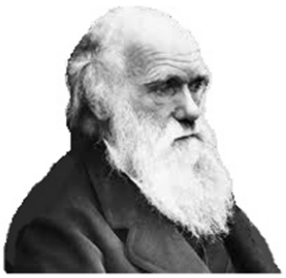
Available online at www.worldnewsnaturalsciences.com



World News of Natural Sciences

An International Scientific Journal

WNOFNS 52 (2024) 12-22 EISSN 2543-5426

Response of Soil Microbial Isolates to the Toxicity of Green Surfactant

Doris I. Iroegbu*, Ifechukwu E. Adieze, Christopher E. Nwanyanwu, Obioma K. Mejeha, Barinuale Sinee

Department of Microbiology, Federal University of Technology, Owerri, Nigeria

*E-mail address: iroegbudoris.20194198118@futo.edu.ng

ABSTRACT

In this study, microorganisms were obtained from hydrocarbon-polluted and unpolluted soil samples using Nutrient agar and Bushnell Hass agar for the enumeration of heterotrophic bacteria and hydrocarbon-utilizing bacteria respectively.The response of hydrocarbons utilizing soil bacterial isolates to the toxicity of green surfactant was assayed. The hydrocarbons utilizing bacterial isolates in soil extract broth were exposed to different concentrations; (1, 2, 3, 4, and 5) % w/v of green surfactants, and their responses (cell growth and viability) were time-dependent and monitored using spectrophotometer and total viable count. These hydrocarbon-utilizing bacteria isolates were two different species of *Micrococcus* (C and D), *Bacillus* sp. (F_2) and *Pseudomonas* sp. (A_2). They showed good response to high concentrations (4 and 5%) of the green surfactant in soil extract broth. This could be a result of a component of the green surfactant such as phosphorus which is an essential nutrient for microbial growth. Hence these tolerated green surfactant concentrations could be recommended to be used as surface active agents to enhance the bioavailability of hydrocarbon to microorganisms for bioremediation practices of hydrocarbon-contaminated sites.

***Keywords*:** Soil isolates, Green surfactant, Concentration, Responses, Hydrocarbon

***Key*:** *Pseudomonas* (A_2) - *Pseudomonas* sp. isolated from Bushnell Hass agar; *Bacillus* (F_2) - *Bacillus* sp (F) isolated from Bushnell Hass agar

(Received 08 October 2023; Accepted 31 October 2023; Date of Publication 02 November 2023)

1. INTRODUCTION

Surfactants, or surface-active agents, are compounds that lower the surface tension between two liquids or between a liquid and a solid. Surfactants are amphilic, meaning that they contain hydrophilic (water-loving) heads and hydrophobic (water-hating) or oil-loving tails.

They adsorb at the interface between oil and water, thereby decreasing the surface tension.

According to [1] hydrocarbon-degrading microorganisms produce biosurfactants of diverse chemical nature and molecular size.

These surface-active materials increase the surface area of hydrophobic water-insoluble substrates and increase their bioavailability, thereby enhancing the growth of bacteria and the rate of bioremediation.

Due to the complex nature of hydrocarbons; emulsifiers and surfactants whose primary function is to facilitate microbial life in environments dominated by hydrophilic-hydrophobic interfaces are introduced into hydrocarbon-contaminated sites so as to enhance the bioavailability and bioaccessibility of such contaminants to microorganisms [2-4].

According to [5] most studies indicate that biosurfactants are completely non-toxic to microorganisms and are unlikely to inhibit biodegradation of PAHs also they are readily biodegradable and pose no additional pollution threat. The production is less expensive and environmental friendly than synthetic surfactants and can be easily achieved in situ at the contaminated sites from inexpensive raw materials [5]. These surface active compounds are produced by varieties of microorganisms and the products having different applications. Numerous microorganisms, especially bacteria have been identified over the past decades which are able to degrade hydrocarbons by producing effective biosurfactants [6-8].

Soaps are among the most commonly used anionic surfactants/emulsifier which are particularly effective in oil cleaning and oil/clay suspension [9]. Soaps and detergents solubilize insoluble contaminants, trap dirt and carry it away with water, dissolve the lipid bilayer membrane enveloping microorganisms, inactivate them, and eliminate viruses and bacteria. The synthetic soap is obtained when a fatty acid is saponified using caustic soda or potassium hydroxide (base) while for the non-synthetic soap, saponification occurs between the natural alkaline liquid derived from palm bunch ash (base) and fatty acid without any synthetic chemical additives. [10] reported that palm bunch ash enhanced the rate of bioremediation of crude oil-polluted soil at low levels of contamination.

