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Screening for Lipase Enzyme Producing Potentials of *Bacillus* Species Isolated from Different Automobile Workshops in Anambra State

Chikaodili Chinezie Ubajekwe[1], E. N. Chukwura[2], S. N. Dimejesi[2], B. O. Uba[2], L. Eleanya[1], J. N. Ezendianafo[2] and N. A. Dibua[1].

[1]Department of Microbiology, Chukwuemeka Odumegwu Ojukwu University, P.M.B.02 Uli, Anambra State, Nigeria.
[2]Department of Microbiology, Faculty of Natural and Applied Sciences, Tansian University, Umunya, Anambra State, Nigeria.

*Corresponding authors e-mail: chinezieubajekwe@gmail.com; Tel.: +234(0)8034281442

Abstract

Lipases are versatile biocatalysts and are used in different bioconversion reactions. Microbial lipases are currently attracting a great amount of attention due to the rapid advancement of enzyme technology and its practical application in a variety of industrial processes. The aim of the present study was to screen for lipase enzyme producing potentials of *Bacillus* species isolated different automobile workshops in Anambra State. In study, soil samples were collected on six different locations with history of oil contamination namely Awka, Nnewi and Onitsha representing the three senatorial zones of Anambra State. Fourteen bacilli strains were isolated from oil contaminated soil samples using Nutrient Agar after heat treated at 80 °C for 15 min prior to serial dilution. The result showed that Onitsha sampling location point B had the highest lipase bacterial count of 23.90 X 10^4 CFU/g while Awka sampling location point A had the least count of 1.80 X 10^4 CFU/g, respectively. Tributylin phenol red test was carried out to determine primary lipase activity and subsequent secondary lipase activity was determined using Basal medium with tributyrin (1%). The results revealed that the crude enzyme extracts of three strains AWK1, NW1 and ON3 out of 14 lipolytic bacterial strains had the highest protein content and enzyme activities of 1.29, 1.40, 1.77 mg/mL and 39.18, 40.59, 28.80 U/mL, respectively and applied for further practical applications. These selected bacterial strains were later characterized and identified physiologically and found to be *Bacillus* sp. AWK1, *Bacillus thuringiensis* NW1 and *Bacillus tropicus* ON2 using Bergey's Manual for Determinative Bacteriology, respectively. The isolation and lipase activities of these strains suggest that the automobile workshops soil samples are great reservoirs lipase producers.

Keywords: *Anambra State, Automobile workshop, Bacillus, Alkaline Lipase, Soil*

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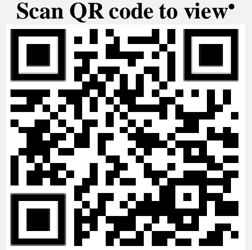

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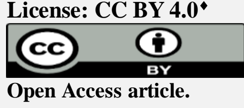

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Introduction

The industrial enzyme producers sell enzymes for a wide variety of applications. The most widely used industrial enzymes include protease, lipase, cellulase and amylase that remove soils based on proteins, lipids, and polysaccharides. The estimated value of world market is presently about US$ 2.7 billion and is estimated to increase by 4 % annually through 2012 (Deb *et al*., 2013). Nowadays, the use of enzymes in detergents is widespread in developed countries and more than half of the detergents contain enzymes. Many laundry detergent products contain at least an enzyme or a mixture of enzymes including proteases, lipases, amylases, cellulases, mannanases and pectinases (Gürkök, 2019).

Lipases (EC 3.1.1.3) are serine hydrolases that break down long chain fatty acid esters of glycerol at the water-oil interface. They constitute the third largest group of industrial

enzymes after proteases and carbohydrates (Niyonzima and Moore, 2015). Lipases are occupying a place of prominence among biocatalysts owning to their ability to catalyze a wide variety of reactions and are an important group of biotechnologically relevant enzymes and they find massive applications (Mohammed, 2013). Lipases are carboxylic ester hydrolases currently attracting an enormous attention due to the fact that they are the most versatile and widely used enzyme in biotechnological applications and owing to their unique properties (Alhamdani and Alkabbi, 2016). The stability at alkaline pH and a broad temperature range are required for any detergent lipase (Niyonzima and Moore, 2015). The important source of lipases is microorganisms as they secrete bulk quantity of enzymes (Niyonzima and Moore, 2015). Lipases are produced by microorganisms (bacteria and fungi), plants and animals. However, microbial lipase especially from bacteria are more useful than their plant and animal origin; since they have great variety of catalytic activities and microorganisms are easy to manipulate genetically and capable of rapid growth on inexpensive media (Veerapagu *et al*., 2014; Yan *et al*., 2014). The microbial lipases are commercially most important mainly because they are secreted into the culture medium by many of microbial species that belong to bacteria, fungi, yeasts and actinomyces (Alhamdani and Alkabbi, 2016).

