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Effects of Nitrogen and Carbon Sources on Biosurfactant Production by Hydrocarbon-utilizing *Stenotrophomonas* sp.

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Authors' contributions

This work was carried out in collaboration among all authors. Authors VE and GCO designed the study. Author VE carried out sample collection. Authors VE and BO carried out the experiments. Authors GCO and IJO coordinated the overall study. Authors VE, IJO, BO and GCO jointly drafted and corrected the manuscript. All authors read and approved the manuscript.

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ABSTRACT

Aim: This study investigated effects of nitrogen and carbon sources on the production of biosurfactant by a hydrocarbon-utilizing bacterium, *Stenotrophomonas* sp.
Methodology: The hydrocarbon-utilizing bacterium was isolated with Bushnell Haas (BH) broth using enrichment method. Biosurfactant production was screened by evaluating the following characteristics: Emulsification index (E-24), oil spreading (displacement), tilted glass slide,

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haemolysis on blood agar, and lipase production. Effects of combination of nitrogen sources (yeast extract and NH_4NO_3, yeast extract and urea, yeast extract and asparagine, yeast extract and peptone, $NaNO_3$ and peptone, $NaNO_3$ and asparagine, and yeast extract and $NaNO_3$) and carbon sources (glucose, fructose, galactose, cassava peel, soya bran, olive oil, sucrose, crude oil, diesel and glycerol) on biosurfactant production were determined with emulsion stability and surface tension as responses. The bacterium was identified based on phenotypic, microscopic, and biochemical characteristics.

Results: The isolate produced colonies on BH agar containing either naphthalene or hexadecane as sole source of carbon after 48-h incubation. Screening characteristics for the production of biosurfactant by the isolate were as follows: 46% emulsification index, 3.1 cm^2 oil displacement, 1.8 cm zone of clearance on tributyrin agar, γ-haemolysis, and positive tilted glass slide. The best carbon source with the highest emulsion stability (51.6%) was fructose whereas the best surface tension reduction (30.85 mN/m) was observed with olive oil as carbon sources after 7 days of incubation. For nitrogen, the combination of yeast extract and NH_4NO_3 gave the highest emulsion stability (60.7%) and the best surface tension reduction (39.58 mN/m). The data obtained were significant at $P<0.05$ and the bacterial isolate identified as *Stenotrophomonas* sp.

Conclusion: This study has demonstrated the ability of the hydrocarbon-utilizing bacterium, *Stenotrophomonas* sp. to produce biosurfactant, indicated by reduction of surface tension and formation of stable emulsion. This method of biosurfactant production can be further scaled up for industrial purpose.

Keywords: Stenotrophomonas sp.; hydrocarbon-utilizing bacterium; biosurfactant; surface tension.

1. INTRODUCTION

Despite the enormous advantages (biodegradability, low toxicity, effectiveness at extreme temperatures or pH values and environmental compatibility) of biosurfactants over chemical surfactants [1,2], the problem of low yield continues to limit their industrial application [3,4]. Commercial interest in biosurfactant is justifiable as industrial applications of biosurfactants cut across the petroleum, cosmetic, pharmaceutical, detergent, paint and food industry [5-10]. Biosurfactants, which are surface active substances that reduce surface tension, help to break the interfacial tension that exists between surfaces. They possess both hydrophilic and hydrophobic ends and thus accumulate at interfaces, decrease interfacial tensions and form aggregates such as micelles. Biosurfactants enhance hydrocarbon mobilization making the hydrocarbons readily available to microbial degradation [11]. This is the basis of their application in biodegradation and bioremediation of hydrocarbons.

Many microorganisms have been implicated in the production of biosurfactants and they include mould, yeast and bacteria. Bacterial isolates seem to dominate biosurfactant production for now but interest in yeast because of their "generally regarded as safe status" [12] continues to gain ground. Among the bacterial isolates, the frequently implicated genera include *Pseudomonas*, *Klebsiella*, *Acinetobacter*, *Arthrobacter*, *Alcaligenes*, *Bacillus*, *Enterobacter,* and *Stenotrophomonas.* These bacteria have been isolated from different environments. Although majority of the biosurfactant-producing bacteria are still isolated from hydrocarbon contaminated environments, reports of isolation from other sources that are not contaminated with crude oil exist. According to reports, about 2 to 3% of the screened population in uncontaminated soils are biosurfactant-producing microorganisms and this value may increase to 25% in contaminated soils [13]. Conversely, enrichment culture techniques specific for hydrocarbon-degrading bacteria may lead to much higher detection of biosurfactant-producers up to 80% [14]. However, dearth of information and limitation in the techniques for screening biosurfactant - producing microorganisms may account for the low estimation of organisms with capacity for biosurfactant production.

