International Journal of Global Science Research
Vol. 3, Issue 6, October 2016, pp. 391-398
Available Online at www.ijgsr.com
© Copyright 2016 | ijgsr.com | All Rights Reserved
ISSN: 2348-8344 (Online)

Research Paper

In vitro Assessment of *Pseudomonas stutzeri's* Biosurfactant Extract on Its Petroleum Hydrocarbon Degradation Potentials

G. I. Ogu[*] and J. C. Igborgbor
Department of Biological Sciences, Novena University, PMB 002 Ogume-Kwale, Delta State, Nigeria.
Department of Biology, College of Education, PMB 2090 Agbor, Delta State, Nigeria.
*Correspondence author email: ogu.gideon@naij.com

Received: 10/07/2016
Revised: 30/08/2016
Accepted: 03/09/2016

Abstract: The assessment of *Pseudomonas stutzeri's* biosurfactant on its hydrocarbon degradation potentials was studied *in vitro*. The reference chemical surfactant was Tween-80. Biosurfactant production was analyzed using emulsification index, oil displacement and drop collapse assay, while the petroleum hydrocarbon degradation potentials was determined in 2 % Escravos Light Crude Oil (ELCO)-amended mineral salt medium for 14 days using the standard gravimetric techniques. *P. stutzeri*'s biosurfactant gave a positive drop collapse test, with an oil displacement and emulsification stability index of 19 mm and 72.5% respectively. The petroleum hydrocarbon biodegradation analysis revealed that *P. stutzeri* respectively yielded 95.71% and 75.43 % in the presence and absence of 2% biosurfactant-amended medium after 14 days incubation. The results were statistically significant ($P<0.05$) when compared with the chemical surfactant which yielded 80.57 % biodegradation. Findings from this study suggest that *P. stutzeri,* isolated from crude oil polluted soil, possesses bioactive biosurfactant that might be exploited for enhanced bioremediation technology.

Keywords: Biosurfactant, Bioremediation, Crude oil, Emulsification index.
Abbreviations: Escravos Light Crude Oil (ELCO), Mineral Salt Medium (MSM)

INTRODUCTION

In recent years, there have been growing public health concerns on the increasing rate of environmental pollution resulting from wastes generated by anthropogenic activities. Despite the fact that the discovery of petroleum products has made life easier by their applications in automobile fuel, jet engines, cooking, lubricants, cleaning solvents, cosmetics among other, their continuous usage are in no small measure impacting negatively on the quality of air, water and soil environment (Achuba and Okoh, 2014). All stages of crude oil production, distribution, processing and usage impact negatively on the environment, and the greatest inevitable environmental

International Journal of Global Science Research ISSN: 2348-8344 (Online)
Vol. 3, Issue 6, October 2016, pp. 391-398
Available Online at www.ijgsr.com
© Copyright 2016 | ijgsr.com | All Rights Reserved

challenge is oil spillage from natural seepage, leakages and accidents.

In Nigeria, petroleum spillage and environmental pollution is aggravated by sabotage and deliberate vandalization of pipelines and storage facilities. The consequences of these have been erosion of ecobiota, extensive environmental degradation, enormous financial loss, and poverty leading to the continuous crises in the Niger Delta Area, that have culminated into kidnapping of indigenous and expatriate oil workers (Imoobe and Iroro, 2009). The attendant environmental consequences of petroleum hydrocarbon pollution in oil producing communities have continued to attract public health attention.

Generally, several potential remediation strategies for petroleum polluted sites have been explored over the years. They include the use of physical, mechanical, chemical and biological agents to reduce or clean up the pollutants and their negative impacts from the environments. Great successes were recorded with these remediation techniques, but the attendant negative consequences of the physicochemical method have stimulated the increasing attention on the use of biological alternative (Okoh, 2006; Ogu et al., 2015; Ogu and Odo, 2015; Srivastava, 2015). Use of biological agent or biodegradation is the deliberate use of plants, animal or microorganisms or their products to complete the mineralization of the organic contaminants to carbon dioxide, water, inorganic compounds, and cell proteins (Ogu and Odo, 2015)

