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Evaluation of Indigenous Biosurfactant-producing Bacteria for De-emulsification of Crude Oil Emulsions

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Authors' contributions

This work was carried out in collaboration among all authors. Author OAF designed the study and wrote the protocol. Authors OAF and AAOO supervised the entire work. Author AAOO gave the experimental designs and lead in microbiological studies while author OAF gave the lead in the demulsification studies. Author MAA performed the laboratory analysis and wrote the first draft of the manuscript. Authors OAF and AAOO managed the literature searches, read and approved the final manuscript.

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ABSTRACT

Conventional method of removing water from crude oil using chemicals is unfavourable from both the economic and environmental perspectives; so, this study aims at formulating economical and environmentally-friendly biosurfactant de-emulsifiers. Biosurfactant-producing bacteria isolated from oil-contaminated soil samples from Nigerian National Petroleum Corporation (NNPC) depot Apata, Ibadan, Oyo State of Nigeria were applied on crude oil emulsions for the purpose of separating water-in-crude oil emulsions. Thirty-five of 41 bacterial strains were further screened for ability to degrade (de-emulsify) hydrocarbon, using vapour transfer method. Highest displayed de-emulsification activities at 24 h were *Pseudomonas* sp. AGO1 (50.0%), *Bacillus* sp. DPK1A

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(50.0%), *Bacillus subtilis* AGO1A (50.0%), *Pseudomonas aeruginosa* DPK3A (55.7%) and *Bacillus subtilis* PMS1B2 (66.0%); and at 48 h were, *Bacillus subtilis* AGO1A (50.0%), *Ps. aeruginosa* DPK3A (60.0%) and *Bacillus subtilis* PMS1B2 (66.7%). Higher de-emulsification activities were recorded on supplementation of growth media, with *Ps. aeruginosa* DPK3A showing the highest de-emulsification activity of 66% when grown on growth media supplemented with glucose and yeast extract, at temperature of 60℃. In comparison with chemical de-emulsifier, microbial de-emulsifier produced 66%, 62% and 60% volume of water, while chemical de-emulsifier produced 63%, 60% and 66.2% volume of water. This study demonstrated that generally regarded as safe (GRAS), hydrocarbon-utilising, biosurfactant-producing bacteria, especially the *Bacillus* species isolated from crude oil-contaminated soils, when cultured on appropriate medium is effective in diesel degradation and treatment of water-in-crude oil emulsion; thus, reducing cost and environmental pollution.

Keywords: Biosurfactant bacteria; chemical de-emulsifiers; crude oil; de-emulsification activities; emulsification test.

1. INTRODUCTION

Surfactants are chemically synthesised surface-active compounds, which are widely used for large number of applications in various industries [1]. However, there has been increasing demand for biological surface-active compounds or biosurfactants, which are products of large number of microorganisms that exert biodegradability, low toxicity and widespread applications compared to chemical surfactants [2]. Biosurfactants can also be used as moistening agents, dispersing agents, emulsifiers, foaming agents, beneficial food elements and detergents in many industrial areas, like organic chemicals, pharmaceuticals, cosmetics, beverages and foods, metallurgy, mining, petroleum, petrochemicals, biological control and management, etc. [3-7].

Biosurfactant-producing microorganisms are commonly found in different environments, such as soil or water samples that are contaminated with hydrophobic organic compounds (i.e., oil contaminated soils) like refinery wastes [8-10]. Furthermore, biosurfactants are presently used as emulsifiers, de-emulsifiers, wetting agents, spreading agents, foaming agents, functional food ingredients, as well as detergents [11]. Due to their unique properties and vast array of applications, sourcing of new biosurfactant-producing microbes is therefore, currently in great demand [5], and a number of studies have thus, described the effect of exogenously added microbial biosurfactants in enhancing the bioremediation of crude oil-polluted soils by indigenous microbes [5,12-14]. Other studies also highlighted how to purify and detect biosurfactants [15-17]. In addition, various experiments at laboratory scales on sand-pack columns and field trials have successfully indicated the effectiveness of biosurfactants in microbial enhanced oil recovery [11].

