Production, Characterization and Purification of Lipase by Bacteria Isolated from Palm Oil Mill Effluent and Its Dumpsites Soil

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Abstract: Lipases (triacylglycerol acylhydrolases, EC3.1.1.3) are water-soluble enzymes that catalyze the hydrolysis(and synthesis) of ester bonds formed from acyl glycerol and long-chain fatty acids at lipid-water interface. Palm oil mill effluents have been reported to be rich in lipolytic bacteria, thus this work aimed at production, characterization and purification of lipase by bacteria isolated from Palm oil mill effluent and its dumpsites soil. From the serially diluted soil and palm oil mill effluent samples, fifteen(15) bacterial isolates were selected based on the zones of clearance (qualitative screening)on selective agar based medium. They were further screened quantitatively for their lipolyticactivities using titrimetric method. Five bacterial isolates showing high lipase activity were selected and identified based on their morphological, biochemical and physiological characteristics as *Bacillus subtilis*, *Staphylococcus aureus*, *Bacillus* sp., *Serratiamarcescens* and *Pseudomonas aeruginosa.*The enzyme exhibited maximum activity at incubation temperature of 45°C at 48hr incubation time and agitation rate of 150rpm. Also, pH 7.0 to 7.5 was found to be best for lipase activity. The lipase produced retained stability up to pH 10 and temperature of 70^0C. Highest stability of the enzyme was observed with $Ca^{2+}$and least with Mn2+ metal ions.The total protein content, enzyme activity and specific activity of the enzymes reduced with each purification step for all the isolates. The lipase produced possessed activity and stability over a range of pH values and high thermal stability at ambient temperatures making them suitable candidates for industrial applications.

Keyword: Palm oil mill effluent, Lipolytic activity, Titrimetry, Enzyme stability

1.0 Introduction

Lipases are a group of water-soluble enzymes that catalyze the hydrolysis (and synthesis) of ester bonds in insoluble acylglycerols at lipid-water interfaces (Jaeger and Eggert, 2002; Treichel *et al*., 2010).Lipases hydrolyze triacylglycerides into diacylglycerides, monoacylglycerides, free fatty acids and glycerol (Gilham and Lehner, 2005; Angkawidjaja and kanaya, 2006). Lipase reaction occurs at the interface between the aqueous and the oil phases because of an opposite polarity between the enzyme (hydrophilic) and their substrates (lipophilic) (Reis *et al*., 2008). Lipases are ubiquitous in nature that is they are found in plants, animals and microorganisms. Lipases from microbial sources are of considerable commercial importance, because of their high versatility and stability, moreover, they have the advantage of being readily produced in high yields (Hasan *et al*., 2006) of which, bacterial lipases are more economical and stable (Mohan *et al*., 2008).

Bacterial lipases are mostly inducible enzymes, requiring some form of oil, fatty acid, fatty acid alcohol or fatty acid ester for their induction (Shah and Bhatt, 2011), though there are reported cases ofconstitutive lipase production by bacteria (Elwan *et al*., 1983; Gao *et al*., 2000) and they are non-specific in their choice of substrate with a few being thermostable (Odu and Akujobi, 2012). Such features are responsible for lipases being extensively used for biotechnological applications in the food technology, dairy industry, cosmetics, textile and detergent industries (Fariha *et al.*, 2006). In the food industry, lipases play a vital role during the fermentative steps of sausage manufacture and to determine changes in long-chain fatty acids liberated during ripening. Lipases remain enzymatically active in organic solvents (Verma *et al*., 2008; Klibanov *et al*., 2001) which enhances their potential and flexibility as biocatalysts against a wide range of unnatural substrates (Ghori *et al*., 2010). Also, they are known to catalyze other reactions like esterification, trans-esterification and inter-esterification between a fatty acid and an alcohol which are the reverse reactions of hydrolysis (Leonov, 2010) making them the most widely used biocatalysts in organic chemistry (Jaeger and Eggert, 2002).In addition to lipases, esterases are also grouped into hydrolases and these two enzymes can often be confused with each other but they are really different from each other in terms of substrate specificity (Zhang *et al*., 2008).

Lipases have been observed to be monomeric proteins, with molecular weight in the range of 19-60 kDa. Generally, bacterial lipases have neutral or alkaline pH value and show activity in a broad pH range (pH 4 to pH 11) (Vakhlu *et al*., 2006). The thermal stability of lipases range from 20ºC to 60ºC (Litthauer *et al*., 2002; Gutarra *et al*., 2009).Lipase activity depends on the presence of large surface area and requires mild to extreme conditions, with stability

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in organic solvents a desirable property in synthetic reactions. Most known bacterial lipases are stable in organic solvents (Gupta *et al*., 2004). The ability of lipases to remain stable in organic solvent without any stabilizer has been found to be in favour of some reactions (Zhao *et al*., 2008) highlighting various biotechnological applications of lipases such as the synthesis of chirally important drugs and drug intermediates (Singh *et al*., 2010a). Reactions catalyzed by lipases usually occur in organic-aqueous interfaces which is desirable because this ensures the separation of enzyme from substrates or products easily (Rahman *et al*., 2005) thus a reduction in cost during downstream processing, which is a highly desired feature in bioprocess engineering.

Palm Oil Mill Effluent (POME) is a highly voluminous liquid waste with high content of carbohydrates, protein, nitrogenous compounds, lipids and minerals, which in turn releases unpleasant smell (Ahmad *et al*., 2003). The high organic content in POME makes it possible to reuse the effluent for biotechnological purposes such as a fermentative medium for lipase production. Especially as oil wastes are considered serious pollutants when discharged into aquatic bodies (Ibegbulam-Njoku *et al*., 2014).This study aimed at production, characterization and purification of lipase from bacteria isolated from Palm Oil Mill Effluent.

