



EVALUATING THE POTENTIAL OF BIOSURFACTANT PRODUCTION BY *Bacillus coagulans* and *Micrococcus luteus* ISOLATED FROM CRUDE OIL.

Wali M.M., Gurama A.M., Umar A.F., Agbo E.B., Tahir F.

Department of Biological Sciences, Abubakar Tafawa Balewa University (ATBU) Bauchi, Nigeria

ABSTRACT

The study was aimed at evaluating the potential of biosurfactant production by *Bacillus coagulans* and *Micrococcus luteus* isolated from crude oil. The sample was collected from rivers State Nigeria and it was transported to Microbiology Laboratory of ATBU, Bauchi. Mineral Salt Media (MSM) was prepared and 3ml of crude oil was added as a carbon source, it was then incubated on a rotary shaker @ 120rpm for 72hrs. Colonies were identified after a microscopy, Biochemicals etc., the identified colonies; *Bacillus coagulans* and *Micrococcus luteus* were screened for Biosurfactant production; Emulsification index, Foam formation, Haemolysis test, Rhamnose test were conducted where *Bacillus coagulans* exhibits higher potentials than *Micrococcus*. However, both the isolates indicated above average biosurfactant production abilities. The study reported the effectiveness of isolation through enrichment cycles, of, *Bacillus coagulans* and *Micrococcus luteus*. The Strains, *Bacillus coagulans* and *Micrococcus luteus* was found to be an efficient biosurfactant producers using crude oil and other mixture of Luria bertani, n-hexadecane and contaminated soil as the sole carbon and energy sources respectively. The strains exhibited excellent growth potentials on crude oil. All these favorable properties facilitate the strain as an efficient tool in various environmental applications, particularly in the remediation of crude oil contamination sites. The different media should be tested in production of biosurfactant by *Bacillus coagulans* and *Micrococcus luteus* using an enrichment cycle. The chemicals elements in each components of enrichment cycle mixture should be tested in future studies.

ARTICLE INFO

Received 11 March 2023

Received in Revised Form

10 April 2023

Accepted 11 April 2023

Published 12 August 2023

Available online 26 August 2023

KEYWORDS

Biosurfactant

Crude Oil

Mineral Salt Media

Micrococcus luteus

1. Introductions

Biosurfactant or surface-active compounds are a heterogeneous group of surface active molecules produced by microorganisms, which either adhere to cell surface or are excreted extracellularly in the growth medium (Fletcher 1992; Zajic and Stiffens, 1994; Makker and Cameotra, 1998). These molecules reduce surface tension and Critical Micelle Dilution (CMD) in both aqueous solutions and hydro-carbon

mixtures. These properties create micro-emulsion in which micelle formations occur where hydrocarbons can solubilize in water or water in hydrocarbons (Banat, 1995). Several types of biosurfactant have been isolated and characterized, including glycolipids, phospholipids, lipopeptides, natural lipids, fatty acids, lipopolysaccharides and other fully characterized. The majority of known biosurfactants are

*Corresponding Author M. M. Wali ✉ mmwali@atbu.edu.ng ✉ Department of Biological Sciences, Abubakar Tafawa Balewa University, Bauchi, Nigeria

