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Heavy metal application of response surface optimized-lipopeptide biosurfactant produced by *Pseudomonas aeruginosa* strain CGA-02 in low-cost substrate

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Abstract

Cost-effective methods of biosurfactant production with minimal environmental impact are needed as global demand continues to increase. This study evaluated lipopeptide biosurfactant production in a *Pseudomonas aeruginosa* strain CGA-02 using a low-cost carbon substrate. The structural properties of the biosurfactant and applicability of the biosurfactant in heavy metal removal were evaluated. Response surface methodology (RSM) involving central composite design (CCD) was used to optimize process parameters to maximize biosurfactant production. The study identified sugar cane molasses and sodium nitrate as carbon and nitrogen sources of choice for bacterial growth and biosurfactant production, with a relatively 2.64-fold increase in biosurfactant yield under optimized conditions. Analysis of the biosurfactant measured a surface tension reduction of water from 72.2 ± 0.26 to 30.5 ± 0.2 mN/m at 40 mg/L critical micelle concentration. GC–MS and FTIR analysis revealed structural properties of the lipopeptide biosurfactant, with fatty acid components predominantly 9-octadecenoic acid (oleic acid), n-hexadecanoic acid, cyclotetrasiloxane and trimyristin, and infrared peaks belonging to amine, carboxyl, nitrile, alkanol, ether and carbonyl groups. Capture of heavy metals using the biosurfactant was evaluated in soil microcosms. Removal rates of 80.47, 100, 77.57, 100, and 97.57% were recorded for As, Pb, Hg, Cd and Cr respectively after 12 weeks of incubation. There was no significant difference ($p < 0.05$) in the removal efficiency of the biosurfactant and an analogous chemical surfactant, sodium dodecyl sulphate. First and second-order kinetic models described heavy metal removal rates by the biosurfactant. We demonstrate the production of a useful biosurfactant using low-cost waste carbon.

Article Highlights

- Lipopeptide biosurfactant was effectively produced by *Pseudomonas aeruginosa* strain CGA-02 in low-cost substrate under optimum conditions of 25g/L sugarcane molasses, 25g/L sodium nitrate, 1ml inoculum size and 60ml medium volume in 250ml flasks.

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- The lipopeptide biosurfactant efficiently enhanced removal of heavy metals (Arsenic, Lead, Mercury, Cadmium and Chromium) at a significant rate in a 12-week soil microcosm experiment.
- First-order and second-order kinetic were tested to describe the heavy metal removal rate by the biosurfactant. Predictions of the models, determined by the correlation coefficients, indicated that the heavy metal removal in this study was best described by the first-order reaction model with a correlation coefficient ranging (r2) from 0.88 – 0.95.

Keywords *Pseudomonas* · Biosurfactant · Lipopeptide · Optimization · Heavy metal · Response surface

1 Introduction

Biosurfactants are surface-active compounds of biological origin. They are commonly excreted by microorganisms during the stationary phase as metabolites [1], however, some studies have reported the production of biosurfactants during the active growth phase of microorganisms [2]. Biosurfactants can reduce interfacial and surface tension between liquids with different levels of polarity and enable immiscible liquids to form stable emulsions [3]. The global industrial interest in biosurfactant production continues to grow and in 2022, the global market for biosurfactants was valued at 1.2 billion USD. This market has been forecast to reach 1.9 billion USD by 2027 at an 11.2% compound annual growth rate (CAGR) [4]. The largest markets for biosurfactants are in cosmetic and personal care, pharmaceuticals, biomedical, agriculture and environment remediation industries [5]. Biosurfactants possess higher functionality as compared to chemical-based surfactants; they have high binding, solubilization, desorption and dispersion capacity, and are excellent emulsifiers and foam producers [6]. Biosurfactants are dependable, non-toxic, eco-friendly, versatile, and highly biodegradable. Regulations on synthetic surfactants and increasing demand for green solutions are market drivers for biosurfactant development.

Biosurfactants are often utilized in sustainable bioremediation of hydrocarbon- and heavy metal-polluted soil. Biosurfactants have been used for metal chelation, with the resultant formation of metal complexes that enhance metal mobility and facilitate metal removal from the soil matrix [7]. They can also transform metals to less toxic forms through detoxification, and eliminate toxic ones via mobilization, emulsification and solubilization [8]. Members of the microbial genera *Pseudomonas, Citrobacter* and *Burkholderia* (Gram-negative bacteria), *Bacillus* (Gram-positive bacteria), and *Candida* (yeast) are widely reported producers of biosurfactants with prospects for heavy metal removal [9–11].

The cost-competitiveness of the biosurfactant production process is a significant drawback to meeting global market demands. Current research has focused on the identification of robust microbial strains, low-cost substrates, and optimized production processes, including efficient bioreactor technology [12]. The use of renewable wastes as substrates for biosurfactant production is economical and contributes to waste management [13]. Common low-cost waste materials that have been used recently for biosurfactant production were identified by Singh et al. [14]. However, the required process parameters for biosurfactant production differ among different microorganisms and the selection of optimum parameters for production can be tedious. Optimization processes can be accelerated using techniques such as one-factor at a time (OFAT), design of experiments [15], artificial neural intelligence [16], and response surface methodology (RSM). The RSM allows multiple parameters to be handled simultaneously and generates a model that best describes the parameter combination giving optimum yield [17].

In this study, we optimized biosurfactant production from a strain of the bacterial species *P. aeruginosa* strain CGA-02 using commonly available, low-cost waste material as a carbon source. The physiochemical properties of the produced biosurfactant were analyzed, and the bioremediation potential of the produced biosurfactant was tested in soil microcosms contaminated with heavy metals.

2 Material and methods

2.1 Chemicals, reagents, and solvents

All chemicals, media and reagents used in this present study were of analytical grade. Kerosene was purchased from the local fuel station in Awka, Nigeria.

2.2 Sample collection

The two sampling sites used in this study are located in the Southern region of Nigeria. The soil sample used for the initial isolation of the biosurfactant-producing *P. aeruginosa* strain CGA-02 was an auto-mechanic workshop soil polluted with spent-engine oil in Awka, Anambra State (6°12′45.68″N; 7°04′ 19.16″E). The sampling site for heavy metal-contaminated soil was a crude oil refinery site in Okarki, Ahoada Local Government Area of River state (5°04′58.08″N; 6°38′ 59.32″E). The soil samples were collected in sterile sampling bags, transported to the Microbiology Laboratory, Nnamdi Azikiwe University, and stored in the refrigerator for further analyses.

