NJBAS

Available online at http://www.ajol.info/index.php/njbas/index
Nigerian Journal of Basic and Applied Science (December, 2016), 24(2): 102-108
DOI: http://dx.doi.org/10.4314/njbas.v24i2.14

ISSN 0794-5698

Full Length Research Article

Comparative Studies on the Biosurfactant Production Capacity of *Bacillus Subtilis* and *Pseudomonas Aeruginosa* Using Engine Oil and Diesel Respectively as Substrate

[1]O.A. Abdulsalam, [1] *M.B. Tijjani, , [1]M.S. Aliyu, , [1]I. Garba, and [2]A. Wada-Kura
[1]Department of Microbiology, Ahmadu Bello University, Zaria- Nigeria
[2]Department of Microbiology, Federal University, Dutse- Nigeria
[*Corresponding Author: Email: mbtijjani@yahoo.com, bashtj@hotmail.com]

ABSTRACT

The study was carried out to compare the the production capabilities and the biosurfactant activity of the bacteria, *Bacillus subtilis* and *Pseudomonas aeruginosa* using engine oil and diesel as the substrates respectively. The test organisms were isolated from engine oil contaminated soil as in the case of the *Bacillus subtilis*, which was collected from an automobile workshop in Samaru, Zaria and hydrocarbon-contaminated water in the case of the *Pseudomonas aeruginosa*. The medium used for the experiment was a mineral medium supplemented with 2% engine oil and 2% diesel as the sole source of carbon and energy for *Bacillus subtilis* and *Pseudomonas aeruginosa* respectively. Production of biosurfactant was assayed by monitoring the increase in cell concentration, biosurfactant concentration, emulsification index and decrease in surface tension. Highest level of cell concentration and biosurfactant concentration (3.3×10^8 CFU/ml and 0.0106mg/ml respectively) were obtained at 144 h for the *Pseudomonas aeruginosa* using diesel as source of carbon and energy while the highest level of cell concentration and biosurfactant concentration (3.2×10^8 CFU/ml and 0.0096mg/ml respectively) were obtained at 120hrs for the *Bacillus subtilis* using engine oil as source of carbon and energy. The research show that *Pseudomonas aeruginosa* using diesel as the sole source of carbon and energy is better for the production of biosurfactant than *Bacillus subtilis* using engine oil as the sole source of carbon and energy.

Key words: Biosurfactant, engine oil, diesel, *Pseudomonas aeruginosa, Bacillus subtilis.*

INTRODUCTION

Diverse types of microbial surface active amphiphilic molecules are produced by a range of microbial communities (Banat *et al*., 2014). They can be produced as part of the cell membrane by a variety of yeast, bacteria and filamentous fungi (Chen *et al.,* 2007). Currently, many biosurfactant-producing microorganisms have been isolated and identified to belong to: *Bacillus*, *Agrobacterium*, *Streptomyces*, *Pseudomonas*, and *Thiobacillus* as producers of amino acids-containing biosurfactants; *Pseudomonas*, *Torulopsis*, *Candida*, *Mycobacterium*, *Micromonospora*, *Rhodococcus*, *Arthrobacter*, *Mycobacterium*, *Corynebacterium*, *Mycobacterium*, and *Arthrobacter* as producers of glycolipids; *Thiobacillus*, *Aspergillus*, *Candida*, *Corynebacterium*, *Micrococcus*, and *Acinetobacter* as producers of phospholipids and fatty acids (Liu *et al*., 2015). Biosurfactants are amphiphilic compounds produced on living surfaces, mostly microbial cell surfaces, or excreted extracellularly and contain hydrophobic and hydrophilic moieties that reduce surface tension and interfacial tensions between individual molecules at the surface and interfaces respectively (Karanth *et al.,* 2005). Rhamnolipids are one of the most intensively studied microbial produced biosurfactants. They lower surface tension of water from 72 to 25–30 mN m^{-1} and exhibit critical micelle concentration (CMC) as low as 10–200 mg L^{-1} (Beuker *et al*., 2016). Production of rhamnolipids is mainly described in the opportunistic pathogen *Pseudomonas aeruginosa* using shake flask, batch, fed-batch or continuous systems (Beuker *et al*., 2016). Researchers have worked with various combinations of carbon and nitrogen sources in biosurfactants production technology (Banat *et al*., 2014).

