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# Potential application of biosurfactant from marine bacteria in bioremediation

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**Abstract.** *Mohanty M, Das S. 2018. Potential application of biosurfactant from marine bacteria in bioremediation. Ocean Life 2: 59-72.* Marine bacteria were screened for their potential ability to produce biosurfactants which can effectively reduce polycyclic aromatic hydrocarbons (PAHs) as the only carbon and energy source. Having many toxic effects, the PAHs are very harmful to flora and fauna as well as affecting humankind adversely. This work aimed at investigating the potential applications of biosurfactant in aerobic degradation of PAHs under stress conditions. The antimicrobial and anti-adhesive capacity of the biosurfactant were also tested against different pathogenic species. Marine bacteria were collected from sediment samples of Paradip Port, Visakhapatnam Port, Rishikulya, Bhitarakanika and screened for their biosurfactant production. Its growth was optimized in carbon and nitrogen sources for maximum biosurfactant production. Naphthalene and PAHs degrading isolates were evaluated for their biodegradative potential through UV-Vis spectroscopy and phenotypical characterization by SEM studies. Five candidate isolates, identified to be *Ochrobactrum, Streptococcus, Pseudomonas* sp., *Pseudomonas aeruginosa* and *Achromobacter xylosoxidans* showing 99.9%, 99.6%, 99%, 99.3%, 98.6% of Phenanthrene degradation (100mg/L) and 99%, 99.1%, 89.75%, 94.01%, 97.02% of Naphthalene degradation (100 mg/L), respectively.

**Keywords**: Bioremediation, biosurfactants, PAHs, polycyclic aromatic hydrocarbons

## INTRODUCTION

Given the fact that 70% of the earth surface is covered by salt water, the marine environment becomes the most significant habitat as compared to other habitats in the biosphere. Industrialization and extraction of natural resources have caused in large-scale environmental pollution. Large amounts of toxic wastes have been scattered in thousands of contaminated sites which spread across our nation whose natural sink is the coastal marine regions. Thus, all of us is being exposed to contamination from past and present industrial practices, emissions in natural resources (water, air, and soil) even in the most isolated regions; hence, the risk to human and environmental health is rising. Our challenge is to develop innovative and cost-effective solutions to clean up polluted environments, to make them safe for human habitation and consumption, as well as to protect the functioning of the ecosystems which support life.

The pollution is mainly originated from various anthropogenic sources like an oil spill, plastic debris, pesticides, fertilizers, chemicals, radioactive substances, heavy metals, biological, solid wastes. Polycyclic aromatic hydrocarbons (PAHs) are the major constituent of oil and are abundant everywhere in nature, for example, in the soil, air, water, flora, and fauna. Most of PAHs are carcinogenic, mutagenic and teratogenic to many organisms including mammals.

About 23,000 metric tons of PAHs are discharged to the marine environment by anthropogenic sources every year (Eisler 1987). PAHs may come into the marine environment by spillage of petroleum and petroleum products, atmospheric deposition of PAHs, domestic and industrial sewage, surface land runoff, biosynthesis. PAHs incorporated with some airborne particles settle down in the bottom of the sea. The petroleum and petroleum products undergo diffusion, evaporation, some chemical changes, sunlight effect (photo-oxidation) (www.intechopen.com/download/pdf/29372).

PAHs are the most toxic pollutants in the hydrocarbon family. The toxicity of PAHs varies substantially in the marine environment. In crustaceans, the level of toxicity is even higher, and in teleosts, it is deficient. Some aquatic plants and animals' uptake the PAHs, then accumulate it. Absorption of the PAHs is highly species specific. The fish and crustaceans readily absorb pahs whereas some algae and mollusks are unable to metabolize these PAHs. Many marine organisms can eliminate these PAHs, so biomagnification is not observed in the food chain (Eisler 1987). Some PAHs are pooled in the cell membrane of the microbes due to their lipophilic nature. Exposure to PAHs, however, leads to cell damage, carcinogenesis, teratogenesis, and mutagenesis as PAHs binds covalently with the macromolecules like RNA, DNA, and protein.

Various physicochemical methods are introduced to remove these contaminations from the environment. But these are too expensive, non-specific and also they introduce the secondary contaminants to the environment. An eco-friendly, cost-effective and bio-based method is adapted to treat these contaminations, called bioremediation. Bioremediation is a way of detoxification and degradation of toxic pollutants either through intracellular accumulation or by enzymatic transformation to less toxic or non-toxic compounds (Singh et al. 2008). Microorganisms exhibit the potential to degrade, transform or chelate the poisonous chemicals, but this transformation process is prolonged. The major components of this bioremediation are the microbes and their products. The critical factors of bioremediation are the availability of contaminants, availability of microbes and a suitable environment — also, the nutrients, pH value, oxygen influence the bioremediation of PAHs (Singh Cameotra and Makkar 2010).

Due to the low water solubility and hydrophobic characteristic of PAHs, many microorganisms have developed several mechanisms to increase the availability of such compounds to utilize them as carbon and energy source. Microorganisms produce two main types of surface-active compounds: biosurfactants and bioemulsifiers. Biosurfactants greatly reduce the air-water surface tension while bioemulsifiers do not decrease as much the surface tension but stabilize oil-in-water emulsions. Example of some biosurfactants: Rhamnolipid, Surfactants, Sophorolipid, Lipopeptide, Trehalose tetra ester.

Biosurfactants in the degradation of heavy metals, PAHs, pesticides in soil and water environment, are of great significance. The microbes generally use organic compounds as the source of carbon and energy for their growth. When a hydrocarbon ($C_xH_y$) or any insoluble substrate is the carbon source, microorganisms regulate their diffusion into the cell membrane by producing a variety of biosurfactants. Some bacteria and yeasts produce ionic surfactants which emulsify the $C_xH_y$ substrate in the growth medium (Karanth et al. 1999). For example, Rhamnolipids which are excreted by different *Pseudomonas* spp. (Guerra et al. 1984), non-ionic trehalose corynomycolates are generated by many *Mycobacterium* spp. and *Arthrobacter* spp.

Biosurfactants are biodegradable, usually low toxic, biocompatible, digestible for which they are used in cosmetics, pharmaceuticals and as functional food additives. They can be produced from industrial wastes and by-products, and this is of particular interest for bulk production (e.g., for use in petroleum-related technologies). Furthermore, biosurfactants can be efficiently utilized in handling industrial emulsions, control of oil spills, biodegradation and detoxification of industrial effluents and in decontamination soil. They are more effective at extreme pH, temperatures, and salinity.

In the last decades, there has been a growing interest in isolating microorganisms that secrete surface active molecules with good surfactant characteristics like low critical micelle concentration (CMC) and high emulsification activity. Such microorganism is simultaneously presenting low toxicity and good biodegradable ability. The type and amount of the biosurfactants generated by microbes depend on the producer organism, carbon and nitrogen, trace elements, and temperature. Biosurfactants are categorized mainly by their microbial origin and chemical composition (a type of polar group present). Based on the structure of their hydrophilic part, biosurfactants are classified primarily into five categories: (i) Glycolipids, (ii) Lipopeptides, (iii) Fatty acids, (iv) Polymer type, (v) Particulate biosurfactants (Desai and Banat,1997; Gautam and Tiagi, 2005).

The objectives of the present study are : (i) Screening, phenotypic and biochemical characterization of biosurfactant producing marine bacteria, (ii) Growth optimization of the isolates in different carbon and nitrogen sources, (iii) Extraction and chemical characterization of a biosurfactant produced from the strains, (iv) Antioxidant, anti-adhesive and antimicrobial activity study of the extracted biosurfactant, (v) Biosurfactant based bioremediation of PAHs.

## MATERIALS AND METHODS

Fourteen bacterial strains were collected from Paradeep port, Vishakhapatnam, Rishikulya, Bhitarakanika marine water. They were streaked in Luria Bertani (LB) agar plate and maintained at pure culture.

### Colony morphology

Colony morphology was observed for twenty-four hours incubated cultures of the isolated bacteria.

### Screening for biosurfactant activity

*Drop collapsing test*

Fourteen bacterial strains were maintained overnight in LB broth with ten mM $MgCl_2$. Next day these were sub-cultured into MMMF media and incubated for 24 h at $23^oC$. 10 µL of the supernatant of each strain was spotted on the polystyrene coated glass plate that was covered by immersion oil (Taguchi et al. 2006).

*Oil spread method*

Bacterial strains were inoculated in LB broth with ten mM $MgCl_2$ for 24 h. Next day 20 µL of immersion oil was layered uniformly to a 20 mL of distilled water that was kept in the Petri plate. 20 µL of the culture was added to a different spot on the immersion oil after doing vertex for 2 minutes. After 30 sec, it was examined whether they are giving a clear zone or not. (Morikawa et al. 2000).

