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Isolation, Screening, Identification, Molecular Characterization and Optimizatiom of Lipase producing Bacteria from Edible Oil Contaminated Soil in Maiduguri, Borno State, Nigeria

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Abstract
Lipase is an important hydrolytic enzyme used for numerous industrial applications due to their qualities such as versatility, specificity, increased sustainability and environmental friendliness. This work focused on the isolation, screening, identification and molecular characterization of lipase producing bacteria isolated from edible oil contaminated soil in Maiduguri, Borno state as well as their optimization for higher lipase production. Bacteria were isolated using standard microbiological techniques and screened for lipase production using tributyrine, rhodamine B and Tween 80 agar media. Secondary screening for high lipase producing bacteria was done in a mineral salt medium. The highest lipase producing bacteria was suspected to be *Pseudomonas sp* which was identified as *Stutzerimonas stutzeri* by sequencing using 16s rRNA gene when subjected to homology search using Basic Local Alignment Search Tool (BLAST). Lipase producing parameters were subjected to varying temperatures and pH for optimal lipase production. The bacteria *Stutzerimonas stutzeri* were identified as good candidates for lipase production in this research. having the highest lipase activity (10.65 U/mL) with coconut oil as the substrate, glycerol as the carbon source, yeast extract as the nitrogen source, pH 9.0, incubation at 40 °C for 48 h, inoculum size of 4000 µL, and agitation at 120 rpm, resulting in a substantial yield increase.

Keywords: isolation, screening, identification, Molecular characterization, optimization, lipase producing bacteria, edible oil contaminated soil

Introduction

Lipase (Triacylglycerolacylhydrolase EC 3.1.1.3) is an enzyme that specifically acts on fats and hydrolyses them into fatty acid and glycerol at water lipid interface (Singh *et al.,* 2017). This enzyme also plays a vital role in different reactions which include acidolysis, esterification and aminolysis. This make it one of the important enzymes for industrial application and it is used in many industries which include diary, detergent, medicine and pharmaceuticals, fat and oleo-chemical, food, cosmetics and perfume industries (Baron et al., 2019). Although lipase is produced by both prokaryotes and eukaryotes, the bacterial lipases are the most important lipases in terms of industrial application as they are easy to cultivate and optimize to get higher yield (Ali *et al.,* 2023; Messaoudi *et al.,* 2011). Lipase producing bacteria include *Bacillus sp, Psuedomanas sp, Serratia sp, Aspergillus sp* among other species (Ezenwelu *et al.,* 2022; Popoola *et al.,* 2021). Natural oil such as olive, coconut, vegetable, and petroleum oils tend to increase lipase production (Sharma *et al.,* 2023), as such lipase producing microorganisms are mostly found in settings such as industrial waste , vegetable oil processing facilities, diaries, oil contaminated soil, oil seeds, decomposing food, compost piles,

coal ash and hot springs (Funke *et al.,* 2019). *Staphylococcus sp., Bacillus sp.* and *pseudomonas sp.* were isolated from edible oil contaminated soil in Nigeria (Fakorede *et al.,* 2019). Abubakar *et al.,* (2024) reported that *Bacillus sp. and pseudomonas sp*. are the predominant lipase producing bacteria in Nigerian soil. Nutritional and physico-chemical factors such as temperature, pH, nitrogen, carbon source and presence of lipids, inorganic salts, agitation and dissolved oxygen concentration boost lipase production by microorganism (Yassin *et al.,* 2023; Pham *et al.,* 2021). The aim of this study is to identify and characterize the highest lipase producing bacteria and to optimize lipase production in bacteria isolated from edible oil contaminated soil in Maiduguri.

Methodology

Collection of Sample

Three Soil samples were collected at Bolori store area with the aid of a soil auger at a depth of 10-15cm depth. About 10g of soil was collected into sterile bags. The samples were labeled as sample 1, sample 2 and sample 3 and were transported to the laboratory and stored at 4°C to reduce bacterial activity and potential degradation.

Isolation of Bacteria

A 5 in 10 folds serial dilution technique was performed. Exactly 1ml of each sample was weight and placed in a sterile test tube with 9ml of distilled water. Fivefold serial dilutions are prepared aseptically. The sterile test tubes were taken and labelled per dilutions ranging from 10^{-1} to 10^{-5}.the tubes were serially diluted till the last tube dilution (10^{-5}). Exactly 0.1ml from the serially diluted samples (10^{-5}) was poured into the sterile petri plates containing nutrient agar. The petri plates were gently shake for uniform distribution of samples and incubated at room temperature for 24 hours. Distinct colonies formed were further sub cultured until pure isolates were obtained and maintained on nutrient agar slants and preserved at 4◦C (Rajeshkumar et al 2013).

Primary Screening For Lipase Producing Bacteria

Pure isolated bacterial cultures were screened for lipase activity using tributyrin agar medium. Rhodamine B agar media and Tween80 agar media.

The tributyrin agar medium

This was prepared with 5g/L peptone, 3g/L yeast extract, 15g/L agar, 10 ml/L tributyrin in distilled water at pH 7.0. The medium was sterilized for 15min by autoclaving with 15lb pressure at 121°C and cooled. The aliquot was transferred to petri dish and allowed to solidify. A loopful of each pure culture was streaked each onto tributyrin agar plates separately and incubated at 37°C for 24 hours. After incubation, the clear zone of hydrolysis around the colonies indicated the presence of lipase activity.

Rhodamine olive oil agar medium

This was prepared with 5g/L peptone, 3g/L yeast extract, 15g/L agar, 4g/L NaCl in distilled water, sterilized for 15 min by autoclaving with 15 lb pressure at 121ºC and cooled to 60◦C. 31.25 ml/L olive oil, 10ml/L sterilized filtered rhodamine dye (1mg/ml) was added and the pH of the media is 7.0. Aliquots were transferred to petri dishes and allowed to solidify. A loopful of each pure culture was streaked onto rhodamine olive oil agar plates separately and incubated the inoculated plates at 37 for 48 hours. After incubation, formation of orange, fluorescent halos around bacterial colonies visible upon UV irradiation indicates lipase production.

Tween80 agar medium

This was prepared at pH 7.0 with 10g/L peptone,20g/L agar, 5g/L NaCl, 0.1g/L $CaCl.2H_2O$, in distilled water, sterilized for 15 min by autoclaving with 15 lb pressure at 121ºC and cooled to 45◦C. 10 ml/L of Tween 80 was added to the cooled media. Aliquots were transferred to petri dishes and allowed to solidify. A loopful of each pure culture was streaked onto Tween80 agar plates separately and incubated the inoculated plates at 37 ºC for 48 hours. After incubation, a white precipitate around colony indicates lipase activity.

Secondary Screening Of Lipase Production

All the selected lipase producing bacterial cultures were further screened for production of lipase. The production media (pH 7.0) was prepared with peptone (20.0g/l); $MgSO_4.7H_2O$ (0.5g/L); KCl (0.5g/L); K_2HPO_4, 2.0g/L; olive oil (10g/L) and added in distilled water, autoclaved for 15minutes at 15lb pressure at 121◦C and cooled to about 60◦C before the addition of 1ml/L olive oil. 1.0ml of overnight grown selected lipase producing bacterial cultures was inoculated in 100ml of production medium in 250ml Erlenmeyer flasks separately and incubated at 30◦C for 24 hours. Culture was centrifuged at 10,000rpm for 10 minutes after incubation, supernatant was used to assay for lipase.

Lipase assay

The crude extracellular lipase enzyme was extracted by centrifugation. Lipase activity was assayed by titrimetric method using olive oil emulsion as substrate (Oyetunde et al 2022). The reaction mixture containing 5ml of olive oil emulsion, 2ml of 0.03% Triton X-100, 2ml of 3M NaCl, 1 ml of 0.075% CaCl and 5ml of phosphate buffer and 1ml of crude enzyme extract was incubated at 37◦C for 15 minutes. The reaction was terminated by adding 20ml of ethanol: acetone (1:1, v/v) to the mixture. The free fatty acids liberated during the reaction was titrated against 0.02 N NaOH using phenolphthalein as indicator. The control containing the reaction mixture and ethanol; acetone solution without the addition of enzyme was titrated against 0.02 N NaOH. Similarly. One unit of lipase enzyme was defined as the amount of enzyme that released 1 µmol of fatty acid per minute under assay conditions. The quantity of fatty acids liberated in each sample was calculated based on the equivalents of NaOH used to reach the titration end point, accounting for any contribution from the reagent, using the following formula:

$$\mu\text{mol fatty acid/ml subsample} = \frac{[(\text{ml NaOH for sample} - \text{ml NaOH for blank}) \times \text{N} \times 1000]}{5\text{ml}}$$

Where N is the normality of the NaOH (0.1 in this case) and 5 ml is the volume of reaction mixture.

Identification And Characterization Of Bacteria Isolates

The isolates were identified morphologically and biochemically described in Berkey's Manual of Systematic Bacteriology (2nd edition) (Sharma, 2015).

Morphological identification of Bacteria Isolates

Colony morphology and characteristics such as the size, shape, colour, elevation, surface, edges of the bacteria isolate on the plates were observed and Smears were prepared for Gram's staining.

Gram's Staining Technique of Bacteria Isolates

A thin smear of the bacterial isolate was made on a clean grease free glass slide from 18 to 24 hours' culture and heat-fixed by flaming. Two drops of crystal violet were added to the smear for 60 seconds after which it was washed with tap water and Gram's iodine was added for another 60 seconds. The stain was then decolorized by flooding the slide with 95% alcohol until no more violet coloration was observed. (This lasted for 10 seconds). Two drops of safranin reagent were then added for 30 seconds to counter stain. It was rinsed again with tap water and allowed to air dry. The stained isolates were examined microscopically

under the oil immersion objective to determine their Gram's reaction and cellular characteristics as well as the different shapes of the bacteria cells. Gram positive cells appeared purple while Gram negative cells were red or pink. (Sharma, 2015).

