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BACILLUS CIRCULANS AS BIOSURFACTANT-PRODUCER DURING CRUDE OIL DEGRADATION

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ABSTRACT

Petroleum and its byproducts are one group of universal environmental pollutants. Microorganisms have over time played significant roles in the clean-up exercise of unwanted substances in the environment. This research was aimed at studying the degradative potentials of biosurfactant-producing bacterial isolates (Bacillus sp.) from palm oil mill effluent (POME) in crude oil degradation. Standard microbiological and analytical methods were applied to ascertain biosurfactant production and degradation of crude oil by Bacillus sp. isolated from palm oil mill effluent discharged points and logging area of effluent bunk at Nigerian Institute for Oil-Palm Research (NIFOR) in Edo State. The bacteria were isolated and subjected to screening for hydrocarbon degradation and biosurfactant production. Biosurfactants characterization by Fourier Transform Infra-Red (FTIR) technique. total viable heterotrophic bacterial count of POME and Bonny light crude oil ranged from 6.6 $x10^6$ – 8.2 $x10^6$ cfu/ml and 4.2 $x10^6$ – 5.8 $x10^6$ cfu/ml respectively. Bacillus sp. that had the highest biodegradative potential and biosurfactant production was identified molecularly as Bacillus circulans. It could be used as bio-stimulants to ameliorate crude oil polluted areas as an efficient and cost- effective technology.

Keywords: biosurfactant, degradation, effluent, petroleum, biostimulants.

INTRODUCTION

The advancement of sustainable biotechnology has motivated the exploration for compounds that are biodegradable and natural to reclaim sites polluted with hydrocarbons. This steered the innovation of naturally obtained surfactants. This Surfactants are molecules of chemical compounds (amphipathic) comprising of both polar and non-polar portions decreases surface tension of viscous aqueous substances and thus forming structural aggregates like micelles (Bodour and Miller-Maier, 2002). Biomolecules exuded by numerous microorganisms (fungi, bacteria and yeast) (Gaur *et al*., 2019). *Bacillus* species are identified for lipopeptide nature of biosurfactants (Felix *et al*., 2019). They have been widely applied as dispersants and emulsifiers in oil-spill sites, agricultural, food processing, pharmaceutical, cosmetics and industrially owing to their natural properties (Brooijmans *et al*., 2009).

Surfactant of microbial origin (biosurfactants) are distinct surface-active compounds exuded by microbes. There are non/less toxic with biodegradability associated to synthetized surfactants and thus suitable for environmental applications like soil degradation, spilled-oil dispersion, oil recovery enhancement processes (Oberbremer *et al*., 1990), A large diversity of biosurfactants have been reported as effective (Desai *et al*., 1993).

Bacillus subtilis has been extensively researched for biosurfactant secretion and identified for proven effectiveness of generating high-performance lipopeptide, called *surfactin* or *subtilisin*. This is unique and most effective biosurfactants identified that reduces (water, temperature or surface tension) from 72 to 27 mN / m, with CMC in water 24 mM (Al-Bahry *et al*., 2013).

Emergence of water and soil pollution by oil and its products is accumulating, as one of the major environmental pollutants. Bases of pollution are: hazards to fuel tankers by vessels and trucks; leaks in underground reservoirs, under rust, oil drilling and handling actions; and insufficient emissions of oil-based waste products manufactured by oil companies during plastics, pharmaceuticals, solvents and cosmetics production. (Lin *et al*., 2010).

Petroleum-contaminated soil can be remediated by microbial isolates that exudes certain oil displacing compounds (lipopeptides) with emulsifying activities and surface tension classified as biosurfactants. They are effective biosurfactant that can be applied in the environment in crude oil recovery, pharmaceutical and food processes (Anyanwu, 2011).

Biosurfactants is applied widely in petroleum industries as demulsifiers, emulsifiers, oil reclamation agents. Microbial Enhanced Oil-Recovery (MEOR) method that utilizes biosurfactant comprising culture broth to release crude oil rapped in reservoirs (Marchant and Banat, 2012).

