



Preliminary Screening of Bio-surfactant Producing Bacteria Isolated from an Oil Contaminated Soil

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Authors' contributions

This work was carried out in collaboration between all authors. Author OYA designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors KAH and YS managed the analyses of the study. Author OYA managed the literature searches. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/AJBGMB/2018/42259

Editor(s):

(1) Dr. Ahmed Medhat Mohamed Al-Naggar, Professor, Department of Agronomy, Faculty of Agriculture, Cairo University, Egypt.

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Complete Peer review History: <http://prh.sdiarticle3.com/review-history/25455>

Original Research Article

Received 27th April 2018

Accepted 3rd July 2018

Published 9th July 2018

ABSTRACT

Spent motor oil represents one of the most prominent and tenacious contaminants of soil within mechanic workshops across Nigeria. Adaptation and natural evolution proffer a rich array of a microorganism capable of producing bio-surfactants which are of high value to industry, particularly in hydrocarbon degradation. This study was undertaken to isolate and screen for bio-surfactant producing bacterial strains isolated from engine oil contaminated mechanic sites. Data obtained revealed that using 0.3% Fluconazole supplemented nutrient agar media, bacterial isolates were obtained from soil samples within Apo mechanic village, Abuja, Nigeria. Hemolytic assay method and foam capacity test were used for screening. The positive strains were grown in liquid medium and the emulsification index (EI₂₄) was determined. A total of 3 bacterial isolates referred to as A2, B1 and C8 were positive for the hemolytic and foam capacity tests, with an emulsification index (EI₂₄) of 40.0, 11.7 and 36.7 respectively. Growth measurements were determined by measuring optical density of the cells in broth using spectrophotometer at 605nm over a 96 hour incubatory period in nutrient broth at 4°C, 37°C, 42°C. All isolates displayed mesophilic characteristics

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with progressive growth. At 37°C, isolate A2 had the highest growth rate via optical density readout 1.95, 2.11, 2.32, 2.55, measured at 605nm. Isolate A2 also the best performing isolate at 42°C with optical density readings of 1.32, 1.00, 0.91 and 1.68, thereby suggestive of thermotolerant ability. All isolates demonstrated good growth in broth medium with pH ranging from 8.97-9.16 at 37°C. All isolates were positive for catalase and citrate, negative for methyl red, Voges-Proskauer and indole tests. Isolate A2 was the only gram-positive, oxidase negative and non-motile bacteria. The bio-surfactants produced by the three different bacterial isolates would possess chemically distinct signatures that can be harnessed for multiple applications ranging from bioremediation to degradable detergent uses.

Keywords: Bio-surfactants; contamination; emulsification; engine oil; soil microorganisms.

1. INTRODUCTION

Crude oil and its associated petroleum products constitute a global nuisance when released to the environment as a result of accidental spills, thereby causing soil contamination [1,2,3,4,5]. Accumulation of these petroleum hydrocarbons in the environment pose a risk to human and animal health as these pollutants end-up in our water-ways and food chain via the consumption of tainted water, crops with large hydrocarbon deposits in their vacuoles, etc all of which may lead to the development of kidney disease, liver problems, possible damage to the bone marrow and even increase the risk of cancer [6,7,8,9,10,11]. Treatment and recovery of petroleum products from soil is a challenging process owing to their adsorption unto the soil surface as well as their confinement in a water-immiscible phase [12].

Amongst the different strategies employed for the treatment of hydrocarbon polluted soils, bioremediation which involves the use of microorganisms is the foremost eco-friendly approach to date [13,14,15,16]. Microorganisms possess genes that enable them to utilise a variety of petroleum products as a source of carbon and energy source, the efficiency of which depends on the contaminants' chemical structure, concentration, toxicity, and the physiological conditions of the immediate environment [17,18,19,20]. This suggests that either the whole or component parts of crude oil serve as food targets for certain micro-organisms thereby transforming the physico-chemical properties of the oil as the organism consumes such parts to complete its metabolic functions. With such findings, environmental biologists consider microbial degradation of oil, via the use of a variety of mechanisms or products secreted extra-cellularly, as one of the foremost routes for the natural removal of such oils from contaminated environments as a cost-effective,

cheap and environmentally friendly treatment [21].