The bioremediation may be due to the addition of nutrients from the ash or a reduction in soil acidity due to the alkaline ash. According to [11] application of soap treatments on soil increases surface-active compounds including rhamnolipids, trehalolipids, sophorolipids, emulsan, liposan, and surfactin.

This also resulted in an increase in the microbial consortia of the contaminated soil which influenced a general reduction in the total petroleum hydrocarbon (TPH) contents of the soil. According to [12] organic soap consists of potassium, phosphorus, and nitrogen. These compounds are vital nutrients for microbes and thus help to remediate oil-polluted soil, as they accelerate biodegradation of the petroleum hydrocarbon in soil [13].

Given the destructive effect of soap on the lipid bilayer membrane enveloping microorganisms, particularly bacteria, this study assayed the response of hydrocarbons utilizing soil bacterial isolates to the toxicity of green surfactant.

2. MATERIALS AND METHODS

2. 1. Sample collection and characterization

The soil samples were collected from Bara Alue Community in Tai Local Government Area in Rivers State at a depth of 10-15 cm using a shovel. The hydrocarbon-contaminated soil sample was collected from a crude oil-polluted site, while the unpolluted soil sample was collected from an adjacent unpolluted site. The contaminated and uncontaminated soil samples were mixed separately to obtain composite samples. Physicochemical analysis was conducted on the soil samples to determine their pH, electrical conductivity, total organic carbon (TOC), total organic matter (TOM), total nitrogen and available phosphorus.

2. 2. Isolation and Identification of soil microbes

The sun-dried soil samples were sieved through a 2 mm pore-sized mesh to remove debris and stones. Thereafter 1 g of each of the soil samples was suspended in 100 ml of sterile distilled water contained in 250 ml conical flask and stirred vigorously. Serial dilution of the solution was carried out up to 10^6 dilution factor. Nutrient agar (NA) and Bushnell Hass (BH) agar were prepared and poured into plates for bacteria and hydrocarbon-utilizing bacteria respectively. One-tenth (0.1) ml of appropriate dilution was spread plated on the various plates.

The nutrient agar plates were incubated for 48 h at 30 °C. For the Bushnell Hass (BH) agar, sterile filter paper (Whatman No.1) saturated with crude oil was aseptically placed unto the covers of the inoculated inverted plates and then incubated for 192 h at 30 °C. The isolates that developed on the plated were purified by sub-culturing on their respective media. They were identified based on their colonial, morphological, and biochemical reactions. Characterization of isolates was performed following the procedures in Bergey's manual of determinative bacteriology [14]. They were maintained on nutrient agar slants for further studies.

2. 3. Production of green surfactant

Palm bunches whose fruits had been removed were obtained from a palm oil mill at Amakohia station in Mgbirichi, Imo State, Nigeria. The palm bunches were sun-dried for three (3) weeks and then completely burnt to ashes on a clean surface. The ashes were allowed to cool and then sieved througha 2 mm pore-sized mesh to remove larger particles. The ash was packed in a black polyethylene bag for later use.

To prepare the green surfactant 2 kg of the ash was mixed with 4 L of distilled water and allowed to stand for 24 hours. The suspension was filtered through a mesh screen and the filtrate boiled till it dries up to give a slurry substance. One hundred (100) ml of palm oil was thoroughly heated and added to the boiling slurry ash filtrate while vigorously stirring the slurry continuously for a homogenous mixture of oil and ash filtrate. Heating continues till a thick slurry is formed.

The slurry is then allowed to cool forming a black solid mass (referred to as natural black soap or green surfactant).

2. 4. Inoculum preparation

Seed cultures of hydrocarbon utilizing organisms selected were grown in 100ml nutrient broth medium (0.8 %) in 250 ml Erlenmeyer flasks, covered with cotton wool wrapped in

Aluminum foil, and incubated on a rotary shaker (150 rpm) for 24 h at room temperature (28 ± 2 °C). Cells were recovered by centrifugation at 4,000 rpm for 15 minutes. Harvested cells were washed twice in phosphate-buffered saline (PSB, 0.02M) to avoid carryover and resuspended in the buffer medium. The resuspended cells were standardized in a spectrophotometer to an optical density of 1.0 at 540 nm. The cell suspensions served as the standardized inoculum for the studies.

