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Lipase Production by *Bacillus cereus* Isolated from Palm Oil Mill Effluent (POME) Polluted Soil.

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ABSTRACT

Hydrolytic enzymes occupied major industrial share such as esterases, lipases, amylases and proteases. Applications of these enzymes are varied in medical, cosmetics, food, dairy, pharmaceutical, leather, detergents, bioremediations and paper industries. So, lipases are gaining more attention now a day. The present study investigated the effects of culture conditions on lipase production by pure culture of Bacillus species isolated from palm oil mill effluent polluted soil. A total of 28 different lipolytic microorganisms were isolated from soil and POME samples. Micoorganisms producing lipases were selected on the basis of clear zone formation in rhodamine B agar medium. One of these strains that expressed the highest relative activity, was biochemically identified as *Bacillus cereus.* The fermentation growth kinetic indicated that the maximum extracellular lipase activity was at 48h of cultivation.

Lipase activity in the presence of lipidic substrates increased in the following order of olive oil > soybean oil = cottonseed oil > palm oil. Palm oil at 1.5 % of concentration was found to be the most suitable concentration to increase lipase production (43,68 micromol/min/ml). The highest lipase activity was observed at an optimum temperature and pH of 25°C and 5.0, respectively.

Keywords: POME, Lipase, *Bacillus cereus*, polluted soil.

INTRODUCTION

Lipases (Triacylglycerol lipases, EC 3.1.1.3) are water soluble enzymes which have the ability to hydrolyse triacylglycerols to release free fatty acids and glycerol. Lipases have been isolated and purified from fungi, yeast, bacteria, plant and animal sources. Of all these, bacterial lipases are more economical and stable [18]**.** Lipases constitute a major group of biocatalysts that have immense biotechnological applications in food processing, organic chemical processing, pharmaceuticals, cosmetics, paper manufacture, detergent formulations and in environmental management [10].

Microbial lipases are of wide interest because they are highly selective, stable and substrate specific. The substrate and reaction specificities of lipases surpass those of any other known enzyme and the application potentials are limitless [2]**.** The use of microorganisms producing lipase or microbial process to detoxify and degrade oil effluents is one of the recent innovative technologies gaining grounds in the last two decades. The oily environment such as palm oil mill effluent may provide a good environment for isolation of lipase producing microorganisms. Bioremediation techniques depend on having the right microbes in the right place with the right environmental conditions for degradation to occur [14]. The right microbes are those bacteria or fungi, which have the physiological and metabolic capabilities to degrade the contaminants. In many instances, these organisms will already be present at the site (indigenous microbes). Once the right microorganisms are present in the right place, the environmental conditions must be controlled or altered to optimize the growth and metabolic activity of the microbes. Bacterial lipases are mostly extracellular being excreted through the external membrane into the culture medium and are greatly influenced by nutritional and physicochemical factors which include: pH, nitrogen and carbon sources, inorganic salts, dissolved oxygen concentration, temperature**,** etc.. [13]. The aim of the present work was to determine some optimum parameters favorable for the growth and lipase production of an extracellular lipase from *Bacillus cereus* isolated from palm oil contaminated sites in littoral region of Cameroun.

Materials and methods

Sample Collection :

Samples of soils and POME were collected from Palm Oil Mill Effluent Polluted Soil of Specialised Oil Palm Research Station and Artisanal Palm Oil Industry situated in the littoral region of cameroon.

Isolation of Lipolytic Bacteria and screening

Soil samples were serially diluted and plated onto minimal medium containing 0.5% (m/v) peptone; 0.02% (m/v) $MgSO_4$; 0.3%(m/v) NaCl; 0.1% (m/v) KH_2PO_4; 0.5% (v/v) olive oil, 2% agar, pH 8 by spread plate method. Plates were incubated at 30°C for 24hours. Pure cultures of the isolates were maintained on minimal media agar and were subcultured every 15 days. [5]

Screening of the Isolates for Lipase Activity :

Lipolytic microrganisms were screened by qualitative plate assay. Isolates were grown on minimal medium containing 0,05% (m/v) of rhodamine B plates and incubated at 30°C for 2 days. Zone of clearance was observed due to hydrolysis of olive oil [5].