2.3 Isolation of biosurfactant-producing bacteria

Biosurfactant-producing bacteria were isolated from the spent engine-oil-polluted soil sample using m-Cetrimide agar by a pour plating technique. Spent-engine oil-polluted soil was serially diluted tenfold and inoculated onto m-cetrimide agar plates. The plates were incubated at 25 ℃ for 48 h. Colonies were sub-cultured to obtain pure cultures.

Isolates were assayed for biosurfactant production in shake flasks with minimal salt medium (MSM) [18]. Isolates were added to 50 mL of MSM in a 250 ml Erlenmeyer flask and incubated at 25 ℃ for 72 h. Extraction of crude biosurfactant from the fermentation broth was performed using centrifugation at 7000 × g for 30 min, as described previously [19]. Supernatants were used for screening biosurfactant activity.

2.4 Screening tests for biosurfactant production

Two methods for biosurfactant assessment were performed, including an Oil Displacement Technique [20] and a Drop Collapse Method [20]. For the oil displacement technique, forty millilitres of distilled water was placed into a large petri dish and 15 µl of crude oil was overlaid on the surface of the water. Cell-free supernatant (10 µl) was gently placed on the surface of the oil film and allowed to stand for 1 min. The diameter of a clear zone on the oil surface was measured. Sodium dodecyl sulphate (SDS) was used as a positive control while distilled water served as a negative control. For the drop collapse method, the surface of a polystyrene microwell plate was first coated with 7 µl of mineral oil and maintained for 24 h at room temperature. 20 µl of cell-free supernatant was added to the well using a sterile micropipette and incubated at room temperature for 1 min. The drops were examined visually: drops containing biosurfactant collapsed and indicated a positive result, whereas drops without biosurfactant remained stable and indicated a negative result [21]. SDS and distilled water were used as positive and negative controls respectively.

2.5 Emulsification index (E24)

A solution containing 2 ml each of the cell-free supernatant and hydrocarbon (kerosene) was evenly mixed using a vortex mixer for 2 min to obtain an emulsion. The height of the emulsion layer was measured after 24 h to determine the emulsification index. The emulsification index (E24) was calculated by measurement of the height of the emulsion layer, divided by the total height, and then multiplied by 100 ($\frac{Height\ of\ the\ emulsion\ layer}{Total\ height\ of\ the\ mixture} \times \frac{100}{1}$), as described in [22].

2.6 Identification of the biosurfactant-producing isolate

The best biosurfactant producing isolate (strain CGA-02) based on emulsification index, drop collapse and oil displacement result was identified. Bacterial identification was performed using near-full length 16S ribosomal RNA (rRNA) gene amplicon sequencing, as described previously [23]. Amplicons were purified and sequenced bi-directionally on an Applied Biosystems 3730XL 96-capillary DNA sequencer. Read merging was performed using the software package CLC genomics workbench (v11.0.1). The merged reads were analyzed using web-based BLAST against the NCBI database [24].

2.7 Optimization of biosurfactant production by *Pseudomonas aeruginosa* strain CGA-02

2.7.1 Medium optimization on various carbon and nitrogen sources

The biosurfactant-producing *P. aeruginosa* was subjected to fermentation on different waste materials, including sugar cane molasses, spent lubricating motor oil, pulverized banana peel, pulverized potato peel, pulverized orange peel, and spent lubricating generator oil, as sole carbon source with different nitrogen sources (KNO_3, NH_4Cl, $NaNO_3$, $(NH_4)_2SO_4$) for the medium optimization using the one factor at a time (OFAT) method [25]. The fermentations were carried out in a minimal salt medium consisting of Basal medium, nitrogen source, 1% (w/v); carbon source, 2% (w/v); pH, 7.2. A 250 ml Erlenmeyer flask containing 50 ml of the fermentation medium was inoculated with 1 ml of the standardized seed inoculum (OD 600–1.00) and incubated on an orbital shaker (150 rpm) at 30 ℃ for 72 h. All incubations were performed in triplicate, and an un-inoculated flask served as a control. After fermentation, biosurfactant activity was determined by measuring the emulsification index (E_{24}).

2.7.2 Experimental design and statistical analysis of critical parameters influencing biosurfactant production

The software package Minitab (version 17) was used for experimental design and statistical analysis. Central composite design (CCD) was used to optimize critical media components. A 2^4 full factorial CCD for four test factors, each at five levels with eight-star points and seven replicates at the center points was employed to fit a second-order polynomial model. The independent parameters evaluated were the concentration of carbon source (X1) in the range of 5–25 g/L, the concentration of nitrogen source (X2) ranging from 5 to 25 g/L, inoculum size (X3) in the range between 1 and 3 ml, and medium volume in 250 ml flask (X4) ranging from 20 to 60 ml. A total of 31 experiments were conducted, with the emulsification index (E_{24}) as the measured experimental response. The results obtained were subjected to Analysis of Variance (ANOVA) to assess the significance of each factor on biosurfactant production. A probability value of < 0.05 was used as the criterion for statistical significance. The extent of variance that could be explained by the model was determined by the multiple regression coefficients (r^2 value). Optimum conditions were predicted by the response optimizer in Minitab, validated and recorded as mean ± standard deviation [26].

2.8 Recovery of the biosurfactant

P. aeruginosa was cultured under optimized conditions for 72 h in MSM for biosurfactant production. The biosurfactant was extracted by acid precipitation [27]. Briefly, cells were removed by centrifugation at 7000 × g for 15 min, and the supernatant was separated with a Whatmann filter paper. The cell-free supernatant was acidified to pH 4.0 using 6 M HCl and held overnight for precipitation. A 1:1 volume of methanol-chloroform was added to the white precipitate at room temperature to obtain crude extracts of the biosurfactant. The precipitate was recovered by centrifugation at 7000 × g for 30 min. The extracted biosurfactant was weighed after air drying on a pre-weighed Whatman filter paper, as described previously [18].