*Emulsification test*

The bacterial strains were inoculated in LB broth with ten mM $MgCl_2$ for overnight. Two mL of the bacterial culture was transferred to a test tube, and n-octane (1 mL) was added to it. The mixture was vortexed for 2 min and kept for 24 h to see the emulsification result (Cooper et al. 1987).

$$\text{Emulsification index} = (\text{Emulsification height}/\text{Total height}) \times 100$$

*Blood Haemolysis Test*

On the blood agar plates, the isolated new colonies were streaked and kept in an incubator for 24 hours at 37°C. The biosurfactant producing organisms was determined from the presence of the clear zones around the colonies in the plates (Satpute et al. 2008).

### Physical characterization of bacterial isolates

*Gram staining*

First, smears of bacterial suspensions were made with one drop of distilled water on a clean and dry slide glass. These slides were fixed by heat, and the fixed smears were flooded with crystal violet solution and kept for 1 min, and were rinsed off with distilled water. These slides were then treated with Gram's iodine solution, allowed to remain for 1 min, then rinsed with distilled water. These slides were treated with 1-2 drops of Gram's decolorizer and kept for 1-5 sec. Then the slides were rinsed with water. Lastly, these were flooded with safranin and maintained for 1 min, then again rinsed with distilled water. These slides air dried and observed under the light microscope at 40X objective. If the cells retained the pink color of safranin, then these were identified to be, and if they kept the violet color of crystal violet, they were defined as Gram-positive.

*Characterization of biosurfactant producing bacteria by Scanning Electron Microscopy (SEM)*

The freshly cultured strains were pooled at 8000 g, $4^0$C for 5 min and the cells were washed with 0.1 mM phosphate buffer saline (PBS) 3 times. Following this step, the cells were fixed by adding 2 % glutaraldehyde prepared in 0.1 M PBS and incubated at room temperature overnight for fixation. Later, the next day the cells were washed thrice with PBS. These cells were centrifuged at 8000 g, 4˚C for 5 min; then dehydration was done of each sample by a series of ethanol concentrations ranging from 30 %, 50 %, 70 %, 90 %, 100 % and incubated for 18 hours. The fixed samples were incubated for one h with ethanol (100 %), air dried and observed at various resolutions under SEM.

### Biochemical tests of bacterial isolates

*Mannitol motility test*

Freshly cultured bacterial strains were inoculated in mannitol motility nitrate agar and incubated for 24 h and were checked for motility. The strains are non-motile if they showed growth along the line of the inoculation. If the bacteria showed growth by spreading over the medium, then they are motile. This mannitol motility test also confirms whether bacterial strains can ferment mannitol or not. A color change from red to yellow indicates a positive result.

*Nitrate reduction test*

In this test, 1-2 drops of sulphanilic acid and 1-2 drops of N, N-Dimethyl- Naphthylamine reagent were added to the kit medium to check whether these bacterial strains can convert nitrate to nitrite or not. If the color immediately changed into the pinkish red color on the addition of reagent indicates a positive reaction. If there is no change in color, it shows a negative result.

*Sulphide Indole Motility (SIM) Test*

Fourteen strains were inoculated in Sulphide indole motility (SIM) media and incubated these to test whether they are motile or non-motile and whether they are producing sulfide or not. If the color of the medium will change from yellow to black, then $H_2S$ production result will be positive.

### Growth curve

Bacterial strains were inoculated in LB broth, and 300 µL of the freshly inoculated bacterial cultures were taken in the microtiter plate (Tarson). Then, the O.D. was measured at 595 nm in the ELISA Reader (Perkin Elmer) at every two h interval for 24 h. The readings were plotted against time to determine the growth curve.

### Growth optimization of the strains in various carbon and nitrogen sources

*Growth optimization in Carbon sources*

Two aliphatic carbon sources (Glycerol and Sucrose) and five aromatic carbon sources (Kerosene, Biphenyl, Naphthalene, Pyrene, Phenanthrene) were drawn to optimize the growth of these strains. Two percent of each aliphatic carbon sources were added to Bushnell Haas Broth (BHB) with NaCl supplementation (19.450 mg/L), and concentration of 100 mg/L of each aromatic source was taken, and the absorbances were monitored at 595 nm (Onwosi and Odibo 2012).

*Growth optimization in Nitrogen sources*

Two percent of each nitrogen sources ($KNO_3$, Urea and Yeast extract) was added to the BHB (supplementation with NaCl- 19.450 gm/l) with the respective optimized carbon sources for the growth optimization of these strains. Absorbance was read at 595 nm (Onwosi and Odibo 2012).

### Extraction of biosurfactant

Five strains were inoculated in BHBroth as described above with respective carbon and nitrogen sources and then incubated at $25^0$C for seven days with shaking conditions. Supernatants were pooled by centrifuging at 6000 rpm, $4^0$C for 20 minutes. The pH of these supernatants was adjusted by adding 1M $H2SO_4$. Then an equal volume of chloroform: methanol was added to this supernatant in the ratio of 2:1. The mixture was shaken well for proper mixing and then left overnight for evaporation. The presence of biosurfactant is confirmed when white colored precipitates were seen in the interface between the two liquid (Dhouha et al. 2012).

### Characterization of biosurfactant

*Carbohydrate and protein estimation*

Estimation of carbohydrate was done by the phenol-sulfuric acid method (Dubois et al. 1956), while the evaluation of protein was conducted by Bradford method (Bradford 1976).

*Surface tension measurement*

Fifty mL of the crude biosurfactant of 5 strains were taken for surface tension measurement concerning distilled water. The surface tensions of each strain were plotted by digital tensiometer. Hence, the surface tensions concerning distilled water were determined (Abu-Ruwaida et al. 1991).

### Fourier Transform Infrared analysis (FTIR)

FTIR spectroscopy was performed using crude biosurfactant extract obtained from the acid precipitation of the cell-free culture supernatant to determine the chemical nature of the biosurfactants. FTIR Prestige- 21 Fourier Transform Infrared spectrophotometer (Shimadzu, Japan) was used to determine the chemical nature of the biosurfactant by the KBr pellet method (Das et al. 2008a, b; Mukherjee et al. 2009).

### Antimicrobial activity test

Twenty mL Muller Hinton Agar media with supplementation of NaCl (19.450gm/l) was prepared each for Petri plates on each of which three wells were made and named as A, B, and C respectively. The plates were swabbed with *Bacillus, Streptococcus, Shigella, Escherichia coli, Proteus, Salmonella.* To the wells A, purified biosurfactants were added. To the wells, B, diluted biosurfactants (10 fold) were added. To the wells, C, only distilled water (control) was added. The plates were incubated at 37°C for 24 hours and checked for the presence of a clear zone which marked the antimicrobial activity of biosurfactant. Mean of the three readings of the clear zone diameter were taken for each well to calculate the actual zone diameter (Rodrigues et al. 2006).

### Antioxidant activity test

The antioxidant potential of the biosurfactant was measured by their scavenging activity of the stable 1, 1-diphenyl-2-picrylhydrazyl (DPPH) free radical. DPPH method is the easiest method to determine the antioxidant activity of compounds and is widely used. The aliquots of the different biosurfactant level were poured to 5.0 mL of a 0.004% (w/v) solution of DPPH. Absorbance at 517 nm and IC50 were determined after 30 min of reaction. IC50 (the half maximal inhibitory concentration) value denotes the level of sample required to scavenge 50% of the DPPH free radicals. The radical scavenging activity at various biosurfactant concentration was calculated by the equation below:

Equation: $S_{DPPH} = 100 \times (1 - A_{sample}/A_{DPPH})$

Where $A_{sample}$ indicates the absorbance of the solution in the presence of test samples, and $A_{DPPH}$ shows the absorbance of the DPPH solution in the absence of the test samples (Yalcin and Cavusoglu 2010).

### Anti-adhesive test

Two-hundred µL of the crude biosurfactants (100 mg/mL in PBS) were filled in the wells, and the control wells were filled only with PBS. The plate was later incubated at 4˚C for 18 h and washed with PBS three times. The pathogenic bacteria cultures, *Streptococcus pneumoniae,* and *Bacillus* sp. were centrifuged, and pellets were collected then resuspended in PBS and added to these wells. The plate was then kept in an incubator at 4˚C for four h. The plate was washed with PBS three times, then fixed by 200 µL methanol for 15 min. The plate was dried and then stained with 2% crystal violet (200 µL) for 5 min. The dye was re-solubilized with 200 µL of 33% (v/v) glacial acetic acid per well. Then absorbance was measured at 595 nm (Rufino et al. 2011).

% of microbial inhibition = $[1- (A_c/A_0)] \times 100$

Where:

$A_C$ = Absorbance of the well with a biosurfactant concentration c.