Culturing and Characterization of the Isolates :

The isolate showing maximum zone of clearance here is selected for further analysis. Macroscopic and microscopic characteristics of the isolate have been studied for the identification of the isolate. Following macroscopic (cultural characteristics) and microscopic (gram and morphology) examinations, the isolates were carefully purified by successive subculturing on nutrient agar for possible biochemical identification of the purified species. The classification criterion based on Gram staining was used to guide the choice of the biochemical identification gallery to be used next. Thus, the isolate was identified using the API50CH (Biomereux, France) gallery [8]. The gallery kits were used according to the manufacturer's recommendations.

Lipase Activity Assay

Extracellular lipase activity was measured by hydrolysis of p-nitrophenyl palmitate (pNPP). The reaction mixture in the hydrolysis experiment of p-nitrophenyl palmitate contained 100 µl of solution A (0.603 g of p-nitrophenyl palmitate in 100 ml of 2-propanol, sonicated for 2 min before use), 900 µl of solution B (0.4 % triton X-100 and 0.1 % gum arabic in 50 mM Tris-HCl, pH 8.0), and 100 µl of culture supernatant. The product was detected at a wavelength of 410 nm after 5 mins of incubation at 30 °C. One unit of lipase activity was defined as the quantity of enzyme required to release 1 µmol of pNP [12].

Inoculum preparation

Bacterial suspensions were prepared to measure their optical densities at a wavelength of 600 nm adjusted to 0.5Mc Farland, which corresponds to a bacterial population of 10^6 to 10^8 CFU whose absorption at a wavelength of 600 nm is between 0.08 and 0.10 [9].

Lipase production kinetics of isolated strain

The growth study of the isolated bacterial strains was carried out in 250 mL flasks containing 25 mL of minimum liquid medium consisting of : 0.5% (m/v) peptone; 0.02% (m/v) $MgSO_4$; 0.3%(m/v) NaCl; 0.1% (m/v) KH_2PO_4, 0,5 % (v/v) oil palm 0.5% (v/v) Tween 80. Incubation was carried out at 30° C for 72 h. For optical density measurements, using a spectrophotometer, 2 mL of fermentation culture was sampled every 8h for 72h. Cells were then separated from the supernatant by centrifugation at 10,000rpm for 30 min, washed twice with 1 mL of 0.85% NaCl solution [20]. The supernatant collected was used as crude enzyme solution and was assayed for enzyme activity.

Effects of Lipidic Substrates

The effect of lipid substrates on the biosynthesis of lipase by the test organism was carried out as described by Gupta *et al* [7] with little modification. Palm oil present in minimum liquid medium was substituted by different oils like olive oil, soybean oil and cottonseed oil. The sterile medium contained in 250 ml Erlenmeyer flasks was then inoculated with the test organism after which the flasks were incubated (30°C) on a rotary shaker (150 rpm) for 48 h. Thereafter, different concentrations (0.5%, 0.75%, 1%, 1.25%, 1.5%) of the palm oil were studied to determine the optimum concentration for maximum lipase production. Cells were removed by centrifugation at 10,000rpm for 30 mins and cell free supernatant obtained was used directly as crude enzyme to determine the lipase activity as described earlier. [6].

Effect of Temperature

The optimum temperature for lipase production was determined by incubating the isolate in the basal medium at 25°C, 30°C, 35°C, 40°C, 45°C with constant agitation. The supernatants obtained were assayed for lipase activity [3].

Effect of pH

The effect of pH on the extracellular production of lipase in the culture medium was determined in the pH value range from 3-11. Citrate buffer was used for pH values of 3.0 and 5.0 ; phosphate buffer was used for pH values of 7.0 and 11.0, and Tris- HCl buffer was used for pH 9.0. The medium was sterilized at 121°C for 15 mins after which it was inoculated with the test isolate and incubated on a rotary shaker (150 rpm) for 48 h. The supernatant obtained after incubation was used to determine the lipase activity. [3].

Statistical analysis

GraphPad v8.03 software (GraphPad PRISM, San Diego, California, USA) was used for statistical analysis. At least three repetitions were used for the statistical treatment of the data. The data are expressed as mean values ; error bars indicate the standard error.

