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Influence of Environmental Parameters on Lipase Production by *Serratia Marcesens*.

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Abstract: *Lipase-producing bacteria were isolated from the soil in Ohuhu Community in Umuahia North Local Government Area of Abia State, The crude extra cellular lipases from cell-free culture supernatant were reacted in an olive oil mixture and Rhodamine B dye and their lipolytic activities examined. 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. Morphological, biochemical and molecular characterization of the isolate identified it as Serratia marcescens. Various physicochemical parameters that were investigated such as pH, temperature ranges, incubation time and rates of agitation were investigated in a shake flask culture and it revealed that pH 6 and Temperature of 40^0C at 150 rpm for 48 hrs gave optimal lipase activity. It could be inferred that at pH of 6.0 and temperature of 40 °C for 48hrs optimum lipase production by Serratia marcescens can be maximized.*

Keywords: *Lipase, Rhodamine B agar, Serratia marcescens, orange fluorescence, UV light*

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I. Introduction

Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) are one of the most important classes of industrial enzymes. They hydrolyse triglycerides into diglycerides, monoglycerides, glycerol and fatty acids. In recent years, there has been an increasing interest in the study of lipases mainly due to their potential applications as medicines (digestive enzymes), food additives (flavour modifying enzymes), clinical reagents (glyceride-hydrolysing enzymes) and cleaners (detergent additives.Additionally, a promising application field for lipases is in the biodegradation of plastics such as polyhydroxyalkanoates (PHA) and polycaprolactone (PCL) (Jager *et al*., 1995; Mochizuki *et al*., 1995). A large number of beneficial thermophiles which produced lipases with good thermal stabilities have been found in diverse habitats (Wang *et al*., 1995). Their thermal stabilities, particularly in the absence of appreciable amounts of water, increases their attractiveness industrially. Thermostable lipases have a lot of applications in the processing of food, domestic, industrial wastes, cosmetic, detergents and pharmaceutical industries (Ghosh *et al.* 1996; Saxena *et al*. 1999). Thermophilic enzymes are sought after due to their stability towards higher temperatures. Also, their high optimal activity temperatures are associated to remarkable processing benefits, since the higher solubility, decreased viscosity and increased diffusion rates allow the use of higher substrate concentrations and usually lead to higher reaction rates. Also, as the reaction temperatures are generally higher than optimal growth temperature of mesophilic organisms, there is a lower risk of contamination.

II. 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. NA 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*. (2007), 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. The assay mixture consisted of 1.0ml of the substrate emulsion (70ml emulsifying reagent with 30ml olive oil homogenized for 5 minutes using a Vortex mixer). 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). 0.2ml of the enzyme substrate mixture was incubated at 37^{0}C for 30min. The reaction was terminated by adding 2ml acetone-ethanol mixture (1:1 v/v). The amount of fatty acid liberated was determined by titration with 0.01N NaOH using phenolphthalein as indicator. 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. All experiments were run in triplicate sets and the mean values were presented. The values were analyzed using analysis of variance (ANOVA).

The quantity of fatty acids liberated in each subsample based on the equivalents of NaOH used to reach the titration end point was calculated using the following equation:

µmol fatty acid/ml subsample =[(ml NaOH for sample − ml NaOH for blank) × *N* × 1000]/5 ml where *N* is the normality of the NaOH titrant used (0.01 in this case).

Measurement of Initial and Final pH

The initial and final pH of the medium was determined using a pH meter before and after each fermentation.

Effect of pH on lipase activity

The effect of pH on the enzymatic activity of the lipase fraction was determined using a wide range of buffers, including 1M citrate phosphate (pH 3.0-7.0), 1M sodium phosphate (6.0-7.0), 1M Tris-HCL (pH 8.0) and 1M glycine-NaOH(pH 9-10) as described by Anjana *et al*. (2009).

Effect of Temperature on lipase activity

The effect of temperature on lipase activity was determined by assaying the enzyme activity at different temperatures ranging from 30^{0}C-80^{0}C at pH 9, as described by Anjana *et al*. (2009).

Effect of static and agitation condition on lipase production

To evaluate the effect of static and agitated condition on lipase production, five sets (in duplicates) of lipase production medium was prepared. In all the sets, the other conditions *e.g.* pH were kept constant. One set of the medium was kept without agitation (static), while the other sets were kept at agitation rates; 50rpm 100rpm, 150rpm,200rpm and 250rpm. Fermentation was carried out as earlier described. After fermentation for 72hrs at 50^{0}C, cell-free filtrates were obtained and assayed for lipase activity.

