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Effects of Carbon and Nitrogen Sources on Biosurfactant Production by Bacteria

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Abstract

Biosurfactants are organic, surface-active compounds that contain hydrophobic and hydrophilic moieties produced by microorganisms. Their production depends on the type of nutrients, microorganisms and culture conditions. This study was aimed at investigating the effects of carbon and nitrogen sources on biosurfactant production by bacteria. *Actinomyces viscosus* ATCC 15987, *Streptomyces carpaticus* N29, *Alcaligenes faecalis* IISR-EPN BC 08 and *Alcaligenes faecalis* BPS II were isolated from hydrocarbon-contaminated soil and screened for biosurfactant production on Kim's medium. The effects of 3% carbon source and 0.1% nitrogen source on biosurfactant production by the isolates were investigated using one variable at a time method. The activity of the biosurfactants were determined by emulsification index measurement on hydrophobic substrates. The carbon and nitrogen sources of choice for biosurfactant production by the investigated bacteria were: starch/sucrose (emulsification index (E24) range = 51.61% to 69.69%) and ammonium sulphate (E24 range = 43.75% to 62.85%) respectively. Very high emulsification index was observed for *Actinomyces viscosus* ATCC 15987 (65.71%), *Streptomyces carpaticus* N29 (77.50%) and *Alcaligenes faecalis* BPS II (63.16%), using starch and $(NH_4)_2SO_4$) as carbon and nitrogen sources respectively. *Alcaligenes faecalis* IISR-EPN BC 08 utilized sucrose and ammonium sulphate as carbon and nitrogen of choice respectively to produce biosurfactant with emulsification index of 70.00%. Palm oil was highly emulsified by *Actinomyces viscosus* ATCC 15987 (81.25%), *Streptomyces carpaticus* N29 (82.00%), *Alcaligenes faecalis* IISR-EPN BC 08 (79.17%), and *Alcaligenes faecalis* BPS II (80.00%), while *Streptomyces carpaticus* N29 achieved the highest activity (90.48%) with engine oil. This study shows that starch/sucrose and ammonium sulphate improved biosurfactant production by the bacteria. The emulsification of the hydrophobic substrates suggests their use in hydrocarbon bioremediation and as emulsifiers in food industries.

Keywords: Biosurfactants, carbon sources, nitrogen sources, emulsification activity, hydrophobic substrates

Introduction

Surfactants are chemical compounds consisting of amphipathic molecules with distinct hydrophilic and hydrophobic moieties that preferentially partition at physical interfaces. The non-polar moieties are often hydrocarbon chains, while the polar moieties can be a cationic, anionic, non-ionic, or amphoteric molecule. This combination of hydrophobic and hydrophilic moieties enables surfactants to reduce surface and interfacial tensions and form microemulsions, in which hydrocarbons are solubilized in water or vice-versa (Farias *et al.,* 2021). Primarily, because of the diverse and extensive application of chemical surfactants, there is a growing concern over their environmental impacts and difficulty in being degraded in the environment. This necessitated the discovery of an equally efficient but environmentally friendly alternative to synthetic surfactants; this class of surfactants derived from microorganisms are referred to as biosurfactants. (Singh *et al.*, 2018).

Biosurfactants are organic, surface-active amphiphilic compounds that contain both hydrophobic and hydrophilic moieties, produced mainly by bacteria, yeast, or fungi on cell surfaces or can be secreted extracellularly (de Oliveira *et al.*, 2021; Sumiardi *et al*., 2018). Biosurfactants demonstrate emulsion behavior due to their ability to stabilize and form emulsions through diverse mechanisms (Walter *et al.,* 2013). Since most environmental contaminants are hydrocarbon derivatives that are hydrophobic and recalcitrant due to their low aqueous solubility, hindering microbial uptake (Zhu, *et al.*, 2025), surface active agents are required to mobilize them from their repositories (Amodu *et al*., 2014).

Biosurfactants are effective in reducing surface and interfacial tensions, solubilizing hydrophobic substrates: hydrocarbons (hexadecane, kerosene, diesel, crude oil), vegetable oils (olive, sunflower, soybean), industrial oils (motor oil, lubricants)) and modulating cell–surface interactions (Alsayegh *et al*., 2026; Napp *et al*., 2026). Due to their biodegradability, low toxicity, renewable origin, eco-friendly properties and functional versatility, they have attracted considerable attention for applications in: bioremediation, enhanced oil recovery, environmental management, and various industrial processes (Kugaji *et al*., 2025; Adetunji and Olaniran*,* 2021).

Despite the advantages of biosurfactants over chemical surfactants, the problem of low yield continues to limit their industrial application (Zheng *et al*., 2012; Menezes *et al*., 2011). For biosurfactants to compete with chemical surfactants, they must be produced at their optimum capacity. The production efficiency and yield of microbial biosurfactants have improved significantly through the development and optimization of different process parameters (Thakur *et al.,* 2024). Biosurfactants yield and productivity depends on many variables, including fermentation conditions (pH, temperature, agitation speed, inoculum size), pressure, and various salt concentrations (salinity), as well as medium nutrition (carbon and nitrogen sources) (Jiang *et al*., 2020). Among these factors, carbon and nitrogen sources are critical in biosurfactant production (Ezebuiro *et al*., 2019).

Carbon source is the primary variable to consider in the production of biosurfactants, as it directly influences the growth of the microorganism as well as the structure and yield of the target biosurfactant molecule (Sarubbo *et al*., 2022). The rate at which the carbon source is metabolized can often influence the

formation of biomass and/or the production of the metabolites (Singh *et al*., 2017). Generally, there are three types of carbon sources being commonly used in biosurfactant productions: carbohydrate, fats and oils, and hydrocarbon groups. Under the carbohydrate group, simple sugar, starch, and plant sugar-based carbohydrates are the major carbon sources used in biosurfactant production (Kanwal *et al*., 2021; Nurfarahin *et al*., 2018).