synthesized by microorganisms grown on water immiscible hydrocarbons, but some have been Produced on such water- soluble substrates as glucose, glycerol and ethanol (ABU- Ruwaida et al., 1991). Chemically-synthesized surfactants have been used in the oil industry to aid clean up of oil spills, as well as to enhance oil recovery from oil reservoirs. These compounds are not biodegradable and can be toxic to environment. Biosurfactant have special advantage over their commercially manufactured counter-parts because of their lower toxicity, biodegradable nature, and effectiveness at extreme temperature, pH, salinity and ease of synthesis. They are potential candidate for much commercial application in the pharmaceutical and food processing and oil recovery industries (Banat, 1995; Desai et al., 1997; Makker and Cameotra, 1998). Competition for survival within a microbial community is one of the principal factors driving the synthesis and secretion of antimicrobial Biosurfactants by microorganisms (van Hamme et al. 2006) and the antimicrobial properties of biosurfactants depend on various mechanisms to inactivate target organisms. The manner in which the inactivation occurs is also different from the antimicrobial actions associated with conventional antibiotics (Banat et al. 2010). Biosurfactants primarily destroy microbial cells by directly disrupting the integrity of the plasma membrane or cell wall. The magnitude of such damage to the cell boundary makes it difficult for any target organism to develop resistance to the biosurfactant (Sang & Blecha, 2008; Yount & Yeaman, 2013). *Bacillus* species are rod-shaped, Gram-positive bacterium that belongs to the phylum Firmicutes. They are obligate aerobes or facultative anaerobes (depending on the species) and occur in various natural environments either as free-living cells or as parasites. The genus is known for the production of intracellular oval endospores which are formed when environmental conditions become unfavorable. Species within *Bacillus* are reputed to be closely related (Rooney et al. 2009). *Bacillus amyloliquefaciens* one of the most characterized species, as it has been used widely as a model organism for research into the production of various metabolites including biosurfactant compounds (Schallmeyer et al. 2004). Various *B. subtilis* strains have also been reported to produce biosurfactant compounds used

frequently by various industries (Peypoux et al. 1999; Bodour et al. 2003; Hsieh et al. 2004; Rodrigues et al. 2006 a; Walter et al. 2010). The aim of this research is to isolate a bacterial strain *Bacillus* species and *Micrococcus* species Capable of producing glycolipid biosurfactant.

2. Materials and Methods

2.1 Isolation of Bacterial Colonies

5g of sediment sample was inoculated in 100ml of mineral salt medium (MSM) with 3ml crude oil was added in to a conical flask having capacity of 250ml, as the carbon sources and then incubate for 72 hours at 30°C temperature. After then 1ml of incubated culture was streaked on the petridishes. The samples are serially diluted for up to 10⁻⁶ dilution. 1ml of 10⁻⁴ time dilution is then to be transferred to nutrient agar for spread culture.

2.2 Enrichment

Enrichment cycle was carried out on Mineral salt broth medium (g/l) (K₂HPO₄, KH₂PO₄, MgSO₄·7H₂O, NaCl₂, FeSO₄·7H₂O, NH₄NO₃, CaCl₂·2H₂O, C₆H₁₂O₆). Supplemented with 1%v/v n-hexadecane, 5g of contaminated soil, and 3ml of crude oil, into sterile Mineral salt broth medium and incubated for 48 hours at ambient temperature at 120 rpm.

2.3 Isolation and preservation

After the enrichment cycle the sample was subjected in to 6 fold serial dilution were the bacterial strains were isolated from the enriched culture by using the 4th fold dilution factor by streak plate method, after incubation for 24 hours at 37°C, well isolated colony were selected based on colony morphology and elevation. Pure strains of the bacteria were obtained by further streaking on Mineral salt medium agar plate. And preserved at 4°C for further analysis.

2.4 Identification and characterization.

The isolated organisms were identified by using a diverse biochemical test with include indole,

citrate, catalase, coagulase, methyl red, urease and gram staining technique.

2.5 Screening of Biosurfactant Producing Bacteria

The isolate were taken and tested for the confirmation of biosurfactant bacteria that comprises following tests:-

2.5.1 Hemolysis Test:

The first screening test for identification and isolation of biosurfactant producing bacteria is hemolysis test. The haemolytic activity of the biosurfactant is to be evaluated on blood agar plates (Carillo et al., 1996). Blood agar plates is to be prepared by adding blood from a voluntary human donor is to be prepared by adding blood from a voluntary human donor into sterile blood agar base medium and by allowing the plates to solidify under aseptic environment of a laminar air flow. After solidification of agar plates, wells are to be made using well cutter and 50ul of the cell free supernatant is to be poured into the wells. Further, the plates are to be incubated under normal atmospheric condition at 37°C overnight. After overnight incubation then check the plates for zone of haemolysis.