The quest for enhanced bioremediation of petroleum pollutants has stimulated the screening of some surface active bio-molecules synthesized from oil degrading microorganisms, with a view to studying the roles they play in facilitated crude oil translocation into the cells. One of such surface-acting agents discovered from biological agents is biosurfactants. Biosurfactants are amphipathic biological compounds with hydrophobic and hydrophilic portions. They act between fluids of different polarities (oil/water and water/oil), allowing access to hydrophobic substrates and causing a reduction in surface tension, thereby enhancing the mobility, bioavailability, and biodegradation of such compounds (Silva et. al., 2014). Currently, they have been explored in petroleum industries in the main oil production stages such as extraction, transportation, storage and remediation of water and soil, because of their ecofriendliness, low ectoxicity, and better performances at extreme conditions over the synthetic or chemical surfactants (Silva et. al., 2014). Based on the potential usefulness of biosufactants, and the fact that previous studies have reported that biosurfactants produced by *Pseudomonans* species could be useful for bioremediation application (Aparna et al., 2011; Pacwa-Płociniczak et al., 2011), we decided to investigate the *in vitro* effect of biosurfactants produced by *Pseudomonas stutzeri,* a prominent bacteria isolated from crude oil polluted sites in Ogoni-land, on petroleum hydrocarbon biodegradation in Niger Delta region of Nigeria.

MATERIALS AND METHODS

Source of Soil Sample

Crude oil contaminated soil were collected randomly and homogenized from five different spots at Eleme and Tai Local Government Area in Ogoni-land, South-East of the Niger Delta basin, Port-Harcourt, River state, Nigeria. Ogoni-land was chosen because of its long history of petroleum pollution (UNEP, 2011).

Source of Crude oil

The Escravos light crude oil (ELCO) (dark brown in colour) used in this study was

© Copyright 2016 | ijgsr.com | All Rights Reserved

obtained from NPDC, NNPC subsidiary, Warri, Delta State, Nigeria.

Isolation of Bacteria

Bacteria able to degrade crude oil were isolated on crude oil amended mineral salts medium (MSM) by enrichment method as described by Odjadjare et al. (2008). Ten grams (10 g) of the soil samples was added separately to 100ml sterile defined growth Mineral Salt Medium (MSM) (pH 7.0) in 250 ml conical flask containing the desired petroleum as the carbon and energy source to a concentration of 1 % (v/v). The cultures were be incubated at ambient temperature (28 ± 1.0°C) on an orbital shaker at 100 rpm. After seven (7) days incubation, samples were then serially diluted and 1ml portions were plated following standard pour plate techniques onto sterile MSM agar containing 1 % petroleum. Isolates were purified and transferred to agar slants (stored at 4°C) as working stock cultures for further use and strain characterization. Pure cultures of bacterial isolates were identified on the basis of their colonial morphology, cellular morphology and biochemical characteristics according to the taxonomic scheme of Bergey's Manual of Determinative Bacteriology (Holt et al., 2002).

Selection of Petroleum Degrading Isolate

Mineral Salts broth and crude oil were autoclaved separately at 121°C for 15 min. Sterile crude oil which served as source of carbon and energy was added at 1 % (v/v) to make up a final volume of 100 ml sterile liquid MSM. Each isolate was subsequently inoculated into separate flask of the medium. Control flask containing the MSM and 1 % (w/v) of crude oil but without organism was also prepared. The flasks were monitored and agitated daily for a period of 10 days. Utilization of crude oil was assessed by monitoring the cell density at 600 nm wavelength with spectrophotometer after 10 days period (Khan and Shukla, 2011). The bacterial isolate that show highest cell density was then selected and used for the biodegradation study. An aliquot of each isolate was transferred to fresh medium containing 1 % (w/v) of crude oil to re-confirm their ability to utilize crude oil.

Inoculum Development and Standardization

Inoculum of the bacterium was prepared by centrifuging a 10 ml overnight nutrient broth culture at 8500x g for 15mins. The supernatant was discarded and the cell pellet suspended in 30 ml of 0.85 % (w/v) saline solution and centrifuged again. This washing step was repeated, and the cells were finally re-suspended in 5ml saline solution and the suspension was adjusted to an optical density at 600 nm of 0.1 which is equivalent to a cell population of about 1.0 x 10^8 cells/ml on the Mc-Farland standard.