Biosurfactants, which for a long time have been confused with bioemulsifiers, derived their name from biologically produced surfactants (Rahman and Randhawa, 2015). They play a primary function, facilitating microbial presence in environments dominated by hydrophilic-hydrophobic interfaces (Paniagua-Michel *et al*., 2014). They are amphipathic molecules with both hydrophilic and hydrophobic moieties that partition preferentially at the interface between fluid phases that have different degrees of polarity and hydrogen bonding, such as oil and water or air and water interfaces. This property explains their broad use in environmental applications. 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 (Liu *et al*., 2015). Research focusing on the microbial production of surfactants increasingly gains attention due to the strong surface activity and specific characteristics of some of these biosurfactants (Willenbacher *et al*., 2015). Bacterial strains belonging to genera *Bacillus* and *Pseudomonas* typically produce lipopeptide biosurfactants (Dadrasnia and Ismail, 2015). The ability to reduce surface tension is a major characteristic of surfactants (Anandaraj *et al.,* 2010).

Generally petroleum hydrocarbon compounds bind to soil components and are difficult to remove or degrade. Biosurfactants can emulsify hydrocarbons, thus enhancing their water solubility, decreasing surface tension and increasing the displacement of oily substances from soil particles. The relatively sudden introduction of xenobiotic chemicals and the massive relocation of natural material to different environmental compartments can often overwhelm the self-cleaning capacity of recipient ecosystems and thus result in the accumulation of pollutants to problematic or even harmful levels.

MATERIALS AND METHODS

Isolation of the Organisms

Bacillus subtilis was isolated from oil spilled soil which was collected from an automobile workshop in Samaru, Zaria. A total of 1.0g of the soil was weighed and was suspended in 9ml sterile distilled water. Serial dilution of 10^1 to 10^4 of the suspension was carried out. Nutrient agar plates were prepared by dissolving 3.36g of the powder into 120ml of distilled water and heated to dissolution of the powder. The preparation was autoclaved at 121^0C for 15 minutes. The solution after cooling was poured into six (6) 20ml sterile standard Petri dishes and was allowed to gel. Aliquot (0.1ml) of 10^{-2} to 10^{-4} dilutions were inoculated in duplicates by spread plate method, and were incubated aerobically at 37^{o}C for 48 hours.

Colonies that show big, cream, wrinkled and spreading colonies which are presumptively typical of *Bacillus subtilis* were picked and sub cultured into nutrient agar slant and nutrient broth. Gram and endospore stainings were carried out for the presumptive identification of the isolates. Biochemical characterization was done to identify the organism using catalase, citarate, indole, starch hydrolysis test, gelatin hydrolysis test and sugar fermentation tests (mannitol, sucrose, lactose).

For the isolation of *Pseudomonas aeruginosa,* water samples were collected in sterile Bijou bottles from car-wash run-off water contaminated with oil. Cetrimide agar was prepared, allowed to cool and dispensed in 20 ml into five (5) agar plates. Four (4) plates were inoculated with the water sample and one was left uninoculated as control. The plates were incubated at 37^0C for 24 h (Murray *et. al.,* 1999). Colonies obtained were subcultured on agar slant and stored in a refrigerator. Overnight culture of the isolates were subjected to the following biochemical tests: catalase, oxidase, motility. Other physiological

and morphological characterizations carried out were: pigmentation on cetrimide agar and growth at 4^0C.

A loopful of 24hrs broth culture of the isolate was streaked on fresh Cetrimide agar and incubated at 37^0C for 48hrs (Vanessa *et. al*., 2009). Colonies with dark blue coloration against the pale blue background (colour of the methylene blue-supplemented medium) indicates biosurfactant production which is due to the formation of surfactant-methylene blue complex which gave the dark blue coloration (Van Hamme *et. al.,* 2003).

Substrate Preparation

The engine oil and diesel used for the purpose of this research were of commercial grades obtained from the local petroleum market in Zaria, Nigeria. The media used for the experiment was a mineral medium supplemented with 2% engine oil and 2% diesel for the growth of *Bacillus subtilis* and *Pseudomonas aeruginosa* respectively, as the sole source of carbon and energy. They were prepared by dissolving 0.4g potassium phosphate, 0.4g of sodium phosphate, 0.2g iron (II) sulphate, 2g ammonium chloride, 0.4g calcium chloride, 0.2 magnesium sulphate in 250ml Erlenmeyer flask containing 200 ml distilled water. Duplicate of the media were prepared and the preparations were sterilized and were plugged with cotton wool as described by Muhammad (2012).

Inoculation and Incubation

One (1) ml of 24hrs broth culture of the isolates was pipetted into each of the mineral media using a sterile syringe. The inoculated flasks were plugged with cotton wool and incubated at 37^0C in a shaker operating at 120rpm for 7 days (Priya *et al.,* 2009).