2. 5. Toxicity assessment of the green surfactant on the isolates

The hydrocarbon-utilizing bacteria obtained from the soil samples were assessed to determine the effect of the green surfactant on their growth using soil extract broth (consisting of: soil extract 17.75; glucose, 1.0; K_2HPO_4, 0.5 in g/l and adjusted to pH 7.2) as the growth medium.

Five different weights (1, 2, 3, 4, and 5 g) of the green surfactant were dissolved in 50 ml of the soil extract broth and made up to 99 ml with soil extract broth to obtain stock of green surfactant-soil extract broth solution. The control flask contained a mixture of 98 ml of soil extract broth and 1 ml of water. Pipettes were used to dispense 9.9 ml from the different stocks of green surfactant-soil extract broth and control into different bijou bottles then sterilized afterward.

Thereafter, 0.1 ml of the standardized isolates were inoculated into the sterile green surfactant-soil extract broth solution to make up 1, 2, 3, 4, and 5 % (w/v) green surfactant in the solution. At intervals of 120 h and 168 h, the microbial growth and viability were assessed by measuring the broth's optical density (OD) using Uv/vis Spectrophotometer @ 600 nm and by microbial total viable count (TVC). Serial dilution of the broth solution was carried out up to 10^4 diluents. Aliquots 0.1 ml were inoculated by spread plating in triplicateson Bushnell Hass (BH) agar and incubated at 30 °C for the appropriate time. Then the colonies that developed were counted and average counts were recorded and used to calculate the colony-forming unit per milliliter (CFU/ml).

3. RESULTS

3. 1. Physicochemical properties of soil samples

The physicochemical analysis conducted on the soil samples is shown in Table 1. The result revealed that the levels of pH, conductivity, total organic carbon and available phosphorus were higher in the unpolluted soil sample than in the polluted soil sample. Whereas the levels of total organic matter and total nitrogen were higher in the polluted soil than in the unpolluted soil sample.

3. 2. Incidence of prominent bacteria in soil samples

Bacteria isolates from polluted and unpolluted soil samples are shown in Table 1 below.

The total heterotrophic bacteria count and the hydrocarbon-utilizing bacteria count from the polluted soil samples were higher than that obtained from the unpolluted soil samples.

Table 1. Physicochemical properties of polluted and unpolluted soil sample

Physicochemical Parameters	Level	
	PSS	USS
pH	4.1	6.3
Electrical conductivity (µS/cm)	132	155
Total Organic Carbon (%)	4.60	15.43
Total Organic Matter (%)	5.36	3.19
Total Nitrogen (%)	0.07	0.04
Available Phosphorus (mg/kg)	0.27	0.36
KEY: PSS - Polluted soil sample; USS- Unpolluted soil sample		

Table 2. Microbial load in polluted and unpolluted soil samples

Microbial groups	Total CFU/ml	Prominent Isolates
Polluted soil sample (PSS)		
Total heterotrophic bacteria	2.65×10^{8}	*Pseudomonas* sp. (A_1) *Corynebacterium* sp. *Bacillus* sp. (E) *Bacillus* sp. (F_1)
Hydrocarbon utilizing bacterial count	1.14×10^{6}	*Pseudomonas* sp. (A_2) *Micrococcus* sp. (C) *Micrococcus* sp. (D)
Unpolluted soil sample (USS)		
Total heterotrophic bacterial count	2.34×10^{8}	*Pseudomonas* sp.(A_1) *Corynebacterium* sp. *Bacillus* sp. (E) *Bacillus* sp. (F_1) *Staphylococcus* sp.
Hydrocarbon utilizing bacterial count	4.70×10^{5}	*Pseudomonas* sp.(A_2) *Micrococcus* sp. (D) *Bacillus* sp. (F_2)
A - *Pseudomonas* sp; C, D – different species of *Micrococcus;* E, F – different species of *Bacillus*; A_1 – *Pseudomonas* (A) from nutrient agar; A_2 –*Pseudomonas* (A) from Bushnell Hass agar; F_1 – *Bacillus* (F) from nutrient agar; F_2 – *Bacillus* (F) from Bushnell Hass agar.		

