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Screening for Biosurfactant-Producing Bacteria Isolated from Abattoir Soil.

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Abstract

Biosurfactants possess essential properties that make them highly indispensable in many industries, such as waste management, food, agriculture, and pharmaceuticals, due to their surface-active and wetting abilities. The search for microorganisms capable of producing this extensively utilised biomolecule is increasing daily. In this study, abattoir soil collected from Awka metropolis, Anambra State, was screened for biosurfactant-producing bacteria using mineral salt medium (MSM) supplemented with engine oil. Three bacterial isolates (NJ1, NJ2, and NJ3) were obtained from MSM agar. These isolates were subjected to four biosurfactant screening assays: haemolysis test, drop collapse test, oil spreading test, and emulsification index (E_{24}). The results indicated that all three isolates showed alpha-haemolytic activity and drop collapse positive reactions. Notably, NJ3 exhibited a complete collapse reaction (+++) in the drop collapse test. Additionally, in the oil spreading test, NJ3 demonstrated the highest displacement of engine oil (10 mm), followed by NJ2 (7 mm) and NJ1 (5 mm). The determination of E_{24} using kerosene for all isolates revealed that NJ1 exhibited the highest E_{24} at 13%, while NJ2 and NJ3 had E_{24} of 2.56% and 2.50%, respectively. Morphological characterisation, Gram staining, and biochemical analyses performed on the isolates identified NJ1 and NJ3 as members of *Streptomyces* spp., while NJ2 was identified as *Bacillus* sp. These findings suggest that abattoir soil could be a potential source of biosurfactant-producing bacteria.

Keywords: Biosurfactant, Abattoir, Oil Spreading, Screening, *Streptomyces* spp

Introduction

Microorganisms can produce secondary metabolites that have applications in the cleanup of polluted environments, especially those impacted by organic pollutants and heavy metals. The application of these metabolites, known as biosurfactants, is gradually reducing the dependency on the synthetic surfactants that otherwise have been heavily relied on for remediating such contaminations. Biosurfactants are products of microbial secondary metabolism distinguished by possessing a hydrophilic head and a hydrophobic tail, a chemical property that enables them to minimise surface and interfacial tensions (Uyar & Saglam, 2021). Biosurfactants are produced by different microorganisms, such as bacteria, fungi, and yeast (Sapute *et al*., 2010). They may be secreted extracellularly or bound to cells during growth on water-immiscible surfaces (Martinho *et al*., 2019). These secretions of biosurfactants have been attributed to the series of processes engaged by microorganisms in the utilisation of organic substrates (hydrocarbon) as a

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source of carbon and energy (Ndibe *et al*., 2018). Biosurfactants have also been reported to be produced from animals and plants (Ruangprachaya & Chuenchomrat, 2018).

The advantage biosurfactants have over synthetic surfactants is that their introduction does not produce or leave behind toxic residues of global health concern. Also, their structural designs make them more suitable for the effective degradation of pollutants as they tend to accumulate more between fluid phases (Nunes *et al*., 2021). They are also known to be very active even in high temperatures, pH, and salt concentrations, and they are very biocompatible and digestible. They also have selective interactions and perform unique activities in their applications (Santos *et al*., 2016). There are various forms of biosurfactants; they include the low molecular weights responsible for the reduction of interfacial/ surface tensions between two immiscible phases (glycolipids, lipopeptides and lipoproteins, phospholipids, and fatty acids). And high molecular weights that have little or no effect on surface tensions; they possess complex structures (mixtures of heteropolysaccharides, lipopolysaccharides, lipoproteins and proteins), and they are mainly emulsifiers (Nikolova & Gutierrez, 2021).

