



Plant growth effect of chromium contaminated effluent treated with Biosurfactant producing *Citrobacter freundii*

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Abstract

The present study was carried out to evaluate removal of chromium heavy metals in effluent waste water by surface active agent. Biosurfactant producing bacterium *Citrobacter freundii* was isolated from soil samples collected from mechanical shed in and around Namakkal. Chromium removal using crude oil utilizing bacteria was tested. Ability of growth on crude oil under shaking condition was studied. Biosurfactant activity production was carried out by oil displacement, hemolytic activity and emulsification index. The experimental data reveals that, out of 7 oil tolerant bacteria, S3 only found to be potent oil tolerant and biosurfactant producer. The isolate is a hemolytic, Gram negative rod showed maximum oil displacement activity than other tested strains. The reduction property of crude biosurfactant tested on leather tannery effluent shows decreased concentration of hexavalent chromium. Toxicity of treated effluent on seed germination and vigor index showed enhanced germination and plant growth potential evidenced that the treated effluent is free from chromium ⁶⁺ toxicity.

Keywords: chromium, biosurfactant, *Citrobacter* sp, toxicity, vigor index

Introduction

Industrial activities, such as mining, production of batteries for vehicles, industrial waste deposits and the dispersal of ash from incineration processes are key source for contamination of soil by heavy metals. Different technologies have been developed and implanted to reduce costs associated with the treatment of contaminated soil by heavy metals. Most importantly, immobilising heavy metals in a solid matrix and desorption and solubilisation are promising methods ^[1]. Biosurfactants enhance metal desorption from soils or in water by forming complexes with the free, non-ionic metals in solution. The mechanisms driving biosurfactant-metal binding include ion exchange, precipitation dissolution, counter-ion association and electrostatic interaction ^[2]. The hydrophobic (non-polar) part of the biosurfactant is insoluble in water and may have a long-chain of fatty acids, hydroxyl fatty acids or α -alkyl- β -hydroxy fatty acids. The hydrophilic (polar) end can be a carbohydrate, amino acid, cyclic peptide, phosphate, carboxylic acid or alcohol ^[3]. Oil displacement and emulsification index methods were routinely used to screen the capability of isolates for producing biosurfactant ^[4]. New approaches and attempts for metal stabilization using biosurfactants have also been recently showed bioremediation of Cr (VI) by a biosurfactant producing marine isolate *Bacillus* sp ^[5]. Glycolipids and lipopeptides are the most widely studied groups of biosurfactant compounds. The glycolipid based biosurfactants include mannosylerythritol lipids, sophorolipids, trehalolipids and rhamnolipids are primarily produced by most *Pseudomonas* species, particularly *P. aeruginosa* strains ^[6]. Surfactin is the cyclic lipopeptide biosurfactant produced by *Bacillus* sp. and it can reduce the surface tension of water from 72 to 27 mN/m concentrations ^[7]. Biosurfactants have fulfilled almost all the regulations and demands of the society regarding the replacement of toxic chemicals and aerobic

effluent treatment ^[8]. The potential of biosurfactant depends on the concentration of biosurfactant required to reduce surface tension by forming a micelle is called Critical Micelle Concentration ^[9].

Materials and Methods

Description of the study site

Petrol and diesel discharged soil was collected from two wheeler mechanic shed, situated in and around Namakkal, in a sterile bottle and processed for microbial studies. Totally 25 hot spots were selected and surface soil samples were collected and processed.

Isolation of bacteria

In order to isolate the biosurfactant producing bacteria the sample was enriched with 100 ml of nutrient broth with 2% crude oil. In a sterile crude oil supplemented broth 1 g of soil was aseptically added and kept under 100 rpm for 48 h. It was serially diluted up to 10⁷ using saline and 1 ml of 10⁷ dilution was poured on sterile petridish. The sample plate was over layered with sterile nutrient agar medium and incubated under aerobic conditions at 37°C for 24 hours. After 24hr colony morphology was recorded and subcultured.