Measurement of Biosurfactant Concentration

The biosurfactant concentration was measured indirectly by measuring the absorbance of biosurfactant-methylene blue complex at wavelength of 500nm. Methylene blue with a concentration of 0.015mg/ml was prepared and it's optical density was measured using spectrophotometer. The solution was used as standard. Two (2) ml of the broth culture was pipetted into a test tube containing 2ml of the standard, and one (1) ml of chloroform was added to the mixture. The mixture was vortexed for two minutes and allowed to stand. The biosurfactant-methylene blue complex was siphoned using Pasteur pipetted into a curvette and the absorbance was measured using spectrophotometer (Rahman *et al.,* 2002).

Measurement of Cell Concentration (Biomass)

McFarland 0.5 was used as the standard for the biomass measurement. One (1) ml of the broth culture was pipetted into a clean curvette and the absorbance was measured using a spectrophotometer (Rahman *et al.,* 2002).

Measurement of Emulsification Index (E_{24})

Four (4) ml of the broth culture was pipettted into a clean test tube, vortexed for 1 minute and allowed to stand at room temperature for 24 h. The length of the emulsion layer and the length of the column were measured using a centimetre ruler after 24hrs. The percentage emulsification index was measured (Muhammad, 2012).

RESULTS

The Organisms

The isolates were characterized and confirmed. The result is presented in Table 1 and Table 2. Growth was observed on nutrient agar after 24hrs of incubation at 24^0C.

The biosurfactant concentration was measured indirectly by measuring the absorbance of biosurfactant-methylene blue complex at wavelength of 500nm. The concentration of the complex was measured using 0.015mg/ml methylene blue as the standard solution which has an optical density of 0.848. The results obtained are shown in Table 2.

Cell concentration was measured indirectly by measuring the absorbance of the broth culture at 650nm using McFarland 0.5 as the standard

(approximate biomass equivalence=1.50x8cfu/ml, light absorbance at 650nm=0.153). The results obtained are shown in Table 3.

Cell concentration was measured indirectly by measuring the absorbance of the broth culture at 650nm using McFarland 0.5 as the standard (approximate biomass equivalence=1.50x8cfu/ml, light absorbance at 650nm=0.153). The results obtained are shown in Table 4.. The growth pattern obtained for the organism show the distinctive phases of microbial growth (i.e. log phase, stationary and the death phase). However, the lag phase was not noticed. Also readings were taken at intervals of 24h, perhaps it may be even absent in this case since the inoculated organism was at the lag phase of growth (a 24hrs broth culture). The biosurfactants concentration (mg/ml) for *Pseudomonas aeruginosa* increases with the time of incubation until it reaches 144hrs and 168 hrs where a decrease in concentration was observed. The concentration of biosurfactants (mg/ml) for *Bacillus subtilis* also increases steadily until at 144hrs and 168 hrs where a decline in biosurfactants concentration was noticed.
Table 5 shows the mean emulsification index at respective time interval for *Pseudomonas aeruginosa* and *Bacillus subtilis,* as the time of incubation increases there was an increased in emulsification index, however, a decrease in emulsification index was noticed as the time of incubation increases, this was observed at 144hrs and 168 hrs respectively for *Pseudomonas aeruginosa* and *Bacillus subtilis.*

DISCUSSION

The highest concentration of biosurfactant obtained for the culture of *Bacillus subtilis* in this research was 0.0096mg/ml and it was obtained at 120hrs, beyond which the level declined continuously. The highest concentration obtained for *Pseudomonas aeruginosa* was 0.0104mg/ml and it was obtained at 144hrs. For the *Bacillus subtilis*, the time for peak in production was concomitant with the biomass and the emulsification index, where the highest biomass obtained was also at 120hrs with a value of $3.20x10^8$CFU/ml. The highest value obtained for emulsification index (also at 120hrs) was 29.0%. For the cultures of *Pseudomonas aeruginosa*, the peak in production was obtained at 144hrs and highest emulsification index was obtained at this time interval, but the peak in biomass was at 120hrs. The emulsification index in this research shows a positive correlation with the concentration of the biosurfactant in solution. This is similar to the findings of Rahman *et al.* (2002).
The declination in growth may be due to depletion in nutrients since it is a batch culture as explained by many studies in microbial growth pattern. Accumulation of toxic waste products in the broth may also contribute to the declination of the growth. The waste may come from metabolism of more complex hydrocarbons such as naphthalene, benzyl rings and other component of engine oil as suggested by Muhammad (2012). Interference with the membrane uptake process and reduced bioavailability of micellar hydrocarbons were also reported by Muhammad (2012) to affect the growth of the organism as well as level of biosurfactant production.