Compared to the chemical methods that need harsh conditions such as high pressure and temperature, using of microorganism is considered in many purposes including heavy metal absorption, gene engineering, digestion, production of novel anti-microbes and particularly for producing of industrial enzymes (Fari *et al*., 2020). As a result, the exploration of diverse sources for isolation of microbes particularly *Bacillus* species with lipase enzyme producing potentials becomes necessary and hence form the thrust of this present study. The present study was undertaken to screen for lipase enzyme producing potentials of Bacillus species isolated different automobile workshops in Anambra State.

Materials and Methods

2.1 Collection of Soil Sample

The soil samples were collected on six different locations with history of oil contamination namely Awka, Nnewi and Onitsha representing the three senatorial zones of Anambra State. On each location, soil samples were taken from three distinct points with five meters between two points. The soil sample was removed from the topsoil and the ground was dug to about 5 - 10 cm depth before samples were taken with sterile hand trowel. The sub samples were mixed together and later packed in sterile polythene bags and properly tied. The samples were taken to the laboratory for analysis and were kept in the fridge at 4 °C until needed (Oyeyiola *et al.,* 2013; Uba et al. 2018a; Uba, 2019a; 2019b).

2.2 Isolation of *Bacillus* Isolates

Fifty-gram soil samples were transferred to 150 mL sterile distilled water and heat treated at 80 °C for 15 min. After that 0.1 mL of soil suspension was spread over pre – sterilized Nutrient agar plates. The inoculated plates were incubated at 30 °C for 24 - 48 hr. The plates were examined after incubation period for rough and abundant colonies with waxy growth (1 – 4 mm diameter) and irregular spreading edge. Suspected *Bacillus* species colonies were stained by Gram staining method. The Gram - positive bacilli were maintained on Nutrient agar slants for additional identification tests (Kumar *et al*., 2020).

2.3 Screening Alkaline Lipase Producing Bacillus Species

A sensitive and specific plate assay for detection of lipase producing species makes use of Tributylin - Phenol red medium. The growth medium contained (g/L): Nutrient broth, 8.0; $CaCl_2$, 1.0 and agar-agar 20. The medium was autoclaved after adjusted to pH 9, and cooled to about 60 ℃, then , 1 % tributyrin and 500 µL of phenol red dye solution (0.01 % w/v distilled water and sterilized by filtration) was added with vigorous stirring. It was then poured into Petri plates under aseptic conditions and allowed to solidify. The *Bacillus* strains were spot inoculated onto these plates. Lipase producing *Bacillus* strains were identified on spread plates after incubation at 37˚C for 48 hr. After incubation, a change of colour from red to orange was observed. This indicated that the release of fatty acids was due to lipolysis (Ilesanmi *et al*., 2020).

2.4 Production of Enzyme

Basal medium with tributyrin (1 %) was used for the production of enzymes from the selected alkaline lipase bacterial isolates. All the isolates were inoculated in the conical flask containing 100 mL basal medium with substrates at pH 9. Flasks were incubated on rotary shaker at 37 °C and 120 rpm for 5 days after which the cells were separated by centrifugation. The culture was centrifuged at 8,000 x g for 90 min and the cell-free supernatant was used as a crude enzyme source.

2.5 Determination of Lipase Activity and Protein Concentration

2.5.1 Lipase assay

In this study, lipase activity was measured using Jaganmai *et al.* (2023) method where 4- para nitro phenol acetate (pNPA) solution was prepared freshly by mixing 100 mg/mL in 1 % Triton X and 2 % Sodium dodecyl sulphate. The reaction mixture contained 3 mL of pNPA substrate solution (freshly prepared), 6 mL of 50 mM Tris-HCl buffer (pH 8.5) and 1 mL of the lipase enzyme (Crude/Purified), and incubated at 40 ºC for 30 min. The resulting solution was extracted using 3 mL acetone/ethanol (1:1) and 0.5 mL Folin reagent added and absorbance was measured at 405 nm.

