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RESEARCH ARTICLE

BIOSURFACTANTS PRODUCTION POTENTIAL OF NATIVE HYDROCARBON DEGRADING BACTERIA STRAINS IN REFINERY EFFLUENT COLLECTED FROM KADUNA REFINERY AND PETROCHEMICAL COMPANY NIGERIA

[1]Zakka Jonathan, [2*]Markus Victor, [3]Paul Abraham [4]Tanta Emmanuel and [5]Jonathan Bege

[1,4]Department of Microbiology, Kaduna State University, PMB 2339, Kaduna, Nigeria
[2, 3]Department of Biochemistry, Kaduna State University, PMB 2339, Kaduna, Nigeria
[5]Department Biochemistry, Ahmadu Bello University, Zaria, Nigeria

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ABSTRACT

The enormous diversity and application of biosurfactants and the increased environmental concerns due to the toxicity of chemical surfactants and their difficulty in being degraded in the environment, coupled with the emergence of more stringent laws and advance in biotechnology have led to biosurfactants being a potential alternative to the chemical surfactants. In this study, native hydrocarbon degrading bacterial strains in refinery effluent collected from Kaduna Refinery and Petrochemical Company Nigeria were isolated, characterized, and screened for their potential to produce biosufactants. Pure cultures of the bacterial strains were obtained by enrichment technique from the refinery effluent collected at three different sites along the affluent site. Isolated strains were characterized by microscopic examination, gram staining reaction, morphological, cultural and biochemical tests. Screening for the potential to produce biosurfactants was by haemolytic activity and emulsification of hydrocarbons. The result showed that the isolated bacteria strains belong to the genera *Bacillus sp, Pseudomonas sp, Lactobacillus sp, Staphylococcus aureus* and *Escherichia coli,* with *Pseudomonas sp, Bacillus sp,* and *Staphylococcus aureus* having the highest biosurfactant production when grown on diesel as sole carbon source. Not all heterotrophs obtained from hydrocarbon polluted environment are capable of producing biosurfactants.

Key Words: Refinery effluent, Native hydrocarbon degrading bacteria, Biosurfactants.

INTRODUCTION

Refinery effluents are wastes originating from industries principally involved in refining crude oil and manufacturing fuels, lubricants and petrochemical intermediates (Esedafe *et al.,* 2015). The pollution of landmass, water bodies and ground water reserves by industrial chemicals and drilling activities in oil field areas is a serious problem to the environment, and the use of bioremediation for the removal of contaminants has been reported to provide a safe, efficient, versatile and economic alternative to commonly used physical and chemical treatment in several aquatic and terrestrial ecosystems (Vasileva-Tonkova and Galabova; Millioli *et al.,* 2009). In chemical treatment, the toxicity of chemical surfactants and their difficulty in being degraded in the environment, coupled with the emergence of more stringent laws and advance in biotechnology have led to biosurfactants being a potential alternative to the chemical surfactants. (Van-Hamme *et al*., 2006; Reis *et al*., 2013). Biosurfactants are amphiphilic biological compounds produced extracellularly or as part of the cell membrane by a variety of bacteria and filamentous fungi utilizing various substances such as sugars, oils and wastes (Mata-Sandoval *et al.*, 1990; Chen *et al.,* 2007; Reis *et al.,* 2013). These biosurfactants are surface-active compounds having diverse chemical nature and molecular size that can aggregate at interfaces between fluids with different polarities such as water and hydrocarbons. (Banat, 1995; Rahman *et al.,* 2002). Biosurfactants are widely applicable today in several industrial processes, such as lubrication, wetting, softening, fixing dyes, making emulsions, stabilizing dispersions, foaming, preventing foaming, as well as in food, biomedical and pharmaceutical industry, and bioremediation of hydrocarbons contaminated sites (Reis *et al.,* 2013). Although biosurfactants have a lot of promising applications in several industrial processes, food, biomedical and pharmaceutical industry, and bioremediation, their industrial scale production is currently difficult due to high raw material costs, high processing costs and low manufacturing output (Henkel *et al.,* 2012; Reis *et al*., 2013). As a result these, the current research challenges are to increase the yield and to reduce the cost of raw materials and production (Mukherjee *et al.*, 2006; Reis *et al.,* 2013), hence the necessity of this research.