Nitrogen source plays an important role in biosurfactant production as protein and enzyme synthesis required for microbial growth depends on the concentration and nature of the nitrogen source (Vandana and Singh, 2018). There are two types of nitrogen sources: organic and inorganic nitrogen. The two can be differentiated clearly based on the unit structure present in them. The unit structure for organic nitrogen will be in molecules such as yeast extract, meat extract, tryptone, or peptone, while inorganic nitrogen will have unit structures that consist of positive and negative ions such as ammonium nitrate and ammonium sulphate. Organic nitrogen may also contain some carbon component and had been reported to significantly support cell growth and polysaccharides formation as compared to inorganic nitrogen (Deepika, *et al*., 2016).

The quality and quantity of biosurfactant produced by microorganisms depends on both the types of carbon source present, and on the types and amounts of other nutrients such as nitrogen source present in the culture growth medium. This implies that the study of the effects of different carbon and nitrogen sources on the growth of biosurfactant-producing microorganisms and the yield of biosurfactant, are of paramount importance (Nwanyanwu *et al*., 2017; Onwosi and Odibo, 2013).

This study was, therefore, aimed at investigating the effects of carbon and nitrogen sources on biosurfactant production by bacterial species isolated from hydrocarbon-contaminated soil in Awka, Southeast Nigeria**.**

Materials and Methods

Study design

This study utilized cross-section design to select biosurfactant-producing bacterial strains. The primary screening was based on haemolysis and oil displacement, while Secondary screening was based on drop collapse test together with emulsification activity. An experimental design based on a Completely Randomized Design (CRD), conducted in triplicate, was employed in separate trials to compare the carbon and nitrogen sources for the biosurfactant production.

Soil sample Collection

A total of 200 samples were randomly collected from hydrocarbon-contaminated soils, which included the abattoirs, mechanic workshops and refuse (waste) dumps, in Awka locality (Lat.6°12'45.7"N, Long.7°4'19.2"E), Southeast Nigeria. The samples were collected in sterile polythene bags, at a depth of 5 -10 cm of the soil, and taken to the Department of Applied Microbiology and Brewing, Nnamdi Azikiwe University, Awka, Laboratory for analysis.

Isolation of biosurfactant-producing bacteria

The biosurfactant-producing bacteria from the soil samples were isolated following the modified methods described by Zaki *et al.* (2013) and Lakshimipathy *et al.* (2010), on starch casein agar medium (Soluble starch, 10g; casein, 0.3g; KNO_3, 2.0g; NaCl, 2.0g; K_2HPO_4, 2.0g; $MgSO_4$. $7H_2O$, 0.05g; $CaCO_3$, 0.02g; $FeSO_4.7H_2O$, 0.01g; Agar, 20.0g; distilled water, 1000 ml; pH 7.0). One gram of the soil sample was serially diluted ten-fold and 0.1ml of 10^{-5}

and 10^{-6} dilutions inoculated on starch casein agar plates. The plates were incubated for 7 days at 30°C, and colonies which show morphological differences were isolated, purified by streak plate technique and maintained at 4°C for further studies.

Fermentation for biosurfactant activity

Each pure isolate was sub cultured at 30°C. After 7 days incubation, it was inoculated into 10ml of sterile distilled water in a test tube and standardized using 0.5 Mcfarland standard (Patel *et al*., 2015). This served as the standard inoculum. Mcfarland standard was prepared by mixing 0.5 ml of 1% BaCl and 9.95 ml of 1% H_2SO_4.

Kim's medium (g/L: $NaNO_3$, 1; KH_2PO_4, 0.1; $MgSO_4$. $7H_2O$, 0.1; $CaCl_2$, 0.1; yeast extract, 0.2%; olive oil, 3%; pH 6) was used as the fermentation medium for the screening of biosurfactant-producing bacteria as described by Kalyani *et al*. (2014). Then 50ml of the medium in sterile 250ml Erlenmeyer flask was inoculated with 2ml of the standardized inoculum. The flask was incubated on a rotary shaker for 7 days at 30°C, and the content centrifuged at 7,000 rpm for 20 minutes. The cell free supernatant obtained was used for haemolysis test, oil spreading assay, drop collapse test and emulsification index measurement to determine biosurfactant activity.

Haemolytic activity

Haemolysis was carried out on plates of blood agar medium supplemented with 5% human blood as described by Carillo *et al.* (1996). Wells of 2cm diameter were made on the blood agar plates using sterile cork-borer and 0.1ml of the cell-free supernatant added with a micropipette. The blood agar plates were incubated at 28°C for 7 days. The plates were then examined for zone of clearance around the wells.

Oil spreading assay

The oil-displacement method was used to detect the activity of biosurfactant as described by Morikawa *et al.* (2000). A 10µL of crude oil was added to the surface of 40 ml distilled water in a petri dish using a micropipette to form a thin oil layer; and 10µL of the cell-free supernatant of the isolate was gently placed on the center of the oil layer and the presence of biosurfactant in the supernatant, displaced the oil, forming a clear zone. The diameter of the cleared zone on the oil surface correlates to surfactant activity.

Drop collapse test

Biosurfactant production by the isolates was screened using the qualitative drop-collapse test described by Youseff *et al.*, (2004). A 2 µL olive oil was added to 96-well microlitre plate and equilibrated for 1 hr at 37°C, after which 5 µL of the supernatant of the isolate was added to the surface of the oil in the well and the shape of the drop on the oil surface observed after 1 minute. Collapse drops indicated positive result, while intact drops indicated negative results. Sterile distilled water was used as control.

Emulsification index measurement

The emulsification index (E24) of the biosurfactant was determined by mixing 2 ml of kerosene and 2 ml of the cell-free supernatant in a test tube as described by Ellaiah *et al*. (2002). The mixture was vortexed for 2 minutes and allowed to stand for 24 hours. Emulsification index was given as percentage (%) of the height of the emulsified layer (cm) divided by the total height of the liquid column (cm).

Molecular Identification of Isolates

The four active biosurfactant producers were identified by 16S rRNA sequence analysis at Bioinformatics services limited, Ibadan.

