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PRODUCTION OF BIOSURFACTANTS USING PSEUDOMONAS AERUGINOSA FOR BIODEGRADATION OF HERBICIDE

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ABSTRACT

This study was aimed at isolating *Pseudomonas aeruginosa* from the soil to produce biosurfactant for degradation of herbicide. The soil sample was collected from oil polluted site in mechanic workshop, Minna, Niger State, Nigeria and transferred to the microbiology laboratory of the University in a sterile polyethylene bag. *Pseudomonas aeruginosa* was isolated from the soil and identified using microbiological and biochemical assays. The isolate was screened for biosurfactant production using haemolytic assay, drop collapse, oil spreading and emulsification test. Biosurfactant production was carried out using mineral salt medium for ten days. The ability of the biosurfactant produced by *P. aeruginosa* in degrading herbicide was tested on five concentrations of herbicide (5%, 10%, 30%, 50%, 70%) and the results showed biodegradation percentages as 56.4%, 53.1%, 32.6%, 29.8% and 26.2% respectively revealing that degradation was higher at lower concentrations. This suggests that herbicide could be biodegraded especially at low concentrations using biosurfactant produced by *Pseudomonas aeruginosa*.

Contribution/Originality: The paper's primary contribution is finding that biosurfactant could be produced from bacteria (*Pseudomonas aeruginosa*) and this could be used for degradation of herbicide that has remained an environmental problem.

1. INTRODUCTION

Contamination of soil is prominent among the most critical environmental issues throughout the world, and it has a hugely harmful impact on people, animals, microorganisms, and plants (Abioye *et al.*, 2017; Abioye *et al.*, 2019). The herbicides, used to fight against the weeds in the agriculture are very toxic to soil biota. To some extent, these herbicides are unrestrainedly used by farmers without considering the long or short term effects in soil medium. It is evident that most of these herbicides may cause the reduction of sensitive populations of certain groups of biota in the soil medium. It is believed that in cases where these herbicides are used to treat soils, they are considered harmful to nematode, earthworms and other biological organisms (Kumar and Kumawat, 2018). Aside from being toxic to the target organisms, it can affect non target organisms and cause serious side effects. This may also reach the level of human consumption through contamination of ground water used primarily for drinking. An ideal herbicide should have the ability to destroy weeds quickly and be degraded into non-toxic components.

Biosurfactants are amphiphilic (consist of hydrophobic and hydrophilic moiety) biological compounds produced extracellularly or as part of the cell membrane by a variety of yeast, bacteria and filamentous fungi (Chen *et al*., 2007). The biosurfactants are complex molecules covering a wide range of chemical types including peptides, fatty acids, phospholipids, glycolipids, antibiotics, lipopeptides, etc. (Chen *et al*., 2007). The ability to reduce surface tension and form micelles is a major characteristic of surfactants so that the biodegradable contaminant can been capsulated by the hydrophobic microbial surface. The mechanism leads to attachment as a result of the micelles binding to the hydrocarbon receptors on the surface of the microbial cells (Jin-Feng *et al*., 2015).