Biosurfactant Production by *P. stutzeri* culture

Two milliliters of the standardized cell culture was aseptically inoculated into a 250 ml flask containing 100 ml mineral salt medium prepared from modified Bushnell-Hans medium as described by Ismail et al. (2014). The flasks were prepared and used in triplicates, each containing 1 % ELCO. Sets of control were un-inoculated. All the flasks were incubated at room temperature on a shaker set at 100 rpm and monitored for 10 days.

Assay for Biosurfactant production

The supernatants obtained from the culture broth by centrifugation at 5000 rpm for 20 minutes were used to test for biosurfactant assay. The common assays done were the drop collapse test, oil spreading and emulsification index (E_{24}).

Drop Collapse Test: A clean grease-free glass slide was carefully coated with a drop of crude oil (ELCO) before using sterile Pasteur pipettes to add a drop each of the

© Copyright 2016 | ijgsr.com | All Rights Reserved

supernatant and distilled water for test and control set-up respectively. They were observed for collapse and spreading over the oil coated surface, which is an indication for the presence of biosurfactant (positive results). A negative result was noted if the supernatant drop remains after 60 seconds (Jain et al., 1991).

Oil Spreading Test: Into three different, sterile, 15cm diameter Petri-dish was added 40 ml of sterile distilled water before using a sterile micro-pipette to carefully layer 15µl of crude oil (ELCO) onto it. Thereafter, 10µl of the supernatant was gently added on the centre of the oil layer and observed for 60 seconds. A positive result occurs when the oil is displaced leaving a clear zone on the water surface (Morikawa et al., 2000). The average diameters of the clearance zones were measured in millimetres (mm) and compared with distilled water control.

Emulsification Index test (E_{24}): Two millilitres (2ml) of the supernatant was mixed with a similar volume crude oil (ELCO) and thoroughly vortexed for 2 mins. This was done in triplicates. The height of the emulsion layer after 24hrs of stabilizing the mixture was measured accordingly (Cooper and Goldenberg, 1987). The emulsification index (E_{24}) was then calculated by finding the ratio of height of the emulsion layer and the total height of liquid. The value was recorded in percentage (%)

Biosurfactant extraction

The biosurfactant produced was extracted as described by (Adamu et al., 2015). The culture broth was centrifuged at 5000 rpm for 20 mins in order remove the bacterial cells. The supernatants collected were dispensed into sterile test-tubes and acidified to a pH of 2.0 with hydrochloric acid (0.1M). A mixture of chloroform and methanol in the ratio of 2:4 (v/v) was added to the supernatants, mixed thoroughly, and waited to stand for spontaneous separation into clear distinct phases. The lower phase containing the extracts were then concentrated in an oven set at 40^0C to a dried crude biosurfactant (Rabah and Bello, 2015).

Crude Oil Degradation Study and Analysis

The method described by Ogu et al. (2015) was used. Fresh overnight culture of *P. stutzeri* at the log phase of growth were adjusted with sterile distilled water to give a bacterial cell count of 1.0 x 10^8 cfu/ml and transferred to four 250 ml conical flasks each containing 100 ml of sterile-defined mineral salts medium with 2 % of crude oil. Biosurfactant at concentrations of 1% and 2% were added into the second and third flasks, while 2% Tween 80 (chemical surfactant) was added into the fourth flasks. The set-up was replicated along with the un-inoculated control flask containing 100 ml of sterile-defined mineral salts medium with 2 % of ELCO. The flasks were then incubated on orbital shakers set as 100 rpm for 14 days at 30^0C. The residual crude oil was extracted to analyze the biodegradation rate using the gravimetric techniques as described earlier (Ogu and Odo, 2015). All the flasks were added 20 ml of n-hexane and repeatedly separated using a separating funnel for complete recovery of the residual oil. Moisture present in the extracts was removed using anhydrous sodium sulfate, before evaporation to dryness on a water bath. The percentage difference in weight between the net initial oil weight added to the medium and the final weight of residual oil was taken as the amount of crude oil biodegradation.