Bio-surfactants are compounds produced and secreted by many yeasts, bacteria and filamentous fungi which serve as a measure to metabolize water-immiscible substrates with a high level of specificity, via the reduction of surface and interfacial tension [22,23,24]. Bio-surfactants are synthesized both intra-and-extra-cellularly such that they promote the transportation of insoluble substrates via the membrane, thereby acting as a biocide agent and aid in substrate solubilization [23,24,25,26,27,28]. Surfactants obtained from microbiological sources are termed bio-surfactants which unlike their chemical counterpart, are biodegradable, non-injurious to the environment and are chemically distinct to their synthetic counterparts based on the nature of their polar grouping [29]. At the turn of the millennium, a considerable amount of emphasis has been placed on bio-surfactant usage owing to peculiar diversity and suppleness of use [30,31]. With these characteristics, all of which have found several industrial, scientific and environmental applications, production on commercial scale is on the increase [30]. With the unique diversity of microorganisms that exist in nature, bio-surfactants obtainable from different microorganisms differ chemically from each other based on their hydrophilic head; allowing for a wide range of variation in their physical and biological properties [32].

Screening and selection of bio-surfactant candidate bacterial strains from diverse habitats is an interesting area of research that enhances both our collective knowledge and cache of microorganisms that can serve industrially significant processes [33]. A number of approaches are employed in the isolation and screening of bacterial isolates that are capable of degrading petroleum hydrocarbons via bio-

surfactant production [25,34,35,36]. One of such simple and common tests involve the formation of an emulsified layer in broth solution supplemented with an oily compound [24,37]. With the surge of spent motor oil on the increase in soils across mechanic workshops as reported in a previous study [5], this study therefore aimed to screening for bio-surfactant producing bacterial isolates from soil contaminated with spent oils from a mechanic workshop in Abuja.

2. MATERIALS AND METHODS

2.1 Isolation of Bacterial Strains

Soil samples were collected randomly in sterile polystyrene sampling containers using a sterile spatula from the center of motor oil-stained patches from three (3) points in the workshops by scooping to about 20 cm from the Apo mechanic village workshop in Abuja, Nigeria. The sampling points were 30 meters away from each other in either direction. The containers were labelled properly and immediately transported to the She da Science and Technology Complex (SHESTCO) laboratories for analysis. The source samples were serially diluted in sterile distilled water to 10^{-6} and inoculated on nutrient agar medium plates by spread plate method. These plates were incubated at 37°C for 24 hours and counted. About five grams (5 g) of soil sample was mixed with 100ml Minimal salt (MS) medium containing (g/L) 0.5 $(\text{NH}_4)_2\text{SO}_4$, 0.1 $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.076 $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ and 40 mM phosphate buffer (pH 7.25). Solid MS medium was made by the addition of 1.8% agar-agar (Difco Laboratories, Detroit, MI USA); and amended with spent engine oil, supplemented with 0.3% fluconazole, then incubated for 7 days at 35°C. After 7 days 17% of the inoculum was added into fresh 100ml MS medium and also amended with spent engine oil (0.04% v/v) and incubated for another seven days.

2.2 Purification and Biochemical Assessment

Pure cultures from the spent engine-oil enriched media was isolated by plating 1.0 mL of the enriched cultures onto minimal salt (MS) agar, sprayed with spent engine oil on the surface. This was incubated in dark at 28 – 32°C for five days. Colonies were periodically transferred to MS agar to obtain the pure culture. Using the morphological examinations, bacterial species were isolated and sub-cultured onto separate agar plates. The pure cultures were incubated at 37°C for 18-24 on nutrient agar slants. The

obtained isolates were subjected to biochemical tests with the following parameters: Gram staining, methyl red, voges-proskauer, catalase, citrate, oxidase, indole and motility.

