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ISOLATION AND CHARACTERIZATION OF BIOSURFACTANT PRODUCING BACTERIA FROM OIL CONTAMINATED SITE

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ABSTRACT

Biosurfactant are surface-active metabolites produced by microorganisms. Biosurfactant tends to solubilize hydrocarbons in the surrounding environment, by reducing surface tension and increasing carbon uptake. In this study, isolation of biosurfactant producing bacteria and effects of biosurfactant were assessed. Oil sample was collected from engine oil contaminated site and six strains were isolated. Strains were screened for biosurfactant production by hemolysis test, drop collapse test, oil spreading assay, lipase activity, and emulsification index. Then strains were further screened for maximum production on different carbon sources e.g. dextrose, sucrose, starch, fructose and glycerol, also different nitrogen sources such as peptone, yeast extract, urea, sodium nitrate, ammonium sulphate. The promising strain was directed for further examination. Among different carbon sources better results were noted in dextrose about 0.23 gm/50ml. further investigation is to explore the effects of biosurfactant on plant growth promotion and its antimicrobial activity.

Keywords: Biosurfactant, bacteria, oil contaminated site

INTRODUCTION

Biosurfactants are amphiphilic compounds produces on living surface, mostly on microbial cell surface or excreted extracellularly and contains hydrophobic and hydrophilic moieties that confer the ability to accumulate between fluid phases, that reduces surface and interfacial tension[21]. The hydrophilic moiety of the biosurfactant can be a carbohydrate, an amino acid, a phosphate group or alike compounds, whereas the hydrophobic moiety is mostly the fatty acid carbon chain**[24]**. They are structurally diverse group of surface active molecules synthesized by microorganisms[6]. Microorganisms those have been stated to produce a number of classes of biosurfactants such as mycolic acid, glycolipids, polysaccharide-lipid complex, lipoprotein or lipopeptides, phospholipids or the microbial cell surface itself[12]. Few of the microorganism species like *Pseudomonas sp.,* is found to produce glycolipid group of biosurfactant called rhamnolipids[22]. Lipopeptides or lipoproteins such as surfactin, produced by *Bacillus subtilis*[5]. These both species of organisms are examined and noted for their capability of producing higher amount of biosurfactants. In addition, these organisms can utilize various renewable resources, especially agro-industrial waste as important carbon sources. These directs to the economical biosurfactant production and helps to reduce pollution caused by hydrocarbon wastes[28]. Due to

advantageous properties like ecological acceptance, low toxicity, potentially high activities, effectiveness and stability at extreme levels of temperature, pH, salinity and possibility of production from renewable substrates, they have been increasingly attracting the attention of scientific and industrial community. The reason, these scientific and industrial communities are very much interested in this biosurfactant product is that it is multifunctional material or product that can be used as emulsifier and demulsifier, wetting agents, foaming agents, spreading agents, functional food ingredients and detergents [23]. Out of most positive influences of the biosurfactant (lipopeptide), the recent one is in agriculture and is mainly considered for its biocompatibility with living organisms. Biosurfactant producing microorganisms enhances plant growth through improvement in plant immunity against organic contaminants in the environment[26]. Researchers verified that plant growth promoting rhizobacteria (PGPR) positively enhances plant development after association with the hydrogen degrading bacteria in the contaminated soil[17]. Increase in awareness among public about the use of environment-friendly and sustainable green products demands new strategies development to cut down the production cost for replacement of toxic synthetic surfactants with biosurfactants. In present study, the intention is to determine the stable potent biosurfactant and to examine the potential of produced biosurfactant for crude oil contaminated soil detoxification and to promote plant growth and development.

MATERIALS AND METHODS

Sample collection and Isolation of biosurfactant producing bacteria

Oil contaminated soil sample was collected from automobile workshop in Navsari district. The sample of soil was enriched for the better isolation of biosurfactant producers into 250 ml Erlenmeyer flask containing 100 ml of Mineral Salt Medium (MSM) broth containing (in g/L): 15g $NaNO_3$, 1.1g KCl, 1.1g NaCl, 0.00028g $FeSO_4 \cdot 7H_2O$, 3.4g KH_2PO_4, 4.4g K_2HPO_4, 0.5g $MgSO_4 \cdot 7H_2O$, 0.5g yeast extract incorporated with different crude oils 5% (V/V) as the carbon source, then the flasks were incubated at 37°C for 7 days at 200 rpm in rotatory shaker incubator. After incubation, the samples were serially diluted and spreaded on Nutrient Agar (NA) plates and the plates were incubated at 37°C for 24-48 hours. Isolates were subcultured and used for further studies.