2.9 Surface tension measurement and determination of critical micelle concentration

The surface tension reduction capacity of the crude biosurfactant was determined with the aid of a Thomas Scientific CSC digital tensiometer [28]. Crude biosurfactant was added to 1 L of sterile distilled water in increasing concentrations (10–80 mg) and the surface tension was read in the tensiometer. Sodium dodecyl sulfate (SDS) was used as a control surfactant. The Critical Micelle Concentration (CMC) was determined by plotting the surface tension as a function of the biosurfactant concentration.

2.10 Characterization of produced biosurfactant

Fourier Transform Infrared Spectroscopy (FT-IR) was used to characterize biosurfactants using the method described in [29]. Briefly, a Buck scientific M530 USA FTIR instrument equipped with a detector of deuterated triglycine sulphate and beam splitter of potassium bromide was used for analysis. One mg of the extracted biosurfactant was mixed with 200 mg

KBr (Spectroscopic Grade), and the infrared spectra (with wave numbers ranging from 4000 to 400 cm^{-1}) were recorded in Shimadzu FT-IR-8400 spectrometer. The data collected were averages of 50 scans over the entire range.

Produced biosurfactants were also analyzed using Gas Chromatography-Mass Spectroscopy (GC–MS). Briefly, 1 µl of the extracted biosurfactant solution was injected into a GC–MS machine (Agilent Technology 5890, with a split detector and Mass Spectrometer Detector). Helium was used as the carrier gas at a constant flow of 1 ml/min and an injection volume of 1 µl, injector temperature 250 ℃, and ion-source temperature 280 ℃. Total GC running time was 90.67 min. Peaks were identified by a combination of references to their mass spectra and the NIST08 mass spectral database [30].

2.11 Activity of biosurfactant in heavy-metal contaminated soil

2.11.1 Heavy metal analysis of polluted soil samples

Heavy metal (cadmium, lead, chromium, arsenic and mercury) contents of the polluted soil were determined using an atomic absorption spectrometer (AAS; Agilent FS240AA) according to the method of APHA [31]. Polluted soil samples were air-dried, passed through a 50 mesh (0.297 mm opening), mixed with distilled water to obtain a 19% water content, and homogenized. Two g of each soil sample was heated in a furnace at 550 °C for 2 h and diluted with 20 ml of 20% H_2SO_4. Whatman filter paper No.1 was used to filter the suspension. The filtrate was placed into the AAS for measurement of cadmium, lead, chromium, arsenic and mercury content. Standard metal solutions of the metals of interest (Spectra AA standard) were obtained from Agilent Technologies, USA, and dilutions in the concentration range (2–6 ppm) were prepared and processed. A metal blank was prepared using all the reagents except the sample. Calibration curves for each metal were prepared by plotting the absorbance of standards versus their concentrations. Sample concentrations were calculated through standard regression analysis.

2.11.2 Enhanced heavy metal removal through biosurfactant application

Heavy metal removal using the biosurfactant produced by *P. aeruginosa* strain CGA-02 was investigated. Soil samples (400 g) were treated with 5% of the extracted biosurfactant in a plastic container by homogenously mixing the soil and a solution containing the crude biosurfactant. The mixture was kept at 25 ℃ for the 12-week experimental period. Sodium dodecyl sulphate and distilled water served as positive and negative controls, and AAS was used to measure concentrations of heavy metals, throughout the experiment at 4 week intervals.

2.11.3 Kinetic modelling of heavy metal removal

Rates of heavy metal removal were modeled by the first-order and second-order kinetics [32, 33]. The rate of heavy metal removal as a function of time were fitted with the models described in the following equations-

First order reaction:

Differential rate law: $\text{Rate} = \frac{-dC}{dt} = kC^1 = kC$

Integral rate law: $\text{Rate} = \frac{-dC}{dt} = kC$

Rearranging [34]

$$\begin{aligned} &= \frac{dC}{C} = -kdt \\ &\ln\left(\frac{C_t}{C_o}\right) = -kt \\ &: C_t = C_o e^{-kt} \end{aligned} \tag{1}$$

Half life $\left(t_{1/2}\right)$ is the time it takes for half of initial concentration of the heavy metal to be removed from the soil and is represented by the equation [34]:

$$C = \frac{1}{2}C_o$$

After a period of one half-life, $t = t_{1/2}$

$$\frac{C_o/_2}{C_o} = \frac{1}{2} = e^{-kt_{1/2}}$$
$$t_{1/2} = \frac{\ln 2}{k} = \frac{0.693}{k} \text{ (first order half – life)} \quad (2)$$

Second order reaction:
Differential rate

$$KC^2 = -\frac{dC}{dt}$$
$$\frac{1}{C_t} - \frac{1}{C_o} = kt \quad (3)$$
$$\therefore \frac{1}{C_t} = kt + \frac{1}{C_o}$$

At half-life [35]

$$kt_{1/2} = \frac{1}{\frac{1}{2}C_o} - \frac{1}{C_o}$$
$$t_{1/2} = \frac{1}{kC_o} \text{ (Second order half – life)} \quad (4)$$

2.12 Statistical analysis of data

All the experiments related to biosurfactant screening (i.e., emulsification index measurement, surface tension, and biosurfactant production) were performed in triplicate. Basic statistical analyses were performed using the software package SPSS (v 23.0), and results were presented as mean ± standard deviation (SD). The software package Minitab (v 17) was used to generate the experimental design for optimization and perform analysis of variance (ANOVA) and regression of the critical parameters affecting biosurfactant production. A p-value threshold of 0.05 was used for significance. The software package Prism (v6; GraphPad) was used for the kinetic modelling of heavy metal removal.

