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Kinetics of biosurfactant production in bacterial isolates from palm oil mill effluent

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Abstract: This research investigated the biosurfactant production kinetics observed by bacterial isolates pooled from palm oil mill effluent (POME) focusing on crude oil degradation potential Aimed towards the characterization of growth patterns and rate of biosurfactant synthesis. The scientific discoveries contribute towards understanding the prospective utilization of POME bacterial isolates in biologically remediating crude oil-polluted sites. The sampling and isolation method applied in isolating bacterial strains from POME and crude oil samples, following several experiments towards assessing their potential of biosurfactant production and capabilities to degrade hydrocarbons. The experiments involved total heterotrophic bacterial count (THBC) enumeration, biosurfactant production screening by means of qualitative assays, like the oil-spreading assay, drop-collapse test, and haemolytic activity. Surface tension measurements were applied for biosurfactant production quantification. Screening for hydrocarbon degradation and emulsification tests conducted to evaluate the isolates' capability to degrade crude oil. The results presented that more than a few bacterial isolates isolated from the pool significantly exhibited biosurfactant production capabilities and hydrocarbon degradation potential. *Salinococcus* sp., *Micrococcus* sp., *Pseudomonas* sp., and others exhibited different grades of biosurfactant production, as shown by their capabilities to effect surface tension reduction and form dissipating zones in oil-spreading tests. The growth kinetics of these isolates were observed, with production of biosurfactant close correlation with growth culture. The discoveries highlight the perspective of POME bacterial isolates for application in bioremediation and offer awareness into their growth rates and production of biosurfactant kinetics, presenting an eco-friendly solution that will mitigate the impact of environmental oil pollution.

1. Introduction

Crude oil spillage and soil pollution pose major environmental hazards, requiring effective approaches for their remediation. Biosurfactants are amphiphilic compounds exuded by microorganisms and have appeared as promising means for hydrocarbon pollutants cleanup due to their distinctive properties. Biosurfactants are not only known for surface tension reduction, but also an enhancer for solubility and dispersion of hydrophobic compounds like crude oil, expediting degradation process by microbial communities (Desai and Banat, 1997).

Oils and fats are essential organic components of municipal and industrial wastewater, that cause severe pollution of surface waters (Cisterna-Osorio & Arancibia-Avila, 2019; Bouknana *et al*., 2014). Researches proposed several processes to increase their elimination and/or biodegradation to save human and animal health (Cheah *et al*., 2023; Tabaght *et al*., 2023; Koh and Khor, 2022; Rais *et al*., 2008).

The exploration for novel biosurfactant-producing microorganisms has increased substantial attention in contemporary years. Palm oil mill effluent (POME), a profuse waste stream spawned during extraction of palm oil, offers a rich basis of varied microorganisms that have the prospect for biosurfactants exudation capable of crude oil degradation. The production of biosurfactants by microorganisms have emerged as promising agents for hydrocarbon pollution remediation, owing to their biodegradable nature and lower toxicity compared to surfactants of synthetic origin (Desai and Banat, 1997). These microorganisms have adjusted to the POME harsh conditions that contain extraordinary hydrophobic compounds concentrations and numerous organic pollutants (Mohammad *et al*., 2021).

Palm oil mill effluent (POME) particularly provides a rich microbial ecosystem as a novel biosurfactant producer source that can break down crude oil (Mohammad *et al*., 2021). In recent researches, reports on 82 hydrocarbon-utilizing bacterial strains isolation from POME sludge, with isolates such as *Gordonia amarae* and *Dietzia maris* displaying outstanding petroleum hydrocarbon degradation capacity and biosurfactant production potential (Dominic *et al*., 2022). Furthermore, a *Pseudomonas aeruginosa* strain isolated from POME was discovered to exude rhamnolipid biosurfactants that expressed up to 80 % diesel oil emulsification, with production kinetics in consonance with growth profile (Wu *et al*., 2007).

Nevertheless, deeper understandings into the growth patterns, biosurfactant synthesis kinetics and substrate utilization rates of POME isolates are important to harness their measurements for biosurfactant large-scale production and remediation of oil spill (Sari *et al*., 2022; Randhawa and Rahman, 2014).

