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Bioremediation of heavy metals (Zn and Cr) using microbial biosurfactant

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Abstract. In the present study ten different strains of bacteria were isolated from the heavy metal contaminated soil and water. The isolated colonies were cultured on Blood-Agar plates and subjected to haemolysis. The strains showing α and β haemolysis were observed positive for biosurfactant production. These strains were characterized biochemically and morphologically. The morphological identification confirms that the isolates were Gram negative *Bacilli*. Further the production of biosurfactant was confirmed with the help of CTAB method which confirms the production of biosurfactant, rhamnolipid. The purified biosurfactant from isolate vb4 was studied for degradation of heavy metal i.e. Chromium and Zinc. The degradation was analysed on X-Ray fluorescence spectrophotometry. In the sample with Chromium conc. of 10 and 20 ppm, the metal was reduced to the concentration of 4.5 ppm and 9 ppm, respectively. While in the sample with chromium at 80 ppm, the remaining amount of heavy metal was 41 ppm. In case of Zinc, the 40 ppm sample was degradated upto 2 ppm and 80 ppm sample was degradated upto 26 ppm.
Key Words: rhamnolipid, *Bacillus*, biosurfactant, heavy metal, spectrophotometry.

Introduction. Bioremediation is the use of microorganisms or their enzymes to break down and thereby detoxify dangerous chemicals in the environment (Obayori et al 2009). It plays a major role in making the environment clean from pollutants and contamination. Biosurfactants refers to any compound from microorganisms that have influence on interfaces i.e. surface acting agents which bring down the interfacial tension between the two liquids. Some of the advantages of biosurfactants over synthetic ones include lower toxicity, biodegradability, selectivity, specific activity at extreme temperatures, pH and salinity, the possibility of their production through fermentation, their potential applications in environmental protection and management, crude oil recovery, as antimicrobial agents in health care and food processing industries. Among the best studied biosurfactants are rhamnolipids that belong to the glycolipid class. Rhamnolipids have been identified predominantly from *Pseudomonas aeruginosa* (Burger et al 1963; Zhang & Miller 1992; Beal & Betts 2000). Present study shows the ability of *Bacillus* sp. to produce biosurfactant and degradation of heavy metals present in medium.

Material and Method

Microorganisms. For present study different microorganism samples were collected from metal contaminated areas in and around Baddi Industrial area. The production of biosurfactant from the isolated bacterial colonies were determined by the ability of the isolates to lyses the erythrocytes. The blood agar plates, inoculated with isolates, were prepared and incubated at 37^{o}C for 24hrs and then the haemolysis was measured.

Characterization of biosurfactant producing organism. The screened biosurfactant producing organism was then characterized by using different morphological and biochemical tests, includes Gram staining, Motility Test, Indole Test, Methyl Red Test, Voges-Proskauer Test, Citrate Test, Spore Staining, Starch Hydrolysis, Casein Hydrolysis, Gelatin Hydrolysis, Lipid Hydrolysis, Gelatin liquefaction Test, Oxidase Test, Catalase Test.

CTAB method for confirmation of rhamnolipid production. Ten isolates on the basis of blood haemolysis, is further screened for rhamnolipid production were streaked on the plates composed of the mineral salt medium with the addition of 200 micro gram per ml cetyl-tri-methyl ammonium bromide (CTAB), 5 microgram per ml methylene blue and 1.5% agar (Siegmund & Wagner 1991). Out of ten colonies six show positive result for rhamnolipid production on CTAB plates.

Production of rhamnolipid from isolates. Out of ten screened bacterial colonies, *Bacillus* vb4 isolate was used for further study as it shows highest rhamnolipid production/colony on CTAB plates. In this study the production of biosurfactant was carried out in water soluble medium containing 1.5% (V/V) cooked vegetable as substrate. The medium was inoculated with isolate vb4 and incubated for seven days.

Extraction of rhamnolipid. During incubation the surfactants was produced and released into the medium. This was extracted by the acid precipitation method. Medium was centrifuged at 5000 rpm for 15 min. The cell free broth containing surfactant was collected in a separate tube. The surfactant in the broth was precipitated at pH 2.0 by adding conc. HCl. The broth was again centrifuged at 5000rpm for 15 min; the surfactant was extracted with dichloromethane. Further, purification was achieved by re-crystallization. The dichloromethane extract was dissolved in distilled water containing sufficient NaOH to give pH 7.0. This solution was filter through Whattman no. 4 filter paper and reduced to pH 2.0 with conc. HCl. The white solid was collected as a pellet after centrifugation and weight was measured separately.

Effect of biosurfactant on metal removal. The extracted biosurfactant from isolate vb4 was used to study the removal of metals i.e. chromium and zinc. The nutrient broth medium containing the salts of chromium oxide and zinc sulphate was prepared and sterilized. The salts of chromium and zinc were added to the medium at conc. of 10 ppm, 20 ppm, 40 ppm, 60 ppm, 80 ppm, in 100 ml per medium, respectively. The pH of the medium was adjusted to 7.0 to 7.2 and sterilized in an autoclave at 15 lbs pressure for 15 min. Then the extracted biosurfactant (about 50 µl/ml) was inoculated in the medium and incubated at 30^{o}C for 24hrs. The medium with rhamnolipid was kept as treatment and medium without biosurfactants served as control. The treatment methods were same for both the metals used. Then the tubes were analyzed for the conc. of metals present after treatment in X-Ray Fluorescence spectrophotometry.

