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Effects of lateral agitation on the yield of biosurfactants produced by environmental strains of *Pseudomonas aeruginosa*

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Abstract
The use of renewable carbon and energy sources and the elimination of specific culture conditions such as agitation can help lower the overall cost of biosurfactant production. The impact of two carbon sources (glycerin and olive oil) and lateral agitation (with and without) on biosurfactant yield in *Pseudomonas* strains isolated from soil and water was examined. Oil-drop technique and drop-collapse test were used to screen the candidates. The biosurfactant production was monitored by calculating the isolates' Emulsification Index (E_{24}) (at $p<0.05$ at 95% confidence level) on various hydrophobic substrates. Eighty per cent (80%) of the data were significantly different, with 37.92% showing a comparatively high E_{24} value for biosurfactants produced under lateral agitation and 62.08% showing a comparatively high E_{24} value for cell culture cultivated without agitation. The structural identification of rhamnolipid biosurfactants from *Pseudomonas* sp. revealed 3-5 types of congeners with variable concentrations. Without agitating the growth media, a large quantity of biosurfactants can be produced.
Keywords: Biosurfactants, Rhamnolipids, Emulsification index, agitation, Hydrophobic.

Introduction

Surfactants are amphipathic compounds with hydrophilic and hydrophobic moieties (Santos *et al.,* 2016). When present in a system at low concentrations, these compounds possess a unique ability to adsorb onto the surfaces or interfaces of such systems and significantly modify the surface or interfacial free energy of those surfaces (Rosen and Kunjappu, 2012). The surfactants' action brings about a decrease in tension in these systems (Vijayakumar and Saravanan, 2015). A large number of surfactants are made from petroleum via chemical techniques, and as a result, they are not biodegradable and are often harmful (Santos *et al.,* 2016). This harmful effect has prompted a quest for alternatives to chemically derived surfactants, with biosurfactants (surfactants derived from microorganisms) emerging as a promising option because they represent little or no environmental risk (Vijayakumar and Saravanan, 2015; Santos *et al.,* 2016).

Biosurfactants are produced extracellularly or as part of the cell membranes of microorganisms (Suresh Chander *et al.,* 2012; Vijayakumar and Saravanan, 2015). The unique properties of biosurfactants make them attractive choices as surfactants. Biodegradability, diversity, environmental friendliness, minimal toxicity, lower critical micelle concentration (CMC), fantastic foaming properties, high selectivity and activity at extreme temperature, pH, and salinity, as well as the possibility of being produced from industrial wastes and by-products (Pacwa-Pociniczak *et al.*, 2011; Fenibo *et al.*, 2019) are just a few of them. Biosurfactants have found use in a wide range of industrial processes, including foaming, emulsification, detergency, wetting, and solubilisation due to their unique qualities (Suresh Chander *et al.*, 2012; Santos *et al.*, 2016). The

production of biosurfactants and their overall yield is affected by many factors such as carbon source, nitrogen source, culture conditions (pH, temperature, agitation), the concentration of elements in medium, dilution rate as well as the microorganisms (Kaskatepe and Yildiz, 2016; Ikhwani *et al.*, 2017).

Biosurfactants are classified primarily based on their chemical structure and microbial source (De *et al.*, 2015). They are amphiphilic in structure, with a hydrophilic moiety (acid, alcohol, peptide cations, or anions, mono-, di-, or polysaccharides) and a hydrophobic moiety (unsaturated or saturated hydrocarbon chains, or fatty acids) (De *et al.*, 2015). Microbial-derived biosurfactants can be categories into two based on molecular mass (Rosenberg and Ron, 1999). The low molecular mass bioemulsifiers include glycolipids, phospholipids, lipopeptides and fatty acids, and high-molecular mass bioemulsifiers include a polymeric and particulate surfactant (Rosenberg and Ron, 1999; De *et al.*, 2015; Vandana and Singh, 2018; Fenibo *et al.,* 2019). Glycolipids are carbohydrates that are covalently linked to lipids. The commonest ones identified are sophorolipids, trehalolipids and rhamnolipids (Rosenberg and Ron, 1999; De *et al.*, 2015). Rhamnolipids are widely studied biosurfactants produced principally by *Pseudomonas aeruginosa* and other species (Kaskatepe and Yildiz, 2016). They possess one or two rhamnose linked to one or two molecules of β-hydroxydecanoic acid (Vandana and Singh, 2018). As a result of their high surface and emulsifying activity, solubilisation, low toxicity level, and biodegradability, rhamnolipids have found numerous applications in different fields such as environmental bioremediation of heavy metals and hydrocarbon pollutants and microbial enhanced oil recovery (Gudiña *et al.,* 2015; Chong and Li, 2017).