2.0 Materials and Methods

Sample collection

Palm oil mill effluents (POME) and soil samples from palm oil mill processing sites were collected from Igbo Elerin and Onidundun (Lagelu and Akinyele Local Governments) in Ibadan metropolis of Oyo State, Nigeria.

Isolation of Lipolytic bacteria

Lipolytic bacteria was isolated from the palm oil mill effluent and soil samples using a chemically defined medium composed of 2.0g/l of castor oil, NaCl 5.0g/l, Calcium Chloride 0.1g/l, peptone 10.0g/l, Agar 20.0g/l, and Congo red 0.2g/l as indicator (Loo *et al*., 2006).

Production of lipase

The five best lipolytic bacteria were selected for lipase production according to a modified method of Sirisha *et al*., (2010) using a medium composed of 100ml(12.8g Na_2HPO_4, 3g KH_2PO_4, 0.5g NaCl, 2mM $MgSO_4.7H_2O$, 10g NH_4Cl, 2g glucose and 10ml castor oil)g/l in a 250ml Erlenmeyer flask. Production was carried out at 37°C on a shaker at 150rpm.

Assay for lipase activity

Lipase activity was measured by titrimetric method using castor oil as substrate according to the method of Kambiz and others (2008).Castor oil emulsion used was prepared by mixing 10% (w/v) gum Arabic solution in water, 25% (v/v) castor oil and 75% (v/v) of the 10% (w/v) gum Arabic solution in water was used in preparing the castor oil emulsion. Cell-free supernatant was obtained by centrifuging at 10,000rpm for 15minutes at 4^0C. The reaction mixture (1ml of 0.02M phosphate buffer (pH 7), 0.5ml of 0.03M Calcium chloride solution, 0.5ml of castor oil emulsion and 0.5ml of enzyme solution) was incubated at 37^0C for 40minutes. The reaction was stopped and fatty acids were extracted by addition of 10ml of acetone: ethanol (1:1). The amounts of fatty acids liberated were calculated by titrating with 0.05M NaOH solution using phenolphthalein indicator. A blank was prepared by incubating the reagent mixture of the above composition without the enzyme solution following the same procedure as described above. One unit of enzyme is defined as the amount of enzyme required to hydrolyze 1µmol of fatty acids from triglycerides (Gombert *et al*., 1999).

Effect of incubation period on lipase activity

Isolates were inoculated into production medium broth containing 12.8g Na_2HPO_4, 3g KH_2PO_4, 0.5g NaCl, 2mM $MgSO_4.7H_2O$, 10g NH_4Cl, 2g Glucose and 10ml castor oil at 37^0C in an orbital shaker at agitation speed of 150rpm. The culture broth was harvested on a 24 hourly basis for 120hours. Enzyme assay was carried out using standard assay procedure as described above.

Effect of different inducers on lipase activity

Castor oil present in the production medium was replaced with different oils such as palm kernel oil, cod liver oil and Olive oil at a final concentration of 1% (w/v) while keeping the other parameters constant. Enzyme assay was carried out using standard assay procedure.

Effect of different carbon sources on lipase activity

Glucose present as carbon source in the production medium was replaced with different carbon sources like sucrose, starch, maltose and lactose at 1% (w/v) final concentration by keeping the other parameters constant. Enzyme assay was carried out using standard assay procedure.

Effect of different nitrogen sources on lipase activity

Different nitrogen sources (peptone, yeast extract, KNO_3, $NaNO_3$ and NH_4Cl) were added to the production medium broth at a final concentration of 1% (w/v) by keeping the other parameters constant. Enzyme assay was carried out using standard assay procedure.

Effect of pH on lipase activity

The optimum pH for the production of lipase was determined by varying the pH of the production medium broth, ranging from pH 6 to pH 8 while the

other parameters are unaltered. Enzyme assay was carried out using standard assay procedure.

Effect of agitation on lipase activity

In the determination of the optimal agitation speeds for peak enzyme activity, the isolates were cultured in an orbital shaker at varying agitation speed from 120 to 250rpm. Enzyme assay was carried out using standard assay procedure as described.

Effect of incubation temperatures on lipase activity

The optimum temperature for the production of lipase was determined by incubating the culture production medium broth at varying temperatures (30^0C to 50^0C) while keeping the other parameters constant. Enzyme assay was carried out using standard assay procedure as described above.

Characterization of Lipase Enzyme Produced

Effect of temperature on the activity and stability of the enzymes

The effect of temperature on activity and stability of the enzymes were carried out by incubating the enzyme mixture at various temperatures of 30^0C, 40^0C, 50^0C, 60^0C, 70^0C and 80^0C, for one hour and assaying for their activity by standard assay procedure using castor oil as substrate (Benattouche *et al*., 2012).

Effect of pH on the activity and stability of the enzymes

Citrate-phosphate buffer was used to prepare assay medium for pH 4, 5, 6 and 7, while Tris-HCl buffer was used to prepare assay medium for pH 8, 9 and 10. 1ml of each enzyme and substrate was mixed and then incubated at temperature of 30^0C for one hour according to the method of Benattouche *et al*., (2012). The residual lipolytic activities were then determined using castor oil as substrate.

Effect of metal ions and inhibitors on the activity of the enzymes produced

The effects of different metal ions on the activity of the enzymes were examined on the purified enzyme by preincubating with metal salts ($CoCl_2$, $BaSO_4$, $MnSO_4$, $CuSO_4$, $CaCl_2$) and inhibitors Ethylenediaminetetraacetic acid (EDTA) and Sodium dodecyl sulphate (SDS) for 1hour at 30°C. The activity was determined using castor oil as substrate. Each metal salts were used at a concentration of 1mM. Enzyme activity was then checked by standard assay procedure.