Apart from aiding bioremediation via detergency and emulsification, biosurfactants also possess antimicrobial properties, and they are also used in the production of high-value products (Nunes *et al*., 2021). They function as wetting agents and are also used in the production of semiconductors (Marchut-Mikołajczyk *et al*., 2021). Although the application of biosurfactants in biotechnology and bioprocessing industries is promising, the cost of their production for industrial application is not sustainable when compared to their chemical counterpart (Alves *et al*., 2019). To bridge the gap, the cost of production must be reduced by seeking renewable materials, especially agro-waste products (Marchut-Mikołajczyk *et al*., 2021). Isolation, screening, and identification of microorganisms involved in biosurfactant production are the first considerations in biosurfactant research workflow (Biniarz *et al*., 2015). Therefore, there is a need for continuous efforts to seek microorganisms from different habitats capable of producing high-yield and efficient biosurfactants. In this study, soil from the abattoir was screened for biosurfactant-producing bacteria using four different assay methods.

Materials and Methods

Sample collection

Soil samples were collected randomly from various points at a depth of 5 -10 cm from an abattoir located at latitude 6.25^0N and longitude 7.14^0 E, at an altitude of 33 metres above sea level, situated on Awka/Enugu road in Amansea, Anambra state, Nigeria. The samples were placed in sterile zip bags and transported to the laboratory for further analysis.

Isolation of biosurfactant-producing microorganisms

Ten grams (10g) of soil sample was inoculated into 90ml of sterile mineral salt medium (MSM) broth ($NaNO_3$ 15g/l; KCl 1.1 g/l; $FeSO_4$. $7H_2O$ 0.00028 g/l; K_2HPO_4 4.4 g/l; KH_2PO_4 3.4 g/l; $MgSO_4.7H_2O$ 0.5g/l; $MnSO_4$ 0.05 g/l; and Na_2HPO_4 0.44 g/l) and 2% engine oil (EO) constituted in 250ml Erlenmeyer flasks at pH 7 and incubated at 35^0C on a rotary shaker at 150rpm for 7 days. Thereafter samples were serially diluted (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}) and plated on mineral salt medium (MSM) agar (Agar 15g/l; $NaNO_3$ 15g/l; KCl 1.1 g/l; $FeSO_4$. $7H_2O$ 0.00028 g/l; K_2HPO_4 4.4 g/l; KH_2PO_4 3.4 g/l; $MgSO_4.7H_2O$ 0.5g/l; $MnSO_4$ 0.05 g/l; and Na_2HPO_4 0.44 g/l) and incubated at 35^0C for 7 days and observed for growth. Isolates were purified by subculturing on MSM agar before isolates were stored on MSM agar slants at 4^0C (Pardhi *et al*., 2020).

Biosurfactant production

Isolates were suspended in MSM media supplemented with 1% engine oil and kept in a rotary shaker (150rpm) at 35^0C overnight. Subsequently, 2% of the culture was now inoculated into a 30ml MSM media incubated for 5 days under the same conditions. These were centrifuged at 4000rmp for 20 minutes, and the cell-free supernatants were used.

Biosurfactant screening tests

The following screening tests were performed:

Haemolytic activity

A haemolytic assay was performed by supplementing agar plates with 5% (v/v) of human blood. Isolates from fresh cultures were spread onto the surface of blood agar plates and incubated at 37^0C for 48 hours. Plates were observed for halo zone clearance (Bicca *et al*., 1999).

Drop collapsed test

This was performed according to Jain *et al* (1991), as modified by Bodour & Maier (1998). To an engine oil-coated glass slide, one drop of cell-free supernatant was dropped and observed for one minute to check for collapse. The same process was repeated using distilled water as a negative control.

Oil spreading test

In a Petri plate containing 50ml of distilled water, 100 µl engine oil was gently added to form a thin layer on the water surface. Afterwards, 10µl of fresh cell-free supernatant was gently added to the centre of the oil layer. This was observed for one minute for any oil displacement and formation of a zone of clearance; distilled water was used as a negative control (Morikawa *et al*., 2000; Youssef *et al*., 2004).