For biosurfactants to compete with chemical surfactants, they must be produced at their optimum capacity. Several factors can affect the production of biosurfactants by microorganisms such as a good culture media cocktail, inoculum size, pH, temperature, nutrient sources, carbon-nitrogen ratio, metallic ions and agitation speed etc. [6]. Among all the above factors carbon and nitrogen sources are critical. Effects of carbon sources on the production of biosurfactants by

diverse microorganism have been investigated by various researchers. Carbon sources for biosurfactant production may be grouped into water insoluble (hydrocarbons) and water soluble. Banat et al. [15] reported that water insoluble substrates support biosurfactant production more than water soluble substrates; although, most microorganisms may have preference for specific substrates. The growth and production of biosurfactant by *Bacillus* on sucrose, glucose, mannose and mannitol but not fructose, arabinose or maltose has been reported [16]. Asha et al. [17] reported that the quantity and quality of biosurfactant produced varied when two different organisms namely *Bacillus licheniformis* BS2 and *Pseudomonas aeruginosa* BS2 were grown in mineral media supplemented with glucose, mannitol, glycerol, hexadecane and oil sludge. The negative regulatory effect of glucose on biosurfactant production has also been reported.

Nitrogen source is one indispensable factor that regulates the yield and efficiency of biosurfactant production. *Arthrobacter paraffineus* ATCC 19558 prefers ammonium to nitrate as inorganic source and urea as an organic source for the production of biosurfactants. Contrarily, Healy et al. [18] reported that *Bacillus cereus* preferred ammonium nitrate (NH_4NO_3) for optimum yield and efficiency while *Pseudomonas aeruginosa* preferred $NaNO_3$ [19,20]. It has also been reported that *Pseudomonas aeruginosa* biosurfactant production is mostly favoured by limiting nitrogen environment [20].

This study investigated the effects of nitrogen and carbon sources on the production of biosurfactant by *Stenotrophomonas* sp.

2. MATERIALS AND METHODS

2.1 Isolation of Hydrocarbon-utilizing Bacterium

Hydrocarbon impacted soil was collected from a crude oil contaminated site in K-Dere, Gokana, Nigeria. The hydrocarbon-utilizing bacterium was isolated using enrichment method previously described by Mittal and Singh [21] and Mnif et al. [22]. Isolation was carried out with Bushnell Haas agar (g/L: $MgSO_4$ 0.2; $CaCl_2$ 0.02; $KHPO_4$ 1.0; K_2HPO_4 1.0; NH_4NO_3 1.0; $FeCl_3$ 0.05; pH 7.0) medium amended with 1% crude oil. The bacterial isolate was tested for its ability to grow on either long chain alkane or polycyclic aromatic hydrocarbon. The isolate was purified on nutrient agar and stored in nutrient agar slant and Bushnell Haas agar slant.

2.2 Screening for Biosurfactant Production

The isolate was screened for biosurfactant production using the following techniques: Emulsification index (E_{24}), lipase production, oil spreading, haemolytic activity and tilted glass slide [23].

2.2.1 Emulsification index (E_{24}) %

Emulsification index (E_{24}) is a quick, consistent, and quantitative measure of biosurfactant production. The E_{24} was carried out following the method described by Nitchke and Pastore [24]. In brief, 2 mL of kerosene was added to equal volume of cell-free broth and vortexed at maximum speed for 2 min. The mixture was kept at ambient temperature for 24 h. After 24 h, emulsification index was calculated. Distilled water was used as the negative control, whereas Tween 80 served as positive control.

$$E_{24}(\%) = \frac{\text{total height of the emulsified layer x 100}}{\text{height of the liquid layer}}$$

