Characterization of Biosurfactant From *Pseudomonas Stutzeri* SJ3 for Remediation of Crude Oil-Contaminated Soil

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Abstract

In the present work, production of biosurfactant was studied from the bacterial strains isolated from the soil samples collected from oil contaminated sites in Karaikal ONGC, Puducherry, India. Six morphologically different hydrocarbonoclastic bacterial strains (SJ1-SJ6) isolated on oil agar plates were further screened for biosurfactant production. Based on the screening methods results of 26 mm oil displacement zone, positive results of drop collapse test, 68.14% emulsification index (E24) and 79.2% of bacterial adherence percentage, the isolate SJ3 was selected as the most potent strain and it was identified as *P. stutzeri* using standard biochemical and 16S rRNA gene sequencing-based methods. Optimization of the *P. stutzeri* strain showed 36 h incubation, 150 rpm agitation, pH 7.5, 37^{o}C, 1% salinity, 2% glucose as carbon source and 1% yeast extract as nitrogen source were the ideal conditions for growth and the biosurfactant production was found to be growth dependent. The crude biosurfactant showed broad range of antibacterial activity against the bacterial pathogens tested. The *P. stutzeri* isolated from oil spill site showed biosurfactant with antibacterial activities.

1. Introduction

The revolution of modern industries and their activities has led the world affecting in many ways like soil and water pollution. Exploration, transportation, consumption, spills and disposal of petroleum hydrocarbons like crude oil, diesel and gasoline in the aquatic and terrestrial ecosystems are frequently contaminated by the petroleum hydrocarbon pollutants cause serious ecological problems (Okoh, 2006). Even though mechanical and chemical methods reduce these issues to some extent, these practices are often expensive, time consuming and not ecofriendly (Mandri and Lin2007). Bioremediation is a method of choice to remove the hydrocarbon pollutants in the environment. Microbes produce surface active compounds as extracellular secretions or cell wall bound molecules (Plaza *et al.*, 2006). Hydrocarbon degrading bacteria effectively degrade the hydrophobic substrates by the production of biosurfactants.

These microbial-derived biosurfactants are amphiphilic molecules produced by bacteria, fungi, yeasts and algae as extracellular component or on microbial cell surfaces. They have both hydrophobic and hydrophilic moieties that reduce the surface tension and interfacial tensions between the surfaces of the molecules and interface. The classes of the biosurfactants are mainly glycolipids, lipopeptides, lipoproteins, fatty acids, phospholipids and polymeric forms (Maneerat and Dikit, 2007) and they are mainly secreted as secondary metabolites and play vital role in the survival of the biosurfactant producer (Singh and Cameotra, 2004).

Biosurfactants are advantageous over its chemical counterpart like low toxicity, higher biodegradability and environmental compatibility and lower critical micelle concentration, easy production and synthesis from renewable sources, higher activities in various extreme conditions (Mukherjee *et al.*, 2006). Biosurfactants are also having their applications in cosmetic, food, pharmaceutics, agriculture aspects (Makkar and Cameotra, 2002). Thus, the present study was on isolation of a potential biosurfactant producing bacterium from the oil spill site.

2. Materials And Methods

2.1. Isolation of hydrocarbonoclastic bacteria

100g of oil contaminated soil samples from different locations in harbor, Karaikal ONGC, Puducherry, India was aseptically collected in sterile containers and transported to the laboratory and processed immediately. 1g of each sample were serially diluted and 10^{-4} dilution was spread on Bushnell Hass agar medium prepared in seawater supplemented with 1% crude oil as the only carbon source (Bushnell and Hass, 1941) and the plates were incubated at 30°C for 7 days. After incubation, morphologically different hydrocarbonoclastic bacterial colonies were streaked on Zobel Marine agar slants and used for further screening.

2.2. Screening for biosurfactant production

The six hydrocarbonoclastic isolates named as SJ1, SJ2, SJ3, SJ4, SJ5 and JS6 were further screened for biosurfactant production using different methods such as oil displacement test (Morikawa *et al.*,1993), drop collapse test (Youssef *et al.*, 2004), BATH (bacterial adhesion to hydrocarbon) assay (Rosenberg *et al.*, 1980) and emulsification index (EI24) (Cooper and Goldenberg. 1987) to select the most potent strain.