Screening for biosurfactant producers

Hemolytic activity

Hemolytic assay was performed on Blood Agar plates. Culture broth of selected isolates were spot inoculated on Blood Agar plates and incubated for 48 hours at 37°C. The plates were examined for clear zone (Hemolysis) around the colony, which is qualitative method used as an indicator of biosurfactant production[14].

Oil Spreading Assay

Oil spreading experiment was performed using the method described by Morikawa *et al*., 1993. In brief, 50 ml of distilled water added to a petri plate followed by addition of 2ml of crude oil to the surface of the water. 500µl of the cell free culture broth was then added to the oil surface. If biosurfactant is present in the cell free culture broth, the oil will be displaced with an oil free clear zone indicates surfactant activity, also called oil displacement activity. A negative control

was maintained with distilled water (without surfactant), in which no oil displacement or clear zone was observed[13].

Drop collapse assay

For drop collapse assay, the glass slides coated with oil were taken and a drop of cell free culture broth placed on it and observed for result after 1 min. The result was considered positive for biosurfactant production when the drop was flat and those cultures that gave rounded drops were scored as negative indicative of the lack of biosurfactant production[29].

Lipase activity

In this assay, overnight culture broth of the selected isolates was spot inoculated on Tributyrin agar medium and after plates were incubated at 37°C for 48 hours. Then the zone of lysis around colonies was observed[7].

Emulsification index (E24)

For determination of the emulsification index, 48 hour grown culture supernatants were collected by centrifugation at 10,000 rpm for 10 minutes. Then 2 ml of supernatant and 3 ml of a selected hydrocarbon source (Kerosene) were mixed in a test tube and vortexed for 2 minutes. The test tube left intact for 24 hours and after, the height of emulsion layer was measured to determine the emulsification index (E24) by using following formula:[4]

$$\mathbf{E24} = \frac{\mathbf{Height\ of\ the\ emulsion\ layer}}{\mathbf{Height\ of\ total\ solution}} \times \mathbf{100}$$

Optimization of Biosurfactant producing microorganisms

Preparation of Inoculum

About 50 ml of Nutrient broth was prepared and sterilized by autoclaving at 121°C temperature and 15 lbs pressure. It was inoculated with maximum positive results showing screened isolate and incubated at 37°C for 24-48 hours in an incubator shaker[15].

Optimization of Carbon source

Total 250 ml of Mineral Salt Medium (MSM)was prepared and dispensed 50 ml of medium in five 100 ml conical flasks and sterilized. Media was incorporated with five different carbon sources (1%) like Dextrose, Fructose, Sucrose, Glycerol and Starch keeping peptone as nitrogen source. About 1 ml of inoculum was inoculated, and then flasks were incubated at 37°C for 24-72 hours in an incubator shaker at 120 rpm. After incubation, the media was centrifuged at 10,000 rpm for 10 minutes. Product was obtained and its dry weight was measured[15].

Optimization of Nitrogen source

Total 250 ml of Mineral Salt Medium (MSM)was prepared and dispensed 50 ml of medium in five 100 ml conical flasks and sterilized. Media was incorporated with five different nitrogen sources (0.5%) like Peptone, Yeast extract, Urea, Ammonium nitrate, Ammonium sulphate. Dextrose was added as the carbon source into all the different nitrogen sources. About 1 ml of inoculum was inoculated, and then flasks were incubated at 37°C for 24-72 hours in an incubator

shaker at 120 rpm. After incubation, the media was centrifuged at 10,000 rpm for 10 minutes. Product was obtained and its dry weight was measured[15].

Optimization of pH

Total 250 ml of Mineral Salt Medium (MSM)was prepared and dispensed 50 ml of medium in five 100 ml conical flasks and sterilized. Prior to addition of inoculum to the solution, the samples were adjusted to different pH viz.pH 5, 6, 7, 8 and 9. Dextrose as the carbon source and peptone as nitrogen sources were added. About 1 ml of inoculum was inoculated, and then flasks were incubated at 37°C for 24-72 hours in an incubator shaker at 120 rpm. After incubation, the media was centrifuged at 10,000 rpm for 10 minutes. Product was obtained and its dry weight was measured[15].

Optimization of Temperature

Total 100 ml of Mineral Salt Medium (MSM)was prepared and dispensed 50 ml of medium in two 100 ml conical flasks and sterilized. Media was incubated at two different temperatures like room temperature and 37°C. About 1 ml of inoculum was inoculated, and then flasks were incubated for 24-72 hours at respective temperature condition. After incubation, the media was centrifuged at 10,000 rpm for 10 minutes. Product was obtained and its dry weight was measured[15].