2.3 Determination of Surface Tension by Capillary Rise Method

Surface tension was determined using the capillary rise method described by Munguia and Smith [25] and Kumar et al. [26]. In brief, 5 mL of the broth medium was transferred into a centrifuge tube and centrifugation performed at 10000 rpm for 15 min. After the centrifugation, 2 mL of the supernatant was gradually decanted into a sample bottle flask and placed in a water bath kept at constant temperature of 25°C. Non-heparinized glass haematocrit capillary tube (Vitrex, USA) was freely suspended in the sample and the height travelled by the sample in the tube recorded. Surface tension of the sample was calculated using the formula:

$$\gamma = \frac{\rho g h r}{2}$$

Where:

γ = surface tension (mN/m),
ρ = density of the sample (mass/volume) (g/cm^3),
g = acceleration due to gravity (980 cm/s^2),
h = height of fluid in the capillary tube (cm), and
r = radius of the capillary tube (cm).

Table 1. Different carbon and nitrogen sources studied

S/N	Carbon Sources	Nitrogen sources
1	Glucose	YE + NH_4NO_3
2	Fructose	YE + Urea
3	Galactose	YE + Asparagine
4	Cassava peels	YE + Peptone
5	Soya bran	$NaNO_3$ + Peptone
6	Olive oil	$NaNO_3$ + Asparagine
7	Sucrose	YE + $NaNO_3$
8	Crude oil	
9	Diesel	
10	Glycerol	

2.4 Effect of Carbon Sources on Biosurfactant Production

Ten different carbon sources (glucose, fructose, galactose, cassava peels, soya bran, olive oil, sucrose, crude oil, diesel, and glycerol) were screened for their effect on biosurfactant production (Table 1). Bushnell Haas broth medium was used except that the NH_4NO_3 was replaced with a combination of yeast extract and $NaNO_3$ for all the set-ups. Each of the broth medium was supplemented with 2% (w/v) of the carbon source to be tested. The medium was autoclaved at 121°C (0.15 mPa) for 15 min. After sterilization, the media were brought to ambient temperature and 5% standard inoculum from the test isolate (standardized using 0.5 Mc Farland standards) used. The inoculated media were incubated at 30°C for 4 days and 7 days and biosurfactant production determined by estimating the emulsification index and surface tension of the cell-free broth.

2.5 Effect of Nitrogen Sources on Biosurfactant Production

Combination of nitrogen sources ($NaNO_3$, yeast extract, peptone, urea, NH_4NO_3, and arginine) was carried out and their suitability as nitrogen sources for biosurfactant production investigated (Table 1). Bushnell Haas broth medium was used with NH_4NO_3 replaced with the nitrogenous compounds to be tested for each of the set-ups. Each of the broth medium was supplemented with 2% (w/v) of the best carbon source. The medium was autoclaved at 121°C (0.15 mPa) for 15 min. After sterilization, the media were brought to ambient temperature and 5% standard inoculum of the bacterial isolate (standardized using 0.5 Mc Farland standards) used. The inoculated media were incubated at 30°C for 4 and 7 days and biosurfactant production determined as previously stated.

2.6 Colonial and Biochemical Characteristics of the Bacterial Isolate

The texture, colour, elevation, edge, shape and size of the colonies on nutrient agar plate were noted. Biochemical tests including sugar fermentation, citrate utilization, methyl red and Voges Proskauer, indole, gelatin, motility, indole were carried out on the isolate to aid its identification. Gram's stain was also carried out on the isolate to determine its Gram's reaction using a microscope. Results obtained from the biochemical test were interpreted using Bergey's Manual for Determinative Bacteriology [27] and other online resources.

2.7 Statistical Analysis

The results were compared by one-way analysis of variance (one-way ANOVA) and multiple range tests to find the differences between measurement means at 5% (p=0.05) significance level using IBM® SPSS® Statistics Version 21.0 (Gailly and Adler, US).

3. RESULTS

3.1 Isolation of Bacterial Isolate

The bacterial isolate grew on Bushnell Haas medium amended with 1% crude oil. The isolate also grew on both long chain and polycyclic aromatic hydrocarbon. The test isolate had a good growth on Naphthalene and Hexadecane amended Bushnell Haas agar after 96 h and 48 h of incubation, respectively.

3.2 Biosurfactant Screening Characteristics

The result of the biosurfactant screening characteristics is shown in Table 2. The isolate gave an emulsification index of 46%, displaced oil by 3.1 cm^2 and showed zone of clearance of 1.8 cm on tributyrin.