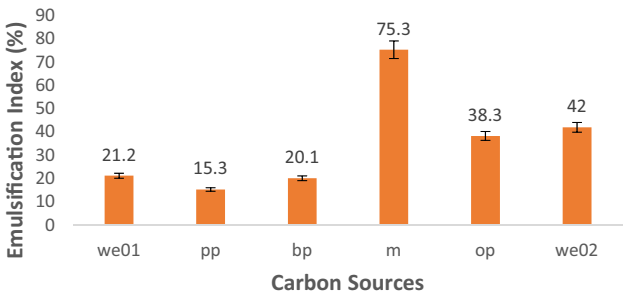

Fig. 1 Screening of waste materials as sole carbon source for biosurfactant production with sodium nitrate as a nitrogen source. Biosurfactants produced by bacterial strain CGA-02 were evaluated by measuring the emulsification index. The biosurfactant produced by strain CGA-02 under growth with sugar cane molasses had a significantly higher emulsification index (p-value = 0.002) relative to all other tested carbon sources. we01 = spent lubricating motor oil, pp = pulverized potato peel, bp = pulverized banana peel, m = sugarcane molasses, op = pulverized orange peel, we02 = spent lubricating generator oil. All conditions aside from carbon source were kept constant and experiments were conducted in triplicate

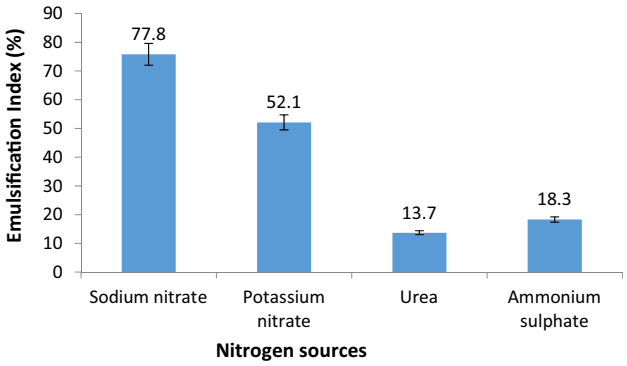

Fig. 2 Screening of nitrogen sources for biosurfactant production with sugarcane molasses as the carbon source. Biosurfactants produced by bacterial strain CGA-02 were evaluated by measuring the emulsification index. The biosurfactant produced by strain CGA-02 under growth with sugarcane molasses and sodium nitrate had a significantly higher emulsification index ($p = 0.001$) relative to all other tested nitrogen sources. All conditions aside from nitrogen source were kept constant and experiments were conducted in triplicate

Table 1 Parameter estimates of the quadratic response surface model for biosurfactant production by *Pseudomonas aeruginosa* strain CGA-02

Model term	Parameter effect	Coefficient	SD	T-value	P-value
Constant		47.87	3.21	14.91	0.000*
x_1	11.48	5.74	1.73	3.31	0.004*
x_2	5.20	2.60	1.73	1.50	0.153
x_3	− 10.95	− 5.48	1.73	− 3.16	0.006*
x_4	− 0.38	− 0.19	1.73	− 0.11	0.913
x_1*x_1	− 4.56	− 2.28	1.59	− 1.43	0.171
x_2*x_2	− 9.43	− 4.72	1.59	− 2.97	0.009*
x_3*x_3	5.19	2.60	1.59	1.63	0.122
x_4*x_4	2.74	1.37	1.59	0.86	0.401
x_1*x_2	14.30	7.15	2.12	3.37	0.004*
x_1*x_3	− 11.35	− 5.67	2.12	− 2.67	0.017*
x_1*x_4	15.35	7.68	2.12	3.61	0.002*
x_2*x_3	− 13.45	− 6.73	2.12	− 3.17	0.006*
x_2*x_4	− 6.35	− 3.18	2.12	− 1.49	0.154
x_3*x_4	2.40	1.20	2.12	0.56	0.580

*Statistically significant values

3 Results

3.1 Isolation of biosurfactant-producing bacteria

Biosurfactant-producing bacteria were isolated from oil-polluted soil using cetrimide agar as the isolation medium. The best biosurfactant-producing isolate (strain CGA-02), as evaluated in screening tests was selected and used in this study. The biosurfactant produced by isolate CGA-02 had a 77.1% emulsification index at 24 h, displaced oil in water by 1.7 ± 0.2 cm diameter and exhibited a positive drop collapse test result.

3.2 Identification of the biosurfactant-producing isolate

The identification of the biosurfactant-producing isolate was performed using16S rRNA gene amplicon sequencing analyses, as described previously *e.g.* in [23]. We identified the isolate as a *Pseudomonas aeruginosa*, with 100% identity to a number of other *P. aeruginosa* strains, including strains PA38182 (GenBank HG530068) and CGA1 (GenBank MT318155). The sequence was submitted to NCBI GenBank and assigned MW242749 as accession number.

Table 2 Analysis of variance(ANOVA) for the quadratic response surface model for biosurfactant production by *Pseudomonas aeruginosa* strain CGA-02

Source	DF	Adj SS	Adj MS	F	P
Regression	14	5987.51	427.679	5.92	0.001
Linear	4	1673.74	418.435	5.79	0.004
Square	4	1130.09	282.523	3.91	0.021
Interaction	6	3183.68	530.613	7.35	0.001
Error	16	1155.33	72.208		
Lack of fit	10	876.58	87.658	1.89	0.226
Pure error	6	287.75	46.459		
Total	30	7142.84			

P-value ≤ 0.05 is significant at 95% confidence level. Model Summary: S = 8.49754, R2 = 92.83%, R2 (adj) = 90.67%

DF Degree of freedom, *SS* Sum of squares, *MS* Mean square

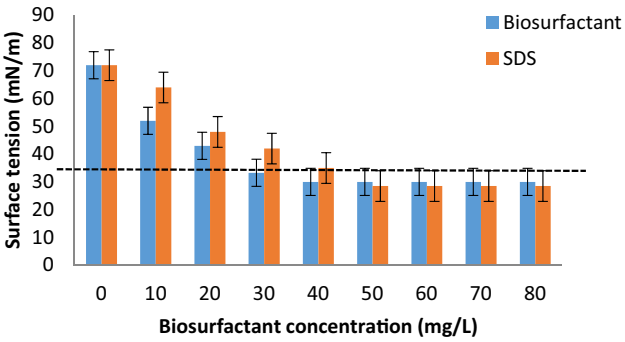

Fig. 3 Surface tension measurement showing the critical micelle concentration (CMC) for the biosurfactant produced by strain CGA-02 and a control compound, SDS. CMC for the biosurfactant was attained at 40 and at 50 mg/L for SDS

3.3 Optimization of fermentation conditions for biosurfactant production

In this study, one factor at a time (OFAT) method of optimization was first used as a preliminary optimization procedure to identify the best carbon (*i.e.*, waste materials) and nitrogen sources for biosurfactant production by *P. aeruginosa* strain CGA-02 in shake flask experiments. In the experiment with different waste materials as indicated in Fig. 1, fermentation with sugarcane molasses yielded a biosurfactant with significantly higher emulsion index (p-value = 0.002) as compared to biosurfactants produced using the other waste materials. As the biosurfactant produced by strain CGA-02 had nearly twice the emulsification index as compared to next highest carbon source, sugarcane molasses was selected as the carbon of choice for biosurfactant production by *P. aeruginosa*. Likewise, sodium nitrate was selected as the nitrogen of choice for biosurfactant production as it significantly (p = 0.001) improved biosurfactant yield well than the other nitrogen sources tested in the fermentation medium (Fig. 2).