Effect of substrates concentration on the activity of the enzymes produced

Various concentrations (5%, 10%, 15%, 25% and 30% (v/v)) of castor oil was prepared in water with gum arabic and 0.2M sodium phosphate buffer at pH 7.0. They were used for enzyme assay without increasing the volume of the enzyme. Enzyme activity was then checked by standard assay procedure.

Purification of the Lipase Enzyme

Enzyme purification was done using Ammonium sulphate, enzyme dialysis and Sephadex G-100 column chromatography. The crude enzyme extract was precipitated out in a salting out process (Dixon and Webb, 1971). This was done using two $(NH_4)_2SO_4$purification steps. 40% saturated solution of ammonium sulphate $(NH_4)_2SO_4$was used to remove hybrid proteins from the fermentation broth by adding the salt. It was then continuously stirred for 15minutes using a magnetic stirrer, the mixture was kept at 4^0C for 24hours and later centrifuged at 4000rpm for 5minutes. The precipitate obtained was dissolved in citrate phosphate buffer. The supernatant was treated with 80% ammonium sulphate and then centrifuged. The precipitates were then pooled together. The pooled protein precipitate from the first and second ammonium sulphate precipitation steps was dialyzed using citrate phosphate buffer solution (pH 7.5) to remove $(NH_4)_2SO_4$.The precipitate was dispensed into dialysis tube, well tightened at both ends to create turgor pressure in the tube and submerged in citrate phosphate buffer pH 7.5 for 48hours in refrigerated condition. This was done to remove the ammonium sulphate salt (Ding *et al*., 2012).The buffer solution was changed until clear solution was obtained. The desalted enzyme solution (dialysate) was applied to a Sephadex gel G-100. Fast Flow column pre-equilibrated with pH 7.5 citrate phosphate buffer solution was used. The column was washed with the buffer and the absorbed proteins were eluted using 0.2mol/L in citrate phosphate buffer at a flow rate of 0.1ml/min (Ding *et al*., 2012).The active fractions that contained lipase enzyme were pooled, dialysed and assessed for protein content. The resulting enzyme was utilized for the characterization of the extracellular lipase.

Activity of the purified Lipase enzyme

Lipase activity and protein content of the enzyme was determined at each stage of the purification (that is crude extract, ammonium sulphate precipitated, dialyzed and the column purified enzymes) so as to determine the effect of purification on the activity of the enzyme. The lipase activity was carried out according to standard procedure as modified by the method of Kambiz *et al*., (2008).

The total protein content was determined by the method of Lowry *et al*. (1951) using Bovine Serum Albumin (BSA) as standard (1mg/ml).

3.0 Results and Discussions

From the serially diluted soil and palm oil mill effluent samples, 15 bacterial isolates were selected based on their zones of clearance (qualitative screening) on selective agar based medium. They were further screened quantitatively for their lipolytic activities and five (5) strains with highest activities were selected for further use. The selected strains were characterized

based on their morphological, biochemical and physiological qualities and identified as *Bacillus subtilis, Serratia marcescens, Staphylococcus aureus, Bacillus species and Pseudomonas aeruginosa.* Reports have shown isolation and purification of lipases from fungi, yeast, bacteria, plant and animal sources, of which bacterial lipases are more economical and stable (Joseph *et al*., 2007).

Effect of different incubation periods on lipase activity

The highest lipolytic activity (5.52U/mL) was recorded with *Staphylococcus aureus* followed by *Pseudomonas aeruginosa* (4.90U/ml), *Serratia marcescens* (4.70U/ml), *Bacillus* spp (4.52U/ml) and the least enzyme activity of 3.55U/mL was observed for *Bacillus subtilis* at 48hr incubation time (Figure 1). Lowest activities for the isolates occurred at 24hours except for *Staphylococcus aureus* which had its lowest activity (2.90U/ml) at 72hours. Higher lipolytic activities observed at 48hr was similar to the work of Sirisha *et al*.(2010) who reported that bacteria lipases are best produced at the early stationary phase but disagrees with the work of Kumar *et al*. (2012).

Effect of different oil inducers on lipase activity

From figure 2,palm oil induced the highest lipase activity for *Bacillus subtilis* and *Bacillus* sp. which is similar to the report of Sirisha *et al*., (2010) while *Pseudomonas aeruginosa* and *Staphylococcus aureus* had their highest activity with Olive oil and *Serratia marcescens* with cod liver oil. Also lowest lipase activity was recorded using castor oil as inducer, which is similar to the report of Glogauer *et al*., (2011) who reported that this could be as a result of the unusual hydroxyl functional group on the twelfth carbon of the ricinoleic acid.