Effect of crude oil on bacterial growth ^[10]

Growth rate of seven isolates were tested independently at two different concentration of oil. Five milliliter of 24 hours old colonies were inoculated in 100 ml nutrient broth medium in 250 ml Erlenmeyer flask containing 2 and 5 % crude oil. The setup was incubated under 200 rpm at 37°C for 24hr. Growth rate was recorded at 600 nm.

Biosurfactant Production ^[11]

Isolates were grown in 500ml Erlenmeyer flasks containing 100ml yeast glucose broth adjusted to pH 7.0 was used as

culture medium. The flasks were incubated at 37 °C on a shaker incubator for 2 days. Another set up was maintained under static condition. To extract the biosurfactant, the bacterial cells were removed by centrifugation and the remaining supernatant liquid was collected. Biosurfactant was obtained by adjusting the supernatant pH 2.0 using 6N HCl and keeping it at 4°C overnight. The precipitate thus obtained was pelleted by centrifugation for 20min, dried and weighed. For further purification, the crude surfactant was dissolved in distilled water at pH 7.0 and dried at 50°C. The dry product was eluted with Chloroform: Methanol (6:1).

Oil displacement Test ^[12]

In this test, 14 ml of distilled water was added to a petri dish which is 90 mm in diameter. 1ml of diesel was added to the water surface. To this setup 50 µl of cell free culture filtrate was loaded on to the oil surface and the diameter of clear halo zone was measured after 30sec.

Foam height

Foaming of biosurfactant in culture medium was determined by shaking vigorously the supernatant (10 ml) and allowed it for 2 min. The height of foaming was calculated using a graduated cylinder. Foaming activity was detected by the equation

$$\text{Height of foam / Total height} \times 100$$

Emulsification capacity

A mixture of 5 ml hydrocarbon and 2.5 ml cell free extract obtained after the centrifugation of sample culture were taken in a test tube and homogenized by vortexing for 2 min. The emulsion activity was investigated after 24 hours and the emulsification index (E₂₄) was calculated by the total height of the emulsion by the total height of the aqueous layer and then multiplying by 100. The results were compared with SDS as positive control.

$$E_{24} = (\text{Height of the emulsification layer / total height of mixture}) \times 100\%$$

Hemolysis test

The bacterial strains producing biosurfactant and its hemolytic activity against sheep blood was checked. Anticoagulant treated whole blood was taken and diluted with Phosphate buffer saline (1:9ratio). 100 µL of blood in 1 mL of tyrode and 100 µL extracted samples are mixed and incubated for 30 min at room temperature. DMSO used as negative control and Triton x100 (1%=70% lysis) used as positive control. After centrifugation OD values were recorded at 540 nm and percentage of hemolysis were calculated.

$$\% \text{ of haemolysis} = \text{S-NC/PC-NC} \times 100$$

Identification of potent biosurfactant producer

Isolated strain is subjected to preliminary Gram staining and KOH test. The polyphasic tests; IMViC, catalase and oxidase tests were used to find out the genera.

Estimation of chromium reduction

Reduction of chromium by biosurfactant was determined by diphenylcarbazide method describe by EPA. 100 ml of leather tannery effluent was autoclaved. To 45 mL of effluent, 5 mL of Biosurfactant was added and kept in a rotary shaker at 120 rpm for 24 hrs. The filtrate containing the residual concentration of Cr was determined

spectrophotometrically at 540 nm followed by filtration using Whatman filter paper (No. 1). To 1 mL of the supernatant, 9 mL of 0.2 M sulphuric acid and 0.2 mL of 0.25% diphenyl carbazide in acetone were added, and the absorbance of the pink colour was read at 540 nm using distilled water as blank. Same procedure was carried for culture filtrate and untreated effluent. The linear regression of the standard graph was used for the estimation of chromium present in the solution. The chromium removal percentage was calculated using the following formula

$$E = (C_i - C_f / C_i) \times 100$$

Where C_i = initial metal ion concentration, OD; C_f = final metal ion concentration OD