Biochemical Characterization of Bacteria Isolates

The biochemical parameters tested include.

Indole Production test

A loopful of a broth culture of bacteria isolates was inoculated into a test tube of tryptone water medium and incubated at 37°C for 5 to 7 days after which 0.5ml of Kovac's reagent was added to the tryptone water culture. A deep red colour developed in the presence of indole, which separates out in the alcohol layer within 10 minutes while no colour change indicated a negative reaction (Koneman, *et al.*, 2019).

Catalase test

This was carried out to test the ability of the isolate to produce coagulase enzyme. A slide was marked with two sections, a loopful of normal saline (0.85% NaCl in aqueous solution) was placed in each section and a small amount of 18 to 24 hours agar culture was suspended in each drop until a homogeneous suspension was obtained. A drop of human plasma was added to one of the suspension and stirred for 5 seconds. The other was left as control. A positive result shows clumping, which will not re-suspend, (Koneman *et al.*, 2019).

Oxidase

Two to three drops of 1% tetramethyl paraphenylene di-amine hydrochloride were applied on a filter paper to moisten it. A smear of each bacterial isolate was made on the moistened filter paper by means of sterile inoculating loop. A positive result was recorded when the moistened filter paper turns purple after smearing within 10 seconds, while delayed reaction without purple colouration was regarded as negative, (Koneman *et al.*, 2019).

Nitrate Reduction test

Nitrate peptone water consisting of peptone water and 0.1% potassium nitrate was used. 5mls portions of the medium were distributed into each test tube, containing inverted Durham tubes. The tubes were sterilised in an autoclave at 121°C for 15 minutes and allowed to cool before inoculation with isolates. Un-inoculated tube served as control. All the tubes were incubated at 30°C for 5 days. The ability of the isolates to reduce nitrate to nitrites, ammonia or free nitrogen was determined by adding to each tube 0.5ml of 1% sulphanillic acid in 5N acetic acid, followed by 0.5ml of 0.6% dimethylnaphthylanine in 5N acetic acid. The development of a red colouration indicated a positive result and the presence of gas in the Durham tube indicated the production of nitrogen gas. Absence of a colour change indicated negative reaction, (Koneman *et al.*, 2019).

Citrate Utilization test

Simmon's citrate medium was prepared and dispersed into screw capped bottles and sterilised. The content of each bottle were allowed to cool and the test organism inoculated into it. It was then incubated at 30°C for 48 hours after which the colour change was observed. A positive result was shown by a change in colour of the medium from green to blue while a negative result was indicated by a change in colour (Koneman *et al.*, 2019).

Methyl Red test

The microbe could ferment mixed acids on supply of glucose. Proportions and types of acids produced by anaerobic fermentation of glucose is a key characteristic which helps to differentiate various genera of enteric bacteria. To a prepared glucose phosphate medium in test tube, loop full of the isolate were inoculated and incubated at 37^{o}C for two days. To the two days old culture, drops methyl red solution were added. Then were shaken slightly and examined for the formation of red ring at the interface. Appearance of red colour at the surface of the reagent layer showed positive methyl red reaction (Koneman *et al.,* 2019).

Sugar Utilization test

Sugar fermentation medium was dispensed into test tubes containing an inverted Durham tube each. These were autoclaved at 115°C for 10 minutes. The tubes were allowed to cool after sterilisation and bacterial isolates were inoculated into the tubes. These were incubated at 37°C for three days. Uninoculated tube served as the control. The tubes were examined daily for change in reaction after which acid and gas (when produced) caused the phenol red to turn to yellow while gas evolution was indicated by displacement of solution in Durham tube (Koneman *et al.,* 2019).

Gelatine Hydrolysis test:

Gelatin is a protein, which can be metabolised only by microorganisms capable of producing proteolytic enzymes that break it down. When broken down, gelatin loses its gelling qualities. Precisely, 15% of gelatin was prepared and mixed with 100ml nutrient broth. The mixture was dissolved in hot water bath with constant swirling. The homogenous solution was then dispensed into test tubes and sterilised; loopful of the isolate was inoculated into the cooled medium and incubated at 30°C for 7days.

Gelatinase production was detected by transferring the tubes into the freezer for 15 minutes. Tubes in which the broth remained liquefied and turned fluidy were recorded as positive. This showed gelatinase production by the isolate (Koneman *et al.,* 2019).

Voges-Proskauer test

Voges-Proskauer test was developed to detect the presence of acetone in the bacterial culture. The test was performed by adding potassium hydroxide and alpha-naphtholto the V-P broth which has been inoculated with bacteria. On inoculation, if cherry red colour is seen then the result is positive, while negative result is yellow-brown colour. It depends on the conversion of glucose to acetyl methyl carbinol. If glucose was broken down, it would react with alpha-naphthol (VP reagent I) and potassium hydroxide (VP reagent II) to give a red colour change (Koneman *et al.,* 2019).

Triple sugar iron (TSI)

The TSI media was prepared according to manufacturer's specifications and slanted in test tubes and 24 hours of the bacterial isolates were inoculated with a sterile transfer using a wire loop and then the butt were stabbed. The needles were withdrawn and the slants were streaked. The test tubes were incubated at 37^{o}C for 24 hours after which they were examined for gas production, hydrogen sulphide, glucose, lactose and sucrose fermentation (Koneman *et al.,* 2019).

Molecular Characterization Of Highest Lipase Producing Bacteria

The following steps were followed to carryout molecular characterization of the selected high lipase producing bacteria.

Genomic DNA isolation of Selected High Lipase Producing Bacteria

The genome of the bacteria was extracted by DNA purification protocol using DNA extraction Kit (Accu prep Genomic DNA extraction kit from Bioneer). Pellets of bacterial cells was re-suspended into a clean 1.5

ml tube containing 200 µL PBS. Exactly 20 µL of Proteinase K was added to tube containing PBS and vortex mixer. Exactly 200 µL Binding buffer (GC) was added to the sample and mixed immediately by vortex mixer and incubated at 60℃ for 10 min to achieve maximum lysis. The sample was completely re-suspended and 100 µL of Isopropanol was added and mixed well by pipetting. After this step, it was briefly spin down so as to get the drops clinging under the lid. Vortexing was avoided here as this might reduce DNA yield. The lysate was carefully transferred into the upper reservoir of the binding column tube (fit in a 2 mL tube) without wetting the rim. The tube was closed and centrifuged at 8,000 rpm for 1 min.

Each Binding column tubes were closed to avoid aerosol formation during centrifugation. It was centrifuged again at a higher speed (>10,000 rpm) until the binding column tube was empty. The tubes were opened and transferred the binding column tube to a new 2 ml tube for filtration (supplied). 500 µL of washing buffer 1 (W1) was added without wetting the rim, closed the tube, and centrifuged at 8,000 rpm for 1 min. The tube was opened and the solution from the 2 ml tube was poured into a disposal bottle and carefully added 500 µL of washing buffer 2 (W_2) without wetting the rim, the tube was closed and centrifuged at 8,000 rpm for 1 min. It was centrifuged once more at 12,000 rpm for 1 min to completely remove ethanol, and make sure no droplet clinging to the bottom of binding column tube. The Binding column tube was transferred to a new 1.5 mL tube for elution (supplied), added onto binding column tube was 200 µL of Elution buffer (EL, or nuclease-free water), and waited for 1 min at RT (15~25℃) until EL was completely absorbed into the glass fiber of binding column tube. To increase DNA yield, it was allowed to stay for 5 min after adding Elution buffer (EL). The volume of EL added was adjusted from 50 µL to 100 µL. The eluted genomic DNA was stable and used directly as template for PCR.

Polymerase Chain Reaction (PCR) of High Lipase Producing Bacteria

For reaction set-up of the PCR (*One* GoTaq PCR premix, New England BioLab), templates were added, specific primers and water to the premix to make a 20 uL reaction needed for the PCR set up, 16 µL of deionized water (dH_2O), 1 µL of Forward: 27F(5'-AGAGTTGATCCTGG-'3) and 1 µL, of Reverse: 1492R (5-'GGTTACCTTGTTACGTT-'3) (Bioneer), 2 µL of template DNA under the PCR Conditions: (Thermal cycler PTC 100, MJ Research, Pre- Denaturation: 5 min at 95^{o}C, Denaturation:40 sec at 94^{o}C, Annealing: 40 sec at 54^{o}C, Extension: 40 sec at 72^{o}C 35 cycles and final extension: 5 min at 72^{o}C. The result was ran on 1. % agarose gel.

Gel Electrophoresis of PCR Amplicon of High Lipase Producing Bacteria

For 1.0 %, 1g of agarose was used. The solution was heated in a microwave until agarose was completely dissolved and allowed to cool in a water bath set at 50^{o}C – 55^{o}C. Gel casting tray was prepared by sealing ends of gel chamber with tape and appropriate casting system. Appropriate number of combs was placed in the gel tray. 5 uL of ethidium bromide was added to cooled gel and poured into gel tray and allowed to cool for 15-30 min at room temperature. The combs were removed, placed in electrophoresis chamber and covered with TAE buffer. DNA and standard (Ladder) were loaded onto the gel. Electrophoresed at a constant voltage of 90 volts for at least 1hour. DNA bands were visualized using UV light box and gel imaging system (Biorad).

