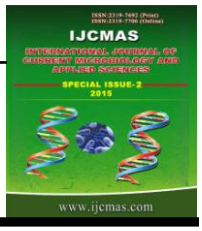
Int.J.Curr.Microbiol.App.Sci (2015) Special Issue-2: 343-356

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Special Issue-2 (May-2015) pp. 343-356**
http://www.ijcmas.com



Original Research Article

Biosurfactant Mediated Plant Growth Promotion in Soils Amended with Polyaromatic Hydrocarbons

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Keywords

Biosurfactant, *Streptomyces*, Polyaromatic hydrocarbons, Plant growth promotion

A B S T R A C T

The present study was carried out with the perspective of obtaining a potent biosurfactant employing molasses as substrate and evaluation of its potential for Plant growth in soils contaminated with polyaromatic hydrocarbons. *Streptomyces* sp. V2was grown in optimized medium containing molasses as the carbon source and purified by acid precipitation followed by solvent extraction. The purified biosurfactant was used to determine its chemical composition, surface active properties, antimicrobial potential and potential to alleviate stress of phenanthrene and anthracene on plantlets. Our results indicate that *Streptomyces* sp. V2 produced a potent biosurfactant. The biosurfactant was amorphous brown, water soluble and proteoglycan in nature. The yield of the biosurfactant was 56.7 mg/L and reduced the surface tension of water by 11.020 ± 0.526 mN/m. It reduced the interfacial tension between kerosene water interface by 4.802 ± 0.186 mN/m and had a CMC of 72 mg/L. The biosurfactant exhibited weak antibacterial activity and was stable over a wide range of pH and temperature. Unpaired "t" test ($p<0.05$) to determine significant difference due to different treatments revealed that addition of biosurfactant to phenanthrene and anthracene amended soils, significantly increased the vitality and growth of *Trigonellafoenum-graecum* and *Triticum* spp. Thus biosurfactant from *Streptomyces* sp. V2 was promising and could find potential in bioremediating PAH contaminated soils. The biosurfactant was efficiently produced using molasses thereby lowering environmental stress.

Introduction

Polyaromatic hydrocarbons (PAH) also known as polynuclear aromatic hydrocarbons are group of most widespread organic pollutants found in soil, sediment and oily substances. These compounds are a major cause of concern recently because some of them are potentially carcinogenic, mutagenic and teratogenic and hence a

major health hazard (Zhang *et al.*, 2010; Kumar *et al.*, 2011; Diab and Sandouka, 2012). So a major task for researchers is focus on efforts in bioremediation of these harmful hydrocarbons.

Increasing agricultural productivity to meet the growing demands of human population is a pressing need of the hour. Equally alarming is the damage caused to the environment by contaminants and pollutants such as polyaromatic hydrocarbons (Pacwa-Płociniczak *et al.*, 2011). Therefore the use of green compounds to disarm these pollutants and thereby improve soil fertility is becoming an urgent necessity.

Biosurfactants can find potential application in improving the soil quality by way of soil remediation. Biosurfactants are usually employed to increase bioavailability and biodegradation rate of hydrocarbons; their several additional advantages over synthetic surfactants viz, absence of toxicity, greater environmental compatibility and ability to be generated from renewable sources make them superior over their chemical counterparts (Zhou *et al.*, 2013). Also raw materials account for about 50% of the final product cost. Better choice of raw material is a way to cut down production costs and make the process economically feasable (Nitschke *et al.*, 2004). In the current study, we used molasses as a carbon source, which is raw material obtained from sugar industry. Molasses has low cost as well as it is a source of carbon and also contains other compounds such as proteins, non sugar organic compounds, inorganic compounds and vitamins (Makkar *et al.*, 2011).

Hence, the present study was carried out with an aim to produce potent biosurfactant employing cheaper renewable resource namely molasses and to evaluate its potential to reduce the stress of PAH and promote plant growth.

Materials and Methods

Strains and media

Actinomycetes strains isolated from soil sample collected from botanical garden of Modern College, Pune, India were used for the present study. Casein Starch Molasses medium (CSMM) prepared by replacing casein by molasses from Casein starch medium was used for screening and production of the biosurfactant. Molasses was added to the medium in a proportion of 1:250 (Vol/Vol). Molasses for the study was obtained from a local sugar factory in Nira, Maharashtra. After initial screening, the best producer strain was used in all further studies. This strain was identified by slide culture technique and cell wall analysis (Doshi *et al.*, 2010).

