FABJ

Food and Applied Bioscience Journal

ISSN : 2286-8615
VOLUME 10 ISSUE 3
(SEPTEMBER - DECEMBER 2022)

FABJ

Food and Applied Bioscience Journal

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ISSN : 2286-8615
VOLUME 10 ISSUE 3
(SEMTEMBER - DECEMBER 2022)

FABJ
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Process parameter studies by central composite design of response surface methodology for production of biosurfactant by *Escherichia coli* khodavandi-alizandeh-2 isolated from hydrocarbon contaminated soil

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Submit: 30 August 2022, Received: 27 September 2022, Revised: 1 November 2022, Accepted: 23 November 2022, Publish online: 25 December 2022

Abstract

Biosurfactants synthesized by microorganisms are surface-active secondary metabolites that have gained industrial significance due to their interfacial tension-reducing properties. Hydrocarbon-polluted soils have proved a major habitat of biosurfactant-producing bacteria. This study explored the isolation of bacteria from petroleum contaminated soil and optimization of process parameters for biosurfactant production from *Escherichia coli* Khodavandi-Alizandeh-2 using response surface methodology (RSM) of Design Expert. Bacterial isolates were screened for biosurfactant production on mineral salt medium containing 1% automobile oil using various screening procedures (hemolysis test, oil displacement test, bacteria adhesion to hydrocarbon and emulsification assay). Parameter conditions (temperature, pH, carbon source, nitrogen source, agitation and inoculum quantity) were optimized for maximum biosurfactant yield. From several bacteria screened only one showed maximum hemolytic activity, 55% bacterial adhesion, and 50% emulsification activity. Molecular evolutionary genetic analysis of the isolate using 16S RNA gene sequence revealed to be *Escherichia coli* Khodavandi-Alizandeh-2. The optimization studies revealed that optimal biosurfactant production was obtained at 30°C temperature, 2 g glucose, 3 g yeast extract, 150 rpm Agitation, 2 mL inoculum quantity and pH 7. RSM further revealed that an increase in temperature at reduced pH will increase biosurfactant yield. The main chemical constituent of the biosurfactant produced as unveiled by Gas Chromatography Mass Spectrometry was Pyrrolo[1,2]pyrazine-1,4-

dione, hexahydro-3-(phenylmethyl) (63.22%). This research can find applications in bioremediation and hydrocarbon degradation.

Keyword: Central Composite Design; Biosurfactant; *Escherichia coli* Khodavandi-Alizandeh-2; Hydrocarbon contaminated soil

1. Introduction

Microbial biosurfactants are surface-active compounds with emulsifying properties that bring about a dissolution of microbial biofilms (Rani *et al.,* 2020). Surface-active chemicals generated by microorganisms can lower the interfacial tension between two immiscible fluid states (Mulugeta *et al.,* 2021). Different groups of microorganisms have been reported to be produced by biosurfactants namely phospholipids, glycolipids, lipopeptides, neutral fatty acids, and polymeric biosurfactants (Henkel and Hausmann, 2019). These utilize a broad range of organic compounds as carbon and energy sources for their growth and metabolism. This includes *Mycobacterium sp, Arthrobacter sp*. and *Rhodococcus erythropolis* that produce non-ionic trehalose monomycolates (Cazals *et al.,* 2020).

The type and quantity of microbial surfactants generated are mostly determined by the producer microorganism, other parameters such as carbon and nitrogen, aeration, temperature, and minor elements also affect the biosurfactant production (Sari *et al.,* 2019). Many studies have used a diverse range of carbon sources in the manufacture of biosurfactants. However, crude oil, glucose, diesel, sucrose, and glycerol have been known as exceptional carbon substrates for the synthesis of biosurfactants. It is obvious that carbon substrate is important in biosurfactant synthesis; however, its importance is organism dependent, as different carbon sources in the medium affect the composition of the biosurfactant production with *Pseudomonas sp*. (Nayarisseri *et al.,* 2018). Moreover, it has been reported that biosurfactants (surface active compounds) are being synthesized when the nitrogen source in the culture medium is depleted especially during the stationary cell growth phase (Adu *et al.,* 2020). Because variations in temperature, pH, aeration, or agitation speed can alter the outcome, optimizing the bioprocess is always important to get large quantities of biosurfactant. The majority of biosurfactant production is said to take place at temperature ranging from 25 to 30°C. However, in *Aspergillus paraffineus* and *Pseudomonas sp*. DSM-2874, the composition of the biosurfactant produced changed as a result of the temperature range. Among glycolipids which are biosurfactants, the prominent ones are trehalolipids and sophorolipids, rhamnolipids etc. Rhamnolipids are glycolipids with one or two molecules of hydroxydecanoic acid joined to one or two molecules of rhamnose (Henkel and Hausmann, 2019; Khare and Verma, 2020). Sophorolipids are glycolipids that are produced by yeasts and consist of a dimeric carbohydrate sophorose linked to a long-chain hydroxyl fatty acid by glycosidic linkage. Sophorolipid consists of carbohydrate sophorose linked to a long-chain hydroxyl fatty acid by glycosidic linkage and is often produced by microbial yeasts. Generally, glycolipid is a mixture of the lactone with six to nine different hydrophobic sophorolipids and has many applications (Nguyen *et al.,* 2020).