Oil displacement assay

20µl of crude oil was added to the surface of 25 ml of distilled water containing Petri plate followed by adding 10µl of cell free culture broth. If biosurfactant is present in the cell free broth; the crude oil will be displayed with the oil free clear zone is formed. The potent strain was selected based on the highest size of zone diameter.

Drop collapse test

A clean glass slide was covered with few drops of crude oil. After complete spread of the oil, a drop of cell free culture supernatant was added, the disintegration of the drop indicates positive result.

Determination of emulsification index

Emulsification index (E24) of the biosurfactant produced by the isolates was measured by mixing equal amount of cell free culture supernatant with crude oil and stirred well for 2 min then the height of the emulsification layer (EL) was measured. E24 is the ratio of the height of the EL and total height of the liquid (THL) after 24h of incubation. E24 = EL / THL×100.

BATH (Bacterial adhesion to hydrocarbons) assay

Initial optical density of cells in the mineral salt medium was determined initially at660nm using the method described by Rosenberg *et al.*, 1980. 0.7ml of crude oil was mixed with 10 ml of suspension containing known density of cells (10^8 CFU/ml). After mixing for 15 min and let it stand for 30 min; the oil

and water layer was allowed to separate. The aqueous phase was removed and OD was measured again. The percentage of bacteria adhered to the oil was calculated by using the formula:

Percentage (%) of bacterial adherence = [1-(OD shaken with oil / OD original)] x 100

2.3. Identification of potential biosurfactant producing SJ3 strain

Based on screening, the isolate SJ3 was selected as the most potent strain and it was identified using biochemical methods using Bergey's Manual of Systematic Bacteriology (Bergey *et al.*, 1974) and 16S rRNA sequencing based identification using universal Eu-bacterial primers 27F (5'AGAGTTTGATCCTGGC TCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'). Sequence similarities were searched using BLAST. Phylogenetic tree was analyzed as described by Kumar *et al.*, 2016 using MEGA software version 7.0.

2.4. Optimization and mass culture of *P. stutzeri* (SJ3)

Growth of the most potent biosurfactant producing *P. stutzeri* (SJ3) strain was optimized using one parameter at a time approach for different physicochemical parameters. As the biosurfactant production was found to be growth dependent, growth was estimated in this optimization processes. Emulsification index (E24) for the production of biosurfactant was estimated. The tested parameters were incubation periods 0–48 h(with 6 h interval), agitation (0-200 rpm), pH 4–10 (with 0.5 interval), temperatures (25, 30, 35, 37, 40 and 45°C), salinity (NaCl concentration – 0.5-3% with 0.5 interval), different carbon sources (sucrose, glucose, maltose, starch and cellulose); ideal carbon source glucose (0.5-3%) and nitrogen sources (beef extract, yeast extract, peptone, ammonium sulphate, ammonium nitrate and sodium nitrate); ideal nitrogen source yeast extract (0.5–2.5%) using crude oil as substrate (1%) were tested. Ideal conditions from the above optimization such as 36 h incubation, 150 rpm agitation, pH 7.5, 37^{o}C, 1% salinity, 2% glucose as carbon source and 1% yeast extract as nitrogen source were used for mass culturing in a 1L conical flask containing 500 ml of medium.

2.5. Effect of different carbon sources on biosurfactant production by *P. stutzeri* SJ3

Apart from crude oil, seven different oil samples (1% each) such as diesel, petrol, vegetable oil, tamanu oil, peanut oil, sesame oil and *Pongamia pinnata* oil were tested as a sole carbon source with optimized conditions in 200ml of medium.

2.6. Recovery of biosurfactant

The cell free culture supernatant from each culture was obtained by centrifuging the culture at 12000 rpm for 20 min. followed by acid precipitation of the biosurfactant at pH 2 by adding 6N HCl and incubation at 4^{o}C for overnight. The acidified biosurfactant precipitate was collected by centrifugation culture at 12000 rpm for 20 min. followed by neutralization using phosphate buffer (pH 7), extraction using equal volume of ethyl acetate and dried using a rotary vacuum evaporator and was tested for biosurfactant activity.