Sequencing of Amplified Genomic DNA using (ABI 3100) sequencer

Sequencing reaction was prepared in a 2.0 mL tube. All reagents were kept on ice while preparing the sequencing reactions and were added in the order listed below: 9.5 uL of dH_2O, 10.0uL of DNA template, 2.0 uL of primers, 8.0 uL of Bigdye master mix. The sequencing reaction was set up in the PCR machine as below: Thermal cycling program: 96^{o}C for 20 sec 50^{o}C for 20 sec X 30 cycle 60^{o}C for 4 min. Ethanol precipitation: prepared a labeled sterile 0.5 ml tube for each sample. Also, prepared fresh stop

solution/glycogen mixture as follows per sequencing reaction: 2 uL of 3M Sodium acetate, 2 uL of 100 mM Na2-EDTA and 1 uL of 20 mg/ml of glycogen (provided in the kit).
To each of the labelled tubes, 5 uL of the stop solution/glycogen mixture were added. The sequencing reaction was transferred to each of the appropriately labelled tube and mixed thoroughly. Exactly 60 uL cold 95 % (v/v) ethanol was added from -20 freezer and mixed thoroughly and was immediately centrifuged at 14,000 rpm at 4^{o}C for 15 min. The supernatant was carefully removed with a micropipette (the pellet was visible). The pellet was rinsed with 200 uL 70 % (v/v) ethanol from -20 freezer, centrifuged at 14,000 rpm at 4^{o}C for a minimum of 2 minutes. All the supernatants were carefully removed with a micropipette and was vacuum dried for 10 min and resuspended the sample in 40 uL of the sample loading solution (provided in the kit). In sample preparation for loading into the instrument, the resuspended sample was transferred to the appropriate wells of the sample plate, overlaid each of the resuspended sample with one drop of mineral oil from the kit. The sample plate was loaded into the instrument and started the desired method.

Optimization Of Fermentation Factors Of Lipase Production Of Selected Isolates

Culture supernatant was used to determine lipase activity, all procedures were carried out using modified method of (Ojo and Ogunbanwo, 2022). The effect of the following parameters on lipase production was determine

Alternative substrate

olive oil which is the main substrate that was present in the growth medium was replaced by Soybean oil, coconut oil, palm oil, and sesame oil and were all tested individually. All other parameters remain unaltered.

Carbon source

The main carbon source in the growth medium was replaced Glycerol, sucrose, glucose and fructose and were tested individually. No was changed other parameter apart from carbon source.

Nitrogen source

The main nitrogen source in the culture media (Peptone) was replaced with yeast extract, potassium nitrate, ammonium nitrate and urea. Other contents of the culture media remain the same as that in the main media.

Alternative pH

Lipase production was tested at pH 3, 5, 7, 9, and 11 to determine the effect of pH on lipase activity without changing any other content in the media.

Alternative temperature

The isolated bacteria were cultivated in the growth medium at temperature range of 25◦C- 60◦C at 5◦C interval in shake flask and incubated for 24 hours. The content of the media remain the same as that of the main media.

Incubation period

The isolated bacteria were cultivated on growth medium and incubated at varying time from 12 hours to 72 hours. Samples were taken periodically at an interval of 12 hours to measure the activity of lipase. No change was made to the content of the media.

Inoculum volume

The growth medium was inoculated with varying amount of pure bacteria cultures ranging from 1000µL to 7000µL and incubated for 24 hours without altering the content of the media.

Agitation speed

The inoculated culture flasks were agitated at varying rpm ranging from 40 to 180 rpm with interval of 20rpm using orbital shaker stuartSI500SSLI. The content of the media remain the same as that of the main media.

Result

Primary screening of lipase producing bacteria

Ten isolates were obtained from the three samples. The positive lipase producing bacteria were selected based on their ability to react with the lipid contained in the screening media.

Positive bacterial isolates with extend lipolysis activity

The positive lipase producing bacteria were selected based on their ability to react with the lipid contained in the screening media (tributyrin agar media pH 7) to form a diameter of halo opaque zone of inhibition (Plate I) around the bacteria colonies which indicate the presence of lipase activity. The result is shown in table I.

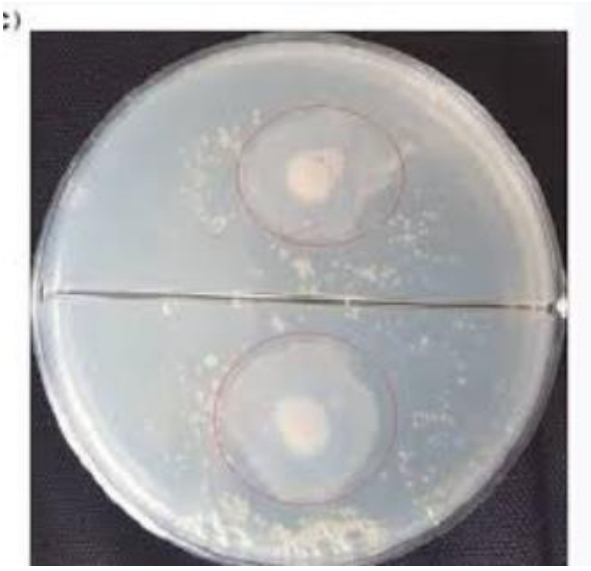

Plate I Positive lipase producing isolate showing zone of inhibition on tributyrin agar media

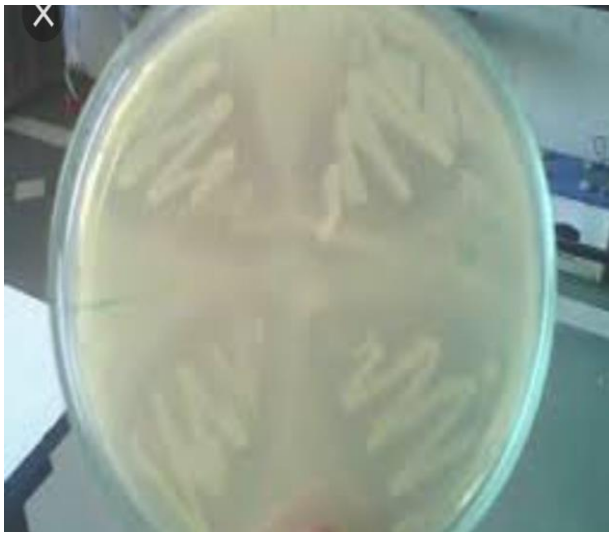

Plate II Positive lipase producing Bacteria showing white Precipitate around colonies

Table I Qualitative screening of lipase producing bacteria isolates.

S/N	Isolates	Zone clearing (mm)
1	Sample 1	8.40
2	Sample 2	12.62
3	Sample 3	6.25

Table I Shows the qualitative screening for lipase producing bacteria isolates after secondary screening of the lipase producing bacteria isolate, with zone of clearing 8.40mm for the isolate in sample1, 12.62mm for isolate in sample 2 and 6.25mm for the isolate in sample 3.

Secondary Screening of Lipase producing bacteria isolates

All selected positive lipase producing bacterial isolates obtained from the primary screening were further cultivated in secondary lipase production media (pH7.0). The result is as shown in Fig I

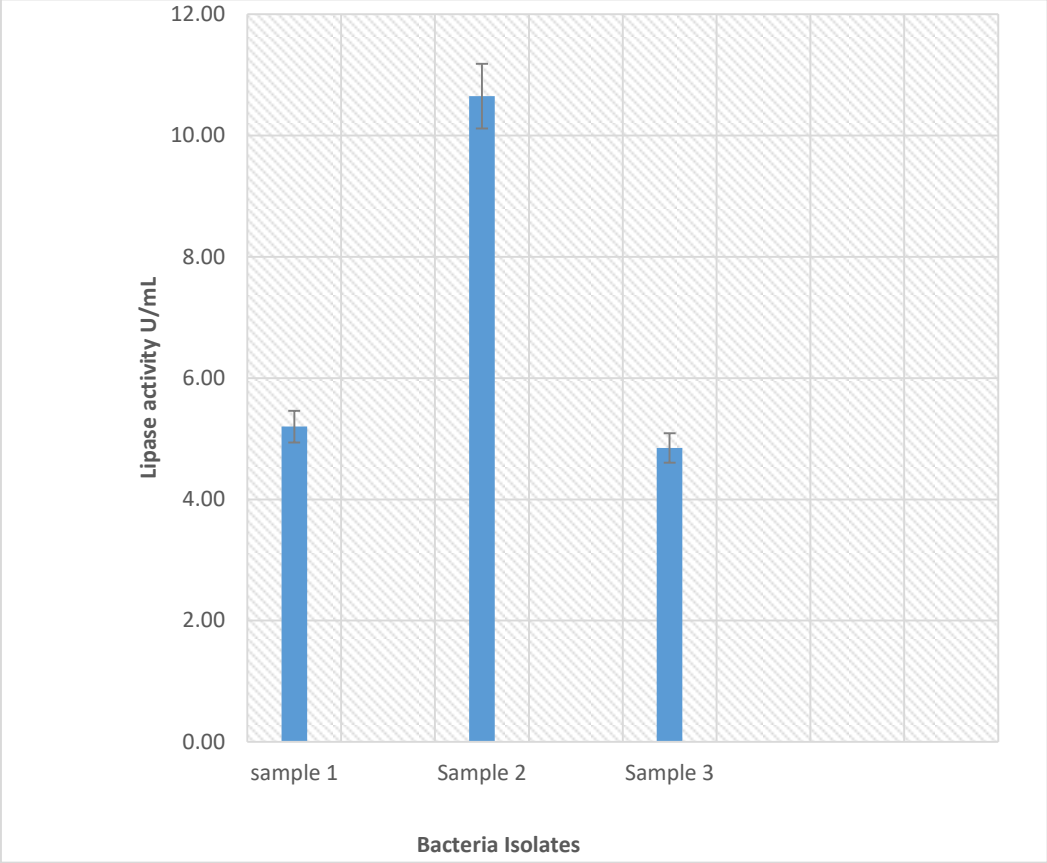

Fig I: List of bacteria isolates with lipase producing capacity

The result in fig I showed that bacteria isolates from the second sample has highest lipase producing capacity of 10.65U/mL followed by the isolates in the first sample with lipase capacity of 5.20U/mL and then that in the third sample which has 4.85U/mL lipase producing capacity

Identification and Characterization of Bacteria Isolates

Table II: Biochemical Characteristics of Lipase producing bacteria isolates

	Sample 1	**Sample 2**	**Sample 3**
Morphological			
Colony	None	None	None
Edge	Entire	Entire	Regular
Surface	Smooth	Smooth	Smooth
Cell shape	Rod	Rod	Rod
Gram stain	+	-	-
Motility	+	+	-
Biochemical			

Spore staining	+	-	-
Catalase	+	+	+
Oxidase	-	+	-
Citrate	+	+	+
MR	+	-	+
Urea	-	-	+
Indole	-	+	-
VP	-	-	-
Nitrate reduction	+	-	+
TSI	A, G, HS	G, HS	NC
Fermentation			
Glucose	A	NC	AG
Manitol	A	NC	AG
Sucrose	NC	NC	AG
Lactose	NC	NC	NC
Possible isolate	*Bacillus sp*	*Pseudomonas sp*	*Proteus Sp*

A= Acid production only, **AG** = Acid and Gas production, HS = Hydrogen Sulphate, **G** =Gas production**,** **NC** = No change, **+** = positive, **-** = Negative.