This dispersants and emulsifiers have been broadly applied as a retort alternative in combating oil spills in maritime locations. They are held as most efficacious approach to expedite maritime oil biodegradation (Rongsayamanont *et al*., 2017).

In this research, the application of biosurfactant in bioremediation of crude oil pollution has been reported to be a new clean up approach and *Bacillus circulans* identified to possess the capacity of enhancing oil-spill remediation.

MATERIALS AND METHODS

Sampling area

Oil palm mill effluent

Effluent from palm oil mill was obtained from the effluent point of palm oil mill operation in Nigerian Institute of Oil Palm Research (NIFOR), Edo state, Nigeria in July, 2019. Duplicate samples were collected representing pooled samples from effluent point and mixed homogenously.

Crude oil

Petroleum hydrocarbon (Bonny Light crude oil) was obtained from Port Harcourt refinery and stored at ambient temperature in dark reagent sample bottle to avoid ultraviolet and infrared radiation.

Microbial isolation

The total viable heterotrophic bacterial count (TVHBC) for palm oil mill effluent was assessed using a modified method of microbial isolation and screening for crude oil biodegradation. Briefly, 1ml of sterilized crude oil sample mixed with 1 g of well-mixed effluent sample was incorporated into 50 mL of mineral salt medium comprising (g/L); 1.1 g KCl, 1.1 g NaCl, 3.4 g KH_2PO_4, 4.4 g K_2HPO_4, 0.00028g $FeSO_4.7H_2O$, 0.5 g $MgSO_4.7H_2O$, 0.5g yeast extract, 15 g $NaNO_3$ at 37°C in shaker incubator (100rpm). Then 1g of well-mixed effluent sample was also incorporated into mineral salt composition as mentioned above. Serially dilution for both with sterile saline (0.85% NaCl) was employed aliquots of each suspension

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inoculated in supplemented nutrient agar plate. After 48h of incubation, bacteria were carefully chosen based on colony appearances on nutrient agar plates. Triplicate flasks were applied while un-inoculated flask served as control.

Screening test biosurfactant

Hemolytic activity

Biosurfactants can bring about lysis of erythrocytes (red blood cells). This principle was applied in the hemolysis assay as established by Mulligan *et al*., 1984. The biosurfactant-producing capacity of *Bacillus circulans* was confirmed by the blood hemolysis method Axenic bacterial culture was grown on blood agar plate newly prepared and incubated at 37°C for 48-72 h. The clear zone formation around the bacterium confirmed biosurfactant production (Ghasemi *et al*., 2019).

Drop collapse test

The drop-collapse method was employed for biosurfactant screening produced using *Bacillus circulans.* The technique was applied to identify the presence of rhamnolipid in the culture broths. Five (5) micro liters were decanted into the polystyrene wells and sustained for 24 hours at 22°C to enable it dry. The midpoint of the oil-coated well was chockfull with filtered cell-free supernatant of the culture broth. Oil drop was flat after 1–2 minutes; the outcomes were detailed positive for bio-surfactant production. This was to observe if the culture broth contained biosurfactant in oil-coated wells collapsed (positive). Absence of alteration in droplets nature indicates negative (Mamta *et al*., 2020).

Blue agar plate method

Supplement of glucose as source of carbon (2%) and cetyltrimethylammonium bromide (CTAB: 0.5 mg/mL) and methylene blue (MB: 0.2 mg/mL) was introduced into mineral salt agar medium to detect anionic biosurfactant. Methylene blue agar with 4mm bored wells were filled with 30µl of cell free supernatant. Plates were incubated at 37°C for 48-72 h. If appearance of darkish blue halo region, surfactant was secreted by the microorganism on growth plate. Thus, anionic biosurfactant production occurred (Thavasi *et al*., 2011).