$A_0$ = Absorbance of the control well

### Biodegradation of Polycyclic Aromatic Hydrocarbon (PAH) by Biosurfactant

*Phenanthrene biodegradation*

First, bacterial cultures were inoculated in LB broth for 24 h. The next day, 100 µL of these bacterial cultures were subcultured in 50 mL Bushnell Haas media (with supplementation of NaCl 19.450 gm/l) with 100 mg/L of phenanthrene for seven days for enrichment culture. On the 7th day, the pellets of each bacterial culture were collected by centrifuging at 6000 rpm, 10 min at $4^0$C. Then these pellets were re-suspended in B H broth (2 mL). Then the O.D. of each bacterial pellet (300 µL) was read at 595 nm in ELISA Plate Reader. As O.D. of each strain was found less than 1, then 50 µL of an enriched pellet of each strain was moved to 5 mL of BHB with Phenanthrene (100 mg/L) and kept in a shaker incubator (in the dark) at 180 rpm, 37˚C. On the Day-1, Day-3, Day-5, Day-7, extraction was done by adding the equal volume of n-Hexane. After adding n-Hexane, the sample was vortexed for 5 minutes, then centrifuged at 6000 rpm for 10 min at $4^0$C to collect the Hexane layer. Then O.D. of the Hexane extract was taken at 292 nm and also scanned from 200 nm to 400 nm (Tao et al. 2007).

*Naphthalene biodegradation*

First bacterial cultures were inoculated in LB broth for 24 h. Next day 100 µL of these bacterial cultures were subcultured in 50 mL Bushnell Haas media (with supplementation of NaCl 19.450 gm/l) with 100 mg/L of naphthalene for seven days for enrichment culture. At 7th day the pellets of each bacterial culture were collected by centrifuging at 6000 rpm, 10 min at 4˚C. Then these pellets were re-suspended in BHB (2 mL). Then the O.D. of each bacterial pellet (300 µL) was read at 595 nm in ELISA Plate Reader. As O.D. of each strain was lower than 1, then 50 µL of an enriched pellet of each strain was moved to 5 mL of BHB (with NaCl 19.450 gm/l) with Naphthalene (100 mg/L) and run in a shaker incubator (dark) at 180 rpm, $37^0$C. Then at Day-1, Day-3, extraction was done by adding the equal volume of n-hexane. After adding n-Hexane, the sample was vortexed for 5 min, then centrifuged at 6000 rpm for 10 minutes at $4^0$C to collect the Hexane layer. The O.D. of the Hexane extract was measured at 254 nm and also scanned from 200 nm to 400 nm (Tao et al. 2007).

## RESULTS AND DISCUSSION

### Cell morphology

Cell morphologies of these five strains (JV502, JV501, JP022, JV201, and JP011) were given in Figure 1.

### Screening for biosurfactant activity

*Drop collapse test*

Ten µL cell suspension of each strain was placed on the polystyrene coated glass plate that was covered by immersion oil. If the cell suspension contains biosurfactant, the drop will collapse or spread due to the reduction of a hydrophobic surface. On the other hand, if no biosurfactant present in the cell suspension, the drops will remain stable as the polar water molecules are repelled from the hydrophobic surface. The stability of the drop depends on the biosurfactant level. Only strains JV801 and NR802 gave negative results and rested gave positive results (Table 1).

*Oil spread method*

Cell-free culture broth of 14 strains was added to the plate that contained distilled water and oil. The 12 strains (JV201, JV501, JV502, JP022, JP011, NE3B01, NE3B02, NP202, NP103, ATCC, NR802, and JV801) showed the zone of displacement in oil. The zone of displacement displayed the biosurfactant production in these strains, and the results were noted down (Table 1).

*Emulsification test*

In the emulsification test, JP022 showed 41% of emulsification activity, and the other strains ranging between 35-40 % of emulsification activity and harmful emulsification activity was observed in NP202, NP103, and JV101 (Figure 2).

*Blood hemolysis test*

Fourteen strains were streaked on blood agar plates. Among the strains, JV501, JV201, JP022, JP011, and ATCC showed hemolytic activity by forming a clear zone around the colonies (Figure 3).

Among these fourteen strains, five strains gave good results in the screening of biosurfactant test, so they proceeded for further study.

### Physical characterization of bacterial isolates

*Gram staining*

Cell morphology of these ten strains was studied by gram staining and observed under oil immersion microscope. Among these strains, JV501 was Gram-positive bacteria, and others were Gram-negative. Similarly, JV501 was coccus, and the others were rods. The results have been given below in Table 2.

**Table 1.** Results of drop collapse test and oil spread method

| Strain name | Drop collapse test | Oil spread method |
|---|---|---|
| JV501 | Positive | Positive |
| JV201 | Positive | Positive |
| JV502 | Positive | Positive |
| JP022 | Positive | Positive |
| JV202 | Positive | Negative |
| JV101 | Positive | Negative |
| JP011 | Positive | Positive |
| JV801 | Negative | Positive |
| NE3B02 | Positive | Positive |
| NE3B01 | Positive | Positive |
| NP202 | Positive | Positive |
| NP103 | Positive | Positive |
| ATCC | Positive | Positive |
| NR802 | Negative | Positive |

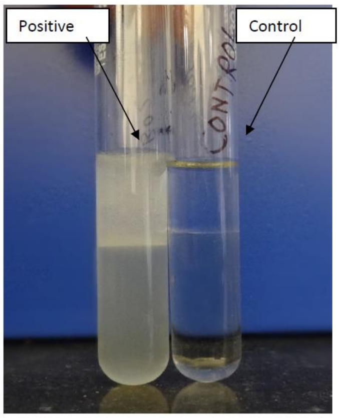


**Figure 2.** The tubes showing emulsification activity

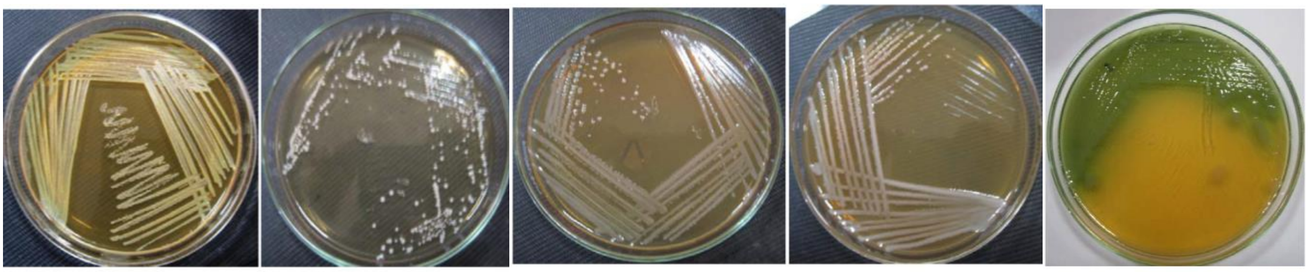
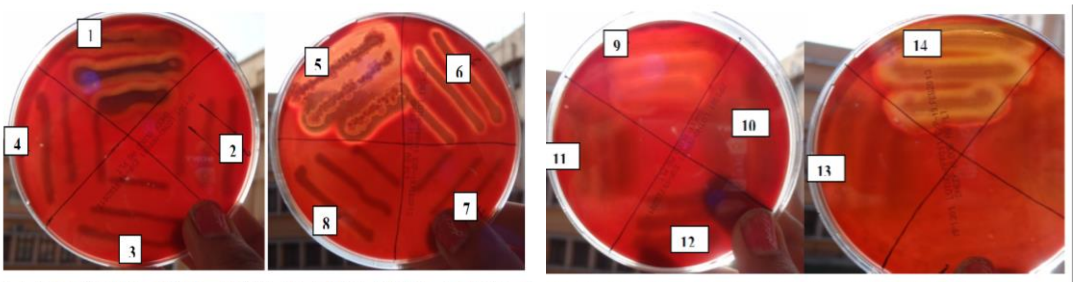
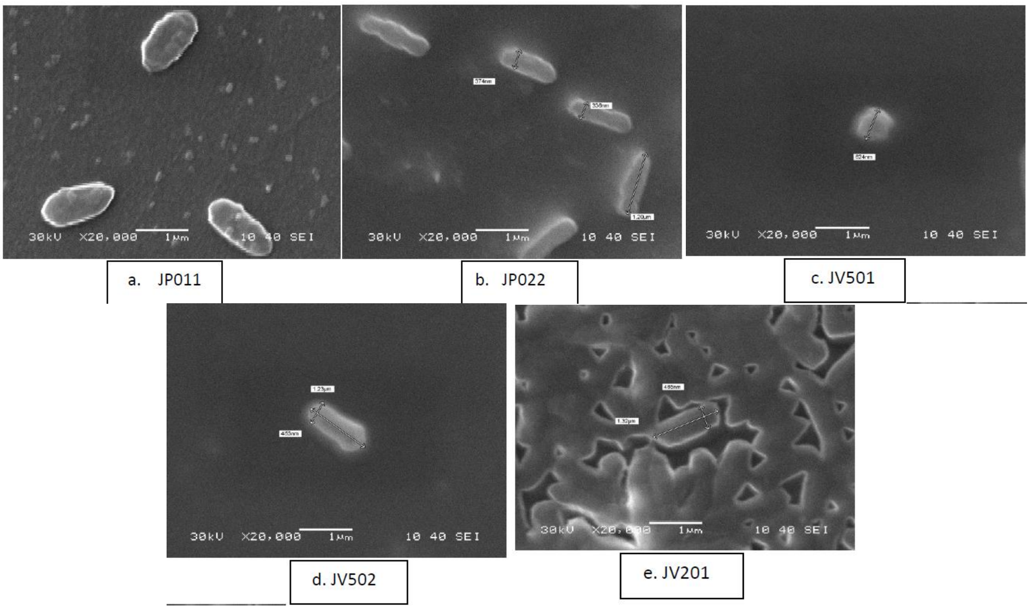

a) JV502 b) JV501 c) JP022 d) JV201 e) JP011

**Figure 1.** Cell morphology of the isolated strains



(a) 1-JP011, 2-JV502, 3-JV101, 4-JV202 (b) 5-JP022, 6-JV201, 7-NE3B01, 8-NE3B02 (c) 9-ATCC, 10-NR802, 11-NP103, 12-NP202 (d) 13-JV801, 14-JV501