3.3 Effects of Carbon and Nitrogen Sources on Biosurfactant Production

The effects of carbon and nitrogen sources are presented in Figs. 1 to 4. Fructose gave the highest emulsion stability (51.6%) whereas the best surface tension reduction (30.85 mN/m) was observed with olive oil as carbon source after 7 days of incubation. The combination of yeast extract and NH_4NO_3 gave the highest emulsion stability (60.7%) and the best surface tension reduction (39.58 mN/m).

3.4 Identification of the Bacterial Isolate

Colonial, microscopic and biochemical characteristics of the bacterial isolate are presented in Table 3. The results revealed the organisms as a Gram negative rod-shaped, obligate aerobe, motile with flagella.

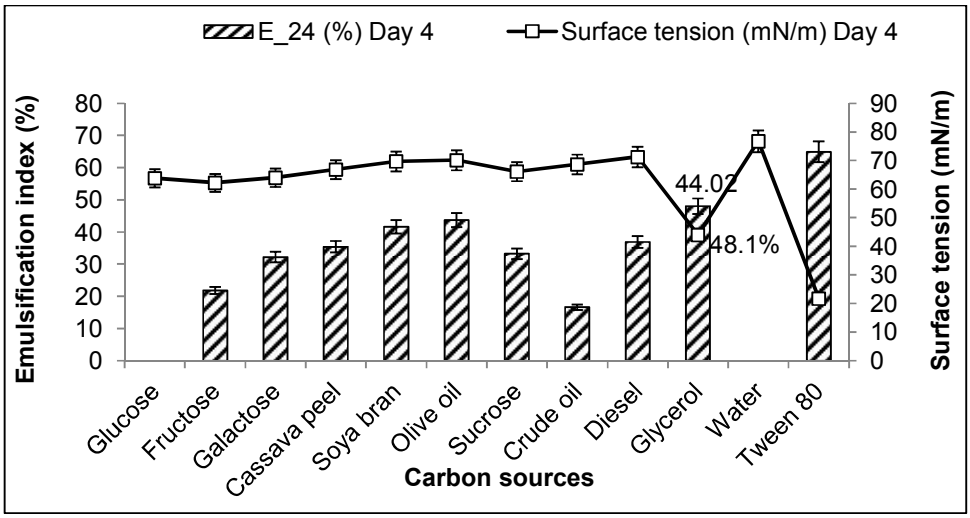

Fig. 1. Effects of carbon surfaces on biosurfactant production (emulsion stability and surface tension reduction) after 4 days of incubation

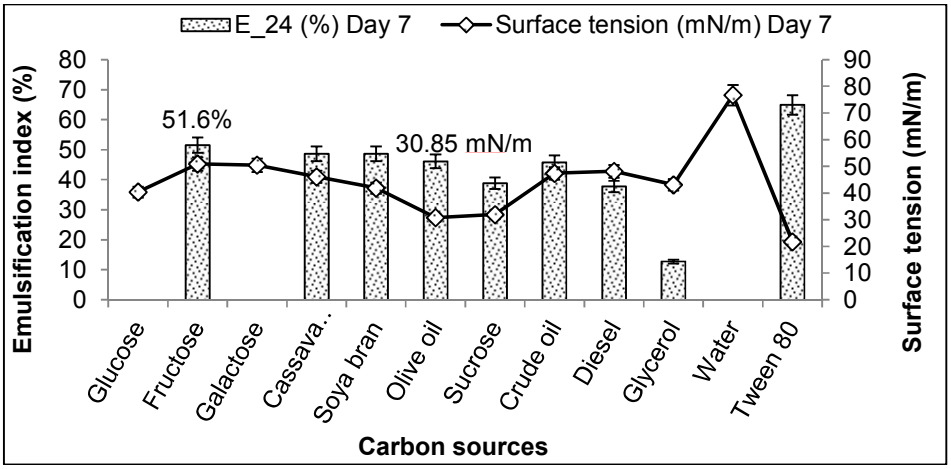

Fig. 2. Effects of carbon surfaces on biosurfactant production (emulsion stability and surface tension reduction) after 7 days of incubation

Table 2. Hydrocarbon-utilizing and biosurfactant-screening characteristics of the bacterial isolate