Oil Spread Activity

Bacterial isolates with ability to degrade crude oil, were subcultured to obtain axenic culture. The bacterial isolates were cultured overnight on nutrient agar, dislodged with sterile distilled water. Inoculum size was standardized by 1.0 Macfarland prior to inoculation. Two millilitres (2ml) suspension of inoculum were added to 98ml mineral salt medium in a 250ml Erlenmeyer flask, gestated in orbital shaker for 7 days at 37˚C, 150 r.p.m. The reaction was carried out in 10:1:0.05 ratio for water; BLCO (Bonny Light Crude Oil); biosurfactant respectively. In other to observe the oil spread activity, 40ml water was placed in petri dish, 4ml of crude oil dispensed on the water surface as well as 400microltre of biosurfactant was applied. The diameter was measured after 60 seconds to ascertain the spreading activity (Plaza *et al*., 2006).

Recovery of Biosurfactants

The extraction method involving the application of acid precipitation and solvent extraction technique was employed. Fermented cell free broth was obtained after spinning at 5000 xg and 25 ^{o}C for 30mins. Acquired supernatant served as crude biosurfactant. Solvent extracted through

reacting 3:1 of cell free material; crude biosurfactant and preserved with solvent mixture extract (methanol/ chloroform/ acetone, 1:1:1 by volume), 40% (w/v) *Zinc sulphate* solution, Ammonium sulphate and acid precipitate. Experimental work was aerated using an orbital shaker, extraction supernatant centrifugation at 10000 rpm, 4^{o}C designed aimed at 30 min using a refrigerated centrifuge. Precipitate was dried and black color paste observed after 24 hrs (Adebajo *et al*., 2020).

Growth Performance

Inoculated cell culture during degradation process were plated out, pH evaluation and optical density (O.D.) measured at 600nm. Successive optical densities obtained frequently throughout the period of degradation. Determination of growth curve was obtained by plotting optical densities against time.

Analysis of biosurfactant extract using FTIR technique

Fourier-transfer Infra-Red absorption spectra was used with Buck science M530 USA FTIR. Analysis at Springboard Laboratory, Awka Anambra province. Buck Science M530 USA FTIR was hired for analysis. The device was reinforced with a deuterated triglycine sulphate sensor with a potassium bromide beam splitter. Gram A1 software used to perform spectra to operate. Approximately 1.0g of well-packaged salt pellet samples. During the measurement, the FTIR spectra was obtained at typical locations of 4,000 - 600 cm-1 and augmented by 32 scans and 4 cm-1 adjustments. The FTIR spectra was shown as transmitter values, UV-VIS Spectroscopic analysis. UV spectrophotometric analysis was applied to the sample using a 2nm optical spectrophotometer (Apel 3000UV) with a diameter of 2nm, using a 10-mm cell at room temperature. The output is tested under UV light visible wavelength from 300-800nm

Crude Oil Degradation (Total Hydrocarbon Content)

The degradation of oil was investigated using 50ml mineral salt medium augmented with 5g/l crude oil decanted into three 100ml Erlenmeyer flasks. First flask inoculated with 1ml of seed inoculum, the second flask contain 1ml seed inoculum and 1% synthetic surfactant, 1ml seed inoculum and 1% of the purified biosurfactant was introduced to third flask. Flasks gestated at 30°C for 7 days using orbital shaker at 150rpm. Residual oil extracted and oil degradation rate determined. Residual TPH was extracted from samples and quantified with Gas Chromatography-Mass Spectrometry (GC-MS) analyzer (Omotayo *et al*., 2012).

Percentage degradation (%) =

$$\frac{\text{CTPH control} - \text{TPH treatment}}{\text{TPH control}} \times 100 \quad - \quad (2)$$

CTPH control=concentration of total petroleum hydrocarbons of the control. TPH treatment = Total petroleum hydrocarbons of the treatment.

Emulsification test (E_{24})

Suspension of uncontaminated culture colonies with 2 mL of mineral salt medium after 48h gestation and 2 mL hydrocarbon (oil) introduced to tubes. Mixture vortexed at high speed for 1min and 24h stance. Emulsion index (E_{24}) signifies height of suspension layer (cm) alienated through total height (cm), multiplied by 100.