Lipase Activity (U/hr/mg of protein) = (Absorbance at 410nm/mg of protein) X Dilution factor

2.5.2 Determination of Protein concentration

Protein concentration was determined by Biuret method as described by Nasry *et al*. (2022). Bovine serum albumin (BSA) was prepared at 150 g/L stock protein standard and after which different concentrations range 0-150 g/L will be made. To all the tubes, 4 mL of 3 % NaOH solution was added to make it up to 5 mL solution. Also, 1 mL of Biuret solution was added, shaken, and incubated at room temperature for 15 mins. Similar procedure was repeated for all samples. The absorbances were measured spectrophotometrically at 540 nm

wavelength using water blank. The amount of protein was determined from the BSA standard curve.

2.6 Characterization and Identification of Microbial Isolate

The *Bacillus* strains with the highest activities for each substrates were selected and identified further by colonial morphology, microscopic technique, biochemical tests and molecular methods using the methods of Willey *et al.* (2008), Cheesbrough (2006) and HPA (2007).

2.6.1 Morphological characteristics

2.6.1.1 Microscopic morphology

2.6.1.1.1 Gram staining

This technique divides bacteria into Gram positive and Gram-negative groups. A smear of the isolate was made on clean dry grease-free slide, using a sterile wire loop. The smear was air-dried and heat fixed by passing over flame quickly three times. It was then covered with 0.5 % crystal violet solution for 1 min and rinsed with distilled water. The slide was flooded with 1 % Gram's iodine (which served as a mordant that fixes the dye inside the cell). The iodine was washed off after 1 min and 95 % ethanol was used to decolorize the smear for 30 sec. The smear was counter-stained with 0.1 % safranin dye solution for 1 min. It was then washed off and the slide air - dried, and observed under the microscope using oil immersion objective lens after placing a drop of oil immersion. Gram positive and negative reactions were indicated by purple and red colours, respectively (Cheesbrough, 2006).

2.6.1.1.2 Spore staining

According to the method of HPA (2007), smears of the isolates were prepared and fixed on a slide. The underside was vapor heated and flooded with 5 % Malachite green solution. Heating was continued until visible water condensate forms under the slide with evaporation at the top. It was washed using distilled water. Smears were counter stained with 0.5 % safranin solution for 10 seconds. Slides were washed, dried and observed under oil immersion objective lens after placing a drop of immersion oil. A green space within the cells would indicate the presence of spores.

2.6.2 Biochemical characteristic

The following biochemical test were carried out on the isolated pure *Bacillus* strains and include: catalase, nitrate reduction, oxidase, indole, motility, methyl red - Voges Proskauer, citrate, urease, gelatin liquefaction, hydrogen sulphide production, and sugar fermentation tests, respectively (Cheesbrough, 2006; Uba 2018b; 2018c).

Results and Discussion

The result of the lipolytic bacterial count obtained from different soil samples is presented in Table 1. From the result, Onitsha sampling location point B had the highest lipase bacterial count of 23.90 X 10^4 CFU/g while Awka sampling location point A had the least count of 1.80 X 10^4 CFU/g, respectively. This revealed that Onitsha sample a higher reservoir of the lipase producing bacterial strains followed by Nnewi and then Awka sampling locations, respectively.

In the present study, the Tributyrin Phenol Red medium (TPR) was utilized to study the activity staining. The formation of yellow halos or zone indicated the tributylin hydrolysis by the alkaline lipase of *Bacillus* bacterial strains. Similarly, the lipases obtained from *Bacillus* bacterial strains were able to hydrolyse tributylin and form yellow halos or zone in the presence of Phenol Red as shown in Figure 1. The formation of yellow clearance zone was due to the release of fatty acids on lipolysis that get complexed with the Phenol Red (Niyonzima and More, 2014).

The results of the protein contents and enzyme activities of the lipase producing strains is shown in Figure 2. The result indicates that strains AWK1, NW1 and ON3 out of 14 lipolytic bacterial strains had the highest protein content and enzyme activities of 1.29, 1.40, 1.77 mg/mL and 39.18, 40.59, 28.80 U/mL, respectively. Previous study by Barik *et al.* (2022) reported that the bacterial strain *Kocuria flava* Y4 (GenBank accession no.MT773277) lipase activity was found to be 41± 0.64 U/mL, which was assessed by the para nitrophenyl acetate (p-NPA) assay. The observation made in their study was similar to the present study.