Results

Screening, Isolation and Identification of Lipolytic Bacteria :

Six different soils samples and Two different POME samples collected from different areas show 28 pure isolates. 15 of them had lipase activity,and the best activity was obtained with isolate D17(**Fig.1**). The colony labelled as D17 showed maximum zone of clearance when plated on synthetic medium based on rhodamine B. The addition of morphological characteristics(**Tab. I**), like positive motility, Gram-positive staining and the presence of spore-forming organisms, enabled isolate D17 to be assigned to the genus *Bacillus*. The identification of the fermentation of major sugars (**Tab. II**) for the identification of *Bacillus* using the 50CH Kit confirmed the biochemical identification of the species *Bacillus cereus.*

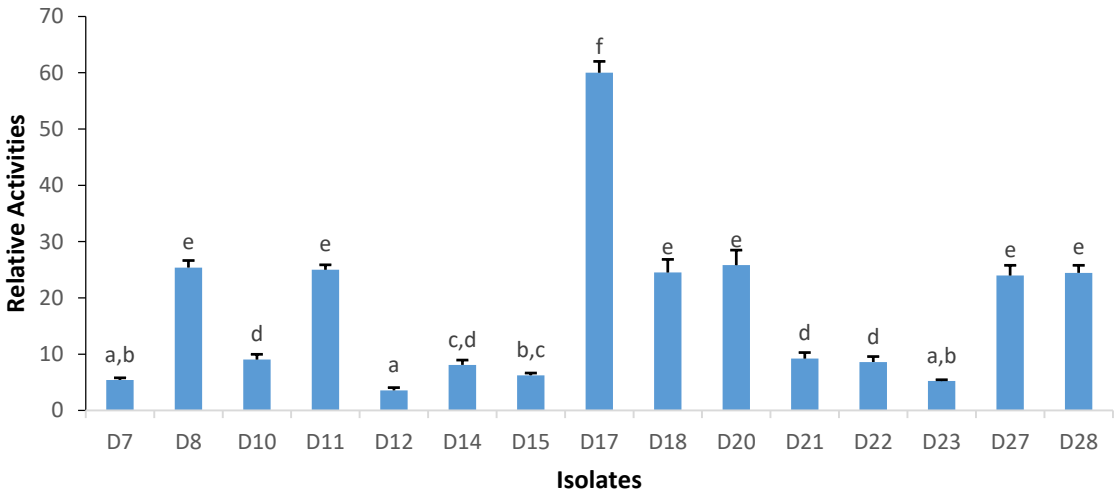

Figure 1 : Relative activities of isolates on agar-olive oil-Rhodamine B medium

Table I : Identification on the bases of morphological characteristics

	D17
Macroscopics characters	Dry whitish colonies, with irregular edges, opaque, bulging, Diameter : 1 to 2 mm
Microscopics characters	Form: Bacillus Bacterial type : Gram-positive Type of grouping: isolated or in pairs Mobility: positive Presence of spores : yes

Table II : Results of 49 biochemical tests of D17 isolate on the bases of API50CH Kit.

0.	Control	-	10. D-galactose	-	20. methyl-α-D-mannopyranoside	-	30. D-melibiose	-	40. D-turanose	-
1.	Glycerol	+	11. D-glucose	+	21. methyl-α-D-glucopyranoside	-	31. D-saccharose	+	41. D-lyxose	-
2.	Erythritol	-	12. D-fructose	+	22. N-acetylglucosamine	+	32. D-trehalose	+	42. D-tagatose	-
3.	D-arabinose	-	13. D-mannose	-	23. Amygdaline	-	33. Inulin	-	43. D-fucose	-
4.	L-arabinose	-	14. L-sorbose	-	24. Arbutine	+	34. D-melezitose	-	44. L-fucose	-
5.	D-ribose	+	15. L-rhamnose	-	25. Esculine	+	35. D-raffinose	-	45. D-arabitol	-
6.	D-xylose	-	16. Dulcitol	-	26. Salicine	+	36. Amidon	+	46. L-arabitol	-
7.	L-xylose	-	17. Inositol	-	27. D-cellobiose	+	37. Glycogen	+	47. Gluconate	-
8.	D-adonitol	-	18. D-mannitol	-	28. D-maltose	+	38. Xylitol	-	48. 2-ketogluconate	-
9.	Methyl-β-D-xylo-pyranoside	-	19. D-sorbitol	-	29. D-lactose	-	39. Gentiobiose	-	49. 5-ketogluconate	-

