



Research Article

Stability and Optimization of Biosurfactant Production by
Enterobacter Cloacae AYP1

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ABSTRACT

Biosurfactants are diverse and heterogeneous group of microbial metabolites produced by certain bacteria and fungi that have both hydrophilic and hydrophobic components. This work was aimed at assessing the production, stability and optimization of rhamnolipid biosurfactant production conditions by *Enterobacter cloacae* AYP1 strain. Biosurfactant production, extraction, purification and optimization of the production conditions were assessed in mineral salt medium followed by stability tests under different ranges of pH, temperatures and salts concentrations using standard procedures. Gas chromatography mass spectroscopy (GC-MS) was used in characterizing the biosurfactant produced. One factor at a time (OFAT) optimization studies revealed that production of biosurfactant by *Enterobacter cloacae* AYP1 was optimal at 3% carbon source concentration (1.5mg/ml), pH 7.5 (1.7mg/ml), temperature 40°C (1.8mg/ml) and incubation time of 96hrs (1.96mg/ml). The biosurfactant produced was characterized using gas chromatography mass spectroscopy (GC-MS). The results revealed the presence of different fatty acids at different peaks. This indicates that the production of rhamnolipid biosurfactant was optimal at the carbon concentration, pH, temperature and incubation time of 3%, 7.5, 40 °C and 96hrs respectively. There is the need to use biotechnological approaches with a view to improving the performance of the bacterial strain.

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INTRODUCTION

Microorganisms produce biosurfactants which are surface active agents having both hydrophobic and hydrophilic moieties. They are classified as glycolipids, lipopeptides, lipoproteins, lipopolysaccharides, and phospholipids based on the chemical components found in these molecules (Ekprasert *et al.* 2020). Bacteria, yeasts, and molds produce biosurfactants, which are a varied and heterogeneous category of microbial compounds with wide ranges of applications.

A number of biosurfactants were studied by many researchers but bacterial biosurfactants (rhamnolipid, surfactin and lichenysin produced from *Pseudomonads* and *Bacillus* species) were the most-thoroughly investigated (Brumano *et al.*, 2016). These microbial compounds are very advantageous because of their structural variety, great biodegradability, low environmental effect and toxicity, high specificity, stability and activity under harsh conditions and have been shown to remove petroleum hydrocarbon pollutants from drill cuttings and other hydrocarbon-contaminated waste streams like soil and refinery effluent in addition to many industrial applications (Luna *et al.*, 2015). It also have applications in many unconventional fields such as cosmetics, pharmaceuticals, petroleum recovery polymerization, foods/beverages and environmental remediation, as opposed to the synthetic surface active agents which are used as laundry detergent and individual care products (Banat *et al.*, 2010).

Studies have shown that some biosurfactants with high capacity for the hydrophobic organic compounds emulsification have above 35 mN/m as medium surface tensions (Fox and Bala 2000; Lai *et al.* 2009; Barros *et al.* 2008; Oliveira *et al.* 2013). This however, depends on the stability of the surfactants which is dictated by the prevailing conditions in the environment it is been applied.

As such, biosurfactants' stability is a significant concern in its usage in different environments including soil. As changes in soil pH, salinity and temperature do affect stability and the rate of biosurfactant production (Fortuny *et al.* 2007), so many interests have recently been shifted towards finding most suitable conditions for its production and stability. In our previous study, *Enterobacter cloacae* AYF1 has been identified as a potent soil-borne biosurfactant producer. The present study therefore, was aimed at characterizing, determining the stability and optimizing the conditions for production of biosurfactant by *Enterobacter cloacae* AYF1 strain.

MATERIALS AND METHODS

Production of Biosurfactant

Mineral salt medium containing the following composition in g/l: 0.6 MgSO₄.7H₂O, 2.2 Na₂HPO₄, 0.05 NaCl, 0.01 FeSO₄. 7H₂O, 1.4 K₂HPO₄, and 0.02 CaCl₂ was used throughout the study. The mineral salt medium was supplemented with 5 ml of stock solution of trace element containing g/l: 0.42 CoCl₂.6H₂O; 1.0 CuSO₄.5H₂O, 0.56 H₃BO₃, 2.32 ZnSO₄.7H₂O, 1.78 MnSO₄.4H₂O and 1.0 EDTA, 0.39 Na₂MoO₄.2H₂O, 0.66 KI and 0.004 NiCl₂.6H₂O and 3% diesel was used as a carbon source. Stock solution was sterilized by filtration and added to the medium. The pH 7.2 was used as the adjusted pH of the medium and the medium was sterilized at 121 ° C for 15 min (Abouseoudet *et al.*, 2008). A stored culture of *Enterobacter cloacae* AYF1 from the Postgraduate Laboratory Department of Microbiology, Bayero University Kano, Nigeria was inoculated into flasks containing 100 ml of the medium and incubated using incubator shaker at 30 ° C for 7 days.