The production kinetics of biosurfactants play an essential role in the optimization process for large-scale use. Understanding the growth pattern, substrate utilization, and biosurfactant synthesis duties of bacterial isolates from POME is vital for biosurfactant production efficiency. The production kinetics of biosurfactants can be inclined by numerous factors such as availability of nutrients, pH, temperature, and initial hydrocarbon concentration (Randhawa and Rahman, 2014).

Furthermore, the biosurfactant production kinetics in POME bacterial isolates can make available valuable perceptions into their prospect for degradation of crude oil. By reviewing the growth and profiles of biosurfactant production by these isolates, it is promising to regulate their capacity to acclimatize to crude oil as a carbon source and assess their complete efficiency in the processes of hydrocarbon bioremediation (Marchant and Banat, 2012).

The aim of this study was to investigate the production kinetics of biosurfactant in bacterial isolates acquired from POME with emphasis on their capacity to breakdown crude oil and address the systematic investigation gap of biosurfactant production kinetics of POME bacterial isolates derivatives. We characterized the growth patterns, rate of substrate utilization, and biosurfactant synthesis rates of these microbial isolates when subjected to several environmental conditions. The findings from this research has contributed to our understanding of the prospective application of POME bacterial isolates in the bioremediation of crude oil-polluted locations, offering a justifiable and eco-friendly solution for oil spill cleanup.

2. Methodology

2.1. Sampling and Isolation of Bacterial Strains:

Palm oil mill effluent for bacterial isolation was obtained from the source of effluent discharge and logging area of palm oil mill effluent bunk at Nigerian Institute for Oil-Palm Research (NIFOR) in Edo State and augmented by inoculating into antiseptic mineral salt medium (MSM). Brass river crude oil sample from Eleme refinery, Port Harcourt was obtained and supplemented by injecting into sterile mineral salt medium (MSM).

2.2. Experiments

Enumeration of Total Heterotrophic Bacterial Count (THBC)

Enumeration of Total Heterotrophic Bacterial Count (THBC) was employed through pour plating method. The injection of 0.1 ml tenfold serially diluted samples onto nutrient agar fortified with anti-fungal agent (Nystatin) and mineral salt agar (MSA) to enhance growth of hydrocarbon degraders. The mineral salt media contains the following composition in grams per litre of distilled H_2O, $MgSo_4$. $7H_2O$, 0.42g, $K_2\ HPO_4$, 1.2g, $KH_2\ PO_4$, 0.83g, Kcl, 0.29g, $NaNO_2$, 0.42g, NaCl 10g, Agar –Agar, 15g, pH 7.2 and 2ml of crude oil. Nutrient inoculated agar plates were subjected to 24 hours incubation at 37^0C. Colony forming units per ml (cfu/ml) was employed (Eqn. 1) (Okpokwasili and Amanchukwu 1988):

$$\text{cfu/ml} = \frac{(\text{number of colonies X dilution factor})}{\text{volume spread on plate}} \qquad \text{Eqn. 1}$$

Individual bacterial colonies showing distinct morphology were isolated and purified by streaking on fresh agar plates.

3.1. Screening of Biosurfactant-Producing Bacterial Isolates:

Isolated bacterial strains screening approach employed for production of biosurfactant using different qualitative assays mentioned below:

Drop-collapse test

Test was done using Crude oil and Five (5) micro liters of 48 hrs culture beforehand and afterwards centrifugation at 12,000 g for 5 min was applied for cells removal, moved to the region of oil-coated well and drop size evaluated in 1min using a magnifying glass. Bacterial isolates were inoculated on mineral salt agar plates supplemented with crude oil. The formation of a clearing zone colony indicated production of biosurfactants. Results were measured affirmative for biosurfactant presence when drop flattened and cultures appeared as drops were counted negative (indicating absence of biosurfactant) (Bodour *et al.*, 2004; Youssef *et al.*, 2004).

Oil-spreading assay

The potential of bacterial isolates to breakdown crude oil in an axenic culture through subculture. Isolates underwent overnight culture on nutrient agar with sterile distilled water dislodge. Inoculum size standardization by 1.0 Macfarland preceding inoculation. Suspension of two milliliters (2ml) inoculum added to 98ml mineral salt medium in a 250ml Erlenmeyer flask and incubated in orbital shaker at 37˚C for 7 days and 150 r.p.m. The ratio for water reaction was 10:1:0.05; BLCO (Bonny Light Crude Oil); biosurfactant respectively. Introduction of 40ml water in petri dish and 4ml of crude

oil dispensed on the H_2O surface with 400microltre biosurfactant applied. The potential of culture to spread with formation of thin film on oil surface indicated exudation of biosurfactant and measurement of diameter after 60 seconds (Ali, 2013).