The growth pattern obtained for the organism show the distinctive stages or phases of microbial growth (i.e. log phase, stationary and the death phase). However, the lag phase was not noticed. This may be because the lag phase is very short, and the readings were taken at intervals of 24h, perhaps it may be even absent in this case since the inoculated organism was at the phase of growth (a 24hrs broth culture).

It could be deduced that the optimum biosurfactant production was at the early stationary phase of growth. The production and the concentration of biosurfactant produced show almost similar pattern with that of the bacterial growth curve. Sabina *et al.* (2010) in their experiment conclude that biosurfactant is a secondary metabolite of microbial metabolism, i.e is produced at the stationary phase of growth,

suggesting that the product is not related to the growth of the organism.

Table 1. Morphological and Biochemical Identification of Bacillus subtilis

Characteristics	Reaction
Colony morphology on nutrient agar	Cream, big, spreading, finely wrinkled and slimy
Microscopy	Gram positive rods
Endospore staining	Positive (Central spores)
Starch hydrolysis test	Positive
Catalase	Positive
Citrate	Positive
Gelatin	Positive
Sucrose	Acid production
Lactose	Gas production
Mannitol	Negative

Furthermore, the fluctuations observed in the growth of the organism, biosurfactant production and the emulsification indices may be due to extrinsic factors that were beyond control in the laboratory during the course of the research. Some of these factors include power failure which is required for constant agitation, variance in particulate spectrophotometric activity, etc. Maintaining these parameters will help in producing a more linear result.

Table 2: Biochemical characterization of *Pseudomonas aeruginosa*.

Characteristic	Reaction
Growth on Cetrimide agar with pigmentation	Positive
Pigment on agar plate	Bright green
Microscopy	Gram negative rods
Motility	Positive
Catalase	Positive
Oxidase	Positive
Pigmentation in broth culture	Bright green at the top layer with dirty brown sediment
Growth at 4^0C	Negative
Colony colour on Cetrimide/methylene blue agar (CTAB-MB)	Intense blue compared to the agar background

Table 3. Biosurfactant concentration at intervals

Time (hrs)	Mean Absorbance (PA)	Biosurfactant Concentration (mg/ml) for PA	Mean Absorbance (BS)	Biosurfactant Concentration (mg/ml) for BS
24	0.188	0.0012	0.166	0.0029
48	0.262	0.0046	0.209	0.0037
72	0.362	0.0064	0.300	0.0053
96	0.385	0.0068	0.322	0.0057
120	0.590	0.0104	0.542	**0.0096**
144	0.628	**0.0106**	0.517	0.0091
168	0.504	0.0094	0.461	0.0082

Keys: PA = *Pseudomonas aeruginosa,* BS = *Bacillus subtilis.*

Table 4. Cell concentration at time intervals

Time (hrs)	Mean Absorbance (PA)	Cell Concentration for PA (CFU/ml)	Mean Absorbance (BS)	Cell Concentration for BS (CFU/ml)
24	0.153	$1.50x10^8$	0.155	$1.52x10^8$
48	0.193	$1.89x10^8$	0.200	$1.96x10^8$
72	0.233	$2.28x10^8$	0.221	$2.17x10^8$
96	0.294	$2.88x10^8$	0.298	$2.92x10^8$
120	0.313	**$3.06x10^8$**	0.326	**$3.20x10^8$**
144	0.272	$2.66x10^8$	0.279	$2.74x10^8$
168	0.253	$2.47x10^8$	0.255	$2.50x10^8$

Keys: PA = *Pseudomonas aeruginosa,* BS = *Bacillus subtilis.*

Table 5. Values of Emulsification indices at the respective time intervals

Time (h)	Mean Emulsification Index (PA)	Mean Emulsification Index (BS)
24	12.9	10.7
48	15.7	14.6
72	21.5	18.1
96	26.9	24.4
120	29.9	29
144	31.5	28
168	30.6	25.2

Keys: PA = *Pseudomonas aeruginosa,* BS = *Bacillus subtilis*

CONCLUSION

Engine oil and Diesel can serve as the sole source of carbon and energy for the production of biosurfactants by *Bacillus subtilis,* if provided with optimum nutrients and conditions necessary for growth. The culture of *Pseudomonas aeruginosa* was found to produce more quantities of the biosurfactants than the culture of *Bacillus subtilis*, though the peak production time for the *Pseudomonas aeruginosa* culture was longer than that of the *Bacillus subtilis*.