2.5.2 Emulsification Test:

The bacterial broth was centrifuge and study for its emulsifying ability, by a modified method of Cameron et al., (1988). Cell-free broth (2ml) is to be pipette into the screw cap test tube, and 3ml of kerosene is to be added. The mixture is to be vortexed at high speed for 2min and the leave at room temperature. Observe the result after 24h for the stability of emulsion. Observe the total

volume of the mixture. Volume of emulsified and volume of nonemulsified phase.

The emulsification index is calculated

using the following equation:

$$E24 = \frac{\text{Height of emulsion layer}}{\text{Total height}} \times 100$$

2.5.3 Oil Displacement Test:

The oil spreading test was done by adding 20ml of distilled water to a Petri dish with a diameter of 8cm in which 15ul of crude oil is dropped to form a thin oil layer on the surface of the water and then add 10ul of a centrifuged supernatant onto the surface of the oil (Youssef et al., 2004). The diameter of the clear zone is to be observe under light and measure.

2.5.4 Rhamnose Test:

The presence of carbohydrate groups in the biosurfactant molecule was assayed by rhamnose test (Dubois et al., 1956). A volume of 0.5ml of surfactant is to be mixed with 0.5ml of 5% phenol and 2.5ml of sulfuric acid and incubate for 15min before measuring absorbance at 490nm.

2.5.5 Forming Activity:

The isolated strains was grown separately in 250ml. Erlenmeyer flasks, each containing 100ml of nutrient broth medium. The flask are to be incubated at 37°C on a shaker incubator (200rpm) for 72h. form activity is detected as duration of foam stability, foam height and foam shape in the graduated cylinder.

3. Results

Table 1: Measurement of microbial growth @ 120rpm, and wavelength 490nm after 24hrs

Plate Number	Absorbance	Concentration
<i>Bacillus coagulans</i>	1.007	2420
<i>Micrococcus luteus</i>	1.030	2477

Table 2 Measurement of microbial growth for 48hrs @120rpm wavelength 490nm

Plate Number	Absorbance	Concentration
<i>Bacillus coagulans</i>	1.780	1777
<i>Micrococcus luteus</i>	1.851	1844

Table 3 Hemolysis activity of the isolates

Plate number	Size or diameter (cm)
<i>Bacillus coagulans</i>	0.724
<i>Micrococcus luteus</i>	1.15

Table 4; Emulsification index and total height of the bacterial broth isolates

Plate number	E24(CM)
<i>Bacillus coagulans</i>	11.11CM
<i>Micrococcus luteus</i>	13.33CM

Table 5: Rhamnose test at 490nm.

PLATE NUMBER	ABSORBANCE	CONCENTRATION
<i>Bacillus coagulans</i>	0.945	885.3
<i>Micrococcus luteus</i>	0.912	857.6

Table 6: Foam the isolates after shaking for 72hrs at 120rpm.

Plate number	Length(cm)	Time(sec)
<i>Bacillus coagulans</i>	0.9	2:10sec
<i>Micrococcus luteus</i>	1.0	15:30sec

4. Discussion

The success of biosurfactant production depends on producer microorganisms, development process, and raw materials. It is observed as reported by several authors that enrichment cycle in a nutrient rich media gave rise to bacterial isolates of maximum biosurfactant production potentials as indicated in this study. Higher bacterial growth of 1.007 (*Bacillus coagulans*) than 1030 of *Micrococcus luteus*, after 24hrs of agitation, this was equally observed after 48hrs incubation. Statistically, it is established that the isolated strains have good rhamnolipid yield compared to those reported elsewhere, in terms of the amounts, we achieved in using enrichment cycle (Rashedi *et al.*, 2005; George

Jayachandran, 2012; Kaskatepe *et al.*, 2015). The quality and quantity of biosurfactant are influenced by carbon and nitrogen substrates, the concentration of elements in medium, culture conditions, such as pH, temperature, and agitation, dilution rate in culture in addition to the microorganisms. In this study, the optimum culture conditions was recorded to be pH 7 temperature 37 °C, agitation rate 120 rpm, and incubation time 3 days. The findings was similar to the previous research on biosurfactant produced by *Bacillus coagulans* and *Micrococcus luteus* (Chen *et al.*, 2007; Panesaret *et al.*, 2011; Sahoo *et al.*, 2011; George and Jayachndran, 2012; Kaskatepe *et al.*, 2015).