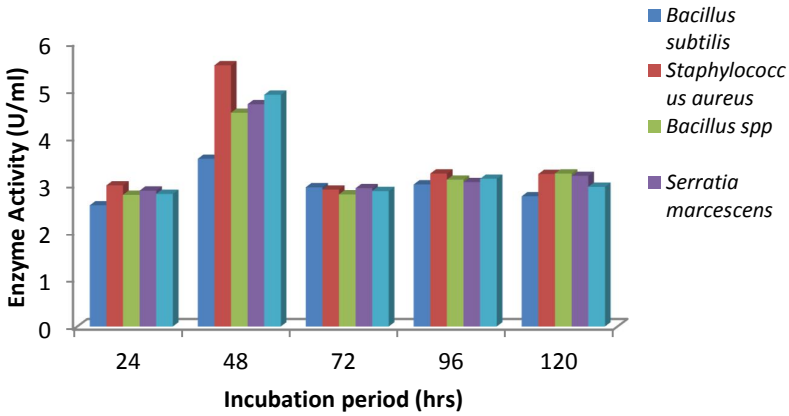


Figure 1: Effect of different incubation periods on lipase activity

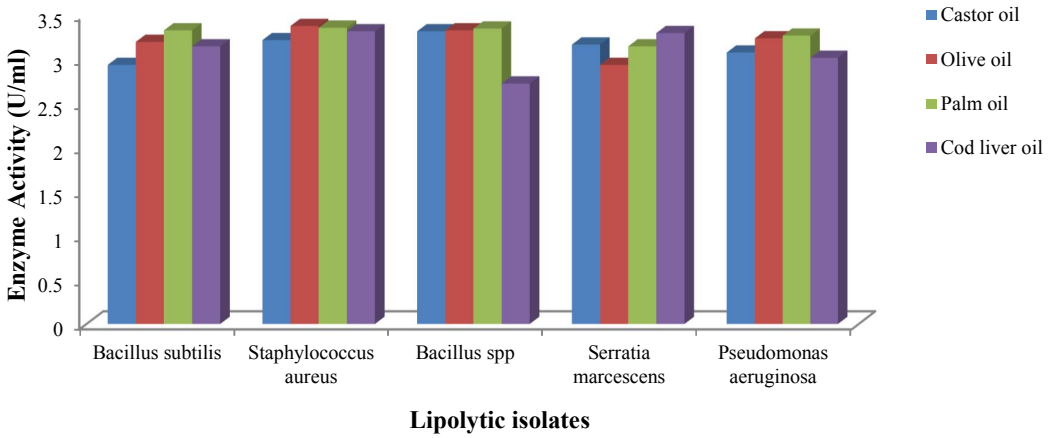


Figure 2: Effect of different inducers on lipolytic activity

3.3 Effect of different carbon sources on lipase activities

Figure 3 shows the effect of different carbon sources on lipase activities, from the figure, *Pseudomonas aeruginosa* (5.65U/ml) and *Bacillus* spp (3.56U/ml) had highest lipase activities using lactose as carbon sources which agrees with the work of Kumar *et al.,* (2012) while highest activities was observed with sucrose for *Bacillus subtilis* (3.69U/ml), maltose for *Serratia marcescens* (4.24U/ml) and glucose for *Staphylococcus aureus* (6.34U/ml) agrees with the work of Lakshmi *et al*. (1999).Although high activity was observed for all the isolates with starch as carbon source but was not the best, an observation similar to the report of Pallari *et al*., (2009) who reported that medium containing starch does not favour the production of lipase enzyme.

3.4 Effect of nitrogen sources on lipase activities

Peptone as source of nitrogen gave the highest enzyme activity for all the isolates except for *Bacillus subtilis* and *Staphylococcus aureus* which had theirs with yeast extract (Figure 4) an observation similar to that of some researchers (Sirisha *et al*., 2010; Kumar *et al*., 2012).Least lipase activities for *Bacillus subtilis, Staphylococcus aureus* and *Serratia marcescens* was recorded with ammonium chloride. *Bacillus* species and *Pseudomonas aeruginosa* had lowest activity of lipase produced with $NaNO_3$ which is in contrast to the report of Sharma *et al*., (2002).

3.5 Effect of different pH on lipase activities

The effect of the medium pH on lipase activity is as shown on Figure 5, *Bacillus subtilis*, *Staphylococcus aureus*, *Bacillus* spp and *Pseudomonas aeruginosa* had their highest lipase activity at pH 7.5 except for *Serratia marcescens* which had its highest activity at pH 8.0. This observation was similar to the results obtained by Glogauer *et al*., (2011) and Odu and Akujobi (2012) and also with the work of some researchers who reported optimum lipase activity at alkaline pH (Prazeres *et al*., 2006; Yuan *et al*., 2010).