Most researches on the production of rhamnolipids by *P. aeruginosa* are centred on aerobic conditions under agitation by orbital shaking. This study investigated the impact of lateral agitation and zero agitation on the production of biosurfactants using two carbon sources as substrates. This was determined by the emulsifying effectiveness of the produced biomolecule on hydrophobic substrates of plant and hydrocarbon origin.

Materials and Methods

Sample Source

Soil and water samples were obtained from oil-contaminated sites, mechanic workshops and Lagoon sediments. The Global Positioning System (GPS) coordinates of the sites are shown in Table 1.

Table 1: Different Sites Samples and their Coordinates

SAMPLE	SAMPLE CODE	GPS COORDINATE
Soil sample from mechanic workshop, Ogba, Lagos.	GI	06.629472ºN, 03.355815ºE
Soil sample from oil-contaminated soil, CMS Garage, Lagos.	CM	06.450105 ºN, 03.390049ºE
Soil sample obtained from Biological garden, University of Lagos, Akoka-Yaba Lagos.	FaM	06.518627 ºN, 03.400556º E
Sample obtained from the water body, CMS, Lagos.	DWS, DSK	06.449380 ºN, 03.389245ºE

Isolation of *Pseudomonas* Species

The isolation of *Pseudomonas* species was done using the procedure described by Aryal (2016). Soil and water samples were plated out using the spread plate technique on Cetrimide Agar at ambient temperature. Pure cultures of bacteria colonies that developed on plates were obtained by sub-culturing on Cetrimide Agar. The bacterial isolates were stored in agar slants at 4°C for further use.

Identification of Bacterial Isolates

The bacterial isolates were identified using a commercially produced 24E Microbact Identification kit. The results were interpreted using the Microbact™ Identification Package.

Inoculum Preparation

The inoculum was prepared by inoculating a loop-full of pure *Pseudomonas* strain in sterilised Mineral Salts Medium (MSM) with olive oil (2%) and incubated overnight at ambient temperature. This was laterally agitated with a flask shaker at 100 oscillations per minute for 5 days. A volume of 2% of the cell culture was subsequently reintroduced into MSM containing 2% olive oil and incubated for 48 h. This was repeated thrice, and the final cell culture from the last batch served as inoculum for further use.

Media and Cultivation Conditions

The composition of the Mineral Salts Medium (MSM) for biosurfactant synthesis include (g/L): Na_2HPO_4 (2.2 g), KH_2PO4 (1.4 g), $MgSO_4.7H_2O$ (0.6 g), Fe $SO_4.7H2O$ (0.01 g), NaCl (0.05 g), $CaCl_2$ (0.02 g), yeast extract (0.02 g) and 0.1 mL of trace element solution containing (g/L): $ZnSO_4{\cdot}7H_2O$ (2.32 g), $MnSO_4{\cdot}4H_2O$ (1.78 g), H_3BO_3 (0.56 g), $CuSO_4{\cdot}5H_2O$ (1.0 g), $Na_2MoO_4{\cdot}2H_2O$ (0.39 g), $CoCl_2{\cdot}6H_2O$ (0.42 g), EDTA (1.0 g), $NiCl_2{\cdot}6H_2O$ (0.004 g) and KI (0.66 g). The pH of the medium was adjusted to 7.0 ± 0.2 (Abouseoud *et al.*, 2008). Carbon and nitrogen sources were added separately. Cultivations were performed in 250 mL flasks containing 100 mL medium at ambient temperature and agitated in a flask shaker at 100 oscillations per minute for four days. This was equally repeated without agitation. The medium optimisation was conducted with a fixed nitrogen source, changing the carbon source while maintaining other conditions. The carbon sources used were olive oil (2% w/v) and glycerin (20 g/L), with $NaNO_3$ as a nitrogen source.