+ : positive test, - : negative test

Effect of incubation period on Lipase Production

The D17 (*Bacillus cereus*) was isolated and inoculated in minimum liquid medium and was harvested at 8 hours interval. Maximum enzyme activity was observed at 48 hours at the staionary state(**Fig.2**) and may be due to the release of intracellular lipase into the medium due to death and lyses of bacterial cells [2].

The activity of the enzyme gradually decreased after 48 hours. The fall in enzyme activity may be as a result of exhaustion of nutrients in substrate which resulted in the inactivation of

enzyme [14]. Kais *et al*. [11] in their study reported that it might be due to the absorption of the enzyme by the substrate or by the proteolytic activity. The result obtained with the test isolate is not in corroboration with the results obtained by Nwanyanwu *et al*. [14] in their works as they found that lipase production by *Bacillus* sp. isolated from Palm Oil Mill Effluent polluted soil was maximum at 72 h.

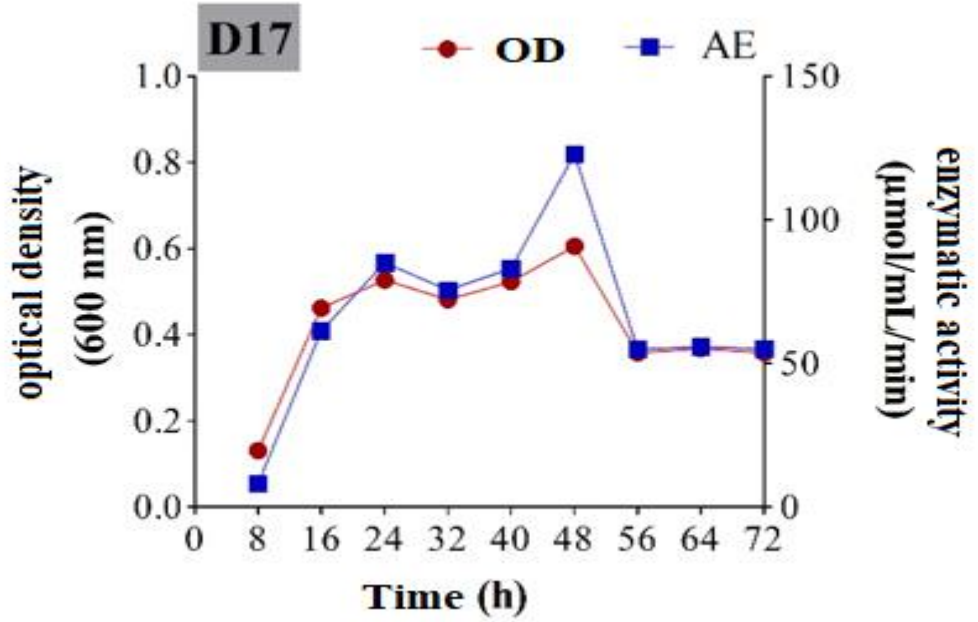

Figure 2: Effect of incubation time on lipase production

Effect of triglycerides on lipase production

Lipases are induced mostly in presence of oils in culture medium. Four different lipid sources were tested to study their effects on lipase production (**Fig.3).** All the lipid substrates enhanced lipase production with particularly high values being obtained with olive oil. This is in line with the work of Fickers *et al*. [4]. with *Bacillus sp -H-91*, *Y. lypolytica* , Abdel-Fattah [1].with *Bacillus sp*., Zouaoui *et al.* [12] with *Pseudomonas aeruginas* who found olive oil to be the best lipase inducer. Salwoom *et al* [17] found that palm oil, an economical and widely available natural triglyceride, generated similar lipase activity. Similar studies conducted on lipase production by *Bacillus* sp., showed maximum yield in olive oil [21]. Various studies revealed that olive oil is more effective to induce production of lipase, due to lipid content in olive oil, which is structurally easily digested by microorganisms [10].