3.16 Effect of incubation time on lipase activity

The amount of lipase produced or the maximum lipase activity was monitored after every 12hr till 154hr (12, 24, 48, 60, 72, 96,120 and 154).

III. Results

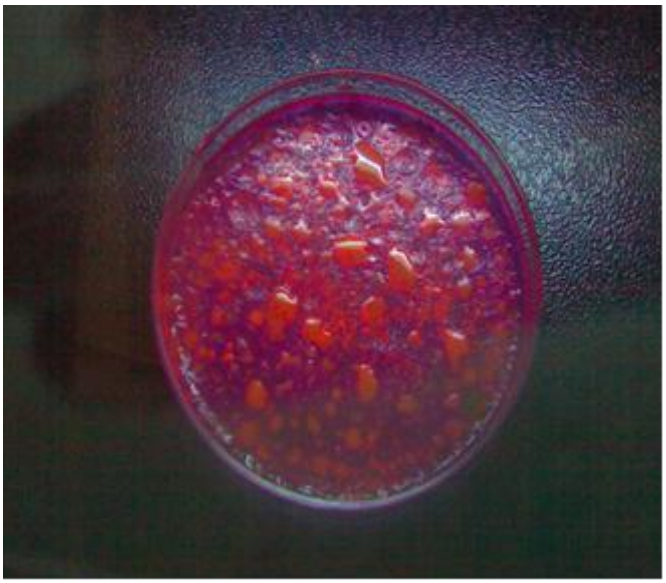

PLATE1: Showing Orange Fluorescence Colonies Of Isolate On Rhodamine B Agar Under Uv Irradiation.