Biosurfactant Production

Biosurfactants activity of the *Pseudomonas* strains was determined by oil spreading technique (Morikawa *et al.*, 1993), drop-collapse Test (Jain *et al.*, 1991) and emulsification index activity (E_{24}) (Nayarisseri *et al.*, 2018). The emulsification index was determined at different times of growth (60, 72 and 96 hours). The cell-free supernatant was used as the crude surfactant.

Preliminary Study

The growth phase of the cell cultures was determined to estimate the duration for culturing, the time at which biosurfactant production commenced and the period of highest yield. Two isolates were subjected to a 3-stage olive oil enrichment before testing for biosurfactant production under two different conditions of lateral agitation and zero agitation and measuring the emulsification index (E_{24}) against kerosene. This was estimated at 18, 24, 48, 60, 72 and 96 hours of growth.

Determination of Biosurfactant Production on Different Hydrophobic Substrates

The production of biosurfactant was followed in batch cultures during a 96 hours incubation at optimum conditions (de Sousa *et al.,* 2011). This was determined by calculating the emulsification index (E_{24}) using biosurfactants from olive oil and glycerin with and without (zero) agitation at different times of growth on nine hydrophobic test substrates; neem seed oil, olive oil, coconut oil, palm oil, palm kernel oil, castor oil, diesel, fresh and spent engine oil.

Biosurfactant Recovery, Partial Purification and Structural Characterisation

The culture broth was centrifuged at 2500 rpm for 40 minutes to remove the cells. The clear supernatant served as the source of the crude biosurfactant. The pH of the supernatant was adjusted to 2.0 by treating with 6N hydrochloric acid. The treated supernatant was again centrifuged at 2500 rpm for 40 minutes. The biosurfactant was recovered from the free culture supernatant by cold acetone precipitation as described by Pruthi and Cameotra (2003). Three volumes of chilled acetone were added and allowed to stand for 10 hours at $4^{o}C$. The precipitate was collected by another centrifugation and evaporated to dryness to remove residual acetone and re-dissolved in sterile distilled water.

High-Performance Liquid Chromatography (HPLC) was used to analyse the biosurfactants recovered by cold acetone precipitation. Twenty millilitres of the precipitated biosurfactant re-

dissolved in distilled water was measured into a separating funnel to which 20 mL of acetonitrile was added, and the mixture shaken for 30 minutes while releasing the funnel cap intermittently to allow the escape of built-up pressure. Two layers were observed in the mixture: the aqueous layer, which was run off and the hydrocarbon layer, which was collected into a 25 mL standard flask, made up to the mark and ready for HPLC analysis.

Standards for rhamnolipid were injected into the HPLC, which generated a chromatogram with a given peak area and peak profile. These were used to create a window in the HPLC to prepare the test samples analysis. An aliquot (5 µl) of the extracted test sample was injected into a UV E254 nm detector HPLC (Shimadzu Nexera mx), which had a 100 mm Ubondapak C_{18} column of 7 µm thickness with an acetonitrile/water mobile phase and a flow rate of 5 mL/min and 0.08 mL/min respectively to obtain a chromatogram of corresponding peak area and peak profile. The peak area of the sample was compared with the standard, relative to the concentration of the standard to obtain the concentration of the sample using the formulae,

$$\text{Concentration of Sample} = \frac{\text{Peak Area of Sample} \times \text{Standard Concentration}}{\text{Peak Area of the Standard}}$$

The various rhamnolipid samples obtained from the bacteria isolates were identified using mass spectrometry (MS), where standards were used to determine their retention times and response factors.

Statistical Analyses

Statistical significance was determined using the independent sample test, T-test, using SPSS for Windows Version 17.0 packet program. The level of significance was defined at $p < 0.05$ at 95% confidence level. The graphs were constructed using GraphPad Prism 5.0. The result of the data was an average of three independent measurements.