Production of Biosurfactant using optimized conditions and nutrient components

Total 100 ml of Mineral Salt Medium (MSM)was prepared using optimized carbon source, nitrogen source, pH and temperature. About 1 ml of inoculum was inoculated, then flask was incubated at 37°C for 24-72 hours. After incubation, the media was centrifuged at 10,000 rpm for 10 minutes. Product was obtained and its dry weight was measured[15].

Extraction of biosurfactant after optimization

The supernatant was taken for biosurfactant extraction. The pH was adjusted to 2.0 using 6N HCl and kept at 4°C for overnight. The extraction performed twice with an equal volume of ethanol. The solvent was removed by evaporation. Obtained white precipitated product was used for further experimentation[1].

Dry weight of Biosurfactant

Sterile petri plate was taken and the weight of the plate was measured. Then the extracted product (precipitates) was poured into the plate. They were placed in the hot air oven for drying at 80°C for 30 minutes. After drying, the plate was weighed. The dry weight of biosurfactant was calculated by using the given formula:

$$\textbf{Dry weight of biosurfactant} = \text{Weight of the plate after drying} - \text{Weight of the empty plate}$$

Weight of the biosurfactant was expressed in terms of **grams/ 50 ml** (Dry weight).

Purification of Biosurfactant

The culture broth was centrifuged for 10 minutes at 10,000 rpm to obtain cell free supernatant. The equal volume of Chloroform: Methanol (2:1) was added. This mixture was shaken well to mix all the liquids and left intact overnight for evaporation. White colored sediment obtained is pure biosurfactant.

Application of Biosurfactant (Functional characterization of the biosurfactant)

Exploration of biosurfactant for plant growth

Mung bean seeds were used and are surface sterilized with 10% Na– hypochlorite for 20 min and then washed with sterile distilled water before use. The biosurfactant was dissolved in de-ionized water. The seed germination experiment was conducted in a petri plate containing 20 seeds positioned on filter paper soaked with two different concentrations (0.3 and 0.5 gm/100 mL) of biosurfactant solution. Distilled water 100% v/v was used as a control. The plates were kept in normal condition at room temperature for 2 days.

Following the germination test seeds treated with biosurfactant solution were transferred in pots (seeds without pre-treatment with biosurfactant were used as control) and kept in regular temperature condition. The emergence of plant seedlings was tested and checked for the morphological characteristic of plants like shoot length (mm) and root length (mm) [8] [27].

Antimicrobial activity of biosurfactant

Biosurfactant for its antimicrobial potential was assessed through well diffusion assay as mentioned before by[24]. Mueller–Hinton agar plates were prepared and it contain *Escherichia coli* and *Salmonella typhi* as test organism. The biosurfactant (10 mg/mL), Tetracycline, Chloramphenicol, Penicillin Streptomycin (1 mg/mL) and biosurfactant in addition with antibiotics (5:1 mg/mL) were poured at a concentration of 100 µL and kept at 37°C for 24 h of incubation. To investigate the additional effect of biosurfactant with antibiotics if there was any increase in the diameter of the inhibition zone was measured in (mm) as compared to antibiotics. Antibiotics without biosurfactants were used as a positive control[27].

RESULT AND DISCUSSION

Isolation of biosurfactant producing bacteria

Soil sample was analyzed by enrichment culture technique for the selection of potential biosurfctant producers, and henceforth, six isolates were selected. Out of six isolates, only one was gram-positive rod, and remaining all were gram-negative rods. All the six isolates were catalase positive, two utilizes citrate, one was urease positive and three hydrolyzed starch.

Screening for Biosurfactant producers

Hemolytic activity

The isolates were streaked on blood agar plate. The plates streaked with isolate-1, showed the β-haemolysis activity. Other isolates showed no hemolysis (Fig: 1).

Drop collapsing assay

Drop collapse is a sensitive test, which can give result with a very small amount of surfactant. Only the isolate-1 gave positive result of high drop collapsing activity. To further confirm the biosurfactant production, the cell free culture broths of organisms were subjected to oil spreading assay (Fig: 2).