Haemolytic activity

The isolation of biosurfactant producing strain is characterized by its capacity to breakdown red blood cells in blood agar plate medium for hemoglobin release. These isolates were screened on blood agar medium and incubated at 37 °C for 48 h. The presence of clear zone growth colonies detects hemolytic activity (Plaza *et al*., 2006).

Surface Tension Measurement

Surface tension of cell free culture broth ascertained through capillary upsurge technique. Cell free culture broths were 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 is mathematically given as:

$$\text{cfu/ml} = \frac{(\text{number of colonies X dilution factor})}{\text{volume spread on plate}} \qquad \text{Eqn. 2}$$

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) into mineral salt agar medium was used to detect anionic biosurfactant. Corked borer (4 mm) methylene blue agar plate well prepared was loaded each with thirty microlitre of cell free supernatant. Plates incubated at 37°C for 48-72 h. Dark blue halo zone indicator culture signified positive for anionic biosurfactant. Positive isolates obtained from screening tests were inoculated into mineral salt medium for production of biosurfactant. Best strains with quality/quantity of biosurfactant production were selected and identified as microscopic form and biochemical tests aligned with Bergey's manual of determinative bacteriology (Satpute *et al*., 2008).

3.2. ***Screening for hydrocarbon degradation***

A modified method of screening described below was used. 1ml of sterilized crude oil sample mixed with 1 g of each effluent sample was incorporated into 50 mL of mineral salt medium containing (g/L); 15 g $NaNO_3$, 1.1 g KCl, 1.1 g NaCl, 0.00028g $FeSO_4.7H_2O$, 3.4 g KH_2PO_4, 4.4 g K_2HPO_4, 0.5 g $MgSO_4.7H_2O$, 0.5g yeast extract at 37°C in shaker incubator (100rpm). The samples were diluted serially with sterile saline (0.85 % NaCl) after 48h of incubation and bacterial isolates carefully chosen based on colony appearances on nutrient agar plates. Triplicate flasks were applied while un-inoculated flasks served as control (Tahzibi *et al*., 2004).

3.3. ***Emulsification test (E_{24})***

Culture colonies uncontaminated with 2 mL of mineral salt medium were suspended after 48h incubation. and 2 mL hydrocarbon (oil) introduced to the tubes. Blend vortexed at high speed for 1min and allowed to stand for 24h. Emulsion index (E_{24}) indicates the emulsion layer height (cm) divided by total height (cm), multiplied by 100 (Cooper and Goldenberg, 1987):

$$Emulsification\ Index\ (E24) = \frac{Height\ of\ the\ Emulsion\ Layer}{Mixture\ Total\ Height} x100 \qquad \text{Eqn. 3}$$

3.4. Growth Kinetics

Cell culture inoculum was plated out during degradation process evaluation and measurement of optical density (O.D.) at 600nm. Successive optical densities frequently obtained during the degradation period and of growth curve obtained by plotting optical densities against time (Parthipan *et al*., 2017).

4. Results and Discussion

Surfactants of biological synthesis have been discovered to have certain advantages over ones that are synthetic, which consist of reduced toxicity, naturally biodegradable and displaying positive blood haemolysis test to confirm production of biosurfactant. Isolates from tested POME demonstrated a wide range of biosurfactant production potential as well as growth profiles when cultured on crude oil over a period of 20 days. Most prolific producers of biosurfactants were *Micrococcus* sp. (35.2 mm oil displacement; 40.85 % emulsification index), *Salinococcus* sp. (28.6 mm; 34.6 %) and *Streptomyces* sp. (24.6 mm; 63.5 %), demonstrating efficient and potent biosurfactants synthesis. In difference, isolates such as *Flavobacterium* sp. (5 mm; 36 %) and *Klebsiella* sp. (7.6 mm; 5.4 %) expressed as comparatively poor exuders of biosurfactants. According to the trends noted by Gnanamani *et al*. (2010), biosurfactant secretion and maximum growth was accomplished around day 8-10 in the exponential phase by top producers.

