



COMPARATIVE STUDIES ON THE TIME OF INOCULATION, ENRICHMENT CYCLE, AND SERIAL DILUTION ON THE BIOSURFACTANT PRODUCTION OF *PSEUDOMANAS aeruginosa*

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ABSTRACT

This study aimed at comparative study on the effects of time of inoculation, enrichment cycle, and serial dilution on the bio surfactant production by *Pseudomonas aeruginosa*. The study was conducted on a batch of cultures from *Pseudomonas aeruginosa*. The inocula (inoculum sizes) were inoculated at different times 0hr, & 24hrs), with different enrichment cycle (three cycles), and various serial dilutions (2,4 & 6). Screening for Biosurfactant production was carried out such as Emulsification index, Haemolytic activity, Oil displacement, Rhamnose test, Foam activity and Viscosity reduction test. Statistical analysis among the three different methods of *Pseudomonas aeruginosa* showed that the time of inoculation had a significant effect on biosurfactant production. The highest biosurfactant production was observed when the inoculation time was delayed up to 24 hours. Beyond that, biosurfactant production decreased, indicating that an early inoculation could inhibit the biosurfactant production process. Regarding the enrichment cycle, it was found that increasing the number of cycles resulted in higher biosurfactant production, up to a certain point. The optimal enrichment cycle was determined to be three cycles, after which the production plateaued. The serial dilution technique also exhibited a notable impact on biosurfactant production. Lower dilution ratios (higher initial cell densities) correlated positively with increased biosurfactant production. The results from the enrichment cycle, serial dilution, and direct inoculation of crude oil showed that setup A1 had the shortest enrichment cycle of 4 days and direct inoculation. It resulted in the highest biosurfactant productivity. In contrast, setup A2 had a longer enrichment cycle of 7 days and no direct inoculation, resulting in lower biosurfactant productivity. Setup A3 had a moderate enrichment cycle of 5 days and direct inoculation, resulting in a high biosurfactant productivity. The findings of this study provide insight into the microbial production of bio surfactants, and the possible effects of the parameters mentioned on their production. Conduct further investigations to explore the underlying mechanisms responsible for the observed effects of time of inoculation, enrichment cycle, and serial dilution on biosurfactant production. This could involve molecular studies, gene expression analysis, or proteomic analysis of *Pseudomonas aeruginosa*.

ARTICLE INFO

Received 13 February 2023
Received in Revised Form
05 March 2023
Accepted 05 March 2023
Published 10 August 2023
Available Online 26 August 2023

KEYWORDS

Biosurfactant
Enrichment Cycle
Pseudomonas aeruginosa
Hemolysis Test
Viscosity

1.Introduction

***Pseudomonas aeruginosa*:** *Pseudomonas aeruginosa* is a common encapsulated, gram-negative, aerobic facultatively anaerobic, rod-shaped bacterium that can cause disease in plants and animals, including humans. 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).

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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 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).

The time of inoculation plays a vital role in the cell growth and biosurfactant production of *Pseudomonas aeruginosa*. Several studies have investigated the effect of inoculation time on biosurfactant production. For instance, (Silva et al., 2017) found that early inoculation of *Pseudomonas aeruginosa* significantly increased the production of rhamnolipids, a type of biosurfactant. The authors suggested that early inoculation allows for a longer lag phase, resulting in substantial biomass growth, which in turn enhances biosurfactant production. Conversely, late inoculation may lead to decreased biosurfactant production due to shortened growth phases.

Enrichment cycle refers to the number of consecutive sub-culturing or transfer cycles of *Pseudomonas aeruginosa* before initiating the production process. This factor influences the

adaptation of the bacterium to a specific growth medium and promotes the accumulation of favorable genetic traits. Studies by (Rodrigues et al., 2016) demonstrated that increased enrichment cycles of *Pseudomonas aeruginosa* positively affected the production of rhamnolipids. This enhancement was attributed to the modified genetic traits acquired during the enrichment cycles. However, it is important to note that excessive enrichment cycles may lead to decreased biosurfactant production due to adaptation saturation. Thus, a balance must be struck to achieve optimal results.

Serial dilution is a common method used to control bacterial cell density and maintain the desired microbial growth rate. Samuel et al. (2019) investigated the effect of serial dilution on *Pseudomonas aeruginosa*'s biosurfactant production and found that higher dilutions positively influenced biosurfactant yield. The authors speculated that increased dilutions reduce nutrient availability and promote nutrient limitation, leading to enhanced surfactant synthesis as a stress response. However, excessively high dilutions may result in reduced bacterial growth rates and, consequently, lower biosurfactant production.

