Optimization and lipase production of *Lysinibacillus sphaericus* in domestic oil rich waste water

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Abstract

The present study investigated the growth and lipase production by *Lysinibacillus sphaericus* in a minimal medium. The growth conditions (i.e oil concentration, nitrogen source, carbon source, metals and non metals) were optimized for maximum production of enzyme. Influence of different culture conditions including varied environmental and nutritional conditions were tested. Lipase activity was determined by colorimetric method based on the activity in cleavage of p-nitrophenylpalmitate (p-NPP). Maximum lipase production (5.1 mM/min) was found on the fifth day of the cultured filtrate at pH 8 and temperature of 30^0C. Two point five percent (2.5%) oil concentration supported highest lipase enzyme production with (6.67mM/min). Ammonium phosphate and glucose encouraged the maximum activity/production. Optimum production of lipase (6.46 mM/mins) was exhibited by medium supplemented when cell growth is 0.286mg/l. Also, sulphate produced lipase maximally among the non metals used. From this work, optimizing the culturing conditions and modifying the composition of the medium dramatically improved the lipase production by *Lysinibacillus sphaericus*. Lipase production by *Lysinibacillus sphaericus* is very promising and could be used for industrial purposes and biotechnology.

[Aderiye B.I, Sulaimon A.M. **Optimization and lipase production of *Lysinibacillus sphaericus* in domestic oil rich waste water]**

Keywords: lipases; enzyme; *Lysinibacillus sphaericus*; optimum growth; optimum lipase production; optimization.

Introduction

Proper management of wastewater in our societies today as a result of anthropogenic activities is a necessity, not an option. The wastewater is usually classified based on their characteristics. Some of these wastewaters with components compatible with municipal wastewater are often recovered from the municipal sewers and many are industrial wastewaters which require pre-treatment to remove some substances prior to discharge into the municipal system. Lipids (characterized as oils, greases, fats, and long-chain fatty acids) are one of the important organic component of domestic oil rich wastewater. Their amount in municipal wastewater is approximately 30-40% of the total composition (Chipasa and Medrzycka, 2006).

There has been many studies over the behaviour and removal of lipids in biological treatment system. Domestic oil wastes either of petroleum or vegetable origin are considered as serious types of hazardous pollutants in aquatic environments, due to their high toxicity to the aquatic organisms (Carvalho *et al.* 2005). Wastewaters containing fat and oils were traditionally treated physically, which is currently considered insufficient if the fat is in its dispersed form. Recently, biological treatment has been found to be the most efficient method for removing fat, oil and grease by degrading them into miscible molecules (Contesini *et al*., 2010). Therefore, manipulation of microorganisms for treatment through optimization and their bioremediation potentials affords a very efficient tool for purifying contaminated effluents and natural water (Contesini *et al*., 2010).

Under certain conditions, it is possible to isolate bacterial strains that are capable of degrading lipids by using a selective medium containing a source of lipid. The use of lipase enzymes (triacylglycerol acylhydrolases) that are produced by these organisms degrading lipids may solve that problem, where they have been reported to catalyze the synthesis or hydrolysis of fats (Kanwar *et al*., 2006).

Lipases occur naturally in plants, animals, and microorganisms (Melo *et al*., 2005; Paques *et al*., 2008; Gangadhara *et al*., 2009; Li *et al*., 2010). Among these sources, microbial lipases prove to be best owing to their substrate specificity, greater stability and lower production costs. Microorganisms which are effective lipase producers include bacteria, fungi and yeast (Abada, 2008). Those lipid-degrading bacteria often secrete and release extracellular lipase enzymes, where these enzymes are generally inducible in the presence of different inducers such as different fatty acid methyl esters, olive oil, palm oil, and oleic acid (Sangeetha *et al*., 2008). The synthesis and secretion of extracellular lipases by microorganisms appear to be controlled in a variety of ways in order to utilize it for industrial purpose (Dutra *et al*., 2008; Khemika *et al.,* 2006).

Microbial lipases constitute an important group of biotechnologically valuable enzymes (Guerzoni *et al*., 2001; Holker *et al*., 2004). Most of the well-studied microbial lipases are inducible extracellular enzymes, and they are synthesized within the cell and exported to its external surface or environment (Kanwar *et al*., 2006).