Corresponding author: Markus Victor,
Department of Biochemistry, Kaduna State University, P.M.B 2339, Kaduna, Nigeria.

MATERIALS AND METHODS

Sample collection

The refinery effluent was collected at the Kaduna refinery, Kaduna Nigeria. This was collected in three sterile containers (75ml) from 3 sites along the effluent site and transported to the microbiology laboratory Kaduna State University, Kaduna, for analysis.

Media

All cultivations were performed in mineral salt medium (MSM) which contains 48g K_2HPO_4, $3H_2O$, 1.5g KH_2PO_4, 1.0g $(NH_4)_2SO_4$, 0.5g $Na_3C_6H_5O_7$, $2H_2O$, 0.2g $MgSO_4$, $7H_2O$, 0.1g yeast extract dissolved in distilled water, pH 7.2 and sterilized by autoclaving at 121^{o}C for 15 minutes. Other media used include: Nutrient Agar, Urea, Peptone Water, Methyl red –Voges Prokauer, Simmon Citrate agar and Triple Sugar Iron media.

Isolation of Bacteria

Serial dilution was done using distilled water as the diluents. Exactly 9ml each of the sterile distilled water was poured into 4 test tubes labeled 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4}. Exactly 1ml of the sample was transferred into the 10^{-1} dilution test tube, this was shaken, and 1ml of the solution was to the next dilution test tube (10^{-2}). This was done until the last dilution factor (10^{-4}) was obtained. Then 0.1ml each of the 10^{-3} and 10^{-4} dilution factors were inoculated on Nutrient agar using the pour plate method. The plates were incubated at room temperature for 24-48 hours. Colonies which developed on the plates were sub-cultured repeatedly to obtain pure cultures. The pure cultures were maintained on agar slants for further characterization.

Identification Bacterial isolates

Bacteria isolates were identified on the basis of microscopic examination, cultural characteristics, and morphological characteristics. Confirmatory identities of the microorganisms were made using Bergey's manual of determinative bacteriology (Buchanan and Gibbons, 1974).

Characterization of Bacterial Isolates

The Bacteria isolates identified were characterized by a combination of gram staining reaction and relevant biochemical tests as described below.

Gram Stain Reaction

Briefly, a smear of the sample was made on a glass slide and stained with crystal violet solution (primary dye) for 60 seconds, after which it was washed with tap water and well drained to avoid diluting the mordant. It was flooded with iodine solution (mordant) for 30 seconds, and washed with tap water, it was then decolorized with acetone alcohol by adding drop wise until all color was removed and washed with tap water. The slide was flooded with safranin (counter stain) for 60 seconds, washed and allowed to dry; it was then examined under oil immersion (Oyeleke and Manga, 2008).

Biochemical tests

The following biochemical tests were carried out according to the procedure described by Oyeleke and Manga, (2008): Catalase test, Indole test, Motility test, Urease test, Citrate Utilization test, Methyl Red Voges- Prokauer Test and Triple sugar Iron (TSI) test.

Screening Isolates for Potentials to Produce Biosurfactants (Haemolytic activity)

The ability of the isolates to produce biosurfactant has been associated with their haemolytic activity on blood agar. Briefly, the isolated strains were inoculated on blood agar (BA) to screen for potential to produce biosurfactants and the inoculated BA plates were incubated at 37^{o}C for 24-48 hours. The Haemolytic activity was then detected by the occurrence of a defined clear zone around the colony as described by Carrillo *et al.,* (1996). This method appears to be a good screening criterion for surfactant-producing strains. This is based on the ability of the isolates to produce hemolysis on blood agar.