The biofilm contains extracellular material products and bacterial cells formed at the surface or confined within the matrix. Such biofilms on food-processing surfaces are possible means of contamination that open to disease transmission and food degradation. A critical step in the production of high quality biosurfactants and ensuring consumers' satisfaction is by controlling microbe adhesion to food-contact surfaces (Da Silva *et al.*, 2021). Certain prospective applications of biosurfactant in environmental control and pollution include hexa-chloro-cyclohexane degradation, microbial enhanced oil recovery (MEOR), hydrocarbon in the aquatic environment, and hydrocarbon degradation in the soil environment and removal of heavy metals from contaminated soil (Patowary *et al.*, 2018). Such applications of biosurfactant in microbial enhanced oil recovery lower interfacial tension, wetting of solid surfaces, reduction of oil pour point and viscosity, stimulating releasing of oil trapped within capillaries, and dissolution of oil (Nikolova and Gutierrez, 2021). The main objective of this research was to produce biosurfactants by microorganisms from petroleum-contaminated soil and optimize the condition parameters for the utmost production of biosurfactant and analyzed the chemical constituents of biosurfactant synthesized using Gas Chromatography Mass Spectrometry (GCMS).

2. Materials and Methods

2.1 Sample collection

Potential microorganisms were isolated from four different soil samples collected from mechanic workshops in Mowe, Obafemi Owode Local Government Area, Ogun State, Nigeria. Soil samples were packed in sterile bottles and transported to the laboratory.

2.2 Screening and isolation of biosurfactant producing bacteria

Isolation of the potential bacterial cultures capable of producing biosurfactant was carried out by using serial dilution technique and pour plate method on nutrient agar Medium. Plates were incubated at 37°C for 24 h. Pure cultures of the isolates were maintained on slants at 37°C and were sub-cultured from time to time to maintain viability in the laboratory.

The isolated bacteria were inoculated into a sterile mineral salt medium (MSM) containing NH_4CL 0.4, KH_2PO_4 1.0, K_2HPO_4 1.8, $MnSO_4.H_2O$ 0.04, $MgSO_4.7H_2O$ 0.04, $FeCl_3$ 0.01, $CaCl_3$ 0.001, and yeast extract 0.002 at pH 7.02 supplemented with 1 mL of automobile oil for enrichment and incubated in a shaking incubator for 24 h. The broth was incubated at 37°C for 168 h.

2.3 Emulsification assay

Culture broth was centrifuged at 10,000 rpm for 15 mins. Three (3) mL of supernatant were mixed with petroleum and vortexed for 2 min to homogenize both liquids. The emulsification activity was observed after 24 h. The emulsification activity was determined according to Bodour *et al.* (2004) adopting the formula given below the E24% was calculated

$$E24 = \frac{height\ of\ emulsion\ layer}{total\ height\ of\ liquid\ column} \times 100\%$$

2.4 Hemolytic activity

The blood agar was made up of nutrient agar containing 5% (v/v) defibrinated rabbit blood. The isolates were inoculated on blood agar plates and incubated for 48 h at 37°C. After incubation, plates were observed for zone of hemolysis to confirm production of biosurfactant.

2.5 Bacteria adherence to hydrocarbon (BATH) assay

Bacterial cells were washed twice with equal volume of buffer solution (K_2HPO_4, KH_2PO_4) at pH 7 and then were suspended in the same buffer salt solution and optical density (OD) taken at 620 nm. Petroleum was added and vortex for 3 min in test tubes. After vortex-shaking the crude oil and aqueous phase were allowed to separate for 2 h. OD of aqueous was then measured at 620 nm using spectrophotometer (Nayarisseri *et al.,* 2018). Percentage of cell adherence was calculated by the following equation:

$$1 - \frac{OD\ of\ aqueous\ phase}{OD\ of\ initial\ cell\ suspension} \times 100$$

2.6 Oil displacement test

Ten (10) µL of engine oil was added to the surface of 40 mL of distilled water in a Petri dish to form a thin layer. Then 10 µL of culture supernatant was gently placed on the oil layer. The presence of biosurfactant displaced the oil and a clear zone was formed (Morikawa *et al.,* 2000).

2.7 Optimization of studies of biosurfactant production

2.7.1 Experimental design

The biosurfactant synthesis was optimized using RSM provided by Design-Expert software 13.0 (Stat-Ease Inc. Minnepolis, USA). A standard RSM design tool known as Central Composite Design (CCD) was applied to study the reaction variables for biosurfactant production. Six independent variables are pH (6-8), agitation (100-200 rpm), temperature (25-35^{o}C); carbon (glucose) source (1-3 g); nitrogen (yeast extract) source (1-3 g); and inoculum quantity (1-3 mL) were employed. A central composite matrix with 5 levels was used and 13 runs were carried out in a random order (Table 1).