Genomic DNA (16S rRNA) was extracted using ZR bacterial DNA miniprep of

Zymo Research. The DNA was subjected to PCR and amplified using Taq 2X Master Mix from New England Biolabs (M0270). The forward and reverse primer used were (27F: AGAGTTTGATCMTGGCTCAG) and reverse (1525R: AAGGAGGTGWTCCARCCGCA). The cycling conditions for the amplification of the 16S rRNA were - Initial denaturation at 94˚C for 5mins, followed by 36 cycles of denaturation at 94˚C for 30sec, annealing at 56˚C for 30secs and elongation at 72˚C for 45sec. This was followed by a final elongation step at 72˚C for 7 mins and hold temperature at 10˚C. The amplified DNA was run on agarose gel for electrophoresis and visualized under UV transilluminator. The amplified fragments were sequenced with a Genetic Analyzer 3130xl sequencer from Applied Biosystems using manufacturers' manual and the sequencing kit used was BigDye terminator v3.1 cycle sequencing kit. Bio-Edit software and MEGA X were used for the genetic analysis. The evolutionary history was inferred using the Neighbor-joining method (Saitou and Nei, 1987), and the evolutionary distances were computed using the maximum composite likelihood method (Tamura *et al*., 2004).

Screening of carbon sources

The effects of five different carbon sources (glucose, sucrose, starch, fructose, glycerol) on biosurfactant production by the biosurfactant producing isolates (*Actinomyces viscosus* ATCC 15987, *Streptomyces carpaticus* N29, *Alcaligenes faecalis* IISR-EPN BC 08, *Alcaligenes faecalis* BPS II), was investigated using one variable at a time method as described by Anaukwu *et al.*, (2020). Kim's medium served as the basal medium. Fermentation for biosurfactant activity was carried out as described above (Kalyani *et al*., 2014) in a medium consisting of basal medium; carbon source, 3% (w/v), and 2ml of the standardized inoculum prepared as described above. The emulsification index (E24%) of the biosurfactant in the cell-free supernatant was determined as described earlier (Ellaiah *et al*., 2002). Triplicate flasks were used and uninoculated flask served as control. The carbon source that gave the best result was used for further fermentation studies.

Screening of nitrogen sources

The effects of four different nitrogen sources (Potassium nitrate (KNO_3), ammonium nitrate (NH_4NO_3), sodium nitrate ($NaNO_3$), ammonium sulphate ($(NH_4)_2SO_4$)) on biosurfactant production by the biosurfactant producing isolates (*Actinomyces viscosus* ATCC 15987, *Streptomyces carpaticus* N29, *Alcaligenes faecalis* IISR-EPN BC 08 and *Alcaligenes faecalis* BPS II), was investigated using one variable at a time method as described by Anaukwu *et al.*, (2020). Kim's medium served as the basal medium. Fermentation for biosurfactant activity was carried out as described above (Kalyani *et al*., 2014) in a medium consisting of basal medium, nitrogen source, 0.1% (w/v) and 2ml of the standardized inoculum prepared as described above. The emulsification index (E24%) of the biosurfactant in the cell-free supernatant was determined as earlier described by Ellaiah *et al*., 2002. Triplicate flasks were used and uninoculated flask served as control. The nitrogen source that gave the best result was used for further fermentation studies.

Fermentation for biosurfactant activity using the choice carbon and nitrogen sources

Fermentation was carried out in modified Kim's medium consisting of basal medium [KH_2PO_4, 0.1; $MgSO_4.7H_2O$, 0.1; $CaCl_2$, 0.1; yeast extract, 0.2%; pH 6], $(NH_4)_2SO_4$, 0.1% (w/v); starch/ sucrose, 3% (w/v); pH, 7.2. A sterile 250 ml Erlenmeyer flask containing 50 ml of the fermentation medium was inoculated with 2ml of the standardized inoculum and the flask incubated on a rotary shaker (150 rpm) at 30°C. Triplicate flasks were used and

uninoculated flask served as control. After 7 days fermentation, the emulsification index of the biosurfactant in the cell-free supernatant was determined as described by Ellaiah *et al*. (2002).

Assessment of emulsification activity of the biosurfactants using hydrophobic substrates

The oil emulsification activity of the biosurfactant produced by the isolates was evaluated by measuring the emulsification index (E24) of the biosurfactant in the cell-free supernatant obtained from the fermentation study using various hydrophobic substrates (kerosene, palm oil, crude oil, diesel, groundnut oil, engine oil). The E24 of the biosurfactant in the cell-free supernatant was determined as described earlier (Ellaiah *et al*., 2002).

Statistical analysis

The data obtained was subjected to one way Analysis of Variance at 95% confidence level. Statistical Package for Social Sciences (SPSS) software version 21 was used for the ANOVA. Tukey's Honestly Significant Difference (HSD) post hoc test was used to separate significant mean differences. Biosurfactant production using the choice carbon and nitrogen sources were compared using Independent Samples t-Test at 95% confidence level.

Results

Isolation, screening and selection of biosurfactant producing bacteria from soil

Eighty-seven different bacterial organisms were isolated from soil samples collected from different mechanic workshops, abattoirs, and refuse dumps in Awka locality. Screening of the 87 recovered isolates using haemolysis test and oil spreading tests showed that 16 (18.39%) of them had biosurfactant activity, with emulsification ability ranging from 19.35 to 74.07%, oil displacement ranging from 2.1-7.0cm and 56.25% were positive for drop collapse and haemolysis tests (Table 1). As can be observed from Table 1, isolates AS1, ASF, DS4 and DS8 with high emulsification index and oil displacement values were the best biosurfactant producers and were, therefore, selected for further studies.

Identification of the most potent biosurfactant producers

The four best biosurfactant producers (isolates AS1, ASF, DS4 and DS8) were identified based on 16S rRNA gene sequencing. Based on the blast analysis in the NCBI, it was revealed that isolate AS1 has 98.07% pairwise similarity with *Actinomyces viscosus* strain ATCC 15987 which has NCBI accession number MH173790, isolate ASF has 84.38% pairwise similarity with *Streptomyces carpaticus* strain N29 which has NCBI accession number HQ132783, isolate DS4 has 93.0% pairwise similarity with *Alcaligenes faecalis* strain IISR-EPN BC 08 which has NCBI accession number MH249778, while isolate DS8 has 98.36% pairwise similarity with *Alcaligenes faecalis* strain BPS11 which has NCBI accession number MH489072. Figure 1 shows the gel image of the 16S rRNA gene for the isolates. The phylogenetic tree showing the evolutionary relatedness of the isolates is shown in Figure 2.