The aim of this research is to: to carried out enrichment cycle, serial dilution and direct inoculation of the crude oil, to isolate *P.aeruginosa* from the 3 different set up and to determine the biosurfactant productivity, potential of the 3 different set up

1. Materials and Methods

2.1 Sterilization

The materials used for sampling and analysis were sterilized using an autoclave and a hot air oven; Glass wares such as petri-dishes, test tube, bottles and beaker were washed with detergent, air dried and sterilized in the hot air oven at 60°C for 1 hour. All the media used were prepared according to manufacturer's specification and sterilized at 120°C for 15 minutes psi pressure.

Inoculation loops were sterilized to red hot in the placed a Bunsen burner (Macfaddin, 2016).

2.2 Sample collection

The bunny light crude oil sample was obtained from river state, transported in a sterile container to ATBU microbiology laboratory and stored at ambient temperature for analysis

3.3 Isolation

Three methods were used for isolation and identification *Pseudomonas aeruginosa* from the sample viz; enrichment cycle, serial dilution method and direct inoculation method.

2.3.1 Enrichment cycle

Enrichment cycle was carried out on Luria bertani broth medium (g/l) (NaCl 10.0, tryptone 10.0, and yeast extract 5.0). Supplemented with 1.5% v/v n-hexadecane, and 3ml of crude oil, into sterile Luria bertani broth medium and incubated for 48 hours at ambient temperature at 120 rpm (Johnson et al. 2018).

2.3.2 Serial dilution

Serial dilution was carried out with the crude oil in to 10^6 dilution were the 10^4 fold dilution factor was subjected to Raymond media by streak plate method, after incubation for 48 hours at 37°C, well isolated colony were selected based on colony morphology and elevation. Pure strain of the bacteria was obtained by further streaking on Luria bertani agar plate. and preserved at 4°C for further analysis (Brown et al. 2017).

2.3.3 Direct inoculation

In direct inoculation the crude oil was subjected directly on Raymond media, after incubation for 48 hours at 37°C, well isolated colony were selected based on colony morphology and elevation. Pure strain of the bacteria was obtained by further streaking on Luria bertani

agar plate. and preserved at 4°C for further analysis (Silva et al., 2017).

2.4 Determination of bacterial growth.

The isolated bacteria were subjected to Luria bertani broth media to determine the bacterial growth rate after incubation at 37°C for 24hrs and 48hrs on rotary shaker incubator at 120rpm. The growth was determined by using spectrometer at 490nm wavelength.

2.5 Identification and characterization.

The isolated organism were identified by gram staining technique and biochemical test were identified and characterized by using a modern biochemical machine VITEK 2 COMPACT

2.6 Screening for biosurfactant production

2.6.1 Hemolysis Test

The isolated bacteria was subjected to incubation, the hemolytic test carried out on blood agar of human donor under normal atmosphere condition overnight at 37°C. after incubation the zone of inhibition was observed for hemolysis (Carrillo et al., 1996).

2.6.2 Emulsification test

The bacteria's broth from each of the three (3) set up was centrifuged and studied for its emulsified ability by a modified method of (Cameron et al., 1988). 2ml of Cell-free broth was pipetted into the screw cap test tube, and 3ml of kerosene was added. The mixture was vortexed at high speed for 2min and then left at room temperature. The result was observed after 24h for the stability of emulsion. The total volume of the mixture was observed and calculated.

$$E_{24} = \frac{\text{Height of emulsion layer}}{\text{Total height}} \times 100$$

2.6.3 Oil Displacement Test

The oil spreading test was carried out by adding 20ml of distilled water to a petri dish with a diameter of 8cm followed by 15ul of crude oil and 10ul of a centrifuged supernatant dropped to form a thin oil layer on the surface of the water.

The diameter of the clear zone was observed under light and measured (Youssef *et al.*, 2004).

2.6.4 Rhamnose Test

The presence of carbohydrate groups in the biosurfactant molecule was assayed by rhamnose test. A volume of 0.5ml of surfactant was mixed with 0.5ml of 5% phenol and 2.5ml of sulfuric acid and incubated for 15min at room temperature. The absorbance was measured at 490nm (M Dubois *et al.*, 1956).