Isolate code	Growth on hydrocarbon			Biosurfactant screening characteristics				
	Crude oil	Hexadecane (48 h)	Naphthalene (96 h)	Lipase (cm)	Haemolysis	E24 (%)	Oil displacement (cm^2)	Tilted glass slide
B7	+++	++	++	1.8	γ	46	3.1	+

Legend: +++: Excellent growth; ++: Good growth; γ: gamma haemolysis (no haemolysis); +: positive

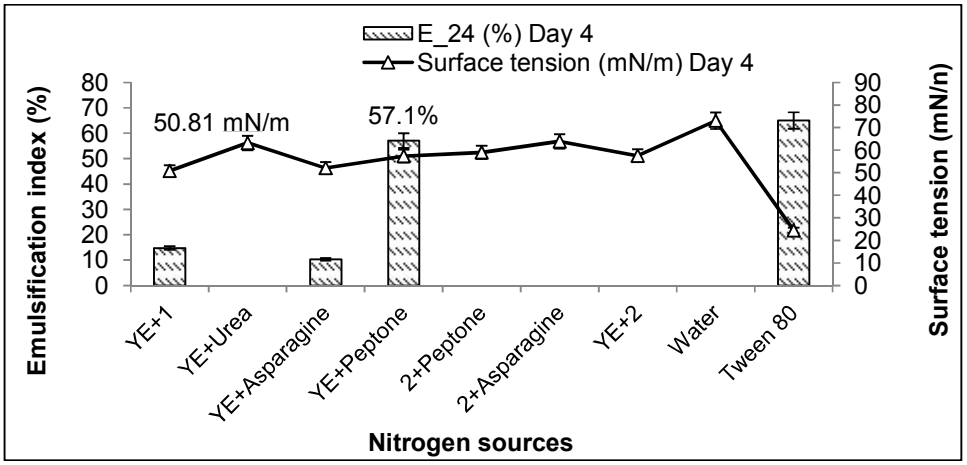

Fig. 3. Effects of nitrogen surfaces on biosurfactant production (emulsion stability and surface tension reduction) after 4 days of incubation

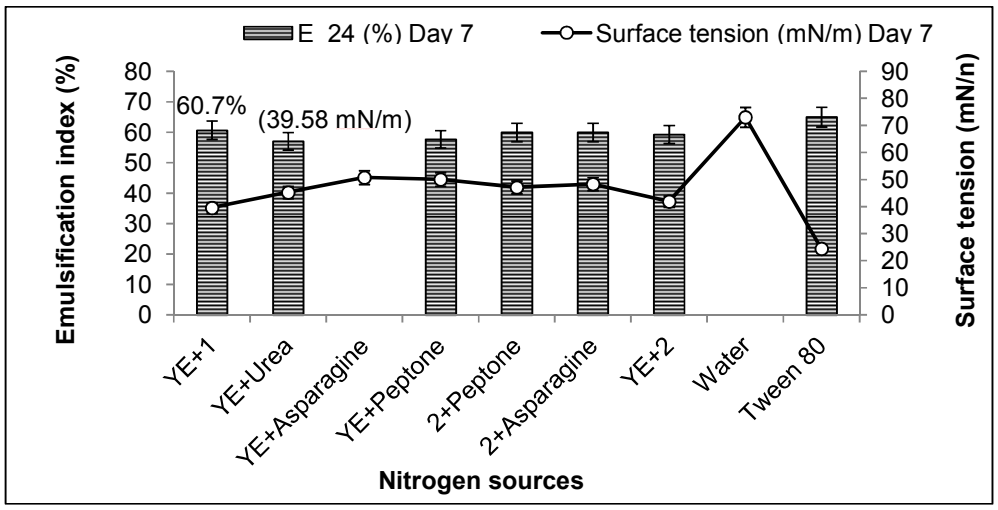

Fig. 4. Effects of different nitrogen surfaces on biosurfactant production (emulsion stability and surface tension reduction) after 7 days of incubation