Biosurfactants lead to an increasing interest on these microbial products as alternatives to chemical surfactants. There are numbers of reports on the synthesis of various types of biosurfactants by microorganisms using water-soluble compounds such as glucose, sucrose, and ethanol or glycerol as substrates. Petroleum related industry was found to be one of the industries that have a great potential in producing microorganisms that may produce biosurfactants. It has been focused here that improving the method of biosurfactants production and characterizing the major properties of the biosurfactants are highly important in the commercial application of biosurfactants (Banat *et al*., 2000). Biosurfactants are a class of microbial metabolites with surface-active properties and they are capable of spontaneous assemblies at the air–water or water–oil interface and thereby reducing surface/interfacial tensions due to their hydrophilic and hydrophobic structural components on the basis of their chemical structures, biosurfactants are divided into five major classes of lipopeptides, glycolipids, phospholipids, neutral lipids, and polymeric compounds (Jin-Feng *et al*., 2015). Currently, many biosurfactant-producing microorganisms have been isolated and identified to belong to *Bacillus*, *Agrobacterium*, *Streptomyces*, *Pseudomonas*, and *Thiobacillus* as producers of aminoacids-containing biosurfactants; *Pseudomonas*, *Torulopsis*, *Candida*, *Mycobacterium*, *Micromonospora*, *Rhodococcus*, *Arthrobacter*, *Mycobacterium*, *Corynebacterium*, *Mycobacterium*, and *Arthrobacter* as producersof glycolipids; *Thiobacillus*, *Aspergillus*, *Candida*, *Corynebacterium*, *Micrococcus*, and *Acinetobacter* as producers of phospholipids and fatty acids (Silva *et al*., 2014). During the last few years, there has been an increasing demand for biosurfactants used as emulsifiers, de-emulsifiers, wetting agents, spreading agents, forming agents, functional food ingredients and detergents. Due to several major advantages over chemical surfactants; lower toxicity, high environmental compatibility, and biodegradability and synthesis from renewable raw materials. Biosurfactants have been becoming the focus of extensive research and applications from food industries to oil industries (Jin-Feng *et al*., 2015). The aim of this study was to screen *Pseudomonas aeruginosa* from the soil with potential to produce biosurfactant useful in the degradation of herbicide.

2. MATERIALS AND METHODS

2.1. Soil Sample Collection

Soil samples were collected from oil polluted site in mechanic workshop, Minna and transferred to the microbiology laboratory of the Federal University of Technology in a sterile polyethylene bag. The samples were collected from depths of 0-15cm for the isolation of bacteria.

2.2. Isolation of Pseudomonas Species

Serial dilutions using the method described by Joanne *et al*. (2011) was used. One gram (1g) of soil sample was suspended in 9ml of sterile distilled water and serially diluted from 10^{-1} to 10^{-5} dilutions. From the dilutions of each sample, 1ml aliquot was transferred aseptically into freshly prepared *Pseudomonas* selective medium agar plates and spread evenly on the medium in duplicates. The inoculated plates were incubated at 37°C for 24 hours after which the plates were examined for growth. The isolate obtained from the above technique was sub-cultured repeatedly on *Pseudomonas* selective medium agar using sterile wire loop and incubated at 37°C for 24hours. Slants containing the isolate were prepared and preserved in the refrigerator at 4°C for further characterization and identification.

2.3. Characterization and Identification of Isolates

Characterization of the isolate was carried out by observing the cultural morphology, microscopy by gram staining and by some biochemical tests (oxidase, catalase, urease, indole and citrate tests). The isolates were identified using Bergy's Manual of Determinative Bacteriology (Bergey *et al.*, 1984).

2.4. Screening of Isolates for Biosurfactant Production

The isolates were screened for ability to produce biosurfactants using the following methods:

2.5. Haemolytic Activity Test

The isolates were streaked on the blood agar and the plates were incubated at 37°C for 24 hours. The plates were examined visually for zone of clearance (haemolysis) around the colonies. Isolates that had ability to lyse red blood cells and form a clear zone around colonies were noted as biosurfactant producers and recorded as positive (+) while those that could not form halo zones were recorded as non-biosurfactant (negative). Complete and incomplete haemolysis was designated as β (beta) and α (alpha) haemolytic activity respectively (Rodrigues *et al.*, 2006).

2.6. Drop Collapse Test

Drop collapse assay developed by Jain *et al.* (1991) was adopted. Two microlitres (2µl) of the cell free supernatant obtained after the centrifugation of 24 hours old broth culture at 6000rpm for 30 minutes using IEC FL 40R centrifuge, USA, were placed on an oil coated solid surface and the shape of the drop was noted after 1 minute. The culture supernatant that collapsed the oil drop was indicated as positive showing the presence of biosurfactant and the culture supernatant which failed to collapse the oil drop and gave rounded drops which appeared like air bubble was indicated as negative showing absence of biosurfactant.