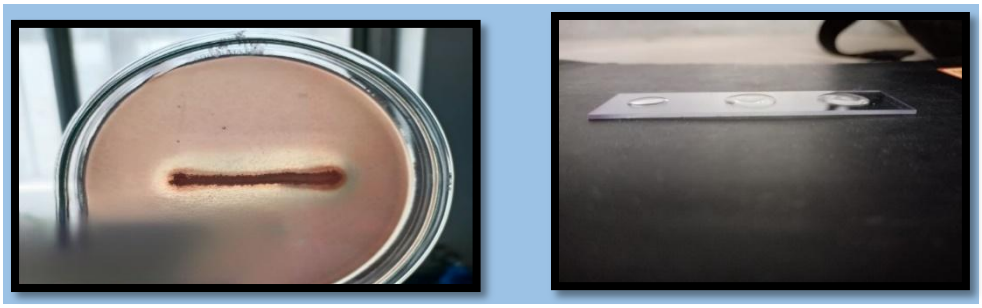

Fig: 1 β-Haemolysis on blood agar plate Fig: 2 Drop Collapsing assay

Oil spreading assay

Oil spreading assay results showed corroboration with the drop collapse assay results in the way that the organisms found positive with drop collapse assay were positive for oil spreading assay as well. Morikawa *et al.* explained that the oil displacement area is directly proportional to the surface-active compound in the solution. The isolate-1 showed the high oil displacement activity. Only biosurfactant producer can only be able to displace the oil. The results were tabulated (Fig: 3).

Lipase activity

Among all six isolates, total three isolated showed positive result as zone of clearance around the colony on Tributyrin agar plates. One isolate showed good clearance zone whereas other two showed moderate zone of clearance. Remaining three isolates gave negative result as no clearing zone observed (Fig: 4).

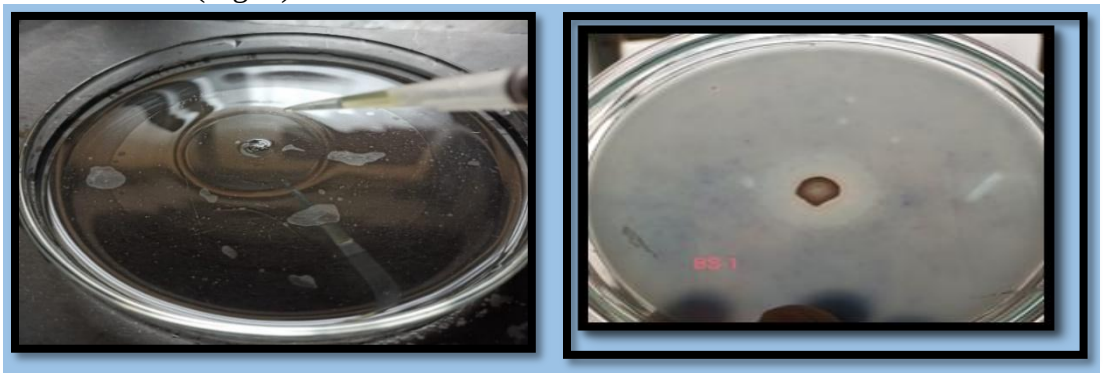

Fig:3 Oil displacement activity Fig: 4 Lipase activity

Emulsification index (E24)

Emulsification assay is an indirect method used to screen biosurfactant production. It was presumed that if the cell freeculture broth contains biosurfactant then it would emulsify the hydrocarbons present. The E24 of all isolates are noted in (Fig: 5). maximum emulsification was observed with Isolate-1 (26.00%) and minimum emulsification (about 4.00%) was of Isolate-5.On screening for biosurfactant production, only isolate-1 that gave positive result for all tests was selected as producer.

Optimization of Biosurfactant production

Among the carbon sources, Dextrose was found most favorable (0.23 gm/50ml)(Fig: 6), similarly, among five nitrogen sources, peptone showed highest biosurfactant production about

(0.22 gm/50ml)(Fig: 7). the optimum pH was confirmed as 8.0 and gave (0.26 gm/50ml) of biosurfactant production(Fig: 8). The optimum temperature was confirmed as 37°C as (0.25 gm/50ml) produced biosurfactant(Fig: 9). All the optimized conditions were taken to prepare the production medium.

Extraction of Biosurfactants and Dry weight

The culture inoculated in optimized mineral salt medium was centrifuged and the supernatant was mixed with Chloroform: Methanol (2:1). White sediment was retained on an empty petri plate and total produced biosurfactnt was measured. According to Singh *et al.*, 2018 maximum amount of biosurfactant produced by *Bacillus subtilis* was 0.324 gm/100ml as compare to that the Isolate-1 found to produce 0.44 gm/100ml of the crude biosurfactant.