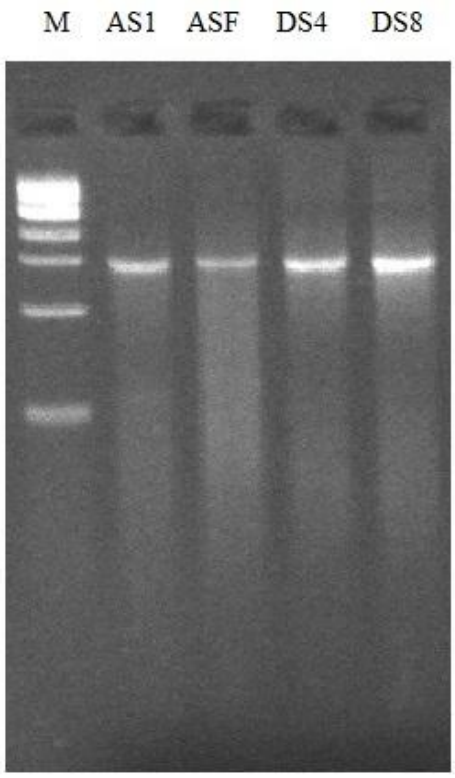


Figure 1: Gel image of the 16S rRNA gene for the isolates

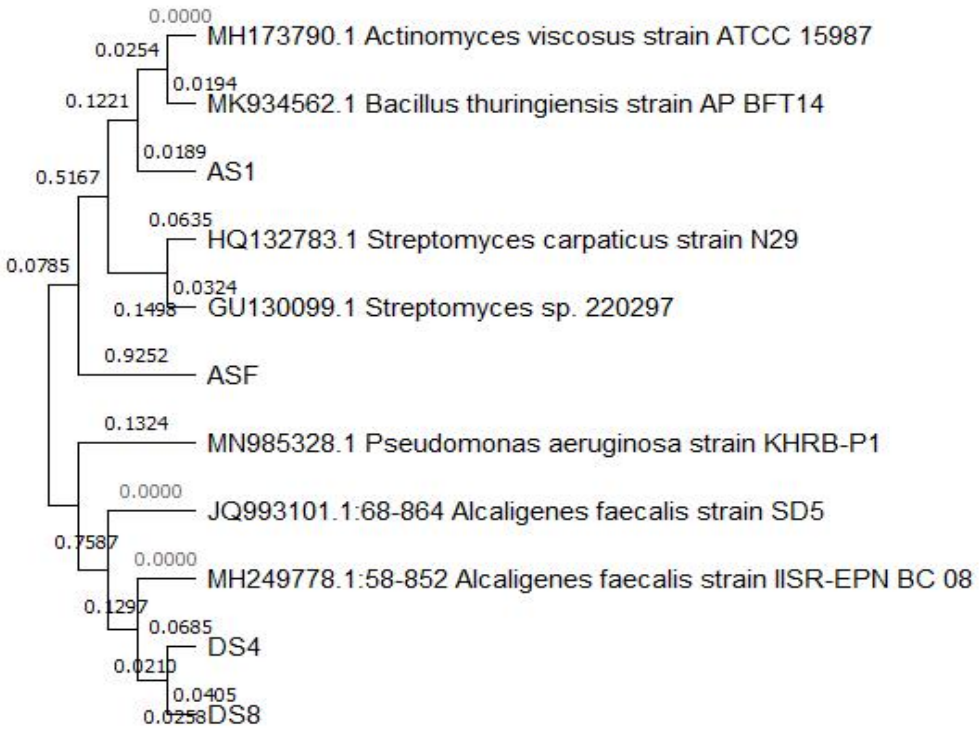


Figure 2: The evolutionary relationship of the isolates using the Neighbor-Joining method

Table 1: Biosurfactant-producing potentials of the isolates

Isolate	E24 (%)	Oil displacement (cm)	Drop collapse	Haemolysis
AS1	60.61	7.0	+	+
AS1(A)	27.59	2.5	-	+
AS2	35.71	4.8	-	-
AS28	19.35	5.5	-	+
ASF	74.07	6.5	+	+
DS1	50.00	4.3	+	+
DS4	66.67	5.3	+	+
DS6	28.57	3.5	-	-
DS7	28.00	5.6	+	-
DSVII	51.72	4.8	+	-
DS8	61.29	5.3	+	+
MS2	22.73	5.6	-	+
MS3	46.88	4.0	+	-
VSB	24.00	2.1	-	-
VS9	26.47	4.9	+	+
VS10	48.78	3.1	-	-

Key: + = positive, - = negative, E24 = Emulsification index at 24hr

Screening of carbon source

The results of the best carbon sources for biosurfactant production is as presented in Figure 3. The result shows starch as the carbon of choice for *Actinomyces viscosus* ATCC 15987*, Streptomyces carpaticus* N29*, Alcaligenes faecalis* BPS 11, while *Alcaligenes faecalis* IISR-EPN BC 08 utilized sucrose as the carbon of choice for biosurfactant production. The highest E24 (69.69%) obtained using starch as carbon source was observed in *Streptomyces carpaticus* N29, while *Alcaligenes faecalis* BPS 11 gave the highest E24 (60.00%) when sucrose was used as the carbon source. There were significant differences in the performances of the carbon sources with the isolates (p-value < 0.05).

