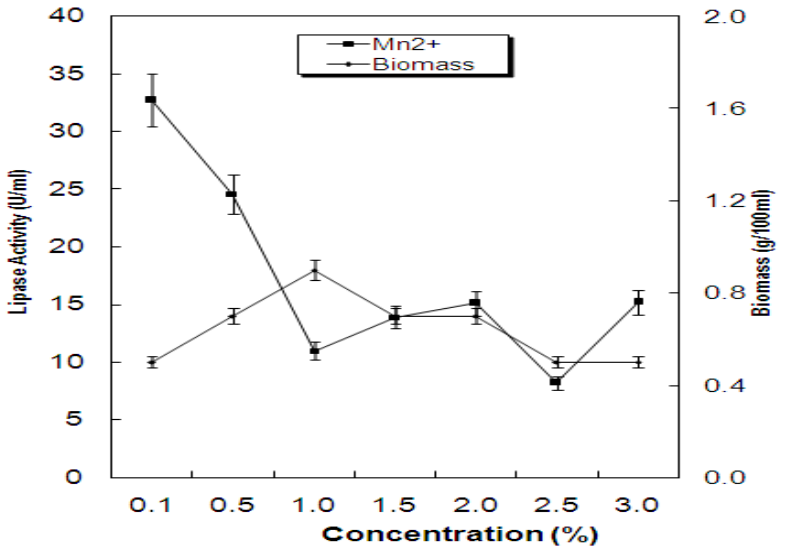


Figure 6: Effect of concentrations of manganese sulphate salt on Growth and Lipase production *Fusarium sp.*

Dheeman et al., (2009) has reported the use of Mn^{2+}, Fe^{2+}, Mg^{2+} and NH^{4+} to enhance lipase production. Increase lipase production was observed when manganesse sulfate was used followed by ferrous sulphate in agreement with the report by Dheeman et al.,(2010) . Increased lipase activity has been found to increase when combinations of sucrose and manganese salts are employed as carbon source and metallic salts respectively (Jagtap et al.,2010). Reports on the use of manganese have been reported by Rajendra et al., 2007;Lotrakul and Dharmsthilil (1997). Manganese sulphate was further subjected to study. Result was shown to be less significant ($p>0.05$). Concentration 0.1% gave the maximum lipase activity and gradually decreased upon increasing concentration; this shows that the effect of manganese sulphate required minimal concentration even though their effects at increase concentration are not negligible. Evidently, manganese is required for maximum lipase production at minute concentrations < 0.1%. The ability of metal ion to stimulate growth is

shown by the various values got on the biomass. After fermentation at 72hrs, $MgSO_4$, NaCl and $MnSO_4$ gave the best growth effect on the growth of the mycelia, this suggests that $MgSO_4$, NaCl & $MnSO_4$ form the major parts of the structural elemental components of the mycelia.

The effect of different concentration manganese on the mycelia growth increased with increasing concentration suggesting or indicating that manganese is required in higher amounts for the growth but not in lipase production *by Fusarium sp.*Manganese salt was selected as the best metal ion. Selection of manganese sulphate could contribute to establish a more profitable process from an economic stand point due to its minimal concentration.result on the effect of various concentrations of manganese sulphate was found to be less significant ($p>0.05$).

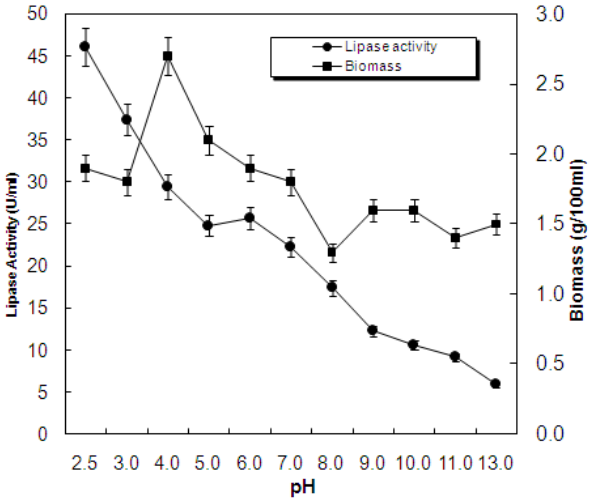


Figure 9: Effect of initial pH on Growth and Lipase production by *Fusarium sp*.