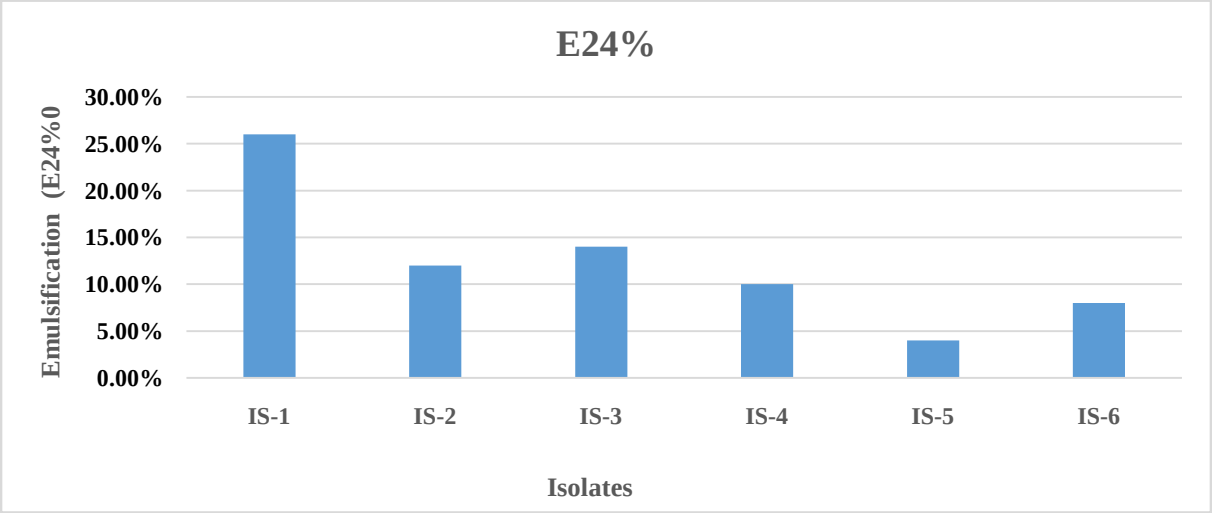

Fig 5: Study of emulsification index of different isolates

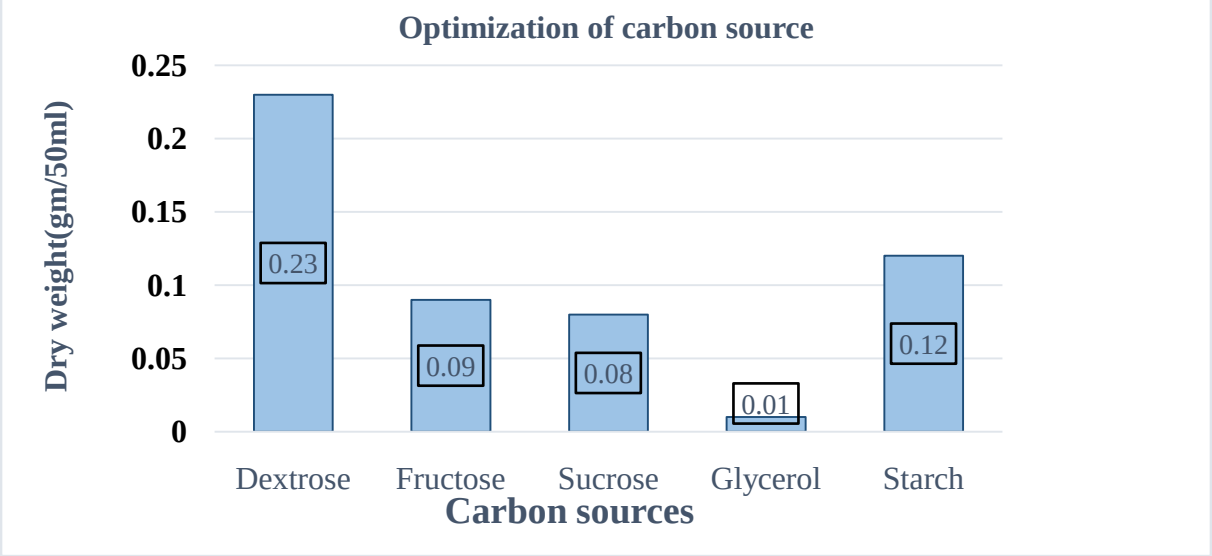

Fig 6: Optimization of carbon source

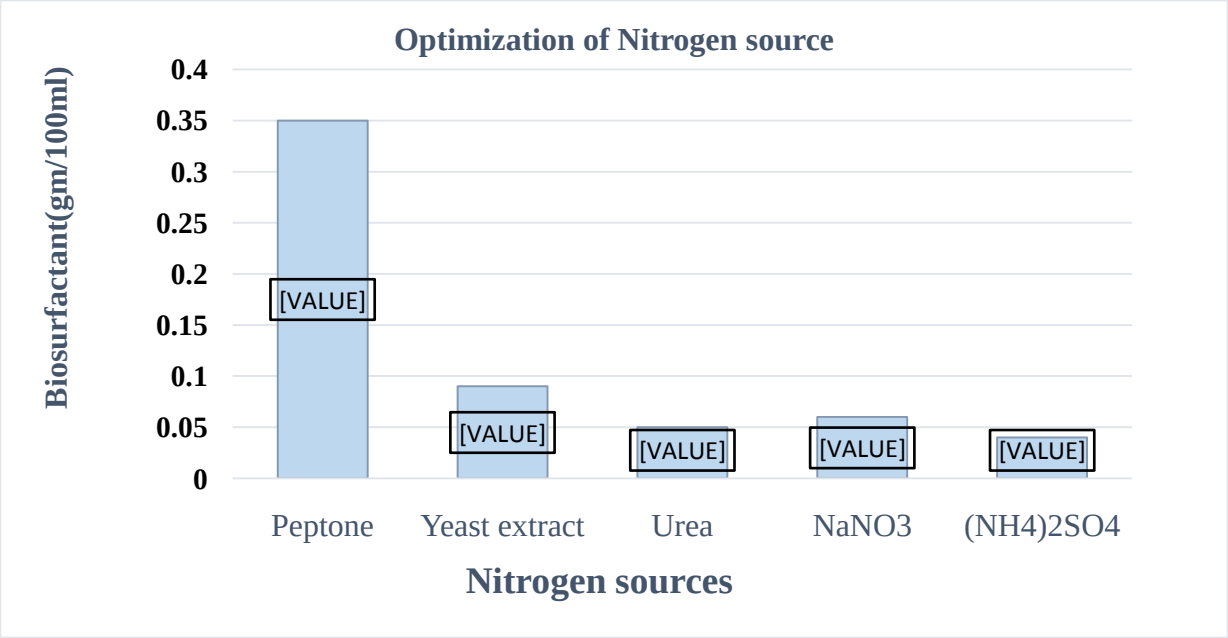

Fig 7: Optimization of nitrogen source

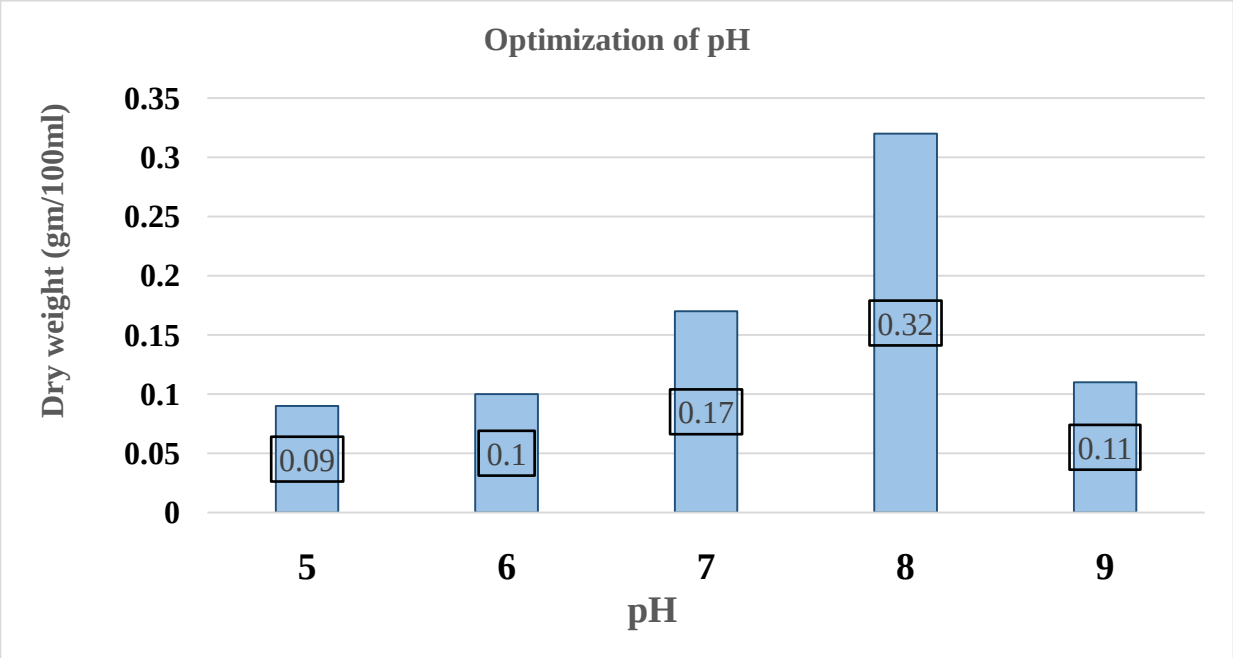