3. 3. Response of isolates on green surfactant

The responses of hydrocarbon-utilizing organisms on different concentrations (1, 2, 3, 4, and 5) % of green surfactant were studied for a duration of 120 h and 168 h. For *Micrococcus* sp. (C) in soil extract broth spiked with the concentrations of green surfactant, the highest optical density (0.985) and total viable count (2.98 CFU/ml) were observed on 4% and 5% green surfactant solution after 120 h respectively (Figure 1). Also, *Micrococcus* sp. (D) in soil extract broth had the highest optical density on 4% green surfactant solution with a value of 0.872 after 120 h while the highest total viable count was on 4% green surfactant solution with a count of 2.94 CFU/ml after 168 h (Figure 2).

Bacillus sp. (F_2) had the highest optical density in 4% green surfactant solution with a value of 0.816 after 120 h while the highest total viable count was in 4% green surfactant solution with a value of 2.92 CFU/ml after 168 h (Figure 3). Results from *Pseudomonas* sp. (A_2) had the highest optical density and total viable count on 4% green surfactant solution with values of 0.842 and 2.95 CFU/ml respectively after 120 h (Figure 4).

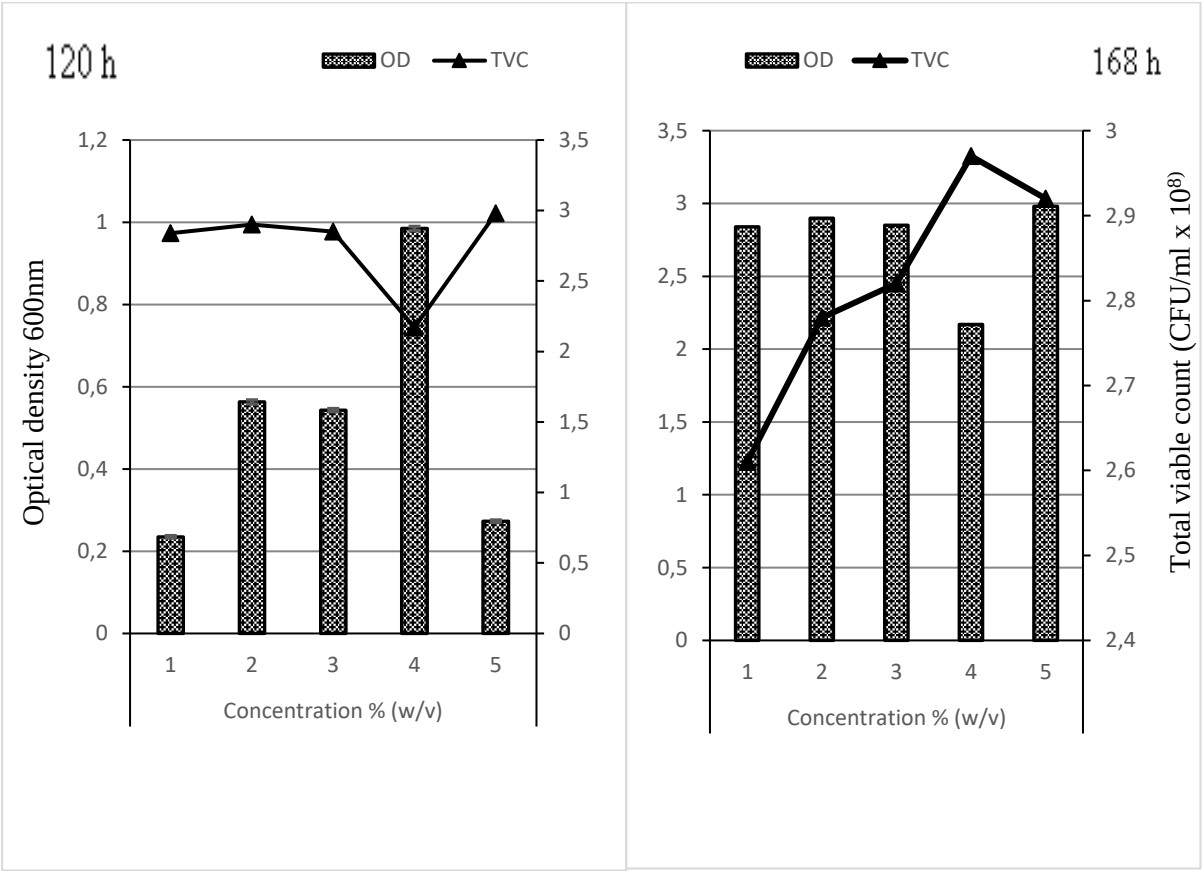


Figure 1. *Micrococcus* sp. (C) in soil extract broth spiked with various concentrations of green surfactant. ± standard error of triplicate