Lipids being one of the most important components of vegetable oil generated by plants, many synthetic compounds and emulsions and mostly found in pharmaceutical and cosmetic industrial effluents. Further, lipids constitute one of the major types of organic matter found in municipal wastewater (Ramachandra *et al*., 2001).

The amount of lipid-rich wastewater increases every year due to urbanization and the development of factories. Suspended lipids can be readily removed from wastewater by physical methods. Nevertheless, chemically and/or physically stabilized lipid/water emulsions should be managed in an appropriate manner. This is necessary because lipids that pass through physicochemical treatment processes contribute to the low levels of biological oxygen demand (BOD) and chemical oxygen demand (COD) in the effluents (Zhang *et al*; 2009). Thus, biological treatment processes will be employed in this study to remove the emulsified lipids from wastewater.

Materials and Methods

Microorganism and inoculums preparation

Lysinibacillus sphaericus isolated from dietary oil rich waste water as characterized and identified molecularly. The isolate was collected from Microbiology department laboratory, Ekiti State University, Ekiti State (Odeyemi and Aderiye, 2011). For this study, Optical density of 0.5 at 550nm which equates (10^6 CFU/ml) was then used as the population size (Mishra *et al*., 2001).

Preparation of Wastewater and Fresh Palm Oil media for Enzyme production

Sterile wastewater was distributed into five portions 100 mL each in 250 mL Erlenmeyer flasks and inoculated with 1 mL bacterial inoculum measured with 600nm absorbance (Gupta *et al.,* 2004). The flasks were kept in shaking incubator with 150 r.p.m at 30℃. Samples were drawn from each of the flasks at intervals of 6 h for a period of 24 h and later centrifuged at 5000 x g for 30 minutes at 4℃. Cell free supernatant corresponding to growth phase was used as the crude enzyme for assay and further analysis. Also, palm oil-containing medium was prepared with 0.2% w/v palm oil, 1.5% K_2HSO_4, 0.05% $MgSO_4$, 1.0% $(NH4)_2SO_4$, 0.2% $CaCl_2$, 0.2% $FeSO_4$ and 0.5% yeast extract, distributed, inoculated and incubated in wastewater culture (Gupta *et al.,* 2004).

Detection and estimation of Lipase Activity

To measure bacterial growth and lipase production, culture samples were removed at designated times and centrifuged at 5000g for 10min. The crude enzyme was usually stored at 4℃ until when needed. Lipolytic activity was determined by colorimetric method based on the activity in cleavage of p-nitrophenylpalmitate (p-NPP) at pH 8.0 (Rajesh *et al*., 2010). The reaction mixture contained 180 µL of solution A (0:062 g of p-NPP in 10 mL of 2-propanol, sonicated for 2 min before use), 1620 µL of solution B (0.4% triton X-100 and 0.1 % Arabic gum in 50 mM Tris-HCl, pH 8.0) and 200 µL of properly diluted enzyme sample. The product was detected at 410 nm wavelength after incubation for 15 min at 37℃. Under this condition, the molar extinction coefficient (410 nm) of p-nitrophenol (p-NP) released from p-NPP was 15000 M^{-1}. One unit of lipase activity was defined as 1 µmol of p-nitrophenol (p-NP) released per minute by 1 mL of enzyme (Cho *et al.,* 2000; Rajesh *et al*., 2010).

Optimization of conditions for Microbial growth and Lipase production

Various process parameters affecting enzyme production were optimized. Such different growth conditions were to be optimized independent of the other. The parameters investigated include (i) incubation time (24 h-12 days), (ii) oil concentration using palm oil-containing medium (2.5-15%), (iii) incubation temperature (20-80 ℃), (iv) pH of medium (4-8), nitrogen source (ammonium nitrate, ammonium nitrite, ammonium sulphate, ammonium chloride, ammonium carbonate and ammonium phosphate; 1%w/v), (vi) supplementary carbon source (glucose, fructose, galactose, sucrose, maltose, lactose; 1%w/v), and (vii) salt ions (Na^+, K^+, Mg^{2+}, Ca^{2+}, Fe PO_4, SO_4, Cl^-). In each case, 1 ml of bacteria culture which equals (10^6 CFU/ml) was added to the medium (Gupta *et al.,* 2004).

Growth and Lipase production of *Lysinibacillus sphaericus* at different incubation periods:

Lipase production was carried out for 12 days and the samples were collected after every 24h to check the production of lipase and growth. Aliquots of the culture broth were withdrawn each day and the cells harvested (10,000g at 4^0C for 30 mins) to determine the optimum growth and lipase production. The supernatant were used for measurement of lipase activity (Francisco *et al.,* 2004; Sharma *et al*., 2009).