Screening of Actinomycete spp for biosurfactant / bioemulsifier production

Screening of biosurfactant production was done by the drop collapse method and determination of emulsification index values (E_{24}). For the drop collapse assay, 10µl cell free supernatant obtained by centrifugation of 10 day old culture in CSMM at 120 rpm, 30 ^{o}C was placed on the slide coated with sunflower oil. The culture was scored positive for biosurfactant production if the drop collapsed. DW and 1% solution of SDS served as negative and positive controls respectively (Bodour *et al.*, 1998). Emulsification index (E_{24}) to quantify the emulsification activity of the culture was determined by the method described by Cooper & Goldenberg, 1987. For the assay, 3ml of kerosene was added to 2 ml of the cell-free broth, vortexed for 2 min and incubated for 24 h. Emulsification Index (E_{24}) was calculated as height of emulsified layer (mm) divided by total height (mm) multiplied by 100.

Effect of physiochemical factors on biosurfactant production

Effect of physiochemical factors such as pH (4-10) and temperature (30, 37 and 45 ^{o}C) on biosurfactant production was evaluated in CSMM. Addition of salts to enhance biosurfactant activity was also evaluated. Salts studied included NaCl, Pb (CH_3COOH), $MgSO_4$, $FeSO_4$ and $NaNO_3$. Salts were amended to CSMM at concentrations of 0.1, 0.5, and 1.0 (% w/v) each.

Partial purification of biosurfactant

For partial purification of biosurfactant *Streptomyces* sp. V2 was grown in CSMM supplemented with 1% w/v Pb (CH_3COOH) and $MgSO_4$ each. pH of CSMM was adjusted at 7.0 and the flasks were incubated at 30ºC at 120 rpm for a period of 10 days. The cell free supernatant obtained by centrifugation at 12,000 rpm for 10 min was acidified to pH 2 using 6N HCl and incubated overnight at 4ºC. The resulting pellet obtained by centrifugation at 10,000 rpm for 10 min was subsequently dissolved in 0.05 M bicarbonate buffer (pH 8.6), reacidified and centrifuged at 10,000 rpm for 20 min. This precipitate was then extracted with chloroform methanol (2:1) and organic fraction evaporated. The resultant crude biosurfactant was redissolved in 0.05 M bicarbonate buffer (pH 8.6) and used for all further experiments (Zhang and Miller, 1992).

Chemical analysis of the Biosurfactant

The partially purified bioemulsifier was anlysed for its chemical composition. Protein content was assayed using the method of Folin Lowry (1951) using Bovine serum albumin as standard. Carbohydrates were quantified according to the protocol of Dubois *et al.* (1956). Reducing sugar content was estimated using the dinitro salicylic acid method (Monreal and Reese, 1969) with glucose as standard. Extraction and quantification of lipids was performed as per the method described by Reddy *et al.* (1983).

Determination of surfactant properties

Determination of Surface tension (SFT) and Interfacial tension (IFT): Surface tension measurements were done by the Wilhemy plate method using a Dynamic Contact angle Surface Tensiometer, DCAT 11 (DataPhysics GmbH, Germany) equipped with a De Noüy Platinum-Iridium plate (length 10mm, width 19.9 mm and thickness 0.2 mm). Interfacial tension measurements were performed between Kerosene water interface. Readings were an average of three independent measurements. All measurements were performed at 25±1^{0}C.

Critical micelle Concentration (CMC): CMCof the surfactantwas determined by plotting the surface tension as a function of biosurfactant concentration. A stock of 1 mg/ml of the partially purified biosurfactant was prepared in distilled water and used for the determination. The CMC was estimated by addition of incremental concentrations of the biosurfactant to distilled water till constant surface tension readings were obtained (Rodrigues *et al.*, 2006).

Stability of Biosurfactant

Temperature and pH stability of the bioemulsifier was determined as described by Joshi *et al.* (2008); for this a 10 mg/ml solution of the partially purified biosurfactant in DW was made and adjusted to different pH values from 1 to 14 using 0.1N HCl and 0.1N NaOH. Emulsification index values with determined at pH value

using Kerosene. For temperature tolerance, the biosurfactant solution was incubated at different temperatures from 10-100^{0}C for one h. and then assayed for quantification of emulisification index using kerosene.