Results

Isolation, Identification and Screening of *Pseudomonas* Species

The bacterial isolates obtained from the environmental samples were identified as *Pseudomonas aeruginosa*. The isolates were positive for biosurfactant production after culturing on olive oil and glycerin as energy and carbon sources under agitation/zero agitation conditions and on screening using the Emulsification index, oil spread and drop-collapse techniques as shown in Table 2.

Preliminary studies to determine the metabolic phase of biosurfactant production

Two *Pseudomonas* strains, CM-II and FaM-I, were selected randomly to determine the time biosurfactant production commenced. The result in Figure 1 shows that biosurfactant was not produced until after forty-eight hours during zero agitation and sixty hours of lateral agitation (stationary phase of growth).

Table 2: Identification and Screening for Biosurfactant Production among Bacterial Isolates

Isolate name	Percentage probability	Oil spread technique	Drop-collapse test	Emulsification Index (E_{24})
Pseudomonas aeruginosa strain CM-II	81.98%	Positive	Positive	Positive
Pseudomonas aeruginosa strain FaM-I	99.16%	Positive	Positive	Positive
Pseudomonas aeruginosa strain GI-A	99.99%	Positive	Positive	Positive
Pseudomonas aeruginosa strain GI-B	99.95%	Positive	Positive	Positive
Pseudomonas aeruginosa strain DSK	99.48%	Positive	Positive	Positive
Pseudomonas aeruginosa strain DWS	99.97%	Positive	Positive	Positive

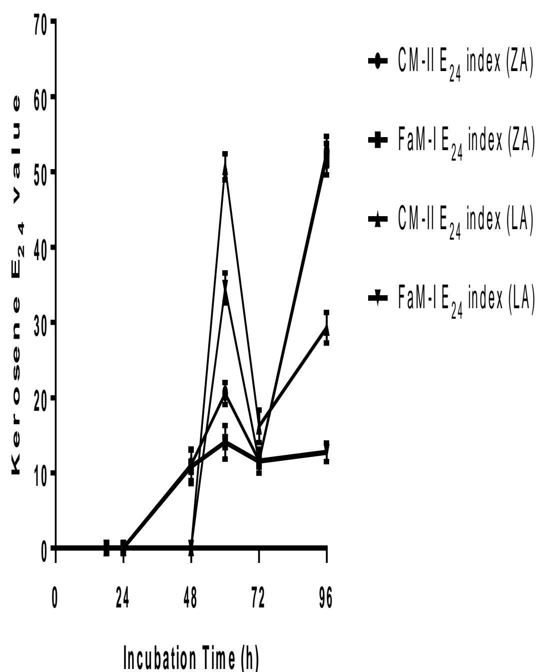

Figure 1: Determination of the onset of biosurfactant production in *Pseudomonas* strains. ZA, Zero agitation; LA, Lateral agitation.

Effect of Agitation on Biosurfactant Yield from different hydrophobic substrates

The E_{24} (%) of the biosurfactant activity of the *Pseudomonas* strains on the different hydrophobic substrates tested is presented in Figures 2 and 3. Both olive oil (Figure 2) and glycerin (Figure 3) supported the growth of the cultures as energy and carbon sources. The biosurfactants produced during lateral and zero agitation conditions in the culture media were effective against the tested substrates. However, in olive oil, as an energy and carbon source, crude biosurfactants of *Pseudomonas aeruginosa* strain GI-A, GI-B, DSK and DWS during lateral agitation did not support the emulsification of diesel and palm kernel oil (Figure 2).

Biosurfactants produced from olive oil and glycerin had E_{24} values ranging from 45.59 – 95.51% and 53.93-92.50%, respectively (Figure 2 and 3). Spent engine oil had the highest E_{24} value, followed by fresh engine oil with vales 40.48-80.64% and 42.12-78.87% for both carbon sources, respectively. The E_{24} value in laterally agitated cultures from olive oil and glycerin ranged from 0 – 90.38% and 8.36 – 92.50%, respectively, while in cultures with zero agitation, it ranged from 6.24 – 95.51% to 8.29 – 92.31, respectively.