**Figure 3.** Hemolytic activity of the isolates, JP011- α hemolysis, JP022- β hemolysis, JV201- β hemolysis, ATCC- hemolysis, JV501- β hemolysis and others are showing a negative result.



a. JP011
b. JP022
c. JV501
d. JV502
e. JV201

**Figure 4.** SEM results of the isolates

**Table 2.** Cell morphology of the isolates

| Strain name | Colour | Gram staining | Shape |
|---|---|---|---|
| JV501 | Purple | + ve | Cocci |
| JV201 | Pink | -ve | Rods |
| JV502 | Pink | -ve | Rods |
| JP022 | Pink | -ve | Rods |
| JP011 | Pink | -ve | Rods |

**Table 3.** Results of all biochemical tests

| Strain name | Mannitol test | Motility test | Nitrate reduction test | Sulfide production test |
|---|---|---|---|---|
| JV501 | + ve | Motile | + ve | - ve |
| JV201 | + ve | Motile | - ve | - ve |
| JV502 | + ve | Motile | + ve | - ve |
| JP022 | - ve | Non-motile | - ve | - ve |
| JP011 | - ve | Non-motile | - ve | - ve |

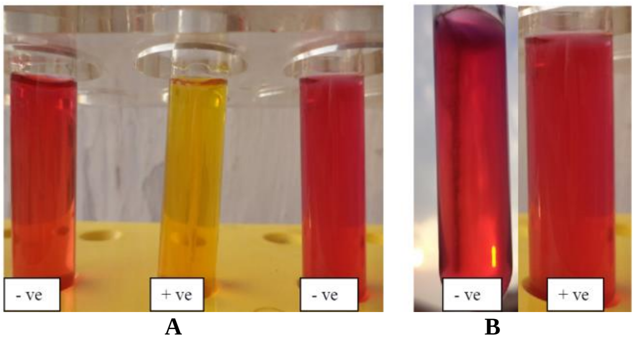


**Figure 5.** A. Mannitol test, + ve showing mannitol fermentation, B. Motility test, + ve showing Motile and - ve showing Non-motile

*Characterization of biosurfactant producing bacteria by Scanning Electron Microscopy (SEM)*

Cell surface topography of the five strains was observed by SEM image (Figure 4).

### Biochemical tests

Table 3 shows the results of all biochemical tests like Mannitol motility test, Nitrate reduction test, Sulphide indole motility test.

#### *Mannitol motility test*

In this test, JV501, JV201, JV502 shows a positive result, and others were showing the adverse effect (Figure 5). In the case of the motility test, all strains were found motile excluding JP022 and JP011.

#### *Nitrate reduction test*

Only JV501, JV502 were showing a positive result, and others were showing an adverse effect.

#### *Sulfide indole motility (SIM) test*

All strains showed a negative result.

### Growth curve

Five strains were inoculated to check their growth curve. The OD was measured and plotted against time in ELISA Reader (Perkin Elmer) (Figure 6).

### Growth optimization in various carbon and nitrogen sources

#### *In different carbon sources*

Two aliphatic carbon sources (glycerol and sucrose) were taken to monitor the growth of these strains.

#### *Aliphatic carbon sources*

Strain JV502, JV201, and JP011 showed robust growth in glycerol (Figure 7). Strain JP011, JV501, JV201, showed good results in sucrose as the carbon source (Figure 8).

#### *Aromatic carbon sources*

JV501, JV201 showed good growth results in Kerosene (Figure 9). JV201, JV501, JV502 showed good growth in Pyrene medium (Figure 10). JV501, JP011, and JV502 grew well in Biphenyl (Figure 11). In Naphthalene, JV501, JV201, and JP022 showed good growth (Figure 12). Meanwhile, the strains JV501, JV201, and JP011 showed the excellent result in Phenanthrene (Figure 13).