Fig 8: Optimization of pH

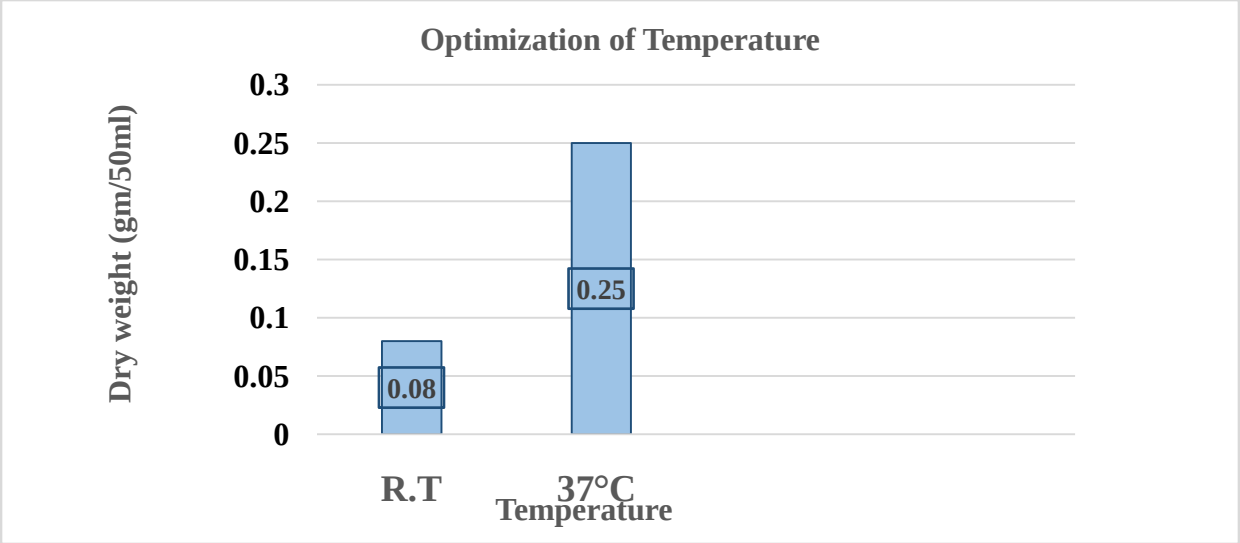

Fig 9: Optimization of temperature

Effect of biosurfactant on plant-growth promotion

In this study, Mung beans were examined to demonstrate the effect of biosurfactant, which showed significantly better effect on seed germination and plant