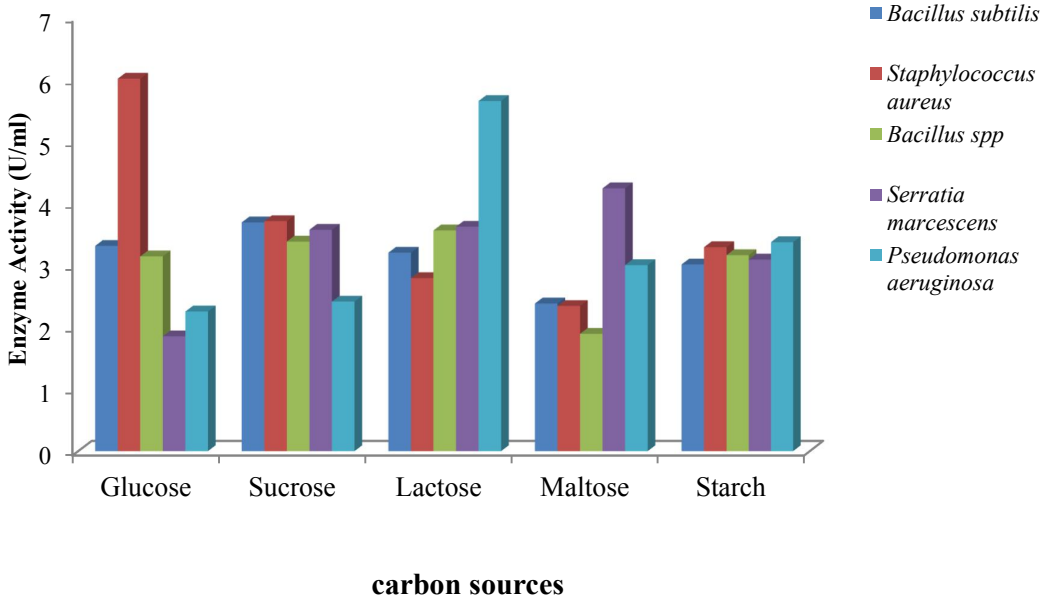


Figure 3: Effect of various carbon sources on the lipolytic activity

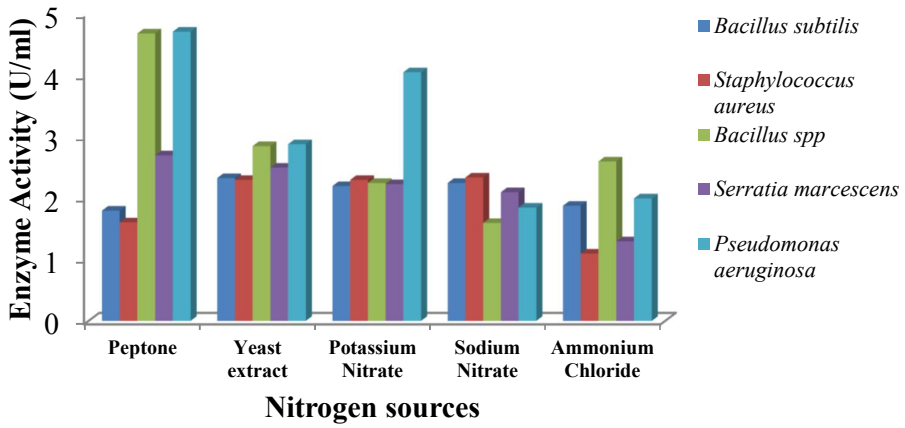


Figure 4: Effect of various Nitrogen sources on lipase Activity

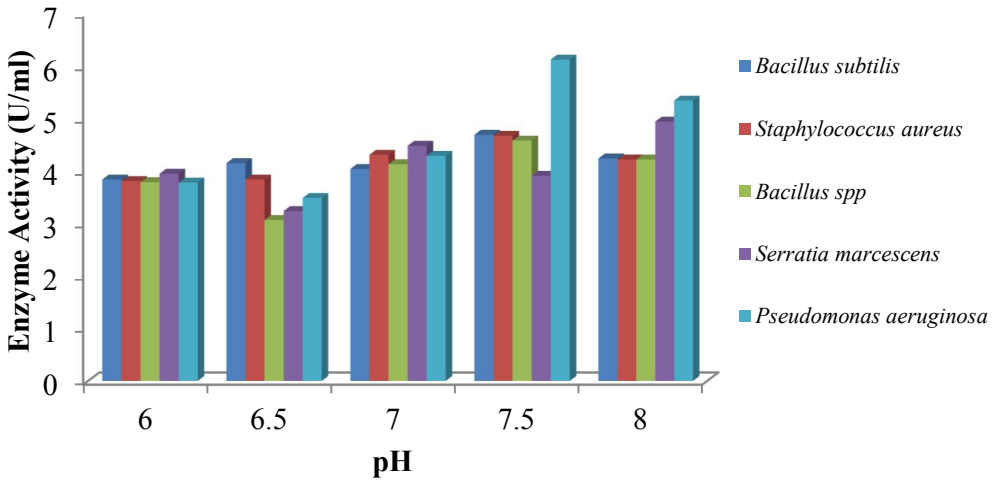


Figure 5: Effect of different pH on the lipase activity

3.6 Effect of different agitation rate (rpm) on lipase activity

Highest lipase activity was observed at an agitation speed of 150rpm for all the isolates (Figure 6) which is similar to the report of Sirisha *et al*., (2010) and Veerapagu *et al*., (2013) that agitation rates of 150rpm best supports lipase production. Review of literatures by Gupta *et al*., (2004) also reported that *Bacillus* species best produced lipase at lower agitation rates. Optimum activity by all isolates was observed at 250rpm.

3.7 Effect of different incubation temperatures on lipase activity

Activities of the lipase enzyme produced by all the isolates increases till 45^0C and decreases by 50^0C.Highest lipase activity was observed at 45^0C for all the isolates, an observation similar to the work of Guzman *et al*., (2008) and Senthilkumar *et al*. (2012) who reported an optimum temperature for lipase activity between 35-45 ^{0}C but is contrary to the works of some researchers (Mohan *et al*., 2008; Sirisha *et al*.,2010; Kumar *et al*., 2012) who reported maximum lipase activity at 35^0C.

3.8 Effect of different pH conditions on Lipase stability

The stability of the lipase enzymes increased with increased pH (Figure 8). The broad pH range observed is similar to the report of Vakhlu and others (2006). The lipases were found to be stable at higher pH conditions, which indicate an alkaline enzyme, an observation similar to the work of Singh *et al*. (2010b) and Glogauer *et al*., (2011) who reported maximum lipase activity and stability at pH 11. Most lipases are alkaline in nature and alkaline lipases are said to be promising catalysts for many industrial processes (Ghori *et al*., 2010).