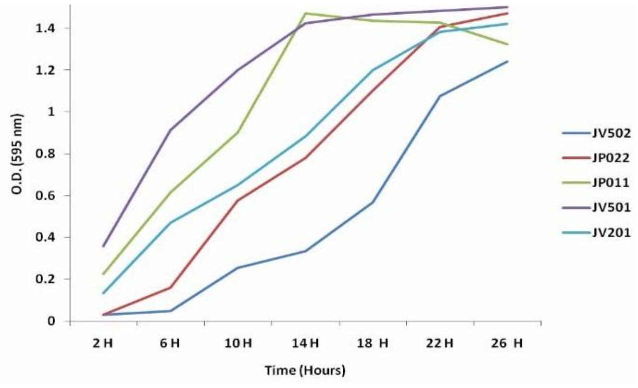


**Figure 6.** Growth curve of the bacterial isolates

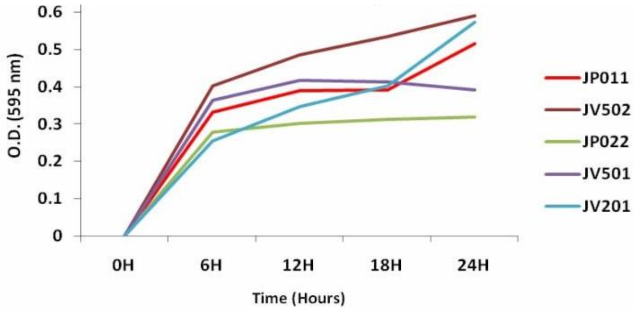


**Figure 7.** Growth optimization of the isolates in Glycerol (2% v/v)

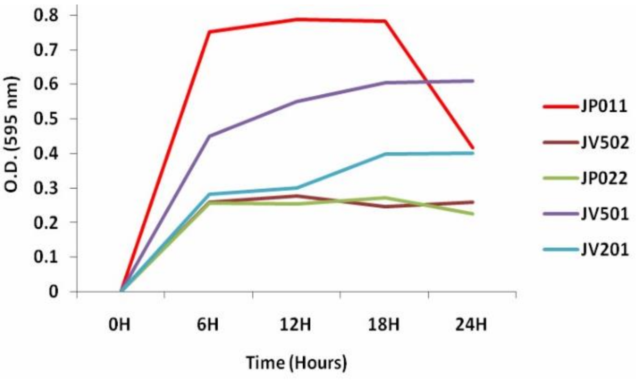


**Figure 8.** Growth optimization of the isolates in Sucrose (2% w/v)

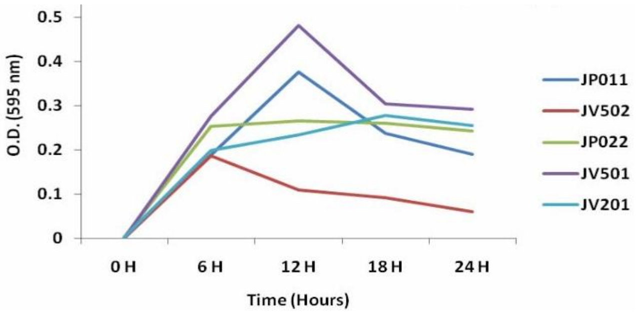


**Figure 9.** Growth optimization of the isolates in Kerosene (2% v/v)

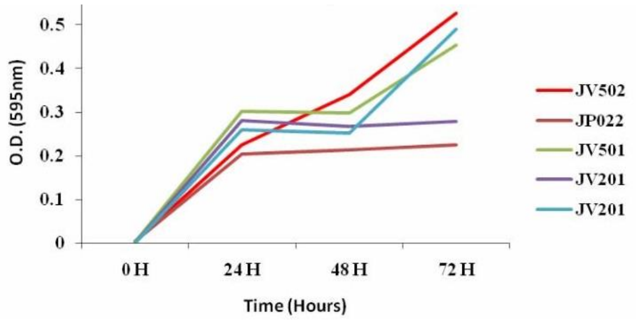


**Figure 10.** Growth optimization of the isolates in Pyrene (100 mg/L)

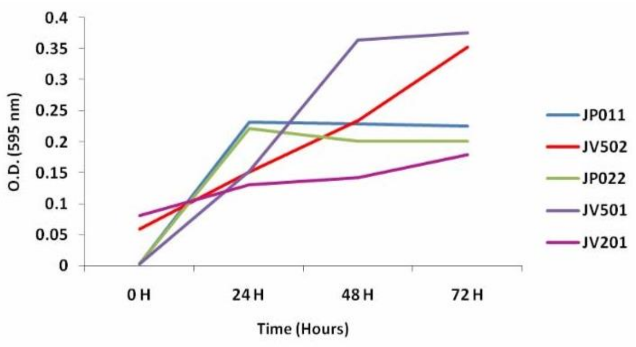


**Figure 11.** Growth optimization of the isolates in Biphenyl (100 mg/L)

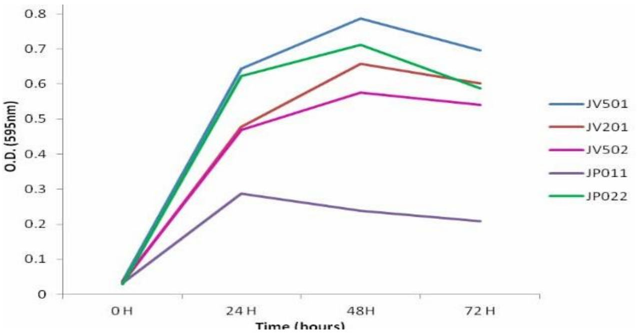


**Figure 12.** Growth optimization of isolates in Naphthalene (100 mg/L)

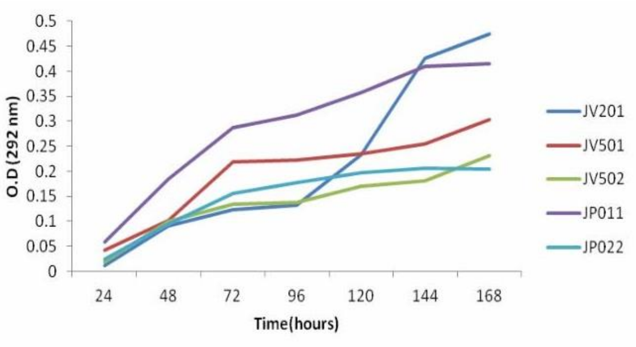


**Figure 13.** Growth optimization of the isolates in Phenanthrene (100 mg/L)

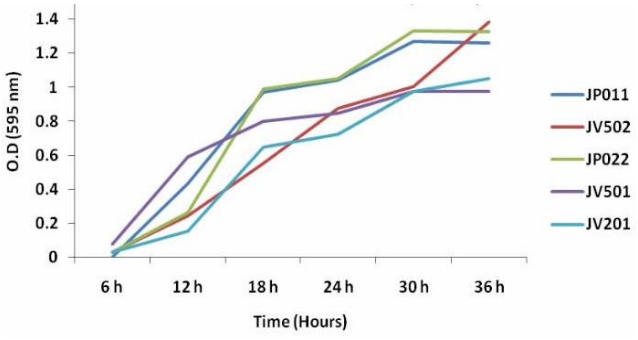


**Figure 14.** Growth optimization of the isolates in Yeast Extract (2% w/v)

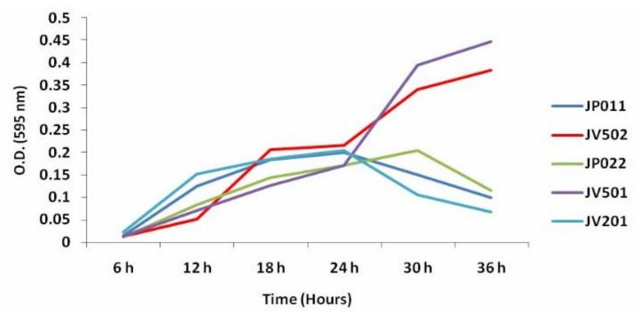


**Figure 15.** Growth optimization of the isolates in urea (2% w/v)

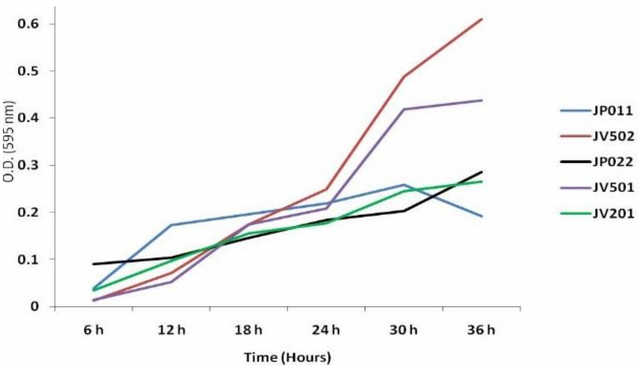


**Figure 16.** Growth optimization of the isolates in potassium nitrate (2% w/v)

*Growth optimization of the strains in different nitrogen sources*

The strains were optimized for better growths in three nitrogen sources yeast extract, urea, potassium nitrate. All the strains showed good growth in the presence of yeast extracts (Figure 14) whereas strain JV501, JV502 showed good growth in urea (Figure 15) and also in Potassium nitrate (Figure 16).

*Extraction of biosurfactant*

The five strains which were inoculated in BHB with respective carbon and nitrogen sources for seven days showed good results. Supernatants were obtained by centrifuging at 6000 rpm at $4^0$C for 20 min and an equal volume of chloroform: methanol (ratio 2:1) was added for acid precipitation with 1M $H_2SO_4$. After the pH was adjusted to 2, the mixture was left overnight for evaporation, and if white colored precipitate were found in between two immiscible liquids, then biosurfactant production was observed. The biosurfactant productions of all five strains were detected (Figure 17).

## Characterization of biosurfactant

*Carbohydrate estimation*

The carbohydrate level present in the biosurfactants was calculated from the standard curve (Figure 18). Table 4 showed that strain JV201 has the maximum carbohydrate content.

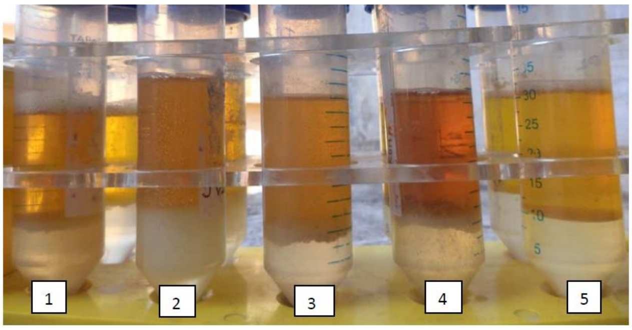


**Figure 17.** Biosurfactant production (1-JV501 2-JV201, 3-JV502, 4-JP022, 5-JP011. All are producing approximately 100mg/25 mL)

*Protein estimation*

The concentration of protein present in the biosurfactants was calculated from the standard curve (Figure 19). JV201 showed the maximum carbohydrate content (Table 5).

*Surface tension measurement*

A digital Tensiometer determined the surface tension of crude biosurfactant for distilled water (Table 6, Figure 20).

*Fourier Transform Infrared analysis (FTIR)*

The determination of the functional group present in the crude biosurfactant by using Fourier Transform Infrared Spectroscopy (Figure 21-29).

**Table 4.** Carbohydrate estimation of the biosurfactants

| Strain name | O.D. at 490 nm | Concentration (µg/mL) |
|---|---|---|
| JV501 | 0.035 | 0.217979 |
| JV201 | 0.172 | 0.709865 |
| JV502 | 0.063 | 0.318511 |
| JP011 | 0.028 | 0.192847 |
| JP022 | 0.048 | 0.264655 |

**Table 5.** Protein estimation of the biosurfactants

| Strain name | O.D. at 595 nm | Concentration (µg/mL) |
|---|---|---|
| JV501 | 0.022 | 1.122537 |
| JV201 | 0.183 | 2.952162 |
| JV502 | 0.015 | 1.042988 |
| JP011 | 0.017 | 1.065717 |
| JP022 | 0.019 | 1.088445 |

**Table 6.** Surface tensions of the isolates

| Strain name | Surface tension |
|---|---|
| JV501 | 55.234±0.028 mN /m |
| JV201 | 55.368 ± 0.028 mN /m |
| JV502 | 51.251 ± 0.028 mN /m |
| JP011 | 43.776 ± 0.029 mN /m |
| JP022 | 52.292 ± 0.028 mN /m |