Growth and Lipase production of *Lysinibacillus sphaericus* by varying oil concentrations:

Different concentrations of palm oil were tested on growth and lipase production of *Lysinibacillus* sp. in broth medium (CFU/v) in (0.0, 2.5, 5.0, 7.5, 10.0, 12.5 and 15.0 in %) at 30^0C in palm oil containing basal medium. This was done by pre-weighing the oil, incorporating it into a known amount of the broth (Sharma *et al*., 2009; Singh *et al*., 2010).

Effect of temperature and pH on growth and lipase production by *Lysinibacillus sphaericus*: The optimum temperature for lipase activity was determined by incubating the assay mixture described above at different temperatures between 20^0C and 80^0C at 10^0C intervals for 24hours and pHs ranging between pH 4 to pH 8. Growth rate by the organism and optimum production of lipase were monitored at a pH interval of 1. The resulting enzyme activity was measured with a Spectrophotometer (PYE Unicam Sp 9, Cambridge, UK) at 600nm (Francisco *et al.,* 2004).

Effect of various nitrogen and carbon sources on growth and lipase production by *Lysinibacillus sphaericus*:

The influence of ammonium nitrate, ammonium nitrite, ammonium sulphate, ammonium chloride, ammonium carbonate and ammonium phosphate and also carbon sources such as glucose, fructose and galactose, maltose, sucrose and lactose (1%w/v), each was examined on the growth and lipase

production of *Lysinibacillus* sp. in the basal medium and then measured with spectrophotometer at 600nm (Lima *et al.,* 2003).

Metallic and non metallic effect on the growth of the isolate: Effect of metals was examined on the growth of the organisms. Metals such as sodium (Na), potassium (K), magnesium (Mg), calcium (Ca), iron (Fe), and non metals such as phosphate (PO_4), sulphate (SO_4) and chloride (Cl) were used. Result of enzyme activity was measured by spectrophotometer at 600nm based on the release of p-nitro phenol (p-NP) (Sharma *et al*., 2009).

Results and Discussion

Optimization of growth and enzyme production by an organism from a natural environment is often needed for industrial applications as found in this work. Lima *et al*. (2003) proposed it could be achieved when microorganisms used for enzyme production is grown in fermenters using an optimized growth medium. Many studies have been undertaken which define the optimal culture and nutritional requirements for lipase production by submerged culture (Carvalho *et al*., 2007). Hydrocarbon degrading bacteria in oily sludge through the decomposition of these compounds reduce the waste volumes and are able to reduce their toxicity (Carvalho *et al*., 2007). The bacteria can also produce biosurfactant and convert heavy oil compounds to lighter compounds and use them as the substrate have also important economically (Carvalho *et al*., 2007). In addition, due to existing toxic and carcinogenic compounds in oily sludge, decomposition of these compounds by the bacteria neutralized these destructive effects. Type and concentration of carbon sources, nitrogen sources, culture pH, growth temperature, oil concentration and incubation period do greatly Influenced lipase production as seen in the present study (Sharma *et al.,* 2002; Kanwar *et al*., 2006).

Effect of incubation period on growth and lipase production by *Lysinibacillus sphaericus*

The lipase production and maximum lipase activity (5.1mM/mins) was observed after 5 days of incubation time (Fig. 1). The organism grew in the medium at the rate of 0.292 mg/day while it produces the lipase at the rate of 0.78mM/min/day. After long incubation time, lipase production was turned down with activity 2.3 mM/min after 12 days of incubation. Lipase enzyme production was highest (5.1 mM/min) on the fifth day of incubation. As the growth of the organisms progressively reduced after the 4th day, enzyme production reduces correspondingly after 5 days of incubation.