Well-known, commonly isolated oil-degrading bacterial species include *Acinetobacter*, *Bacillus cereus*, *Bacillus licheniformis*, *B. megaterium*, *B. subtilis*, *Branhamella catarrhalis*, *Citrobacter intermedius*, *Corynebacterium kutscheri*, *C. xerosis*, *Enterobacter aerogenes*, *Escherichia coli*, *Flavobacterium* sp., *Klebsiella ozaenae*, *Lactobacillus casei*, *L. delbrueckii*, *Micrococcus*, *Proteus inconstans*, *Pseudomonas aeruginosa*, *Ps. fluorescens*, *Ps. diminuta*, *Ps. mallei*, *Rhodococcus* and *Staphylococcus aureus*, *Virgibacillus salaries* [5,7,8,18-24]. Commonly reported oil-degrading fungal flora are *Aspergillus*, *Penicillium*, *Fusarium*, *Amorphoteca*, *Neosartorya*, *Paecilomyces*, *Talaromyces*, *Graphium*, as well as yeasts, like *Candida*, *Yarrowia*, *Pichia*, etc. [25]. However, considering that studies on biosurfactants are significantly sparse in Nigeria; whereas, most of the very unique biosurfactants are geographic-dependent, as well as species and strain specific, due to physiological conditions of the biosurfactants, as well as the adaptive environmental conditions.

The present study therefore, tries to isolate and investigate the de-emulsification abilities of indigenous biosurfactant bacterial species on crude oil emulsions.

2. MATERIALS AND METHODS

2.1 Crude Oil Properties

Crude oil sample used in the study was obtained from Forcados terminal in the Niger-Delta area of Nigeria. The crude oil properties were determined at the Petroleum Engineering

laboratory, Department of Petroleum Engineering, Faculty of Technology, University of Ibadan, Oyo State, Nigeria.

2.2 Sampling

Three sets of oil-contaminated soil samples were collected from NNPC depot, Apata, Ibadan, Oyo State, Nigeria at three different times (bi-monthly). They were collected in sterile bottles, labeled appropriately and transported to the laboratory for further studies.

2.3 Isolation of Bacterial Isolates from Soil Samples

In this study, soil samples were screened for bacteria by slightly modifying the culture media and screening procedures [8] for recovery of biosurfactant-producing isolates. A 5-g sample of each soil was placed in a 250-ml flask containing 50 ml of sterile tap water and incubated at 23℃ in a shaker at 200 rpm for 3 days. On day 3, a sample from each soil slurry was serially diluted and plated on nutrient agar (Lab M, England). Three-fold serial dilutions were carried out for pour plate method of bacterial isolation. One (1 ml) of 10^{-3} dilution of each soil sample was inoculated on sterile Petri dishes in triplicates, after which sterilise nutrient agar was poured aseptically into the inoculated plates. The seeded plates were then incubated at 35℃ for 24 h and 48h, while representatives of each different bacterial colony types were randomly picked from all the primary plates and subcultured repeatedly on sterile nutrient agar plates by the four-corner streaking method to obtain pure cultures. Selected pure bacterial isolates were grown and stored on sterile nutrient agar slants as bench and stock cultures.

2.4 Screening for Bacterial Hydrocarbon Deterioration

Crude oil utilisation test was carried out on bacterial strains obtained from preliminary isolation, as confirmatory identification of actual petroleum-utilising bacteria. Compounded Bushnell Hass agar was used for microbial hydrocarbon deterioration test [26]. Each suspected petroleum-utilising bacterial isolate was streaked on Bushnell Hass agar plate with a sterile filter paper (Whatman No. 1) saturated with crude oil placed in the inside of the Petri dish cover. This was aimed at supplying hydrocarbons as sole sources of carbon and energy for the bacterial growth on the mineral salt agar medium surface through vapour phase transfer. All the plates were inverted and incubated at room temperature for 7– 14 days [27].

2.5 Culture Medium for Biosurfactant-producing Bacteria

The medium for biosurfactant production was composed of mineral salts containing (g/l) KH_2PO_4 (1.0), K_2HPO_4 (1.0), $MgSO_4$ (0.2), $FeSO_4$ (0.05), NH_4NO_3 (1.0), $CaCl_2$ (0.020), supplemented with yeast extract (0.6) as nitrogen source and glucose (0.6) as the carbon source.

2.6 Screening of Biosurfactant Producing Bacteria

The isolated bacterial colonies were tested for their biosurfactant production by carrying out the emulsification activity test. The ability of the biosurfactant to emulsify crude oil was determined following the method described by Salehizadeh et al. [28]. Two ml of crude oil was suspended in test tubes containing 2 ml of cell-free supernatants. The mixture was vortexed at high speed using a vortex mixer for 2mins and the test tubes were left to stand for 24h, after which the emulsification index was measured. The emulsification index (E24) is given as the percentage of the height of the emulsified layer (cm) divided by the total height of the liquid column (cm) and multiplied by 100 [28].

$$\mathrm{E24} = \frac{Height\ of\ emulsfied\ \ layer}{Total\ \ height} \times 100$$

2.7 Characterisation of Biosurfactant Producing Bacteria

The screened biosurfactant producing organisms were characterised by using phenotypic taxonomic tools, which included cultural and microscopic (Gram's) identities, as well as various biochemical tests. General keys used in bacterial identities were according to standard phenotypic taxonomic tools [29,30] by reference to Bergey's Manual of Systematic Bacteriology [31-34].