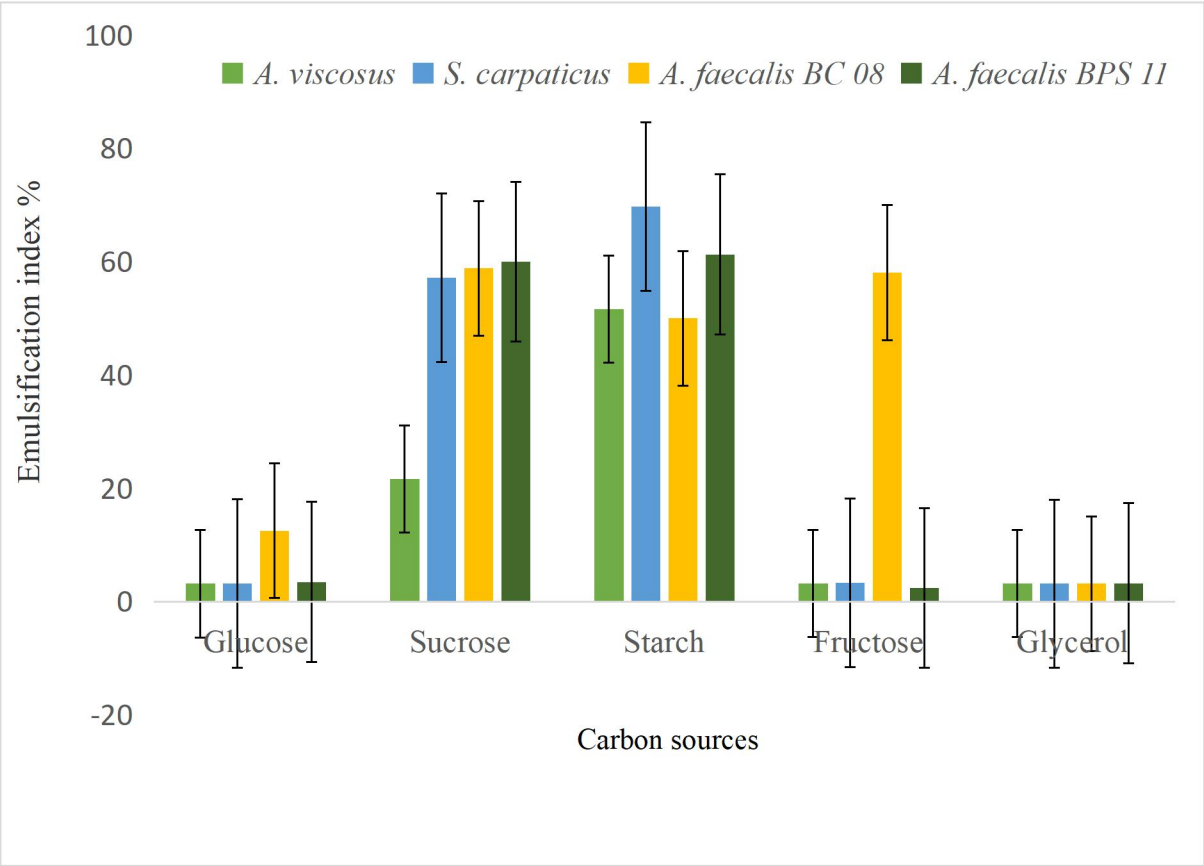


Figure 3: Screening of different carbon sources for biosurfactant production

Screening of nitrogen source

The results of nitrogen sources for biosurfactant production is as presented in Figure 4. The result show that ammonium sulphate was the choice nitrogen for biosurfactant production by the isolates. The highest emulsification index of 62.85% of the biosurfactant produced was observed in *Alcaligenes faecalis* BPS 11 when ammonium sulphate was used as the nitrogen source. The differences in the performances of the nitrogen sources were significant (p-value < 0.05).

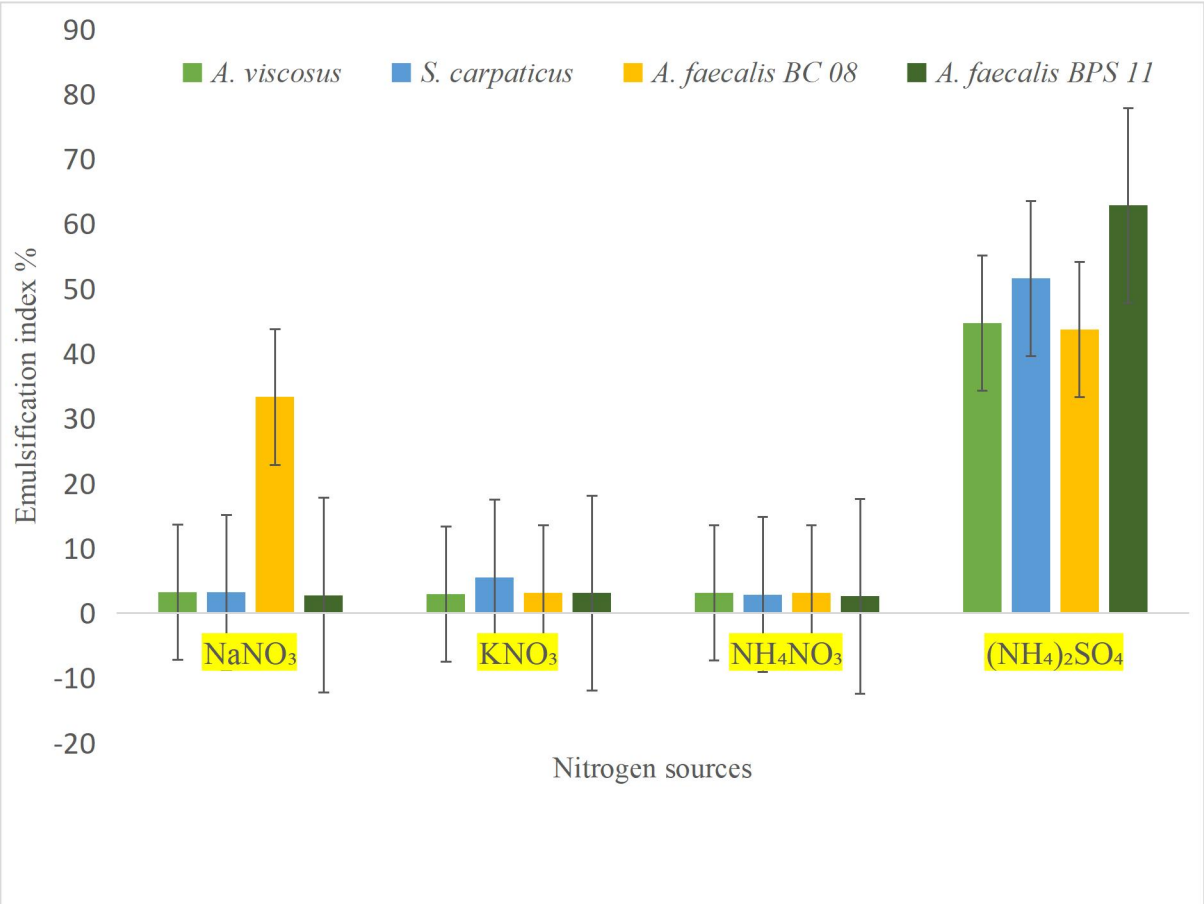


Figure 4: Screening of different nitrogen sources for biosurfactant production

Fermentation for biosurfactant activity using the choice carbon and nitrogen sources