These bacterial strains were later characterized and identified physiologically (Table 2) and found to be *Bacillus* sp. AWK1, *Bacillus thuringiensis* NW1 and *Bacillus tropicus* ON3 and previous studies reported Bacillus genera as a dominant producer of lipase enzyme (Kandala *et al*., 2010; Prabhavathy *et al*., 2013)..

Table 1: Lipolytic bacterial count obtained from different soil samples

Area	Sample	10^{-2} Dilution	10^{-4} Dilution	Mean count (CFU/g)
Awka	A	35	16	2.55 X 10^4
	B	23	13	1.80 X 10^4
Nnewi	A	89	67	7.80 X 10^4
	B	31	22	5.30 X 10^4
Onitsha	A	150	80	11.50 X 10^4
	B	230	248	23.90 X 10^4

Key: CFU/g = Colony forming unit per grams; Awka A and B = Mechanic workshops close to Amaku Teaching Hospital, Awka; Nnewi A and B = Mechanic workshops close to Okpuneze Bus Stop Uruagu Nnewi and Onitsha A and B = Mechanic workshops close Amanasa Mgbuka Obosi off Onitsha.

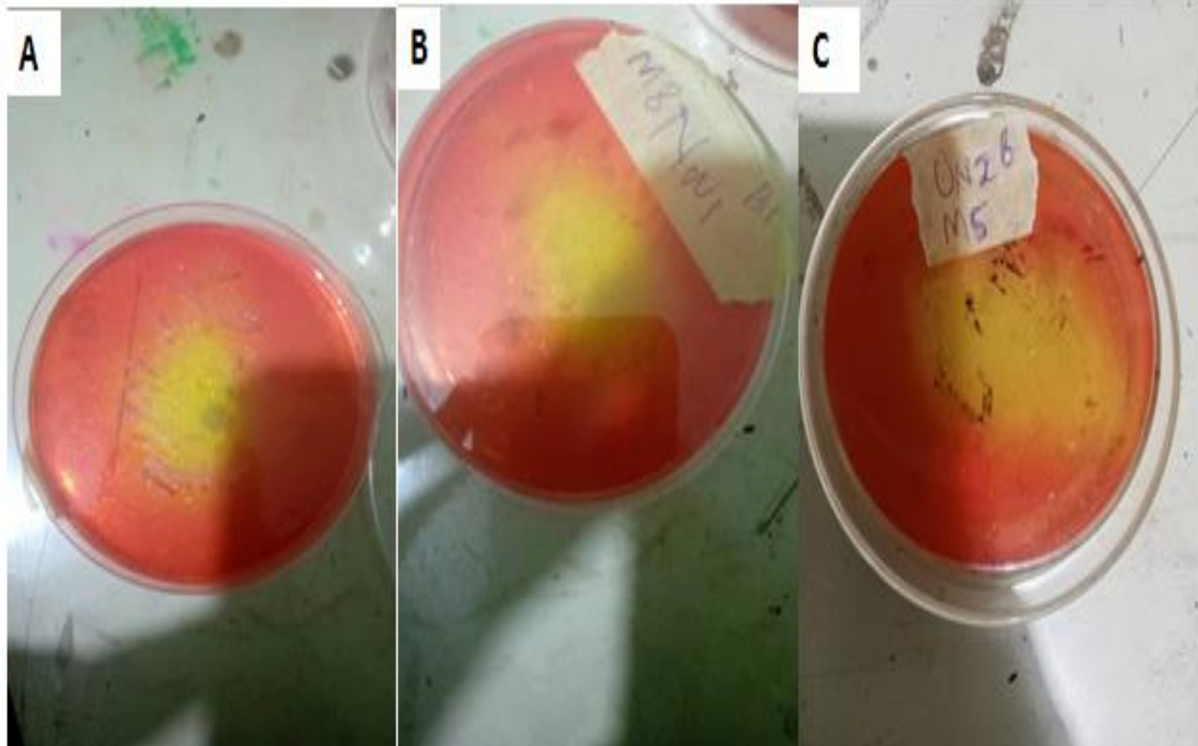

Figure 1: Phenol red-agar plates showing lipase activity of the three selected lipase producing strains

