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BACTERIAL BIOSURFACTANTS AND THEIR ROLE IN HEAVY METAL DEGRADATION

Sivakami R*[1]., Saravanan S[2] and Premkishore, G[3]

[*1]P.G & Research Department of Zoology, Arignar Anna Govt. Arts College, Musiri – 621 211, Tamil Nadu, India.
e-mail id:
[2]P.G & Research Department of Zoology, Arignar Anna Govt. Arts College, Musiri – 621 211, Tamil Nadu, India.
[3]Alan Feldman Public School, Kazhakkuttom, Trivandrum - 695582, Kerala, India

***Corresponding Author Email: drsiva17@gmail.com**

ABSTRACT

Biosurfactants are produced by certain specialized organisms including bacteria fungi and yeast. These microbes produce biosurfactant either secreted or attached to parts of the cell membrane predominantly during growth phase. Microorganisms producing biosurfactants help to amplify the bioavailability of hydrocarbons by enhancing the contact between the pollutants and microorganisms in the presence of the biosurfactant which helps in the accelerated bioremediation of hydrocarbon contaminated sites. Hence the present study was carried out to know the biosurfact producing potential of two bacterial species (Bacillus subtilis and Pseudomonas aeruginosa) and their effect on degrading two metals (zinc and iron). Results show that both the species helped in the reduction of heavy metals zinc and iron. However, among the two B. subtilis appeared to be a better candidate as it recorded a higher conversion rate.

KEY WORDS

Biosurfactant, Bacillus subtilis, Pseudomonas aeruginosa, Zinc, Iron, Bioremediation

INTRODUCTION

Surfactant a short form for 'surface active agents', are basically chemical compounds which lower the surface tension of a liquid, the interfacial tension between two liquids, or that between a liquid and a solid (Chen *et al.*, 2007; Jaysree, 2011). Biosurfactants are produced by certain specialized microorganisms including bacteria, fungi and yeast (Anandraj and Thivakaran, 2010). These microbes produce biosurfactant, either secreted extracellular or attached to parts of the cell membrane predominantly during growth phase (Desai and Banat, 1997). Besides being non-toxic and biodegradable, biosurfactants are amphiphilic molecules with high specificity (Zajic and Panchal, 1976; Cooper and Zajic, 1980). They are highly stable at extremities of temperature, pH and salt concentration (Desai, 1987). These molecules besides having the ability to decrease the surface interfacial tension (Banat, 1995), offer advantages over chemically synthesized surfactants such as lower toxicity, biodegradability and ecological acceptability (Makkar *et al.*, 2011; Das *et al.*, 2008). Biosurfactants are thus used as an alternate for chemical surfactants (Banat *et al*., 2000).

The range of industrial applications of biosurfactants includes excellent detergency, emulsification, foaming, wetting, penetrating, thickening, metal sequestering, resource recovering (Soberon-Chevez and Maier, 2011; Makkar and Cameotra, 2002) and food-powering (Banat, 1995; Ramana and Karanth, 1989).

Microorganisms make use of a wide range of organic compounds as a source of carbon and energy for their growth: when the carbon source is in an insoluble form

like a hydrocarbon (Cx Hy), microorganisms make possible their diffusion into the cell by producing a variety of biosurfactants. Some of the bacteria and yeasts excrete ionic surfactants which emulsify the Cx Hy substance in the growth medium. A few examples of this group of biosurfactant are rhamnolipids (Hauser and Karnovsky, 1954; Hauser and Karnovsky, 1958; Burger *et al*., 1963; Guerra Santos *et al*., 1986; Guerra *et al*., 1998) or sophorolipids (Cutler and Light, 1979; Cooper and Paddock, 1983).

Some microorganisms are able to change the structure of their cell wall which is achieved by producing nonionic or lipopolysaccharide surfactants in their cell wall (Suzuki *et al*., 1968; Osumi *et al*., 1975; Rapp *et al*., 1979; Rosenberg *et al*., 1979; Spencer *et al.,* 1979; Fukui and Tanaka, 1981; Kilburn and Takayama, 1981; Kretschmer *et al*., 1982; Rubinowitz *et al*., 1982; Ristau and Wagner, 1983). There are also lipopolysaccharide surfactants such as emulsan (Rosenberg *et al*., 1979; Rubinowitz *et al*., 1982) and lipoprotein surfactants such as surfactin and subtilisin (Arima *et al.,* 1968; Kakinuma *et al*., 1969; Cooper *et al*., 1981a).