Emulsification Index

Isolates that produced beta hemolysis on blood agar were then inoculated at room temperature for 24 hours. Accurately, 1ml each of the nutrient broth grown isolates were then separately inoculated into 20ml of mineral salt medium having 0.1mg diesel, and then incubated at room temperature for 4 days. At every 24hours, the medium was centrifuged and the supernatant was collected for each isolate. A 2ml volume of each supernatant was added to 2ml of diesel and mixed vigorously by shaking. This was allowed to stand and was examined after 24 hours for emulsification of the hydrocarbon and index of emulsification was determined according to the procedure described by Abbasi and Amiri (2008).

Table 1. Identification and Characteristics of Bacterial Isolates in Kaduna Refinery Effluent

Code Isolates	Morphology	Gram reaction	Coagulase	Catalase	MR	VP	Urease	Citrate	Motility	Indole	TSI	Organisms
A_{01}	Rods, in chains	-	-	+	+	-	+	-	+	-	+K/A	*Pseudomonas sp*
B_{02}	Rods, in chains	+	+	+	+	+	+	-	+	+	+K/A	*Bacillus sp*
C_{03}	Short rods, in chains	+	-	+	+	-	+	+	-	-	+K/A	*Bacillus sp*
D_{04}	Cocci, in clusters	+	+	+	+	-	+	-	-	-	+K/A	*Staphylococcus aureus*
E_{05}	Short rods	+	-	-	+	-	+	-	+	-	+K/A	*Lactobacillus sp*
F_{06}	Long rods	+	-	+	-	+	+	-	-	-	+K/A	*Bacillus sp*
G_{07}	Short rods, in pairs	+	-	+	-	+	+	-	-	-	+K/A	*Bacillus sp*
H_{08}	Rods in pairs	-	-	+	+	-	+	-	+	-	+K/AG	*Escherichia coli*
I_{09}	Long rods	+	-	+	-	+	+	-	+	-	+K/AG	*Bacillus sp*
J_{10}	Rods, in chains	-	-	+	+	-	+	-	-	-	+K/AG	*Pseudomonas sp*

+ means positive, - means negative, +K/A means only glucose is fermented, + K/AG means only glucose is fermented accompanied by gas formation

Table 2. Bacteria Species Isolated From Kaduna Refinery Effluent and Their Hemolytic Properties

Organism	Pattern of Haemolysis
Pseudomonas sp A_{01}	Beta
Bacillus sp B_{02}	Beta
Bacillus sp C_{03}	Beta
Staphylococcus aureus D_{04}	Beta
Lactobacillus sp E_{05}	Beta
Bacillus sp F_{06}	Alpha
Bacillus sp G_{07}	Alpha
Escherichia coli H_{08}	None
Bacillus sp I_{09}	Alpha
Pseudomonas sp J_{10}	Beta

Emulsification Activity = Height of emulsion layer/Total height

RESULTS AND DISCUSSION

A total number of ten isolates were obtained from the sample. The result showed that the isolated bacteria strains belong to the genera *Bacillus sp, Pseudomonas sp, Lactobacillus sp, Staphylococcus aureus* and *Escherichia coli* as shown in Table 1. Out of the ten bacterial isolates identified and characterized from the refinery effluent, 9 (90%) gave positive result by producing hemolysis on blood agar. Six of the isolates (60%) produced beta hemolysis and 3 (30%) produced alpha hemolysis as shown in Table 2. It was noted that one of the bacterial isolates identified as *E. coli* did not produce haemolysis on the blood agar, an indication that it does not any produce biosurfactants. It was reported that not all heterotrophs are capable of utilizing hydrocarbons as sole carbon and energy source during growth (Esedafe *et al.,* 2015). The result of the utilization of diesel by the isolates showed that *Bacillus sp* C_{03} and *Pseudomonas sp* J_{10} has maximum growth in diesel medium as indicated in Table 3. It has been reported that the occurrence of hydrocarbon degrading bacterial strains in samples is an indication of hydrocarbon contamination (Al-thani *et al*., 2009 Esedafe *et al.,* 2015).