2.7. Oil Displacement Technique

Oil displacement method according to Jaysree *et al.* (2011) was used to determine the diameter of the clear zone, which occurred after adding surfactant-containing solution on an oil-water interphase. In this test, 25mlof distilled water was added to a Petri dish which was 90mm in diameter and 100µlof crude oil was added to the water surface followed by the addition of 20µlof cell free culture supernatant obtained after the centrifugation of eighteen hours old broth culture at 600rpm for 30minutes. The diameter of the oil as displaced by the cell free supernatant and the clear zone formed were visualized under visible light and this was measured after 30seconds.

2.8. Emulsification Capacity (E_{24})

Emulsification capacities of the isolates were tried utilizing the strategy of Cooper and Goldenberg (1987). Two milliliters (2ml) of crude oil and 2ml cell free supernatant acquired after the centrifugation of eighteen hour broth culture at 6000rpm for 30 minutes. It was included into a test tube; the mixture was homogenized by vortexing at fast speeds for two minutes. The homogenized blend was permitted to remain for 24 hours undisturbed. Following 24 hours, the height of the steady emulsion layer and aggregate height of the mixture were measured by utilizing a meter rule; the qualities acquired were used to figure the emulsification index (E24), utilizing the relation

$$E_{24} = \frac{\text{height of emulsion layer}}{\text{total height of aqueous layer}} \times 100$$

2.9. Biosurfactant Production

The potential biosurfactant producing isolates were innoculated into a clean nutrient broth and incubated at 37°C for 12hours, at that point one milliliter of the 12 hours old culture was moved into 1000ml of newly arranged mineral salt medium of Jacobucci *et al.* (2001) containing 1ml of raw petroleum. The medium was then incubated at 25°C for 10days with shaking at 300 oscillations for every minute utilizing an incubator shaker.

2.10. Biosurfactant Extraction

Extraction of biosurfactant was carried out using basic precipitation method according to Ibrahim *et al.* (2013). In this method, the bacterial isolates were removed after 10 days of incubation by centrifugation at 6000rpm, using a centrifuge for 30 minutes. The cell free culture supernatant was treated with 1M of freshly prepared NaOH to obtain a pH of 11.0. The treated cell frees supernatants were then utilized for the extraction of the biosurfactant. To each 100ml of the treated cell free supernatant, equal volume of Diethyl ether was included. The blend was permitted to respond for 30seconds, after which it was shaken overwhelmingly and left overnight in a refrigerator until the point where two phase separation was gotten. The upper layer containing significantly the regent was emptied and the lower layer containing the biosurfactant was concentrated utilizing a hot air oven at 100°C, where the majority of the solvent vanished and the leftover residue was transferred to a test tube and centrifuged at 6000rpm for 20 minutes. A whitish residue was acquired as the biosurfactant.

2.11. Determination of Dry Weight of Biosurfactant

The weight of an empty sterile petri plate was taken, and after that the extracted biosurfactant was transferred into the plates. This was set in the hot air oven at 100°C for 30 minutes. Subsequent to drying, the plates and contents were reweighed. The weight of the biosurfactant produced was resolved by utilizing the formula:
Weight of biosurfactant = weight of the plate after drying – weight of the empty plate.

2.12. Determination of the Herbicide Degradation Rates of the isolates

Physical and chemical parameters were studied to observe changes in different concentrations of herbicide undergoing biodegradation. The concentrations used were prepared in volume/volume ratio of herbicide to distilled water. Concentrations of herbicide used were; 5%, 10%, 30%, 50% and 70%in 50ml of distilled water. 1gram of biosurfactant was kept constant in application to all concentrations of herbicide used.
Parameters studied included:

a) pH change
b) Optical density
c) Color change
d) Comparative plant effect

2.13. Study of pH Change

A comparative changein pH when compared to the control was observed over a 15 day period. The different concentrations of herbicide in solution where tested for pH change using a calibrated pH meter.