Antimicrobial activity

Antimicrobial activity of the partially purified biosurfactant was evaluated by the agar well diffusion method (Kiran *et al.,* 2010). MIC values were evaluated by the routine broth dilution method. *Pseudomonas aeruginosa* NCIM 5029, *Escherichia coli* (NCIM 2931), *Candida albicans*, *Staphylococcus aureus* NCIM 5021, *Bacillus subtilis* (MTCC 2422) for the assay were procured from NCIM, Pune.

Potential bioremediation activity of the biosurfactant

For the assay, soil was randomly collected as bulk soil. This soil was first homogenized and passed through sieve. This soil was autoclaved at 121°C and 15 psi for 1 h for three times prior to use. Aromatic hydrocarbons namely anthracene and phenanthrene were added at final concentration of 0.5 g/Kg to the soil. Healthy seeds of *Trigonella foenum-graecum* and *Triticum* spp. were selected. These seeds were surface sterilized by 0.1% $HgCl_2$ followed by washing (three times) with distilled water. 5 seeds were sown in each pot. (Zhang *et al.,* 2010: Diab and Sandouka, 2011).

Partially purified biosurfactant was amended to the soil at a concentration of 500 mg/Kg of soil. Other treatments included control pots with neither PHA's nor the biosurfactant and a second set containing only PAH's. Plants were kept in sunlight and watered daily. Effect of *Trigonella foenum-graecum* and *Triticum* vitality was evaluated in terms of length of shoot and roots. Unpaired "t" test ($p<0.05$) to determine significant difference in treatments was done using the GraphPad Prsim version 5.01.

Results and Discussion

Screening of suitable strain for study

A total of 19 actinomycetes were screened for biosurfactant/bioemulsifier production. Out of 19 strains screened, only 7 tested positive. Strain V2 exhibited maximum activity and was used for further study. This strain was identified as *Streptomyces* by cell wall analysis and slide culture technique. *Streptomyces* strain V2 emulsified different hydrocarbons and exhibited maximum E_{24} of 46.66 for kerosene in CSMM.

Effect of physicochemical parameters on emulsification activity

Molasses served as a good substrate for the production of biosurfactant by *Streptomyces* sp. Strain V2. Emulsification of kerosene differed as the parameters were varied. Maximum activity (E_{24}) was observed at pH 7 (E_{24} value 70) while lowest emulsification was obtained at pH 5 with an E_{24} value of 40 (Fig. 1). NaCl was detrimental while Ferrous, magnesium and lead salts enhanced emulsification activity. NaCl at a concentration of 1 g% was the most detrimental with a total absence of emulsification activity.

Maximum emulsification was obtained with Pb having E_{24} value of 66.66 (Fig. 2). Temperature did not have a very profound influence on bioemulsification activity and activity was almost comparable at all temperatures tested. However, maximum E_{24} of 64.33 was obtained when *Streptomyces* species strain V2 was grown at 30 ^{o}C.

Chemical nature and surfactant properties

Streptomyces sp. strain V2 produced a water soluble proteoglycan biosurfactant (17% protein, lipid 51% and sugar 32%) when grown using molasses as the substrate. The yield of the biosurfactant was 56.7 mg/L and reduced the surface tension of water by 11.020 ± 0.526 mN/m. It reduced the interfacial tension between kerosene and water interfaces by 4.802 ± 0.186 mN/m and had a CMC of 72 mg/L.

Stability and antimicrobial property of the biosurfactant

Good emulsification activity was retained at all values of pH and temperatures tested (Fig. 3 & 4). Maximum activity was observed at 30^{o}C (E_{24} of 68) and pH 6 (E_{24} of 80.95). Lowest activity was retained after heating to 100 ^{o}C (E_{24} of 16.66) and pH 14 (E_{24} of 14.28). Extreme acidic or alkaline pH was detrimental for activity, whereas values between 4 and 8 were optimal. Similarly, temperatures greater than 50 resulted in a decrease in the activity. Partially purified biosurfactant exhibited antimicrobial activity against *E. coli* and *Pseudomonas with a m*inimum inhibitory concentration of 0.05 µg/ml. No activity was found against Gram positive bacteria or yeast.