Out of 324 comparisons of the effect of both lateral and zero agitation on biosurfactant activity on different hydrophobic substrates tested at different incubation time intervals of 60 h, 72 h and 96 h, only 300 results were statistically analysed using T-test due to non-data generated from the effect of biosurfactants from olive oil on diesel and palm kernel oil (Figure 2). From the analyses in Table 3, 21% (63 comparisons) were not significantly different (NSD), and 79% (237 comparisons) were significantly different. Among the significantly different analyses, 37.55% (89 comparisons) of the 237 comparisons were significantly different in terms of high E_{24} values obtained in favour of lateral agitation, and 62.44% (148 comparisons) were significantly different with regards to high E_{24} values obtained when cultivated without (zero) agitation. Among the E_{24} index values that were NSD, 49.21% (31) had higher E_{24} values under lateral agitation and 50.79% (32) without (zero) agitation (Table 3).

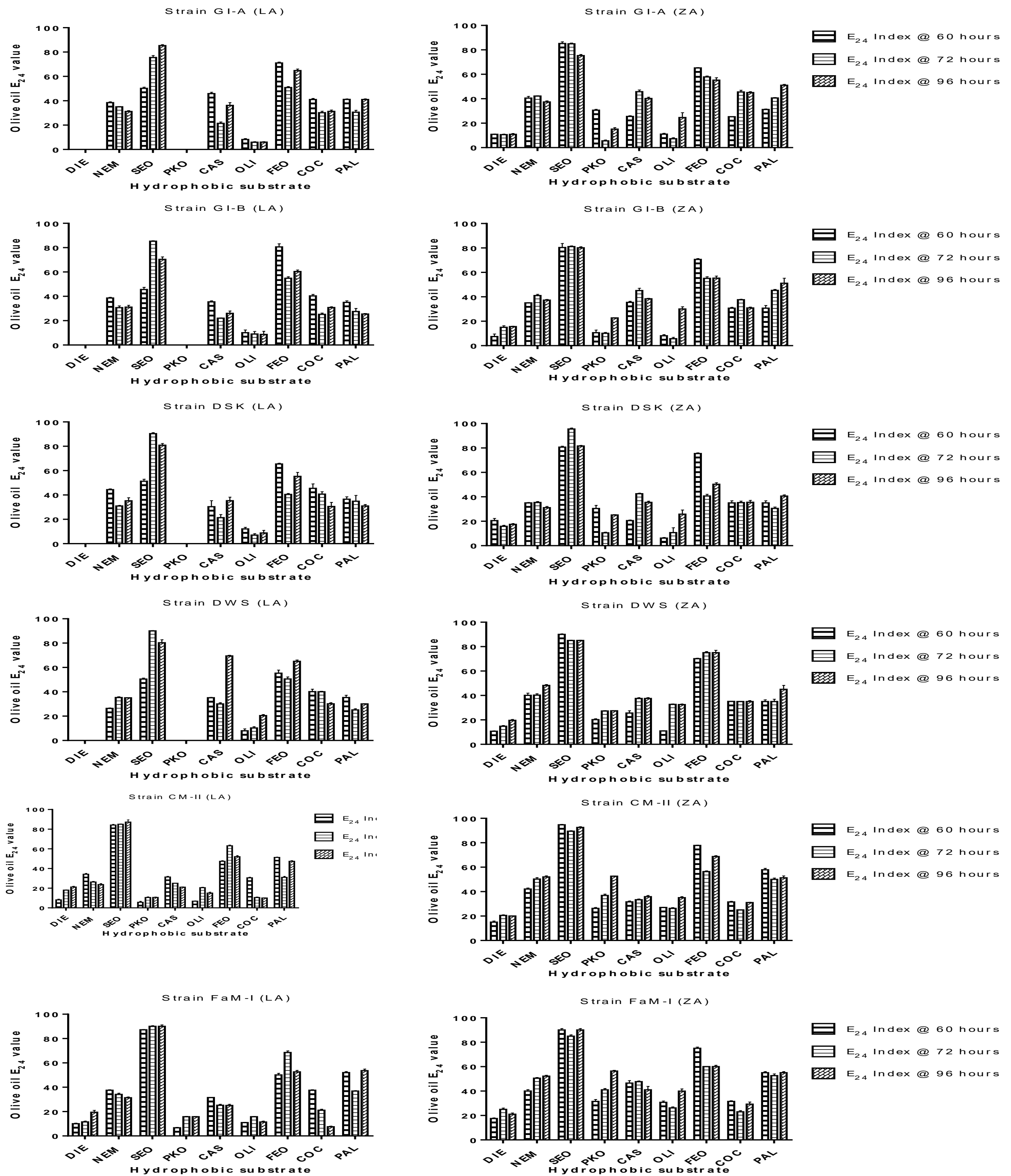

Figure 2: Emulsification Index (E_{24}) % of crude biosurfactants of *Pseudomonas aeruginosa* strains cultured in olive oil as energy and carbon source on different hydrophobic substrates. DIE, diesel; NEM, neem seed oil, SEO, spent engine oil; PKO, palm kernel oil; CAS, castor oil; OLI, olive oil; FEO, fresh engine oil; COC, coconut oil; PAL, palm oil; ZA, Zero agitation; LA, Lateral agitation.