2.14. Optical Density Of the Herbicide after Degradation

The optical density of the degraded herbicide was taken using spectrophotometer at 540nm wavelength. The optical densities of the herbicides after degradation experiment were compared with the optical density of the control to know the amount of herbicide degraded.

$$\text{Degradation rates (\%) of isolate (DR\%)} = \frac{\text{OD1 (control)} - \text{OD2 (degraded)}}{\text{OD1 (control)}} \times 100$$

Where OD_1, (control) implies optical density of the concentration used as control, OD_2 (degraded) means optical density of the herbicide after degradation by the isolates for 15 days and DR% is the degradation rate.

2.15. Colour Change of Degraded Herbicide

The comparative change in colour of the degraded herbicide concentrations as compared to the control was duly noted and observed.

2.16. Comparative Plant Effect

The comparative effect of degraded herbicide on plants up to a two hour mark was observed and recorded.

3. RESULTS

3.1. Identification of Isolates

Table 1 shows the biochemical and morphological characteristics of isolates from the soil. Pure isolates were obtained and identified as *Pseudomonas aeruginosa*. The organisms showed greenish pigmenton nutrient agar and were Gram negative rods. They were positive for oxidase, citrate and urease tests.

Table-1. Morphological and biochemical characteristics of bacterial Isolate.

P. AERUGINOSA ISOLATE	
CHARACTERISTICS	**Results**
GRAM PIGMENTATION	Greenish
REACTION	Negative
SHAPE	Rod
OXIDASE	Positive
CATALASE	Negative
UREASE	Positive
CITRATE	Positive
INDOLE	Negative

3.2. Biodegradation Capacity of Isolate

Table 3 shows the herbicide biodegradation capacity of biosurfactant produced by *P. aeruginosa* isolated from the study area.

Table-2. Screening of isolate for biosurfactant production.

P. aeruginosa	Haemolysis	Drop Collapse	Oil Spreading	Emulsification Index (%)
	B	+++	5.0cm	25.0

+++ = Drop collapsein less than 1minute, control
Emulsification capacity: 0 = No emulsification, 10-20% = Minimum, 20-30% = Average, 30-40%= Maximum.

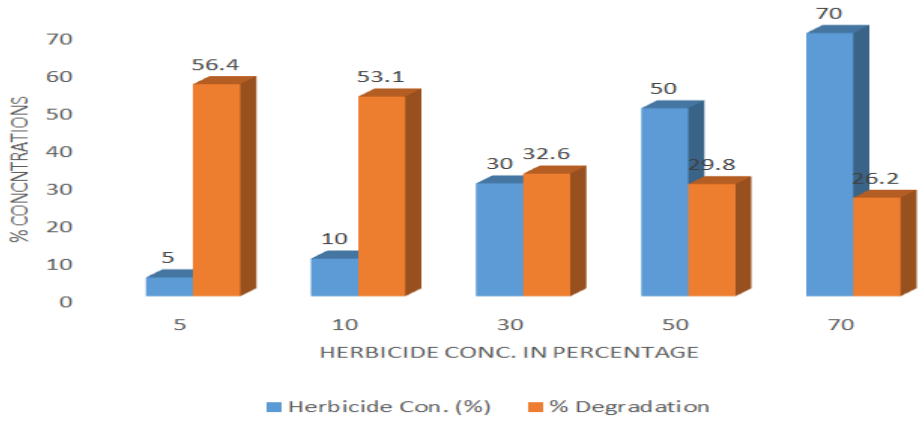

Figure-1. Biodegradation rates of different concentrations of paraquat dichloride herbicide biodegraded using biosurfactant from *Pseudomonas aeruginosa*.

Figure-2. (a) Test plants to study comparative effect of degraded herbicide; (b): Two hours after application of biodegraded herbicide.

Table-3. pH study.

Concentration of herbicide	5%	10%	30%	50%	70%
Day 0	9.49	8.91	8.63	6.71	6.55
Day 5	9.26	8.55	8.33	6.40	6.33
Day 10	8.96	8.13	7.90	6. 08	5.84
Day 15	8.86	8. 02	7.88	5.97	5.80

Control = 4.33.

Table-4. Optical density at 540nm.