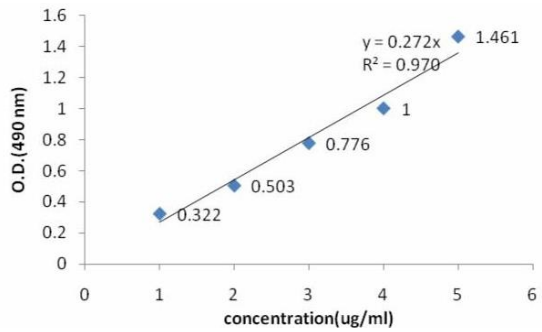


**Figure 18.** A standard curve of D-glucose

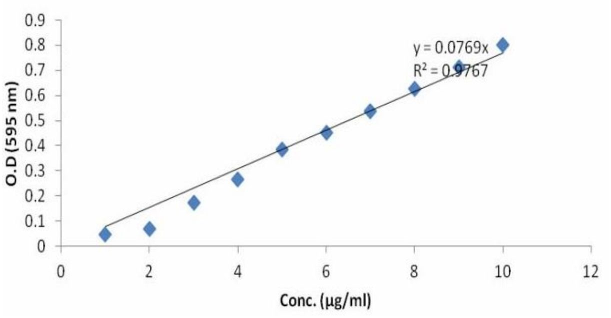


**Figure 19.** A standard curve of protein (BSA)

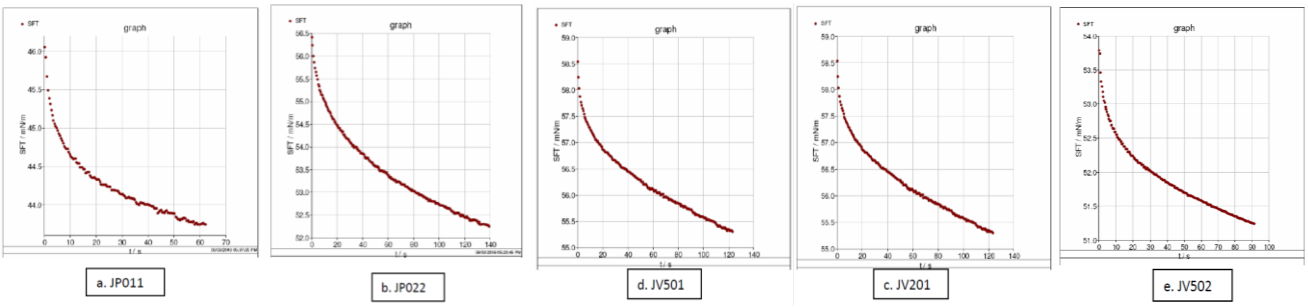


**Figure 20.** Surface tension measurement of the isolates

## Antimicrobial activity

The antimicrobial activities of the biosurfactants found in 5 strains against six pathogenic strains. These were *Bacillus, Shigella, Streptococcus, Escherichia coli, Proteus and Salmonella)* are shown in Figure 25-32.

## Antioxidant activity test

The biosurfactant extracted were checked for their antioxidant activity showing negative results (Table 7).

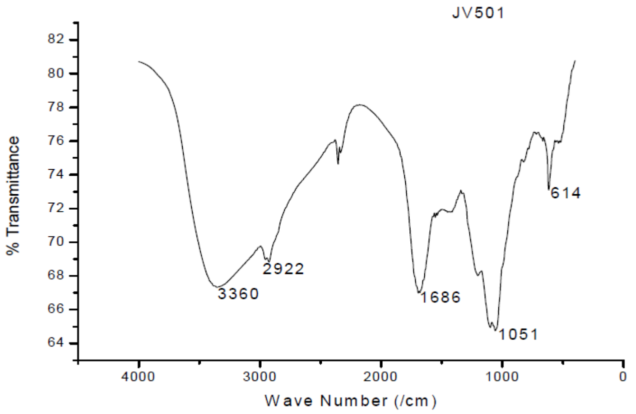


**Figure 21.** FTIR result of JV501

Inference

| Wave number | Bonds | Functional group |
|---|---|---|
| 3360 | N-H stretch | Primary, Secondary amines, Amides |
| 2922 | C-H stretch | Alkanes |
| 1686 | C=O stretch | Carbonyls (general) |
| 1051 | C-N stretch | Aliphatic amines |
| 614 | C-Cl stretch | Aalkyl halides |

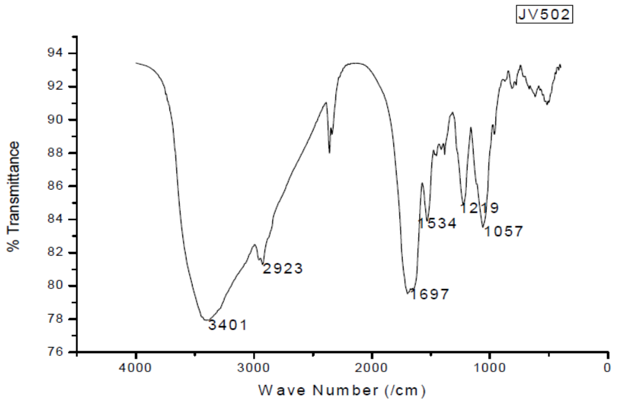


**Figure 22.** FTIR result for JV502

Inference

| Wavenumber | Bond | Functional group |
|---|---|---|
| 3401 | O-H stretch, H-bonded | Alcohols, phenols |
| 2923 | C-H stretch | Alkanes |
| 1697 | C=O stretch | Carbonyls (general) |
| 1534 | N-O asymmetric stretch | Nitro compounds |
| 1219 | C-H wag (-CH$_2$X) | Alkyl halides |
| 1057 | C-N stretch | Aliphatic amines |

## Anti-adhesive test

*Anti-adhesive test against* Bacillus

Anti-adhesive property mainly depends upon the level of the biosurfactant and the microorganisms used. Here, the crude biosurfactants were extracted from five strains. *Bacillus* was taken as pathogenic strain to test the anti-adhesive property of these five biosurfactants, and PBS (phosphate buffer saline) was used as a control that contained no biosurfactant. Our findings demonstrated that biosurfactant extracted from JV201 showed anti-adhesive value 22.61% (Table 8) for the microorganisms *Bacillus* at a minor concentration (1.87mg/mL) means 22.61% adhesiveness inhibited.

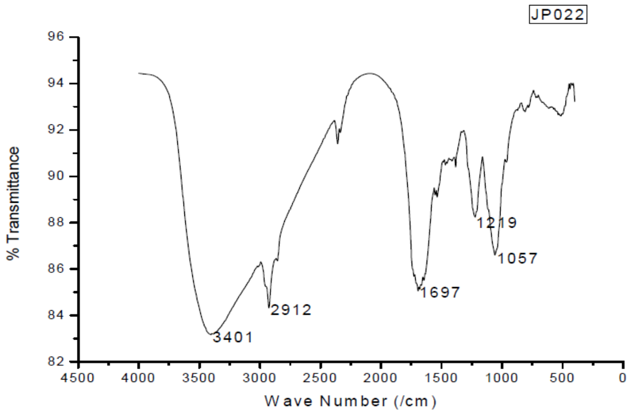


**Figure 23.** FTIR result of JP022

Inference

| Wavenumber | Bond | Functional group |
|---|---|---|
| 3401 | O-H stretch, H-bonded | Alcohols, phenols |
| 2912 | C-H stretch | Alkanes |
| 1697 | C=O stretch | Carbonyls (general) |
| 1219 | C-O stretch | Alcohols, carboxylic acids, esters, ethers |
| 1057 | C-N stretch | Aliphatic amines |

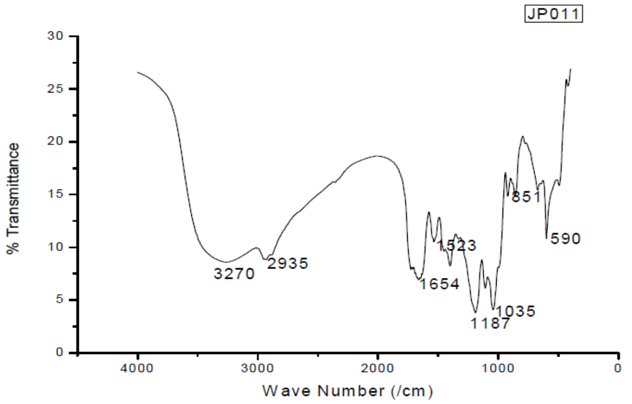


**Figure 24.** FTIR result of JP011

Inference

| Wavenumber | Bond | Functional group |
|---|---|---|
| 3270 | N-H stretch | Primary, Secondary amines, Amides |
| 2935 | C-H stretch | Alkanes |
| 1654 | -C=C- stretch | Alkenes |
| 1523 | N-O asymmetric stretch | Nitro compounds |
| 1187 | C-H wag (-CH2X) | Alkyl halides |
| 1035 | C-O stretch | Alcohols, Carboxylic acids, Esters, Ethers |
| 851 | C-H "oop" | Aromatics |