Emulsification index (E_{24})

This was performed according to Iqbal *et al*. (1995), as described by Bicca *et al*. (1999). To a broth culture, kerosene was added at 2:2 (v/v), vortexed for 2 min, and allowed to stand for 24h. The height of the emulsion was measured by taking the layer formed between the aqueous and kerosene layers. The emulsification index was calculated by measuring the emulsion height and dividing the emulsion layer's height by the mixture's total height, multiplied by 100.

$$\text{Emulsification index } (E_{24}) = \frac{\text{Height of Emulsion}}{\text{Total Height of Mixture}} X100$$

Identification of biosurfactant-producing bacterial isolates

Morphological characterisation and Gram's staining were performed on isolates; in addition, biochemical tests such as catalase test, oxidase test, urease test, indole test, methyl red test, Voges-Proskauer test, citrate test, sugar fermentation test were performed to identify the isolates tentatively.

Results

The morphological characteristics of the three bacteria isolated from abattoir soil are shown in Table 1. The results of Gram's staining showed that all three isolates were Gram-positive, motile organisms, with biochemical results (Table 2), aided by Bergey's Manual of Determinative for Bacteriology, tentative names were assigned to the isolates (see Table 3). The results of screening for biosurfactant production by the test isolates, using four different assay techniques (as shown in Table 4), indicated that all three isolates exhibited alpha-haemolytic activity. Additionally, all three isolates tested positive in the drop-collapse test, with NJ3 showing complete collapse. In the oil spreading test, NJ3 produced the largest diameter of 10 mm, followed by NJ2 with 7 mm and NJ1 with 5 mm. Furthermore, E_{24} (%) results showed that NJ1 had the highest value at 13.5%, followed by NJ2 at 2.56% and NJ3 at 2.50%.

Discussion

The bacteria used in this study were isolated from abattoir soil using a mineral salt medium supplemented with engine oil as a carbon source, specifically to selectively isolate bacteria capable of producing biosurfactants/bioemulsifiers. Soil from abattoirs is impacted with animal wastes (organic pollutants) as well as heavy metals, which may influence the microorganisms that might inhabit the environment, and engine oils are inducers of biosurfactant production. Various habitats have been screened for biosurfactant-producing microorganisms: Ndibe *et al*. (2018) successfully isolated biosurfactant-producing bacteria from rivers using Bushnell Haas Agar (BHA) supplemented with 0.1% crude oil, Cai *et al*. (2015) isolated biosurfactant-producing micro-microorganisms from offshore platforms, da Silva *et al*. (2022) investigated biosurfactant production from fruit waste fermentation, and Rani *et al*. (2020) isolated biosurfactant-producing microorganisms from oil well batteries. Researchers have reported *Pseudomonas* species to

Table 1: Morphological characteristics of the biosurfactant–producing isolates.

Isolate	Form	Surface	Colour	Margin	Elevation	Shapes	Appearance
NJ1	Irregular	Smooth	Ash	Undulate	Flat	Cocci	Chains
NJ2	Irregular	Rough	White	Lobate	Flat	Rod	Chains
NJ3	Punctiform	Smooth	Pale-Grey	Undulate	Flat	Cocci	Threadlike

Table 2: Gram's reaction and biochemical tests results of the isolates

Test	**Isolates**		
	NJ1	NJ2	NJ3
Gram	+	+	+
Catalase	+	+	+
Motility	+		
Indole	-	-	-
Methyl-Red	+	-	-
Voges-Proskauer	-	+	+
Citrate	-	+	+
Oxidase	-	Var	+
Urease	+	+	-
Sugar fermentation			
Lactose	AG	AG	AG
Glucose	AG	AG	AG
Sucrose	A	AG	-
Fructose	A	AG	AG
Maltose	AG	AG	AG

A = positive for sugar fermentation with acid production only; **AG** = positive for sugar fermentation with acid and gas production; **+** = positive; **-** = negative; **var** = varies.