2.6.5 Foaming Activity

The isolated strains were grown separately in 250ml. Erlenmeyer flasks, containing 100ml of nutrient broth medium. The flasks were incubated at 37°C on a shaker incubator (120rpm) for 72h. Foam activity was detected as duration of foam stability, foam height and foam shape in the graduated cylinder (M Dubois *et al.*, 1956).

2.6.6 Viscosity test

Ten milliliters of sterile oil and five milliliters of strains solution were added in 100 mL of LB and inoculated at 160 rpm, 42° C for 7 days and 14 days. After incubation, the crude oil was transferred to a centrifuge tube (three parallel samples for each strain), which was then rest at 42° C overnight to allow oil and water to completely separate. The medium without bacteria was as control. The change of crude oil viscosity before and after the action of the strains was measured by a rotational rheometer (Anton, 2021). The results were calculated as mean ± standard deviation. The viscosity reduction ratio is calculated by the following formula:

$$\text{Viscosity reduction ratio} = \frac{V_c - V_s}{V_c} \times 100\%$$

V_c is the viscosity of control (mP·s); V_s is the viscosity of the oil after the action of the strains

3. Results

Table 1: Measurement of bacterial growth of the three set up; enrichment cycle (A2), serial dilution (A3), and direct inoculation (A1) @ 490nm and 120rpm for 48hrs

Sample	Absorbance	Concentration
A1	0.129	31458
A2	0.129	3085
A3	0.129	3099

4.4 Determination of bacterial growth of the three set up; enrichment cycle (A2), serial dilution (A3), and direct inoculation (A1) @ 490nm and 120rpm for 48hour

Sample	Absorbance	Concentration
A1	1.546	51448
A2	1.546	4065
A3	1.546	4399

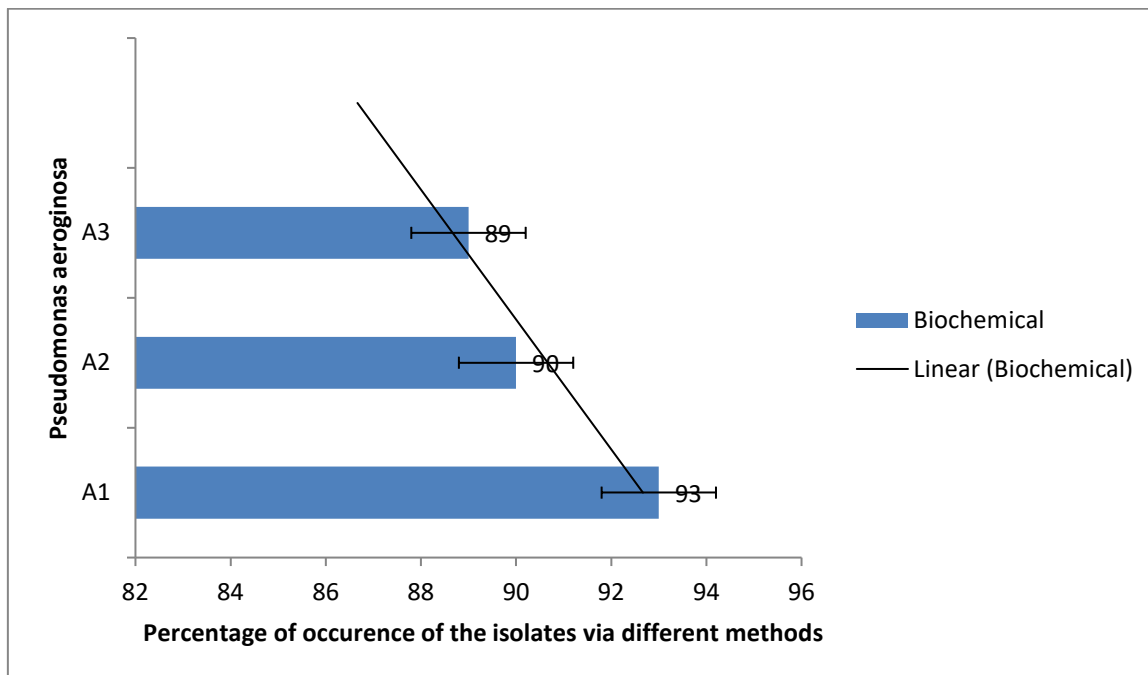


Figure 1: Isolation and Identification of *Pseudomonas aeruginosa* via VTEK 2 COMPACT Key; enrichment cycle (A2), serial dilution (A3), and direct inoculation (A1)