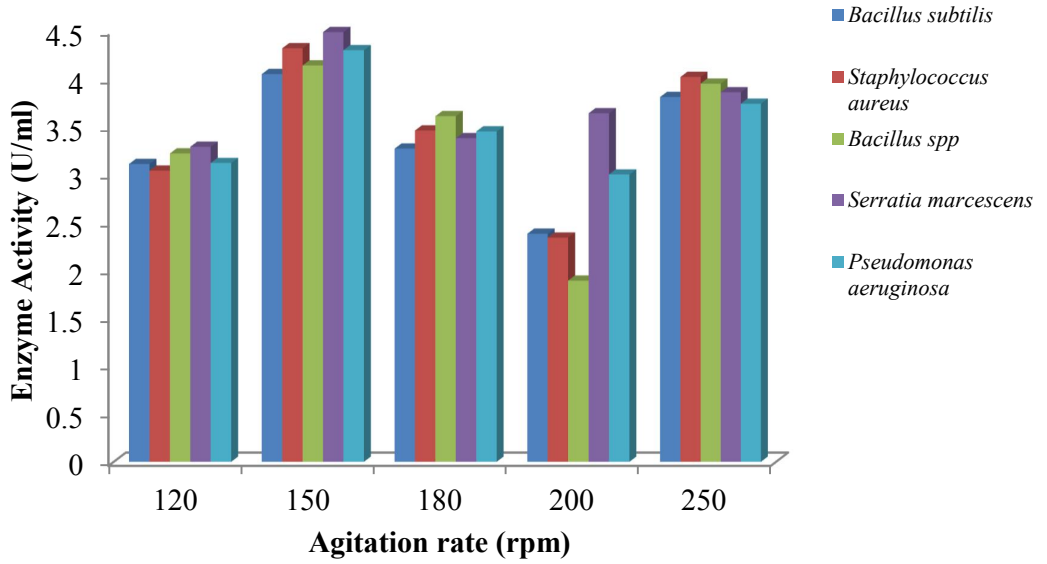


Figure 6: Effect of different agitation rate (rpm) on lipase activity

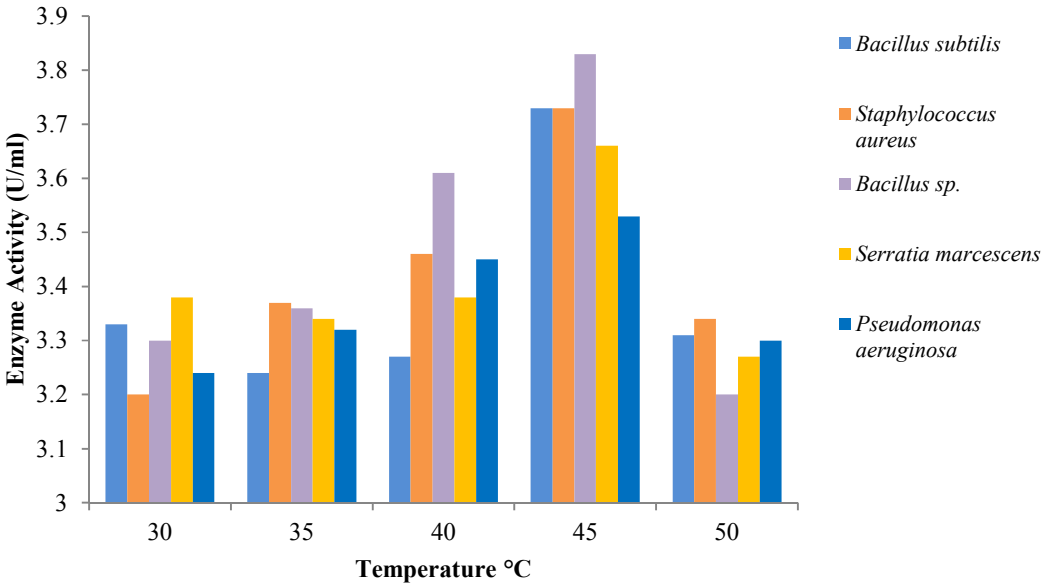


Figure 7: Effect of different incubation temperatures on lipase activity

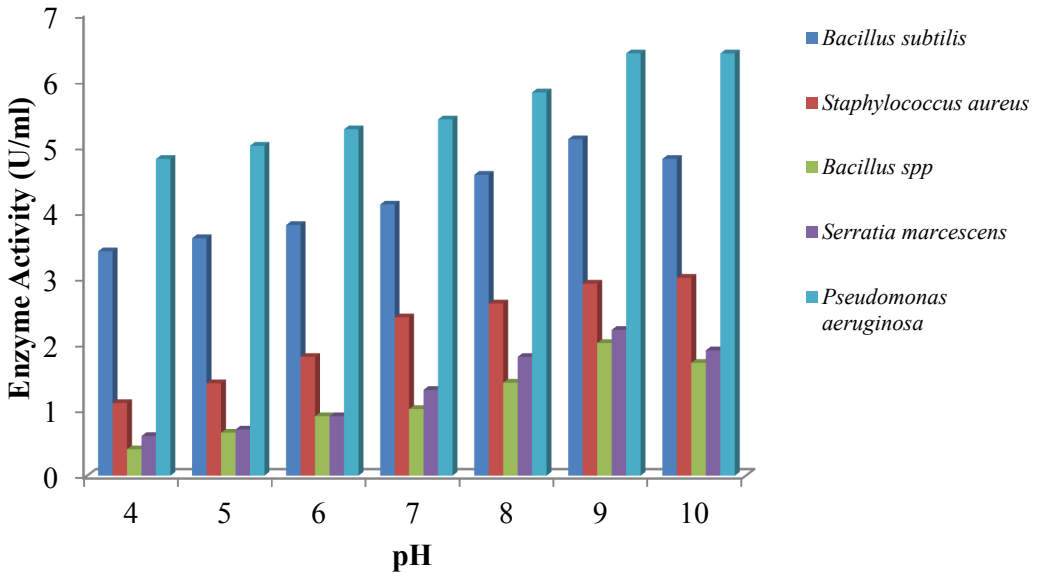


Figure 8: Effect of different pH on Lipase stability

3.9 Effect of different temperatures on lipase stability

The results from this study showed that the lipases remained stable at different temperatures between 30^{0}C to 70^{0}C, observation similar to that of Litthauer *et al*. (2002) and Gutarra *et al*. (2009) (Figure 9).*Bacillus subtilis* had its highest activity at 70^{0}C which reduced with further increase in temperature, this observation is similar to the reports of Wang *et al*., (1995) and Glogauer *et al*., (2011) who reported lipase stability at 70^{0}C. Higher activity was observed at 60^{0}C for *Bacillus* spp and *Serratia marcescens* suggesting that they could be thermostable enzymes and is in agreement with the reports by Nawani *et al*. (2006) and Odu and Akujobi (2012).Thermostability is a desirable characteristic in lipases for application in different industrial processes operating at high temperatures (Janssen *et al*., 1994). *Staphylococcus aureus* and *Pseudomonas aeruginosa* had their highest activity at 50°C and 30°C respectively which is similar to the reports of Joseph *et al*. (2007) which reduced with further increase in temperature. Reduction in activity at higher temperatures observed could be due to the denaturation of the enzyme at high temperatures.