Statistical Analysis

The data were using SPSS 16.0 version for Windows program (SPSS, Inc.) and presented as mean ± standard deviation, significance test was taken at $P<0.05$

International Journal of Global Science Research
Vol. 3, Issue 6, October 2016, pp. 391-398
Available Online at www.ijgsr.com
© Copyright 2016 | ijgsr.com | All Rights Reserved
ISSN: 2348-8344 (Online)

RESULT AND DISCUSSION

The search for cost effective, efficient and enhanced eco-friendly methods for the degradation and removal of petroleum hydrocarbon pollutants in the environments has continued with promising breakthroughs. It is established that virtually all types of hydrocarbons are susceptible to microbial degradation and hence the relevance of using the biotechnological approach (microbial capability) for bioremediation of the hazardous waste is justified (Atlas, 1991; Head, 1998). In this report, the *in vitro* petroleum degradation potential of *P. stutzeri* in the presence and absence of its crude biosurfactant extract was studied.

The characteristics of the bacterium are shown in Table 1.

Table 1. Biochemical characteristics of test bacterium

Isolate code	Colony Colour& Surface	Colony Form Margin, & Elevation	Gram stain	Catalase test	Motility test	Oxidase test	IndoleProduction	Methyl red	Voges-Proskauer	Nitrate Reduction	Starch Hydrolysis	Gelatin Hydrolysis	Lipid Hydrolysis	Sugar fermentation test					Probable Isolate
														Glucose	Fructose	Lactose	Sucrose	Mannitol	
T_3	Ligh/ green	Wrinkled, filiform, Undulated Raised	Gram - ,Cocco bacilli	+	+	-	-	-	-	-	-	+	+	A/ G	-	-	+	+	*Pseudomonas stutzeri*

+ = Positive, - = Negative, A/G= Acid and Gas production

P. stutzeri has been reported to be abundant in hydrocarbon rich environments and thus, well adapted to thriving in such adverse conditions (Sebiomo et al., 2010). Results from this study (Table 2) revealed that *P. stutzeri* is an efficient biosurfactant producer. *P. stutzeri* gave a positive drop collapse result, an oil displacement diameter and emulsification index values of 19.2 mm and 72.5% respectively. The values obtained in this study were relatively higher than those reported for *Bacillus licheniformis, Bacillus alvei*, *Pseudomonas paucimobilis*, and *Citrobacter murliniae* AF025369 with biosurfactants assay results of 18 mm, 20 mm, 15 mm, 18 mm oil displacement diameter and 11.00%, 67.00%, 71.00%, 66.67% emulsification indices respectively (Rabah and Bello, 2015; Anaukwu et al., 2016). This finding further buttresses the

Table 2: Biosurfactant production study of *P. stutzeri*

Assay	*P. stutzeri*[*]	Control
Drop collapse	++++	-
Oil displacement (mm)	19.2 ± 0.01	0.0 ± 0.00
Emulsification index (E_{24}%)	72.5 ± 0.11	0.0 ± 0.00

[*]Values are the mean ± standard deviation of three replicate determinations

International Journal of Global Science Research **ISSN: 2348-8344 (Online)**
Vol. 3, Issue 6, October 2016, pp. 391-398
***Available Online* at www.ijgsr.com**
© Copyright 2016 | ijgsr.com | All Rights Reserved

earlier claims that *Pseudomonas* species are generally efficient biosurfactant producers (Aparna et al., 2011). The oil displacement diameters from this study were, however, lower than those reported for some halophilic bacteria and certain *Candida* strains, which yielded oil displacement diameters ranging from 28 mm – 42 mm (Hesham et. al., 2012; Jaysree et al., 2013). The observed differences could be attributed to the variability in their genetic and environmental responses to available oil type. These biosurfactant assay methods used in the study have been confirmed to be effective and efficient for screening biosurfactant-producing microorganisms (Jain et al., 1991; Satpute et al., 2008; Thavasi et al., 2011).
The effect of addition of previously extracted biosurfactant of *P. stutzeri* showed an increased hydrocarbon utilization rate. *P. stutzeri*, alone, gave a percentage crude oil degradation of 75.43%, as against 81.43% and 95.71% obtained when 1-2% of biosurfactant extracts was included in the medium (Figure 1).