According to Table II that base on the morphological and biochemical characteristics of the bacterial isolates they could possibly be *Bacillus sp* (sample 1), *Pseudomonas sp* (sample 2) and *Proteus sp* (sample 3).

Molecular Characterization of the Highest Lipase Producing Bacteria

The bacteria isolate from sample 2 which was the highest lipase producer was further subjected to molecular characterization as follows

Genomic DNA isolation and Quantification of the Bacterial Isolate

Table III showed the total genomic DNA isolated including quantity and quality of selected Lipase producing Bacterial isolate using a NanoDrop 2000 UV Visible spectrophotometer.

Gel Electrophoresis analysis of 16S rRNA gene of the bacterial isolate

Fig II show a successful amplification of 16S rRNA gene of highest lipase producing bacterial isolate on Gel electrophoresis with target gene size of about 1,470bp using a 100 base pair DNA ladder and a negative control.

16s rRNA Nucleotide sequence (FASTA Format) of the Bacterial Isolate

Table IV show the sequence obtained when 16s rRNA nucleotides sequence of the selected Lipase Producing Bacteria Isolate was subjected to homology search using BLAST, indicating nucleotides sequence of organism with related sequence obtained from the National Centre for Biotechnology Information (NCBI, 2023).

Phylogenetic tree Analysis of BLAST nucleotides sequence

A phylogenetic tree was constructed using information obtained from nucleotides BLAST on the NCBI data base using the computer software application program (MEGA11) as shown in Fig III.

Table III: Genomic DNA isolation of Highest Lipase Producing Bacteria

Sample ID	Nucleic Acid Concentration	Unit	260/280
Sample 2	85	ng/µL	1.86

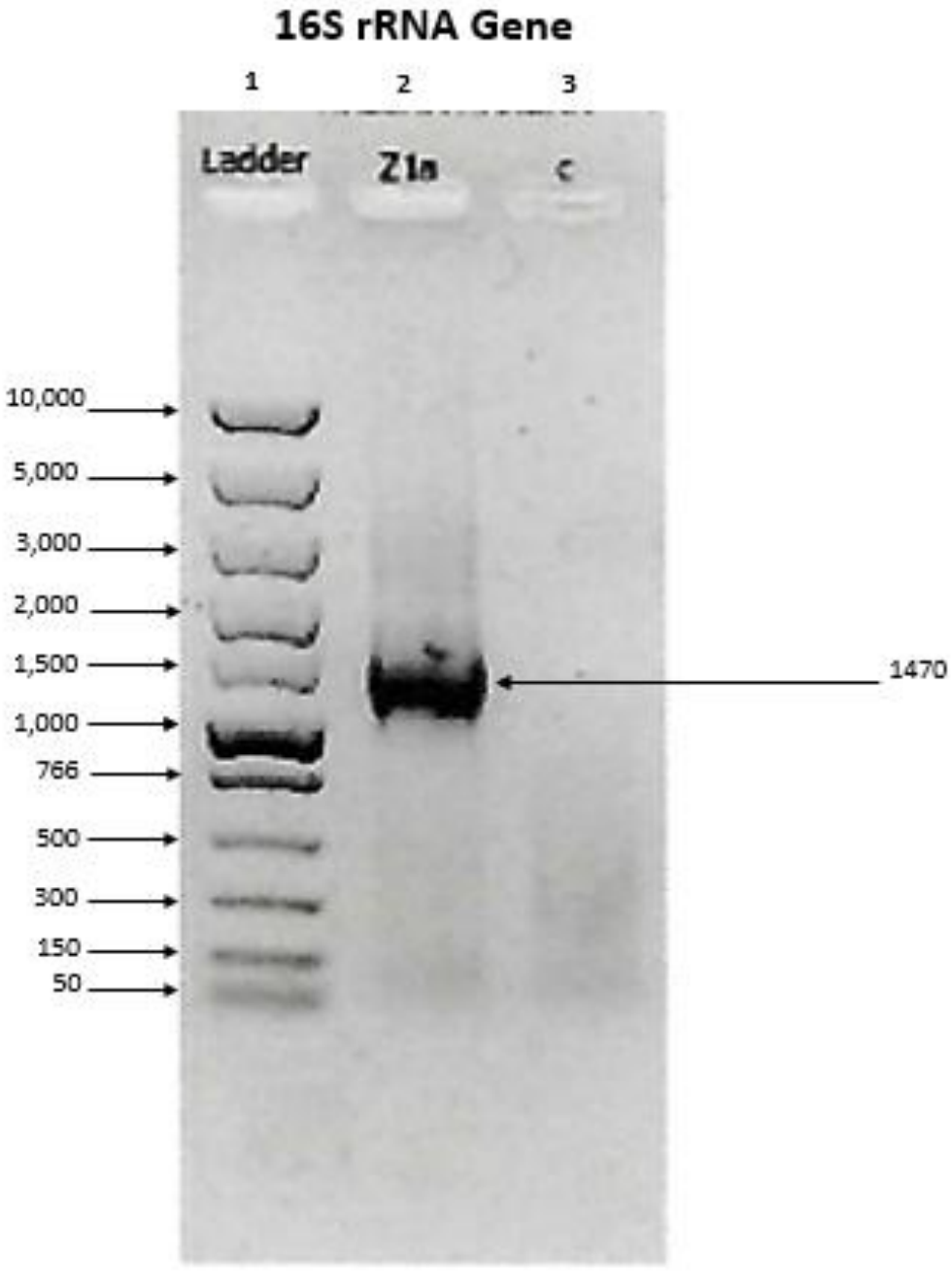

Fig II: Image showing Agarose Gel Electrophoresis separation of selected lipase producing bacteria isolate

Table IV BLAST in FASTA format Sequence 16S Ribosomal RNA Gene for Bacteria (BS2)

S/N	Organism of significance sequence alignment	Total Score	E Value	Percentage identity	Accession Lenth	Accession Number
1	Stutzerimonas stutzeri	2507	0.0	99.07	1499	MF555700.1
2	Stutzerimonas stutzeri	2507	0.0	99.07	1502	JX094167.1
3	Stutzerimonas stutzeri	10015	0.0	99.07	4438731	LR134319.1

4	Stutzerimonas stutzeri	10099	0.0	99.07	4454378	CP027664.1
5	Stutzerimonas stutzeri	2505	0.0	99.07	1537	PP140924.1
6	Stutzerimonas stutzeri	2505	0.0	99.07	4572525	CP140298.1
7	Stutzerimonas stutzeri	2505	0.0	99.07	4572900	CP140616.1
8	Stutzerimonas stutzeri	2505	0.0	99.07	1469	OR764547.1
9	*Pseudomonas* Sp. HGLP 7	2505	0.0	99.07	1461	KX001820.1
10	*Pseudomonas* Sp. HGLP 6	2505	0.0	99.07	1459	KX001819.1

Source: NCBI, (2023)

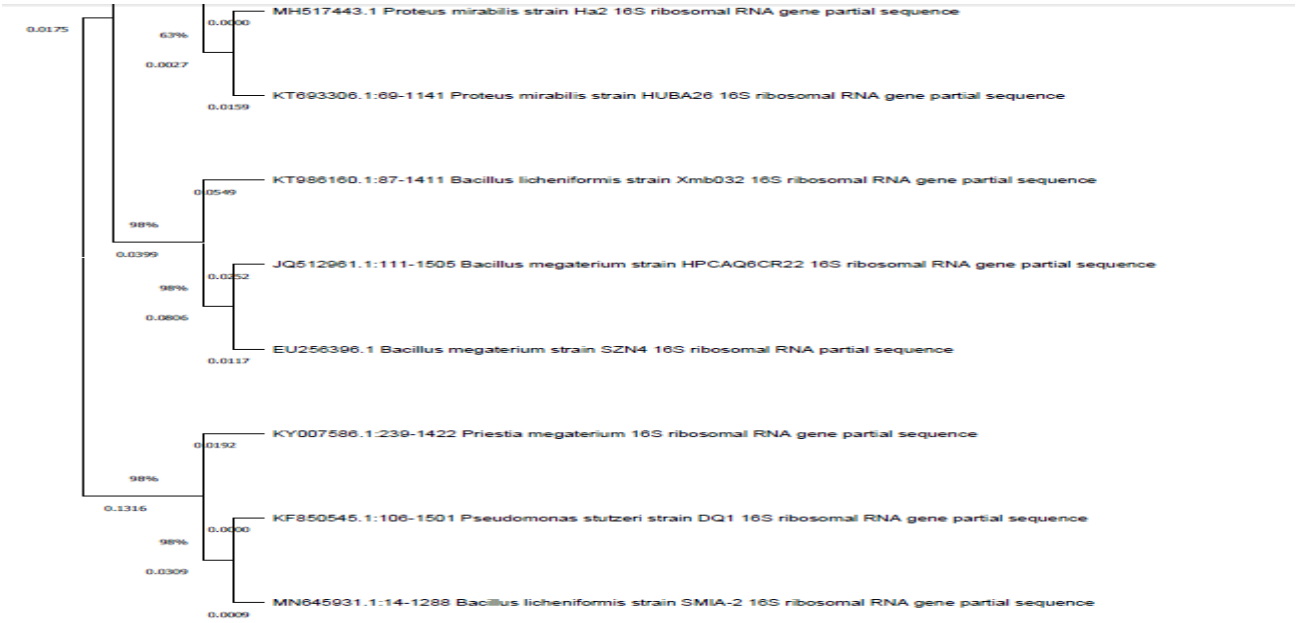

Fig III: Phylogenetic tree constructed using nucleotide sequence obtained from BLAST