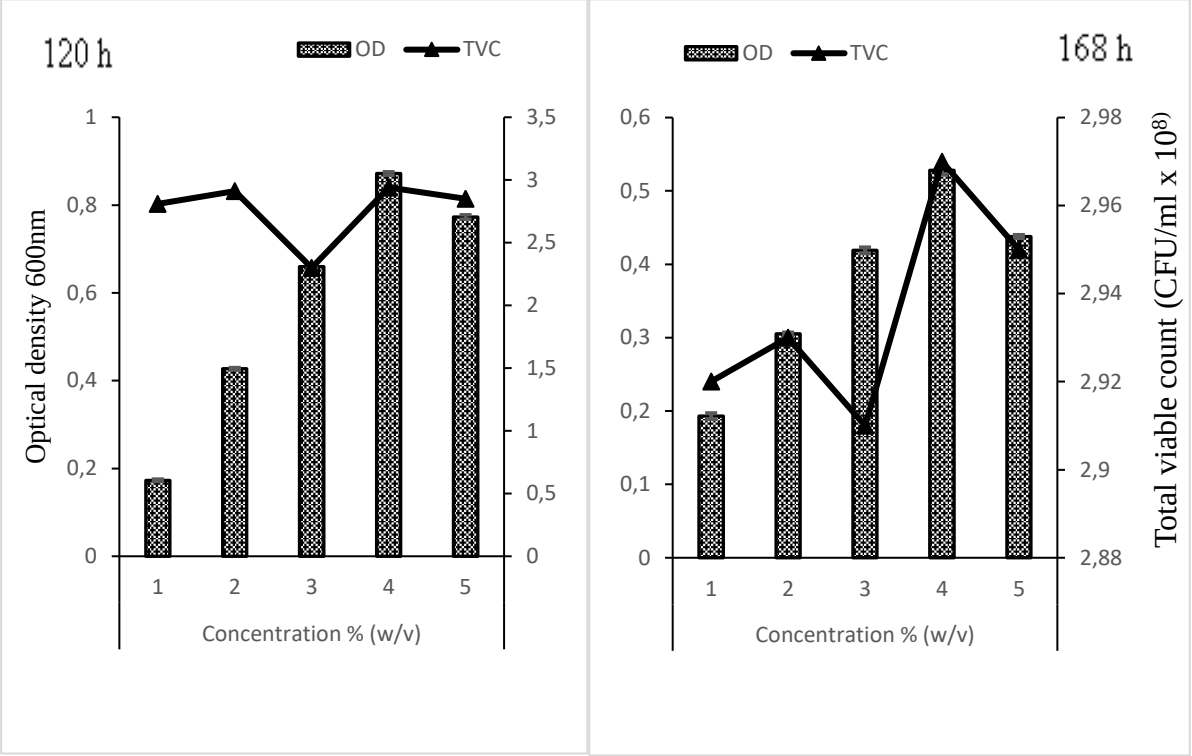


Figure 2. *Micrococcus* sp. (D) in soil extract broth spiked with various concentrations of green surfactant. ± standard error of triplicate