Seed Germination Test ^[13]

The seeds of Tomatoes were surface sterilized with sodium hypochlorite solution (1%) for 10 seconds and rinsed with water. These seeds were then soaked into chromium treated artificial effluent with neutralized pH 15 minutes and subsequently the excess suspension was decanted. After this, these seeds were air dried on tissue paper under shade and cool condition. The seeds similarly treated with only sterilized water were kept to serve as positive control and untreated chromium as negative control. Then the seeds were sown in different pots containing sand and irrigated.

$$\text{Germination rate (\%)} = (\text{number of seeds germinated / total number of seeds}) \times 100$$

$$\text{Vigor index (\%)} = (\text{mean root length} + \text{mean shot length}) \times 100$$

Results and Discussion

Isolation and characterization of bacteria

Soil samples were collected from crude oil rich region and the bacterial populations analysed by enrichment culture technique for the selection of potential biosurfactant producing bacteria. Most of the colonies are translucent, circular and few were irregular, rhizoidal opaque in nature. The margins were predominantly circular and rarely irregular in nature. Totally 25 colonies were isolated and 5(s1-s5) were significantly grow under 2 % oil and two were moderate. Among the five, secondary screening shows four were moderate growth under 5% oil and isolate S3 only showed significant growth rate. Figure 1 reveals that the growth rate on 2 and 5 % oil enriched broth. All the tested strain showed significant growth rate under 2% but increasing the oil concentration to 5%, only one isolate showed significant growth rate. The maximum growth rate 0.04 OD U was recorded in S3. The isolate S3 biochemical characters is given in table 1 and the data shows the strain is indole negative, MR positive, VP negative, citrate positive, catalase positive, oxidase negative, nitrate reducing and Gram negative rod bacterium identified as *Citrobacter* sp. The strain is capable to survive on 5% oil was selected for further studies.

The extracellular biosurfactant from cell free culture filtrate produced under shaking and static condition were extracted and quantified. Good foaming activity (52%) which indicates the presence of biosurfactant in culture filtrate. Data given in table -2 reveals oil displacement and emulsification capacity of *Citrobacter* sp. The recovery of biosurfactant from cell free culture filtrate was done by the classical solvent extraction for product recovery. The yield of the biosurfactant was relatively low under static condition

(max 1.3 g/l) and greatly enhanced 3 fold and estimated as 3.6 g/L. The crude culture filtrate of *Citrobacter* sp showed positive result on oil displacement 16 mm and the emulsification index was recorded as 58%. The data of solvent extracted compound showed higher oil collapse (19 mm) and emulsification index (74 %) than culture filtrate. In oil displacement test there is direct relationship between diameter of clear zone and the concentration of biosurfactant was confirmed between culture filtrate and solvent extract. It was further confirmed both culture filtrate and Biosurfactant are found to be haemolytic in nature. The percentage of hemolytic activity was 58% by solvent extracted compound and 26% by culture filtrate. Primary screening such as oil spreading and emulsion activity is performed and found to be effective method [14]