Legend: 1: NH_4NO_3; 2: $NaNO_3$; YE: Yeast extract

4. DISCUSSION

This study investigated the effect of different carbon and nitrogen sources on the production of biosurfactant by a hydrocarbon-utilizing *Stenotrophomonas* sp. *Stenotrophomonas* sp. is a non-fermenting Gram negative, rod-shaped motile bacterium. It is an obligate aerobe and has been regularly implicated in biosurfactant production and hydrocarbon degradation. *Stenotrophomonas* sp. was first classified under the genus *Pseudomonas*; later it was transferred

to *Xanthomonas* genus but it was in 1993 that the *Stenotrophomonas* genus was fully recognised and properly classified [28,29]. Singh et al. [30] reported pyrene degradation by biosurfactant-producing bacterium, *Stenotrophomonas maltophilia* isolated from crude oil contaminated soil from Vadodara oil refinery in India. Their report corroborates the findings of this study that *Stenotrophomonas* sp. was able to grow on both long chain alkane and polycyclic aromatic hydrocarbons. Another study by Gargouri et al. [31] reported the production of biosurfactant and the degradation of hydrocarbon by *Stenotrophomonas* sp. Similarly, Larik et al. [32] applied *Stenotrophomonas maltophila* in the biodegradation of diesel and engine oil. They also reported that the organism was an efficient biosurfactant producer. The highest emulsion stability achieved with the crude biosurfactant in this study was 60.7%. This is higher than the 56% emulsification index reported by Tripathi et al. [33] with a *Stenotrophomonas* sp. but lower than the reported 70% emulsification index by Gargouri et al. [31] with the same bacterium.

Table 3. Biochemical characteristics of the isolate

Test	Characteristics
Isolate code	B7
Gram Stain	- (rods)
Citrate	-
Motility	+
Oxidase	-
Catalase	+
Indole	-
Urease	-
MR	-
VP	-
TSI	
Slant	Pink
Butt	Yellow
H_2S	-
Gelatin hydrolysis	+
Sugar Fermentation	
Maltose	+
Glucose	+
Lactose	+
Mannitol	-
Sucrose	-
Probable genus	*Stenotrophomonas*

In this study, out of the 10 carbon sources used as substrates for biosurfactant production, the following showed good prospects: Fructose (although the biosurfactant produced could only yield high emulsification index but could not effectively reduce surface tension), cassava peel, soya bran, olive oil and crude oil. Amongst these, the biosurfactant produced with olive oil showed high emulsification index and capacity to reduce surface tension. Lowest surface tension reduction (30 mN/m) was achieved with olive oil as the carbon source. A similar surface tension reduction (29 mN/m) was observed with *Pseudomonas aeruginosa* IITR48 according to the report of Tripathi et al. [33], although the biosurfactant-producing isolate was grown on naphthalene and diesel as sources of carbon. The *Stenotrophomonas* sp. used in this study produced biosurfactant with most of the carbon sources except glucose (at both days 4 and 7) and galactose at day 7. This finding may be due to the fact that the isolate is a non-fermentative bacterium [34]. Abouseoud et al. [20] studied the use of Olive oil as substrate for biosurfactant production by *Pseudomonas fluorescens* and reported that the biosurfactant produced reduced surface tension from 72 mN/m to 32 dyme/cm. Another study that supports the suitability of olive oil as substrate for biosurfactant production is the work of Tan and Li [35].

Different nitrogen sources were studied for use in biosurfactant production. A combination of yeast extract and NH_4NO_3 was best for both emulsion stability and surface tension reduction. Other studies have reported similar findings [36]. Makkar and Cameotra [37] investigated the effect of nitrogen sources (urea, peptone, yeast extract, beef extract tryptone, nitrogen-free medium, potassium nitrate, sodium nitrate, ammonium nitrate and ammonium sulphate) on biosurfactant production by *Bacillus subtilis*. Their findings showed marked differences in production of biosurfactant with different nitrogen sources. Their findings agree with the result obtained from this study.

5. CONCLUSION

This study has demonstrated the ability of the hydrocarbon-utilizing bacterium, *Stenotrophomonas* sp. to produce biosurfactant, indicated by reduction of surface tension and formation of high stable emulsion. The utilization of readily available substrate by the isolate for biosurfactant production will be critical to industrial scale-up of the biosurfactant produced. Moreover the finding that the biosurfactant-producing bacterium grew well on different hydrocarbon sources enhances the prospects of applying the produced biosurfactant in bioremediation of hydrocarbon contaminated

media. The study further revealed that nitrogen source was important in the production of biosurfactant by the *Stenotrophomonas* sp.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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