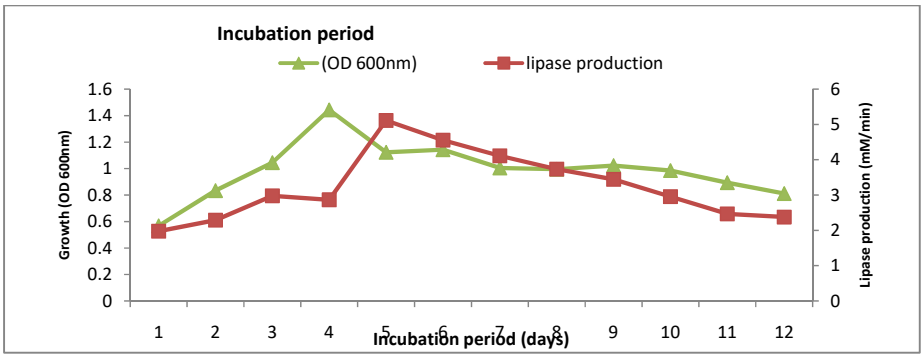

Figure 1: Growth of *Lysinibacillus sphaericus* and its lipase enzyme production at different incubation periods.

Effect of oil concentration on growth and lipase production by *Lysinibacillus sphaericus*

Lipases are generally produced in the presence of a lipid such as oil or any other inducer, such as triacylglycerols, fatty acids, hydrolysable esters, bile salts and glycerol (Sharma *et al*., 2009). Lipidic carbon sources serve as inducers and olive oil with high contents of oleic acid is a well-known inducer for the lipase production by many bacterial and fungal strains (Wang *et al*., 2008). During this study, maximum lipase activity was achieved in the presence 2.5% dietary palm oil with 6.6667mM/mins as shown in figure 2, which is in line with the work of Misbah and Haq (2004) that says 2% olive oil serves as an inducer with substrate. This indicated that additional lipid source is more effective to enhance the lipase production than any other carbohydrate source. In this work, increasing oil concentration brings about significant reduction in the growth of the organisms and the higher the growth of the organism so also the higher the lipase production. The maximum production of lipase at low oil concentration may be due to availability of oxygen for the organism to thrive in the basal medium; this is in agreement with the work of Odeyemi *et al*. (2011) who reported that oxygen becomes much available as an electron acceptor, because *Lysinibacillus sphaericus* thrives well wherever oxygen is available. The microbe is versatile in utilizing the limited nutrient and has the ability to adapt to the toxic condition of detergent contained wastewater (Odeyemi *et al.,* 2013). The growth of the organism reduced with increase in the oil concentration of 5% but reduced progressively at 10% to 15% of the concentration of oil used. Also enzyme production reduced correspondingly with increases in concentration of oil. The microbe grew in the medium at the rate of 0.03 mg/2.5% while production of lipase at this growth rate was (0.6356 mM/min) per 2.5% of oil concentration. It was observed that further increase in oil concentration did not favor to boost the lipase activity. This might be due to poor oxygen transfer at higher level which could modify the microbial metabolism leading to less lipase production (Gombert *et al*., 1999).

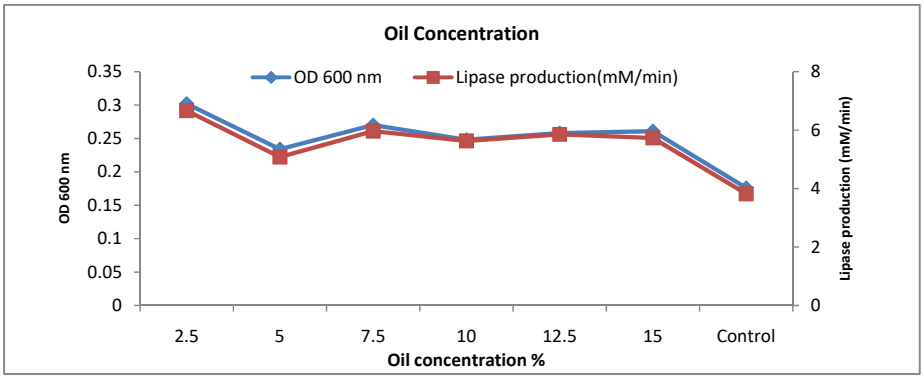

Figure 2: Growth of *Lysinibacillus sphaericus* and lipase enzyme production by varying oil concentration.