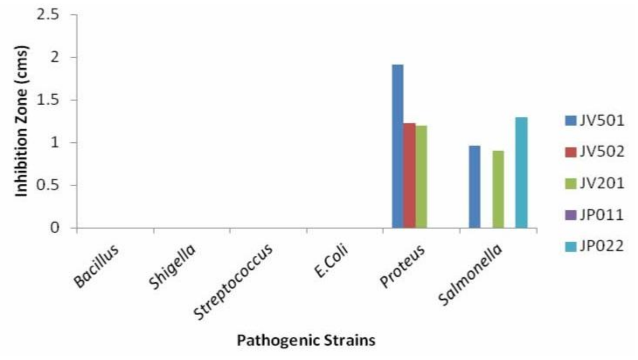


**Figure 25.** Antimicrobial activity of crude biosurfactant against pathogenic strains

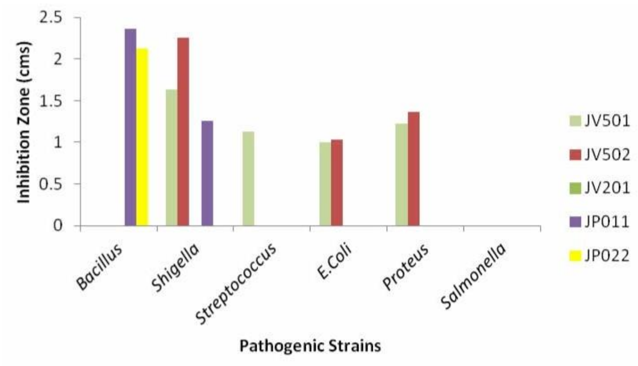
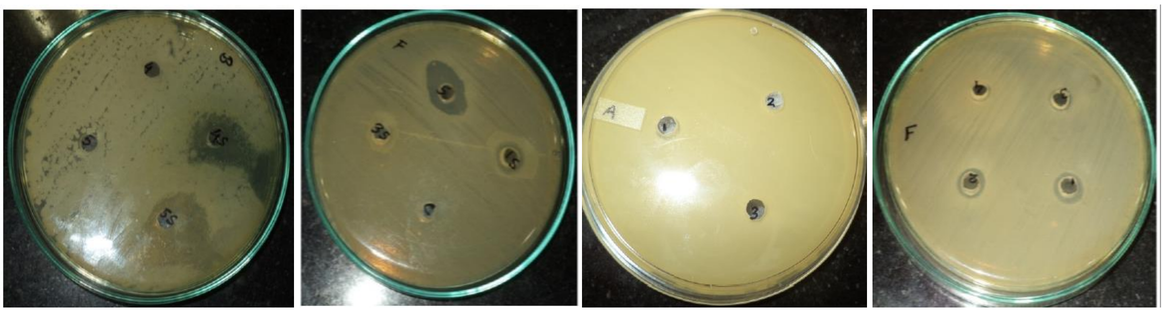


**Figure 26.** Antimicrobial activity of supernatant (biosurfactant) against six pathogenic strains

**Table 7.** The O.D. at 517 nm showing negative antioxidant activity

| Strain name | Control | O.D. after 30 min | O.D. at 1 h | O.D. after 1.30 h |
|---|---|---|---|---|
| JV501 | 1.290 | 1.434 | 1.412 | 1.376 |
| JV502 | 1.290 | 1.834 | 1.757 | 1.706 |
| JV201 | 1.290 | 1.717 | 1.525 | 1.480 |
| JP011 | 1.290 | 1.760 | 1.577 | 1.517 |
| JP022 | 1.290 | 1.629 | 1.457 | 1.328 |



A. Positive B. Nebative

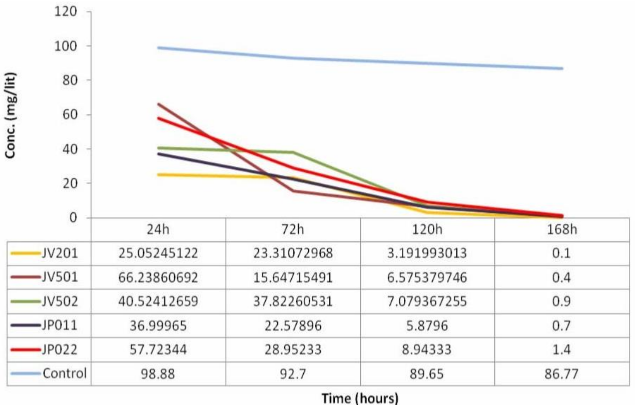
**Figure 27.** Plates showing Antimicrobial Activity by the biosurfactant extracted from the strains

**Table 8.** Percentage of Anti-adhesiveness against *Bacillus*

| Strain name | Biosurfactant conc. (mg/mL) | | | | | | | | | | Control |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 50 | 25 | 12 | 7.5 | 3.75 | 1.87 | 0.93 | 0.46 | 0.23 | 0.11 | |
| JV501 | 15.47 | 11.90 | 16.66 | 9.52 | 10.71 | 8.33 | 10.71 | 4.76 | 8.33 | 14.28 | 0.084 |
| JV201 | -59.52 | 19.04 | 21.42 | 8.33 | 20.23 | 22.61 | 21.42 | 14.28 | 10.71 | 9.52 | 0.084 |
| JV502 | 9.52 | -7.14 | 20.23 | 13.09 | 17.05 | 13.09 | 1.19 | 9.52 | -2.38 | -3.57 | 0.084 |
| JP022 | -8.33 | 4.76 | 11.9 | 8.33 | 14.28 | 13.09 | 4.76 | -9.52 | -17.85 | -3.57 | 0.084 |
| JP011 | -126.19 | -70.23 | -48.80 | -25 | -22.61 | -44.04 | -48.80 | -29.76 | -21.42 | 4.76 | 0.084 |

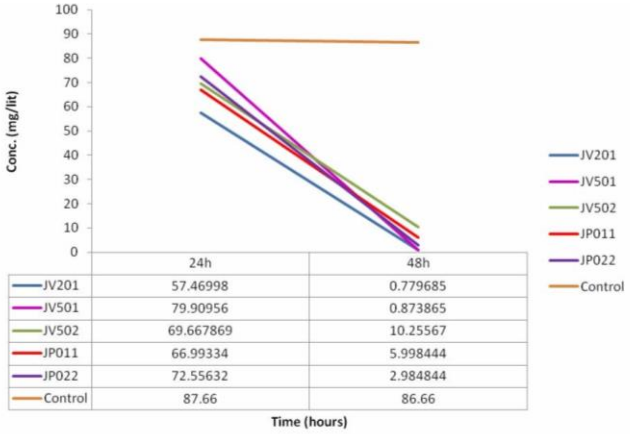
**Table 9.** Percentage of anti adhesiveness against *Streptococcus*

| Strain name | Biosurfactant conc. ( mg/mL) | | | | | | | | | | Control |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 50 | 25 | 12 | 7.5 | 3.75 | 1.87 | 0.93 | 0.46 | 0.23 | 0.11 | |
| JV501 | -16.66 | 8.33 | 7.14 | 3.57 | 2.38 | -8.33 | -25 | -94.04 | -140.4 | -129.7 | 0.084 |
| JV201 | -85.71 | 20.23 | 21.23 | 22.61 | 22.61 | 22.61 | 23.8 | 2.38 | -4.76 | -40.47 | 0.084 |
| JP022 | -70.23 | -26.19 | 1.19 | 2.38 | 2.38 | -2.38 | 3.57 | 14.28 | -13.09 | -51.19 | 0.084 |
| JP011 | 19.26 | 8.55 | 25.89 | 7.98 | 6.55 | -7.54 | -43 | -94.8 | -2.98 | -6.89 | 0.084 |
| JV502 | 3.12 | 6.77 | 15.28 | 28.94 | 18.78 | 4.89 | 5.99 | 3.14 | -5.89 | -3.29 | 0.084 |



|  | 24h | 72h | 120h | 168h |
|---|---|---|---|---|
| JV201 | 25.05245122 | 23.31072968 | 3.191993013 | 0.1 |
| JV501 | 66.23860692 | 15.64715491 | 6.575379746 | 0.4 |
| JV502 | 40.52412659 | 37.82260531 | 7.079367255 | 0.9 |
| JP011 | 36.99965 | 22.57896 | 5.8796 | 0.7 |
| JP022 | 57.72344 | 28.95233 | 8.94333 | 1.4 |
| Control | 98.88 | 92.7 | 89.65 | 86.77 |

**Figure 28.** Isolates showing phenanthrene degradation



|  | 24h | 48h |
|---|---|---|
| JV201 | 57.46998 | 0.779685 |
| JV501 | 79.90956 | 0.873865 |
| JV502 | 69.667869 | 10.25567 |
| JP011 | 66.99334 | 5.998444 |
| JP022 | 72.55632 | 2.984844 |
| Control | 87.66 | 86.66 |

**Figure 29.** Isolates showing naphthalene degradation

### Anti-adhesive test against *Streptococcus*

*Streptococcus* was used for the anti-adhesive analysis. Like above PBS was used as a control. Here biosurfactants from JV501.JV201, JP022 were taken to test the anti-adhesive property against *Streptococcus.* It was observed that JV201 showed the anti-adhesive value for *Streptococcus* was 23.80% at a shallow concentration (0.93 mg/mL) and JP011 and JV502 showed anti-adhesive value for *Streptococcus* at 25.89% at 12 mg/mL concentration and 28.94% at 7.5 mg/mL concentration. These were given in Table 9.