Table 1 Optimization parameter for the production of biosurfactant using experimental design

Std	Run	Factor 1 A:pH	Factor 2 B:Temp. °C	Factor 3 C:Glucose (g)	Factor 4 D:Yeast extract (g)	Factor 5 E:Agitation rate (rpm)	Factor 6 F:Inoculum quantity (mL)	Biosurfactant yield (g/mL)
12	1	7	30	2	2	150	3	3.453
13	2	7	30	2	2	150	2	1.234
4	3	7	35	2	2	150	2	2.435
9	4	7	30	2	2	100	2	1.674
10	5	7	30	2	2	200	2	2.298
8	6	7	30	2	3	150	2	4.253
6	7	7	30	3	2	150	2	2.296
3	8	7	25	2	2	150	2	1.098
2	9	8	30	2	2	150	2	0.424
5	10	7	30	1	2	150	2	0.896
7	11	7	30	2	1	150	2	0.456
1	12	6	30	2	2	150	2	1.567
11	13	7	30	2	2	150	1	0.678

2.8 Production and extraction of biosurfactant

MSM was prepared with 1% engine oil as supplement and inoculated with bacterial culture in a 100 mL conical flask and was incubated for 144 h. After incubation period extraction and emulsification assay was done. The production medium was centrifuged at 7000 rpm for 10 min and supernatant was collected in sterile flasks. Organic solvents i.e. chloroform and methanol (2:1 v/v) and 0.5 mL 6N HCl were added to the supernatant and kept at room temperature for 30 min. After centrifugation, the supernatant was collected in sterile flasks and placed on the rotary evaporator to obtain the dried crude biosurfactant.

2.9 GCMS procedures for identification of various structural analog

2.9.1 Sample preparation*:* Biosurfactant mixtures were separated and identification of various structural analog were done using GCMS (Agilent Technologies QP-2010SE and 6410 Triple Quad MS, USA). Samples were dissolved completely in methanol and were made up to the mark with methanol. They were transferred to GC vials prior to GCMS screening.

2.9.2 GC conditions: Shimadzu GCMS QP-2010SE was used. The stationary phase of separation of the compounds was HP-5 capillary column coated with 5% phenyl methyl siloxane (30 m length × 0.32 mm diameter × 0.25 µm film thickness) (Agilent Technologies). The carrier gas was helium gas was used at constant flow of 1.88 mL/min at an initial nominal pressure of 1.49 psi and average velocity of 35.9 cm/sec.

2.9.3 Procedures*:* Samples (1µL) were injected in split less mode at an injection temperature of 280°C and a total flow of 42.6 mL/min, gas saver mode was switched off. Oven was initially programmed at 90°C then ramped at 9°C/min to 290°C and held at 290°C for 6 min. Run time was 28.22 min with a 4.5 min solvent delay. The mass spectrometer was operated in electron-impact ionization mode at 70eV with ion source temperature of 260°C and transfer line temperature of 280°C.

2.10 Statistical analysis

The experimental data obtained from CCD were analyzed using RSM of Design-Expert Software 13 (Stat-Ease Inc. Minneapolis, USA). The second-order polynomial equation model for prediction of the optimal point between the response yield (biosurfactant yield) and the independent variables. The quality of fit model was evaluated by the coefficients of determination (R2) and its regression coefficient significant by analysis of variance (ANOVA). Response surface (3D) and contour plots were developed using the linear equation obtained from regression analysis of experimental data by keeping two of the independent variables at a constant value while changing the other four variables.

3. Results and Discussion

3.1 Isolation, screening and identification of microorganisms

From several bacteria isolated and screened based on the oil displacement, hemolytic, emulsification and BATH activity, only one (1) was detected to be a biosurfactant producing strain. The isolate was identified using 16S rRNA gene sequence and phylogenetic analysis. The genome sequences submitted using the FINCH TV program to Gen Bank at the NCBI database matched the nucleotide sequence. The nucleotide sequences showing similarity to the query were retrieved and a neighbor-joining phylogenetic tree was constructed.

The query organism was identified as *Escherichia coli* Khodavandi-Alizandeh-2 with Accession No MN186856 (Fig 1). The ability to isolate biosurfactant-producing microorganisms from petroleum-contaminated soil is taken to be evidence that those microbes are active components of the environment. This agrees with the earlier report of Hasan *et al.* (2018) which stated that bacterial strains, namely *E. coli, Pseudomonas flourescens, P. aeroginosa, Alcaligenes sp., Acinetobacter iwoffi, Flavobacterium sp., Bacillus subtilis, Corynebacterium sp.* and *Micrococcus roseus* are among microorganisms from the oil-polluted area. *E. coli* is a genus of non-spore-forming, gram-negative, rod-shaped bacteria, facultatively anaerobic (Westfall and Levin, 2018). Plate 1 shows the agarose gel electrophoresis of DNA fragment amplified by PCR prepared from the positive lipolytic organism. Amplicon contains a PCR product of DNA of *E. coli* Khodavandi-Alizandeh-2. Several bacteria were isolated from the petroleum contaminated soil samples, but after screening processes i.e., hemolysis test, oil displacement test, emulsification assay and BATH. *E. coli* Khodavandi-Alizandeh-2 was able to produce optimum amount of biosurfactant as compared to others. Different factors which affect the production of biosurfactant including pH, temperature, nitrogen source, carbon source were investigated for optimization. A preliminary determination of the biosurfactant production using an oil displacement test revealed the largest clear zone with maximum bacterial adherence to hydrocarbon of 55% (Fig 2) and emulsification activity of 50% (Fig 3). The isolated bacterial cultures from the soil were inoculated on MSM with 1% engine oil as its carbon source. This is with the agreement Yaraguppi *et al.,* (2020) that reported that the bacterial isolates utilize automobile oil as the sole source of carbon.