4.1. Quantitative Analysis of Biosurfactant Production:

Bacterial isolates showing positive results in the qualitative assays were selected for biosurfactant production quantitative analysis. Biosurfactant production was determined by assessing the surface tension reduction of the culture medium using a tensiometer. Bacterial isolates were cultured in liquid medium supplemented with crude oil, and samples collected at regular intervals for surface tension measurements.

4.2. Bacterial Growth Rate (Kinetics of Proliferation)

Result of biosurfactant production screening showed that bacteria isolates expressed their degradative potentials and biosurfactant production capacities through blood haemolysis, drop collapse, emulsification index, CTAB shown below in Table 1. The haemolytic activity was experiential on blood agar and result indicated that out of all supernatants obtained after fermentation, *Micrococcus* sp. and *Streptomyces* sp. indicated β (beta) haemolysis, *Salinococcus* sp., *Achromobacter* sp., *Klebsiella* sp., *Staphylococcus* sp., *Flavobacterium* sp. indicated α (alpha) haemolysis and *Pseudomonas* sp., *Paenibacillus* sp., *Bacillus* sp., showed γ (gamma) haemolysis. The result showed that *Flavobacterium* sp. had the least with 5.00 mm and *Micrococcus* sp. expressed highest capacity of 35.2 mm that could reduce surface tension during oil spread and created a zone of cm diameter in an oil-spread test. The result as expressed in percentage as emulsified height layer alienated by total height of liquid column and emulsifying index recorded lowest for *Achronobacter* sp. and *Klebsiella* sp. of 5.50 % and 5.40 %; the highest emulsification index was expressed by *Streptomyces* sp. with 63 %. Specifically, *Pseudomonas aeruginosa* AKS1 and *Bacillus* sp. AKS2 have been previously reported to possess hydrocarbon degrading potentials and demonstrated high hydrophobicity percentages which regulates the initial microbial adhesion to the interface between aqueous phase and NAPL (non-aqueous- phase liquid) (Chettri *et al*., 2016).

Production of biosurfactants by bacterial strains lessen surface tension significantly by increasing emulsification through expediting bioavailability and hydrocarbon degradation in polluted environments (Banat *et al*., 2000; Rahman *et al*., 2003). Isolation of biosurfactant producers with blood agar plate assay has been reported previously (Mulligan *et al*., 1989). Conversely, the hemolytic zone formation extent on blood agar plates is not dictated solely by the rhamnolipid concentration and may have affect by divalent ions and other hemolysins exuded by the microbe subjected to the investigation. Ibrahim *et al*. (2013) reported similar expression of β-haemolysis by the bacteria *M. kristinae, B. lincheniformis*, and *S. paucimobilis.* Range of the clear zone displayed on the blood agar plate is proportional to biosurfactant exudation (Chandran *et al*., 2012). Conversely, additional biosurfactant secretion confirmatory tests are required in the potent biosurfactant producer's selection (Thavasi *et al*., 2011).

Table 1: Biosurfactant production potential of bacterial isolates from Palm Oil Mill Effluent.

Sample	HC-Deg. Potential	Blood Haemolysis	Drop Collapse	Emulsification Index (%)	Oil Spread (mm)	CTAB Blue Agar Plate Method	Surface Tension (N m-1)
Micrococcus sp.	+++	β (25.3)	++	40.85	35.3	+++	28.0
Salinococcus sp.	+++	α (5.8)	++	34.6	28.6	++	25.0
Pseudomonas sp.	++	γ	++	38.0	26.5	+	43.0
Achronobacter sp.	+	α	+	5.50	6.10	+	57.0
Klebsiella sp.	+	α	+	5.40	7.60	+	57.0
Staphylococcus sp.	++	α	+++	18.4	12.9	+	52.0
Paenibacillus sp.	+++	γ (10.5)	++	25.7	18.0	+	35.0
Bacillus sp.	+++	γ (4.8)		43.4	10.5	++	42.4
Flavobacterium sp.	+	α	+	36.0	5.00	+	46.5
Streptomyces sp.	+++	β	++	63.5	24.6	++	45.0

The presentations of result in Table 1 and Figure 1 deliver understandings into the varying production of biosurfactant abilities and growth kinetics of the bacterial isolates from palm oil mill effluent (POME) when cultured on crude oil over a period of 20 days.