Purification and Extraction of Biosurfactant

Extraction and purification

Bacterial cells were removed by centrifugation (12,000 x g, at 4 ° C for 30 min). Culture supernatant was acidified with 6 N HCl to obtain

the pH of 2.0. The extraction was performed twice with a mixture of chloroform: methanol (2:1 v/v) was added to the culture medium, after being vigorously shaken, this was allowed to stand until phase separation. Extracts were concentrated by rotary evaporation and then anhydrous sodium sulfate was added to remove water (Thampayaket *et al.*, 2008). The crude residue was purified on a silica gel 120 mesh column eluting with a chloroform/methanol gradient ranging from 20:1 to 2:1, collecting ten fractions. Then fractions eluted were pooled and the solvents were evaporated. The resulting residue was dialyzed against distilled water and lyophilized (Li *et al.*, 1984).

Characterization

Gas chromatography mass spectrophotometer was carried out with triple axis detector and auto injector (10µl syringe). As a carrier gas, helium was used. All chromatographic separations were carried out on a capillary column with a length of 30 m, an internal diameter of 0.2 m, and a thickness of 250 m that had been treated with phenyl methyl silox. Other GCMS conditions were an ion source temperature (EI) of 2500°C, an interface temperature of 300°C, a pressure of 16.2 psia, and a 1.8 µl injector in split mode with a split ratio of 1:50 and a temperature of 300°C for injection. The column temperature was set at 35°C for 5 minutes before gradually increasing to 150°C at a rate of 4°C/min. The temperature was raised to 250 degrees Celsius at a pace of 20 degrees Celsius per minute for 5 minutes. The total elution was 47.5 minutes. Mass spectra solution software provided by the supplier was used to control the system and to acquire the data. Identification of the compound was carried out by comparing the mass spectra obtained with those of the standard mass spectra from GC library (Christova *et al.*, 2004).

Biosurfactant Stability Test

Stability studies were carried out as described by Obayoriet *al.* (2009). Cell-free broth was obtained by centrifuging the crude biosurfactant produced

by *Enterobacter cloacae* AYP1 at 5000rpm for 20 minutes and the cell free supernatant obtained was used for the stability tests. The stability tests of the biosurfactants against temperature (45, 55, 65, 75 and 85°C), Sodium chloride salt (1, 3, 6, 9 and 12%) and pH (2, 6, 7, 8 and 12) were determined. The pH of the cell free supernatant was adjusted to acidic (2, 4 and 6 using HCl) and alkaline (8, 10 and 12 using NaOH). Emulsification index (E₂₄) was later determined after 24 hours. Emulsification index of cell free broth was determined by adding 2 ml of oil to 2ml cell free broth and vortexed for 2 min, and it was left undisturbed for 24hr. The E₂₄ index is given as percentage of height of emulsified layer (mm) divided by total height of the liquid column (mm) (Ibrahim *et al.*, 2013).

The total height of liquid, as given by the expression:

$$E_{24} = \frac{H_{\text{emulsion}}}{H_{\text{total}}} \times 100$$

Where H emulsion is the height of the emulsion layer and H total is the total height.

Optimization of Biosurfactant Production Parameters

Different parameters that include carbon source concentration, pH, temperature and incubation time were optimized using one factor at a time (OFAT) method.

Determination of Optimum Carbon Source Concentration for Biosurfactant Production

Different concentrations of diesel 1%, 2%, 3%, 4%, 5%, 6% and 7% were added to the mineral salt broth in different conical flasks thereby serving as the production media. The optimal concentration of the carbon source for biosurfactant production was determined by measuring the quantity of biosurfactant produced as described by Imran *et al.* (2012).

Determination of optimum pH for Biosurfactant Production

The mineral salt media were prepared by using different pH range including 6.0, 6.5, 7.0, 7.5, 8.0, 8.5 and 9. The pH of the medium was adjusted by adding 1% NaOH or 1N HCl (Imran *et al.*, 2012).