Using the choice carbon and nitrogen sources, *Actinomyces viscosus* ATCC 15987, *Streptomyces carpaticus* N29 and *Alcaligenes faecalis* BPS 11 produced high amounts of biosurfactants with Starch/$(NH_4)_2SO_4$ while *Alcaligenes faecalis* IISR-EPN BC 08 produced higher biosurfactant when grown on Sucrose/$(NH_4)_2SO_4$ than in starch/$(NH_4)_2SO_4$ (Table 2). There were no significant differences in biosurfactant production using either starch or sucrose combined with ammonium sulphate in biosurfactant production by the isolates (p-value > 0.05).

Table 2: Biosurfactant production using the choice carbon and nitrogen sources

	Emulsification index (E24) (%) Mean ±SD	
Isolates	**Starch/$(NH_4)_2SO_4$**	**Sucrose/$(NH_4)_2SO_4$**
A. viscosus	65.71±0.02	56.75±0.00
S. carpaticus	77.50±0.20	56.67±0.06
A. faecalis BC 08	48.39±0.01	70.00±0.17
A. faecalis BPS 11	63.16±0.01	45.45±0.02

t-value = 0.83, P-value = 0.439, P > 0.05

Assessment of emulsification activity of the biosurfactants using hydrophobic substrates

The results of the emulsification activity of the biosurfactants using hydrophobic substrates are shown in Figure 5. The biosurfactant of *Streptomyces carpaticus* N29 although had the highest emulsification activity with engine oil (90.48%), showed very high activities with all other substrates. The emulsification activity of biosurfactants produced by *Actinomyces viscosus* ATCC 15987 (81.25%), *Alcaligenes faecalis* IISR-EPN BC 08 (79.17%), and *Alcaligenes faecalis* BPS II (80.00%) were quite high when palm oil was used as substrate. With the exception of *Streptomyces carpaticus* N29, very low emulsification activity was observed in other bacteria when diesel was used as the substrate. One way analysis of variance shows that there were significant differences in the emulsification activities of the biosurfactants produced by the isolates with the hydrophobic substrates (p-values < 0.05).

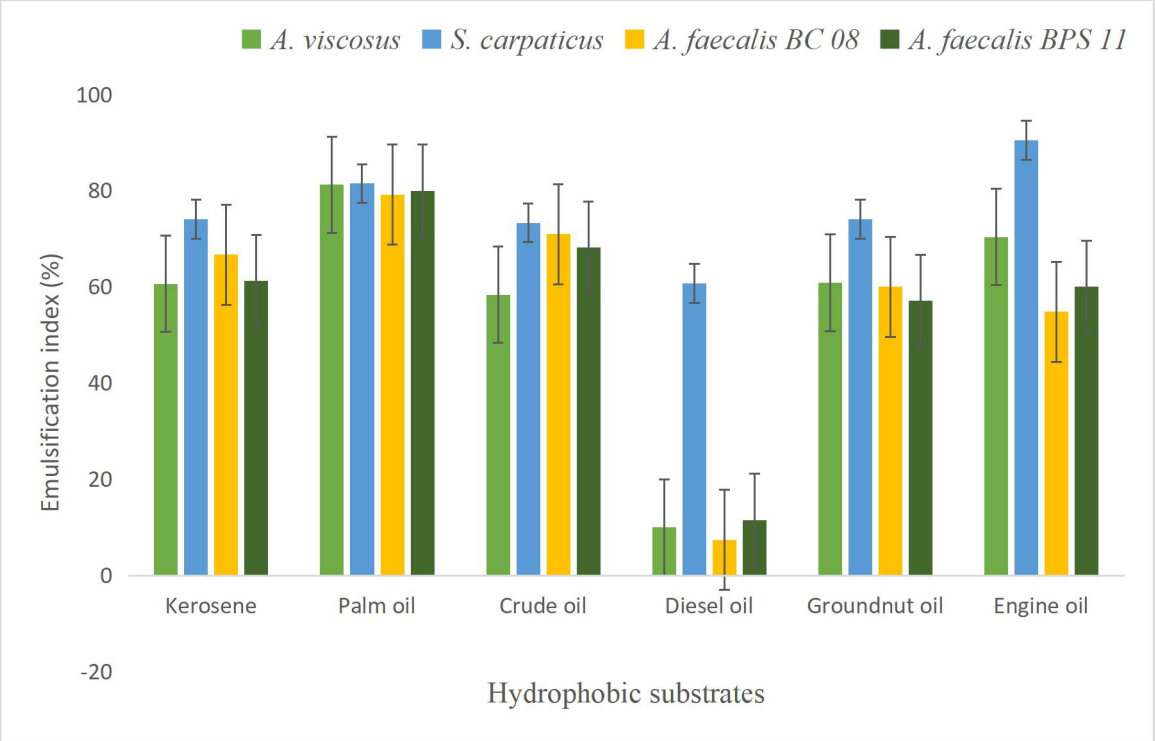


Figure 5: Emulsification activity of the biosurfactants produced by the isolates using hydrophobic substrates

Discussion

Bacterial organisms capable of producing biosurfactants are widely distributed across various natural ecosystems but are mostly isolated from places rich in organic hydrophobic contaminants (Jiang *et al*., 2024; Ali *et al*., 2021). In this study, eighty seven different bacteria species were isolated from different hydrocarbon contaminated soils in Awka locality. This indicates that there are diverse bacterial organisms capable of degrading hydrocarbons and their derivatives (Faisal *et al*., 2023; Ebakota *et al.,* 2017). The isolates were examined by various screening methods including haemolysis test, oil displacement test, drop collapse test and emulsification indices since previous reports have recommended the use of multiple techniques to screen for determination of efficient biosurfactant-producing strains (Soltanighias *et al*., 2019; Nwaguma *et al*., 2016). The primary screening was based on haemolysis and oil displacement tests of which 16 isolates

were recovered for further selection (Sidkey *et al.,* 2016). Secondary screening based on drop collapse test together with emulsification activity was used in the selection of four potent strains from the 16 isolates.