Response surface methodology (RSM) optimization was used for the multiple regression analysis to investigate the impact of concentration of sugarcane molasses and sodium nitrate, medium volume and inoculum size on biosurfactant production by strain CGA-02. RSM simulation of the responses of 31 experiments (see supplementary information 1) performed during the response surface optimization of fermentation conditions yielded a regression model which described the interaction between the test parameters and the response as $Y(E24) = 47.87 + 5.74\ X_1 + 2.60\ X_2 - 5.48\ X_3 - 0.19\ X_4 - 2.28\ X_1^2 - 4.72\ X_2^2 + 2.60\ X_3^2 + 1.37\ X_4^2 + 7.15\ X_1X_2 - 5.67\ X_1X_3 + 7.68\ X_1X_4 - 6.73\ X_2X_3 - 3.18\ X_2X_4 + 1.20\ X_3X_4$ where, Y is emulsification index, X_1 is the concentration of sugarcane molasses, X_2 is the concentration of sodium nitrate, X_3 is inoculum size and X_4 is medium volume. The effects of the test parameters on the biosurfactant yield by strain CGA-02 are presented in Table 1. Concentration of sugarcane molasses (X_1) and inoculum size (X_3) individually affected biosurfactant production significantly ($p < 0.05$), while the concentration of sodium nitrate (X_2) and medium volume(X_4) did not affect biosurfactant production ($p > 0.05$). Interactions of concentration of sugarcane molasses and the other test parameters (concentration of sodium nitrate [X_1X_2] inoculum size [X_1X_3] and medium volume [X_1X_4]) showed significant effects on biosurfactant yield ($p < 0.05$) (Table 1).

Table 3 Fatty acid components of the biosurfactants by GC–MS

Fatty acid components	Relative abundance (%)	Formula
9-Octadecenoic acid	80.8	$C_{18}H_{34}O_2$
n-Hexadecanoic acid	4.5	$C_{16}H_{32}O_2$
Cyclotetrasiloxane	0.47	$C_8H_{24}O_4Si_4$
Trimyristin	3.94	$C_{45}H_{86}O_6$

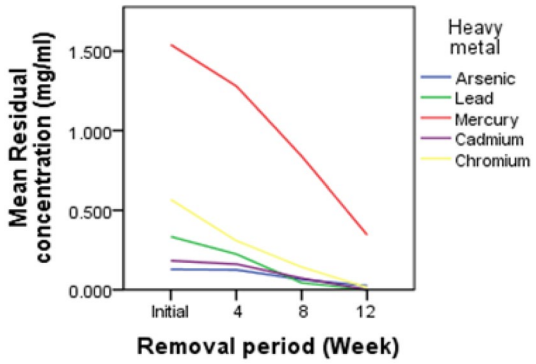

Fig. 4 Residual heavy metal removal patterns in the presence of lipopeptide biosurfactant. Lead and cadmium were removed most rapidly and completely. Starting concentrations of metals were: 0.128 ± 0.02 mg/ml for arsenic, 0.334 ± 0.04 mg/ml for lead, 1.54 ± 0.04 mg/ml for mercury, and 0.183 ± 0.02 mg/ml and 0.568 ± 0.07 mg/ml for cadmium and chromium respectively. Removal kinetics is shown in Table 4

The significance and validity of the regression model were tested using Analysis of Variance (ANOVA). The ANOVA results (Table 2) indicated that the regression model, linear, squares (quadratic terms) and interactions of the variables were statistically significant ($P < 0.05$). The 'lack of fit' test had a p-value = 0.226; hence, the regression model was adequately fitted to explain the interactions of the test variables. The regression coefficient (R^2), indicative of the validity of the regression model, was 92.83%. The R^2 adj had a value of 90.67%, suggesting agreement of the model with the obtained values for output responses.

Following the regression model, a response optimizer algorithm (Minitab) was used to predict optimum fermentation conditions. This yielded recommended conditions of 25 g/L sugarcane molasses, 25 g/L sodium nitrate, 1 ml inoculum size and 60 ml medium volume in 250 ml flasks. Using these optimized conditions, we obtained a biosurfactant yield of 9.28 g/L. This output was a 2.64-fold increase yield over the 3.52 g/L produced under un-optimized conditions of 10 g/L sugarcane molasses, 10 g/L sodium nitrate, 1 ml inoculum size and 50 ml medium volume in 250 ml flasks.

3.4 Surface tension measurement and determination of critical micelle concentration

The biosurfactant produced by strain CGA-02 under optimized conditions reduced the surface tension of distilled water from 72.2 ± 0.26 to 30.5 ± 0.2 mN/m (Fig. 3). There was no significant difference ($P < 0.05$) in surface tension reduction ability of the produced biosurfactant and SDS. Critical micelle concentration (CMC) was attained by the produced biosurfactant at 40 mg/L, no further surface tension reduction was observed at higher concentrations.