2.7. Antibacterial activity of the biosurfactant from *P. stutzeri* SJ3

The recovered each biosurfactant was tested for antibacterial against six different clinically important bacterial pathogens like *Vibrio cholerae, Shigella boydii, Vibrio fluvialis, Shigelladysenteriae, Salmonella typhi* and *Salmonella paratyphi*. Each strain was cultured in Muller-Hinton broth at 37^{o}C for overnight and the cell density was adjusted to 10^{8} CFU/ml (0.5 McFarland standard) and the activity was tested using well diffusion method on Muller-Hinton agar plates. 6mm wells were made with the sterile pipette tips. 50µl of crude cell free supernatant containing biosurfactant was loaded into the each well. Plates were incubated at 37^{o}C for 18–24 hrs and the formation of clear zone around wells were measured and noted.

Fourier transform infrared (FT-IR) spectroscopy analysis of biosurfactant

The chemical structure and the types of functional groups (bonds) of the crude biosurfactant were determined using FT-IR analysis according to the method described by Bezza and Chirwa (2015). 1 mg of the crude biosurfactant was dried in a freeze dryer and ground with 100 mg of KBr and pressed for 30 sec. to get translucent pellets. Then it was analysed in a FTIR instrument (Thermo Niocolet, AVATAR 330 FT-IR system), with the spectrum range of 450–4000 cm^{-1} at a resolution of 4 cm^{-1}. The data obtained from the analysis were corrected for the background spectrum.

3. Result And Discussion

3.1. Isolation of hydrocarbonoclastic bacteria

In the present work soil samples collected from the oil contaminated harbour sites in Karaikal ONGC, Puducherry, India was used for the isolation of hydrocarbonoclastic bacteria using oil agar plate. Six morphologically different colonies on the oil agar medium were further selected for the screening of biosurfactant production. As in the present work, several other has reported the production of biosurfactants from marine environment (Wu *et al*., 2019; Kurniati et al., 2019 and Domingues et al., 2020). Microbial communities in the contaminated area are well adopted to utilize and convert that contaminant for their survival (Mancnaughton *et al*., 1999).

3.2. Screening for biosurfactant production

Screening for selection of potential biosurfactant producing isolate from the six hydrocarbonoclastic isolates named as SJ1, SJ2, SJ3, SJ4, SJ5 and JS6 were done using different screening methods like as oil displacement test, drop collapse test, BATH (bacterial adhesion to hydrocarbon) assay and emulsification index (E24). The results showed that the isolate SJ3 was the most potential one based on the following results; oil displacement showed 26mm of zone, positive for drop collapse test, 68.14% emulsification activity (E24) and BATH assay showed 79.2% bacterial adherence percentage and hence it was selected for further study. Emulsification activity is considered as one of the important criteria for the screening of biosurfactant producing strains (Rosenberg *et al*., 1979; Juwarkar and Khirsagar, 1991 and

Carrillo et al., 1996). Molecules from microbe which exhibits emulsifying and high surface activity are classified as biosurfactants and these molecules are considered as potential bioremediation agents because of their surface and interfacial tensions reducing properties in both aqueous solutions and hydrocarbon mixtures (Banat et al., 2000). Thavasi *et al.*, 2008 used BATH assay-based screening for the isolation of biosurfactant producers.

3.3. Identification of potential biosurfactant producing SJ3 strain

Based on the various biochemical methods and 16S rRNA gene sequencing based molecular method, the most of potential biosurfactant producing isolate SJ3 was identified as *Pseudomonas stutzeri* (Table 1 and Fig. 1)

Table 1
Biochemical identification of *Pseudomonas stutzeri* (SJ3)

Test	Results
Gram´s staining	Negative
Morphology	Rod
Motility	Positive
Catalase	Positive
Indole	Negative
Methyl red	Positive
Voges Proskauer	Negative
Citrate utilization	Positive
Urease	Negative
Starch hydrolysis	Positive
Gelatin hydrolysis	Positive
Esculin hydrolysis	Negative
Glucose	Positive
Lactose	Negative
Sucrose	Positive
Maltose	Negative
Mannitol	Positive
Oxidase	Positive
Mannitol	Positive
Galactose	Negative

