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Research Article

LIPASE PRODUCTION POTENTIALS OF AUTOCHTHONOUS BACTERIAL SPECIES ASSOCIATED WITH WAXY CRUDE OIL

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Abstract

Wax deposition is one of the problems of flow assurance. As a result, operational and remedial costs rise while oil output declines. One of thenovel mitigation measures is the use of pour point depressants such as lipase. The study evaluated the lipase producing potentials of autochthonous bacterial species associated with waxy crude. Four bacterial species were isolated characterized and identified as *Bacillus* sp., *Bacillus subtilis, Micrococcus* sp and *pseudomonas aeruginosa.* Analysis of the lipase producing potentials of the isolates revealed that *P. aeruginosa* exhibited the widest halo-zone (1.4 mm) this was followed by *B. subtilis* (1.1 mm), while *Bacillus* sp. and *Micrococcus* sp had a halo zone of 0.9 mm and 0.7 mm respectively (Table 3). The lipase activity of the culture supernatant was measured as the amount of free fatty acids released per ml enzyme solution per min at 37°C at a regular interval of 24 hours. Maximum lipase activity was observed from *P. aeruginosa* (10.3 ± 0.2 U/mL) followed by *B. subtilis* (9.8 ± 1.2 U/mL). The findings have asserted the fact that most lipases identified in microorganisms are secretory extracellular enzymes and can be isolated at high purity and mass produced for wider industrial application.

Keywords: lIpase production, Bacteria, Waxy crude.

INTRODUCTION

The issue of wax deposition is recognized as a continuous challenge (Golczynski and Niesen, 2001), where paraffin gradually accumulates on the inner walls of pipes when the internal surface temperature falls below the WAT for an extended period of time (Liu *et al.,* 2023). Petroleum waxes have a negative effect on the transportation of crude oil, such as restriction of crude oil flow, thus creating pressure abnormalities and leading to a reduction or interruption in production; higher pumping costs due to addition pressure drop caused by wax deposition; increases capital, operational, and maintenance costs. The primary cause of wax formation in wells, tubing, flowlines, and pipelines is typically attributed to a decrease in temperature, influenced by the crude oil's pour point (PP) and the wax appearance temperature (WAT), also referred to as the cloud point (CP) (Li *et al.,* 2021). Despite the properties of these waxes, studies have shown that they contain microbial species that can break down higher molecular weight hydrocarbons. Effective and rapid degradation of non-volatile high molecular weight hydrocarbons, such as waxes, is a critical factor in addressing wax precipitation and enhanced oil recovery (EOR) during crude oil production and transportation. Biocatalysts such as lipase are enzymes that catalyze the hydrolysis of triglycerides to glycerol and free fatty acids over an oil-water interface and reverse the reaction in aqueous and non-aqueous media (Abada, 2008). Most microorganisms are able to produce lipase extracellularly. These enzymes are resistant to solvents and are exploited in a broad spectrum of biotechnological applications. Microbial lipids have been widely used in oil industry due to their stability, selectivity, and broad substrate specificity (Sharma *et al.,* 2001; Dutra *et al.,* 2008).

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The number and variety of lipases available has increased enormously with much success attributed to exploitation of microbial potential (Vulfson *et al.,* 1994; Sharma *et al.,* 2001).Studies have also shown that microbial products and their enzymes are used as wax suppressants in the oil and gas industry (Liu *et al.,* 2012; Wenjie *et al*., 2012; Sun *et al.,* 2015; Fan *et al.,* 2020). Lipase has been widely reported to have wax depressant potentials (Afdhol *et al.,* 2019; Azeem *et al.,* 2019; Eke *et al.,* 3021). The main mechanisms of microbial wax degradation include reduction of interfacial tension (IFT), wettability alteration, oil-water emulsification, and oil viscosity reduction. There is a need to investigate the cost-effective sources of lipase-producing bacteria that can be exploited for oil and gas sectors. This study was designed to investigate the lipase producing potentials of waxy crude oil autochthonous culturable bacterial communities. The objectives of this study were to isolate and characterize bacterial strains from waxy crude-oil samples and screen their potential to produce lipase enzymes.

