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Bioprospecting for Potential Antimicrobial-Producing Bacterial Isolates from Petroleum-Contaminated Soil in Port Harcourt, Rivers State

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Abstract

The growing threat of antimicrobial resistance and dearth of novel antimicrobial products from natural soil indicates the need to explore alternative environments. The study screened for potential antibiotic-producing bacterial isolates in soil from a petroleum-contaminated environment. Petroleum-contaminated soil sample (10 g) from Port Harcourt, River State was cultured on nutrient and Czapek-Dox agar media for bacteria isolation using pour plate technique. Isolates were screened for antimicrobial potential against clinical strains *Staphylococcus aureus* and *Escherichia coli* using cross-streak method. Cell-free supernatant (CFS) of bioactive isolates collected at 24-hour intervals during a 3-day fermentation was used for secondary screening via agar well-diffusion technique. Antimicrobial compound was extracted through solid-liquid extraction using diaion HP-20 beads and a three-solvent mixture, and bioactivity of extracts confirmed using agar well-diffusion method. Bacteria with antimicrobial production potential were identified via their morphological and biochemical properties. Nine (9) distinct colonies were isolated, four (4) isolates had antimicrobial potential against *S. aureus*, two (2) against *E. coli*, while two (2) isolates inhibited both strains. The CFS of isolates two isolates retained antimicrobial activity against both clinical isolates, while their bioextracts produced 24 and 35 mm inhibition zones respectively against *S. aureus* only. Both were identified as *Bacillus sp.* and *Citrobacter sp.* respectively. This research showed that there is presence of antibiotic-producing bacterial strains in petroleum-contaminated soil and the environment could be better explored as a source of novel antibiotic compounds.

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Introduction

Antibiotics are chemical substances produced as secondary metabolites of microorganisms and selectively inhibit bacteria. Penicillin and Streptomycin were discovered and introduced into clinical practice in the 1930-40s,

and several other compounds were discovered afterwards to cure previously life-threatening infections. However, there is a dearth of new compounds with over 90% of antibiotics approved for clinical applications being synthetic derivatives of existing compounds (Muteeb et al.,

2023). Coupled with growing threat of antimicrobial resistance, new products are scarcely being reported.

The history of antibiotics discovery has described production of new compounds from the screening of naturally selected isolates from various habitats (Overby & Barrett, 2006). Thousands of antimicrobial compounds investigated as antibiotic leads have been purified from different bacteria, fungi and *Actinomycetes* spp. sourced from natural soils (Alvarez-Martinez et al., 2020). Continued search in these environments might results in previously purified compounds (Spellberg, 2014); therefore, underexplored and unusual environments are being explored towards increasing the likelihood of discovering novel antimicrobial compounds (Quinn & Dyson, 2024).

Petroleum-contaminated environment is one of such unnatural and unexplored environments that can be considered for bioprospecting potential antibiotic-producing organisms. The microbes from this environment have been reported for their possession of specialized functions, especially in bioremediation and biosurfactant production (Pal et al., 2019). Ability for specialized functions indicate the presence of specialized metabolism, and isolates from such environment can also be investigated for antimicrobial compounds production. There is also a dearth of information on prospecting this environment, hence, the need to bioprospect this habitat for microorganisms and their products with antimicrobial potential. This study investigated the antimicrobial potential of extracts of bacteria species isolated from petroleum-contaminated soil environment.

MATERIALS AND METHODS

Clinical Isolates

Antibiotic-sensitive clinical isolates *Staphylococcus aureus*, *Enterococcus faecalis*, *Enterobacter sp.* and *Escherichia coli* were obtained from the culture collection of the Microbiology laboratory, Department of Microbiology & Biotechnology, McPherson University. Strains were sub-cultured on Nutrient Agar (NA) plates to obtain fresh isolates for identification and antimicrobial assay.

Sample Collection and Bacteria Isolation

Soil sample (5 g) was aseptically collected at 10 cm depth from different points in the petroleum-contaminated environment in Port Harcourt, Rivers State, Nigeria (4° 46' 34.5684"N, 6° 57' 28.8216"E). Sample was thoroughly mixed and 1

g aseptically transferred into sterile test tubes, with serial dilution to the sixth diluent. Aliquot (1 mL) was thereafter inoculated on Czapek-Dox agar (CDA) and Nutrient Agar (NA) respectively using the pour plate method, and seeded plates incubated at 37 °C for 24-72 hours. Distinct colonies were sub-cultured on NA to obtain pure isolates, and stored on NA slants at 4 °C for further studies. Fresh isolates obtained via subculture were used for antimicrobial and identification studies.

Primary Screening for Potential Antibiotic-Producing Isolates

Primary screening was carried out via the standard cross streak method as previously described (Balouiri et al., 2016). Test isolates were inoculated by streaking in a straight line at the centre of the plate and incubated for 24 hours at 37 °C. Thereafter, clinical isolates were introduced at perpendicular angles, and plates were incubated for a further 24 hours at 37 °C. Inability of clinical isolates to grow close to test isolate indicated its antibiotic production potential.