Optimization of parameters the highest lipase producing isolates

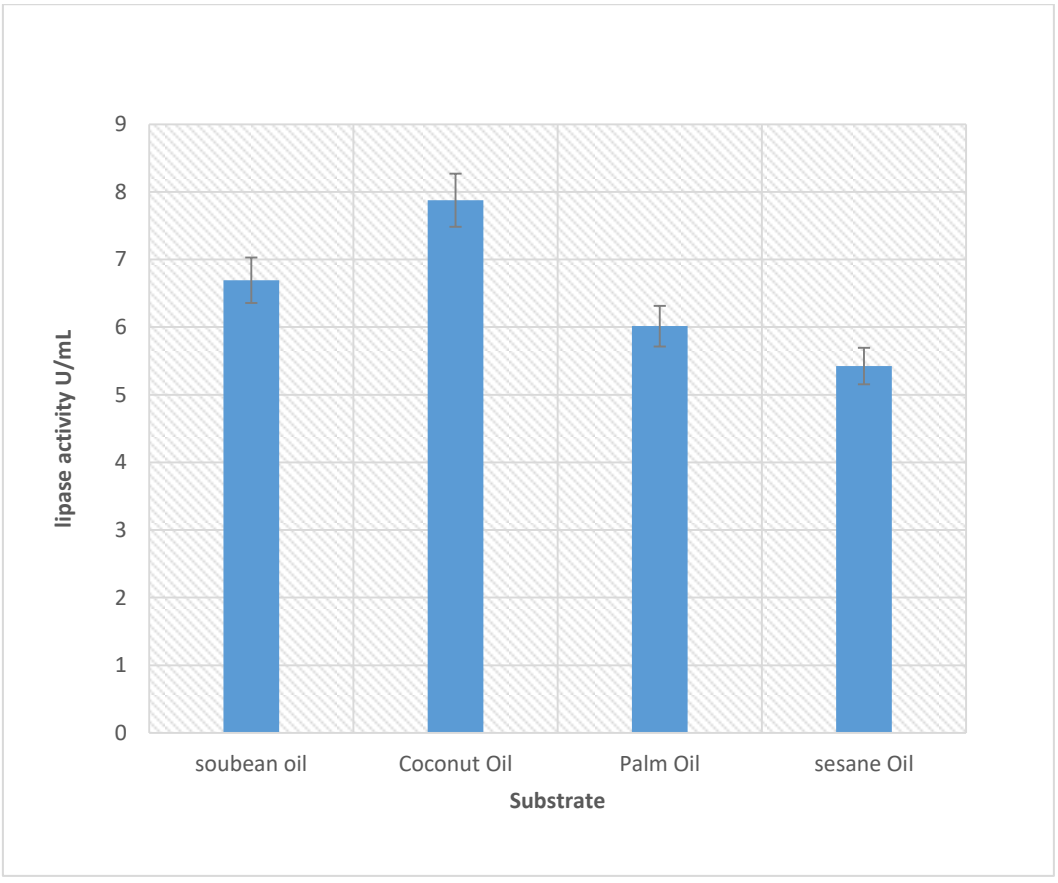

Fig IV: Effect of substrate source on lipase activity

Coconut oil has the maximum lipase activity of 7.876U/mL and the minimum lipase activity of 5.425U/mL was obtained in the media that contains sesame oil substrate as shown in fig IV.

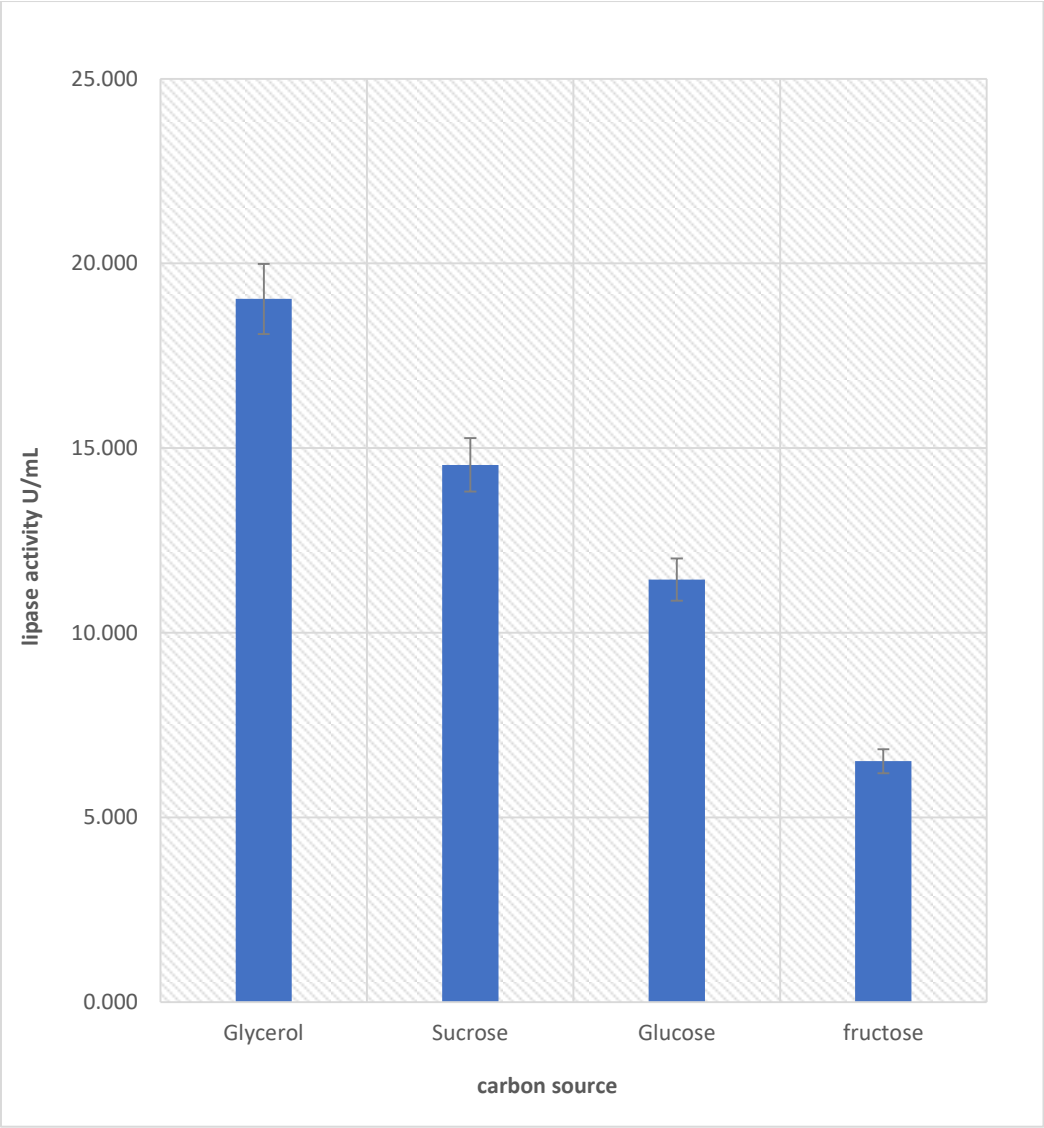

Fig V: Effect of carbon source on lipase activity

According to fig V Bacterial isolate in the culture media that contains glycerol as a source of carbon tend to produce more lipase with the lipase activity of 19.038U/mL than that of sucrose, glucose and fructose