The haemolysis test result showed 56.25% of the isolate positive and 43.75% negative. Zobaer *et al.* (2024), made similar findings in their work on isolation of biosurfactant-producing bacteria from oil-spilled soil and characterization of their secreted biosurfactants in pathogen inhibition and oil-emulsification. On the contrary, Ali *et al.* (2021) and Faisal *et al.* (2023) reported a much lower positive haemolysis test result of 30.76% and 34% respectively. The nature and type of isolates may have contributed to the variations.

The isolates screened produced oil displacement diameter ranging from 2.1cm – 7.0cm. This result agrees with the findings of Faisal *et al.* (2023) and Jiang *et al* (2024) who obtained oil displacement values ranging from 0.3cm – 7.7cm and 2.3cm – 5.9cm respectively but disagrees with the work of Zobaer *et al.* (2024) who obtained lower oil displacement diameter of 1.63cm and 1.93cm in their work on isolation of biosurfactant-producing bacteria from oil-spilled soil. Again, the nature and type of isolates may have contributed to the variations.

The drop collapse result showed 56.25% positive result and 43.75% negative result. This is in line with the works of Sun *et al*, (2018) and Ndibe *et al.* (2018) who reported 53.84% and 54.54% positive results respectively in their study. In contrast, Ali *et al.,* (2021) obtained a lower percentage positive result (46.15%) in their work on production, optimization, stability and oil emulsifying potential of biosurfactants from selected bacteria isolated from oil-contaminated sites. The difference is likely to be due to strain-specific biosurfactant production capacity of the isolates and environmental growth conditions.

Emulsification activity (E24) is an indirect test used for screening biosurfactant production and is considered a simple, reliable and rapid test that measures biosurfactant quantity (Nayarisseri *et al.,* 2019). In this study, kerosene was used as the hydrophobic substrate and the results obtained showed a variable emulsification ability ranging from 19.35% to 74.07%, indicating the ability of the bacterial isolates to produce different amounts of biosurfactants. Hassanshahihan (2014) obtained a similar range of E24 values (10% - 65%) from oil contaminated site of Persian Gulf. Jiang *et al.* (2024), also reported a higher E24 values (81-85%) in their work on isolation and characterization of biosurfactant-producing bacteria for enhancing oil recovery. This indicates that the efficacy of bacterial biosurfactants in emulsification differ based on microbial source and chemical structure of the biosurfactant they produce.

The four active isolates were identified based on 16S rRNA gene sequencing as *Actinomyces viscosus* ATCC 15987 (AS1), *Streptomyces carpaticus* N29 (ASF), *Alcaligenes faecalis* IISR-EPN BC 08 (DS4), and *Alcaligenes faecalis* BPS11 (DS8). All the isolates recovered except *Actinomyces viscosus* ATCC 15987 have been reported as biosurfactant producers (Emeanuru *et al.,* 2024; Hamed *et al.,* 2021; Ndibe *et al*., 2018; Zambry *et al*., 2017; Atuanya *et al.,* 2016; Jeph and Mathur, 2015; Korayem *et al*., 2015; Hayder *et al*., 2014; Khopade *et al.,* 2012b). This consistency confirms the report that biosurfactant production is a widespread trait among hydrocarbon-degrading microorganisms, supporting their role in crude oil bioremediation. Biosurfactants facilitate hydrocarbon solubilization and uptake, thereby enhancing microbial access to otherwise recalcitrant substrates. However,

Actinomyces israelii and *Actinomyces viscosus* have been reported to produce peroxidases, extracellular enzymes used by crude oil degrading microbes (Olajuyigbe *et al*., 2015). The detection of biosurfactant activity in *Actinomyces* viscosus ATCC 15987, therefore suggests that this species may possess dual functionality, combining extracellular enzymatic activity with surfactant-mediated hydrocarbon solubilization, highlighting the possibility of strain-specific variation or previously overlooked metabolic capabilities.

The production of biosurfactant is dependent on the composition of the medium (Korayem *et al*., 2015). According to Singh *et al.* (2017), the carbon consumption of microorganisms during cultivation often regulates growth and metabolites production including biosurfactants. *Actinomyces viscosus* ATCC 15987*, Streptomyces carpaticus* N29 and *Alcaligenes faecalis* BPS II utilized starch as their choice carbon for biosurfactant production. As suggested by Tagami (2024), the choice of starch reflects both the enzymatic capabilities of these bacteria and the metabolic advantages of using a complex carbohydrate that avoids repression, and sustains biosurfactant production. This result agrees with the findings of Jain *et al*. (2013), who reported that among the several carbon substrates tested in their study, production of the crude biosurfactant was found to be the highest with starch. *Alcaligenes faecalis* IISR-EPN BC 08 utilized sucrose as the best carbon source for biosurfactant production. This finding agrees with the works of Nayarisseri *et al*. (2018); Parthipan *et al.* (2017); Hayder *et al.* (2014) and Khopade *et al.* (2012) who observed in their various studies that the maximum biosurfactant production was obtained using sucrose as the sole carbon source. On the contrary, Fouda *et al.* (2016) reported that the lowest production of biosurfactant was observed with starch as carbon source, while Das *et al.* (2009) in their study, reported that sucrose was the least utilized carbon source for biosurfactant production. The variation of the findings in other studies and the findings in this study may be due to the differences in the enzymatic capabilities of the bacterial species studied, which adapt their metabolic pathways according to the available carbon sources (Nikhil and Ansari, 2024). This underscores the importance of selecting appropriate carbon sources in microbial fermentation processes to optimize biosurfactant production (Sarubbo *et al*., 2022; Sharma *et al* 2020; Velioglu and Ozturk Urek, 2014).