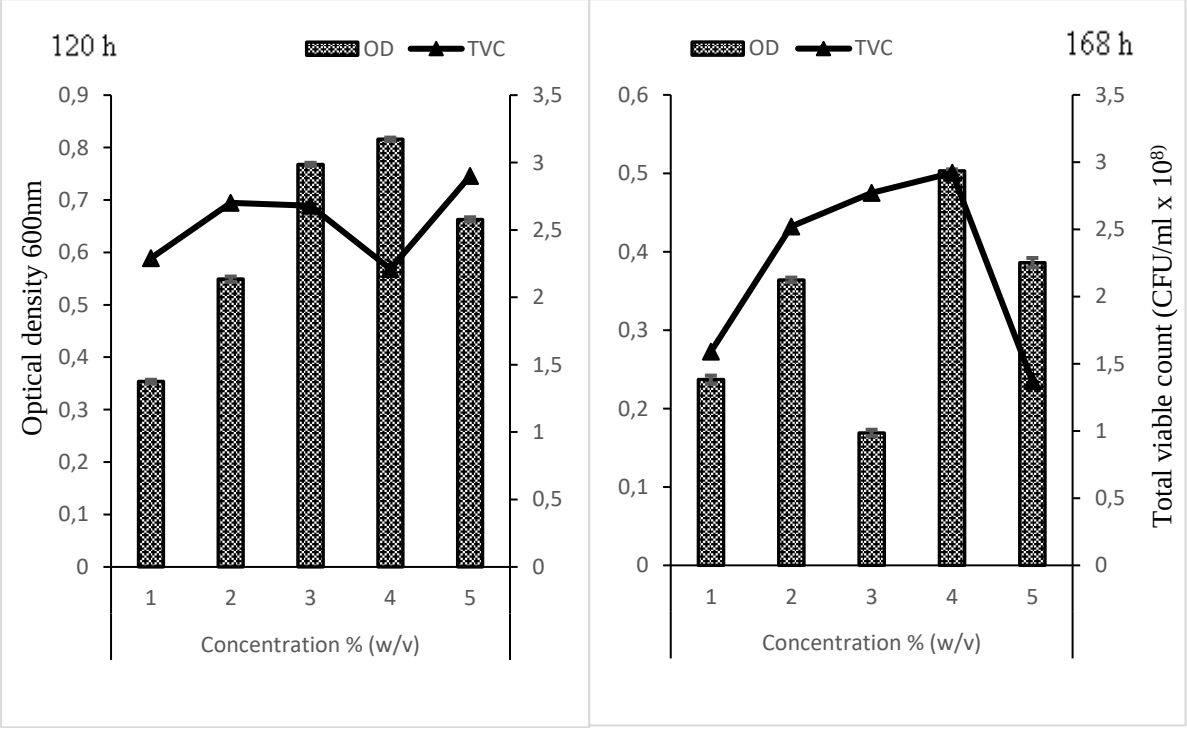


Figure 3. *Bacillus* sp. (F_2) in soil extract broth spiked with various concentrations of green surfactant. ± standard error of triplicate

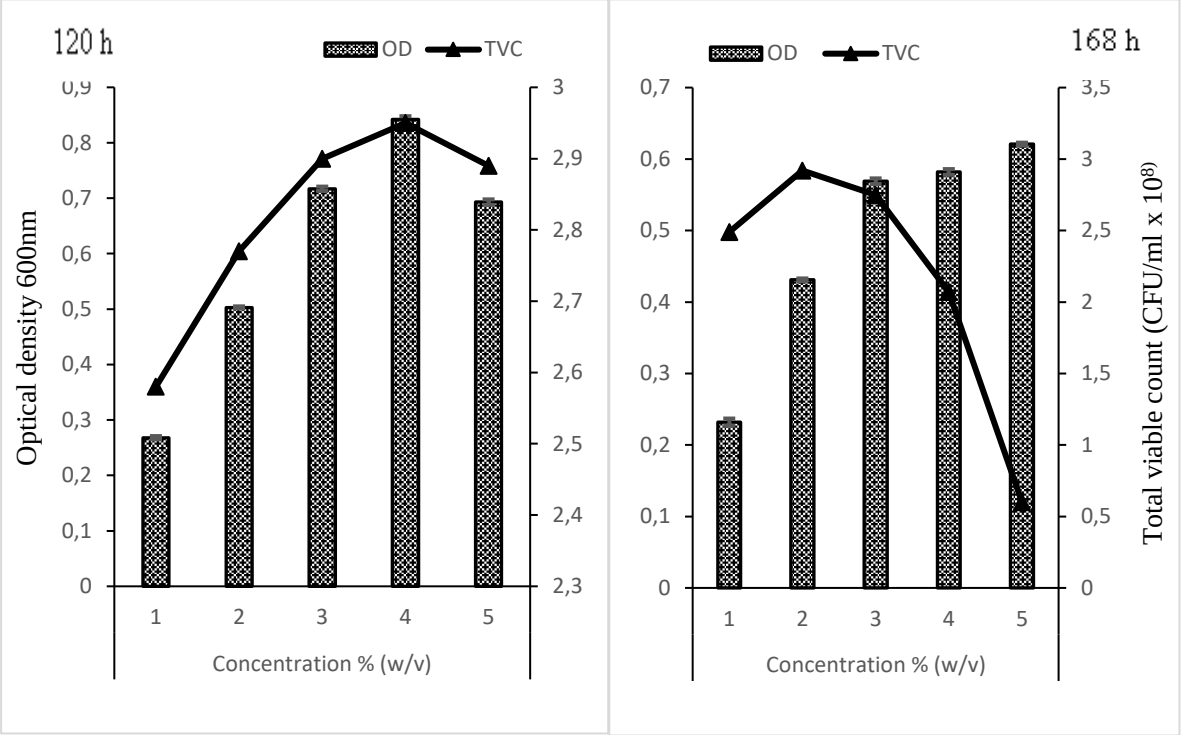


Figure 4. *Pseudomonas* sp. (A_2) in soil extract broth spiked with various concentrations of green surfactant. ± standard error of triplicate