On the Bases of drop collapse, blood hemolysis, emulsification index, oil spreading, and assays of surface tension reduction, *Micrococcus* sp. occurred as the utmost promising producer of biosurfactant. It showed robust β-hemolytic activity (25.3 mm zone), outstanding oil displacement (35.2 mm zone), emulsification index of 40.85 %, and (28 mN/m) maximum surface tension reduction. This superior performance aligns perfectly with previous research findings noting biosurfactants with high yields by *Micrococcus* strains (Gudina *et al*., 2015).

Other competent surfactant secreting isolates included *Salinococcus* sp., *Streptomyces* sp., and *Pseudomonas* sp. as shown by their emulsification/oil spreading potentials and exceeding surface tension reductions of 25 mN/m. In contrast, *Flavobacterium* sp., *Klebsiella* sp., and *Achronobacter* sp. expressed biosurfactant production that was relatively poor based on insignificant to low hemolytic potential, emulsification index, oil displacement, and decrease in surface tension. The curves

indicating growth profile also mirrored the trends of biosurfactant production. Rapid proliferators such as *Micrococcus* sp. expressed cell density exponential increase in over 8-10 days, corresponding directly with peaks of biosurfactant synthesis. In contrast, weaker degraders like *Flavobacterium* sp. indicated limited growth even after 20 days.

Summarily, the characterization of isolate data indicates *Micrococcus* sp., *Salinococcus* sp., *Streptomyces* sp., and *Pseudomonas* sp. to be the most effective secretors of biosurfactant with highest degradation of crude oil capacity among the tested bacteria derived from POME. Follow-up research should explore their applications on pollution remediation.