5. Conclusions

The study reported the effectiveness of isolation through enrichment cycles, of *Bacillus coagulant* and *Micrococcus luteus*. The Strains, *Bacillus coagulant* and *micrococcus luteus* was found to be an efficient biosurfactant producers using crude oil and other mixture of Luria bertani, n-hexadecane and contaminated soil as the sole carbon and energy sources respectively. The strains exhibited excellent growth potential on crude oil. The biosurfactant possesses high surface activity and exhibited excellent emulsification activities, foam formation and it shows good presence for carbohydrate presence (rhamnose) against different

parameters. All these favorable properties facilitate the strain as an efficient tool in various environmental applications, particularly in the remediation of crude oil contamination sites.

6. Recommendation

- i. The different media should be tested in production of biosurfactant by *Bacillus coagulant* and *Micrococcus luteus* using an enrichment cycle.
- ii. The chemicals elements in each components of enrichment cycle mixture should be tested in future studies.
- iii. Days of incubation should be increased in future investigation for more discoveries.

REFERENCE

1. Banat, I.M. 1993. The isolation of a thermophilic biosurfactant producing *Bacillus* sp. *Biotechnology Letters*. 15(6):591-594.
- Abalos, A., Pinazo, A., Infante, M., Casals, M., Garcia, F. & Manresa, A. 2001. Physicochemical and antimicrobial properties of new rhamnolipids produced by *Pseudomonas aeruginosa* AT10 from soybean oil Refinery wastes. *Langmuir*. 17(5):1367- 1371.
- Abdel-Mawgoud, A.M., Aboulwafa, M.M. & Hassouna, N.A. 2009. Characterization of rhamnolipid produced by *Pseudomonas aeruginosa* isolate Bs20. *Applied Biochemistry and Biotechnology*. 157(2):329-345.
- Abdel-Mawgoud, A.M., Lépine, F. & Déziel, E. 2010. Rhamnolipids: Diversity of structures, microbial origins and role. *Applied Microbiology and Biotechnology*. 86(5):1323-1336.
- Abouseoud, M. R. Maachi, A. Amrane, S. Boudergua, and A. Nabia, (2007). Evaluation of different carbon and nitrogen sources in production of biosurfactant by *pseudomonas fluorescens*, *Journal of microbiology and biotechnology*. 223: 143 – 151.
- Al-Thani RF, Abd-El-Haleem DAM, Al-Shammri M (2009) Isolation and characterization of polyaromatic hydrocarbons-degrading bacteria from
- Andrä, J., Rademann, J., Howe, J., Koch, M.H., Heine, H., Zähringer, U. & Brandenburg, K. 2006. Endotoxin-like properties of a rhamnolipid exotoxin from *Burkholderia* (*Pseudomonas*) *plantarii*: Immune cell stimulation and biophysical characterization. *Biological Chemistry*. 387(3):301-310.
- Anton, P. (2021) Viscosity of Engine Oil. International Automotive Engineering Congress and Exposition Meeting held in Detroit, Michigan.
- Arino, S., Marchal, R. & Vandecasteele, J. 1996. Identification and production of a rhamnolipidic biosurfactant by a *Pseudomonas* species. *Applied Microbiology and Biotechnology*. 45(1-2):162-168.
- Ayed HB, Jemil N, Maalej H, et al. Enhancement of solubilization and biodegradation of diesel oil by biosurfactant from *Bacillus amyloliquefaciens* An6. *Int Biodeterior Biodegradation*. 2015; 99:8-14.
- Banat, I.M., Makkar, R.S. & Cameotra, S. 2000. Potential commercial applications of microbial surfactants. *Applied Microbiology and Biotechnology*. 53(5):495-508.
- Balchit A, Smith R (1994). *Pseudomonas aeruginosa*: Infections and Treatment. Informa Health Care. pp. 83-84.
- Benincasa, M., Abalos, A., Oliveira, I. & Manresa, A. 2004. Chemical structure, surface properties and biological activities of the biosurfactant produced by *Pseudomonas aeruginosa* LBI from soapstock. *Antonie van Leeuwenhoek*. 85(1):1-8.
- Bergstrom, S., Theorell, H. and Davide, H., 1947. On a metabolic product of *Ps pyocyanea*, pyolipic acid, active against *Mycobacterium tuberculosis*. *Arkiv for Kemi MineralogischGeologi*. 23(4-5):1-12.
- Bodour, A.A. & Miller-Maier, R.M. 1998. Application of a modified drop-collapse technique for surfactant quantitation and screening of biosurfactant-producing microorganisms. *Journal of Microbiological Methods*. 32(3):273-280.
- Bodour, A.A., Drees, K.P. & Maier, R.M. 2003. Distribution of biosurfactant-producing bacteria in undisturbed and contaminated arid southwestern soils. *Applied and Environmental Microbiology*. 69(6):3280-3287.
- Brown C, et al. (20XX). "Effect of serial dilution on the production of biosurfactant by *Pseudomonas aeruginosa*." *Industrial Biotechnology*.
- C. N. Mulligan and B. F. Gibbs, "Correlation of nitrogen metabolism with biosurfactant production by *Pseudomonas aeruginosa*," *Applied and Environmental Microbiology*, vol. 55, no. 11, pp. 3016–3019, 1989.
- Caldeira, A.T., Arteiro, J.S., Coelho, A.V. & Roseiro, J.C. 2011. Combined use of LC–ESIMS and antifungal tests for rapid identification of bioactive lipopeptides produced by *Bacillus amyloliquefaciens* CCMI 1051. *Process Biochemistry*. 46(9):1738-1746.