Calculation. Metal removal percentage (n) was calculated on the basis of following formula:

$$n = (CM-CMF) \times 100/CM$$

where CM is initial concentration of metal, CMF is final concentration of metal after the effect of rhamnolipid.

Results. In the present study, biosurfactants producing bacterial isolates were isolated from metal contaminated areas in and around Baddi Industrial area. All ten isolates were Gram negative and morphologically rod shaped. The biochemical characters of the isolated strains were studied as described in Bergey's Manual of Determinative Bacteriology (Holt et al 1994) and it is found that all isolates belongs to genus bacillus.

On blood agar plates, bacterial isolates with greater zone of haemolysis were selected for biosurfactant production. These selected ten colonies were further streaked to obtain pure cultures on NAM. Highest rhamnolipid producer strain Bacillus vb4 was screened on the basis of CTAB method and selected for further study.

Effect of biosurfactant on metal removal. High amount of degradation was observed when the sample was analyzed under X-Ray fluorescence spectrophotometry. It was observed that the pattern of Cr and Zn removal from media using the biosurfactant is impressive. The results of X-Ray fluorescence spectroscopy shown that rhamnolipid

biosurfactant is able to degrade Zinc and Chromium effectively in Lab conditions. It is recorded that after incubation, percentage of Cr degradation is approximately same at all concentration of Cr in medium (approximately 47 to 55%) while degradation of Zn is effectively higher at lower concentration (10, 20 and 40 ppm) in comparison to higher concentration (60 and 80 ppm) as shown in Table 1.

Table 1

Showing degradation of Zinc and Chromium by biosurfactants

Initial Conc. (in ppm)	*Zinc ($ZnSO_4$ as source in ppm)*	*Chromium (CrO_3 as source in ppm)*
10	3	4.5
20	2	9
40	2	21
60	20	30
80	26	41

Amount of degradation. On calculating the percentage of amount of degradation for zinc and chromium showed that zinc is degraded more as compare to chromium (Table 2). The maximum amount of Zinc removed is 95% while minimum is 67.5%. In case of chromium, maximum degradation was recorded at 10 ppm and 20 ppm (55% each) and 47.5% at 40 ppm concentration.

Table 2

Percentage degradation of metals

Initial Conc. (ppm)	*Amount of metal degradation (in %)*	
	Zinc	*Chromium*
10	70	55
20	90	55
40	95	47.5
60	66.6	50
80	67.5	48.75

Discussion. Industrial waste and sewage pollute more than 2/3 of India's water resources. The increasing contamination in aquatic resources with pollution including heavy metals (like chromium, lead, cadmium, zinc, nickel etc.) caused dangers for aquatic lives. Biological methods for the removal of heavy metals from industrial waste may provide an attractive alternative to the physico-chemical process; biosurfactants are one of the compounds that aid in alleviating the heavy metals.

Biosurfactants are produced by diverse group of microorganisms and have variation in their chemical structures and surface properties. On the basis of their different chemical structures the biosurfactants are used in various processes like environmental protection, crude oil recovery, agriculture, mining, health care and food process industries etc.

In the present study the sample was collected from heavy metal contaminated water and soil. The collected sample was subjected to serial dilution and streak-plating methods and ten different strains were isolated from the samples. The activity of the isolates for haemolysis was studied on the Blood-Agar plates. The colonies that showed haemolysis were found to be biosurfactant producing (Johnson & Boese 1980). The isolated strains were studied for its biochemical and morphological characteristics. The isolated strains were identified to be Gram Negative *Bacilli.*

The dark-blue colonies were observed by CTAB method, which confirmed the biosurfactant rhamnolipid produced by the isolates. Rhamnolipid are class of glycolipid biosurfactant, produced by *Pseudomonas* spp.

Several medium components influenced the formation of rhamnolipids by the cells. Additionally, there was no unique pattern of how a particular component affected the performance of the cells (Hisatsuka et al 1977; Walter et al 2010; Wang et al 2007).

The rhamnolipids enhances the biodegradation of heavy metals in many ways (Elouzi et al 2012). Same type of results recorded from our experiments where isolated biosurfactant have a good metal removal ability when used in-vitro experiments. The rhamnolipids increases bacterial growth and metabolism by emulsification of some compounds (Burger et al 1963).

Conclusions. The isolated bacterial strain vb4 from metal contaminated areas in and around Baddi was found to be the potent producer of biosurfactant as well as have efficient removal ability of zinc and chromium. The biosurfactant having higher hydrophobicity, emulsification activity, surface tension reduction and wide range of hydrocarbon emulsification activity, can be feasibly used towards *in situ* bioremediation of industrial wastes.

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