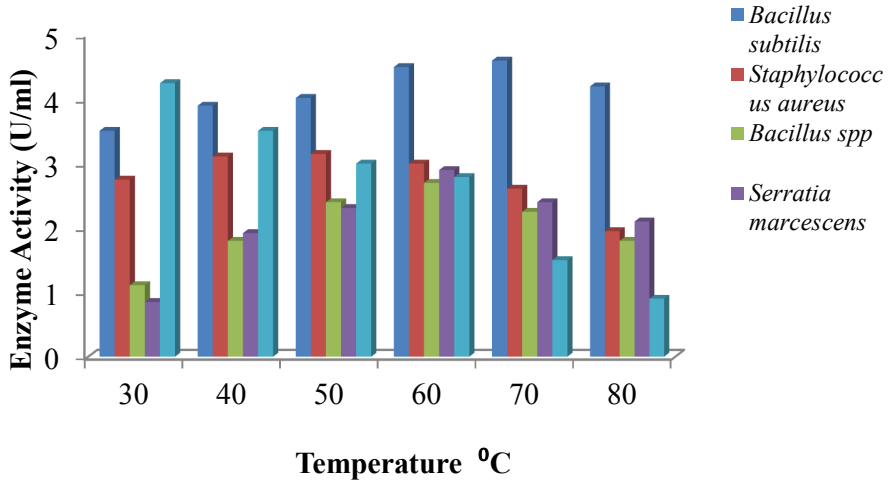


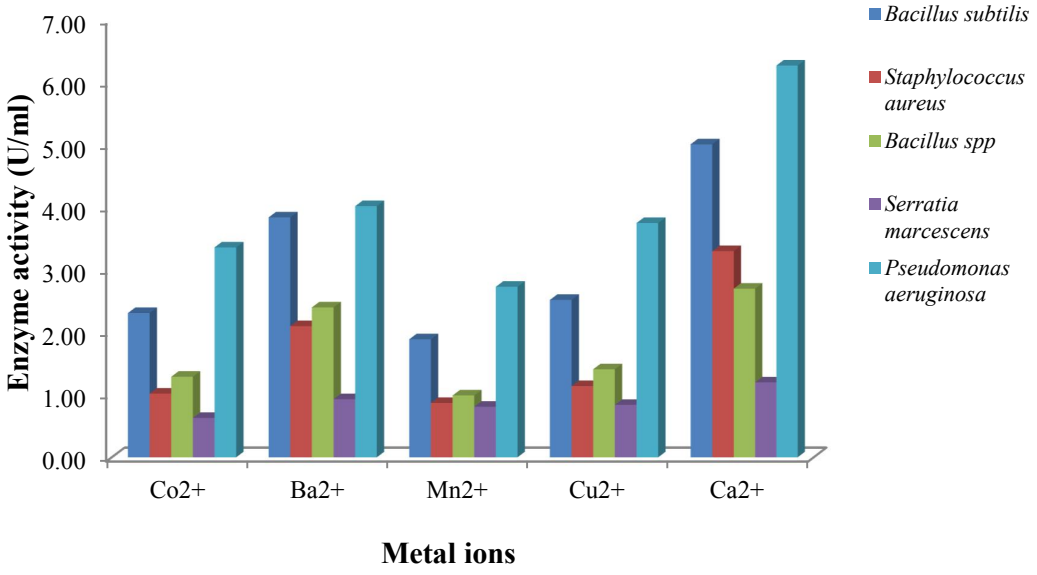
Figure 9: Effect of different temperatures on Lipase stability

Effect of metal ions on lipase stability

The effect of metal salts on the lipase enzyme stability is as shown on Figure 10. Ca^{2+}, Ba^{2+}, Cu^{2+} and Co^{2+} stimulated the activity of lipase produced by most isolates, an observation similar to the reports of Chakraborty *et al*. (2008). Ahmed and others (2009) also reported that lipases could be metallo-dependent and that metal ions have a stimulatory effect on the enzymes. The highest lipase activity observed with Ca^{2+}agrees with the work of Glogauer *et al*. (2011) and hence lipases are called Ca^{2+} dependent metallo-enzyme (Lee *et al*., 2003).

3.11 Effect of enzyme inhibitors on lipase activity

The effect of Sodium Dodecyl Sulphate (SDS) and Ethylenediaminetetraacetic acid (EDTA) on the activity of the lipase produced by the isolates is as shown on Figure 11. Sodium Dodecyl Sulphate (SDS) was found to have an inhibitory effect on the lipases which agrees with the work of Quyen *et al*., (2003) and Mobarak-Qamsari *et al*., (2011) that reported a total loss of activity in the presence of SDS but disagrees with the work of Gogoi and Dutta (2009) which reported that SDS exhibited stimulatory effect on lipases. Also Ethylenediaminetetraacetic acid (EDTA) was observed to inhibit the activity of *Bacillus* spp, *Staphylococcus aureus* and *Serratia marcescens*, which agrees with the report of Glogauer *et al*., (2011) where activity decreases in the presence of the chelating agents EDTA, although this does not agree with the findings of Mase *et al*., (1995) where EDTA had no effect on the lipase