Fermentation and secondary screening for antimicrobial production potential

Tryptone Soya Broth (TSB) was used as fermentation medium for antimicrobial production. Sterilized TSB Medium in a fermentation flask was inoculated with potential antimicrobial-producing isolates (12-24hrs old; 1% broth volume). Flasks were incubated at 37 °C and 150 rpm for 96 hours in a shaker incubator. At 24-hour intervals, aliquot (1 mL) of fermentation broth was centrifuged at high speed for 10 mins to obtain cell-free supernatant (CFS) to determine possible antibacterial activity using the agar well-diffusion protocol. Briefly, Muller-Hinton agar (MHA) was inoculated with clinical isolates adjusted to 0.5 McFarland's standard, and wells were bored on seeded plates using sterilized cork borer (6 mm diameter). CFS of antibiotic-producing bacteria isolates (100 µL) were added to appropriately labeled wells, and plates were incubated at 37 °C for 18-24 hours. Zones of inhibition (ZI) around the wells were measured and recorded appropriately.

Extraction and antimicrobial activity of bioextracts
Antimicrobial component of CFS was extracted using solid-liquid extraction method as previously described (Okudoh & Wallis, 2012). Diaion HP-20 beads (5g) activated in 100% methanol solution was added to CFS and mixture allowed to interact in a conical flask at 30 °C and 150 rpm. After 3

hours, beads were washed with excess deionized water and bounded compounds were extracted in methanol-ethyl acetate-ethanol solvent mixture at 150 rpm for 1.5 hours. Extracted solution was concentrated on rotary evaporator (9-12 °C chiller temperature, 35-45 °C water bath temperature, 140 mmPa vacuum pump pressure, 60 rpm) to remove solvent from crude extract used to determine antimicrobial activity as previously described.

Bacterial Identification

Morphological and biochemical tests were used to determine probable identity of only antibiotic-producing isolates. Gram staining

All tests were done according to standard microbiology techniques.

Results & Discussion

Nine (9) distinct bacterial colonies were isolated from the petroleum-contaminated soil. Six (6) isolates were obtained on CDA, while three (3) distinct colonies were sub-cultured from NA medium. Bacteria are ubiquitous and can be found in different types of environment. Their presence in petroleum-contaminated soil is further confirmation.

Table 1: The primary screening of bacteria isolate for the potential to produce antibiotic

S/N	Isolates	<i>S. aureus</i>	<i>E. coli</i>
1	CDA01	+	-
2	CDA02	-	-
3	CDA03	+	+
4	CDA04	-	-
5	CDA05	-	-
6	CDA06	-	-
7	NA01	-	-
8	NA02	+	-
9	NA03	+	+

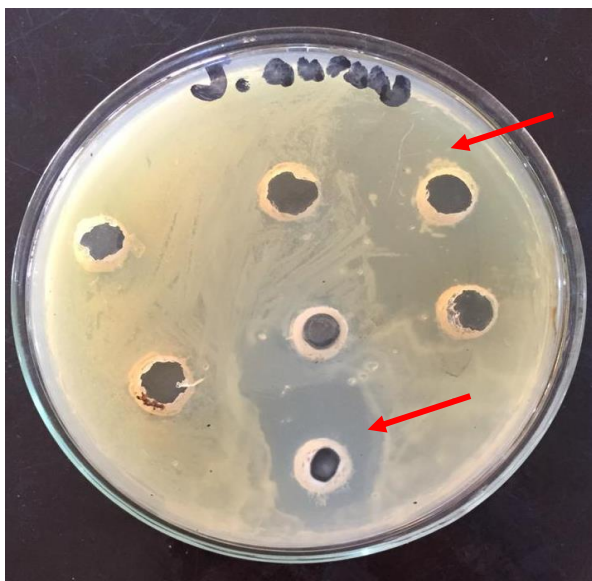


Figure 1: Clear zones (arrows) showing the potential antimicrobial producing isolate

technique was used to determine the morphological properties while biochemical tests including Citrate, Nitrate, Voges-Proskauer, Urease, Catalase, Oxidase, Indole and Hydrogen sulphide (H₂S) utilization tests were carried out for presumptive identification.

Isolation of nine distinct colonies from the polluted environment in this study is comparable with a previous report (Mashoria et al., 2014). It however higher than four bacterial isolates reported from petroleum-contaminated soil in Abuja and India (Khan & Rizvi, 2011; Ugo & Moneke, 2011; Sandhya et al., 2015).