2.8 Preparation of Crude Oil Emulsion

Water in oil emulsion was prepared according to the method described by Falode and Aduroja [35]. Water in oil emulsion was prepared by mixing crude oil with synthetic oilfield brine. The synthetic oilfield brine was prepared by dissolving NaCl in deionised water to obtain the

required salinity similar to the average Niger-Delta field. The emulsification was carried out by using a vortex mixer set at medium speed for 2.5 minutes to get a stable emulsion.

2.9 Oil Field Brine

The synthetic oilfield brine was prepared by dissolving NaCl in deionised water in order to obtain the required salinity similar to the average Niger-delta field, which is about 2.4% [35] by using the equation below:

Salinity equation; Y = 8.3566X – 0.3582

Where:

Y = Salinity (%w/w); % in per thousand
X = NaCl concentration (g/100 ml)
2.4 = 8.3566x – 0.3582
x = 3.30 g

So, about 3.30 g of NaCl was dissolved in 100 ml of deionised water to obtain 2.4% salinity of the synthetic oilfield brine.

2.10 Measurement of De-emulsification Activity

Measurement of de-emulsification activity was carried out by slight modification of the method of Hossein et al. [36]. One ml of each bacterial culture broth was added to 5 ml hydrocarbon (water in oil emulsion) in a test tube, and vortexed for 60s to form a homogenous culture emulsion mixture, which was allowed to stand for about 20 minutes in an incubator at a room temperature. For the control experiments, 1 ml of uninoculated culture medium and 5 ml of water in oil emulsion was used. The de-emulsification activity of biosurfactant de-emulsifier was calculated as follows:

$$\text{De-emulsification activity (\%)} = \frac{\text{Volume of separated water, ml}}{Original\ volume\ of\ water\ in\ the\ emulsion, ml} \times 100$$

3. RESULTS

Table 1 is the result of the properties of the assayed crude oil which was determined in the laboratory.

Table 1. Properties of test crude oil sample

Properties of crude oil sample	
Viscosity (cp)	21.98 cp
Density (kg/m^3)	0.916 kg/m^3
Specific gravity	0.900
API gravity	25.72

Results of emulsification test in this study revealed that only 10 of the screened 20 bacterial strains that were isolated from hydrocarbon-contaminated soil samples showed positive emulsification properties. *Bacillus subtilis* PMS1B2 (66.0 and 66.7), *Ps*. *aeruginosa* DPK3A (57.0 and 60.0) and *B*. *subtilis* AGO1A (50.0 and 50.0) gave the best emulsification properties at 24 h and 48 h respectively, when incubated at 30℃ and 60℃ (Table 2).

The results of effect of carbon and nitrogen sources on de-emulsification activity are as presented in Table 2. Determination of effect of carbon and nitrogen sources on de-emulsification activity by the selected biosurfactant-producing

Table 2. Emulsification and de-emulsification activities of biosurfactant-producing bacteria

Lab codes of bacterial strains	Emulsification activity [Temp.]		De-emulsification activities [carbon source]			
	(24 h)	(48 h)	[Glucose]		[Yeast extract]	
			(30°C)	(60°C)	(30°C)	(60°C)
Bacillus subtilis PMS1B2*	66.0	66.7	50.0	56.0	58.0	62.0
Ps. *aeruginosa* AG01	50.0	13.0	30.0	43.0	46.0	56.0
Ps. *aeruginosa* DPK3A*	57.0	60.0	54.0	56.0	62.0	66.0^
Bacillus sp.DPK1A	50.0	33.0	40.0	49.0	49.0	58.0
Ps. *fluorescence* PMS6	33.0	33.0	33.0	49.0^	51.0	56.0^
Bacillus sp. DPK2	40.0	33.0	33.0	40.0	50.0	57.0^
Bacillus sp. PMS5A	46.0	40.0	40.0	40.0	50.0	57.0^
Bacillus sp. PMS6	33.0	17.0	33.0	48.0	48.0	56.0^
Bacillus licheniformis DPK2A	35.0	35.0	46.0	46.0^	48.0	58.0^
B. *subtilis* AGO1A*	50.0	50.0	50.0	54.0	54.0	60.0^

*Keys: * = best biosurfactant-producing bacterial candidates; ^ = slight or moderate better biosurfactant-producing activities*

bacteria, grown on culture medium containing glucose as carbon source, and yeast extract as nitrogen source inferred that *Bacillus subtilis* PMS1B2 and *Ps. aeruginosa* DPK3A produced higher de-emulsification activities, while *Pseudomonas aeruginosa* AGO1A produced the highest de-emulsification activity, when grown on culture medium supplemented with glucose and yeast extract, at 30℃ and 60℃.