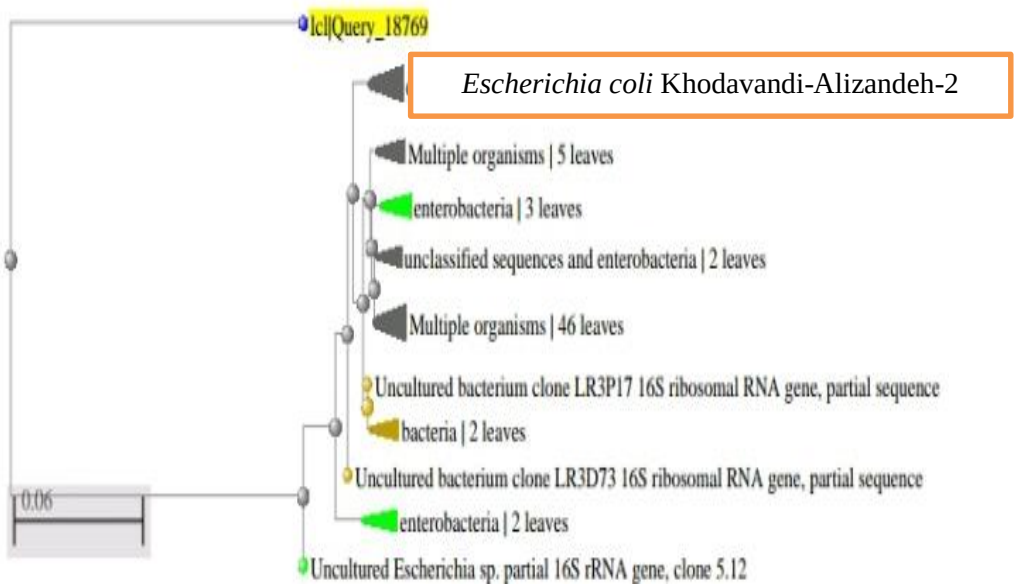

Fig 1 Molecularly identification by 16S rRNA gene sequencing and phylogenetic analysis of *Escherichia coli* Khodavandi-Alizandeh-2

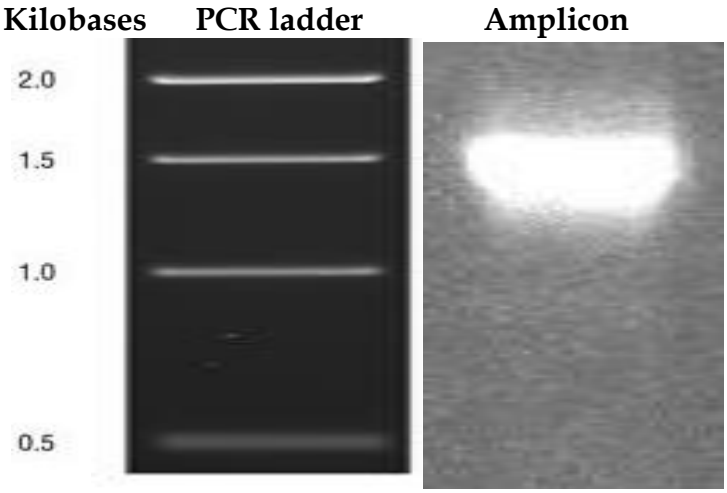

Plate 1 Agarose Gel Electrophoresis of DNA fragment amplified by PCR Amplicon contained PCR product of DNA of *Escherichia coli* Khodavandi-Alizandeh-2