Methodology

Source of Waxy Crude Oil

Waxy crude oil was collected from the Niger Delta oil field and transported to the Microbiology laboratory, University of Uyo, Nigeria

Isolation of Autochthonous Lipase Producers

For efficient isolation of the culturable autochthonous bacterial community, 3.27 g/L of sterilized Bushnell and Haas medium (BHM) (BDH Chemical Ltd.) supplemented with 2% of waxy crude (v/v) as the sole carbon source (pH 7.0) was incubated in a shaker incubator at grown at 25± 2°C for 18–24 h. After incubation, 1 ml of the aliquot was plated on Tributyrin Agar

Plates (TBA) containing per litre of peptone, 5g; beef extract, 3g; tributyrin, 10ml and agar-agar, 20g followed by incubation for 24-48 hours at 37 °C to screen the microorganisms producing lipase. Tributyrin was added into the medium after autoclaving when the medium reached a temperature of 40-45°C.The lipase producing isolates produced a zone of clearance around them, hence, zone of clearance producing colonies was identified as lipase producing microorganisms and isolated. The zone size was measured after 24 and 48 hours of incubation at 37°C (Dutra et al., 2008). Colonies producing largest zone of clearance were selected from each plate, and streaked on TBA plates to obtain pure isolated colonies.

Preservation of isolates

Isolated colonies from streaked TBA plates were picked and streaked on TBA slants. Cultures were stored at 4°C and subcultured at regular intervals of time i.e.15 days.

Characterization and Identification of Positive Isolates

Standard characterization tests (such as Gram staining, catalase, coagulase, motility, starch hydrolysis, methyl-red, Vogues Proskauer, indole, citrate utilization, urease, spore staining, hydrogen sulfide production, and sugar fermentation) were performed. The pure culture was identified based on its cultural, morphological, and physiological features with those in Bergey's Manual of Determinative Bacteriology (Cowan, 1974; Buchanan and Gibbons, 1974). Details of the methods used in characterization of the isolates are as presented below;

a. Gram Staining

This differential staining was used to group bacteria into two major groups: Gram positive and Gram-negative bacteria based on their cell wall composition. In this staining process, a heat-fixed smear was prepared and air-dried. The smear was covered with crystal violet for 60 seconds, rinsed gently with running water stained with iodine and allowed for 60 seconds, before being rinsed off again. The smear was decolorized with 70% alcohol for 10 seconds and rinsed off immediately. The smear was counterstained with safranin for 30 seconds and finally washed with clean water and blotted dry. The stained smear was observed under oil immersion objective lens (100x) microscope. Gram positive bacteria-stained purple (the colour of the primary stain) while the Gram-negative bacteria stained red (the colour of the counter stain).

b. Citrate Utilization Test

This test is used to determine the potential of the bacterial isolate to use citrate as a carebon source and ammonia as its only nitrogen source. The test organisms were inoculated on the surface of sterile molten Simon citrate agar and incubated for 24 hours at 28^{o}C. Colour change from green to blue around the growth streak indicates a positive reaction for the organism.

c. Catalase Test

This test was carried out to demonstrate the presence of catalase in the organism. Catalase is an enzyme that catalysis the release of oxygen from hydrogen peroxide (H_2O_2). Two to three drops of hydrogen peroxide were placed on a clean grease free slide. The test organism was transferred to the slide with a sterile loop. Positive result was indicated by an immediate gas bubbling or effervescence off the mixture.

d. Oxidase Test

Oxidase enzymes play a significant role in the processes carried out by the electron transport system during aerobic respiration. Cytochrome oxidase uses O_2 as an electron acceptor during the oxidation of reduced cytochrome to form water and oxidized cytochrome. A filter paper was placed on a clean petri dish. Two drops of oxidase reagent were added on the filter paper. With a sterile glass rod, the fresh test organism was smeared on the filter paper. A blue colouration after 10 minutes indicated a positive result.