2.3 Growth Profile Studies

The isolates were tested for their ability to grow at 4°C, 37°C and 42°C in nutrient broth for 96 hours. 10 mL of nutrient broth tubes were inoculated with 1% of bacterial cultures. Periodic (24 hour) measurements of growth was achieved using a UV-VIS spectrophotometer, measurements were taken at 605 nm to determine their viability [38]. pH measurements were also taken daily using a digital pH meter (Jenway model 3015) with a glass-calomel electrode combination with vigorous stirring whilst cell viability was determined by the level of turbidity [39]. All tests were performed in triplicate.

2.4 Emulsification Studies

Emulsification activity was measured by growing 24 hour culture in fresh MS medium supplemented with 0.2% v/v spent engine oil. Incubated in a shaker for 72hrs at 220 rpm at 35°C. It was centrifuged and the cell free media (5 ml) and 5 ml of spent engine oil was added and the mixture was vortexed at high speed for two minutes left to stand for 48 hours at room temperature. The emulsification activity was calculated by

$$EI_{24} = \frac{\text{height of emulsified layer (mm)}}{\text{overall height of mixture (mm)}} \times 100$$

Where (EI_{24}) is the emulsification index [40]. This experiment was performed in pyrex test tubes.

2.5 Hemolytic Test

Pure cultures of the obtained isolates was streaked on a solidified blood agar medium (Nutrient agar with 5% v/v human blood) to check for the ability of the isolate to lyse blood cells. Plates were incubated at 37°C for 24hrs, thereafter observed for cleared zone around wells created. Zone of clearance indicate positive and if none indicates negative [25].

2.6 Foam Activity Test

Isolated strains were grown separately in 250 mL Erlenmeyer flasks, each containing 100 mL of nutrient broth medium. The flasks were incubated at 37°C on a shaker incubator (200

rpm) for 72 h. Foam activity is detected as duration of foam stability, foam height and foam shape in the graduated cylinder.

2.7 Statistical Analysis

The data analysis was carried out with one-way ANOVA.

3. RESULTS AND DISCUSSION

Of a total of 9 isolates, 3 visually distinct colonies, one from each sampling point were successfully isolated using fluconazole supplemented nutrient agar and given the designations A2, B1, and C8 respectively (Fig.1). Morphological examination of these three isolates revealed a creamy white, cream and green coloured bacterial isolates, all of which were round shaped, smooth textured with transparent opacity when observed (40X) microscopically (Figs.1-3, Table 1).



Fig. 1. Pictures of white coloured bacteria isolates on Nutrient agar



Fig. 2. Pictures of cream coloured bacteria isolates on Nutrient agar

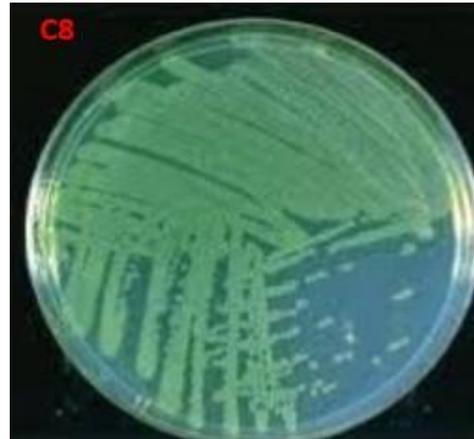


Fig. 3. Pictures of green coloured bacteria isolates on Nutrient agar

Numerous reports have indicated that a lot of *Alcaligenes*, *Arthrobacter*, *Bacillus*, *Corynebacterium*, *Klebsiella*, *Micrococcus* and *Pseudomonas* species are the most predominantly isolated species around petroleum contaminated sites, particularly at mechanic villages across Nigeria as an array of gram positive and gram negative rods as well as cocci [41,42,43,44]. However, confirmation of their identities was asserted through biochemical and physiological deductions, primarily of which include but not limited to growth temperature, cell morphology, cell arrangement, gram reaction, motility, pigment formation, catalase production, indole production, etc [38]. In line with the research efforts mentioned above, this study revealed the distinguishable presence of *Pseudomonas sp.*, (isolate C8) which characteristically produces a green pigment when grown on nutrient agar, coupled with its negative reaction to gram staining, catalase positive, motile, and negative to indole production (Table 1) feature that are synonymous with all known species of *Pseudomonas* [42,43].