Determination of Optimum Temperature for Biosurfactant Production

Optimal temperature for biosurfactant production was determined by growing the bacteria in mineral salt media incubated at different temperature of 25, 30, 35, 40, 45, 50 and 55⁰C (Khokharet *al.*, 2012).

Determination of Optimum Incubation Time for Biosurfactant Production

Optimum incubation time for biosurfactant production was studied by setting the cultivation at different period of time that include 24, 48, 72, 96, 120, 144 and 168 hours. The best range of incubation period where high production of biosurfactant occurs was identified (Khokharet *al.*, 2012).

RESULTS

In this study, characterization, stability and optimization of biosurfactant production by *E. cloacae* AYP1 was investigated. Results in Figure 1 shows the chromatogram of rhamnolipid produced by *Enterobacter cloacae* AYP1 strain and the result revealed the presence of different fatty acids at different peaks. Different fatty acids at different concentrations including hydroxydecanoic acids, decanoic acid, trans- 2-dodecenoic acid, n-pentadecanol, 3-decanoic acid, dichloro acetic acid, Butyl fluoroformate 2-butanolic, 2- methoxy methyl ester, methyl chloride acid and 3-hydroxydecanoic acid were detected.

The result of biosurfactant stability characteristics was presented in Table 1. Emulsification index was above 55% at various temperatures used. Emulsification index for the biosurfactant produced had shown stability at various sodium chloride salt concentrations and pH ranges (Table 1).

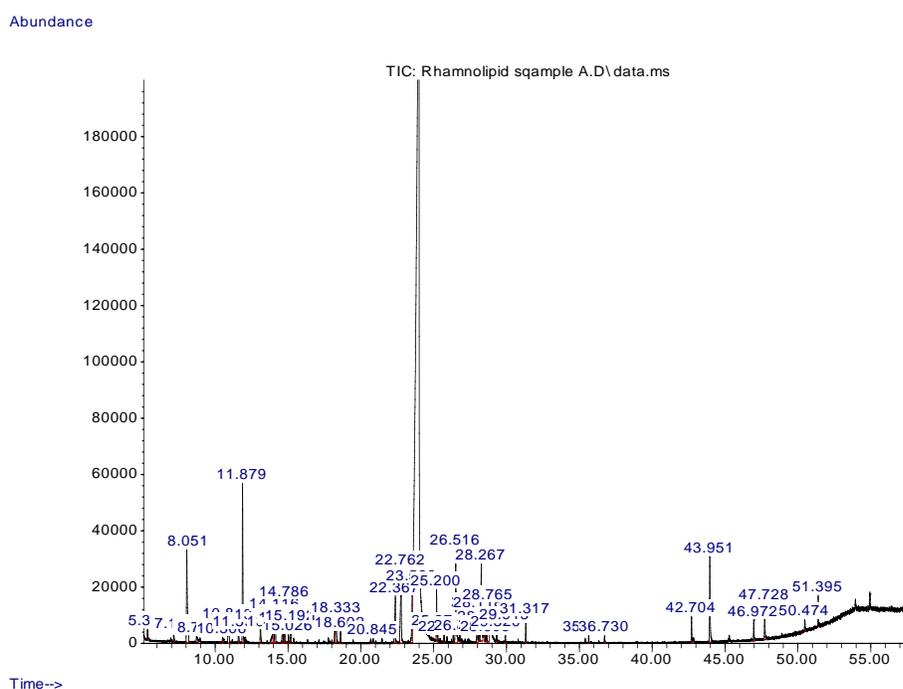


Figure 1: GC chromatogram of Rhamnolipid produced by *Enterobacter cloacae* AYP1 strain

Optimization Results Using OFAT

The one-factor-at-a-time method was employed in determining the effect of different biosurfactant production process conditions (carbon source concentration, pH, temperature and incubation period) on *Enterobacter cloacae* AYP1 strain (Figure 1,2,3 and 4).

Effect of Carbon Source

Figure 1 shows the effect of carbon source on the biosurfactant production that ranges from 1 to 7 (%v/v). As the carbon source concentration increased biosurfactant production increased until the optimum activity of 1.5 mg/ml at 3% v/v diesel carbon source. Biosurfactant production then decreased at 4, 5, 6 and 7% of diesel carbon source.