Concentration of herbicide	5%	10%	30%	50%	70%
Day 0	0.801	0.982	1.312	1.503	1.572
Day 5	0.773	0.927	1.254	1.338	1.303
Day 10	0.744	0.860	1.105	1.141	1.192
Day 15	0.700	0.753	1. 081	1.126	1.183

Control = 1.604 nm.

Table-5. Colour change and comparative plant effect after application.

Concentration of herbicide	5%	10%	30%	50%	70%
Day 0	Green	Green	Green	Deep Green	Deep Green
Day 15	Yellow	Amber	Brown	Light Green	Deep Green

4. DISCUSSION

Pure *P. aeruginosa* isolate was screened and found to be positive for haemolysis (β-haemolysis). Therefore, in this study drop collapse, oil displacement and emulsification assays were included to confirm biosurfactant production by the isolate Table 2. *P. aeruginosa* is indigenous to the soil studied (oil contaminated soil) and other investigations have reported the occurrence of *P. aeruginosa* in diverse habitats (Rashedi *et al*., 2005; Obayori *et al*., 2009). The widespread nature of *P. aeruginosa* might be because they are not fastidious and pigmentation may give upper hand to the life forms (Chen *et al*., 2007).

The haemolytic assay was used in this study as a criterion for biosurfactant production. Many biosurfactants produced by microorganisms particularly bacteria cause lyses of red platelets and has been accepted that there is no bacteria that produce biosurfactant without being haemolytic (Walter *et al*., 2010). Be that as it may, Youssef *et al*. (2004) detailed that a few strains of microorganisms with positive hemolytic movement were discovered negative for biosurfactant generation. In addition, not all biosurfactants have haemolytic activity and compounds other than biosurfactants may cause haemolysis

In this study, cell free culture broth was used as the biosurfactant source. For the isolates with extracellular biosurfactant there was a drop collapse activity what's more, this rule does not include isolates having haemolytic activity yet no biosurfactant production and furthermore the specimen volume required to check the drop collapse was little which permits the conduct of duplicate measurement. The precision and unwavering quality of the result gotten in drop collapse test in this investigation was comparative to the results obtained by Bodour and Miller-Maier (1998); Morikawa *et al*. (2000) reported that the area of oil displacementin oil spreading assay is directly proportional to the concentration of biosurfactant in the solution. In any case, in this examination there was no quantitative investigation directed on biosurfactant concentration versus oils preading movement yet a subjective report to check the presence of biosurfactant in the cell free culture broth was in agreement with above mentioned earlier report. In addition, cell free broth culture of isolates with 4.5-6.5cm clear zone may contain high concentration of biosurfactant. Emulsification assay is an indirect assay method for biosurfactant production. It depends on supposition that if the cell free culture broth contained biosurfactant, it will emulsify the hydrocarbon present in the test solution. In this study, diesel oil was used as the hydrophobic substrate.

P. aeruginosa produced 8.12g/L of biosurfactant after ten days. Compared to previous research (Rashedi *et al*., 2005; Reis *et al*., 2013) the quantity of biosurfactants produced in this study was high. The purpose behind this higher sum might be that the *P. aeruginosa* strains used as a part of this investigation had greater capacity to produce biosurfactant and variability possibilities observe red in pH and optical density Tables 3, 4 & 5. It may in part be due to the type of carbon source used.

The result analysis revealed that biodegradation rates Figure 1 using biosurfactant produced by *P. aeruginosa* were higher at lower concentration of herbicides indicating that concentration of herbicide is inversely proportional to the biodegradation rate using biosurfactant. Therefore, the biosurfactant produced by *P. aeruginosa* isolates may be used as herbicide degraders which can be recommended for use in remediation of herbicide polluted sites Figure 2.

5. CONCLUSION

The *P. aeruginosa* isolate utilized diesel oil as a source of carbon and energy and produced biosurfactant when grown on diesel oil as carbon substrate which was shown to be able to biodegrade herbicide with higher rates at lower concentrations of herbicides. Biosurfactant produced using *P. aeruginosa* can be recommended for use in herbicide bioremediation.

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