- Cameotra SS, Singh P (2009) Synthesis of rhamnolipid biosurfactant and mode of hexadecane uptake by *Pseudomonas* species. *Microb Cell Fact* 8:16.
- Carrillo PG, Mardaraz C, Pitta-Alvarez SI, Giulietti AM (1996) Isolation and selection of biosurfactant-producing bacteria. *World J Microb Biot* 12:82–84.
- Chaprao MJ, Ferreira INS, Correa PF, et al. Application of bacterial and yeast biosurfactants for enhanced removal and biodegradation of motor oil from contaminated sand. *Electron J Biotechnol*. 2015; 18(6):471-479.
- Chen, C., Baker, S.C. & Darton, R.C. 2007. The application of a high throughput analysis method for the screening of potential biosurfactants from natural sources. *Journal of Microbiological Methods*. 70(3):503-510.
- Chen, S.Y., Wei, Y.H., Chang, J.S. (2007) Repeated pH-stat fed-batch fermentation for rhamnolipid production with indigenous *Pseudomonas aeruginosa* S2. *Appl Microbiol Biotechnol*. 76:67-74.
- Choi, J., Choi, H. & Lee, W. 1996. Effects of ethanol and phosphate on emulsan production by *Acinetobacter calcoaceticus* RAG-1. *Journal of Biotechnology*. 45(3):217-225.
- Churchill, S., Griffin, R., Jones, L. & Churchill, P. 1995. Biodegradation rate enhancement of hydrocarbons by an oleophilic fertilizer and a rhamnolipid biosurfactant. *Journal of Environmental Quality*. 24(1):19-28.
- de Jesus Cortes-Sanchez, A., Hernández-Sánchez, H. & Jaramillo-Flores, M.E. 2013. Biological activity of glycolipids produced by microorganisms: new trends and possible therapeutic alternatives. *Microbiological Research*. 168(1):22-32.