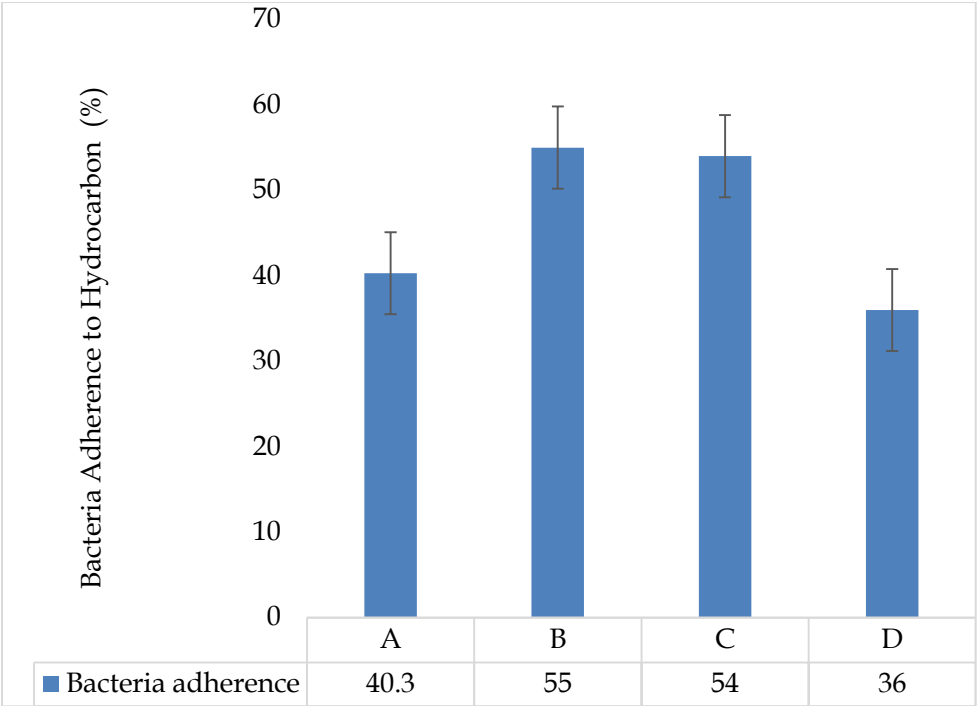

Fig 2 Bacteria adherence to hydrocarbon (BATH) of *E. coli* (B) and other strains. Error bar (standard deviation) of three samples

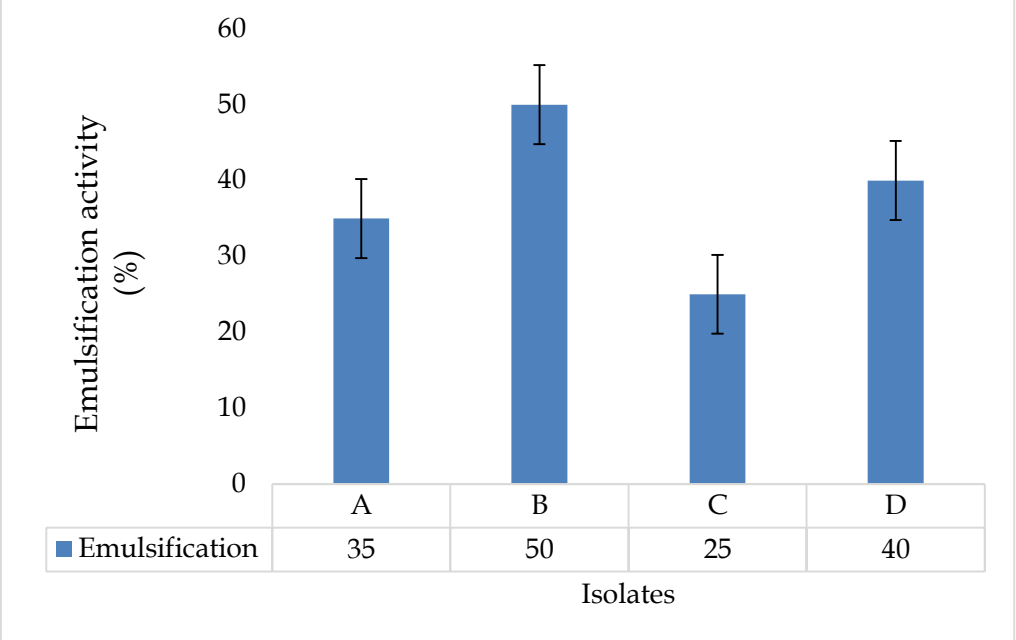

Fig 3 Emulsification activity of *E. coli* (B) and other strains. *Error bar (standard deviation) of three samples*

3.2 Characterization of the biosurfactant by GCMS

Fig 4 shown the GCMS chromatograph of biosurfactant produced by *E. coli* Khodavandi-Alizandeh-2. Structural components of the biosurfactant were identified in positive ion mode. It revealed five rhamnolipid congeners. The most prominent was pyrrolo[1,2]pyrazine-1,4-dione, hexahydro-3-(phenylmethyl) with 63.22%; methyl 8-methyl-decanoate - 8.42 %; l-(+)-ascorbic acid 2,6 dihexadecanoate, 14.82%; 8-ethyl-6,7-dimethyllumazine, 4.76% and bis-(2-ethylhexyl) phthalate, 8.78%. These were in comparison as obtained in earlier literatures (Abdel-Mawgoud *et al.,* 2010; Pantazaki *et al.,* 2011; Patowary *et al.,* 2017). Biosurfactant from *E. coli* Khodavandi-Alizandeh-2 strain that utilized glucose as sole carbon source produced mono-rhamnolipid and dirhamnolipid congeners of rhamnolipid biosurfactants which was quite similar to the one produced by *P. aeruginosa* PG1 strain of Patowary *et al.,* (2017) that utilized crude oil. Das *et al.* (2014) revealed that these congenators of rhamnolipid biosurfactants increased biodegradation of hydrocarbon pollutants and other composition of crude oil based on their physicochemical and microbiological properties on the available contaminants.