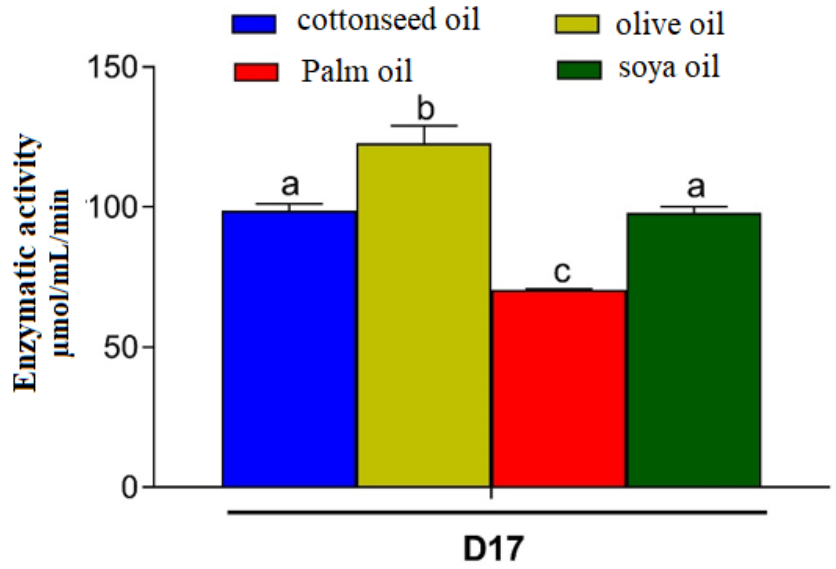

Figure 3 : Effect of triglycerides on lipase production

Effect of different concentrations of palm oil

The effect of different concentrations of palm oil on lipase production showed an increase (10,88-43,68 micromol/min/ml) as the concentration of olive oil increased from 0.5 to 1.5% (**Fig.4**). These results correlate with those obtained by Pandey *et al*. [15]., who showed that *Candida rugosa* lipase production increased proportionally with increasing olive oil concentration.

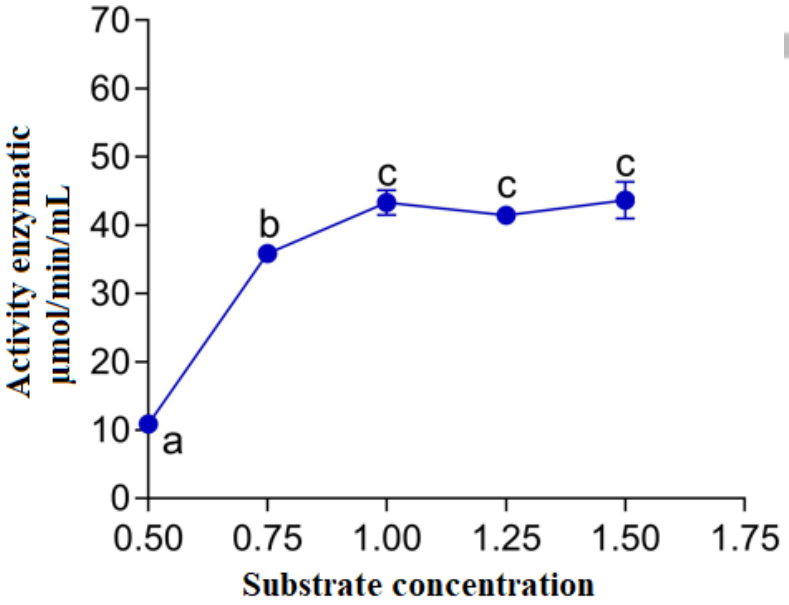

Figure 4 : Effect of substrate concentration on lipase production

Effect of pH on lipase production

Effect of pH on enzyme activity was shown in **Fig. 5**. Maximum production of lipase was obtained at pH 5. The lowest lipase activity was observed in pH value of 7 with lipase activity of 5,02 ± 0.1 µmol/min/mL. This may be as a result of changes in the optimum external pH

which may alter the ionization of nutrient molecules of the medium hence reduced their availability to the organism [19]. The highest lipase activity obtained in this work at pH of 5 is different to the result obtained by Prasad [16] in his work at pH of 7.0 in his assessment of production of extracellular lipase by *Serratia marcescens* isolated from industrial effluent.