Emulsification Index (E_{24}) =

$$\frac{\text{Height of Suspension Layer} \times 100}{\text{Mixture Entire Height}} \quad - \quad (3)$$

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Surface Tension Measurement

Surface tension of culture broth free cells ascertained through capillary upsurge technique. Cell free culture broth introduced to 1L of sterile distilled water cumulative concentration (1-8mg). Capillary tube (0.01cm diameter) and immersed in water. Surface tension quantifier from height of water in capillary tube with equation as follows below: The surfactant tension (γ) is mathematically given as

$$\gamma(\text{mN/m}) = \frac{rh\delta g}{2} \qquad \text{- (4)}$$

g= gravitational acceleration (9.8m/s^2); h=height of column; r= radius of the capillary tube 0.01 cm.

Identification of isolate

This isolate was subjected to molecular assessment and the genetic identity was ascertained by isolation and amplification the DNA sequence from 16S rRNA. Sequence was recognized as the prime bacterial barcode (Schoch, *et al*. 2012).

RESULTS AND DISCUSSION

Figure 1. revealed the Total viable heterotrophic bacterial count (TVHBC) for palm oil mill effluent and palm oil mill effluent supplemented with crude oil indicated growth response as 75.3±6.7 and 44.6±51 respectively. The palm oil mill effluent had higher counts than the POME supplemented with crude oil and shows no toxicity on the microbial growth. This represents a stress-resistant and naturally-occurring bacteria with potential of surviving in environment that is heavily contaminated with crude oil employed as productive means for bioremediation. Hence, higher counts in the palm oil mill effluents than the crude oil supplemented culture. This was in consonance with findings according to Nwankwegu *et al*. (2016) where they testified that palm oil mill effluent has certain bacterial isolates with potentials in crude oil degradation.

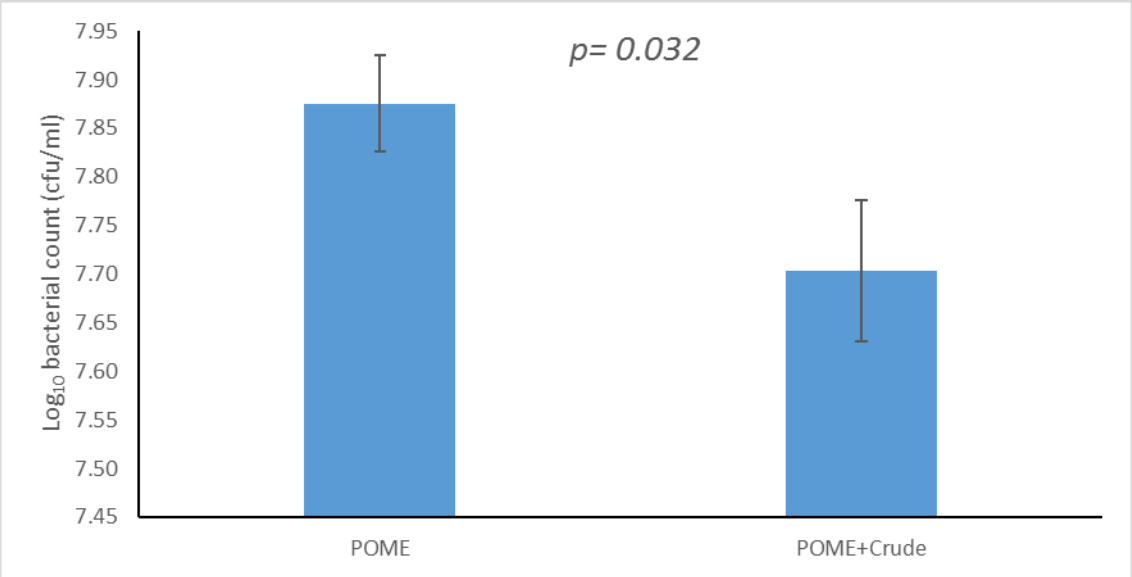

Figure 1: Bacterial growth response on mineral salt medium and crude oil-supplemented medium.