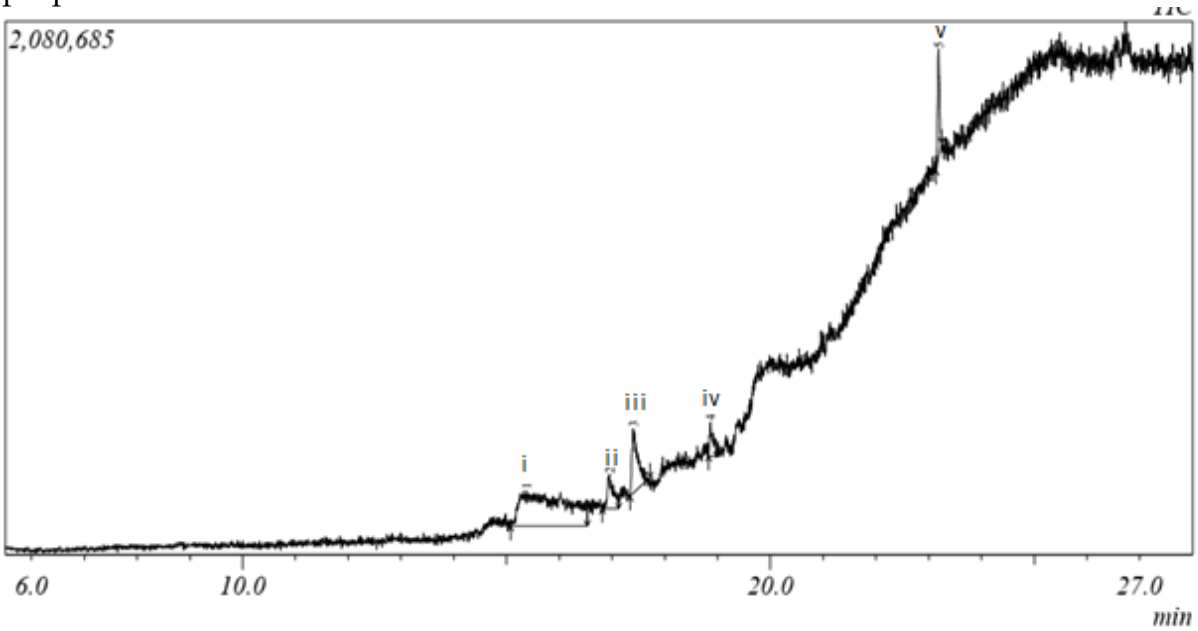

Fig. 4 GCMS chromatograph of biosurfactant produced by *E. coli* Khodavandi-Alizandeh-2. Peak (i). 8-ethyl-6,7-dimethyllumazine; (ii). methyl 8-methyl-decanoate; (iii). 1-(+)-ascorbic acid 2,6 dihexadecanoate; (iv). bis-(2-ethylhexyl) phthalate; (v) pyrrolo[1,2]pyrazine-1,4-dione,hexahydro-3-(phenylmethyl)

3.3 Optimization studies

3.3.1 Analysis of statistical model

The results obtained from the 13 experimental runs according to Design Expert 13.0 software are presented in Table 1, the results were analyzed and fitted to a second polynomial equation given by Equation 1.

$$Y = \beta o + \beta iXi + \beta iiXi^2 + \ldots\ldots\ldots\ldots\ldots\ldots \text{ (Equation 1)}$$

Where Y is response of (biosurfactant yield); β (0=intercept; i=linear; ii=quadratic and ij=interaction) and Xi, Xj (i=1, 4; j=1, 4; i ≠ j represents the coded independent variable) are the model coefficients.

Final equation in term of coded factors

Biosurfactant yield = $+1.80 -0.3652x_1 +0.4271x_2 +0.6485x_3 ++1.21x_4 +0.1994x_5 +0.8865x_6$

The Statistical Model Fit Summary suggested the quadratic model as a best fit-model. The data value was slightly aligned to the unit slope in the quadratic model implying that the model was the best model to represents the factor affecting biosurfactant yield. The actual vs. predicted biosurfactant yield plot (Fig 5) showed a close association between actual vs. predicted yield as the actual values were scattered near to the straight line.