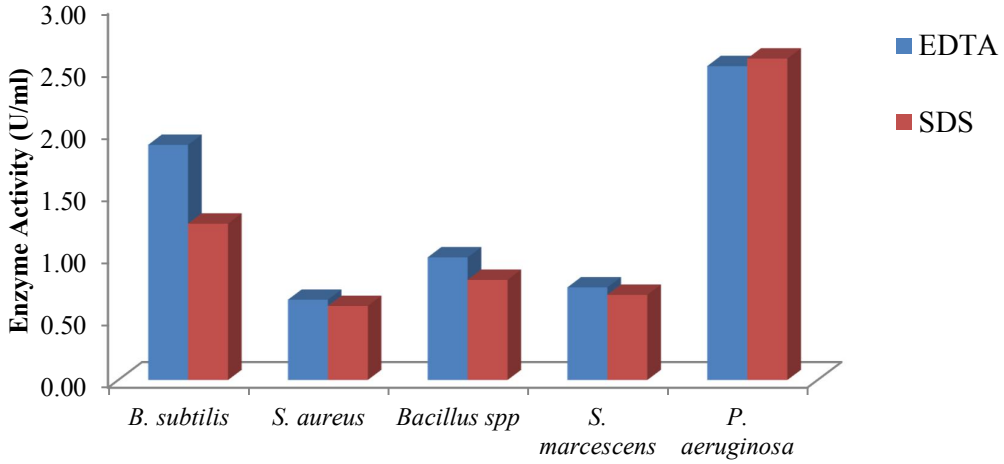


Figure 11: Effect of EDTA and SDS on lipase activity

Purification profile of the lipase enzyme

The extracellular lipase activities of each isolate achieved at optimal conditions was used in production. The cell free supernatants were precipitated by $(NH_4)_2SO_4$ after centrifugation followed by dialysis. Total protein content reduced with each purification step for all the isolates except for *Bacillus subtilis*. Total enzyme activity reduced at each purification stage. The purification fold was higher at the chromatography stage for *Bacillus subtilis* (1.623) and *Staphylococcus aureus* (0.984), while it was higher at the dialysis stage for *Bacillus* spp (0.939), *Serratia marcescens* (0.859) and *Pseudomonas aeruginosa* (1.171). Lipase activity and the total protein content of the enzyme at each purification stage differed significantly ($P<0.05$) within each isolate. Also a yield of 81.45% at the dialysis stage was observed for *Bacillus subtilis*, 82% at ammonium sulphate purification stage for *Staphylococcus aureus*, 67.69% at chromatography stage for *Bacillus* spp, 68.42% at chromatography stage for *Serratia marcescens* and 82.96% at dialysis stage for *Pseudomonas aeruginosa*. Highest lipase yield was recorded with the precipitated enzyme of *Staphylococcus aureus* (82.03%) and the dialysate of *Bacillus subtilis* (81.45%) and *Pseudomonas aeruginosa* (82.96%). The dialysate of the bacterial lipase wee further purified by column chromatography, the stage at which highest yield was observed for *Bacillus* sp. (67.69%) and *Serratia marcescens* (68.42%).

The total protein contents, total enzyme activity and specific activity reduce with each purification step for all the isolates.

Table 1: Analysis of purification profile of lipase enzyme by bacteria isolates

Isolates	Purification steps	Total protein (mg/ml)	Total enzyme activity (U/ml)	Specific activity (U/ml)	Purification fold	Yield (%)
Bacillus subtilis	Crude extract	0.222(0.00)^{d}	20.10(0.30)^{c}	90.54	1.000	100
	$(NH_4)_2SO_4$	0.192(0.00)^{c}	12.41(0.11)^{b}	64.58	0.713	61.69
	Dialysis	0.177(0.01)^{b}	10.10(0.39)^{a}	57.06	0.884	81.45
	Chromatography	0.161(0.01)^{a}	13.50(0.30)^{b}	46.29	1.623	65.33
Staphylococcus	Crude extract	0.196(0.01)c	18.49(0.35)^{d}	94.38	1.000	100

aureus						
	$(NH_4)_2SO_4$	0.135(0.00)[b]	10.80(0.15)[c]	80.00	0.848	82.03
	Dialysis	0.119(0.02)[b]	7.50(0.30)[b]	74.26	0.928	56.00
	Chromatography	0.078(0.05)[a]	5.70(0.15)[a]	73.08	0.984	68.43
Bacillus spp	Crude extract	0.193(0.00)[d]	14.28(0.18)[d]	73.99	1.000	100
	$(NH_4)_2SO_4$	0.187(0.00)[c]	6.41(0.11)[c]	34.41	0.465	23.13
	Dialysis	0.133(0.00)[b]	4.31(0.11)[b]	32.33	0.939	51.16
	Chromatography	0.113(0.00)[a]	3.26(0.15)[a]	29.02	0.897	67.69
Serratia marcescens	Crude extract	0.195(0.01)[c]	11.21(0.05)[d]	57.73	1.000	100
	$(NH_4)_2SO_4$	0.173(0.00)[b]	6.00(0.12)[c]	34.68	0.601	13,33
	Dialysis	0.152(0.00)[a]	4.50(0.00)[b]	29.80	0.859	66.67
	Chromatography	0.137(0.00)[a]	3.42(0.12)[a]	24.96	0.837	68.42
Pseudomonas aeruginosa	Crude extract	0.285(0.00)[d]	22.26(0.24)[d]	78.10	1.000	100
	$(NH_4)_2SO_4$	0.256(0.00)[c]	15.81(0.21)[c]	61.96	0.793	59.11
	Dialysis	0.186(0.00)[b]	13.50(0.30)[b]	72.58	1.171	82.96
	Chromatography	0.151(0.00)[a]	10.26(0.06)[a]	67.95	0.941	68.29

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