e. Spore Formation

Spore forming is one of the modes of survival for organisms during unfavourable conditions. Spore staining is used to identify spore forming organisms. A heat fixed smear was prepared from 24 hours old culture of the test organism. The slide was placed over boiling beaker and the smear was flooded with Malachite green staining solution for about 5-6 minutes until steam rises. Saturation was avoided by continuous flooding with the solution. The slide was cool before rinsing with water for 30 seconds. The smear was then counter stained with safranin for 60 to 90 seconds and rinsed again water for 30 seconds. The slide was then blot dried from the edge with filter paper and examined under oil immersion. The spores stained green while the vegetative cells-stained red.

f. Motility

This test demonstrates the ability of an organism to move from one place to the other with the aid of a locomotive structure like the flagella. The test tube method was used where nutrient broth was stabbed inoculated with fresh test isolate in a straight line. The test tube was covered with a cover and incubated for 3-5 days. The test tube was examined for spread from the line of stab. An observable spread indicated a positive result.

g. Urease Test

This test demonstrates the ability of an organism to elaborate urease enzyme. The enzyme is responsible for the breaking down urea to produce ammonia and carbon dioxide. 24.5g of Christiansen urea agar was diluted in 1,000 ml of water and autoclave for 15mins at 121^{o}C. 10ml of 20% urea was dissolved into agar and shaken to mix; it was then poured into Petri dishes. The test organism was then inoculated via streaking on the media and incubated for 24 hours at 28^{o}C. Development of pink coloration indicated a positive urease test while not pink color indicated negative urease test

h. Starch Hydrolysis

This test demonstrates the ability of an organism to hydrolyze starch. To determine this, the test isolates were streaked on starch supplemented agar plates and incubated for 24 hours. After the 24-hour incubation time, iodine is added to the plate and observed. The appearance of transparent clear zones around the colonies indicated positive results, i.e., the test organism is able to hydrolyze starch while dark blue coloration indicates negative result i.e., the unhydrolyzed starch forms the colored complex with starch.

i. Hydrogen Sulphide (H_2S) Test

This test demonstrates the ability of the isolate to reduce sulphur containing compounds to hydrogen sulphide during metabolism. Here, the test isolates were inoculated into test tubes containing Klinger Iron Agar (KIA) and incubated for 24 hours. After 24 hours of incubation the test tubes were observed. Development of blacken spots indicated positive results while absence of blacken spot indicated negative result.

j. Sugar Fermentation Test

This test demonstrates the ability of microorganisms to ferment carbohydrates by utilization of sugar as source of carbon to produce acid and gas. Exactly 1 gram of sugar was added to 1% peptone water. Then 0.01% phenol red indicator was added into the medium and 10 ml were dispensed into the test tubes with inverted Durham's tubes. The test tubes and their contents were sterilized in the autoclave. Each test organism was inoculated into each sugar tube and incubated at 28^o C for 24 hours. Tubes with yellow colouration indicated acid production, while tubes with yellow colouration and gas bubbles in the inverted Durham's tubes indicated both acid and gas production.

Lipase Enzyme Production

The method described by Mahale*et al.,* (2015) was adopted. A production medium comprising 1.5g molasses, 1g yeast extract, 2.2ml waste groundnut oil, 100ml d/w, 0.5g KH_2PO_4, 0.1g $CaCo_3$, 0.1g $(NH_4)_2SO_4$ and 0.1g $MgSO_4.7H_2O$ was used. Overnight cultures of the test isolates were inoculated in 100 ml of the production medium via the submerged microbial culture in 250 ml Erlenmeyer flasks. Inoculated flasks were incubated in a rotary shaker (150 rpm) at 37°C for 24 hours. After 24 hours. The culture was centrifuged at 10,000 rpm from 10 min at 4^{o}C and the cell free culture supernatant fluid was used as the source of extracellular enzyme. The lipase activity in the supernatant was determined by the titrimetric method.

Lipase Assay: Titrimetric Method

One milliliter (1ml) of the crude enzyme was mixed with 9.9ml of distilled water and 0.1ml of oil. The mixture was incubated at room temperature for 15 minutes and then titrate against 0.05N NaOH using phenolphthalein indicator. The micromoles of free fatty acids obtained from the oil per mL crude lipase enzyme was used to calculate lipase activity:

$$Activity = \frac{(V_s - V_B)x\ Nx\ 1000}{S}$$

Where, V_S is the volume of NaOH solution used by the enzyme-substrate cocktail (mL), V_B is the volume of NaOH used in control cocktail (mL), N is the molarity if NaOH (0.05M), S is the volume of substrate cocktail solution (10 mL). One unit (U) is the amount of lipase enzyme necessary to liberate 1 mol of fatty acids from triglycerides (Baltaci *et al.,* 2018).