growth promotion. Statistical data about plant growth obtained is shown in (Fig: 11). The best results of plant growth were obtained at higher concentration (0.5 mg/ml) of biosurfactant tested.

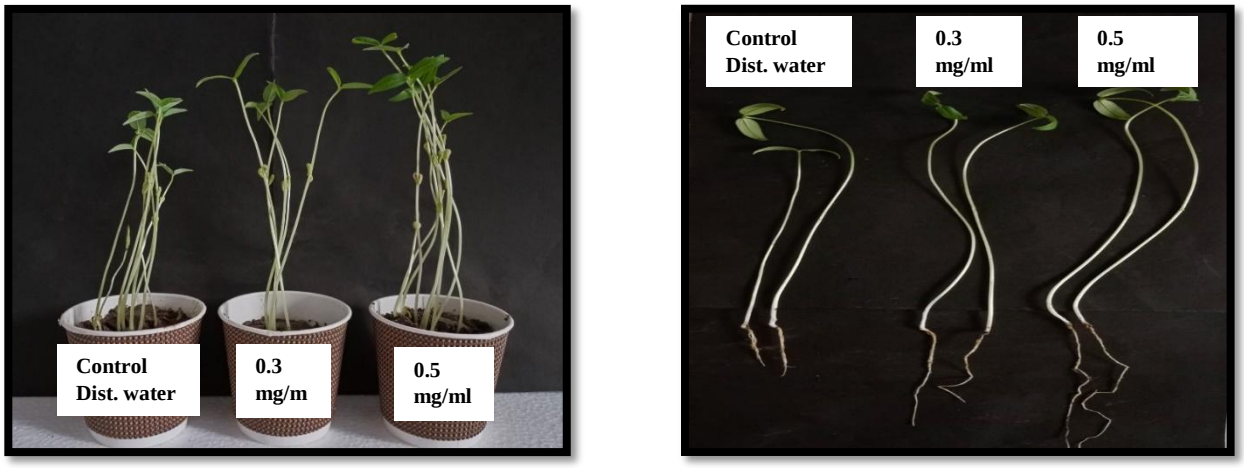

Fig: 10 Mung bean was examined to check the effect of biosurfactant.