Chromium reduction

Bacterial mediated Cr(6+) reduction into Cr(3+) is also a mechanism of specific significance as it transforms toxic and mobile chromium derivatives into reduced. The reduction of Cr (VI) to Cr (III) by biosurfactant is estimated as 26 mg/L from 100mg/L where as in crude culture filtrate treated it was estimated that 44 mg/L. the percentage of reduction was calculated as 74 and 58% (table 3). Reaction of chromium by *Citrobacter freundii* culture alone was 58% and 78% by extracted biosurfactant. Compare to the work of Divyasree *et al.* [15] our finding shows better result by *Citrobacter freundii*. Testing of toxicity among seed germination shows that sample treated with chromium reduced sample shows 70 % seed germination with maximum vigor index 1344 than control. The stem and root length was comparatively higher than control. Experiment tested with untreated effluent showed 20% germination rate and least vigor index 116 indicates that toxicity of Cr⁶⁺ (figure 2). Low concentration of chromium 3⁺ in effluent does not affect the chlorophyll content, and plant growth unlike Cr⁶⁺ [16]. Previous studies have also isolated and reported chromium reduction activity was best among biosurfactant produced by *Citrobacter freundii* [17]. Adsorption is a widely used method for the treatment of industrial wastewater containing color, heavy metals and other inorganic and organic impurities. The rate of heavy metal removal is strongly depends on its chemical composition and quantity of biosurfactant produced. Das *et al.* [18] investigated the possibility of using the biosurfactant produced by chromate tolerant bacterium for removal of heavy metals from solutions. Gnanamani *et al.* [19] reported that biosurfactant producing, bacteria can capable to manage oxidative stress. Production of surfactin from *Bacillus subtilis*, rhamnolipids from *P. aeruginosa*, and sophorolipid from *Torulopsis (Starmerella) bombicola* and concluded that removal of metals from sediments. Di-rhamnolipid produced by *P. aeruginosa* BS2 selectively removed chromium, lead, cadmium, copper, and nickel from a multi-element contaminated soil. Biosurfactant are compounds efficiently restrict heavy metals and detoxify them by complex formation [19, 20]

Conclusion

The surfactin isolated from *Citrobacter freundii* is capable of removing oil and chromium by forming insoluble precipitate is an advantage in effluent treatment process. The application of *Citrobacter freundii* treated effluent

showed no toxicity and recommended for use of effluent for agricultural use.

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Table 1: Colony morphology of hydrocarbon tolerate isolates

COD	Colony	KO	Gra	IMVi	Nitrat	Catalas	Oxidas
S3	White small,	+	- rod	-/+/-/+	+	+	-

Table 2: Biosurfactant production properties among isolates

Strain code	Hemolytic activity (%)	Oil collapse (mm)	Emulsification index (%)
<i>Citrobacter freundii</i> (CF)	28	14	58
Compound	58	18	74

Table 3: Chromium reduction ability of extracted Biosurfactant

Genera of isolates	chromium reduction	
	Mg/L	Percentage
Culture alone	44	56
Extracted biosurfactant	28	72

Table 4: Seed germination and vigor index test for chromium toxicity

	Germination index (%)	Mean root (cm)	Mean shoot (cm)	Vigor index
Untreated	20	2.2	3.6	116
Treated	70	5.8	13.4	1344
Control	80	4.6	11.6	1296

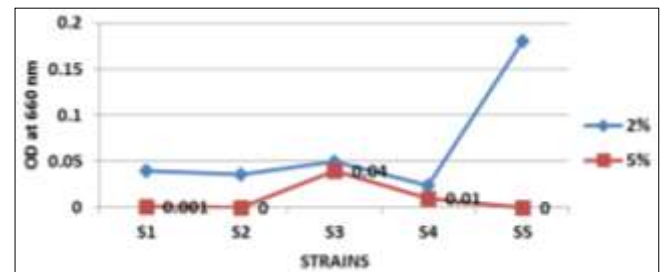


Fig 1: Growth rate of isolated bacterial colonies on crude oil medium

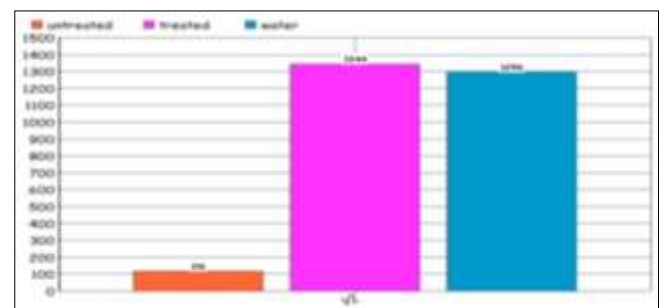


Fig 2: Vigor index of chromium treated water on plant growth

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