Screening for biosurfactant experiment

Table 1. Characterization of biosurfactant produced by *Bacillus circulans*

Sample	HC-Deg. Potential	Blood Haemolysis	Drop Collapse	Emulsification Index (%)	Oil Spread (mm)	CTAB Blue Agar Plate Method	Surface Tension (N m-1)
Bacillus circulans	+++	γ (+)	+++	43.4	10.5	++	42.4

Blood haemolysis and CTAB activity

Hemolytic action of biosurfactant-producing bacterial (*Bacillus circulans*) and categorized as γ-gamma haemolysis by the production of halo about associated colony (Table 1.). Activity evaluation was useful as a prime technique to screen biosurfactant production (Nogueira *et al.*, 2011). Similar technique applied by Abouseoud *et al*., 2008 for biosurfactant action of *Pseudomonas fluorescens* with diameter of hemolytic zone as indicator. The CTAB agar plate technique which is similar to the blood haemolysis activity is semi -quantitative assay to detect presence of extra cellular glycolipids or anionic surfactants showed positive for experiment of *Bacillus circulans* as recorded in Table 1. This was established by Siegmund and Wagner with dynamic colonies delimited by dark blue halos.

Drop collapse test

Assay depends on biosurfactant destabilizaton of liquid droplets. In this study, liquid contained surfactants and drops spread showed positive (Table 1.) collapse force as a result of interfacial tension between liquid drop and hydrophobic surface according to the research by Sumathi *et al*., 2016.

Oil spread activity

Oil displacement investigations exhibited a perfect clear diameter of 10.5 mm as an indicator of biosurfactant production by *Bacillus circulans* (Table 1.) Morikawa *et al*. described the oil displacement area to have direct proportionality to the surface-active compound in the solution. However, in this experiment only the qualitative study to ascertain the presence of surfactant was done.

Biosurfactant characterization

The FTIR spectrum of column biosurfactant discovered significant bands at 3693.43047, 3821.21696, 3393.16566, 3248.5386, 3001.97158, 2800.5929, 2357.833704, 2274.57177, 2157.24735, 2048.95349, 1934.68505, 106775017, (Figure 2). For analysis of numerous functional groups present in the biosurfactant and spectrum compared with Pornsunthorntawee *et al.* (2008).

The biosurfactant component of *Bacillus circulans* from the FTIR study showed C-H expandable bands of CH_2 (methylene) experiential in region 2800.593cm $^{-1}$. Band of carbonyl stretch found at 2048.953 cm^{-1}. Ester carbonyl group established band at 1036.692 cm^{-1} associated with vibration of C-O deformation. The wavelengths obtained were associated with biosurfactant lipopeptide moieties (Fig. 3). This FTIR biosurfactant study was in consonance with results obtained from previous researchers (Kuiper *et a*l. 2004; Das and Mukherjee 2007) with FTIR investigative device to classify biosurfactants.