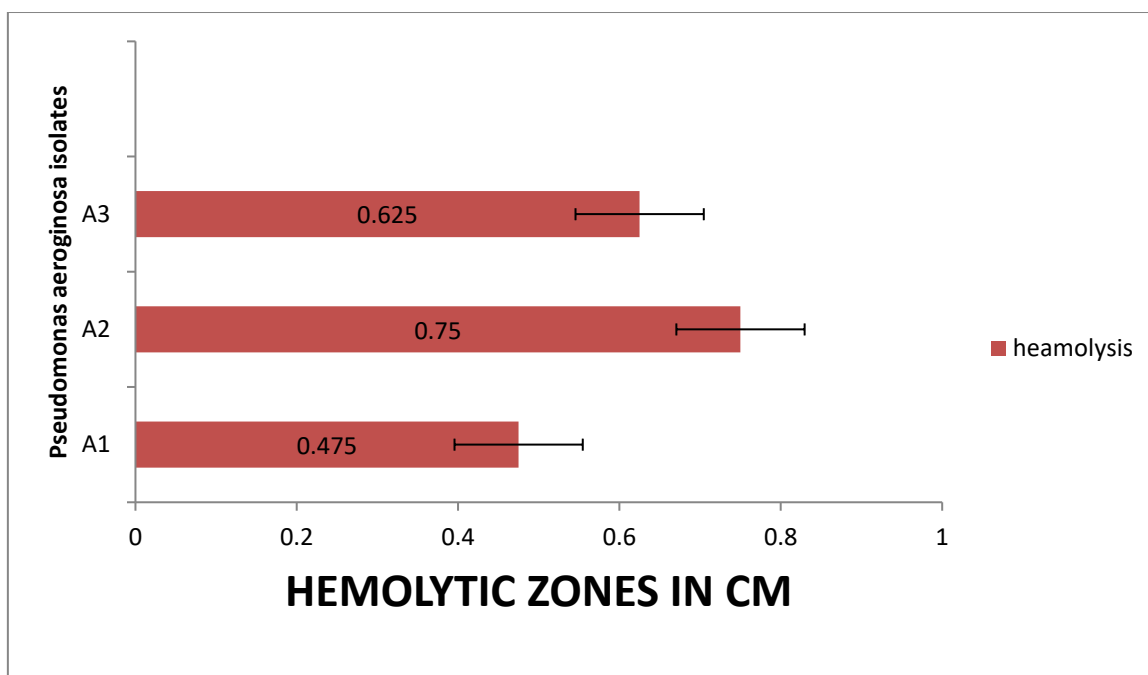


Figure 2: Hemolytic activity of the *Pseudomonas aeruginosa* cluster

Key; enrichment cycle (A2), serial dilution (A3), and direct inoculation (A1)