Bacterial strains with high emulsifying activities also exhibited high de-emulsification activity. Overall, higher de-emulsification activities were recorded when the bacterial strains were cultured in yeast extract supplemented medium than in glucose supplemented medium. Also, higher de-emulsification activities were exhibited at temperature of 60℃. Slight or moderately higher biosurfactant-producing activities were recorded for biosurfactant-producing bacterial strains in supplemented culture medium for growth (Table 2).

3.1 Economic Evaluation

The following assumptions were made for the cash flow analysis: oil production is according to the data (petrowiki.com), price of crude oil is $40 per barrel of oil and the investment is five billion dollars. These assumptions were made for both the microbial and chemical de-emulsifiers, and the results are presented in Table 3.

Table 3. Economic evaluation of de-emulsifiers

Component	Cost for microbial de-emulsifier	Cost for chemical demulsifier
Total capital investment	$5 billion	$5 billion
Total revenue	$19.9 billion	$19.9 billion
Total production	497.9 million barrel	497.9 million barrel
Total operating cost	$18.01 million	$26.7million
NPV (10%)	$6.3279 billion	$6.3217 billion
IRR	20.28%	20.26%
Payback period	3 years	4 years

From the result presented above, the microbial de-emulsifier has a higher (NPV) and (IRR) and shorter payback compared to chemical de-emulsifier, which means that the application of a more environmentally friendly microbial de-emulsifier would yield more profits than the chemical de-emulsifier, despite the exclusion of environmental costs.

4. DISCUSSION

It has been reported that petroleum-derived hydrocarbons are among the most persistent soil contaminants but that some hydrocarbon-degrading microorganisms can produce biosurfactants to increase bioavailability and degradation [20]. In the current study, selected biosurfactant-producing bacterial species (*Bacillus licheniformis*, *Bacillus subtilis*, other *Bacillus*, spp. *Ps*. *aeruginosa* and *Ps*. *fluorescens*) were isolated from soil samples contaminated with petroleum on Bushnell Haas agar, which is recommended for the microbial examination of fuels and for studying microbial hydrocarbon deterioration [26,37]. These bacterial species are also similar to those earlier reported in previous studies [36,37-41]. But there is paucity of related studies in the country for comparison; considering that the identified indigenous bacterial flora having biosurfactant potentials can be geographic-dependent.

Water is usually present in crude oil as a result of mixture, during production operations but the formation of water-in crude oil emulsion leads to production and transportation problems [42]; there is therefore, the need to break oil/water emulsions system through de-emulsification processes. Out of the 41 bacterial strains obtained from oil contaminated soil samples, and tested for the ability to degrade hydrocarbon using vapour transfer method in this study, 20 bacterial strains that showed ability to degrade hydrocarbon were further screened for biosurfactant activity by employing emulsification test method of Salehizadeh et al. [28], and only 10 strains showed biosurfactant activity. Biosurfactant-producing microorganisms have been found to possess the ability to break crude oil emulsions efficiently [39, 43], so, comparison of biosurfactant de-emulsifiers with chemical de-emulsifier for treating water-in-crude oil emulsions [34] showed that chemical de-emulsifier (Schlumberger with product code W054) gave 63%, 60% and 66.2% volume of water, while the biosurfactant de-emulsifier in this study gave 66%, 62% and 60% volume of water. The findings of this study therefore, is in accordance with some previous findings [36,44] which reported production of slightly higher volume of water by biosurfactants. Thus, as indicated in this study, addition of cells and supernatants of de-emulsifying bacterial cultures like, *Pseudomonas and Bacillus* species, will promote de-emulsification of crude oil emulsion.

Breaking of emulsion or de-emulsification is the separation of a dispersed liquid from the liquid in which it is suspended. So, the reason for de-emulsification is to destroy the interface and drive the surfactant to either the oil side or the water side; thus, allowing the oil particles and sediments to coalesce and rise to the surface, as in creaming. Decreasing water phase viscosity or increasing oil viscosity can therefore, enhance de-emulsification; thereby, increasing the diameter of oil droplets, although, lowering the density of oil to water is also appropriate [45]. The biosurfactant-producing bacterial strains in this present study must have produced certain biosurfactants that reduced the surface tension of water and the interfacial tension, which led to de-emulsification of the crude oil emulsion. The bacterial strains were all capable of de-emulsifying the emulsion at varying rates but the three finally selected bacterial strains (*Bacillus subtilis* PMS1B2, *Ps*. *aeruginosa* DPK3A and *B*. *subtilis* AGO1A) possessed better de-emulsification abilities.