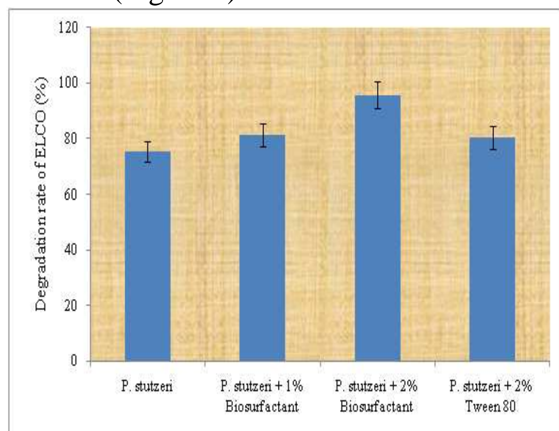

Figure.1: Degradation Rate of ELCO by *P. stutzeri* and effect of its biosurfactant

This finding is in concordance with the reports of earlier workers that biosurfactant produced by isolates relatively enhances their *in vitro* (Aparna et al., 2011; Anaukwu et al., 2016) and *in vivo* crude oil hydrocarbons biodegradation potentials (Jain et al., 1992; Chaprão et al., 2015). Over the years, researchers have studied and documented *Pseudomonas* species as one of the outstanding group of bacteria with high versatility in respect of hydrocarbon utilization. Thus, the observed crude oil hydrocarbon degradation potential of *P. stutzeri* further laid credence to the claim. Biosurfactants are reportedly produced by many microbes in response to stress of hydrocarbon-rich environment, such as in crude oil polluted sites. The released biosurfactant help to reduce the interfacial tension between the cell and hydrocarbon molecules, thereby enhancing their dispersion and bioavailability. Hence, in this current era of modern technology, the study of biosurfactant producing micro-organisms and effect cannot be overemphasized.

Conclusion: In this study, *P. stutzeri* demonstrated profound *in vitro* petroleum hydrocarbon degradation based on the observed percentage reduction in the used crude oil. Also, It has shown to be a potent biosurfactant producer, a finding that support the currently available arsenal of information that *Pseudomonas* species are good biosurfactant producers. The positive effect in hydrocarbon degradation suggests that the biosurfactants of *P. sturtzeri* possess great potentials *in vitro* stimulation of biodegradation of crude oil hydrocarbons. Therefore, further purification and field trials are essential with a view to exploiting them in bioremediation technology.

REFERENCE

Achuba F. I. and Okoh P. N. (2014) Effect of Petroleum Products on Soil Catalase and Dehydrogenase Activities. Open J. Soil Sci. 4, 399-406.

International Journal of Global Science Research **ISSN: 2348-8344 (Online)**
Vol. 3, Issue 6, October 2016, pp. 391-398
***Available Online* at www.ijgsr.com**
© Copyright 2016 | ijgsr.com | All Rights Reserved

Imoobe T. O. T. and Iroro T. (2009) Ecological restoration of oil spill sites In the Niger Delta, Nigeria. J. Sust. Dev. Afr. 11(2), 54-65.

Okoh A. I. (2006) Biodegradation alternative in the clean-up of petroleum hydrocarbon pollutants. Biotechnol Mol. Biol. Rev. 1(2), 38-50

Ogu G. I., Ehiobu J. M. Diete-spiff I. F. and Ndego C. E. (2015) Biodegradation of Crude Oil using Hydrocarbonoclastic Bacteria Isolated from gut of Earthworms in crude oil polluted regions of Niger Delta, Nigeria. Int. J. Chem. Biol. Sci. 2 (7), 6-14.

Ogu G. I. and Odo B. B. (2015) Crude Oil Bioremediation Efficiency of Indigenous Soil Fungal Community Spiked with Cassava peels in Niger Delta Region, Nigeria. Int. J. Sci. Technoledge. 312, 19-26

Adamu A., Ijah U. J. J., Riskuwa M. L., Ismail, H. Y. and Ibrahim, U. B. (2015) Study on Biosurfactant Production by two *Bacillus* species. Int. J. Sci. Res. Knowledge, 3(1), 013-020.

Srivastava S. (2015) Bioremediation of contaminated soil and water. Int. J. Glob. Sci. Res. 2(3), 199-212.

Silva R. F. S., Almeida D. G., Rufino R. D., Luna J. M., Santos V. A. and Sarubbo L. A. (2014) Applications of Biosurfactants in the Petroleum Industry and the Remediation of Oil Spills. J. Mol. Sci. 15, 12523-12542

Aparna A., Srinikethan G., Hedge S. (2011) Effect of addition of biosurfactant produced by *Pseudomonas* sp. on biodegradation of crude oil. In *International Proceedings of Chemical, Biological & Environmental Engineering*. Proceedings of the 2nd International Proceedings of Chemical, Singapore, Singapore, 26–28 February 2011; Vol. 6, pp. 71–75.