Effect of pH

Figure 2 shows the effect of pH on the production of biosurfactant at pH levels of 6.0, 6.5, 7.0, 7.5, 8.0, 8.5 and 9. As the pH increased biosurfactant production increased until the optimum concentration of 1.7 mg/ml at pH of 7.5 and biosurfactant production then decreased at pH of 8.0, 8.5 and 9.0.

Effect of Temperature

Figure 3 shows the effect of temperature on biosurfactant production at temperature levels of 25, 30, 35, 40, 45, 50 and 55°C. As the temperatures increased biosurfactant production increased until the optimum concentration of 1.8 mg/ml at temperature of 40°C was attained. Biosurfactant production then decreased at temperature of 45-55°C.

Effect of Incubation Time

Figure 4 shows the effect of incubation period on biosurfactant production at incubation period of 24hr– 168hr. As the incubation period increased biosurfactant production increased until the optimum concentration of 1.96mg/ml at incubation period of 96hr. Biosurfactant production then decreased at incubation periods of 120-168hr.

Table 1: Stability Characteristics of the Biosurfactant Produced by *Enterobacter cloacae* AYP1

Temp. (° C)	Mean E24 (%)	NaCl%	Mean E24 (%)	pH	Mean E24 (%)
45	71	1	57	2	59
55	65	3	54	6	69
65	62	6	59	7	72
75	60	9	49	8	69
85	56	12	51	12	59

Key: E₂₄= Emulsification Index.