Effect of temperature on growth and lipase production by *Lysinibacillus sphaericus*

Slight changes in growth temperature may affect enzyme production (Saxena and Tanner, 2011). Optimum temperature for lipase production was 30°C with a value of (3.2 mM/min) and growth rate of 0.025mg/6h (Figure 3). This is in agreement with the work of Bhatti *et al*. (2007) who says at higher temperature, due to the production of large amount of metabolic heat, the fermenting substrate temperature shoots up, thereby inhibiting microbial growth and enzyme formation. An increase in

temperature increased the number of effective collision between the enzyme and substrate to form the activated complex and thus the rate of reaction increased. At higher temperature of 40^0C, the enzyme lost 18.8% of the maximum production and with only 61.9% lipase production at the highest temperature (80^0C). There is a limit to the increase in enzyme activity with the increase in temperature (Bayoumi *et al.,* 2007). When the rate of enzyme catalysed reactions is measured at several temperatures, there is an optimal temperature at which the reaction is most rapid, but above that temperature, the reaction rate decreases sharply mainly due to the denaturation of enzyme by heat (Murray *et al*., 2003). Temperature also influences secretion of extracellular enzymes by changing the physical properties of the cell membrane (Pirt, 1975). It may be correlated with the increased production of protease at higher temperatures which lead to deactivation of lipase (Palma *et al.,* 2000).

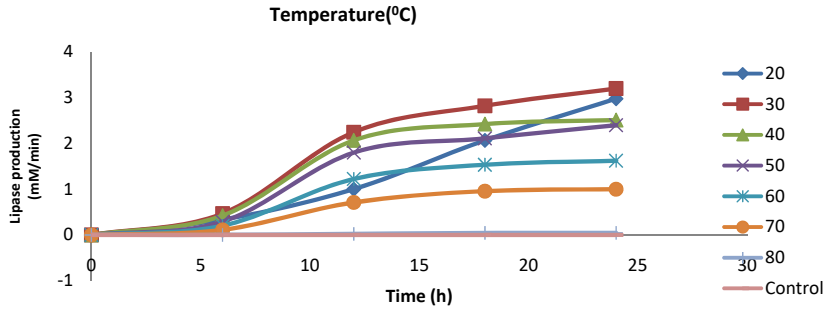

Figure 3: Effects of different temperature on Lipase enzyme production by *Lysinibacillus sphaericus*

Effect of pH on growth and lipase production

One of the major physical parameters which can influence bacterial growth and lipase production is media pH. Thus, the pH of the fermentation medium had a marked effect on the cell growth and enzyme production. Furthermore, the optimal pH values may be affected by the incubation temperature in many thermophiles.

In this present research, lipase exhibited activity in the pH range of 4.0 to 8.0 and the optimum activity was observed at pH 8.0 (Fig. 4). As the acidity of the medium increases, so also significant reduction in enzyme production was observed. For instance, at pH 6.0 the lipase enzyme production was 2.1mM/min while the maximum production of (1.355mM/mins) and (1.1555mM/mins) were observed at pH 5 and pH 4 respectively. The growth of the organism in the medium with maximum activity of pH 8 was at a rate of 0.021mg/6h while its lipase enzyme production was at a rate of (0.470 mM/mins). This work is in agreement to the results of Hassan *et al.* (2006) who found that, the optimum conditions for production of a thermostable lipase by *Bacillus* sp. at temperature 30 ºC and pH 8.0. (Ghanem *et al.,* 2000) also found, the maximum lipase production by *Bacillus* sp. FH5 was obtained after 48 h at pH 8.0. This is not in conformity with the work of Benattouche and Abbouni (2012) who reported that the *Pseudomonas aeruginosa* was able to grow in the pH range from 6 to 8 and produced maximum lipase (38.5 U/ml) at pH 7. Yuzo and Sakaya (2003) also reported that maximum lipase activity from *Pseudomonas fluorescens* HU 380 was detected at pH 7.

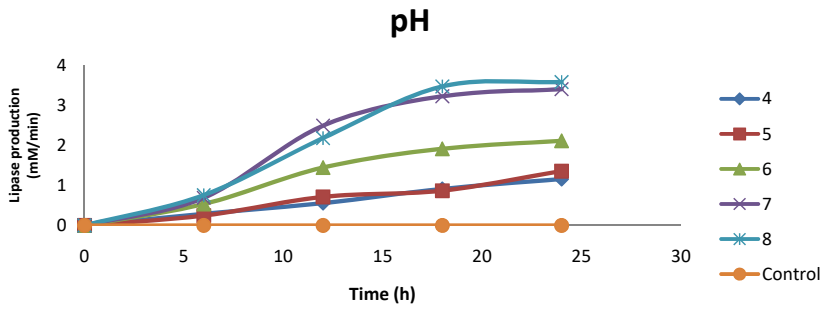

Figure 4: Effects of varying pH on Lipase enzyme production by *Lysinibacillus sphaericus*