Pacwa-Płociniczak M., Płaza G. A., Piotrowska-Seget Z. and Cameotra S. S. (2011) Environmental Applications of Biosurfactants: Recent Advances. Int. J. Mol. Sci. 12, 633-654.

UNEP, (2011) Environmental Assessment of Ogoniland. Available on line at: http://postconflict.unep.ch/publications/OEA/UNEP/OEA.pdf. Assessed October 23rd, 2012, pp.1-262

Odjadjare E. E. O., Ajisebutu S. O., Igbinosa E. O., Aiyegoro O. A., Trejo-Hernandez M. R. and Okoh A. I. (2008) Escravos light crude oil degrading potentials of axenic and mixed bacterial cultures. J. Gen. Appl. Microbiol. 54, 277-284

Holt J. G., Krieg N. R., Sneath P. H. A., Staley J. T. and Williams S. T. (2002) Bergey's Manual of Determinative Bacteriology, 9th edition. Lippincot: Williams and Wilkins. Philadelphia, USA. pp. 131-542.

Khan J. A. and Shukla R. U. (2011) Application of Oil Degrading Bacterial Isolates for Remediation of oil Contaminated Soil. J. Pharm. Biomed. Sci. 12 (12), 1-4

Jain D. K., Thompson D. L. C., Lee H. and Trevors J. T. (1991) A drop collapsing test for screening surfactant producing microorganisms. J. Microbiol. Meth. 13, 271-279.

Morikawa M., Hirata Y. and Imanaka T. (2000) A study on the structure-function

International Journal of Global Science Research **ISSN: 2348-8344 (Online)**
Vol. 3, Issue 6, October 2016, pp. 391-398
***Available Online* at www.ijgsr.com**
© Copyright 2016 | ijgsr.com | All Rights Reserved

relationship of lipopeptide biosurfactants. Biochim. Biophys. Acta (BBA)-Molecular and Cell Biology of Lipids; 1488 (3), 211-218.

Cooper D. G. and Goldenberg B. G. (1987) Surface-active agents from two *Bacillus* species. Appl. Microbiol. Biotechnol. 53(2): 224-229.

Rabah A. B. and Bello H. (2015) Production and Characterization of Biosurfactants from Abattoir Wastewater. J. Bio. Agric. Healthcare. 5(7), 187-190

Atlas R. M. (1991) Microbial hydrocarbon degradation–biodegradation of oil spills. *J.* Chem. Technol. Biotechnol. 52**,** 149-156.

Head I. M. (1998) Bioremediation: towards a credible technology. Microbiol. 144(3), 599-609

Sebiomo A., Bankole S. A and Awosanya A. O. (2010) Determination of the ability of microorganisms isolated from mechanic soil to utilize lubricating oil as carbon source. *Afr. J. Microbiol. Res.* 4 (21), 2257-2264.

Anaukwu C. G., Ezemba C. C., Anakwenze V. N., Agu K. C., Okeke B. C., Awah N. S. and Ekwealor I. A. (2016) Effect of biosurfactant produced by *Citrobacter murliniae* AF025369 and a synthetic surfactant on degradation of crude oil. Edorium J. Microbiol. 2, 1–6.

Hesham M., Mahdy M. A. and Mohamed N. H. (2012) Production of biosurfactant from certain *Candida* strains under special conditions. Researcher. 4(7), 39-55.

Jaysree R. C., Rajam C. and Rajendran N. (2013) Biosurfactant production by halophilic bacteria. Int. J. Pharm. Biol. Sci. 4(4), 904-912.

Jain D. K., Lee, H. and Trevors, J. T. (1992) Effects of addition of *Pseudomonas aeruginosa* UG2 inocula or biosurfactant on biodegradation of selected hydrocarbons in soil. J. Ind. Microbiol. 10, 87-93.

Satpute S. K., Bhawsar B. D., Dhaephalkar P. K. and Chopade B. A. (2008) Assessment of different screening methods for selecting biosurfactant producing marine bacteria. Ind. J. Marine Sci. 37(3), 243-250.