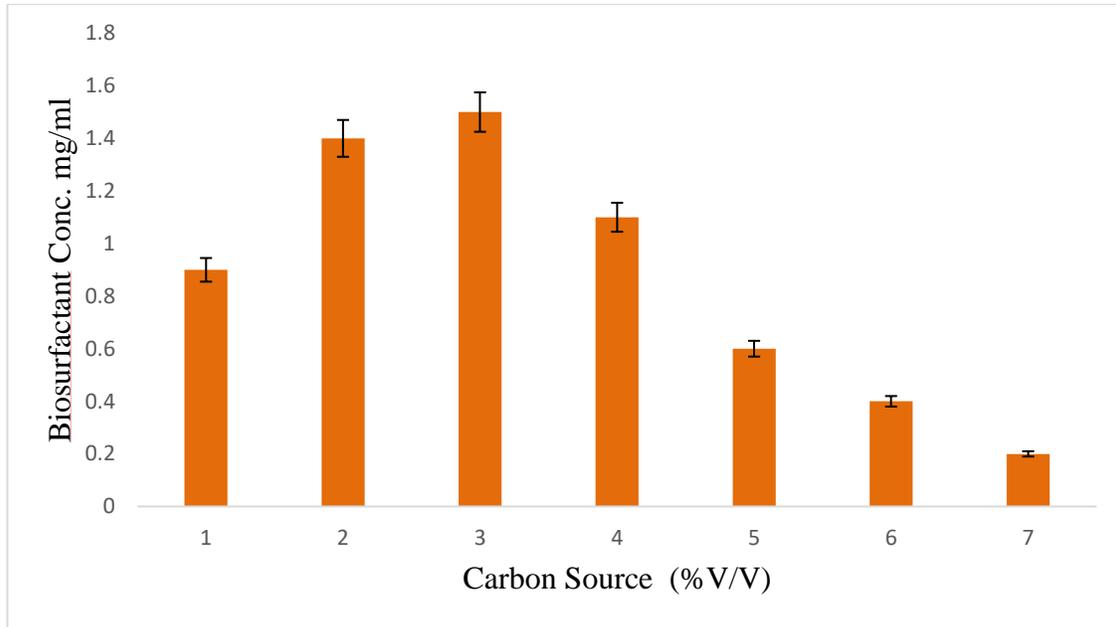


Figure 1: Effect of carbon source on the production of biosurfactant

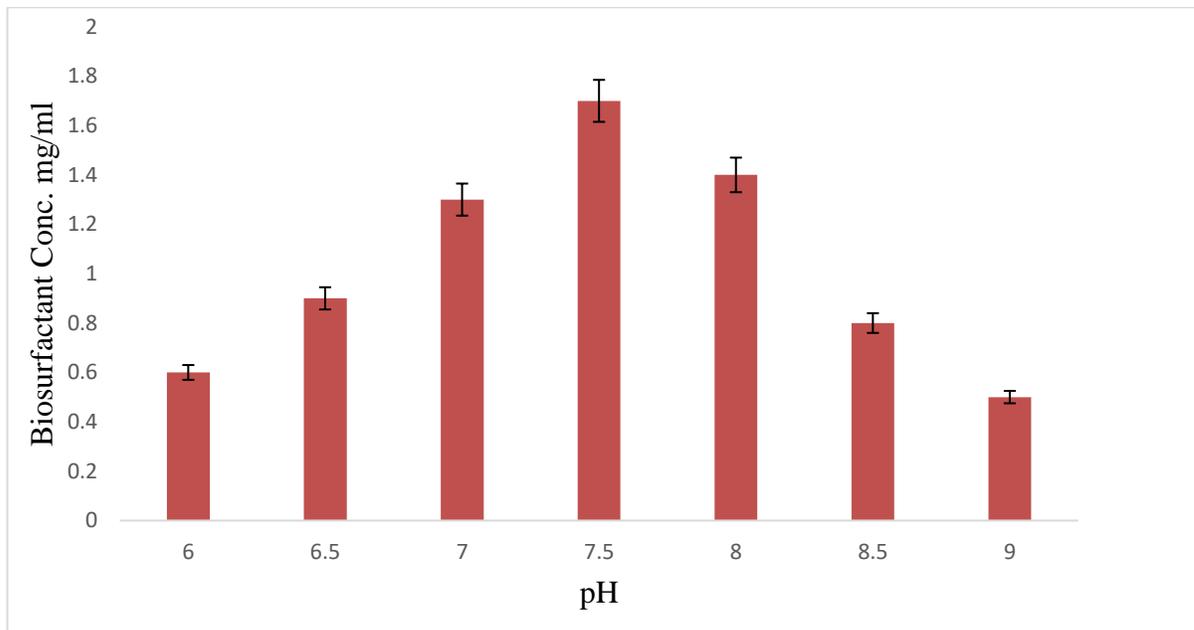


Figure 2: Effect of pH on the production of biosurfactant

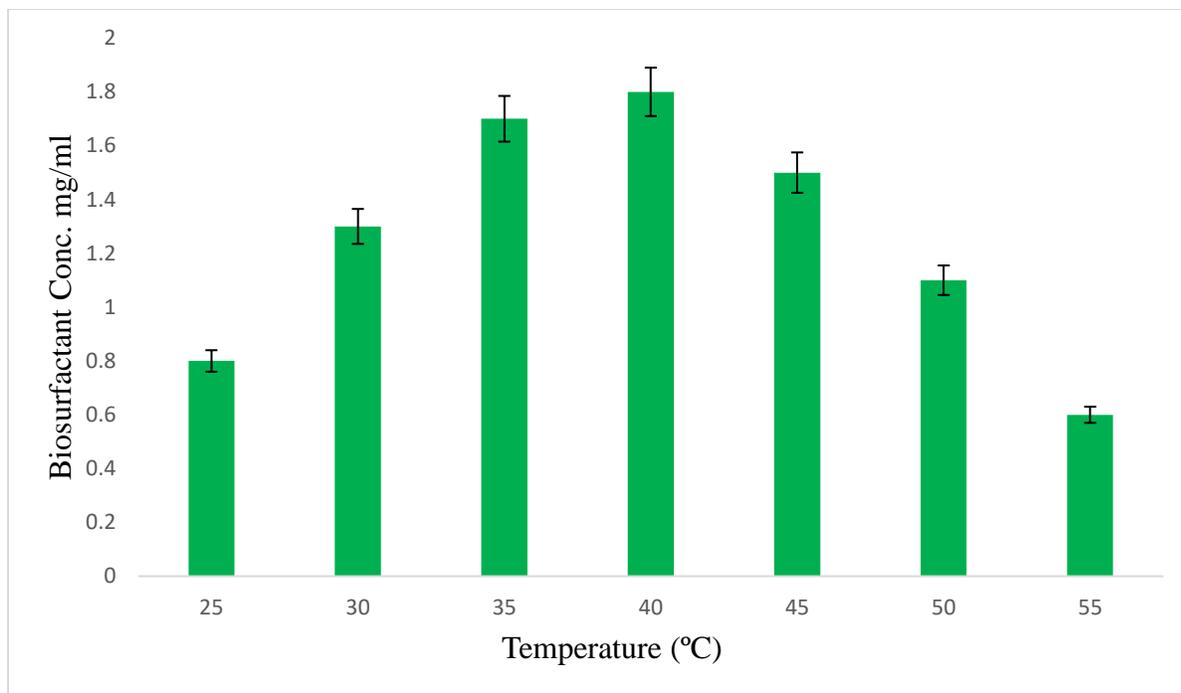


Figure 3: Effect of temperature on the production of biosurfactant

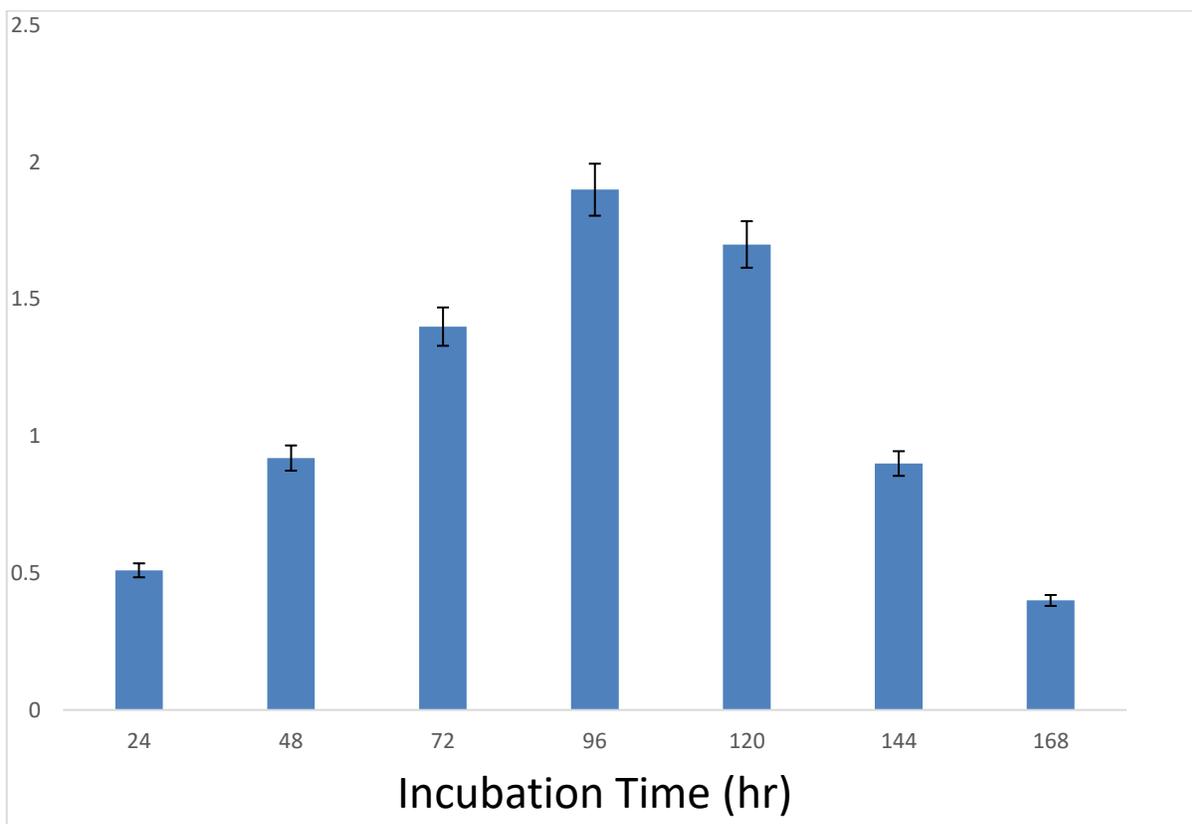


Figure 4: Effect of incubation time on the production of biosurfactant

DISCUSSION

Biosurfactant production is among the frontier research areas in environmental and industrial biotechnology. In this study, biosurfactant produced by *Enterobacter cloacae* AYP1 were characterized and optimum conditions for its production and application were assessed. The results of gas chromatography mass spectroscopy (GC-MS) of the biosurfactant revealed the presence of different fatty acids at different concentrations including hydroxydecanoic acids, decanoic acid, trans- 2-dodecenoic acid, n-pentadecanol, 3-decanoic acid, dichloro acetic acid, Butyl fluoroformate 2-butanoic, 2- methoxy methyl ester, methyl chloride acid and 3-hydroxydecanoic acid. The chromatogram was also in conformity with reference library standard of rhamnolipid. Rhamnolipids are glycolipids with a long chain of aliphatic acids or its derivatives. Rhamnolipids are the best studied and industrially important class of biosurfactants mostly obtained from Gram negative bacteria like *E. cloacae* AYP1. This agreed with the findings of Gamez *et al.* (2017), who screened and characterized biosurfactant producing bacteria isolated from soil contaminated with oily waste. It also agreed with finding of Abbasi *et al.* (2012), who conducted research on production of biosurfactant by *Pseudomonas aeruginosa* MA01 isolated from spoiled apples and its characterization. Recently, there is increased interest in rhamnolipid production and its commercial production is being explored rapidly due to its application as detergents and as agent of soil bioremediation. Reis *et al.* (2013) reported that more than 95% of oil removal in the United State was achieved using commercial rhamnolipids (JBR215, Jeneil Biosurfactant Company, USA).