Nitrogen source plays an important role in the production of biosurfactants since it is essential for microbial growth and enzyme production (Pardhi *et al.,* 2020). It is clear that the isolates' ability to produce biosurfactants varies with different nitrogen sources. Ammonium sulphate was observed to be the nitrogen source of choice for all the isolates. This is similar to the works of Mahmoud *et al.* (2024) and Hamed *et al.* (2021), who reported ammonium sulphate as the best nitrogen source for biosurfactant production by their isolates. Contrary to our findings, lower yields of biosurfactant were obtained using ammonium sulphate as nitrogen source by Shagufta and Dharani, (2022) and Nayarisseri *et al*. (2018) when bacterial isolate SPTSS1 and *Bacillus subtilis* strain ANSKLAB03 respectively were used in the production of biosurfactant. The differences in biosurfactant yield by various bacteria in the presence of different nitrogen sources may be as a result of differences in microbial physiology and media composition. It is therefore important that strain-specific media design is used in biosurfactant production (Nikhil and Ansari, 2024).

Using the choice carbon and nitrogen sources together, improved the biosurfactant productivity, as higher yields of biosurfactants were obtained for all the isolates. This result is in line with the work of Kanna *et al.* (2014) and Haddad *et al.* (2009) who noted an increase in biosurfactant production when sucrose and ammonium sulphate are used as carbon and nitrogen sources respectively. However, contrary to our findings, Ekpunobi *et al*. (2025), reported lower yields of biosurfactants using starch/ammonium sulphate and sucrose/ammonium sulphate. They observed higher yields of biosurfactant using sucrose and ammonium nitrate as carbon and nitrogen sources. Fonseca *et al*. (2007) also obtained higher yields of biosurfactant using sucrose and ammonium nitrate, while Nayarisseri *et al*., 2018 obtained higher yields of biosurfactant using sucrose and yeast extract. Ezebuiro *et al*., (2019) reported that the carbon and nitrogen sources for optimal production of biosurfactants by *Stenotrophomonas* sp. are glucose and a combination of yeast extract and NH_4NO_3, while Jiang *et al*. (2024) observed that *Psedomonas aeruginosa* N33 and *Bacillus paralicheniformis* Nian2 utilized glucose and sodium nitrate for optimal production of biosurfactant. In *Stenotrophomonas nematodicola* T10, beef extract and peptone were observed as a good combination for biosurfactant production (Jiang *et al*., 2024). Optimizing these nutrient sources is essential in industrial applications to enhance biosurfactant yield and cost efficiency (Purwasena *et al*., 2024; Phulpoto *et al*., 2020; Parthipan *et al*., 2017; Gurjar and Sengupta, 2015; Santa Anna *et al*., 2002).

The emulsification activity results show that the biosurfactants formed stable emulsions with the hydrophobic substrates. The biosurfactants maximally emulsified all the hydrophobic substrates except diesel oil. This observation is in line with the work of other researchers, who demonstrated the ability of some biosurfactant-producing bacteria to emulsify various hydrophobic substrates (Oliveira *et al*., 2025; Ali *et al*., 2021; Borah and Yadav, 2017). Oliveira *et al.* (2025) reported that the biosurfactant of *Starmerella bombicola* ATCC 22214 emulsified engine oil, kerosene, soybean oil and diesel oil. Ali *et al.* (2021) observed that the biosurfactants produced by *Bacillus, Burkholderia, Providencia and Klebsiella* species were well-capable of emulsifying kerosene, diesel and soya bean oil, while Borah and Yadav (2017), noted that the biosurfactant of *Bacillus cereus* DRDU1 emulsified kerosene, crude oil and used engine oil. The poor emulsification of diesel oil by most bacteria in this study agrees with the work of Bajelani *et al*. (2023), who reported that the emulsification index in diesel was significantly lower than that using crude oil. Suryanti *et al.* (2021) observed that the biosurfactant produced by *Lactobacillus lactis* emulsified soybean oil, palm oil, sunflower oil and olive oil better than diesel. Lima *et al*. (2020) also reported low emulsification activity when diesel was used as hydrophobic substrate by *Bacillus* sp. L26 and L30. Although, all the bacteria in this study were observed to have poor emulsification activity with diesel oil, *Streptomyces carpaticus* N29 biosurfactant, however, showed relatively higher emulsification index of 60.71%, thus appearing to have comparably broader substrate specificity. In another study, contrary to our findings, Amodu *et al.* (2014), noted that the use of agrowaste in biosurfactant production by *Bacillus licheniformis* STK01, exhibited a better emulsification tendency for all the hydrocarbons tested, except kerosene. This indicates that the different metabolic activities of biosurfactant-producing microorganisms likely affect the chemical structure and functional groups of the biosurfactant produced, and thus the

emulsification activity (de Oliveira *et al*., 2013).

Conclusion

This study shows that starch and sucrose are the choice carbon sources, while ammonium sulphate is the preferred nitrogen source that can be used by the isolates for improved biosurfactant production. The biosurfactants were observed to efficiently emulsify hydrocarbons and vegetable oils, and thus can be utilized for cleaning up of hydrocarbon contaminated sites and as emulsifiers in food industries.

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Authors contributions

Stella Chidimma Nnabugwu and Ikechukwu Amaechi Ekwealor conceptualized and designed the study. Sample collection and laboratory analyses were performed by Stella Chidimma Nnabugwu and supervised by Ikechukwu Amaechi Ekwealor. Data analysis and interpretation were conducted by Tobechukwu Maximilian Cajetan Ajogwu, Josephine Chigbolum Ohuche, and Chidimma Osilo. The manuscript was drafted by Stella Chidimma Nnabugwu and critically revised by Ikechukwu Amaechi Ekwealor and Chito Clare Ekwealor. All authors read and approved the final manuscript.

Conflict of interest

The authors declare no conflict of interest.

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