The highest activity was seen at pH 2.5 (an extreme pH) a slight increase in lipase activity was also found at pH 5.0-6.0, followed by a drastic decrease at increase alkalinity from 7.0-8.0 and the least at 13.0. this shows that the lipase produced by this organism is an extreme acidic lipase. The ability of organism to produce slightly acidic lipase has been reported by other workers; (Armas et al., (2008); Pogaku et al., (2009); Sekhon et al., (2008); Rajesh et al.,(2010); Falony et al., (2006) Armas et al., (2010) also reported the decreased trend in lipase production from pH 6-7 and subsequently. The ability of lipase production to remain increased at highly acidic pH suggests that the acidic nature of lipase (Rajesh et al, 2010),

The resultant change in the pH of the fermentation medium during the various Investigation depend largely on the products of metabolism under given conditions. The pattern of change in the pH of any medium depend largely on the medium composition and the end-product of their metabolism.

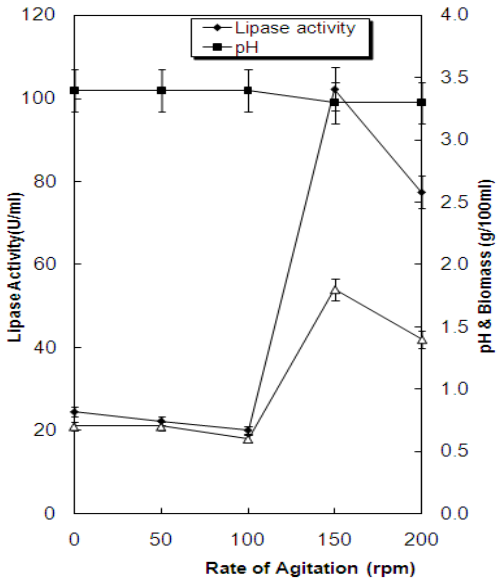


Figure 10: Effect of various rate of agitation on

Optimum shaking conditions required for lipase production was found to be 150rpm, 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 former can digest lipase enzymes. (Rajesh et al, 2010).

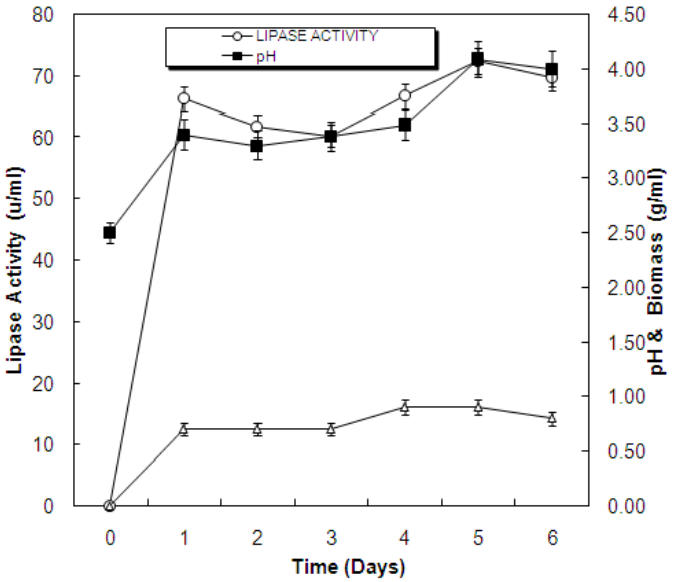


Figure 11: Effect ot Time on initial pH, Growth and lipase production

The time for maximal lipase production was studied in days. Result is found to be significant ($p<0.05$). A high yield of lipase was observed after 24hours of fermentation. This suggests that lipase is produced in the course of growth; during the lag phase of growth, when the cells are adapting to their new environment, the effect of time against growth showed the same trend with increasing lipase production

IV. Conclusion

Fusarium sp. Isolated from the soil in the University of Port Harcourt was confirmed a good producer of lipase. Optimum production of lipase by the isolate was shown to be attained when 1.5% sucrose, 2.5% NH_4Cl , 0.1% $MnSO_4$, 0.9% crude oil were employed as nutritional options at pH 2.5 under cultural conditions of 150 rpm agitation rate at 30^oC for 120 hrs(5days) , which yielded enzyme activity, which is 50-60% the total volume of the fermentation broth. This result therefore, suggests that the production of lipase from *Fusarium* sp. using the above mentioned nutritional and cultural conditions renders this lipase attractive for potential biotechnological applications in various industries.

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