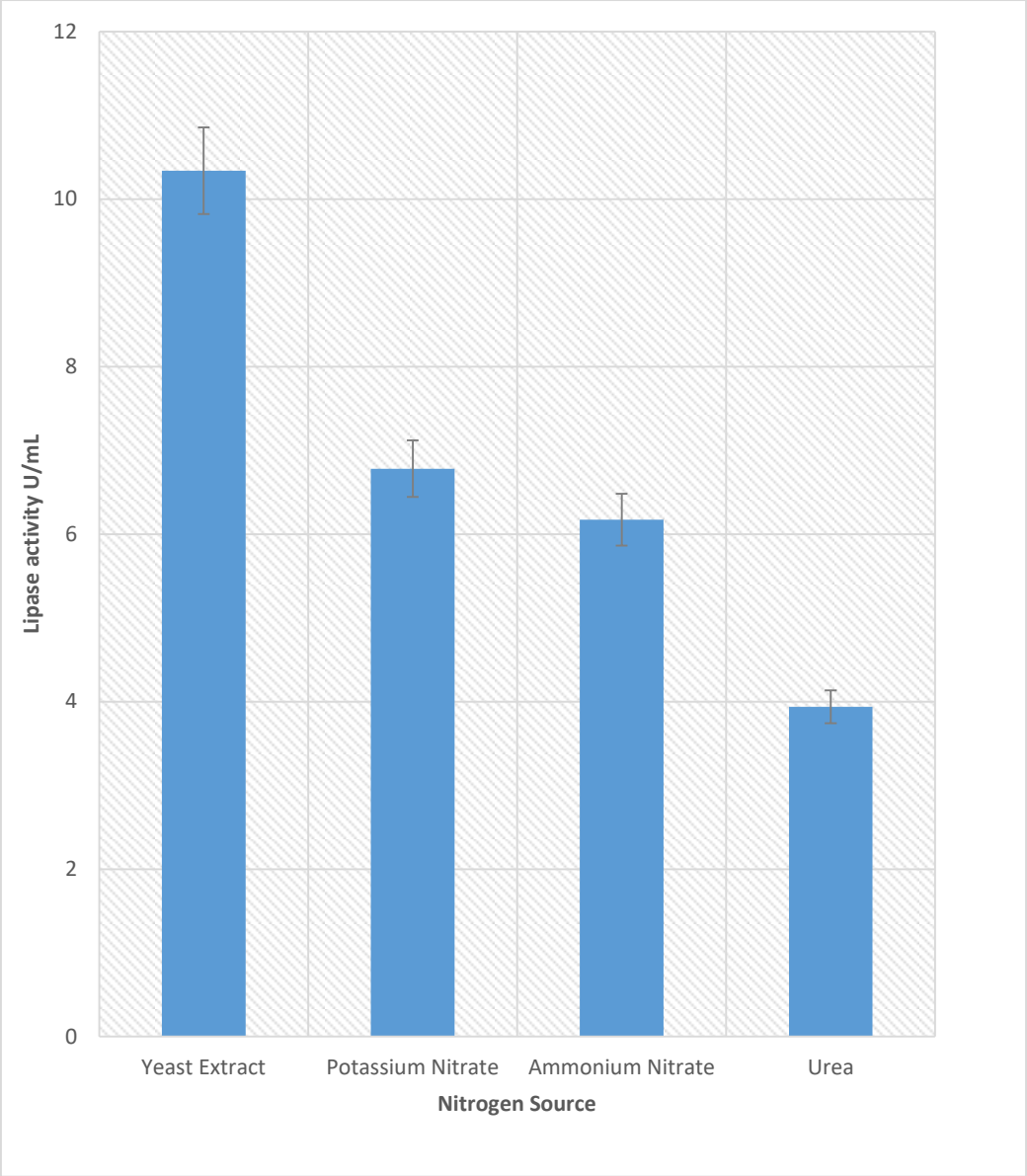

Fig VI: Effect of nitrogen source on lipase Activity

Yeast extract has the highest lipase activity of 10.340U/mL and urea has the minimum lipase activity of 3.938U/mL as shown in fig VI.

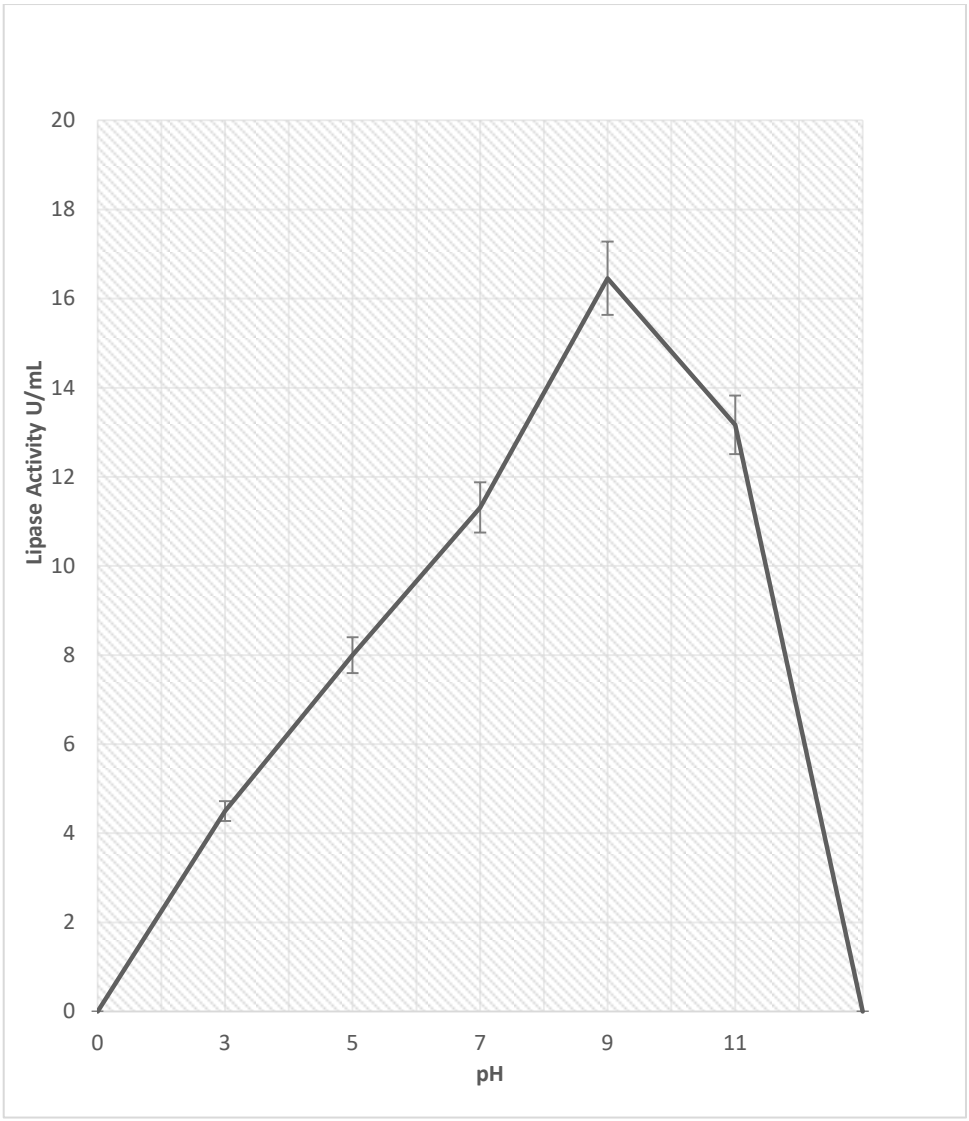

Fig VII: Effect of varied pH on lipase activity

According to fig VII pH 9 has the highest lipase activity of 16.457U/mL and lowest lipase activity of 4.498U/mL was seen at pH 3.

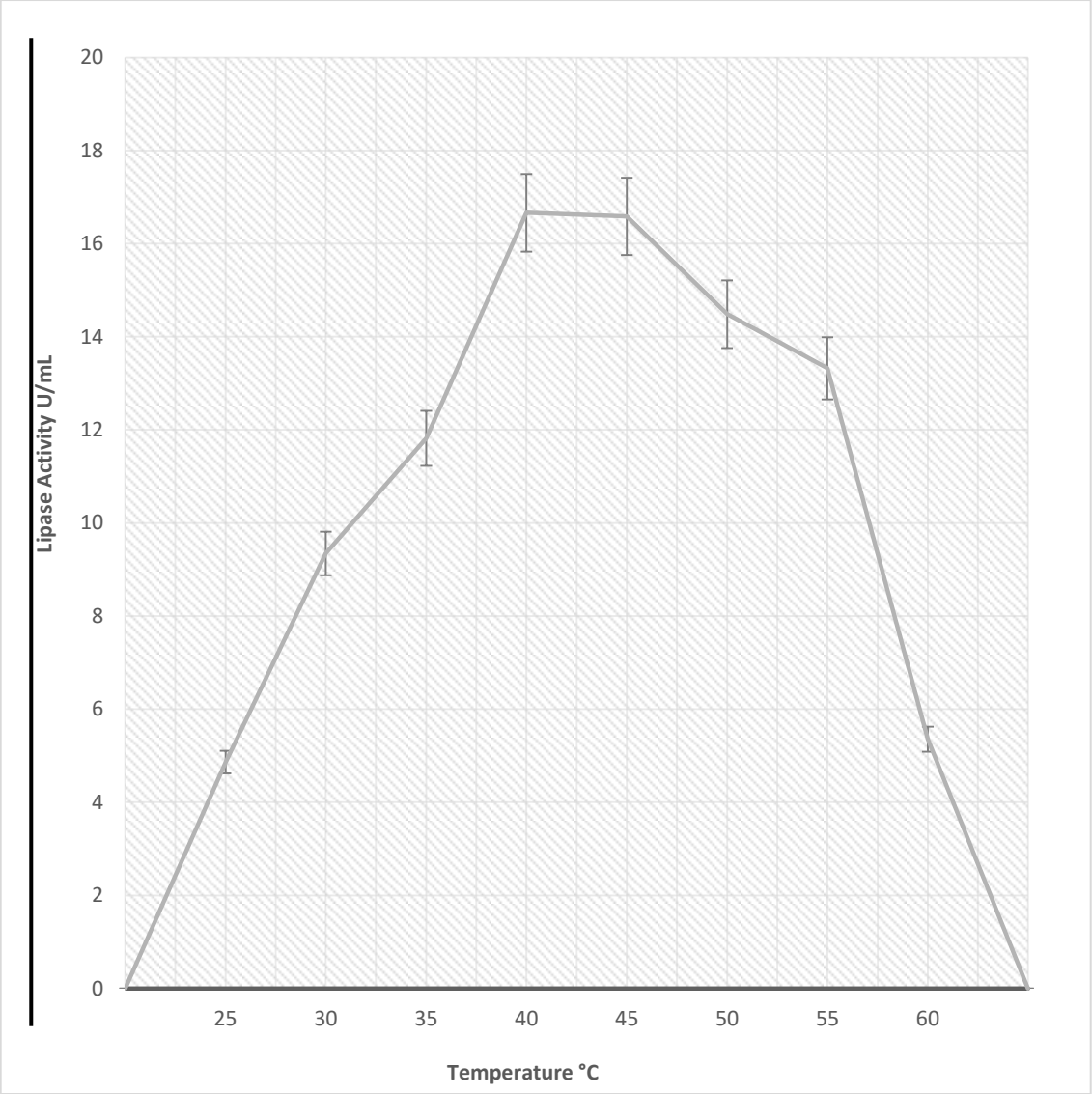

Fig VIII: Effect of temperature on lipase activity

The result on fig VIII shows that higher lipase production with activity of 16.663U/mL was seen at 40◦C and lower lipase activity of 7.860u/mL was measured at 25◦C