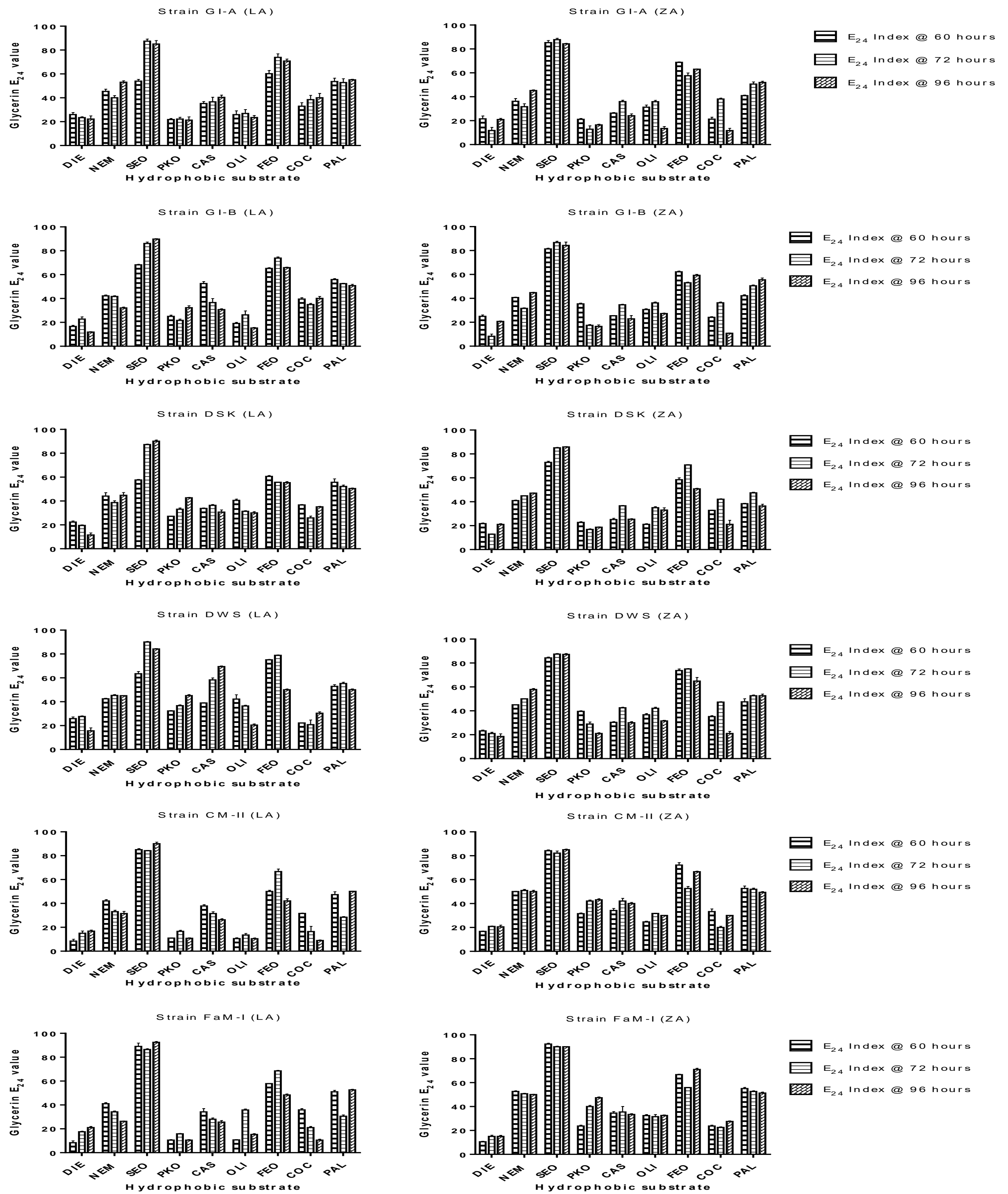

Figure 3: Emulsification Index (E_{24}) % of crude biosurfactants of *Pseudomonas aeruginosa* strains cultured in glycerin as energy and carbon source on different hydrophobic substrates. DIE, diesel; NEM, neem seed oil, SEO, spent engine oil; PKO, palm kernel oil; CAS, castor oil; OLI, olive oil; FEO, fresh engine oil; COC, coconut oil; PAL, palm oil; ZA, Zero agitation; LA, Lateral agitation.

Table 3: T-test analysis of biosurfactant activity from olive oil and glycerin on different hydrophobic substrates

Agitation (E_{24})				**T-test $p<0.05$ (2-tailed)**					
No. of negative activity with Higher E_{24} value		**No. of positive activity with Higher E_{24} value**		**E_{24} Index values**	**Olive oil *(both conditions)**	**Glycerin *(both conditions)**	**Olive oil and Glycerin combined Lateral**	**Zero**	**TOTAL**
Lateral	0	**Lateral**	120	**SD**	112	125	89 (37.55%)	148 (62.44%)	237 (79%)
zero	24	**zero**	180	**NSD**	26	37	31 (49.21%)	32 (50.79%)	63 (21%)
TOTAL	24	**TOTAL**	300	**TOTAL**	138	162	120	180	300
					300		300		