From the data obtained, isolate B1 is *Alcaligene ssp* due to the unique preliminary biochemical signature the isolate in this study exhibited, in line with recent reports found in crude oil polluted soil within the Niger Delta region of Nigeria [44,45]. Based on the same assessment, isolate A2 was found to be *Enterococcus sp.*, based on the initial biochemical assessment as confirmed by the Bergley's manual of Bacteriology [46,47,48].

Growth optimum studies coupled with pH measurements within the nutrient broth over the

period of incubation for isolate designated A2 revealed fluctuations in pH levels roughly between pH 8.97-9.03 by way of turbidity measurements over time. This addresses the veracity of the cell membrane coupled with the cells acidification activity (Table 2). The same data also revealed a maximum growth rate which was attained at 37°C (1.95, 2.11, 2.32, 2.55) for the incubatory duration measured daily thereby pointing to the mesophilic character of isolate A2 (Table 2). Analysis of the data obtained indicates growth fluctuations by way of turbidity measurements over time. This addresses the veracity of the cell membrane coupled with the cells acidification activity.

Data revealed that the growth profile and the associated pH fluctuations for isolate B1, incubated at 4°C, 37°C, 42°C, revealed that the isolate grew best at 37°C with daily readings of 1.8, 2.33, 1.51, 2.07, thereby suggesting a preference for temperatures lower than room temperature although it hints towards a weakly mesophilic trait (Table 3). Fluctuations in its pH measurement ranged between 9.02-9.16 (Table 3).

In a similar fashion, the data obtained from broth samples inoculated with isolate C8 revealed that the optimum growth measurements (1.78, 2.37, 2.07, 1.76) were obtained with cultures incubated

at 37°C over 96 hours (Table 4). Likewise, pH variations ranged from 8.99-9.16.

With the exception of isolate A2 (Tables 2-4), isolates B1 and C8 displayed inhibited growth patterns post 48 hours of incubation at 37°C (Tables 2-4). This period of microbial activity could be due to the release of excess metabolites which in turn thwarts or stresses the growth rate for which survival depends on the activation of certain resistant genes that would overcome the influence of such exogenous by-products [49].

The data obtained also corroborates reports that suggest that the growth rate for most bio-surfactant producing bacteria is best at basic to alkaline pH 7-9 and also between 28-33°C [50]. Research has shown that surfactants secreted by microorganisms not only enhance the growth of the organisms in peculiar environments, they (bio-surfactants) increase the surface area between oil and water by emulsification, and by increasing pseudo-solubility of hydrocarbons by partitioning micelles [51].

From the data obtained in this study, it was deduced that isolate A2 was the better bio-surfactant producing microorganism via the use of particular screening methods (Fig. 4, Table 5). In particular, the emulsification index, 40.0% for

Table 1. Biochemical characterization of isolates

Biochemical test	Isolates		
	C8	B1	A2
Gram stain	-ve	-ve	+ve
Methyl Red	-ve	-ve	-ve
VogesProskauer	-ve	-ve	-ve
Catalase	+ve	+ve	+ve
Oxidase	+ve	+ve	-ve
Citrate	+ve	+ve	+ve
Indole	-ve	-ve	-ve
Motility	+ve	+ve	-ve
Pigment	+ve	-ve	-ve
Texture	Smooth	Smooth	Smooth
Opacity	Transparent	Transparent	Transparent

Key: +ve = positive, -ve = negative

Table 2. Growth profile on varying temperature isolate A2

Time (Hrs)	Temperature			pH
	4°C	37°C	42°C	
0	N/A	N/A	N/A	N/A
24	0.21 ± 0.3	1.95 ± 0.1	1.32 ± 0.2	9.03± 0.3
48	0.32 ± 0.9	2.11 ± 0.5	1.00 ± 0.1	9.00± 0.5
72	0.40 ± 0.6	2.32 ± 0.3	0.91 ± 0.5	8.98± 0.2
96	0.52 ± 0.5	2.55 ± 0.1	1.68 ± 0.6	8.97± 0.7