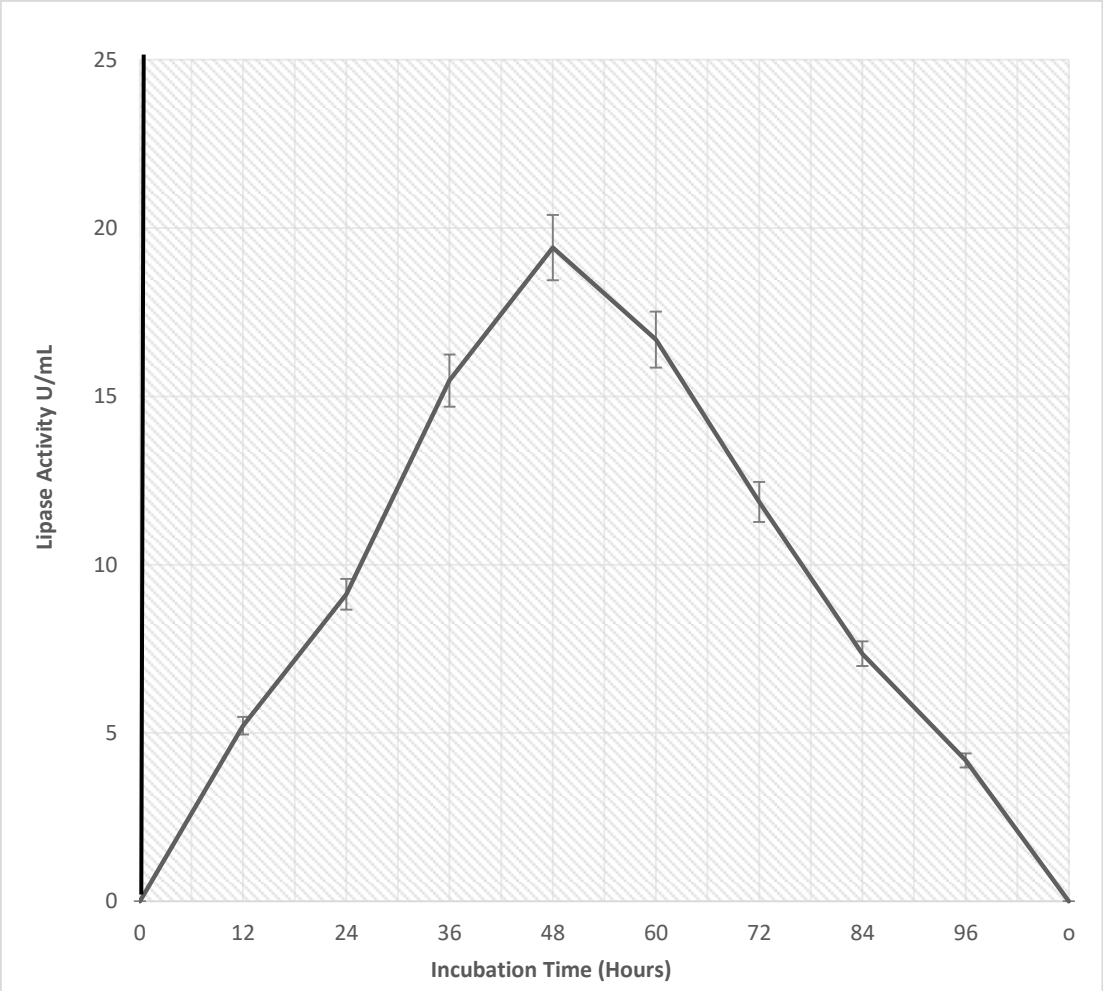

Fig IX: Effect of incubation Time on lipase activity

Maximum lipase activity of 19.423U/mL was measured at 48 hours and minimum lipase activity of 4.185 was measured at 96 hours. The result is shown in figure IX.

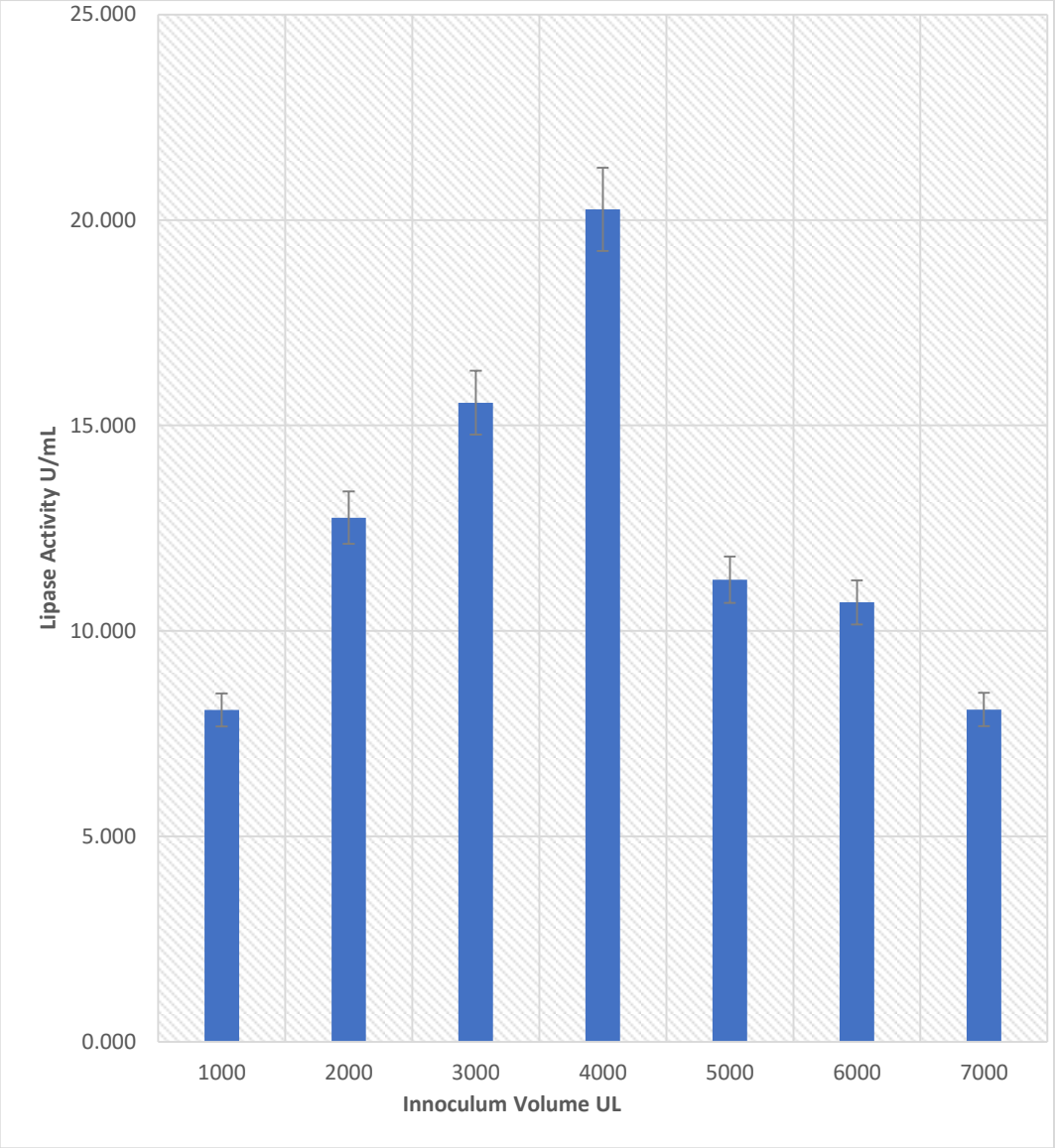

Fig X: Effect of Inoculum volume on lipase activity

As depicted in fig X, maximum lipase activity of 20.258U/mL was measured at 4000µL inoculum volume and lower lipase activity of 8.077U/mL was measured at 1000µL inoculum volume.

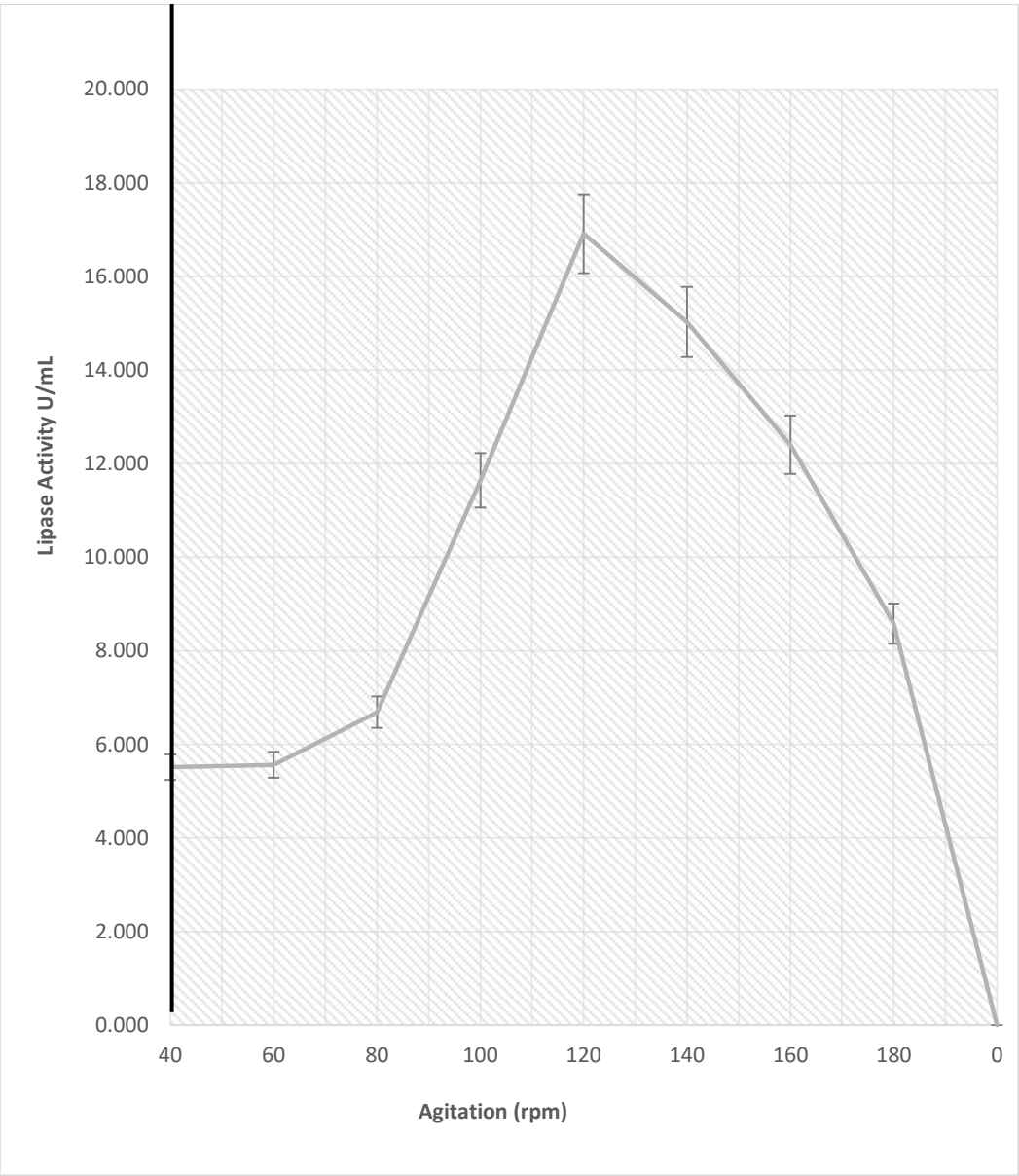

Fig XI: Effect of culture agitation on lipase activity from selected bacterial isolate.