Growth conditions play a key role on de-emulsification activity of the biosurfactant strains; hence, the principle of enrichment culture is to provide growth conditions that are very favourable for the microorganisms of interest, and as unfavourable as possible for any competing microorganisms. Biosurfactant-producing microorganisms when cultured on/in appropriate medium can be used to treat water-in-crude oil emulsions; thus, reducing cost and environmental pollution. It has also been confirmed that temperature and growth conditions play a key role on de-emulsification activity of biosurfactant microorganisms. In this study, more (slight or moderate) emulsifying and de-emulsifying potentials were recorded when the bacterial strains were cultured in Bushnell-Haas medium supplemented with glucose, as well as yeast extract agar, incubated at 30℃ and 60℃. As earlier inferred [36], the addition of glucose and yeast extract to the liquid medium must have had a positive effect, with an increase in growth of the bacterial strains, leading to appreciable higher percentages of emulsification and de-emulsification activities, as well as, better de-emulsifying ability. Therefore, apart from direct isolation of microbial strains by appropriate cultural methods, enrichment of microbial cultures with hydrophobic substrates are very promising for the isolation of biosurfactant-producing microbes [10].

Better treatment of crude oil emulsions is necessary to meet the ever growing demand for fuel and it derivatives without jeopardising quality and environmental safety [46]. Efficient separation of crude oil and water is an important operation in order to ensure not only the quality of crude oil but also the quality of the separated water phase at the lowest cost. The produced water must be well separated from the oil, treated and disposed appropriately. However, Failure to remove water from crude oil emulsions include high cost of pumping, pipelines corrosion and increase in the cost of transportation [47]. Since it is well-known that most of the chemical products used as chemical de-emulsifiers for treating crude oil emulsion are toxic to the environment; hence, the increase in environmental constraint makes it necessary to develop safer formation in order to replace the toxic chemicals and to reduce the cost incurred from importing chemical de-emulsifiers.

Biosurfactants from *Bacillus* spp. have been reported to possess additional property of functionality under extreme conditions of pH, temperature, and salinity [21,48,49]. Similarly, the fact that the selected biosurfactant-producing emulsifiers in this study can withstand a wide temperature (in the mesophilic to the thermophilic) range is of additional physiological importance, since biosurfactant production has been reported under thermophilic condition [50]. Potent biosurfactant-producing *Bacillus* species from natural habitats, such as oil reservoirs have been reported but their diversity in various habitats has not been studied [21]. Findings of this study is very significant to the oil industries for the reason that de-emulsification abilities by biosurfactant microorganisms aid in reducing dependence on imported chemical de-emulsifiers, and also promises potential biotechnology application for use in the oil companies. These are highly suggestive of ability to recover novel indigenous microbial emulsifiers, which are unique in hydrocarbon industrial processes.

Biosurfactants are considered as one of the most-valued microbial products that have gained considerable interest in recent years, and have also become an important product of biotechnology for industrial and medical applications [51,52]. As the usage of petroleum hydrocarbon products increases, soil contamination with diesel and engine oils is becoming one of the major global environmental problems [25], so, it is important to have diverse but promising eco-friendly microorganisms with biosurfactant properties. Three bacterial strains

identified as *Bacillus subtilis* PMS1B2, DPK3A and *Pseudomonas aeruginosa* AGO1A produced higher de-emulsification activities, although *Pseudomonas aeruginosa* DPK3A exhibited the highest de-emulsification activity of 66% when grown on culture medium supplemented with glucose and yeast extract at the temperature of 60℃. It was noted in the current study that bacterial strains with high emulsifying activity showed the high de-emulsification activity, although growth conditions played a key role on de-emulsification activity of the biosurfactant bacteria, since they were able to grow, and also had better de-emulsification activity on culture media supplemented with glucose and yeast extract, than strains grown on glucose alone. Temperature also played a key role on de-emulsification abilities of the biosurfactant bacteria because de-emulsification rate was enhanced with increasing temperature, while the highest rates of de-emulsification were observed at 60℃.

5. CONCLUSION

Recovery of generally regarded as safe biosurfactant-producing bacterial species, with potentials for de-emulsification of water-in-crude oil emulsions were recovered form hydrocarbon contaminated soils in this study, which is an additional advantage for the petroleum industry and the environment, most especially from the public, community and global health perspective.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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