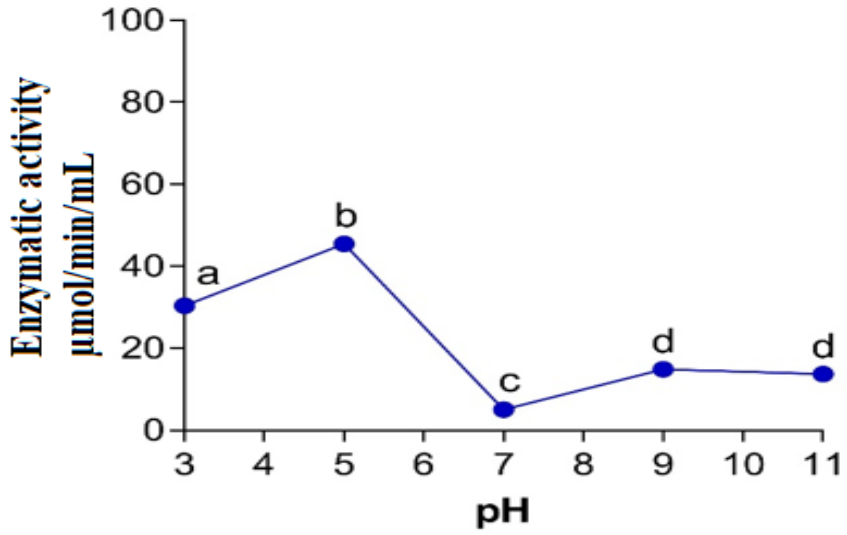

Figure 5 : Effect of PH on lipase production

Effect of Temperature on lipase production

Production of lipase at different temperatures (25 - 50°C) was observed for 48 hrs. Lipase activity was maximum at 25°C. Similarly, a psychrophilic lipase from the Bacillus F19L strain shows a peak in enzymatic activity at 10°C. On the other hand, Joyruth and Growther [10], found maximum lipase production at 40°C for *Bacillus sp., Serratia sp., Stenotrophomonas sp., Halomonas sp., Enterobacter sp*. The temperature for optimal lipase production varies according to the bacterial species and depends on the environmental conditions of the bacteria in question [12].

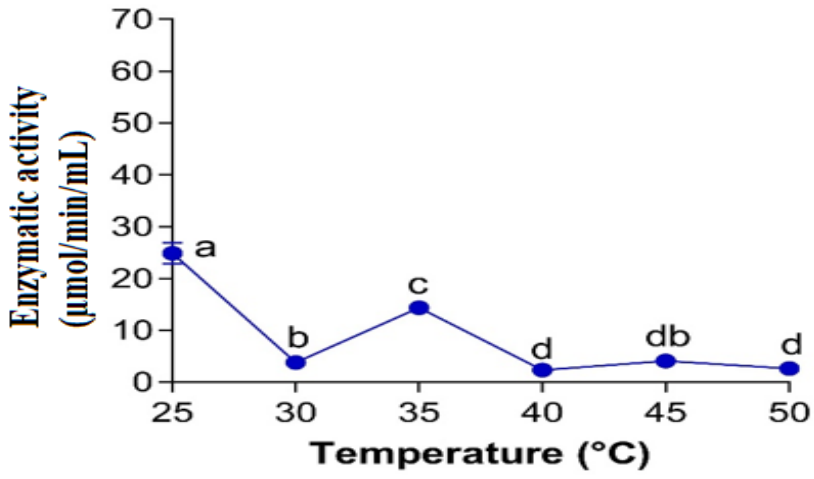

Figure 6 : Effect of temperature on lipase production

Conclusion

Lipase is one of the most valuable industrial enzymes. In present study, lipase producing organisms are isolated from soil and POME sample. 28 microorganisms were isolated with D17, identified as Bacillus cereus as the best purifyers due to its best activity. It was optimized with various physico–chemical parameters for lipase production. It was observed that culture conditions greatly influenced lipase production. These results suggest that *Bacillus cereus* could be used as a potential industrial microbe in the future for the treatment of POME.

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