PAH bioremediation potential of the biosurfactant

The biosurfactant from *Streptomyces* sp. V2 exhibited good plant growth promotion ability for both *Trigonellafoenum-graecum* as well as *Triticum* spp (Table 1 & 2). There was a significant increase ($p<0.05$) in the root and shoot lengths of both plant with supplementation of biosurfactant over that of PAH treated and untreated controls. In general, *Triticum* sp. grew faster over that of *Trigonella* (Fig. 5–8). It could be concluded from the statistical analysis that the presence of the biosurfactant increased the vitality of both plants by alleviating the stress of the phenanthrene and anthracene. There was also a significant reduction in root and shoot lengths on supplementation of the PAH's over that of untreated controls. The mechanism could be probably due to a detoxification of the hydrocarbons by means of degradation in presence of the biosurfactant which can be evaluated in greater detail with advanced studies using HPLC or GCMS to detect biodegradation products obtained.

It is well known that biosurfactants are usually employed to increase bioavailability and biodegradation rate of hydrocarbons, additionally they several additional advantages over synthetic surfactants make them superior candidates in bioremediation (Chandraja *et al.*, 2014; Zhang *et al.*, 2010). The present study was conducted to evaluate potential of the biosurfactant from *Streptomyces* sp. V2 to bring about plant growth promotion in PAH contaminated soils. The aim was to produce a cost effective biosurfactant using cheaper substrate like molasses.

In the current study, different species of actinomycetes were screened for their biosurfactant production ability. In the recent years, large number of groups is working on production of surface active agents from actinomycetes. Although, number of studies on biosurfactant/ bioemulsifiers production is rapidly increasing, reports from *Streptomyces* sp. are not enormous. Maniyar *et al.* (2011) and Deepika and Kannabiran (2010) have production of potent biosurfactants from *Streptomyces* species. Gandhimathi *et al.* (2009) reported production of a

bioemulsifier by a marine *Streptomyces*. Actinomycetes from coastal region of Alibag, Janjira and Goa exhibited production of lipase in addition to the production of a bioemulsifier (Kokare *et al.,* 2007).

Molasses was used as an alternative substrate for production of biosurfactant by *Streptomyces* sp. V2 as economy is the major bottleneck of a biotechnological process. Various cheaper substrates like molasses, whey, oils and fats, agro-industrial wastes etc. have been used by a number of research groups world-wide for production of surface active agents. Makkar *et al.* (2011) have extensively reviewed the recent advances in the utilization of renewable substances for biosurfactant production. Al-Bahry and co-workers (2013) have reported that the biosurfactant from *Bacillus subtilis* B20 producing molasses as substrate could find potential application in enhanced oil recovery.

In our study we found that Pb salt amendment enhanced production, while addition of NaCl was detrimental for activity. There have been several reports suggesting such increase in production of surface active agents on addition of metal salts. In case of marine Actinomycetes, it has been shown that addition of NaCl increases production. However in our case the reduction of activity after addition of NaCl can be explained by the fact that *Streptomyces* sp. V2 is a soil isolate. Temperature and pH also have shown to influence biosurfactant from *Lactobacillus pentosus* production by Bello *et al.* (2012). Biosurfactant from *Streptomyces* sp. strain V2 was harvested and extracted after ten days of incubation in CSMM medium. This is in agreement with the recent data on isolation of rhamnolipid biosurfactant from *Streptomyces* by Kalyani *et al.* (2014). Purification of the biosurfactant was done by acid prercipitation followed by solvent extraction. This has been a method of choice for purification of biosurfactants by large number of researchers (Donio *et al.,* 2013; Farias *et al.,* 2014; Raza et al, 2007). Thampayak *et al.* (2008) have used solvent extraction for purification of biosurfactant from *Streptomyces griseofulvus* and *Streptomyces fradiae*. The yield of biosurfactant obtained from *Streptomyces* sp. V2 was less that that reported by Al-bahry *et al.* (2013) which was around 2.29 g/L from *Bacillus subtilis.*

Some biosurfactants are suitable alternatives to synthetic medicines and antimicrobial agents and may be used as effective therapeutic agents (Gudina *et al.,* 2013). Despite this, very little work has been done to evaluate potential application of surface active agents from actinomycetes in biomedics. Biosurfactant from *Streptomyces* sp. V2 exhibited moderate antimicrobial activity against *E. coli* and *Pseudomonas with a m*inimum inhibitory concentration of 0.05 µg/ml. Donio *et al.* (2013) in their recent study have reported that biosurfactant from *Halomonas* sp. BS4 exhibit good antimicrobial and antifungal activity.