Table: 1 Data of effect on plant growth

	SHOOT LENGTH	ROOT LENGTH
CONTROL	15.0 cm	4.2 cm
0.3 µl/ml	20.0 cm	8.0 cm
0.5 µl/ml	22.7 cm	9.7 cm

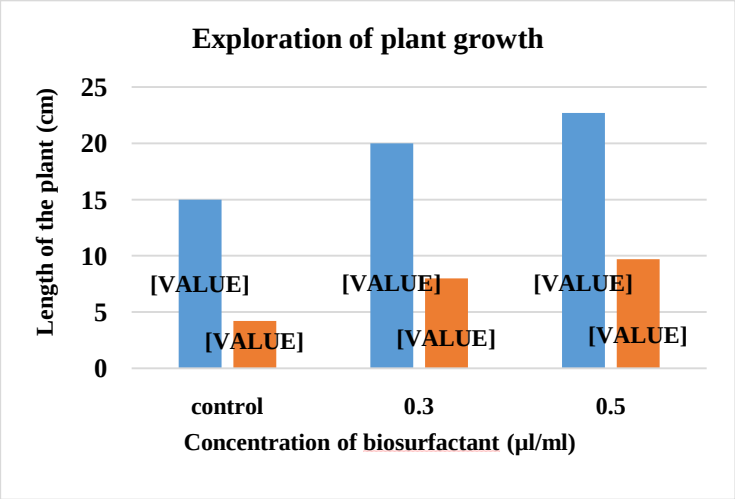

Fig: 11 Statistical data of effect on plant growth

Screening of biosurfactant for Antimicrobial activity

According to Umar *et al.*, 2018, lipopeptide produced by *Bacillus subtilis* showed antimicrobial and synergistic effects with antibiotics against *Escherichia coli* [Fig-12] and *Salmonella typhi* [Fig-13].

In this study, in combination with antibiotics, the results obtained showed an increase in zone of inhibition. The growth of *Escherichia coli* was affected maximum by the combination of produced biosurfactant and chloramphenicol [table- 2]. Whereas *Salmonella typhi* was affected by the combined effect of produced biosurfactant and penicillin [table- 3].

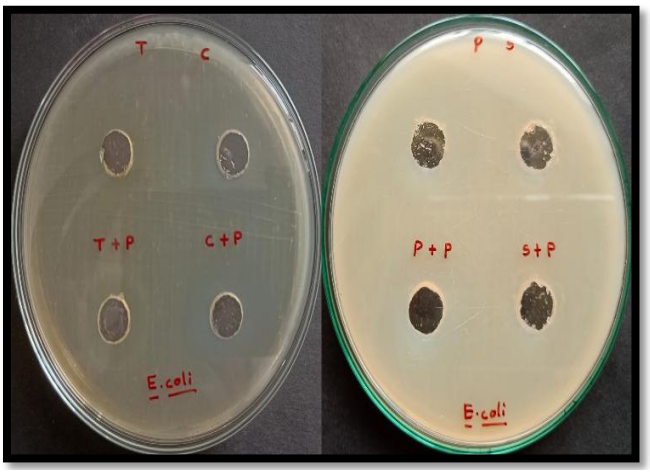

Fig 12: Antibacterial activity against *E. coli*

Table: 2 Antibacterial activity against *E. coli*

Tetracycline	**Control**	16mm
	Product + Antibiotic	20mm
Chloramphenicol	**Control**	25mm
	Product + Antibiotic	29mm
Penicillin	**Control**	24mm
	Product + Antibiotic	25mm
Streptomycin	**control**	16mm
	Product + Antibiotic	18mm

Table: 3 Antibacterial activity against *S. typhi*

Fig 12: Antibacterial activity against *S. typhi*

Tetracycline	**Control**	19mm
	Product + Antibiotic	21mm
Chloramphenicol	**Control**	29mm
	Product + Antibiotic	29mm
Penicillin	**Control**	25mm
	Product + Antibiotic	27mm
Streptomycin	**control**	16mm
	Product + Antibiotic	19mm

CONCLUSION

The microorganisms were isolated from the oil contaminated soil sample. Total six isolates were selected and screened for biosurfactant production. Morphological and Biochemical characterization of this six isolates were determined. Based on screening tests, among six isolate only one Isolate-1 gave all the screening tests positive and selected for further investigation. The media was optimized manually for the production of biosurfactant by the screened isolate, the optimization parameters were carbon source, nitrogen source and physical optimization parameter such as pH, temperature. By these optimized parameters, the optimized media includes carbon source- glucose, nitrogen source- peptone, pH- 8 and temperature- 37°C.The yield of product increased to 0.44 gm/50 ml after the optimization. After optimization the application of the biosurfactant (product) was studied. These applications includes the exploration of the product on the plant growth and antimicrobial activity of product with the combination of antibiotic. The conclusion of the applications performed is that the product enhances the root length of plant compared to the control and in antimicrobial activity, the power of antibiotics like tetracycline, chloramphenicol, and streptomycin were enhanced after the product was added in to it.

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