3.4. Optimisation studies

Optimization revealed that the ideal conditions favored the maximum growth of the potential biosurfactant producing isolate *P. stutzeri* were 36 h incubation, 150 rpm agitation, pH 7.5, 37^{o}C, 1% salinity, 2% glucose as carbon source and 1% yeast extract as nitrogen source (Fig. 2). Tripathi *et al.*, 2019 reported 96 h as the ideal incubation period for the growth of a rhamnolipid biosurfactant producing *Marinobacter* sp. MCTG107b strain. Bicca *et al.*,1999 observed the ideal conditions required for the production of a biosurfactant producing *R. ruber* AC 239 isolate were 37°C, 200 rpm, initial pH 7.0 and 1% Diesel (v/v). In the present study, biosurfactant production was found to be growth dependent. When

different oil sources were tried for the production of biosurfactant using the SJ3 isolate crude oil was found to be best source of followed by diesel, petrol and pongamia oil, peanut oil, sesame oil and tamanu oil. Moshtaghet al. 2018 produced biosurfactant using brewery waste as the sole carbon source. **Previous studies have reported the production** of biosurfactant from *Pseudomonas* species; *P. aeruginosa* (Cooper et al., 1981, Camara *et al.*, 2019 and Khademolhosseini *et al.*, 2019).

3.5. Recovery and FTIR analysis of biosurfactant

The recovery of biosurfactant from the mass culture medium was recovered by using acid precipitation followed by ethyl acetate extraction gave high recovery rate and biosurfactant activity. The recovered crude biosurfactant was analyzed using FTIR spectroscopy and the results showed the presence of different functional groups. Wave numbers 3325.13, 3298.28 and 3267.60 showed N-H stretching represents a strong H bonding whereas the wavelength at 1624.23 showed presence C = O bond presence of carbonyl group. Wave numbers 759.40, 698.11, 652.01 and 623.25 showed the methylene scissoring vibrations from the proteins (Fig. 4). Wu *et al.*, 2012 and Silva *et al.*, 2017 showed that, the biosurfactants produced by Pseudomonas have the capacity to utilize n-alkanes and aromatics. Wu *et al.*, 2019 found the characteristic of peptides bands at 3415 – 3449 cm^{-1} with the N – H stretching and C = O bond stretch absorbance at 1654 cm^{-1} showed carbonyl group for the biosurfactant produced from bacterial isolate of marine environment.

3.6. Antibacterial activity of the biosurfactant from *P. stutzeri*

The crude biosurfactant was tested for the antibacterial activity; it showed a broad range of activity against the six bacterial pathogens tested. The biosurfactant obtained from crude oil as a substrate showed highest activity against the tested pathogens such as *Vibrio cholera*e (20 mm), *Shigella boydii* (18 mm), *Vibrio fluvialis* (16 mm), *Shigelladysenteriae* (17 mm), *Salmonella typhi* (19 mm)and *Salmonella paratyphi* (21 mm) followed by the other sources of biosurfactants (Fig. 3 and Table 2).As in the present work Balan et al., 2016 have reported antimicrobial activity of a lipopeptide biosurfactant produced by *C. Pontifactin* against *Streptococcus mutans*, *Micrococcus luteus*, *Salmonella typhi* and *Klebsiellaoxytoca*.

Table 2
Antibacterial activity of biosurfactants produced by *P. stutzeri* SJ3 using different oil sources against different bacterial pathogens

Pathogens tested	Zone of inhibition (in mm)						
	Crude oil	Diesel	Petrol	Sesame oil	Peanut oil	Tamanu oil	*Pongamiapinnata* oil
Vibrio cholerae	20	5	4	-	13	-	10
Shigella boydii	18	-	11	-	-	3	-
Vibrio fluvialis	16	10	-	-	3	11	8
Shigelladysenteriae	17	13	10	14	10	-	11
Salmonella typhi	19	10	16	4	-	-	5
Salmonella paratyphi	21	10	18	10	14	-	11
'-'= No zone/activity							

4. Conclusion

The present study on production of biosurfactant from *P. stutzeri* SJ3 isolated from oil polluted sites ONGC, Karaikal, Puducherry. The growth optimization of the potent strain for biosurfactant production showed excellent productivity and antimicrobial activity against the tested bacterial pathogens. Hence, the strain can be used in bioremediation applications.

Declarations

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ETHICAL APPROVAL

This article does not contain any studies with human participants or animal performed by any of the authors.

CONSENT TO PARTICIPATE AND PUBLISH

Informed consent was obtained from all individual participant included in the study. The participant has consented to the submission of the case report to the journal. Patients signed informed consent regarding publish their data and photographs.

COMPETING INTERESTS

Authers are requested to disclose interests that are directly or indirectly related to the work submitted for publication.

AVAILABILITY OF DATA AND MATERIALS

The authors confirm that the data supporting the findings of this study are available within the article and its supplementary material.

COMPETING OF INTERESTS

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper and no conflict of interests.