4. DISCUSSION

The polluted soil sample had a lower physicochemical level in comparison with the unpolluted soil sample except for the total organic matter which could be a result of the dead and decayed soil flora and fauna. The total nitrogen was also higher in the polluted soil sample. This could be a result of the presence of nitrogen-fixing bacteria in the soil. According to [15], the presence of petroleum hydrocarbons in the soil has an effects on the soil properties.

In the polluted soil sample, the heterotrophic bacteria isolates obtained were identified as *Pseudomonas* sp. (A_1), *Corynebacterium* sp., *Bacillus* sp. (E), and *Bacillus* sp. (F_1), while the hydrocarbon-utilizing bacteria isolates were *Pseudomonas* sp. (A_2), *Micrococcus* sp. (C) and *Micrococcus* sp. (D). Similarly, the heterotrophic bacteria isolates obtained from unpolluted soil samples were identified as *Pseudomonas* sp. (A_1), *Corynebacterium* sp., *Bacillus* spp. (E and F_1) and *Staphylococcus* sp. while the hydrocarbon-utilizing bacteria isolates were *Pseudomonas* sp. (A_2), *Micrococcus* spp. (C and D) and *Bacillus* sp. (F_2). According to [16], their study also isolated similar organisms; *Bacillus* and *Pseudomonas* sp from virgin soil. The similarity in the isolates obtained in both samples agrees with the statement of [17] that bacteria with the ability to degrade a wide range of crude oil components exist ubiquitously in the environment and rapidly respond in the presence of petroleum.

In this research, the responses of the different hydrocarbon-utilizing bacteria had a good tolerance recorded in concentrations 4 and 5%. These tolerances observed might be a result of the higher quantity of the green surfactant used to produce these concentrations.

It could also be that the palm bunch ash which was a natural raw material for the production of the green surfactant provided more phosphorus from these higher concentrations of green surfactant in the medium.

According to [18] ash yields from palm fruit bunch and plantain peel contain high macro minerals with the order of their concentrations being K>Na>P>Mg>Ca. Phosphorus is an essential nutrient and according to [5] phosphorus provides nutrients for microbial growth. On the contrary, a study on the effect of different concentrations (0.001, 0.01, 0.1, and 1%) of "Meril" (synthetic) detergent on the total number of bacteria, soil fungi and oligonitrophiles conducted by [19] reported that the highest detergent concentration (1%) produced the highest inhibition on the different organisms. This implies that increased synthetic detergent concentrations or their accumulation in the soil bring about a rapid reduction in the number of microorganisms. This suggests that naturally produced soap can improve the tolerance of microorganisms in the environment over synthetic soaps which are produced with complex mixtures having toxicity potentials.

5. CONCLUSION

Soaps are widely used as a dispersant/surfactant. Synthetic soaps are often hardly biodegradable and reported to have toxicity potentials unlike the naturally produced soap (green surfactant) which had no chemical additive. The hydrocarbon-utilizing bacteria; *Micrococcus* spp. (C and D), *Bacillus* sp. (F_2) and *Pseudomonas* sp. (A_2) showed good tolerance to higher concentrations (4 and 5%) of the green surfactant. Since the green surfactant has been found to be tolerant to hydrocarbon-utilizing bacteria, these reported concentrations can be applied as emulsifiers, dispersants, or surfactants in the bioremediation of hydrocarbon-polluted sites. This may enhance the bioremediation of hydrocarbon-polluted sites by improving the bioavailability of the hydrocarbon to the indigenous microbial species.

Acknowledgement

The authors appreciate Ndaa Onyewuchi, I. A. for his guidance in the production of the green surfactant used in this study. They also thank Prof. Wesley, Braide; director, of Anthony Van Leeuwenhoek Research Center in Nekede, Imo State whose laboratory was used for the microbiological analysis.