RESULTS AND DISCUSSION

Microorganisms are distributed in the biosphere (Atlas and Bartha, 1992). One of the indices of microbial adaptation in any ecosystem is its biomass. The culturable autochthonous heterotrophic bacterial density in the waxy samples ranged between $1.3 \pm 0.3 \times 10^4$ and $2.6 \pm 0.3 \times 10^4$ CFU/ml (Table 1). The bacterial density in the waxy crude was lower than the density reported by Henry *et al.*(2022) for crude oil-contaminated soil (4.6×10^7 CFU/g). Four distinct bacterial colonies were obtained, purified, and screened for biosurfactant production. Two of the isolates (WAS-1, WAX-2) were Gram-positive rods, one (WAX-3) was Gram-positive cocci, while the last (WAX-4) was a Gram-negative rod. Biochemical characterization of the isolates revealed the identities (Table 3).

Table 1. Density of culturable autochthonous heterotrophic bacteria in the waxy crude

Samples	THBC (x 10^4 CFU/ml)
Waxy crude 1	2.6 ± 0.3
Waxy Crude 2	1.3 ± 0.3

All the isolates were able to elaborate lipase and exhibited luxurious growth on TBA with colonies surrounded with clear zones. *P. aeruginosa* exhibited the widest halo-zone (1.4 mm) this was followed by *B. subtilis* (1.1 mm), while *Bacillus* sp.and *Micrococcus* sp had a halo zone of 0.9 mm and 0.7 mm respectively (Table 3).The lipase activity of the culture supernatant was measured as the amount of free fatty acids released per ml enzyme solution per min at 37°C at a regular interval of 24 hours. Maximum lipase activity was observed from *P. aeruginosa* (10.3 ± 0.2 U/mL) followed by *B. subtilis* (9.8 ± 1.2 U/mL).

Table 2. Biochemical Characterization and Identification of Bacteria Isolated from Waxy crude and synthetic crude samples

Isolate Code	Gram Reactions	Catalase	Motility	Starch hydrolysis	Citrate	Urease	Spore formation	H_2S	Oxidase	Glucose	Maltose	Xylose	Lactose	Fructose	Sucrose	Mannitol	Galactose	Probable organisms
WAX-1	+ve	+	+	+	-	-	+	-	-	AG	A	-	-	A	A	-	A	*Bacillus* sp.
WAX-2	+ve	+	+	+	+	-	+	-	-	AG	A	A	-	A	-	-	A	*Bacillus subtilis*
WAX-3	+ve	+	-	+	+	+	-	-	+	-	A	A	-	A	-	A	A	*Micrococcus sp*
WAX-4	-ve	+	+	-	+	-	-	+	-	A	-	-	AG	AG	AG	AG	AG	*Pseudomonas aeruginosa*

Table 3. Lipase Activities of the Bacterial Isolates

Isolate	Diameter of halo zone (mm)	Lipase Activity (U/mL)
Bacillus sp	0.9	7.4 ± 0.2
Bacillus subtilis	1.1	9.8 ± 1.2
Micrococcus sp	0.7	5.3 ± 0.5
Pseudomonas aeruginosa	1.4	10.3 ± 0.2

The findings of this study agree with previously reported data. Kanimozhi and Perinbam (2015), while investigating lipase producing potentials of bacterial isolates from oil contaminated sites, reported that the isolate with the highest lipase activity was *Pseudomonas* sp. strain Lp1 based on its biochemical, cultural and morphological characteristics.

Conclusion

This study demonstrated the ability of culturable autochthonous bacteria species (*P. aeruginosa, B. subtilis, Bacillus* sp. *and Micrococcus* sp.) associated with waxy crude oil to elaborate lipase. The findings have asserted the fact that most lipases identified in microorganisms are secretory extracellular enzymes and can be isolated at high purity and mass produced for wider industrial application.

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