3.5 Characterization of produced biosurfactant

3.5.1 Fourier transform infrared spectroscopy (FT-IR)

The characteristic structural groups of the produced biosurfactant were identified using FTIR (See Supplementary Information 2). The absorption bands at 1072.255 and 1233.435 cm^{-1} were assigned to CO stretching vibration of ether. Strong bands at 3329.574, 3519.622 and 3805.485 cm^{-1} were assigned to OH stretching vibration of 1 and 2^0 alcoholic compounds respectively. Medium bands at 1647.643 and 3416.455 cm^{-1} were due to NH stretching vibration of 1 and 2^0 amine compounds. The absorbance bands at 2150.114, 2318.939 and 2444.273 cm^{-1} were assigned to COO stretching vibration of carboxylic acid, CO vibration of carbonyl compound and CN anti-symmetric vibration

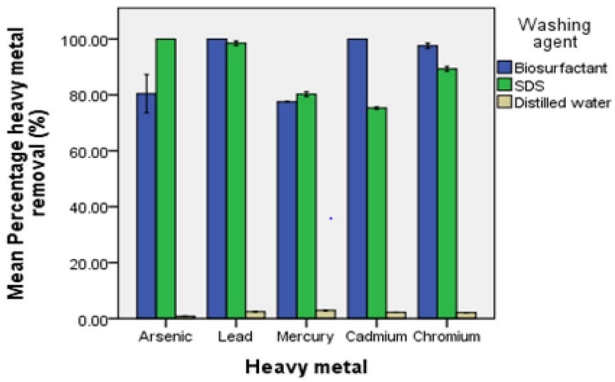

Fig. 5 Percentage heavy metal removal after 12 weeks. Removal efficiencies of the lipopeptide biosurfactant and SDS were 80.47 ± 3.41% and 100 ± 0.00%, 100 ± 0.00% and 98.47 ± 0.42%, 77.57 ± 0.08% and 80.3 ± 0.44%, 100 ± 0.00% and 75.33 ± 0.21% and 97.57 ± 0.49 and 89.37 ± 0.44% for arsenic, lead, mercury, cadmium and chromium respectively. % heavy metal removal was calculated by $= \frac{initial\ concentration - final\ concentration}{initial\ concentration} \times \frac{100}{1}$

Table 4 Kinetic model and linear regression analysis

Heavy metal	k_1 (w^{-1})	r^2	$t_{1/2}$(w)	k_2($kgmg^{-1}w^{-1}$)	$r_2{}^2$	$t_{1/2}$ (w)
As	0.14 ± 0.04	0.88	4.97	2.67 ± 1.00	0.78	0.26
Pb	0.29 ± 0.04	0.95	2.42	6.19 ± 2.19	0.79	0.11
Hg	0.12 ± 0.03	0.91	5.65	0.18 ± 0.07	0.79	3.88
Cd	0.29 ± 0.05	0.88	2.33	5.46 ± 2.78	0.73	0.13
Cr	0.19 ± 0.07	0.89	3.61	4.13 ± 1.79	0.66	0.17

K1 = first order kinetic biodegradation constant obtained as slope of the plot of ln(Co/Ct); k2 = second order kinetic biodegradation constant obtained as slope of the plot of reciprocal of Ct i.e. (1/Ct); Co = initial concentration, Ct = concentration at time (t) in weeks, r2 = correlation coefficient of first order kinetic,r22 = correlation coefficient of second order kinetic, t1/2 = half-life in weeks

of nitrile compound, respectively, whereas the weak bands at1388.193 and 1472.444 cm^{-1} were both assigned to C=C stretching vibration of ethene compound. The characteristic ether group, and the amine, nitrile, alkanol, carboxyl, carbonyl, and alkene composition of the biosurfactant suggest that it belongs to the lipopeptide group.

3.5.2 Gas chromatography-mass spectroscopy (GC–MS)

The relative abundance of fatty acid components of the biosurfactant were analyzed by GC–MS (Table 3). They include 9-octadecenoic acid, n-hexadecanoic acid, cyclotetrasiloxane and trimyristin. The dominant component was 9-Octadecenoic acid (oleic acid; 80.8% relative abundance), while cyclotetrasiloxane had the lowest relative abundance (0.47%). The mass spectrum from GC–MS analysis of the biosurfactant showing the peaks of abundant fatty acid components is shown in Supplementary Information 3.

3.6 Heavy metal analysis of soil samples

Atomic absorption spectrometry (AAS) was used for monitoring of the removal of heavy metals from contaminated soils incubated in the presence of the produced biosurfactant. The initial heavy metal concentrations of the polluted soil were As: 0.128 ± 0.02 mg/ml, Pb: 0.334 ± 0.04 mg/ml, Hg: 1.54 ± 0.04 mg/ml, Cd: 0.183 ± 0.02 mg/ml, Cr: 0.568 ± 0.07 mg/ml. Heavy metal concentrations over time are plotted in Fig. 4. The residual concentrations after 12 week treatment with biosurfactant were As: 0.025 ± 0.004 mg/ml, Pb: 0.012 ± 0.00 mg/ml, Hg: 0.350 ± 0.001 mg/ml, Cd: 0.011 ± 0.00 mg/ml, Cr: 0.014 ± 0.003 mg/ml. Removal efficiencies in the presence of biosurfactant and SDS after 12 weeks experiment were 80.47 ± 3.41% and 100 ± 0.00% for arsenic, 100 ± 0.00% and 98.47 ± 0.42% for lead, 77.57 ± 0.08% and 80.3 ± 0.44% for mercury, 100 ± 0.00% and 75.33 ± 0.21% for cadmium and 97.57 ± 0.49% and

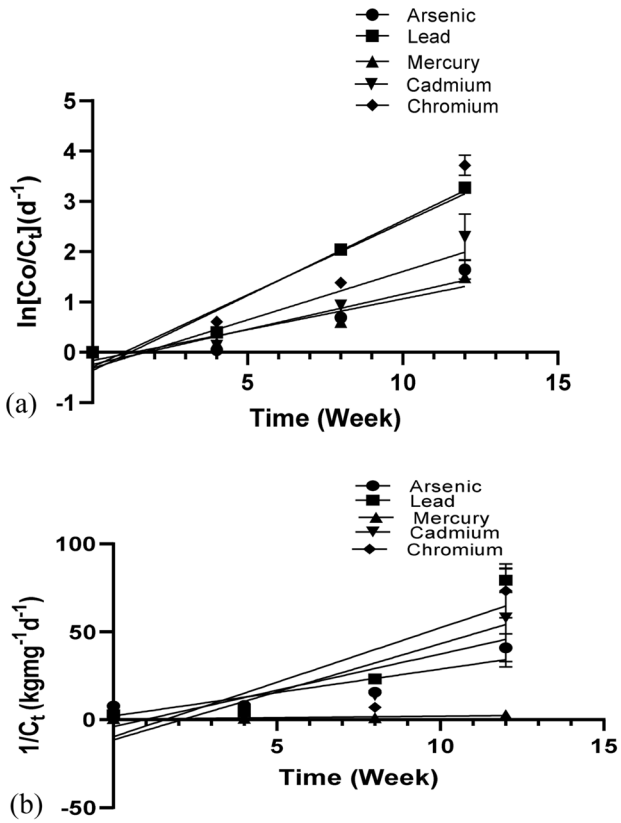

Fig. 6 (**a**) First order and (**b**) Second order kinetic of the heavy metal removal in the presence of biosurfactant

89.37 ± 0.44% for chromium respectively (Fig. 5). Lead and Cadmium were completely removed from the soil in the presence of biosurfactant, while arsenic was removed by SDS completely from the soil. There was no significant difference between heavy metal removal potential of the biosurfactant and the chemical surfactant (SDS) tested.