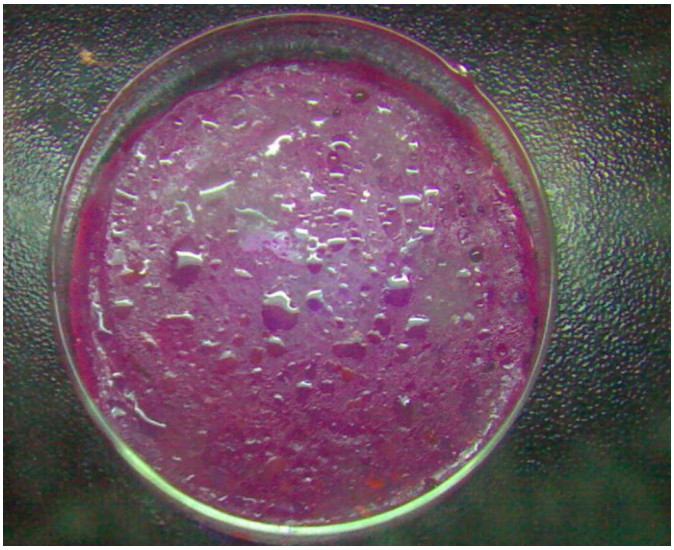

PLATE 2: Showing No Visible Orange Fluorescence Colonies Under Uv Irradiation

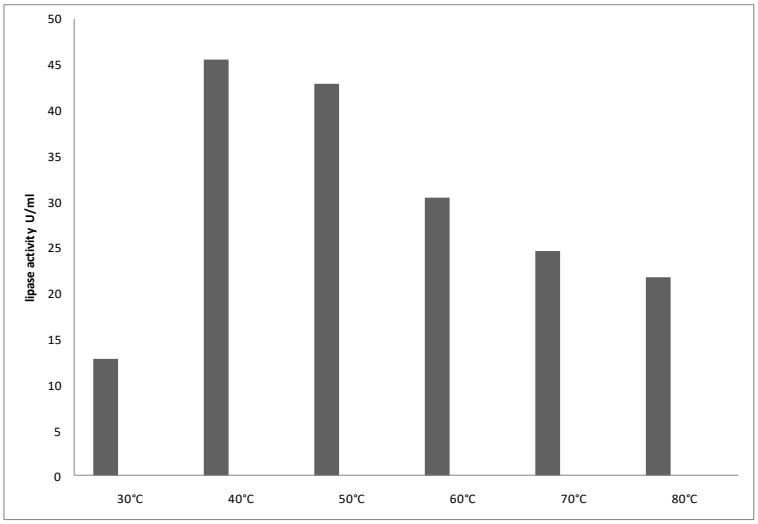

Fig 1: Effect of temperature ranges on lipase activity

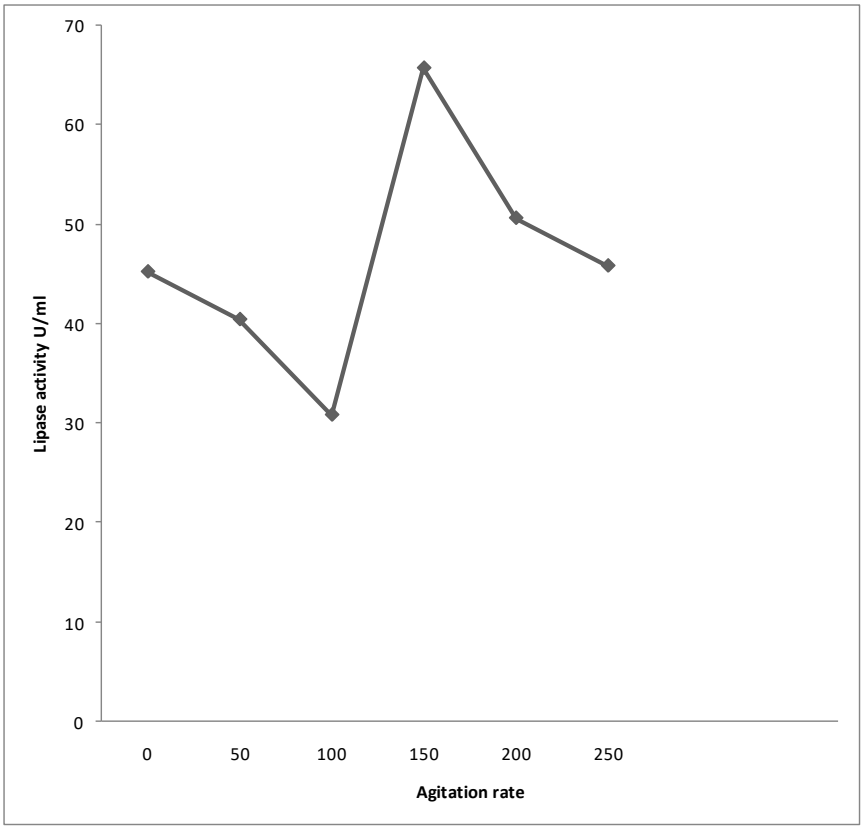

Fig 2: Effect of Agitation Rates on Lipase Activity.