The biosurfactant from *Streptomyces* sp. V2 showed good stability over wide range of pH and temperatures tested. However at low and high pH values a reduction in the activity was observed. This is exactly in agreement with the recent study on the biosurfactant from *E. faecium* conducted by Sharma *et al.* (2015). This biosurfactant exhibited a good stability in the pH range from 6 to 10. Bioemulsifier from *Actinopolyspora* A18 was stable at 10 and 40 $^{\circ}$C while that from *Streptomyces* sp. S22 was stable at room temperature but unstable at lower and higher temperatures (Doshi *et al.,* 2010, Maniyar *et al.,* 2011). However in

these studies only a narrow range of temperatures was tested.

Streptomyces sp. V2 produced a water soluble proteoglycan biosurfactant (17% protein, lipid 51% and sugar 32%) when grown using molasses as the substrate. Proteoglycan type of bioemulsifiers has also been reported by Jagtap *et al.* (2010) from *Acinetobacter* species isolated from healthy human beings. The marine actinomycete *Nocardiopsis alba* MSA10 isolated from a marine sponge has been shown to produce a Lipopeptide biosurfactant (Gandhimati *et al.,* 2009). The yield of the biosurfactant from *Streptomyces* sp. V2 was 56.7 mg/L and reduced the surface tension of water by 11.020 ± 0.526 mN/m. It reduced the interfacial tension between kerosene and water interface by 4.802 ± 0.186 mN/m and had a CMC of 72 mg/L. A biosurfactant has been reported from *Streptomyces* sp. 22 by Maniyar *et al.* (2011) which reduced the surface tension by 23.09 mN/m, however the authors did have not determined the CMC of the biosurfactant. The authors also have a higher yield of 1.6 g/L.

Phenenathrene and anthracene have been designated as two of the 16 priority pollutants by U.S EPA (Arun *et al.,* 2011) hence studies on their bioremediation are utmost important. Our studies indicate that biosurfactant from *Streptomyces* sp. V2 did not exhibit any phytotoxicity to either *Trigonella foenum-graecum* or *Triticum* spp; instead its presence was stimulatory for the vitality of the plants. The addition of biosurfactant to anthracene and phenanthrene containing soils was seen to promote plant growth thereby indicating a protective as well as stimulatory role of the biosurfactant.

Table.1 Effect of biosurfactant on the vitality of *Triticum* measured in terms of shoot and root length in the presence of polyaromatic hydrocarbons

Treatments	**Parameters**	
	Shoot Length (cm)	Root Length (cm)
Control	17.8±0.115	14.8±0.201
Phenanthrene alone	16.9±0.102*	11.2±0.201*
Phenanthrene plus biosurfactant	19.3±0.032*	20.2±0.871*
Control	17.7±0.424	11.9±0.360
Anthracene alone	15.2±0.135*	15.1±0.106*
Anthracene plus biosurfactant	20.1±0.526*	18.2±0.434*

Results are expressed as Mean ± SEM: n=5; *Significance level with respect to control ($p<0.05$)

Table.2 Effect of biosurfactant on the vitality of *Trigonella foenum-graecum* measured in terms of shoot and root length in presence of polyaromatic hydrocarbons

Treatments	**Parameters**	
	Shoot Length (cm)	Root Length (cm)
Control	7.8±0.114	5.7±0.050
Phenanthrene alone	6.6±0.015*	5.2±0.092*
Phenanthrene plus biosurfactant	8.7±0.081*	7.7±0.196*
Control	8.4±0.067	5.5±0.136
Anthracene alone	6.7±0.684*	4.4±0.020*
Anthracene plus biosurfactant	9.4±0.124*	7.3±0.153*

Results expressed as Mean ± SEM: n=5; *: Significance level with respect to control ($p<0.05$)