Effect of nitrogen and carbon source on growth and lipase production:

Generally, microorganisms produce high amount of lipase when organic nitrogen sources such as peptone and yeast extract used as nitrogen source for lipase production by various *Bacillus* spp (Sharma *et al.,* 2002; Kanwar *et al*., 2006). In this present study inorganic nitrogen such as ammonium nitrate, ammonium nitrite, ammonium sulphate, ammonium chloride, ammonium carbonate and ammonium phosphate were used. It was discovered that among the six inorganic nitrogen tested for, the optimum growth and lipase production by *Lysinibacillus sphaericus* was exhibited in ammonium phosphate $(NH_4)_2PO_4$ supplemented medium (fig 5). Glucose had maximum activity and lipase production among the carbon sources used. Each microorganism requires a different carbon source to produce lipase at its maximum level. The major factor for the expression of lipase activity has always been reported as the carbon source, since lipases are inducible enzymes (Gupta *et al*., 2004). These enzymes are generally produced in the presence of lipid or any other inducer and glycerol (Sharma *et al*., 2009). After studying the efficacy of six various carbon sources on the lipase(s) productivity of the bacterial strain, it was obvious that glucose had a great inductive effect on *L. sphaericus* productivity with lipase(s) yields of 5.311 M/mins as revealed in fig 6.

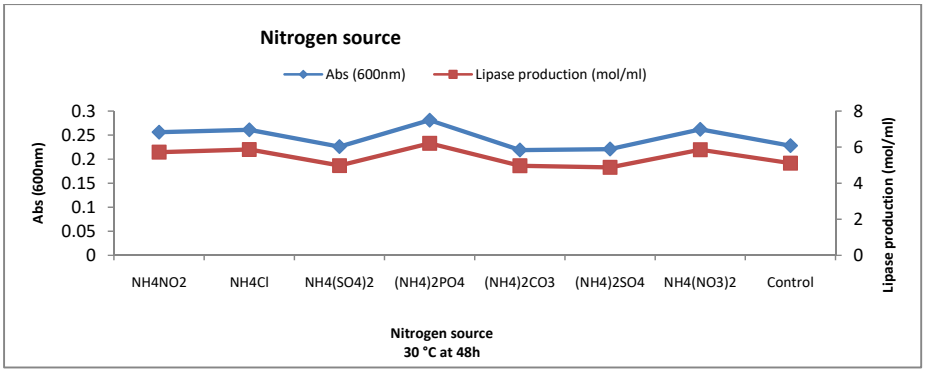

Figure 5: Growth of *Lysinibacillus sphaericus* and its lipase production with different nitrogen sources

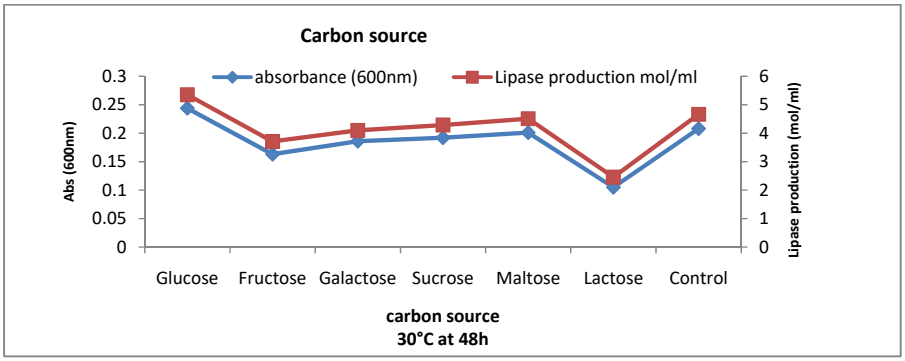

Figure 6: Growth of *Lysinibacillus sphaericus* and its lipase production with different carbon sources

Metallic and non metallic effect on the growth of *Lysinibacillus* sp and its lipase production