AUTHOR CONTRIBUTIONS

Conception, design collection of data and analysis were done by Sekar Harikrishnan, Data interpretation was done by Sekar Harikrishnan, Mohammad S. Alsalhi, Alager Kartick, Sandhanasamy Devanesan and Aruliah Rajasekar drafted the article. Singaram Jayalakshmi was responsible for funding arrangement final approval of the article. All authors discussed the results and commented on the article.

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Figures

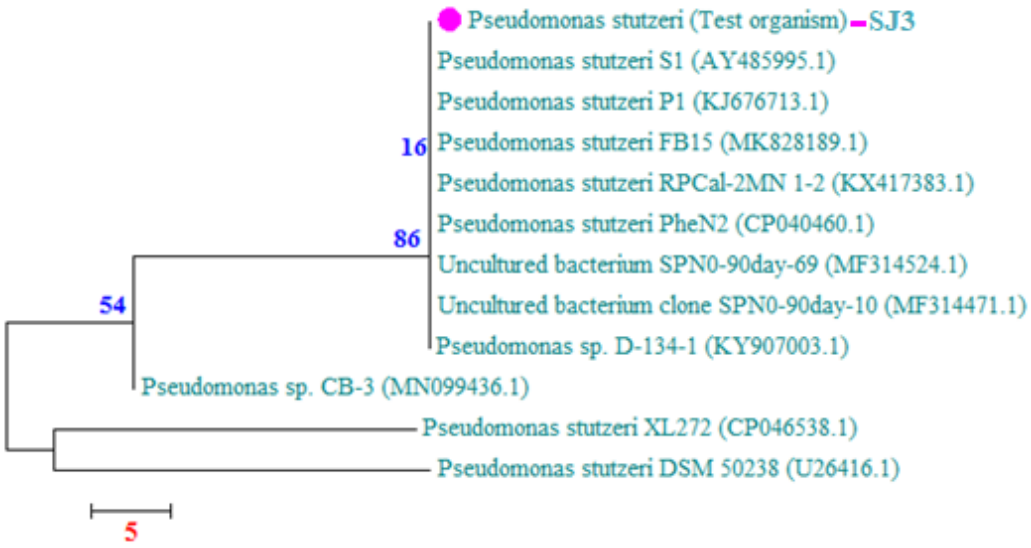

Figure 1

Evolutionary analyses of Pseudomonas stutzeri and phylogenetic tree construction using neighbor-joining method