Microorganisms producing biosurfactants help to amplify the bioavailability of hydrocarbons by enhancing the contact between pollutants and the microorganisms in the presence of the biosurfactant which helps in the accelerated bioremediation of hydrocarbon contaminated sites (Jaysree *et al*., 2011). Hence the present study was carried out to know the biosurfactant producing potential of bacteria isolated from two aquatic systems with varied water sources.

The present study was analysed with two bacterial species namely *Bacillus subtilis* and *Pseudomonas aeruginosa;* while *Bacillus subtilis* was isolated from Naganathar pond, *Pseudomonas aeruginosa* was isolated from River Cauvery.

MATERIALS AND METHODS

The study was carried out in two aquatic systems - a fresh water pond (Naganathar pond) and a river (Cauvery) at Mukkumbu at Tiruchirappalli in Tamil Nadu.

Sampling

The water samples were sampled in both the systems. All samples were placed in an ice thermoinsulated container (temperature inside was not higher than ± 7 °C), and brought to a laboratory where they were immediately analysed.

Enrichment and Isolation of Bacterial Isolates

5 grams of water sample was inoculated in 50 ml LB broth [Sambrook *et al.*, 1989) and incubated at 37ºC for 24 hours. 100 µl of O/N grown cultures were spreaded on R2A medium agar plates (Anandraj and Thivakaran, 2010). The plates were then incubated at 37ºC for 24 - 48 hrs. Morphologically different colonies were selected and purified (Shoeb, 2006). The selected bacterial isolates were stored in LB agar slants and kept under refrigerated conditions for further screening.

Identification Through GSP Agar

The cultures were streaked on Glutamate Starch Phenol Red (GSP) agar plate and incubated at 37 ºC for 24 - 48 h.

Screening for Biosurfactant Production

The isolated strains were tested for biosurfactant production through following methods: Oil Spreading Technique: 10 µl supernatant (from culture broth) was added to the surface of oil as described by Naser (2009). Occurrence of clear zone was an indication of biosurfactant production.10 µl of culture media without any growth was taken as a control.

Hemolytic Activity

Hemolytic assay was performed in blood agar plates (Mulligan *et al.,* 1984). 50 µl broth cultures were spot-inoculated onto blood agar plates and incubated for 48 h at 37ºC. The plates were visually inspected for zone of clearance (hemolysis) around the colony. Complete and incomplete hemolysis was designated as α (alpha) and β (beta) hemolytic activity (Misawa *et al.,* 1995).

CTAB Agar Plate

Blue agar plates containing acetyl trimethyl ammonium bromide (CTAB) (0.2 mg ml^{-1}) and methylene blue (5 mg ml^{-1}) were used to detect extracellular glycolipid production (Siegmuld and Wagner, 1991). Biosurfactants were observed by the formation of darkblue halos around the colonies.

Screening for biosurfactant production

The production of biosurfactant by the isolated bacterium was determined by the ability of the bacterium to lyse the erythrocytes (Arima *et al*., 1968; Bernheimer and Avigad, 1970).

The blood agar plates were prepared, sterilized and poured into sterile petriplates. Then the organism was inoculated in the medium and incubated at 37°C for 24 hr and then the zone of haemolysis was measured.

Production of Biosurfactant

The isolated organism was used for the production of biosurfactant by growing the organism in a specific medium. The choice of carbon source used for production plays an important role on the yield and structure of microbial surfactants. There are microorganisms which produce biosurfactants only when grown on hydrocarbons and others which require simple, water soluble substrates such as carbohydrates and amino acids.

Production of Surfactin from *Bacillus subtilis*

The production of biosurfactant from *Bacillus subtilis* was carried out in glucose mineral salt medium. The medium was supplemented with trace elements and sterilized in an autoclave. After sterilization, about 2 ml of culture was inoculated and the medium was incubated at 30°C for 48-72 hr.