REFERENCES

Anandaraj, B. and Thivakaran, P. (2010). Isolation and Production of biosurfactant producing organism from oil spilled soil. *Bioscience Technology*, 1(3):120-126

Banat, I.M., Satpute, S.K., Cameotra, S.S., Patil, R., and Nyayanit,N.V. (2014). Cost effective technologies and renewable substrates for biosurfactants' production, *Frontiers in Microbiology*, 5:697.

Beuker, J., Barth,T., Steier, A., Wittgens, A., Rosenau, F., Henkel , M. and Hausmann, R. (2016). High titer heterologous rhamnolipid production.AMB Express, 6: 124.

Cheesbrough, M. (2006). *District Laborstory Practice in Tropical Countries* (2nd ed.). India: Cambridge University Press, pp. 64-65

Chen, S.Y., Y.H. Wei and J.S. Chang (2007). repeted pH- stat fed-batch fermentation for rhamnolipid production with indigenous *Psedomonas aeroginosa* S2. *Applied Microbiol Biotechnology*, **76**(1):67-74.

Dadrasnia, A. and Ismail, S. (2015). Biosurfactant production by *Bacillus salmalaya* for lubricating oil solubilization and biodegradation. *International Journal of Environmental Research and Public Health,* **12(8)**:9848-9863

Karanth, N. G. K., P. G Deo and N. K. Veenanadig (2005). Microbial Production of Biosurfactants and their Importance in

Central Food Technology Research Institute.

Liu, J.F., Mbadinga, S.M., Yang, S.Z., Gu, J.D. and Mu, B.Z. (2015). Chemical structure, property and potential applications of biosurfactants produced by *Bacillus subtilis* in petroleum recovery and spill mitigation. *International Journal of Molecular Science*, **16(3)**:4814-4837

Muhammad, I.A. (2012). Use of Petroleum Products as Substrate for the Production of Biosurfactive Amphiphiles by *Pseudomonas aeruginosa.* An unpublished thesis submitted to the Department of Microbiology, Ahmadu Bello University, Zaria.

Murray, M.R., Bason E.J., Pfaller M.A., Tenover F.C. and Yolken, R.H. (1999). *Manual of Clinical Microbiology* (7th ed.). New York. American Society of Microbiology. Pp517-521.

Nwaogu, L. A., G.O.C. Onyeze and R.N. Nwabueze (2008). Degradation of diesel oil in a

polluted soil using *Bacillus subtilis. African Journal of Biotechnology*, **7**(12):1939-1943.

Paniagua-Michel, J.J., Olmos-Soto, J., Morales-Guerrero, E.R. (2014). Algal and microbial exopolysaccharides: new insights as biosurfactants and bioemulsifiers, *Advanced Food and Nutrition Research,*73:221-57.

Priya, T. and G. Usharani (2009). Comparative Study for Biosurfactant Production by Using *Bacillus subtilis* and *Pseudomonas aeruginosa*. *Botany Research International,* **2**(4): 284-287.

Rahman, K.S.M, Thahira-Rahman, Maclean J., Marchant S. And Bent R.I.M (2002). Rhamnolipid Biosurfactant Production by Strains of *Pseudomonas aeruginosa* Using low Cost Raw Materia. *Biotechnology Program,* **18**(6): 1277-1281.

Rahman, P.K.S.M and Randhawa, K.K.S. (2015). Microbiotechnology based surfactants and their applications. *Frontiers in Microbiology,* **6**:1344.

Sabina, V.R., Martha Cristina P.R, Maria De Lourdes B.C, Jose Viruciot M., Blanca Estela R.C and Gundalupe V.N.M (2010). Selection of Biosurfactant/Bioemulsifier-producing Bacteria from Hydrocarbon Contaminated Soil. *Brazilian Journal of Microbiology,* **41**(3):668-675.

Van Hamme, J. D., Singh A. and Ward O. P. (2003) Recent advances in petroleum microbiology *Microbiology and Molecular Biology Reviews*, **67(4)**: 503-549

Vanessa, W., Christoph S., and Rudolf H. (2009) Concept of the isolation of biosurfactant producing microorganisms. *Madame Curie Bioscience Database*. NCBI Bookshelf

Willenbacher, J., Yeremchuk, W., Mohr, T., Syldatk, C. and Hausmann, R. (2015). Enhancement of surfactin yield by improving the medium composition and fermentation process, *AMB Express,* 5:57.