Table 3: Probable organisms

Isolates	**Probable organisms**
NJ1	*Streptomyces* species
NJ2	*Bacillus* species
NJ3	*Streptomyces* species

Table 4: Biosurfactant assays of test isolates

Isolates	**Haemolysis**	**Drop collapse**	**Oil-spreading (mm)**	**Emulsification index E_{24} (%)**
NJ1	Alpha α	+	5	13.5
NJ2	Alpha α	+	7	2.56
NJ3	Alpha α	+++	10	2.50

Collapse "+"; complete collapse "+++"; no collapse "**-**"

be notable producers of biosurfactants due to their ability to utilise recalcitrant compounds (Sharma *et al*., 2015). Other microorganisms known to produce various types of biosurfactants include *Bacillus* species, *Acinetobacter* species, *Arthrobacter* species, *Corynebacterium* species, *Streptomyces* species, *Rhodococcus* species, *Candida* species, *Rhodotorula* species, *Aspergillus* species (Pardhi *et al*., 2022; Ambaye *et al*., 2021; Derguine-Mecheri *et al*., 2021; Dwivedi *et al*., 2019; Sohail & Jamil, 2020; John *et al*., 2020; Silva *et al*., 2019). In this study, members of *Streptomyces* spp and *Bacillus* sp were bacteria that were studied for biosurfactant production.

Multiple assayed techniques are recommended by researchers to efficiently ascertain that screened microorganisms are producers of biosurfactants (Sapute *et al*., 2008). In this study, four different assay techniques were explored on the test isolates for biosurfactant production. They include the haemolytic test, drop collapse test, oil spread test and E_{24}. Similarly, Patowary *et al*. (2017) utilised the drop collapse test and surface tension reduction of the culture medium, in addition to the emulsification test, in their work involving the characterisation of biosurfactants produced by bacteria isolated from the oil logging area. In this study, all tested isolates were positive for haemolysis, with the isolates producing alpha-haemolysis. Studies have reported negative results or gamma haemolytic activities in the haemolytic assay but positive results in other assay techniques (Astuti *et al*., 2019). The haemolytic assay, though simple and useful for screening large numbers of isolates, can produce false results due to the complexity of media and the presence of proteolytic enzymes capable of causing clearance observed in the haemolytic test (Uyar & Saglam, 2021). The drop collapse test performed on our test isolates showed that they were all positive, with NJ3 exhibiting complete collapse. The drop collapse test could be described based on the degree of the collapse achieved in the assay, which is a slight (+), average (++), and complete (+++) collapse. The drop collapse test is considered more reliable than the haemolytic assay for biosurfactant testing; it is fast but somewhat less sensitive (Youssef *et al*., 2004; Raj *et al*., 2021).

NJ3 demonstrated the highest oil-spreading test ability compared to the other two isolates (NJ2 and NJ1). Of the biosurfactant assay techniques adopted in this study, the most reliable assay for biosurfactant production is the oil spreading test, which measures the spread diameter, indicating the biosurfactant's ability to reduce surface/interfacial tension—the primary characteristic of biosurfactants (Raj et al., 2021; Walter *et al*., 2010). Also, the E24 values were relatively low for all isolates tested, which is indicative of biosurfactants with low emulsifying tendencies. High emulsification index values do not necessarily indicate superior biosurfactant production by the microorganisms but rather their ability to reduce surface or interfacial tensions (Samsu *et al*., 2020). It has been observed that emulsification index/activity screening methods experimental reports do not correlate with surface tension measurements (Uzoigwe *et al*., 2015). Despite their low emulsifying tendency, these biosurfactants can still be applied in bioremediation, food, pharmaceutical, and other biotechnological industries due to their ability to reduce surface and interfacial tensions (Bjerk *et al*., 2021).

In conclusion, bacteria isolated from the soil of an abattoir were screened using different assay techniques to determine their ability to produce biosurfactants. It was observed that all the isolates were able to produce biosurfactants in their culture broth. However, the emulsification index of all the isolates was low, suggesting that the tested isolates could only reduce surface and interfacial tension but may not function effectively as bioemulsifiers. This study validates the potential of identifying biosurfactant producers from abattoir soil.

Conflict of interest

The authors declare that there is no conflict of interest.

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