In this research work, stability studies indicated that the biosurfactant produced was stable at different temperature, salt concentration and pH. Formation of stable water-oil emulsion was a

reflection of the biosurfactant stability. Although stable emulsions were obtained at the various temperatures used in this study, a steady decline in the emulsification index was observed as temperature rises from 45°C to 85 °C. Despite the decline, the emulsification index was still > 50%. Recent studies reported by (Purwasena *et al.*, 2019) showed that heating of rhamnolipid biosurfactant at high temperature up to 120 °C caused no significant effect on its performance. Thermostability of biosurfactants have been identified as an important quality that renders the product very attractive especially for environmental and industrial application. The result of stability studies due to temperature in this study agrees with that of Agarry *et al.* (2015).

Results in Table 1 showed that the biosurfactant was most active at neutral pH and 6% NaCl concentration. Although there were variations in the stability of the surfactant at the various pH and salinity ranges used in this study, the emulsification indices were in a close range, which may suggest that the two factors had little effect on its stability. Consequently, it can be inferred that the compounds present in the biosurfactants are not degraded within the range of the physicochemical conditions. Previous studies by Ibrahim *et al.* (2013) have made similar observations. There are several reports on the stability of biosurfactants at different conditions of pH, temperature and NaCl that make biosurfactant stable and ideal for industrial application (Nitschke and Pastore, 2006; Collaet *al.*, 2010; Ibrahim *et al.*, 2020).

The result for the optimization of biosurfactant production using one factor at a time (OFAT) revealed that the biosurfactant production was optimal at 3% diesel concentration (1.5mg/ml), pH 7.5 (1.7mg/ml), temperature 45°C (1.8mg/ml) and 96hrs incubation period (1.96mg/ml). Biosurfactant-producing microorganisms are frequently aerobic and as