Table 2: Antimicrobial activity of bacterial isolates against selected clinical isolates from 24 to 96 hours

Isolates	Zone of inhibition (mm)							
	24 hours		48 hours		72 hours		96 hours	
	<i>S. aureus</i>	<i>E. coli</i>	<i>S. aureus</i>	<i>E. coli</i>	<i>S. aureus</i>	<i>E. coli</i>	<i>S. aureus</i>	<i>E. coli</i>
CDA01	-	-	-	-	-	-	-	-
CDA02	-	-	-	-	-	-	-	-
CDA03	-	-	16	-	-	-	-	-
CDA04	-	-	-	-	-	-	-	-

CDA05	-	-	-	-	-	-	-	-
CDA06	-	-	-	-	-	-	-	-
NA01	-	-	-	-	-	-	-	-
NA02	-	-	-	-	-	-	-	-
NA03	-	-	20	-	-	-	-	-

Table 3: Bioactivity of extracts from potential antimicrobial compound-producing isolates

Zone of inhibition (mm)		
	<i>B. cereus</i>	<i>E. coli</i>
CDA03	24	-
NA03	35	-

Presence of fewer strains could be due to poor growth and environmental parameters in general growth media used. Natural or contaminated soil is home to a very diverse bacterial community, described by a recent study that identified thirty nine (39) species on on zone of inhibition in petroleum-contaminated soil using Bushnell Hass medium (Ahmed & Singh, 2025).

Primary screening outcome for antimicrobial potential by isolates is described in Table 1. Four (4) isolates inhibited the growth of *S. aureus*, while two (2) inhibited *E. coli*. Two (2) isolates

described retention of crude extracts of both CDA03 and NA03 based on zone of inhibition described retention of antimicrobial activity against *S. aureus*, while extract from both isolates lost activity against *E. coli* (Table 3). Furthermore, NA03 had higher inhibition zone (35 mm) than CDA03 (24 mm) (Figure 1). Table 4 described outcomes of morphological and biochemical test carried out on the antimicrobial-producing isolates and their presumptive identities. Both isolates are rod-shaped but CDA03 is Gram positive, while NA03

Table 4: Biochemical characteristics of bacterial isolates exhibiting antimicrobial production potential

Isolate code	Cell shape	Gram Reaction	Catalase	Urease	Oxidase	H ₂ S	Indole	Citrate	Voges-Proskauer	Nitrate	Probable Organism
CDA03	Rod	+	+	-	+	-	+	+	+	+	<i>Bacillus sp.</i>
NA03	Rod	+	+	+	-	+	-	+	-	+	<i>Citrobacter sp.</i>

(CDA03 and NA03) however inhibited the growth of both clinical strains. Outcome of secondary screening after 96 hours revealed lack of antimicrobial activity at 24, 72 and 96 hours respectively for majority of isolates following antimicrobial activity assay (Table 2). Bioactivity was only observed in CFS of two isolates (CDA03 and NA03) at 48 hours. Both isolates retained antimicrobial activity against only *S. aureus* but not *E. coli*. Also, the zone of inhibition of NA03 (20 mm) was higher than that of CDA03 (16 mm). Antimicrobial activity of crude extracts of both CDA03 and NA03 based is Gram negative.

Differences were observed in their reactions to urease, oxidase, hydrogen sulphide and Voges-Proskauer tests. They were therefore identified as *Bacillus sp.* and *Citrobacter sp.* respectively. Identification of *Bacillus sp.* and *Citrobacter sp.* from soil is commonplace and both have also been reported from petroleum-contaminated environments in different parts of Nigeria (Ibrahim et al., 2016; Kawo & Faggo, 2017; Okoye et al., 2020; Anaukwu et al., 2021). Exhibition of antimicrobial potential by both *Bacillus sp.* and *Citrobacter sp.* against clinical isolates could be due to adapted metabolism to

their contaminated environment (Peric-Concha & Long, 2003). However, low number of isolates with antimicrobial potential reiterates knowledge that petroleum-contaminated soil is more of an environment for biodegrading microorganisms (Brooijmans et al., 2009). Antimicrobial ability against both *S. aureus* and *E. coli* indicates capability of the isolates to produce broad spectrum compounds active against both Gram +ve and Gram -ve clinical strains. However, loss of activity on fermentation could be due to a return to a nutrient-rich environment. This means there is absence of stress previously posed by co-isolation and competition for resources by both potential antimicrobial producer and clinical strains (Hibbing et al., 2010; Cornforth & Foster, 2013).

Zone of inhibitions describes the antibacterial activity of extracts from potential antimicrobial producers, and the sizes observed for solution from *Bacillus sp.* and *Citrobacter sp.* indicates potent bioactivity. Similar inhibition zone has been previously reported for activity of antibacterial-producing bacteria and fungi isolated from both natural and petroleum-contaminated soil against *S. aureus* (Ogbonna et al., 2013; Gislin et al., 2018). *Bacillus sp.* from this study also has higher antimicrobial activity against *S. aureus* compared to previous report of its antimicrobial activity where it was isolated from soil samples in Okitipupa, Ondo State (Adeoye et al., 2020). Furthermore, loss of inhibitory effect against *E. coli* by extract from both strains could be due to loss of bioactive compounds during extraction, and presence of more than one compound being produced by the environmental isolates. Antimicrobial activity against *S. aureus* only could also indicate that the active component possesses narrow spectrum activity.

Conclusion

This study isolated nine bacterial species from the petroleum-contaminated environment, and this can be improved in future investigations using growth media enriched or selective for the environment. The study also described isolation of antimicrobial compound-producing bacterial strains *Bacillus sp.* and *Citrobacter sp.* from petroleum-contaminated soil with both broad and narrow-spectrum activity, indicating this potential alternative soil environment could be a source of novel compounds.

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