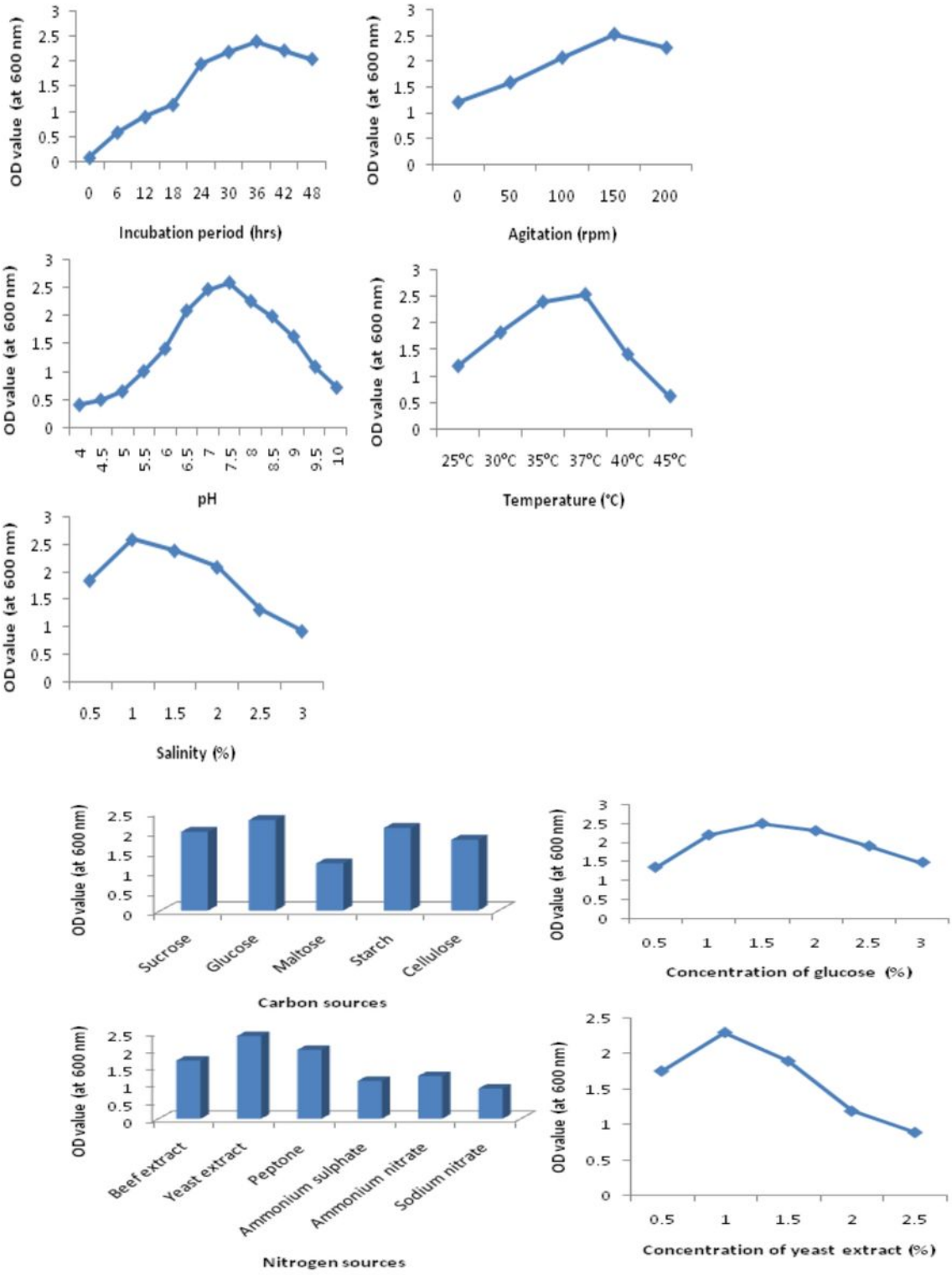

Figure 2

Effect of various physicochemical parameters on growth of potent biosurfactants producing isolate P. stutzeri SJ3