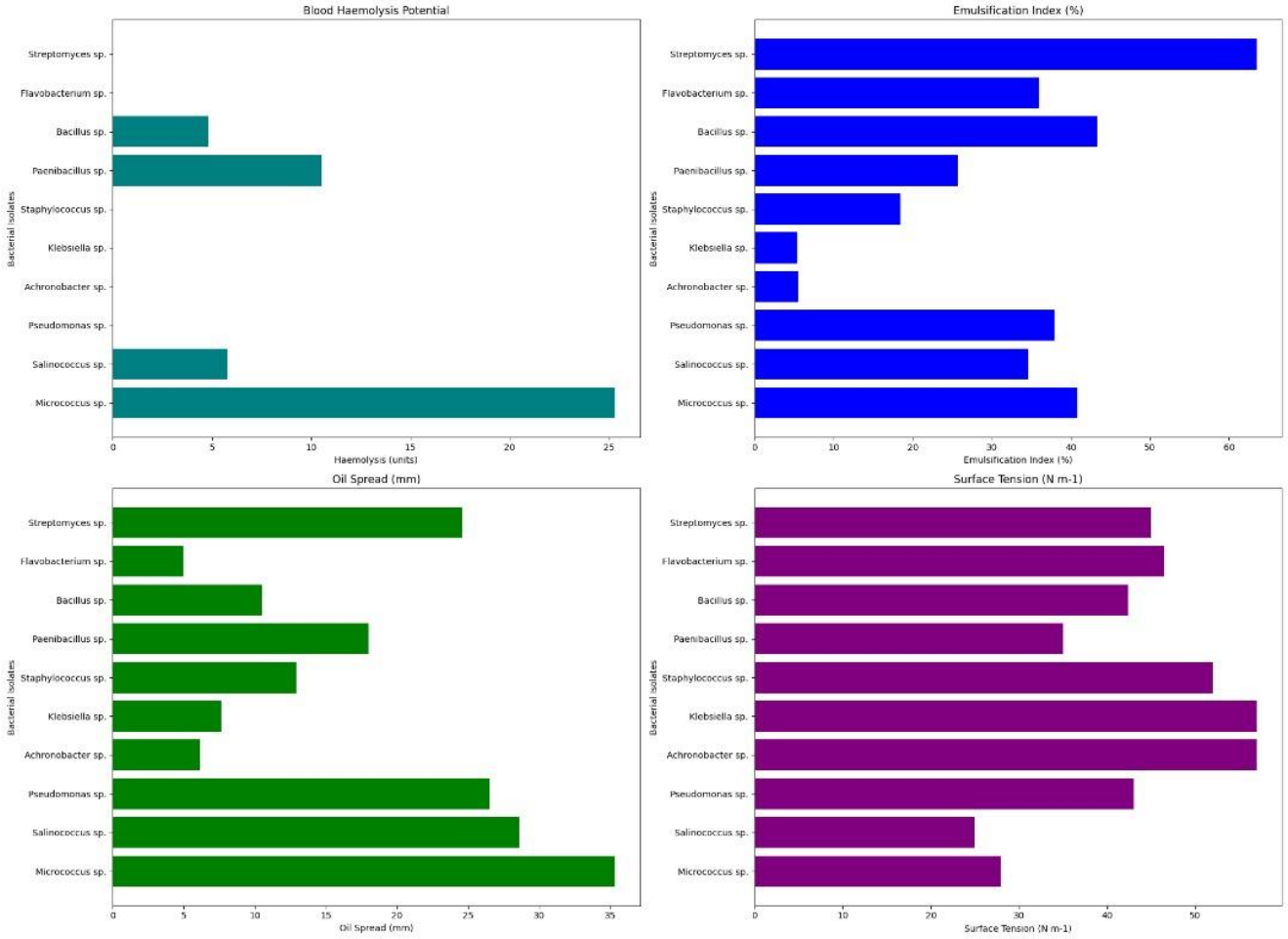

Figure 1.: Bacterial Growth Rate (Kinetics of Proliferation)

Similar research findings according to Gnanamani *et al.* (2010) who reported that reduction in surface tension of samples was the measure of accessing the production of biosurfactants and directly dependent on cell growth. Production of biosurfactants commenced in the primary lag phase and exponentially increased in 10 days after inoculation and bacterial growth decline phase (Fig 1).

During the experimental time-course, bacterial growth rate was monitored through total viable count (TVC). The results are shown in Fig. 1. The population density of *Micrococcus* sp. indicated a significant period of lag phase to death phase within 20 days of incubation. The population density steadily increased from $1.32x10^{8}$ cfu/mL^{-1}to $2.01x10^{8}$ cfu/mL^{-1} of medium. The population density of *Micrococcus* sp. increased steadily in 4 orders of magnitude from $1.32x10^{8}$ cfu/mL $^{-1}$(lag phase), $1.86x10^{8}$ cfu/mL^{-1}(log phase), $2.52x10^{8}$ cfu/mL^{-1}(stationary phase) and $2.35x10^{8}$ cfu/mL^{-1}(death phase).

The result of the evaluation indicated that four of the ten bacteria isolates, *Salinococcus* sp., *Micrococcus* sp., *Bacillus* sp. and *Clostridium* sp. expressed high turbidity, crude oil degradation. These isolates were subjected to analytical evaluation which proved them the best biosurfactant producers and hydrocarbon utilizers.

Biosurfactant synthesis and decrease rate in surface tension correlates with culture growth in the fermentation medium as similarly reported by Bellebcir *et al*., 2023. Following the increased culture growth, surface tension continuously dropped as observed after 10 days (Fig. 1). As observed in the 4th day of incubation, growth and biosurfactant concentration increased for (*Micrococcus* sp., *Pseudomonas* sp., *Klebsiella* sp., *Achronobacter* sp. and *Staphylococcus* sp.) attaining its peak on 8th day. On the 9th day, decline in surface tension was not observed, indicating end of biosurfactant synthesis, perhaps as a result of secondary metabolites release. The production of secondary metabolites in medium might be a resultant effect of biosurfactant synthesizing enzymes interference. The results also indicated production of biosurfactant from palm oil mill effluent bacterial isolates mostly occurs in exponential growth phase proposing that biosurfactants are synthesized with primary metabolite and following cellular biomass increase. This discovery suggests that biosurfactant could be synthesized successfully by immobilized cells subject to chemostat condition.

5. Conclusion

In the present research studies, biosurfactants isolated from palm oil mill effluent (POME) indigenous bacterial isolate expressed growth and biosurfactant production kinetics. This indicated its industry-oriented importance in detergent industries due to its oil displacement potential as well as bioremediation of oil contaminated environments for total recovery. In conclusion, *Micrococcus* sp., *Salinococcus* sp. and *Streptomyces* sp. isolated from POME established exceptional biosurfactant-mediated potential for bioremediation of oil spill, deserving additional pilot-level examination. With optimization process, incessant biosurfactants production possibly will offer eco-friendly agents to mitigate crude oil pollution effectively, both in the lithosphere and hydrosphere. Furthermore, the enzymatic pathways for biosurfactant production in these strains should be explored over omics techniques to understand better and modulate the kinetics of production.

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