References

[1] Ron EZ, Rosenberg E. Biosurfactants and oil bioremediation. *Current Opinion in Biotechnology.* 2002; 13(3): 249-52

[2] Barkay T, Navon-Venezia, S, Ron E, Rosenberg E. Enhancement of solubilization and biodegradation of polyaromatic hydrocarbons by the bioemulsifier alasan. *Applied Environmental Microbiology.* 1999; 65: 2697-2702

[3] Paria S. Surfactant-enhanced remediation of organic contaminated soil and water. *Advances in Colloids and Interface Sciences.* 2008; 138: 24–58

[4] Tadros T. Colloids, polymers and surfactants. *Advances in Colloids and Interface Science.* 2009; 1(3): 281–299

[5] Makarau J. Bioremediation of oil-contaminated soil using emulsifier (liquid soap), NPK fertilizer and microbial (Bacillus sp) treatment (Master's thesis, Ahmadu Bello University, Zaria, Nigeria). Retrieved from https://kubanni.abu.edu.ng/items/704e7315-f299-4dd7-b423-cd7a53fe05df/full. 2015

[6] Al-Wahaibi Y, Joshi S, Al-Bahry S, Elshafie A, Al-Bemani A, Shibulal, B. Biosurfactant production by Bacillus subtilis B30 and its application in enhancing oil recovery. *Colloids Surfaces B: Biointerfaces.* 2014; 114: 324–333

[7] Cai Q, Zhang B, Chen B, Zhu Z, Lin W, Cao T. Screening of biosurfactant producers from petroleum hydrocarbon contaminated sources in cold marine environments. *Marine Pollution Bulletin.* 2014; 86: 402–410

[8] Chandankere R, Yao J, Cai M, Masakorala K, Jain AK, Choi MMF. Properties and characterization of biosurfactant in crude oil biodegradation by bacterium Bacillus methylotrophicus USTBa. *Fuel* 2014; 122: 140–148

[9] Zhang H, Dang Z, Zheng LC, Yi XY. Remediation of soil co-contaminated with pyrene and cadmium by growing maize (Zea mays L.). *International Journal of Environmental Science and Technology.* 2009; 6(2): 249–258

[10] Onyelucheya OE, Osoka EC, Onyelucheya CM. Modeling palm bunch ash enhanced bioremediation of crude oil contaminated soil. *International Journal of Science and Engineering Investigations.* 2013; 2(13): 8–12

[11] Anoliefo GO, Ikhajiagbe B, Okoye PC, Omoregie O. Utilizing local soap-derived biosurfactant for degradation of petroleum hydrocarbon polluted soils: sustainable remediation in focus. *Annuals of Science and Technology -B* 2016; 1(1): 43-51.

[12] Akpokodje OI, Uguru H. Bioremediation of hydrocarbon contaminated soil: Assessment of compost manure and organic soap. *Society for Science and Education,* 2019; 7(5): 2054-7390.

[13] Ijah UJJ, Safiyanu H. Microbial degradation of Escravos light crude oil in soil amended with chicken dropping and NPK fertilizer, 10th Annual Conference of Biotechnology Society of Nigeria. (1997).

[14] Holt JG, Krieg NR, Sneath PHA, Staley JT, Williams ST. Bergey's Manual of Determinative Bacteriology. (9thedition. pp: 518-537). Williams and Wilkins, Baltimore. (1994).

[15] Moses E, Uwah EI. The effect of crude oil pollution on some soil fertilities parameters in Ikot Oboreyin, Ikot Abasi Akwa Ibom State Nigeria. *Merit Research Journal on Environmental Science Toxicology.* 2015; 3(2): 17–24

[16] Olawale O, Obayomi KS, Dahunsi SO, Folarin O. Bioremediation of artificially contaminated soil with petroleum using animal waste: cow and poultry dung. *Cogent Engineering.* 2020; 7(1).

[17] Adieze IE, Nwabueze RN, Onyeze GOC. Effect of poultry manure on the microbial utilization o hydrocarbons in oil-polluted soil. *Nigerian Journal of Microbiology.* 2003; 17(1): 12-16.

[18] Duruanyim VO, Achonwa CC, Ohanaka AUC, Aladi NO, Okoli IC, Okeudo NJ. Physicochemical characteristics of ash derived from palm fruit bunch and plantain peels. Tapas *Institute of Scientific Research and Development.* 2016; 12: 1-8

[19] Mandic L, Djukic D, Kalinic S, Pesakovic M. Effect of different detergent concentrations on the soil microorganism's number. *Acta Agriculturae Serbica,* 2006; 11(22): 69-74