Lysinibacillus sphaericus lipases hydrolyze domestic oil in the presence of various metals. KCl, NaCl, $MgCl_2$, $CaCl_2$, and $FeCl_3$ displays enhanced hydrolysis capability. When KCl, NaCl, $MgCl_2$ were used, hydrolysis of oil rich waste water was three times faster than that of Fe^{3+} and 1.5 times faster with regards to Ca^{2+}. Interestingly, Mg^{+2} ions resulted in a maximal increase in lipase activity The growth rate of the organism at the maximum production of 6.4555 mM/mins of enzyme revealed 0.286mg/l but Fe^{3+} ions showed as antagonistic effect. This is in agreement with the work of (Sharma *et al*., 200; 2001) who says lipase activity was enhanced in the presence of K^{+} and Ca^{+2} and Mg^{+2} ions, but inhibited by Mg^{+2} and Hg^{+2} ions. The addition of Mg^{+2} significantly stimulate lipase production. Also Annamalai *et al*. (2011) found that, metal ions, Ni^{2+}, Mn^{2+}, Hg^{2+}, Fe^{2+}, Fe^{3+} and Co^{2+} usually slightly inhibited enzyme activity and no effect was found with Cu^{2+}. The activity of lipases may be inhibited or stimulated by cofactors. Divalent cations such as calcium and magnesium often stimulate enzyme activity due to the formation of calcium salts of long chain fatty acids (Macrae and Hammond, 1985). Calcium stimulated lipases have been reported in the case of *Acinetobacter* sp. RAG1 (Snellaman and Colwell, 2004). In contrast, the lipase from *Pseudomonas aeruginosa* 10145 is inhibited by the presence of calcium ions. Also, SO_4^{2+} had the highest lipase production (Saxena and Tanner, 2011).

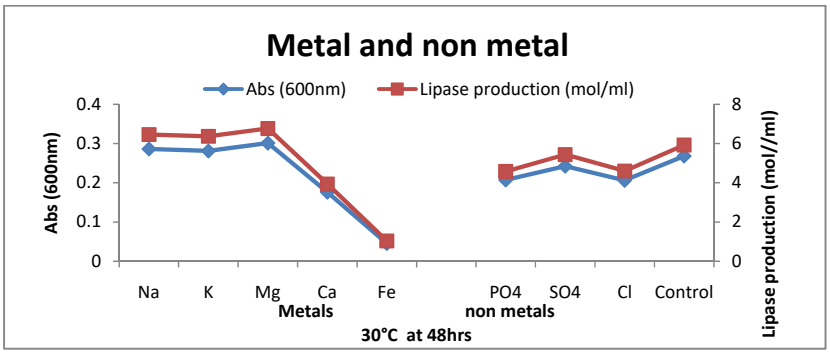

Figure 7: Growth of *Lysinibacillus sphaericus* and its lipase production with different metals and non metals

The present study demonstrated that optimization of culture medium recipe which plays critical role in enhancing the lipase production by newly isolated *Lysinibacillus sphaericus*. The optimum culture pH and temperature for producing maximum extracellular lipase by *Lysinibacillus sphaericus* is 8.0 and 30°C respectively. In case of the temperature, the organism grew very well between 20°C to 50°C. This study indicates that *Lysinibacillus sphaericus* could exert growth in a mesophilic and thermophilic conditions and it could tolerate higher temperature.

Effect of different Concentration of best Nitrogen Source (Ammonium phosphate) on the production of Lipase

Among various nitrogen sources tested, ammonium phosphate performed best. Determination of the concentration of ammonium phosphate that would give optimal growth and enzyme production by *Lysinibacillus sphaericus* was done. It was discovered that when *Lysinibacillus sphaericus* was incorporated into basal medium containing different concentrations (ranging from 0.5mg/Mol to 3mg/Mol) of the ammonium phosphate, 1mg/Mol had the highest production of lipase. It was noticed that as the concentration of the ammonium phosphate incorporated increases, there is gradual decrease in the amount of lipase produced (Fig. 8). This

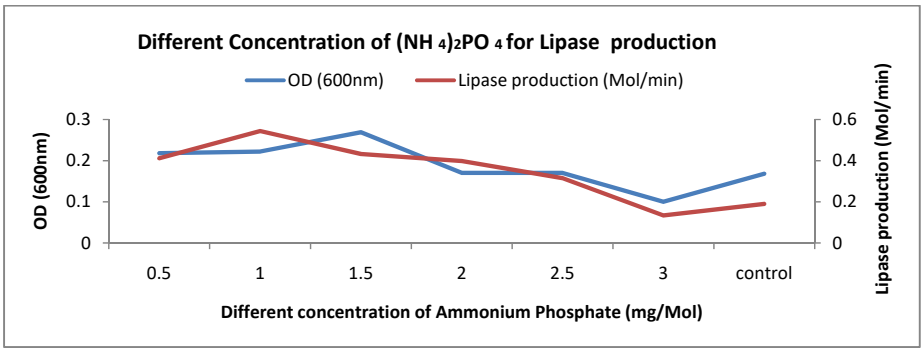

Figure 8: Growth of *Lysinibacillus sphaericus* and Lipase enzyme production at different concentrations of Ammonium phosphate