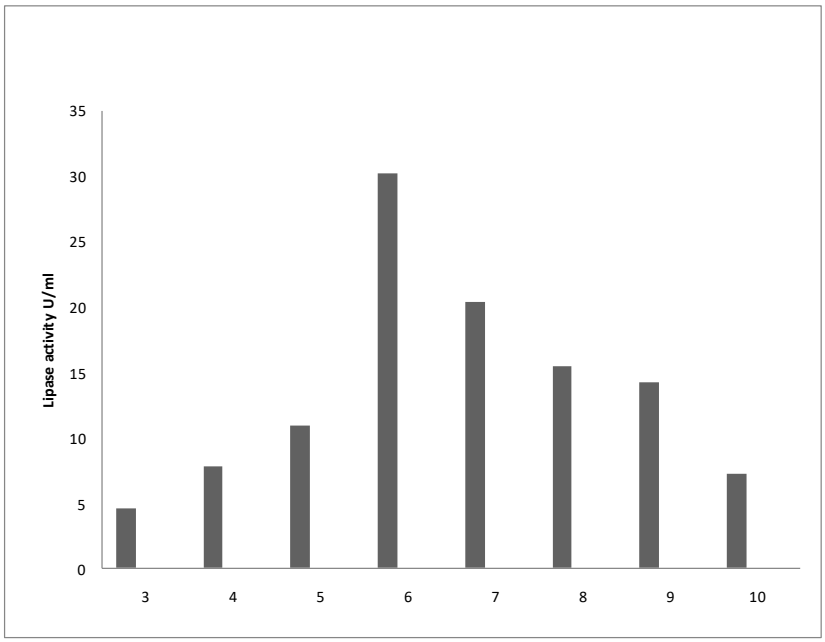

Fig 3: Effect of pH on Lipase Activity

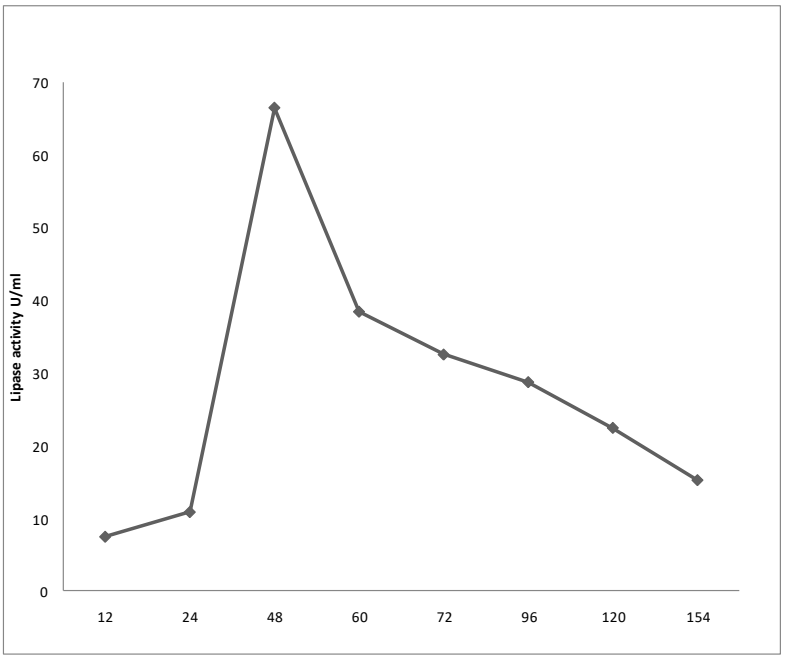

Fig 4: Effect of Incubation Time (hours) on Lipase Activity

❖ Values are mean values of triplicate determination

IV. Discussion

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

In the present study, lipase activity showed gradual increase in temperature from 30^{o}C- 40^{o}C and further increase of temperature beyond 40^{o}C, the production decreased. This is in agreement with the work reported by Gryzyna *et al*.,(2007). Thus the action of protease may be responsible for the remarkable thermal stability of the crude enzyme. This may be ascribed to the poor stability of extracellular lipase, the release of more protease from dead cells, and higher protease reaction rate at higher temperature (Kulkarni *et al.*,2002). Statistical analysis by one-way ANOVA test for the data on lipase activity as a function of medium temperature change indicated a highly significant variation ($p < 0.05$).

The pH of the medium is important for lipase production. Lipase activity and growth is affected by the pH of the medium. Enzyme is very sensitive to the operating pH medium because it might change the ionization states of the enzyme, which affect its activity and selectivity. Molecular charges and consequently molecular interactions and functions are directly related to medium pH (Dheeman *et al*., 2010); thus any change in medium affects any biological functions (Moon & Parulekar, 1991). The results of lipase production at increasing pH showed decreasing lipase across the pH gradient with the highest production found at pH 6.0; followed by pH 7.0 and the least at pH 3.0. This shows that the lipase produced by this organism is an acidic lipase. The ability of organisms to produce slightly acidic lipase has been reported by Pogaku *et al*.,(2010) and Rajesh *et al.*,(2010). There is an indication that proteases which are known to be secreted from dead cells may be more active at pH greater than 7.00 according to Hong *et al.*,(2002). One-way ANOVA test conducted to obtain the data on lipase production as a function of medium pH revealed a highly significant variation ($p < 0.05$). Determination of the effect of agitation of production media, were kept in static and different shaking conditions. At static conditions lipase activity was much more lower than that of subsequent 50rpm - 100rpm agitation. Optimum shaking conditions required for lipase production was found to be 150rpm. At 200rpm shaking condition, lipase production was slightly decreased this maybe due to cell disruption and resulting in the release of intracellular enzymes such as protease and esterase that can digest lipase enzymes (Rajesh *et al,* 2010). Shaking speeds, 100 and 50rpm seemed to disrupt the microaerophilic or near aerobic conditions required for maximum lipase production. This reveals the ability of the organism to produce lipase enzyme maximally at high increase in the availability of oxygen.

The amount of lipase produced was observed after every 12 h till 60 h. The maximum lipase activity was observed after 48 h of fermentation listed in Table 2. After that, although the bacterial growth rate

went on increasing but the specific growth rate decreased. After 48 h, the growth showed divergence from the exponential because in place of homogeneous growth, bacterial pellets began to form in which nutrients and oxygen supply became the growth limiting. After that lipase yield got reduced due to the consumption of nutrient materials. This is in agreement with the findings of Manoj *et al*., (2010) while working on lipase Production by *Bacillus subtilis* OCR-4 in Solid State Fermentation Using Ground Nut Oil Cakes as Substrate

V. Conclusion

The present study revealed that extracellular lipase production by *Serratia marcescens* isolated from soil samples was found to be accelerated at optimized culture conditions such as medium pH, temperature, rate of agitation and time of incubation. From the results, it could be concluded that the medium pH of 6.0 and temperature of 40 °C were optimum for maximizing lipase production by *Serratia marcescens*. It also revealed an effective method to isolate and identify a new microorganism capable to grow and produce lipase at low and moderate temperatures. 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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