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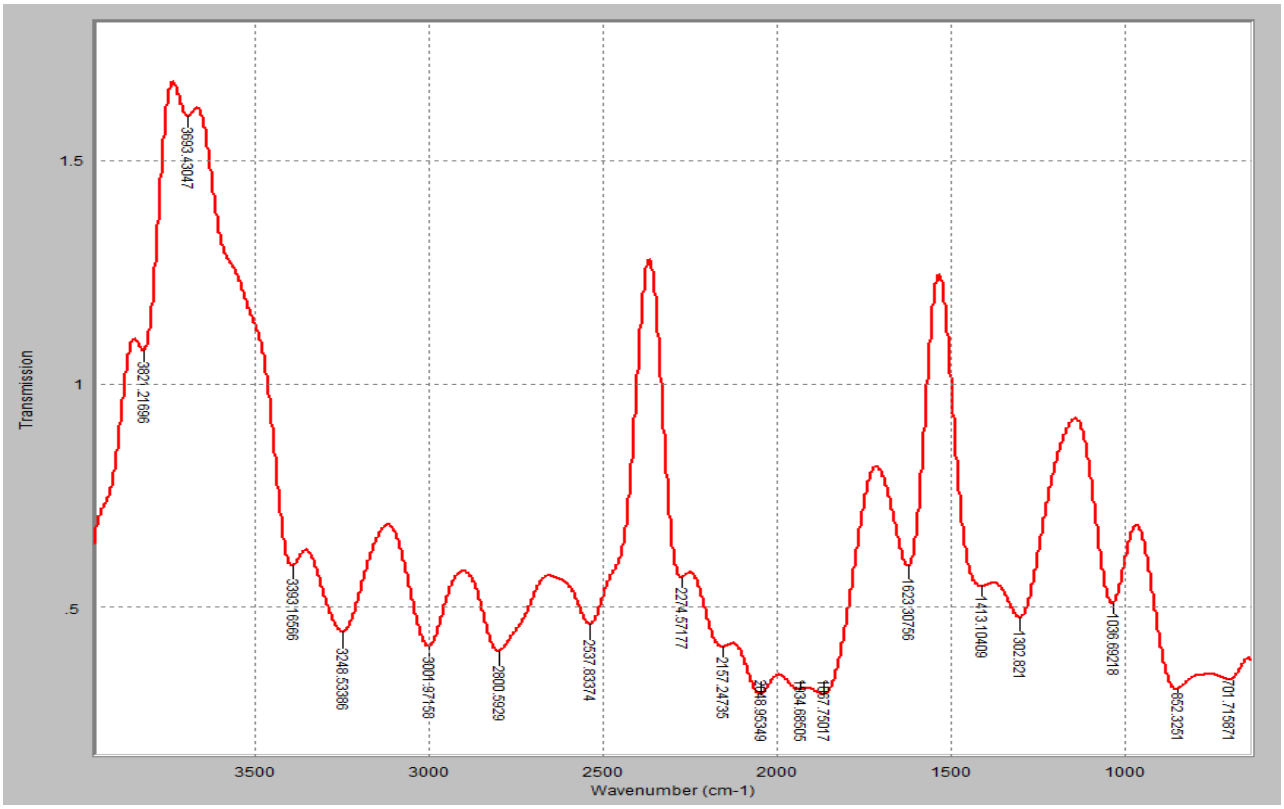
Biosurfactant analysis showed ionized compounds containing molecular weight that may reflect protein and lipid molecules. Analogous outcomes were achieved indicating the lipopeptide biosurfactant by *Bacillus pumilus*.

Figure 3. FTIR spectrum of biosurfactant by *Bacillus circulans*



Bacillus sp. are noted to be biosurfactant-producing, spore-former with ability to survive harsh condition in the environment (Nitschke and Pastore, 2004). This study is in consonance with the findings of Shafi and Khanna (1995) who reported that *Bacillus* sp. possesses the potential of producing biosurfactants when grown in carbohydrate and nitrogen-base mineral salt medium as sole carbon source.

Bacillus circulans when subjected to biosurfactant production showed zone of γ (gamma)-haemolysis. Satpute *et al*. (2008) put forward that organisms should be screened for biosurfactant activities using drop collapse, hemolytic test and emulsification index test. In table 1. Test isolate revealed positive effect for oil spreading test with diameter of 10.5mm and positive for drop-collapse. Drop-collapse test showed positive indication. The hydrophobic surface of oil reduction indicates the incidence of biosurfactant in cell supernatant and the significance of these tests is to conduct biosurfactant-production capacity of microbial isolate. Test isolate showed ability to displace oil on water surface. This suggest biosurfactant production that destabilizes liquid droplets with drop of cell-free supernatant on solid microplate. The drop-collapse ability is on biosurfactant concentration correlating surface tension (Morikawa *et al*., 2000).