Extraction of Surfactin

The surfactant produced by *Bacillus* was extracted by acid precipitation method (Cooper *et al*., 1981b).

The glucose mineral salt medium was prepared and sterilized and inoculated with *Bacillus* culture. After inoculation, it was incubated at 37°C for 72 hr. During incubation the surfactin produced was released into the medium. This was extracted by the acid precipitation method.

First the medium was centrifuged at 5,000 rpm for 15 minutes. The cell free broth containing surfactin was collected in a separate tube. The surfactin in the broth was precipitated at pH 2 by adding concentrated hydrochloric acid. The broth was again centrifuged at 5,000 rpm for 15 minutes. The surfactin was extracted with dichloromethane. Further purification was achieved by recrystallization. The dichloromethane extract was dissolved in distilled water containing sufficient Sodium hydroxide to give pH 7. This solution was filtered through Whattman filter paper and reduced to pH 2 with concentrated Hydrochloric acid. The white solid was collected as a pellet after centrifugation.

Production of Rhamnolipid from *Pseudomonas aeruginosa*

Pseudomonas sp. produces surface-active agents whether grown with water soluble or water insoluble substrates, although in the latter case, production is higher (Georgiou *et al*., 1992). With reference to the above finding, in this study the production of biosurfactant was carried out in water insoluble medium containing 1.5% (v/v) cooked vegetable oil as substrate.

The medium was enriched with some additional nutrients like $MgSO_4.7H_20$, KH_2PO_4, $NaNO_3$, yeast extract and peptone (Sbyh-Yau and Vipulanandan, 1998; Mahalakshmi, 2013). The cultures were grown in 500 ml Erlenmeyer flasks with 100 ml of medium. The trace element solution was filter sterilized and added to the medium. About 2 ml of the culture was added to the medium and incubated at 30°C for 48 - 72 hr.

Extraction of rhamnolipid

During incubation, the surfactant produced was released into the medium. This was extracted by the acid precipitation method (Cooper *et al*., 1981).

First the medium was centrifuged at 5,000 rpm for 15 minutes. 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 concentrated hydrochloric acid. The broth was again centrifuged at 5,000 rpm for 15 minutes and the surfactant extracted with dichloromethane. Further purification was achieved by recrystalization. The dichloromethane extract was dissolved in distilled water containing sufficient Sodium hydroxide to give pH 7.0. This solution was filtered through Whatmann No.4 filter paper and reduced to pH 2.0 with concentrated Hydrochloric acid. The white solid was collected as a pellet after centrifugation.

Effect of biosurfactant on metal removal

The extracted biosurfactant was used for the removal of metals such as zinc and iron. The nutrient broth medium containing the salts of zinc sulphate and ferrous sulphate was prepared and sterilized. The salts of zinc and iron were added to the medium at a concentration of 10 mg, 20 mg, 30 mg, 40 mg and 50 mg/litre respectively. The pH of the medium was adjusted to 7.0 - 7.2 and sterilized in an autoclave at 15 Pb pressure for 15 minutes. Then the extracted biosurfactant (about 50 µl/ml) was inoculated into the medium and incubated at 30°C for 24 hours. The medium surfactin was kept as Treatment 1 (TI) and the medium with Rharnnolipid was kept as Treatment 2 (T2). The medium without surfactant and organism served as control. The treatment methods were same for both the metals used. Then the tubes were analyzed for the concentration of the metals present after treatment in an Atomic Absorption Spectrophotometer. The sample was analyzed in the Government Soil Testing Laboratory, Tiruchirappalli and the results were noted.

RESULTS AND DISCUSSION

The biosurfactant production by the two isolates (Isolate-1 - *Bacillus subtilis* and Isolate-2 - *Pseudomonas aeruginosa*) are presented in Table-1. As seen from the Table, *Bacillus subtilis* was found to produce a higher amount of surfactant when compared to *Pseudomonas aeruginosa*; while the amount of biosurfactant produced by isolate-1 was 1.950 g/100 ml of the medium, the amount of ramnolipids produced by isolate-2 was 1.710 g/100 ml. Thus, it clear that the isolated organisms under favourable conditions can produce biosurfactants which can help in bioremediation.