3.7 Kinetic studies of heavy metal removal in the presence of biosurfactant

The correlation coefficients (r^2) of the first order kinetics (ranging from 0.88 to 0.95) for the removal of the heavy metals were higher than those of the second order kinetics, which ranged from 0.66 to 0.79 (Table 4). Hence, the first order kinetics adequately fitted the data generated in this study. The data plots of $\ln C_o/C_t$ and $1/C_t$ (C_o = initial contaminant concentration, C_t = contaminant concentration at time (t) versus time in weeks (Fig. 6) resulted in linear functions in both reaction kinetic orders. The removal rate constant of cadmium (K_{Cd}) and lead (K_{pb}) of the first order kinetics obtained were relatively high with half-lives ($t_{Cr½}$) and ($t_{pb½}$) of 2.33 and 2.42 weeks respectively. The removal rate constant of the second order kinetics also showed that cadmium (K_{2Cd}–5.46) and lead (K_{2pb}–6.19) were removed relatively rapidly from the soil with half-lives of 0.13 and 0.11 weeks respectively. The rate of removal of mercury, in both reaction kinetic orders, had the lowest removal rate constants (1st order = 0.12 w^{-1}, 2nd order = 0.18 w^{-1}) and highest half-lives ($t_{Hg½}$) of 5.65 and 3.88 weeks for the first and second order kinetics respectively.

4 Discussion

In this study, a robust biosurfactant producing *P. aeruginosa* strain CGA-02 was isolated from contaminated soil. Growth optimization on various waste carbon streams identified sugarcane molasses as the optimal carbon source among available resources, and the produced biosurfactant was determined to be viable for sequestration of heavy metals from contaminated soil. We demonstrate here the viability of using process optimization for rapidly and inexpensively identifying valuable waste streams for microbial growth and biosurfactant production. Process conditions play a critical role in microbial production due to the vulnerability of microorganisms to environmental factors. For

biosurfactant production, detailed characterization of the process conditions can be used to determine relevant process factors influencing productivity and optimal yield by any given organism [36]. Carbon and nitrogen sources are basic factors for consideration in biosurfactant production, owing to their direct influence in the growth and production of biosurfactant molecules [37, 38]. In OFAT experiments, all factors but one are fixed, in order to obtain the condition of the free factor that gives the best yield [39]. In this study, the effects of six different waste materials as sole carbon source and four different nitrogen sources on biosurfactant yield was evaluated using OFAT. The selection of sugarcane molasses and sodium nitrate as carbon and nitrogen is consistent with prior studies [40, 41]. Sugarcane molasses, a waste from sugar production factories, is a low-cost substrate for biosurfactant production and contains high sugar content of approximately 48–56% [42].

Sodium nitrate provides an easily usable nitrogen source for microorganisms and has been previously used in biosurfactant production [43–45].

Although the use of OFAT is highly effective, when optimizing multiple conditions simultaneously, other approaches are preferred. We employed RSM to address further optimization once optimal available carbon and nitrogen sources had been selected. RSM is used to design experiments that simultaneously evaluate the interactions of several process factors and their impact on production yield [46]. We used RSM to optimize carbon and nitrogen concentrations, reactor volume, and inoculum size, leading to significantly increased yield in biosurfactant production (2.64-fold increase) relative to initial growth conditions. Optimizations such as these are extremely valuable and have previously been shown to be effective. For example, Abbasi and colleagues performed RSM optimizations leading to a 1.46-fold increase in biosurfactant yield by *Pseudomonas aeruginosa* MA01 [47]. In this study, optimization led to a final production of 9.28 g/L of biosurfactant, among the highest yields reported in the literature. For example, Rufino and co reported lipopeptide yield of 8.0 g/L by *Candida lipolytica* [48] and another study reported biosurfactant yields of 5.43 and 4.12 g/L by *Pseudomonas* species under optimized conditions using olive oil and soyabean oil as carbon sources respectively [49]. Optimization is essential as the yield of biosurfactant differs among microorganisms and lies heavily on identifying specific conditions of growth and production by each microorganism.

Biosurfactant ability to lower surface tension of aqueous solutions is an important property of surface-active agents [50]. Biosurfactants reduce both interfacial and surface tension and catalyze interaction between molecules with different polarity [51]. At critical micelle concentration of the biosurfactant, the lowest possible surface tension reduction is achieved, above which there is no observable tension reduction [1]. In this study, the surface tension of distilled water was reduced from 72.2 ± 0.26 to 30.5 ± 0.2 mN/m and at 40 mg/L, the biosurfactant attained CMC, indicating that application of biosurfactant produced by the isolate requires 40 mg/L to reduce about half the surface tension of distilled water. This was lower than the approximately 50 mg/L required for the control compound, SDS. In a similar study, biosurfactant produced by *P.putida* MTCC 2467 reduced medium surface tension from 74 to 35 mN/m^{-1} [52]. Another recent study reported that biosurfactant produced by a *Gordonia* spp. reduced the surface tension of aqueous media from 61.06 to 36.82 mN/m and attained a CMC at 250 mg/L concentration [53]. Thus, the biosurfactant produced by *P. aeruginosa* strain CGA-02 can be credited for excellent surface tension reduction.