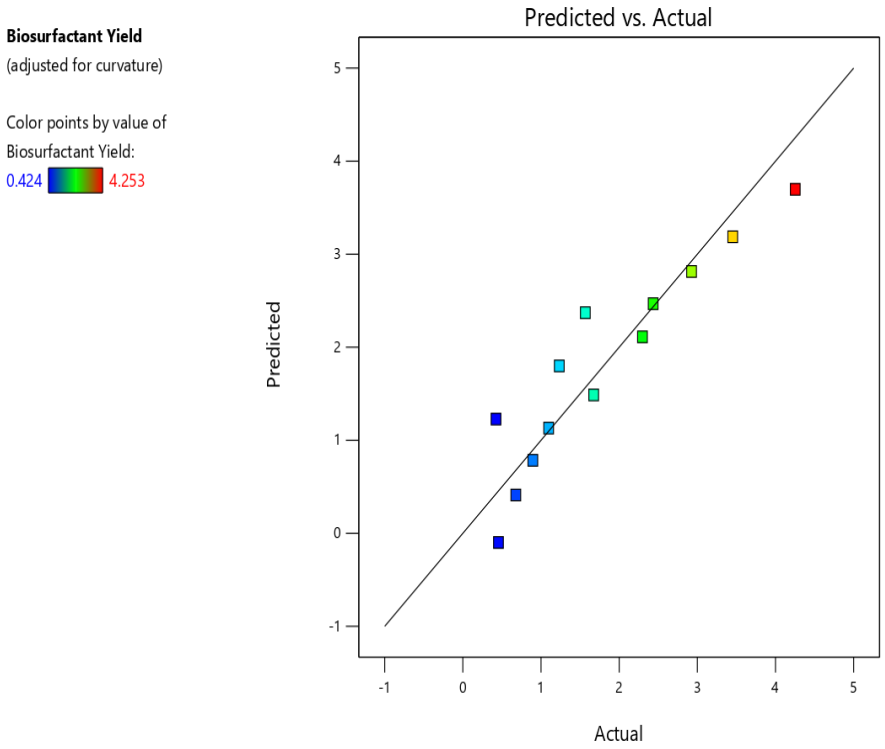

Fig 5 Predicted yield vs. actual yield of biosurfactant produced from *E. coli*

3.4 Influence of operating variables on biosurfactant yield

3.4.1 Influence of main effect variables on biosurfactant yield

Statistical analysis of experimental range studied identifies that temperature, nitrogen (yeast extract) source and inoculum quantity were all important with pH, carbon (glucose) source and agitation as the least significant factor on the biosurfactant yield as shown in Fig 6. pH as a single factor produced a negative effect on the yield as shown in Fig 6 (a), as pH increases the yield decreases, Fig 6 (b) shows that temperature has significant influence on the yield, the highest yield was observed at 35°C. Fig 6 (c) shows that carbon source (glucose) has significant effect on the yield, the highest yield was observed at 3 g. Nitrogen source (yeast extract) 3 g has significant influence on the yield as shown in Fig 6 (d), Fig 6 (e) indicated that agitation

does not have a pronounced effect on the yield, inoculum quantity has the highest influence at 3 mL as shown in Fig 6 (f).

The influence independent variable as well as the interactive variable on biosurfactant yield was studied using RSM provided by Design-Expert software 13.0. The study indicated that all independent variable and interactive variable have important significance on the increase and decrease of the biosurfactant yield. This corresponds to the research of (Al-Dhabi *et al*., 2020) where all the production variables are of significance in the production of biosurfactant. The presence of yeast extract as a nitrogen source increased the biosurfactant yield which agrees with the research of (Parthipan *et al.,* 2017). In this study, maximum biosurfactant yield was at 35°C and pH 7 which corresponds with the study of Asgher *et al.* (2020), this is possible because the optimum temperature for E. *coli* growth is 35-37°C according to Noor *et al, (*2013). Increase in temperature elevated biosurfactant yield, pH also influenced the biosurfactant production as maximum yield was at pH 7 which is similar to a study by Yaraguppi *et al*. (2020).

E. coli was isolated from petroleum contaminated soil by using NA and selected for the production of biosurfactant. The biosurfactant was produced using broth fermentation of MSM with 1% engine oil as a carbon source. The maximum biosurfactant yield was optimized using RSM design. The interaction of different factors influencing total surfactant yield were described and the biosurfactant was found to be produced optimally at a temperature of 35^{o}C and pH 7. The extraction of the biosurfactant was carried out at optimum condition to a yield of 4.3 g/mL. From literature study, it was found that gram negative bacteria are able to produce rhamnolipid type biosurfactant; therefore, *E. coli* is a rhamnolipid biosurfactant producing bacteria. Limited literature was found regarding biosurfactant production by *E. coli*, which indicates the fewer studies on the capability of this isolate in producing biosurfactant.

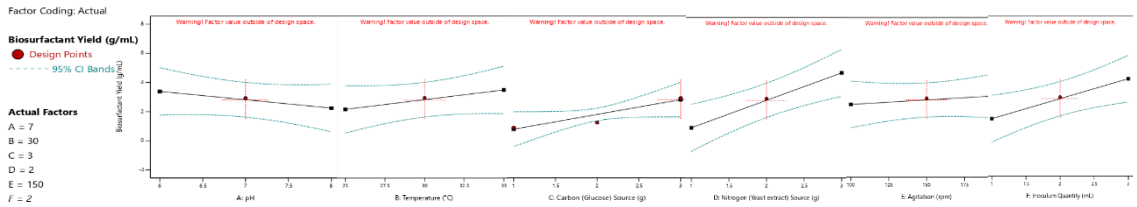

Fig 6 (a-f) Plots of main effects of the biosurfactant yield Cube plot (Fig 7a) shows how three factors combine to affect the biosurfactant yield response. All values shown are predicted values, thus allowing plots to be made even with missing actual data. Because the factors of interest are A (pH), B (temperature) and C (carbon source (glucose)). Biosurfactant yield was maximum at settings A+ (6), B- (35°C) and C- (3 g) (upper left corner with predicted response 4.05438 g/mL). Fig 7b showed the 3D plot for the interaction between temperature and pH. The 3D response surface revealed the increment of temperature from a low level to high level leads to increase of biosurfactant yield with reduced pH. The simultaneous dependence of biosurfactant yield on pH and temperature was shown in Fig 7b. The other four process parameters, carbon source (glucose), nitrogen source (yeast extract), agitation and inoculum quantity were fixed at 2 g, 3 g, 150 rpm and 2 mL respectively. At pH 6 to < 6.5 and