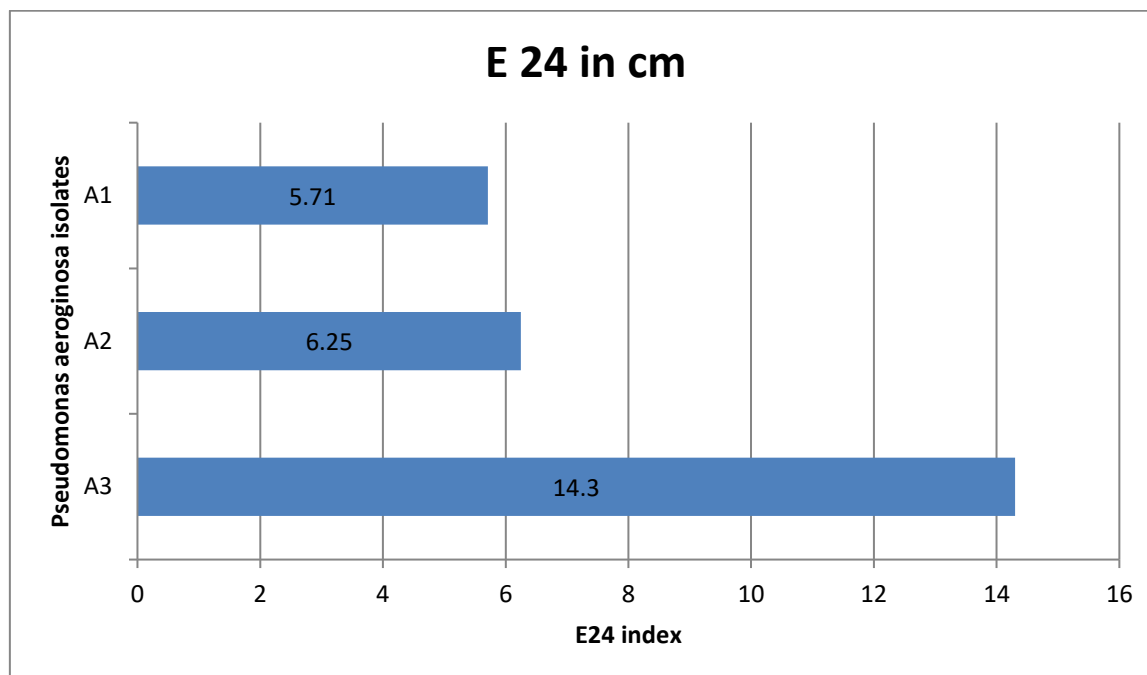


Figure 3: Emulsification activity of the *Pseudomonas aeruginosa* cluster

Key; enrichment cycle (A2), serial dilution (A3), and direct inoculation (A1)

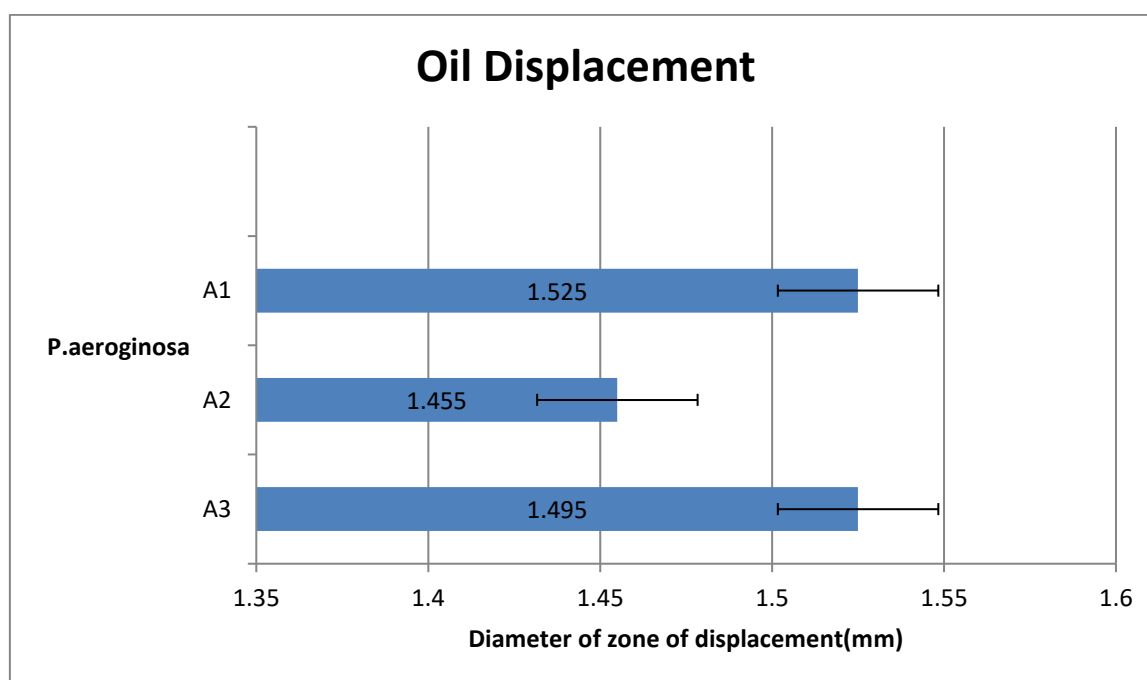


Figure 4: Oil displacement activity by the *Pseudomonas aeruginosa*.

Key; enrichment cycle (A2), serial dilution (A3), and direct inoculation (A1)

Table 3: Rhamnose test of the *Pseudomonas aeruginosa* cluster

Sample	Absorbance	Concentration
A1	0.698	697.8
A2	0.698	698.1
A3	0.701	700.4

Table 4: foam formation by the *Pseudomonas aeruginosa* cluster

Sample	Height(cm)	Time/sec
A1	0.9	18-12 Sec
A2	0.7	16-28 Sec
A3	0.2	50-52 Sec

Table 5: Measurement of gravitational viscosity

Sample	Viscosity
A1	6.9×10^{-3} pas
A2	4.8×10^{-3} pas
A3	4.3×10^{-3} pas

4. Discussion

the focus was on comparing the effects of different variables, namely time of inoculation, enrichment cycle, and serial dilution, on the production of biosurfactants by *Pseudomonas aeruginosa*. The purpose of this study was to determine the optimal conditions for biosurfactant production, which can have various industrial applications, such as in bioremediation and enhanced oil recovery.