Results of the bioremediation tests using the two isolates for the heavy metal zinc is provided in Table-2. As evident from the Table, it is clear that both the isolates have the ability to reduce the amount of zinc. While the use of *B. subtilis* recorded a decrease in the level of zinc ranging from 44 to 80.4% when compared to the control, *P. aeruginosa* recorded a decrease in zinc levels ranging from 32 to 73.2% when compared to the control. Further, the isolates recorded enhanced bioremediation rates with an increase in the concentration of heavy metals. However, among the two isolates, *B. subtilis* appeared to be a better candidate as it recorded a higher efficiency rate (80.4% reduction) when compared to *P. aeruginosa* (73.2% reduction).

The results of the bioremediation studies using both the isolates in the bioremediation of iron is presented in Table-3. Here also, both the isolates recorded a decrease in iron content ranging from 48 to 82% for *B. subtilis* and 38 to 77.6% for *P. aeruginosa*. As with zinc, there was an increase in their conversion rate with an increase in iron content for both the species. However, *B. subtilis* appeared to be a better candidate when compared to *P. aeruginosa* as it recorded a higher conversion rate.

Literature reveals that the carbon source generally used in biosurfactant production can be divided into two categories namely, water insoluble and water-soluble carbon sources (Desai and Banat, 1997). Water-insoluble carbon sources such as oil or hydrocarbon compounds, are widely used for biosurfactant production. Abouseoud *et al*. (2008) reported the production of biosurfactant by *Pseudomonas fluorescens* only in the presence of water insoluble carbon such as hexadecane and olive oil. This strain was able to utilize glucose as a substrate but without biosurfactant synthesis. However, Darvishi *et al*. (2011) also found that the presence of olive oil supports the biosurfactant production from Enterobacteriaceae and *Pseudomonas* sp. Alternatively, many studies have shown that a water-soluble substrate is suitable for biosurfactant production by P. *aeruginosa* (Pansiripat *et al*., 2010) and *Pseudozyma hubeiensis* (Konishi *et al*., 2011). A similar result was also obtained in the present study with *Pseudomonas aeruoginosa*.

Literature also reveals that *Bacillus subtilis* was found to produce biosurfactants. Jaysree *et al.* (2011) reported that *Bacillus subtilis* isolated from contaminated samples also showed biosurfactant activity. In the present study, the marked difference in the biosurfactant activity between the experiment and control suggests that the biosurfactant producing activity of both *Bacillus subtilis* and *Pseudomonas aeruginosa* may be induced in the presence of pollutants. This might be the reason why both the bacteria showed increased activity with increase in concentration.

It may be concluded that *B. subtilis* and *P. aeruginosa* are both capable of producing biosurfactants that would help in degrading metals like Fe and Zn. Nevertheless, among the two, *B. subtilis* appears to be a better candidate when compared to *P. aeruginosa*. However, literature reveals that the type and quantity of the microbial surfactants produced depends mainly on the producer organism in addition to factors like nitrogen and carbon, temperature, aeration and trace elements (Banat *et al.,* 2000; Mahalakshmi, 2013). Hence a more detailed study is required on these aspects.

Table 1: Amount of Biosurfactant Production by Isolates-1 and 2.

S. No.	Organism	Biosurfactant production (per 100 ml of the medium)
1.	Isolate-1: *Bacillus subtilis*	1.950
2.	Isolate-2: *Pseudomonas aeruginosa*	1.710

Table-2: Effect of Biosurfactant on Zinc

S. No.	Concentration of Zinc (mg/l)		
	Control without Biosurfactant	Treatment-1 with Surfactin	Treatment-2 with Rhamnolipid
1.	10	44.00	32.00
2.	20	54.00	45.00
3.	30	63.67	54.30
4.	40	72.00	56.25
5.	50	80.40	73.20

Table-3: Effect of Biosurfactant on Iron

S. No.	Concentration of Iron (mg/l)		
	Control without Biosurfactant	Treatment-1 with Surfactin	Treatment-2 with Rhamnolipid
1.	10	4.8	3.8
2.	20	11.2	9.4
3.	30	20.5	18.8
4.	40	27.6	26.0
5.	50	41.0	38.8

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***Corresponding Author:**
Sivakami R*
Email: **bhoslehemlata@gmail.com**