Table 3. Growth profile on varying temperature isolate B1

Time (Hrs)	Temperature			pH
	4°C	37°C	42°C	
0	N/A	N/A	N/A	N/A
24	0.16± 0.3	1.80± 0.1	1.59± 0.6	9.06± 0.3
48	0.17± 0.7	2.33± 0.4	0.47± 0.2	9.08± 0.5
72	0.21± 0.9	1.51± 0.1	0.41± 0.4	9.11± 0.3
96	0.34± 0.2	2.07± 0.6	0.47± 0.1	9.16± 0.2

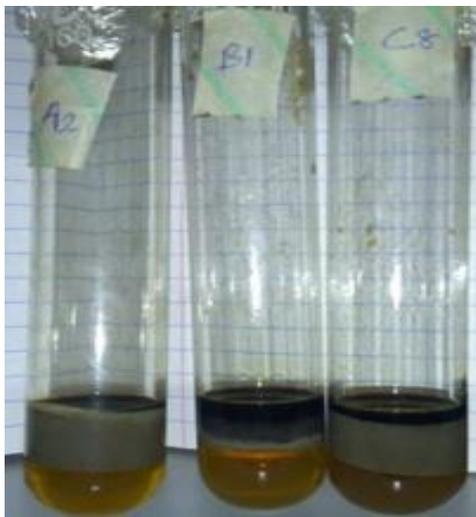
Table 4. Growth profile on varying temperature isolate C8

Time (Hrs)	Temperature			pH
	4°C	37°C	42°C	
0	N/A	N/A	N/A	N/A
24	0.20± 0.4	1.78± 0.3	1.74± 0.7	8.99± 0.4
48	0.15± 0.1	2.37± 0.8	0.76± 0.9	9.01± 0.6
72	0.22± 0.6	2.07± 0.6	0.57± 0.4	9.07± 0.3
96	0.23± 0.2	1.76± 0.2	1.03± 0.1	9.16± 0.7

Table 5. Screening for bio-surfactant activity

Isolate	Emulsification index (EI ₂₄)	Hemolytic test	Foam activity	Inference
A	40.0%	+ve	+ve	Bio-surfactant producing
B	11.7%	+ve	+ve	Bio-surfactant producing
C	36.7%	+ve	+ve	Bio-surfactant producing

isolate A2 was the highest of the three isolates which was followed by 36.7% for isolate C8 and 11.7% for isolate B1 at the initial screening. All isolates tested positive for foam and hemolytic activities. The culmination of these results were consistent with other reports [52,53].

**Fig. 4. Emulsification of engine oil**

These results confirmed the presence of bio-surfactant in the cell free culture supernatant.

After the confirmation of bio-surfactant synthesizing capability of the strain *B. subtilis* A1 culture condition was further optimized.

The results obtained thus far is in tandem with other reports that have also characterized the growth profile of bio-surfactant producing microorganisms with respect to foaming capability, thermo-stability, salinity, etc with organisms like *Acinetobacter* sp., *Bacillus licheniformis*, *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Rhodococcus erythropolis* and *Torulopsis bombicola* [25,54,55,56,57].

4. CONCLUSION

Data obtained in this study has shown that indigenous bacteria isolated from petroleum-rich environments were bio-surfactant producers, exhibiting characteristic features with regards to emulsification, hemolytic activity and foam capacity (Table 5). The organisms also displayed strong mesophilic (33-37°C) properties and a preference for growth at alkaline pH 9 (Tables 2-4). Their ability to create emulsions with engine oil strongly suggests that these microorganisms could be applied towards the bioremediation of engine oil contaminated soils via the use of bio-surfactants amongst a host of tools in their arsenal.

ACKNOWLEDGEMENTS

The authors would like to thank Mr. Olawore Samuel Bolaji, Mr. Godwin Etuk-Udo and Mr Salisu Abubakar for their varied support.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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