Emulsification assay is an indirect technique employed in biosurfactant screening. Bonilla

et al. (2005) revealed that emulsification activities (E_{24}) is used to ascertain the production of biosurfactant as expressed in percentage (%) height of suspended level divided by actual height of liquid portion. This research revealed that *Bacillus circulans* expressed 43.4% emulsification potential.

Hydrocarbon Degradation

Evaluation of the hydrophobic surface according to Omotayo *et al*., 2012. The result stated that *Bacillus circulans* showed adherence and positive cell hydrophobicity conveyed indicator of biosurfactant production (Table 1.).

Growth Performance

Analysis of pH during biosurfactant exudation

Growth during biosurfactant production trailed similar arrangements on crude oil as reported in different studies (Abdel-Mawgoud *et al*. 2009). pH is an important variable to be considered during crude oil degradation triggered by biosurfactant as it has important effect on biodegradation rates. This study shows that biosurfactant production occurred when pH was 8.05-8.20. This in consonance with the research done by Trupti *et al*. (2007) and observed that extremes pH has effect on microbe's ability to breakdown hydrocarbons with reduction in breakdown proportions at pH 6 and 9 based on their observation. Conversely, pH range of 7.6-8.1 and thus, with no appearance of significant effect on rate of biodegradation.

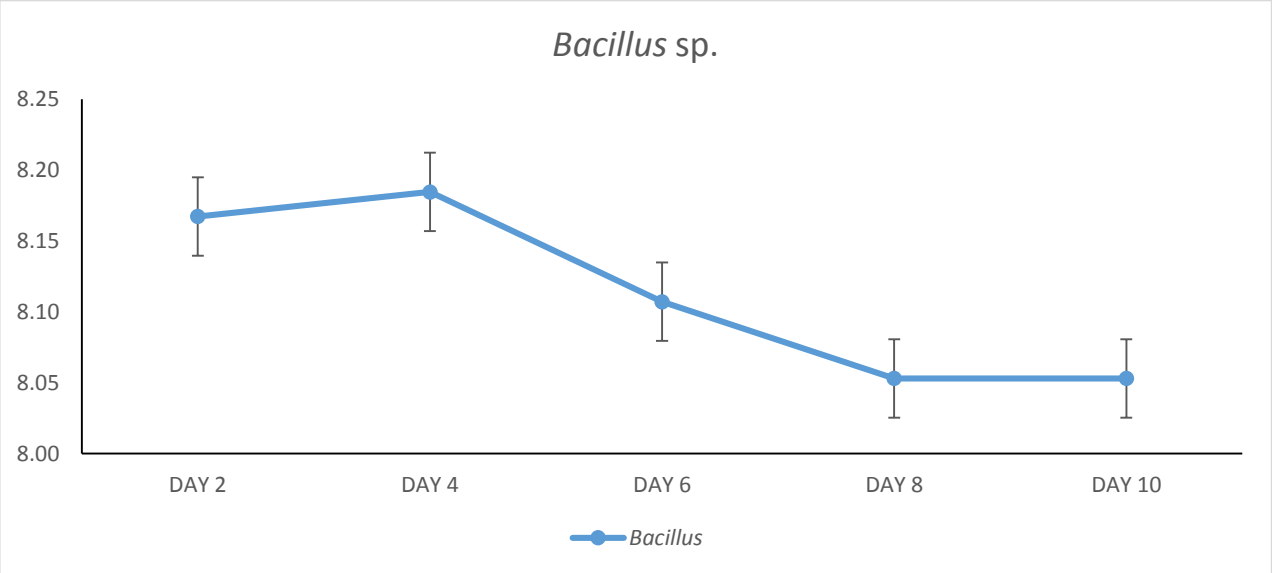


Figure 2: Analysis of pH during biosurfactant exudation by *Bacillus circulans*.