SD, Significantly Different; NSD, Not Significantly Different; *(Both Conditions), Statistical comparison between lateral agitation and zero agitation.

Structural characterisation of biosurfactant

The HPLC-MS analysis showed the *Pseudomonas* spp. biosurfactants to be rhamnolipids of 3-5 types of congeners with varying concentrations (Table 4). The congeners were: Rha-C_{10}, Rha-C_{12}, Rha-C_{10}:1-C_8, Rha-C_{10}-C_{10} and Rha-C_{10}-C_{12}:1/1. The five congeners were found in *P. aeruginosa* strains GI-A, GI-B and DWS. All the congeners were also present in *P. aeruginosa* strain FaM-1except for Rha-C_{10}-C_{12}:1/1. Congeners Rha-C_{10}:$_1$-C_8 and Rha-C_{10}-C_{12}:1/1 were not observed in strains CM-II and DSK. *Pseudomonas aeruginosa* strain GI-B had the highest yield, while *Pseudomonas aeruginosa* strain DSK had the lowest yield. The overall concentration of rhamnolipid congeners produced by each isolate is given in descending order of GI-B (43.563 mg/L) > GI-A (37.131 mg/L) > DWS (28.773 mg/L) > FaM-I (22.843 mg/L) > CM-II (18.439 mg/L) > DSK (13.936 mg/L).