Table 3. Utilization of Diesel by Bacterial Isolates

Organism	Extent of Diesel Utilization
Pseudomonas sp A_{01}	++
Bacillus sp B_{02}	++
Bacillus sp C_{03}	+++
Staphylococcus aureus D_{04}	++
Lactobacillus sp E_{05}	++
Pseudomonas sp J_{10}	+++

Table 4. Emulsification of diesel by Biosurfactant producing bacterial Isolates

Organism	Emulsification index (%)			
	Time (days)			
	1	2	3	4
Pseudomonas sp A_{01}	46.2	50.0	50.0	50.0
Bacillus sp B_{02}	46.2	50.0	50.0	50.0
Bacillus sp C_{03}	50.0	50.0	53.9	53.9
Staphylococcus aureus D_{04}	46.2	50.0	50.0	53.9
Lactobacillus sp E_{05}	46.2	50.0	50.0	50.0
Pseudomonas sp J_{10}	50.0	50.0	53.9	53.9

Different kinds of bacteria have been employed by many researchers in producing biosurfactants using culture media, most of which are isolated from contaminated sites usually containing petroleum hydrocarbon byproducts and/or industrial waste (Rahman *et al.,* 2002; Benincasa, 2007). All the bacterial isolates tested for the emulsification assay were capable of utilizing hydrocarbon. They all gave a positive result for biosurfactant production as indicated by the result of their emulsification assay (Table 4). The emulsification indices of biosurfactant producing bacteria ranged from 46.2% to 53.9% after 4 days. The highest percentage emulsification was produced by *Staphylococcus aureus* D_{04}, *Bacillus sp* C_{03} and *Pseudomonas sp* J_{10} with 53.9% emulsification index. This however may not mean that other isolates that the emulsification percentages were low did not produce much biosurfactants. Esedafe *et al.,* (2015) reported that not all hydrocarbon utilizers give a positive result for emulsification capacity assay. It was noted that biosurfactants produced by microorganisms in the course of their growth and metabolism either solubilize, mobilize or emulsify the hydrocarbon substrate and as such are grouped on the basis of their functions. Hence it was noted that, all emulsifiers are surfactants but not all surfactants are emulsifiers. One of the limiting factors in bioremediatio is the bioavailability due to low solubility of the oil in water (Rosenberg, 1993). The first step in hydrocarbon degradation involves a membrane-bound oxygenase, which is important for hydrocarbon degrading bacteria to come in direct contact with the hydrocarbon substrates (Rosenberg, 1993; Ron and Rosenberg, 2001).

Biosurfactants therefore help to disperse the oil, increase the surface area of hydrophobic water-insoluble substrates, area of hydrophobic water-insoluble substrates, increase their bioavailability thereby enhancing the growth of bacteria and the rate of bioremediation, reduce surface tension, Critical Micelle Concentration (CMC) and interfacial tension in both the aqueous solution and hydrocarbon mixture, and help detach the bacteria from the oil droplets after the utilizable hydrocarbon has been depleted (Rosenberg, 1993; Banat, 1995; Banat *et al.,* 2000; Ron, 2000; Ron and Rosenberg, 2001; Rahman *et al.,* 2002; Padhi *et al.,* 2012).

Conclusion

This study showed that not all heterotrophs obtained from a hydrocarbon polluted environment are capable of producing biosurfactants. It also corroborate the work of some other researchers that native microbes from industrial and/or municipal wastewaters can be harnessed to develop a robust, cost effective and environmentally friendly process for the treatment of hydrocarbon contaminated aquatic and terrestrial ecosystem, and application in other industrial, agricultural and health care process.

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