### Biodegradation of Polycyclic Aromatic Hydrocarbons (PAHS) by biosurfactant

*Phenanthrene biodegradation*

Phenanthrene was added to the BHB in the concentration 100 mg/L for five strains (JV201, JV501, JV502, JP011, and JP022). O.D. was read at 292 nm for four days (1st, 3rd, 5th and 7th day) by extracting it with n-hexane. From five strains, JV201 and JP011 degraded phenanthrene from initial of 100 mg concentration to 3.19 and 8.94 mg within 120 hours. The other strains also displayed excellent degradation results (Figure 28).

*Naphthalene biodegradation*

Naphthalene was added to the BHB in the concentration 100 mg/L for five strains- JV201, JV501, JV502, JP011, and JP022. Absorbance was taken at 254 nm for four days (1st day, 3rd day, 5th day and 7th day) by extracting it with n-hexane. Among these five strains, it was observed that JV201 and JP011 had shown higher efficiency in the degradation of naphthalene and strain JV501 showed complete degradation of naphthalene very quickly (Figure 29).

## Discussion

Fourteen bacterial strains were isolated from Paradeep port, Vishakhapatnam, Rishikulya, Bhitarakanika marine water and streaked in LB agar plates and maintained at pure culture. These strains were screened to check the biosurfactant production.

In the oil displacement test, twelve strains showed positive results, and two strains (JV801 and NP802) were negative. In drop collapse method, all strains showed positive results except JV202 and JV101. In emulsification assay, JP022 showed 41% of emulsification activity and the other strains ranging between 35-40%, except for NP202, NP103, and JV101, they gave negative results. In the hemolytic blood test, JP011 showed α hemolysis, JP022- β hemolysis, JV201- β hemolysis, JV501- β hemolysis, ATCC- α hemolysis and others were showing negative results.

From these biosurfactant screening assays, five strains named JV501, JV201, JV502, JP011, and JP022 were showing positive results of producing biosurfactant. Therefore, these five strains were taken for further study. SEM result showed that four strains are rod except for JV501 (cocci). Based on biochemical tests, mannitol motility test, nitrate reduction test, and sulfide indole motility tests, it was found that JV501, JV201, JV502 were showing mannitol fermentation and also motile. The remaining showed negative results in mannitol fermentation and also non-motile. Similarly, in the nitrate reduction test, JV501 and JV502 showing in the reduction of nitrate to nitrite and others displayed negative results. In sulfide production tests all were showing negative results.

Despite numerous report on the antimicrobial activities of biosurfactants, the biosurfactants are produced mostly by the micro-organisms of terrestrial origin. The number of reports on marine antimicrobial biosurfactant molecules is negligible. Therefore, their antimicrobial potentials have not been explored in details. This problem was addressed in the present work, and the biosurfactants isolated from marine bacteria as well as petrochemical wastes were tested for antimicrobial action against a battery of pathogenic test organisms. Six pathogenic strains namely *Proteus, Bacillus, Shigella, Escherichia coli, Streptococcus, Salmonella* were used for the antimicrobial test. Biosurfactants produced from JV501 showed antimicrobial activity against *Proteus*, *Salmonella*, JV502 against *Proteus*, JV201 against *Proteus*, *Salmonella* and JP022 against *Salmonella*. Supernatants of these five strains proceeded to the antimicrobial test. Among these, JP011 demonstrated antimicrobial activity against *Bacillus* and *Shigella*, JP022 against Bacillus, JV501 against *Shigella, Streptococcus* and *Escherichia*, JV502 *Shigella, Escherichia coli*.

Antimicrobial activity of the tested strains is evident in this study and thus can be useful in many domestic and commercial uses. The isolated biosurfactant showed activity against both Gram-positive and Gram-negative bacterial strains. The result is quite in contrast to earlier reports on antimicrobial actions of the biosurfactants where the lipopeptide biosurfactants were reported to be active mostly against Gram-positive bacteria (Singh and Cameotra 2004).

The growth pattern of the isolates was usually between 16-20 hrs having good biosurfactant production during this period. The chemical characterization of the produced biosurfactant using FTIR demonstrated that the peak obtained through this analysis usually corresponds to primary and secondary amines functional groups, also having carboxylic acid stretch, alkane stretch as well as aromatic groups present. The carbohydrate estimation using Phenol-sulphuric acid test and protein estimation using Bradford assay showed that there were much carbohydrate and protein content in the extracted biosurfactant. Different aliphatic and aromatic carbon sources were utilized as a substrate for the growth of biosurfactant producing bacteria to have an optimization study of which references are widely utilized. All the five strains could easily use both glycerol and sucrose. The growth was also seen in different aromatic compounds like naphthalene, pyrene, phenanthrene, biphenyl, kerosene suggesting that the bacteria could efficiently utilize naphthalene and phenanthrene more readily, and then comes biphenyl. The rationale behind biosurfactant production on hydrocarbon utilization must have stimulated itself by enhancing the substrate availability. In some literature, it was mentioned that biosurfactant production in the presence of hydrocarbons showed better production of biosurfactants (Kumar et al. 2006). Here the result was the same; the bacterial strains were showing better production of biosurfactant by utilizing the PAHs as a carbon source. Biosurfactants usually lower the tensioactive force between the two phases. The surface tension of this fraction of the strains JP011, JP022, JV201, JV501, JV502 was found to be in the range of 40-55 mN/m, with the lowest (43.776 mN/m) being from the strain JP011 indicating its powerful surface tension-reducing property.

The anti-adhesive nature of biosurfactant was also tested for the five strains against *Bacillus* and *Streptococcus* of which few strains producing biosurfactant showed good anti adhesiveness. This property can be attributed for the cleaning of pathogenic organisms present in medical equipment and efficiently used in medical uses. The antioxidant potential of the biosurfactant was determined by their scavenging activity of the stable 1, 1-diphenyl-2-picrylhydrazyl (DPPH) free radical. DPPH method is widely used and the easiest way to measure the antioxidant activity of compounds. But the isolated strains did not show any positive antioxidant results.

Biosurfactant utilized in bioremediation has been harnessed relentlessly for biotechnological purposes. We have isolated and identified five potent strains having high surface tension reducing property: *Ochrobactrum, Streptococcus, Pseudomonas* sp., *Pseudomonas aeruginosa*, and *Achromobacter xylosoxidans* which have 99.9%, 99.6%, 99%, 99.3%, 98.6% of phenanthrene degradation (100mg/L) and 99%, 99.1%, 89.75%, 94.01%, 97.02% of naphthalene degradation (100 mg/L) respectively having good antimicrobial and anti-adhesive properties.

To conclude, nowadays the production of biosurfactant is increasing due to its properties like low toxicity, biodegradability, digestibility and biocompatibility and also due to its vast applications in bioremediation of various toxic substances like PAHs. It is produced on living surfaces mainly microbial cell surfaces or synthesized extracellularly amphiphilic compounds reduces the interfacial tension between the surfaces and interface respectively. When bacteria are present in stress conditions like hydrophobic environment, they utilize these hydrophobic substances like carbon and energy sources. Bacteria produce biosurfactant which helps in conversion of the hydrophobic layer into small micelles which can be easily engulfed as a carbon source which is the primary nutritional requirement. PAHs are released into the environment by various sources. These can be classified into natural sources and anthropogenic sources, but anthropogenic sources produce higher PAHs than natural sources. The anthropogenic sources are the waste products of various industries (Petroleum, Diesel), domestic sewage, the oil spill in the marine environment, smoking, by burning of coal, diesel, and petrol (fuel for energy). The hydrocarbons contaminate the subsoil and groundwater. It enters the food chain and disturbs it. Some of the light polyaromatic hydrocarbons bind to the dust particles in the atmosphere and persist for a long time. It enters into the human body through inhalation, food, skin and causes mutagenic and carcinogenic effects. These compounds are highly toxic, even a low amount of them present in the soil may cause serious problems.

Eco-friendly technologies such as degradation by microorganisms must be used to clean the environment. Bioremediation has been accepted as an essential method for the treatment of oil pollution by biosurfactant excreted by bacterial colonies. Under certain conditions, living microorganisms especially bacteria can metabolize many classes of hydrocarbons compound. Since hydrocarbons carry high organic matter, it can be assimilated by the bacteria as a carbon source. There are many ways used to clean up the organic contaminants. Some non-biological methods such as excavation and discharge of contaminated soil to landfill sites are employed. Biological methods are the processes that utilize plants (phytoremediation) or microorganisms (bioremediation) to remove these pollutants from soil. Therefore, employing biobased techniques like the production of biosurfactant in large quantities through bioreactors can be efficiently commercialized in industries and can be applied in highly polluted areas for complete biodegradation of the toxic polycyclic aromatic hydrocarbons.

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