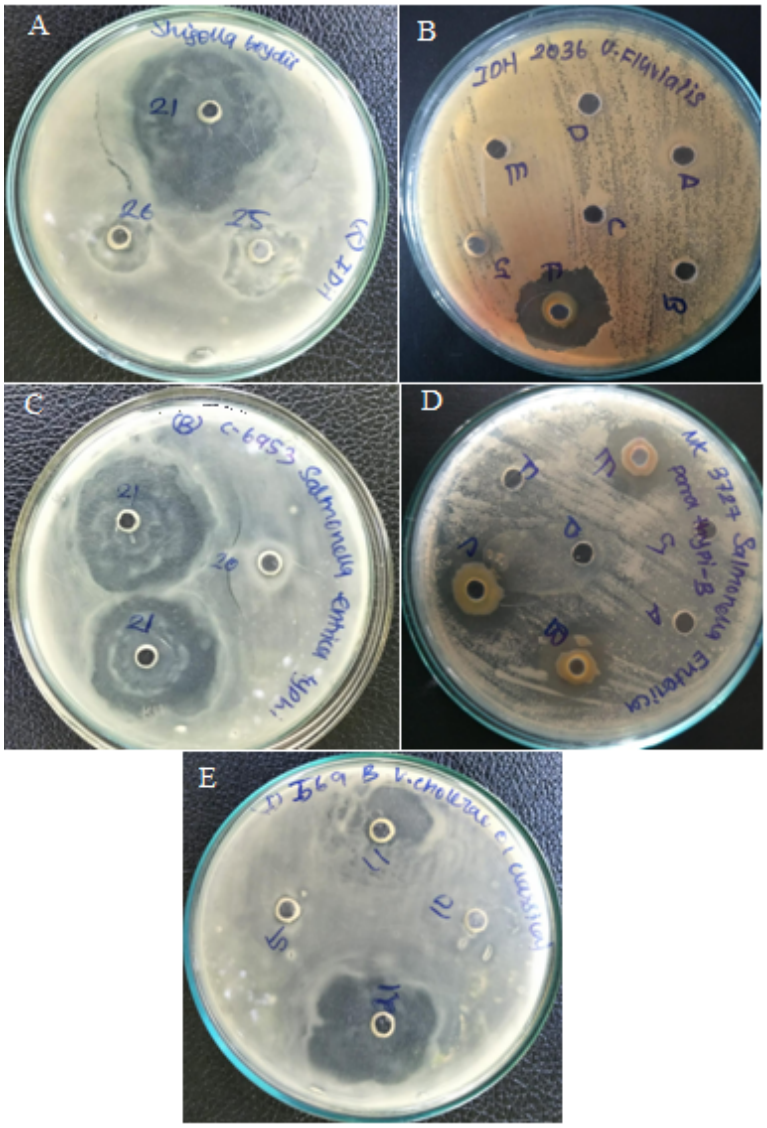

Figure 3

Antibacterial activity of biosurfactants produced by P. stutzeri SJ3 against different bacterial pathogens (A-E)

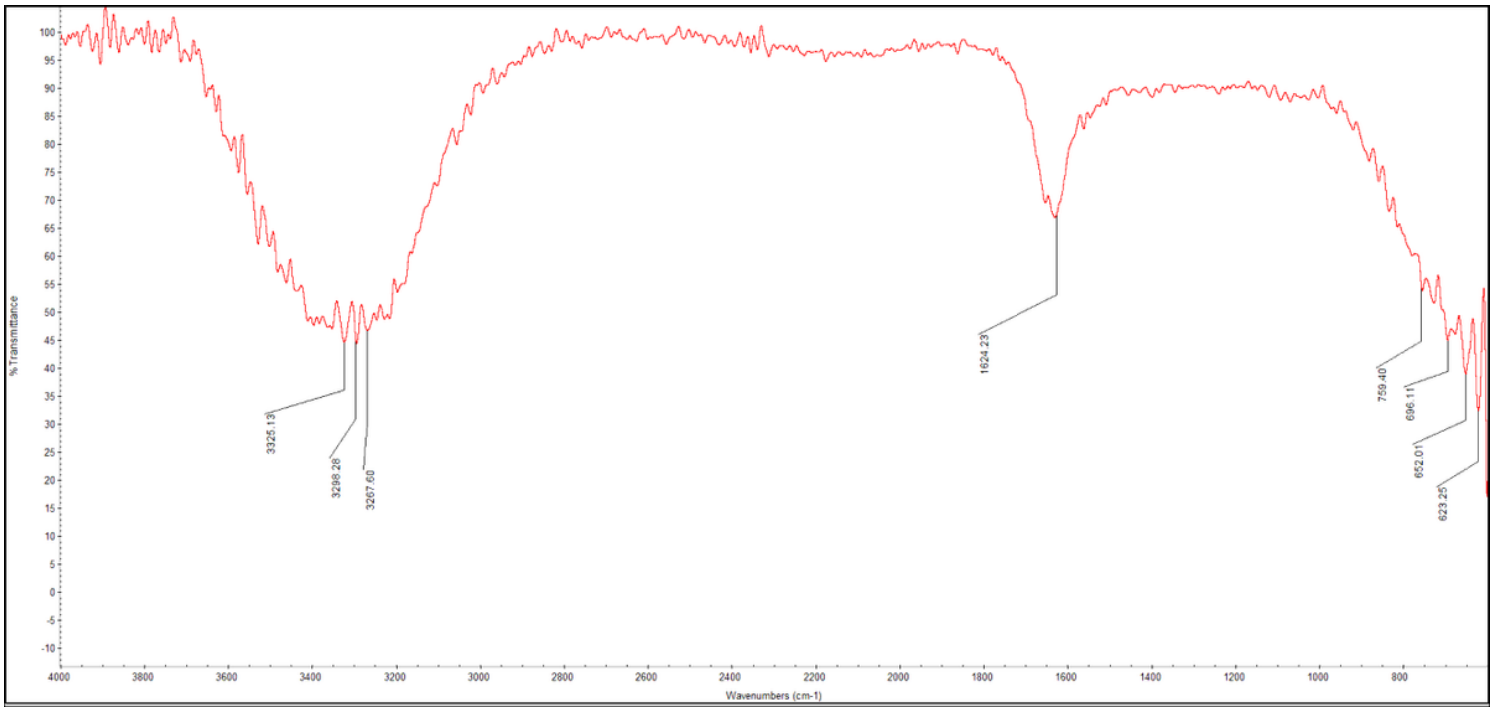

Figure 4

FTIR analysis of crude biosurfactant produced by P. stutzeri