diesel was used in this study, higher diesel concentrations would invariably produce lower biosurfactants. This is due to the fact that high diesel concentration creates relatively anoxic condition because of the low capacity of oxygen to dissolve in oil which restricts the microbial metabolism. To facilitate oxygen dissolution, the use of soluble substrates as carbon sources have been suggested (Hu *et al.*, 2015). The lower or higher pH conditions led to decrease in biosurfactant production which might be due the fact that the pH below or above the optimum

CONCLUSION

It can be concluded that the biosurfactant produced by *Enterobacter cloacae* AYP1 strain was rhamnolipid and have shown good stability at different conditions of pH, temperature and NaCl salt concentrations. During the stability testing highest emulsification activities were recorded at pH of 7, temperature of 45 °C and at of 6% NaCl. The production of rhamnolipid biosurfactant occurred at different physicochemical conditions and the optimum carbon concentration, pH,

precipitate the biosurfactant; causing its structure to become distorted and its activity lost. This is agreement with the work of Purwasena *et al.* (2019), who made similar observations. The optimum pH, temperature and incubation period used in this study have several precedence including Adamu *et al.* (2015) where pH 8, 45°C and 4% NaCl were observed to be optimum for biosurfactant production using *Bacillus sphaericus* EN3. This result is also in conformity with the findings of Colla *et al.* (2010).

temperature and incubation time of were 3%, 7.5, 40 °C and 96hrs respectively. Biosurfactant production is highly controlled by multiple factors and better knowledge of different production conditions can greatly improve their commercial production. Employing metabolic and cellular engineering approaches can lead to the improvement of bacterial strains for large-scale biosurfactant production.

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