temperature of 33.3°C to 35°C caused significant increment in biosurfactant yield to 4.5 and then decreases as the pH increases from 6.5 to 8 this indicates that the best yield can be ascertained at pH of 6 to < 6.5 and temperature of 33.3°C to 35°C (Fig 7c).

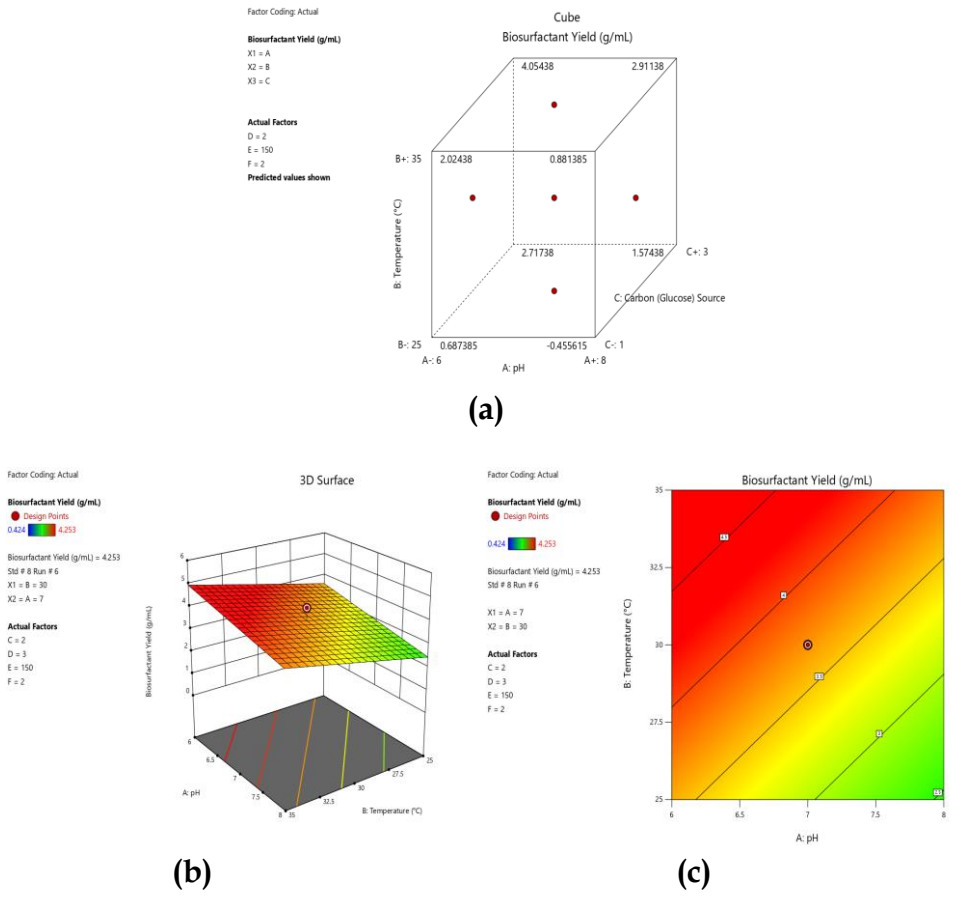

Fig 7 (a) Cube plot showing the effect of pH, temperature, and carbon (glucose) source on biosurfactant yield at the center point of nitrogen (yeast extract) source (2 g), agitation (150 rpm); (b) 3D surface plot and (c) interactive contour plot of biosurfactant yield as a function of pH and temperature: glucose (2 g), yeast extract (3 g), agitation (150 rpm), inoculum quantity (2 mL).

4. Conclusion

Biosurfactants, mostly produced on microbial cell surfaces are active and amphiphilic molecules that have several applications in cosmetics, petrochemical and food industries. Their important role also in control of oil spillage and detoxification of oil contaminated industrial effluent confers significance for its production and characterization. This study optimized parameter studies of response surface methodology for biosurfactant production using *E. coli* Khodavandi-Alizandeh-2 isolated from hydrocarbon contaminated soil. As a result of disparity in the pH, carbon and nitrogen sources, temperature, agitation rate that can change the after-effect, optimizing the process parameters was engaged to get large quantities. Chemical analysis of the compound was determined to ensure compliance of their bioactivity thereof. The biosurfactant produced during the degradation process has potential to reduce the surface tension of the culture medium.

Acknowledgement

The authors wish to thank the technical staff of the Department of Biological Sciences in person of Mr. David Adeagbo, Mr. Steve Jacob for their assistance.

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