As shown in fig XI, the maximum lipase activity of 16.912U/mL was measured at 120rpm and lower activity of 5.515U/mL was measured at 40 rpm.

Discussion

A total of ten bacteria were isolated from the three samples of which all were found positive for lipase production after screening. Screening of lipase producing bacterial isolates was done using Tributyrine, rhodamine B and tween 80 agar medium. Eight (08) bacteria were found positive for lipase production and suspected to be *Bacillus* sp., *Pseudomonas* sp., *Proteus sp* as identified by biochemical and morphological characterization of the isolates.

A similar type of isolation was reported by Sagar *et al.* (2013), who obtained similar isolates from vegetable oil contaminated soil in Tamil Nadu, India. Babatunde and Olayemi (2023) has also reported the isolation of *Bacillus* sp., *Pseudomonas* sp., and *Staphylococcus* sp., from edible oil contaminated soil in Nigeria. According to Umaru and Ahmad (2022) and Ibrahim and Adamu (2023) *Bacillus* sp, and *Pseudomonas sp* are the most dominant lipase producing bacteria in Nigerian soil.

Molecular characterization of the selected lipase producing bacteria was done by

Amplifying the16S rRNA region of the bacterial genome. The highest lipase producing bacteria from sample 2 which was suspected to be *pseudomonas sp* by morphological and biochemical characterization

was identified as *Stutzerimonas stutzeri* (formerly classified as *Pseudomonas stutzeri*) when the 16S rRNA region sequence of the bacteria was subjected to homology search using BLAST to obtain similar bacterial sequence which was used to construct the phylogenetic tree using neighbor joining method. Han, *et al.* (2020), reported that identification of bacteria by molecular techniques was found to be the most reliable method of bacterial identification.

In similar studies, highest lipase producing bacteria as identified by nucleotide sequence obtained with 16s rRNA gene include *Pseudomonas aeuriginosa* (Oluwasaye et al 2020), *Bacillus subtilus* (Baruwa et al 2021), *Anoxybacillus sp* (Bakir and Metin, 2017), *Bacillus subtilis* and *Bacillus Fumarioli* (Chouhan, 2020), *Bacillus cereus* (Kattatheyil et al 2024). *Bacillus sp* and *Stenotrophomonas sp* (Joyruth and Growther, 2020). According to a study by (Soyombo, 2024), thermophilic lipases produced by *Stutzerimonas stutzeri* in Nigeria are highly stable and have high catalytic ativity at elevated temperature and are promising biocatalyst for wide range of industrial applications.

However, the highest lipase producing bacteria was further subjected to optimize the level of lipase production by the bacteria using different substrates sources. In this study, Coconut oil tend to have higher lipase activity of 7.876U/mL than the other substrate used and. This is contrary to the work of Oliveira et al. (2013). In their study, olive oil has a lipase activity of 14.5U/mL as it supports lipase production in *Pseudomonas aeruginosa*. Olive oil contains long chain carbon (C18:1) which takes time to dissolve in media and thus gives the bacteria time to take up substrate at different time thus making it a better substrate for lipase production. In a study by Yassin *et al.,* (2023), maximum lipase activity of 8.88U/mL was attained when olive oil was used as substrate source. However, tween 80 and olive oil were the suitable substrate sources for optimum lipase production for all the bacteria in a study conducted by Pham *et al.*, (2021).

Carbon as a substrate has been a major factor of lipase production (Burcu Bakir and Metin 2017). Maximum lipase activity was obtained with glycerin as a carbon source and fructose as a carbon source shows lowest lipase activity of 3.995U/mL in this study. However, fructose tend to act as lipase production repressor and supports lipase production only in the medium containing sugar as source of carbon. On the contrary, highest lipase production was seen when sucrose was used as a carbon source in the study by Sangheeta *et al.,* (2012) and Savalia and Dungrechiya (2022).

Out of all the nitrogen sources used in this study, yeast extract stimulated lipase production most. This is in agreement with the work of Sirisha et al. (2010), where better lipase production by *Staphylococcus* was seen in medium that contains yeast extract than those that contained peptone and tryptone and Pham *et al.,* (2021) which shows yeast extract as the best nitrogen source for most bacteria. According to Gupta et al. (2004), bacteria prefer peptone and yeast extract as nitrogen sources for maximal lipase production. In a study by Yassin et al. (2023), optimum lipase activity of 9.1U/mL was achieved when peptone was used and lower activity of 1.2U/mL with sodium nitrate. Inorganic nitrogen sources such as ammonium chloride and di-ammonium hydrogen phosphate have also been reported to be effective in some microbes (Jayarama *et al.*, 2020).

A study by Yassin et al. (2023) showed Optimum lipase activity of 8.1U/mL at pH 7.0, this is supported by the studies of Murtius et al. (2022), Pham et al. (2021), Oluwasaye et al. (2020) and Gupta et al. (2004) where lipase production was best at pH 7.0 and decline as the pH increases. In a recent study by Ikhwani et al. (2024), highest lipase activity of 3.173±0.070U/mL was observed at pH 8.0. In this study, maximum lipase production activity of 16.457U/mL was measured at pH 9.0. This is similar to the work of Maythan et al. (2016) which shows that the lipase activity of *Bacillus sp* was higher at pH 9 during the 24 hours culture period. Bacteria that produce lipase at higher pH could be good candidates for industrial application.

Optimum temperature promotes binding of enzymes and substrate while temperature changes give rise to cleavage of hydrogen bonds between substrate and enzyme active sites (Gupta et al 2004; Robinson 2015). In this study, optimal lipase production activity was obtained at 40◦C. similarly, optimum lipase activity was

also recorded at 40◦C in studies by Murtius et al. (2022) and Yassin et al. (2023). This is contrary to the work of Pham et al. (2021) with optimum lipase production at 35◦C and highest lipase activity was also observed at 35◦C in a study by Ikhwani et al. (2024), in a dramatic way, the lipase activity decreased at 40◦C and 45◦C before slightly increasing at 50◦C. According to Bharathi et al. (2019), the ideal temperature for lipase is determined by the source and type of lipase.

Optimal lipase activity was seen at 48 hours of incubation while minimum lipase production activity was observed after 96 hour of incubation in this study. This indicates that the organism was at the lag phase during maximum lipase production and decrease in lipase production at 96 hour of incubation could be due to depletion of nutrients, accumulation of toxic end products, and change in pH of the medium or loss of moisture. This is in line with the study by Yu et al. (2023) were lipase production activity depleted at 96 hour of incubation. The longer the incubation time the higher the enzyme produced until it reaches it optimal point and decline (Murtius et al 2022).

Ikhwani et al. (2024) in their 60 minutes of observation found that the best lipase activity of 2.810±0.106U/mL was observed at 15^{th} minute which decline as the time increases.

Maximum lipase production with activity of 16.912U/mL was recorded at higher agitation of 120rpm. Pham et al. (2021) in their study also observed increased in lipase production under shaking condition. An agitation speed of 150rpm was found to be optimal for lipase activity due to enhanced oxygenation in the culture (Kim et al 2011). This indicates that the bacterial isolates in this study are aerobic and require large amount of dissolved oxygen for their growth and multiplication. Higher agitation (more than 150rpm) may cause enzyme denaturation and damage the byproducts due to accumulation of hydrogen peroxide (Russi and Brown 2016; Salwoom et al 2019). According to Eprahimpour et al. (2008), static culture with moderate aeration produce more lipase than shake cultures. A,yuni and IImi (2021) also emphasized that lipase productivity and enzyme specific activity decreases as agitation speed increases and high lipase production was obtained at static condition and 96 hours of incubation.

Increase inoculum volume increases lipase activity. According to a report by Salihu et al. (2012), as inolucum volume increases, so as lipase activity. This is supported by the study of Oluwasaye et al. (2020) where highest lipase activity was reported with the highest inoculum volume of 4000µL, similar to the findings in this study as higher lipase production was obtained at the highest inoculum volume of 4000µL. The study of Savalia and Dungrechiya (2022) also supported that increase in lipase activity is directly proportional to increase in inoculum volume. However, according to the report by Thakur et al. (2014), as inoculum volume increases, lipase activity decreases due to cells struggling for nutrient.

Conclusion

The lipase highest producing bacteria isolated which was identified as *stutzerimonas stutzeri* are good candidates for lipase production and certain factors such as pH, temperature, and incubation time and aeration rate play a very vital role during enzyme production and metabolic activity.

Declarations

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Competing interests

The authors have no relevant financial interests or non-financial interest to disclose.

Ethical approval

No human or animal was used in the stu

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