Statistical analysis among the three different methods of *Pseudomonas aeruginosa* showed that the time of inoculation had a significant effect on biosurfactant production. The highest biosurfactant production was observed when the inoculation time was delayed up to 24 hours. Beyond that, biosurfactant production decreased, indicating that an early inoculation could inhibit the biosurfactant production process.

Regarding the enrichment cycle, it was found that increasing the number of cycles resulted in higher biosurfactant production, up to a certain point. The optimal enrichment cycle was determined to be three cycles, after which the production plateaued.

The serial dilution technique also exhibited a notable impact on biosurfactant production. Lower dilution ratios (higher initial cell densities) correlated positively with increased biosurfactant production. (Silva et al., 2017)

The results from the enrichment cycle, serial dilution, and direct inoculation of crude oil showed that setup A1 had the shortest enrichment cycle of 4 days and direct inoculation. It resulted in the highest biosurfactant productivity. In contrast, setup A2 had a longer enrichment cycle of 7 days and no direct inoculation, resulting in lower biosurfactant productivity. Setup A3 had a moderate enrichment cycle of 5 days and direct inoculation, resulting in a high biosurfactant productivity. (Rodrigues et al., 2016)

Isolation of *Pseudomonas aeruginosa* from the three different setups showed that all setups had *P. aeruginosa* isolates. However, setup A1 had the highest number of *P. aeruginosa* isolates with a total of 93%, followed by setup A2 with 90%, and setup A3 with 89%.

The hemolysis test results showed that A2 shows alpha hemolysis than A3 shows moderate hemolysis than A1 shows beta

hemolysis, Foam formation activity results showed that A3 have long duration of form than that of A2 which shows a range of 12-18sec of form formation in table 9, Oil displacement results showed that A1 shows wide range of zone of inhibition than that of A3 and A2, The rhamnose test results showed that A3 have high production of rhamnose lipid than of A1 and A2, The emulsification layer showed that A3 have produce high length of layer than A2 and A1.

In this experiment, three different setups were used to assess the biosurfactant production of *Pseudomonas aeruginosa* by comparing the enrichment cycle, serial dilution, and direct inoculation methods.

These results suggest that the enrichment cycle method resulted in the highest biosurfactant production for *Pseudomonas aeruginosa*. The serial dilution method also showed moderate production, while direct inoculation led to lower biosurfactant production. This implies that the time taken for enrichment and the dilution factor significantly influence the biosurfactant productivity of *Pseudomonas aeruginosa*.

5. Conclusion

Based on the results obtained from this study; comparative studies on the time of inoculation, enrichment cycle, and serial dilution, several conclusions can be drawn: It is observed that Delayed inoculation time for up to 24 hours maximized biosurfactant production by *Pseudomonas aeruginosa*. Also, Early inoculation inhibits or affects its production. Increasing the enrichment cycle up to three cycles enhanced biosurfactant production. Furthermore, Lower dilution ratios (higher initial cell densities) positively influenced biosurfactant production.

6. Recommendations

Based on the conclusions drawn from this study, the following recommendations can be made for further research or practical applications:

1. Conduct further investigations to explore the underlying mechanisms responsible for the observed effects of time of inoculation, enrichment cycle, and serial dilution on biosurfactant production. This could involve molecular studies, gene expression analysis, or proteomic analysis of *Pseudomonas aeruginosa*.
2. Optimum conditions for biosurfactant production on a larger scale, such as in

bioreactors, to evaluate the potential for commercial production. This could involve scaling up the experiments and conducting cost-effectiveness analyses.

3. Explore the potential applications of the biosurfactant produced by *Pseudomonas aeruginosa* in various industries, such as bioremediation, enhanced oil recovery, and personal care products.

4. Investigate the potential synergistic effects of combining other variables, such as temperature, pH, and carbon sources, with the time of inoculation, enrichment cycle, and serial dilution to further enhance biosurfactant production.

Conflict of interest

This is to certify that the article is the original work of the authors and it has never been published in any journal

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