Table 4: Rhamnolipid Congeners and Concentrations in *Pseudomonas* aeruginosa from the environment

S/N	***Pseudomonas aeruginosa* strain**	**Congener concentration (mg/L)**					**Total**
		Rha-C_{10}	**Rha-C_{12}**	**Rha-$C_{10:1}$-C_8**	**Rha-C_{10}-C_{10}**	**Rha-C_{10}-$C_{12:}$1/1**	
1	GI-B	4.115	2.697	2.805	29.284	4.667	43.563
2	GI-A	3.602	1.714	1.094	28.224	2.497	37.131
3	DWS	2.201	0.382	0.453	25.208	0.529	28.773
4	FaM-I	1.562	0.337	0.225	20.719	0	22.843
5	CM-II	1.279	0.340	0	16.820	0	18.439
6	DSK	0.611	0.340	0	12.985	0	13.936

Discussion

Biosurfactant production by *Pseudomonas aeruginosa* strains in the environment, is well established in the literature (Kaskatepe and Yildiz, 2016; Liu *et al.*, 2018; Câmara *et al.*, 2019) and the strains from this study corroborate these findings. The

biosurfactant was produced during the stationary phases of the strains' growth, making them a secondary metabolite. This substantiates that biosurfactants are secondary and not primary metabolites, as confirmed by Saleh *et al.* (2014).

Studies indicate that the yield of biosurfactants varies depending on the carbon source and the nutrient medium (Soberón-Chávez *et al.*, 2005; Kaskatepe and Yildiz, 2016). This was evidenced in the study when comparing the biosurfactant production yield from glycerin and olive oil as carbon sources on different hydrophobic substrates. The results showed glycerin (the commercial name of glycerol, which usually contains a small percentage of water and not less than 95% of glycerol) as a better carbon source with higher emulsification index. Glycerin is a cheap and good source of carbon for the production of rhamnolipids (Rashedi *et al.*, 2006; Silva *et al.*, 2010; Eraqi *et al.*, 2016), and the higher emulsification index could be due to its heterogenous composition that provide important nutrients for cell growth and production of glycolipids (de Sousa *et al.,* 2011).

The result of lateral agitation and zero agitation dynamics on biosurfactant production revealed that the E_{24} obtained under conditions of no (zero) agitation were higher than lateral agitation. The speed and method of agitation applied during the incubation of microorganisms are important in producing biosurfactants to ensure oxygen transfer from the gas phase to the aqueous phase (Kaskatepe and Yildiz, 2016). According to Pimienta *et al.* (1997), a higher concentration of rhamnolipid was achieved by rotatory shaking (orbital agitation) when compared to the thermoregulated bath (lateral agitation) and the control without agitation. They went further to say that a system without agitation does not present a good time-oxygen transfer which increases the process time for an efficient production. However, the result of this study suggests that substantial quantities of biosurfactants can be produced without the agitation of the culture medium when compared to lateral agitation.

Characterisation of biosurfactant from the isolates revealed that they were rhamnolipids. Rhamnolipids are mainly produced by *Pseudomonas aeruginosa* (Abdel-Mawgoud *et al.,* 2010; Rikalović *et al.,* 2015; Hassan *et al.,* 2016). Rhamnolipids are a diverse group of molecules with more than 60 reported congeners (Abdel-Mawgoud *et al.,* 2010; Chong and Li, 2017), and they have four known homologues, which are formed by one or more rhamnose units linked to one or two chains of fatty acids with eight to fourteen carbon atoms, which can be saturated or not (Hörmann *et al.,* 2010; Müller *et al.,* 2010). The rhamnolipids from this study are mono-rhamnolipids with β-hydroxy-fatty acids side chain lengths from C8 to C12 and Rha–C10–C10 as the predominant congener produced by the strains. This has also been reported by Rikalović *et al.* (2015) and Rendell *et al.* (1990). According to Rikalović *et al.* (2015), the complexity of the composition of rhamnolipids mixtures is found to depend on various factors such as the origin of the bacterial strain, type of carbon substrate, culture conditions, age of the culture, the *P. aeruginosa* strain itself, as well as the method of rhamnolipids isolation and purification.

The attributes and potency of biosurfactants make them preferable to commercial surfactants. However, commercial large scale production of biosurfactants remains a challenge because of high cost of production. Therefore, it is important to seek ways of reducing all costs associated with making the final product. This study has highlighted the possibility of getting higher biosurfactant yield without incurring the expenses of keeping cell cultures agitated, thus eliminating the use of sophisticated and expensive culture vessels that will consume much power to run.

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