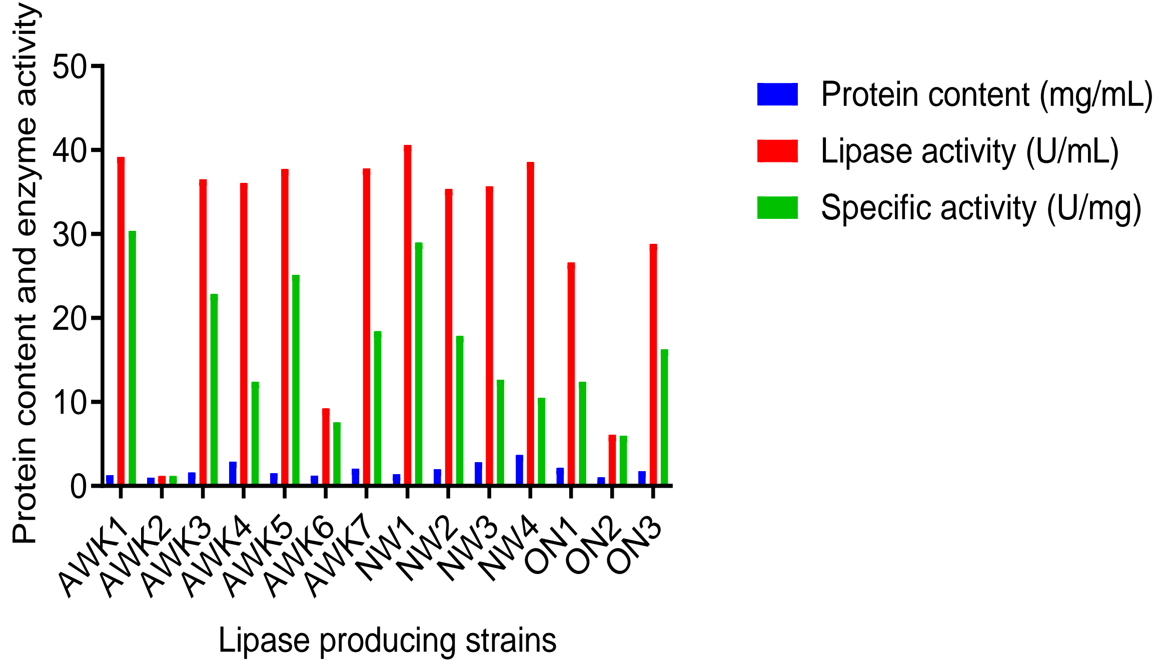

Figure 2: Protein content and enzyme activities of the lipase producing strains

Table 2: Biochemical profile of the selected lipase producing strains

Test parameter	AWK1	NW1	ON2
MR	+	+	+
MOT	+	+	+
CIT	+	+	+
H_2S	+	+	+
URE	-	-	-
IND	-	-	-
VP	+	+	+
GEL	+	+	+
GLU	+	+	+
MAN	+	+	+
INO	+	+	_
SOR	-	+	-
RHA	+	+	+
SAC	+	+	+
ARA	+	+	+
SUC	+	+	+
XYL	+	-	+
MAL	+	-	+
OX	+	+	+
CAT	+	+	+
NIT	+	+	+++
COAG	-	-	-
Gram reaction	+ Rod	+ Rod	+ Rod
Spore reaction	+	+	+
Tentative identity	*Bacillus* sp.	*Bacillus* sp.	*Bacillus* sp.

Key: + = Positive; - = Negative; +/- = Variable; MR = Methyl red; MOT = Motility; CIT: Citrate; H_2S: Hydrogen sulfide production; URE: Urease; IND: Indole; VP: the Voges-Proskauer test; GEL: Gelatinase; GLU: Glucose; MAN: Mannitol; INO: Inositol; SOR: Sorbitol; RHA: Rhamnose; SAC: Sucrose; ARA: Arabinose; MAL = Maltose; OX = Oxidase; CAT = Catalase; NIT = Nitrate reduction; COAG = Coagulase

Conclusion

The whole study revealed that automobile workshops in Anambra State are great reservoirs of lipase producers. The study further revealed that Onitsha sample a higher reservoir of the lipase producing bacterial strains followed by Nnewi and then Awka sampling locations, respectively. The selected strains were characterized and found to belong to Bacillus genera as dominant producer of lipase enzyme in this study. The isolation of these lipase producing strains further signify that they could be exploited for several biotechnological prospects.

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