Most species of *Pseudomonas* are found to produce glycolipid biosurfactants [54–56], while only a few has been reported to produce lipopeptides [18, 57, 58]. In this study, we investigated properties of the produced biosurfactant. FT-IR revealed hydrophilic moieties of the biosurfactant, and the ether, amine, nitrile, alkanol, carboxyl, carbonyl and alkene groups identified in the biosurfactant are characteristic components of lipopeptides [59]. Lipopeptide biosurfactants are the second largest group of biosurfactants [60] and have attracted attention due to low toxicity and biodegradability [61]. Although bacteria from the genus *Bacillus* are the most frequently recovered lipopeptide producers [62], a number of studies have characterized lipopeptides produced by *Pseudomonas* isolates. For example, Chauhan and colleagues [63]. Identified a number of different types of lipopeptides produced by *Pseudomonas* species, including viscosin, amphisin, tolaasin, milkisin, putisolvin and syringomycin. Similarly, we previously identified a lipopeptide from *P.aeruginosa* strain CGA1 which reduced surface tension of water to 35 mN/m and attained CMC at 60 mg/L [18]. In another study, a lipopeptide produced by *P. fluorescens* BD5 exhibited high emulsification activity and stability more than Triton X-100 and Tween 20 and reduced tension of waster from 72 to 31.5 mN/m [64]. Elsewhere, lipopeptide bioemulsifier produced by *P. nitroreducens* TSB.MJ10 emulsified xylene and petroleum alongside other hydrocarbons under environmental stress of pH, temperature and NaCl, and was effectively applied for the bioremediation of hydrocarbons in the marine environment [65].

GC–MS analysis was used to characterize the hydrophobic group composition of the biosurfactant in this study. Several fatty acid components were recovered. Recovery of these components as the fatty acid contents of the biosurfactants produced by *P. aeruginosa* in this study is consistent with prior studies [65–67]. The components produced by

P.aeruginosa strain CGA-02 are beneficial products applied in petroleum, cosmetic, food and pharmaceutical industries. For example, 9-octadecenoic acid (oleic acid) is a good emulsifying agent in soap, a moisturizer in creams [68], and a solubilizer in aerosol products [69]. n-hexadecanoic acid (palmitic acid), has excellent surface reducing properties [70]. Cyclotetrasiloxane is used as hair conditioner, skin conditioner and in other cosmetics as foaming agent [71]. Thus, the components produced by *P. aeruginosa* strain CGA-02 are valuable materials, not only in bioremediation and sustainable environment, but in pharmaceuticals, biomedicals, food and cosmetic industries. The biosurfactant produced by *P. aeruginosa* strain CGA-02 are most similar to that reported in another study [72] in which oleic acid was recorded as the most dominant fatty acid component in biosurfactant produced by *Pantoea alhagi* (76.26% abundance). Other microorganisms such as *E. coli* [73] and *Candida* [74] can biosynthesize oleic acid, but the *P. aeruginosa* strain CGA-02 in this study, produced biosurfactant with relatively higher abundance of oleic acid (80.8%). Further study is recommended on the direct syntheses of oleic acid and other organic acids from biosurfactants.

The heavy metal removal ability of the biosurfactant was compared with a chemical surfactant, SDS. SDS is an anionic surfactant and has shown excellent performance in heavy metal removal [75]. The biosurfactant produced by strain CGA-02 performed competitively well with the SDS, and this is attributed to the anionic nature of SDS and lipopeptides. Anionic lipopeptides readily react with metal ions, forming lipopeptide-heavy metal complexes, thereby enhancing metal mobility and resultant removal of the metal from the soil matrix [76]. There was no direct measure of the lipopeptide-metal complexes in this study; however, we recommend a direct quantification of the complexes in reaction mixtures as a confirmation of the removal activities by the biosurfactant. In a study [77], biosurfactant produced by *Candida* removed Pb, Zn and Cu at rates ranging from 30 to 80%. Removal of cadmium, lead, copper and zinc in the presence of a lipopeptide biosurfactant at rates of 44.2, 40.3, 26.2 and 32.07% respectively were recorded in another study [78]. Lipopeptide biosurfactant by *Paenibacillus* sp. D9 significantly removed heavy metals from wastewater and polluted sands and dispersed oil better than chemical surfactants [79]. In this study, the lipopeptide from *P. aeruginosa* strain CGA-02 removed heavy metals (arsenic, lead, mercury, cadmium, chromium) at relatively higher rates ranging from 75.33 to 100%.

Kinetic models describe the order of removal activity and evaluate the rate of heavy metal removal during bioremediation [32]. The removal half-life and kinetic orders were also the basis for assessment of efficiency of heavy metal potential of the biosurfactant. The removal constants (K), half-lives ($t½$) and correlation coefficients (r^2) vary in this study among the heavy metals tested, indicating the removal potential of the biosurfactant varies by heavy metal type. The half-lives for the various metals in both first order and second order reaction indicate fastest and most complete removal of lead and cadmium from the soil relative to other measured metals. A linear function was generated in the two reaction orders (Fig. 6); at increasing concentration of the biosurfactant, increased removal of the metal was observed. Therefore, predictions of the models, determined by the correlation coefficients, indicated that the heavy metal removal in this study was best described by the first order reaction model with correlation coefficient ranging (r^2) from 0.88 to 0.95. Several authors have reported the suitability of first order reaction for explanation of degradation and heavy metal removal kinetic [77, 80–82].

5 Conclusion

The results obtained in this study have proven that *P. aeruginosa* strain CGA-02 is an excellent lipopeptide producer, and that high-value biosurfactant can be produced using low-cost materials as growth substrates. Growth optimization through one-factor-at-a-time and response surface modelling dramatically increased biosurfactant yield, leading to a local optimum of 9.28 g/L of biosurfactant. The produced surfactant was largely composed of oleic acid and was effect in sequestration of multiple heavy metals from contaminated soil.

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Author contributions All authors contributed to the study conception and design. Material preparation, data collection were performed by CGA, ABI and VNA. Data analysis was done by CCE. The first draft of the manuscript was written by CMO, VIA and CCO. Review of the manuscript was done by IAE and SG, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Data availability The bacterial 16S rDNA sequence data was deposited to the NCBI GenBank database under accession number MW242749, and is available at the URL: https://www.ncbi.nlm.nih.gov/nuccore/MW242749.

Declarations

Competing interests The authors have no relevant financial or non-financial interests to disclose.

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