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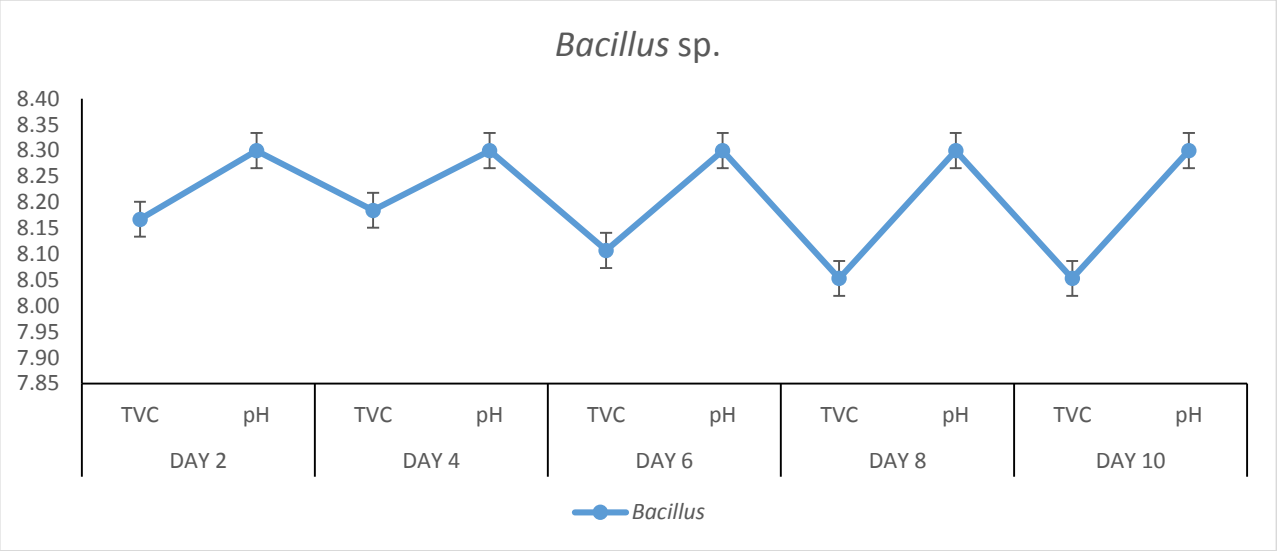

Figure 3: Growth performance of *Bacillus* sp. during biosurfacant production.

Surface tension determination

Results obtained from surface tension was 42.4 N m^{-1} in the study using *Bacillus circulans.* The efficacy of surfactant was ascertained by capacity to decrease the interfacial and surface tension. This was in consonance with the surface tension reduction related with an amphiphilic molecule in biosurfactant production from marine *Streptomyces* species (Khopade *et al*., 2012). In our study, data on surface tension action and emulsification index measurements (Table 1.) indicated a correlation amid emulsification index and surface tension activity at 43.4% and 42.4 N m^{-1} respectively.

Identification of isolate

Bacillus circulans remains a novel biosurfactant producer not stated in antiquity according to literature. extracted from the refined impurities of palm oil and was shown to have the potential to grow in crude oil as a basis of living carbon. It was identified as in consonance with Bergey's Manual of Systematic Bacteriology (Sneath *et al*., 1986) and is molecularly identified as *Bacillus circulans* strain ATCC 4513.

Biosurfactants have been the biochemical of intensive research over decades ago, in small quantities compared to microbes and the investigation yield engrossed on production. *Bacillus circulan* has been tested for the secretion of high-performance particles in both biosurfactant and bioemulsifiers. Biosurfactants produced by palm oil mill effluent bacterial isolates can play vital roles in variety of environmental and industries activities. This is a promising biotechnological approach that highlights bacterial isolates significance obtained in palm oil waste materials (mill effluent) for environmental clean-up.

CONCLUSION

The present research engrossed on studying biosurfactant production by means of palm oil mill effluent bacteria. Selectively, *Bacillus circulans* expressed potency as biosurfactant producer and can be applied in oil spill remediation.

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