Effect of different Concentration of best Carbon Source (Glucose) on the production of Lipase

The maximum lipase activity activities were obtained at 0.5mg/Mol substrate concentration. Substrate concentration may be a dynamic influencing factor that affects the product yield and enzyme activity. The higher the concentration of glucose in the sample brought about decrease in the amount of lipase produce as shown in figure 9, such that between 0.5mg/Mol gave the highest yield of enzyme.

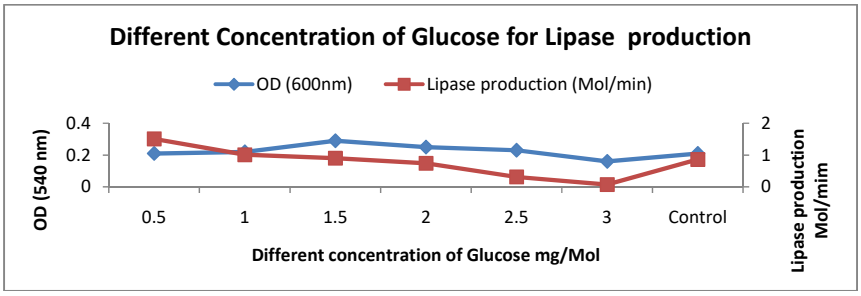

Figure 9: Growth of *Lysinibacillus sphaericus* and Lipase enzyme production at different concentrations of Glucose.

Effect of different Concentration of best Metal nutrient (Magnesium and phosphate) on the production of Lipase

The increase in concentration of both ions (Mg^{2+} and phosphate) from 10mM to 50mM in the production medium caused reduction of enzyme activity. Ten millimole of Mg^{++} had the highest production of enzyme but strongly affected production by *Lysininbacillus sphaericus* when the concentration increased at 30^0C for 24 hours as shown in figure 10.

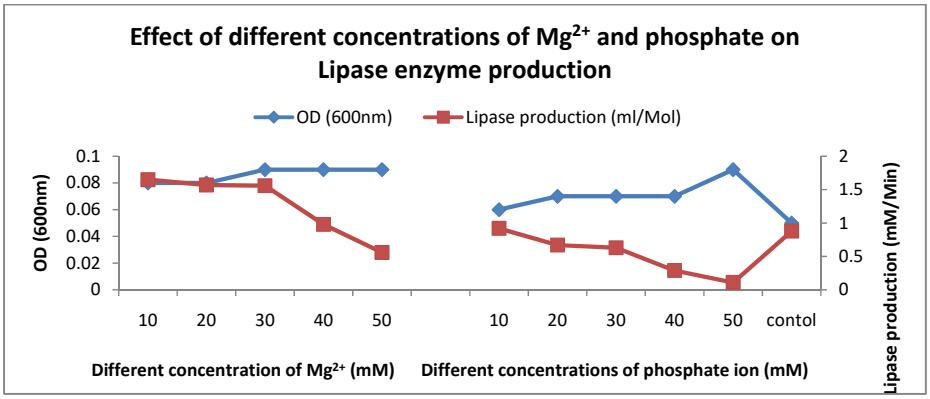

Figure 10: Growth of *Lysinibacillus sphaericus* and Lipase enzyme production at different concentrations of Mg^{2+} and phosphate.

Conclusion

Optimizing the culturing conditions and modifying the composition of the medium dramatically improved the lipase production by *Lysinibacillus sphaericus*. Base on the present findings, realizing the best optimal concentration and range from the nutritional parameters and environmental respectively gave a clue towards setting up a medium that could give the best yield of lipase production. These could be use for further studies. it is concluded that, the nutritional, environmental factors and the lipase production are interdependent and give useful basic information to achieve the large scale production of lipase by *L. sphaericus*.

Acknowledgements

I sincerely appreciate the assistance of Dr. A. Odeyemi and Mr. Bamidele for the technical assistance during the course of this research.

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