Fig.1 Effect of pH on production of biosurfactant by *Streptomyces* sp. V2

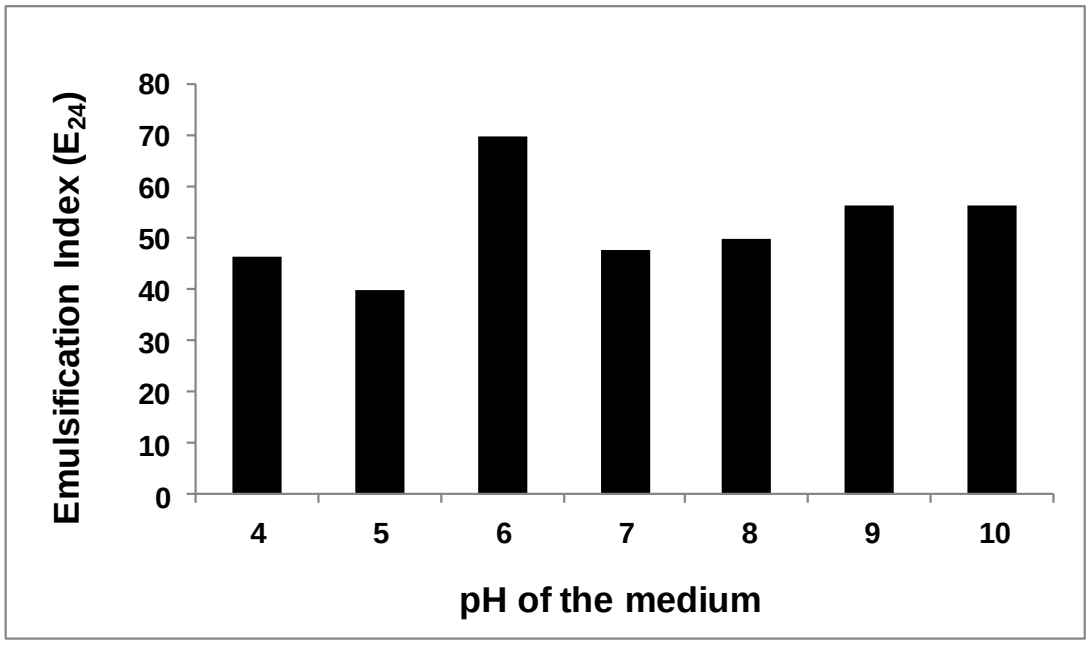

Fig.2 Effect of Salt supplementation on production of biosurfactant by *Streptomyces* sp. V2

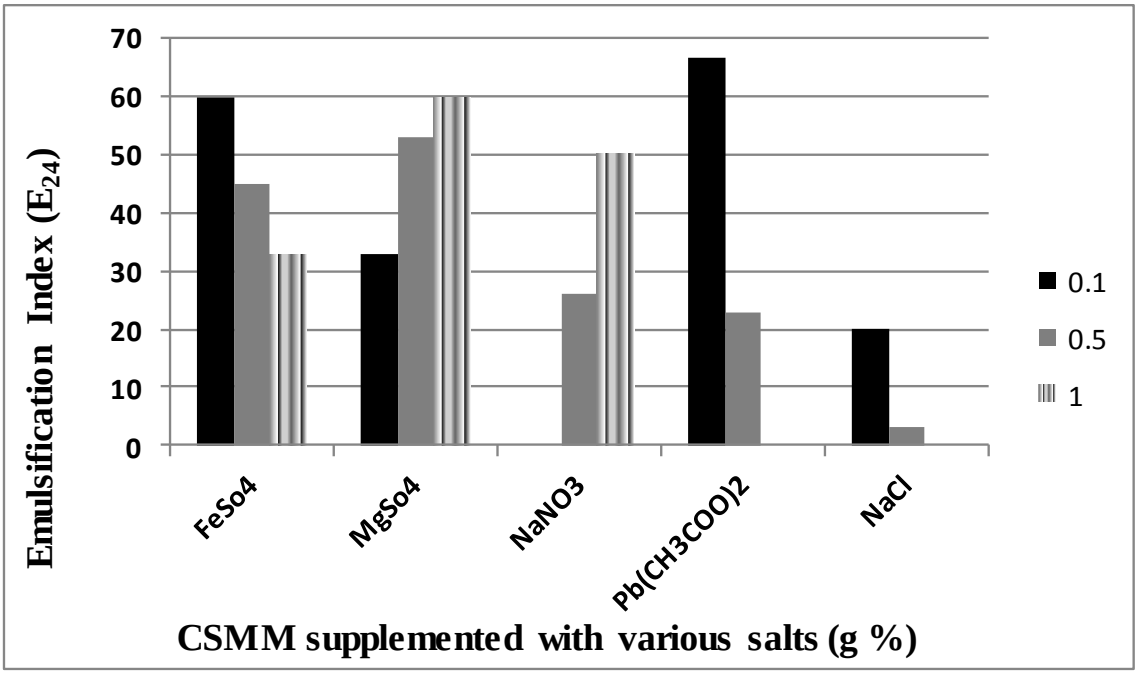

Fig.3 Effect of temperature on the stability of biosurfactant from *Streptomyces* sp. V2

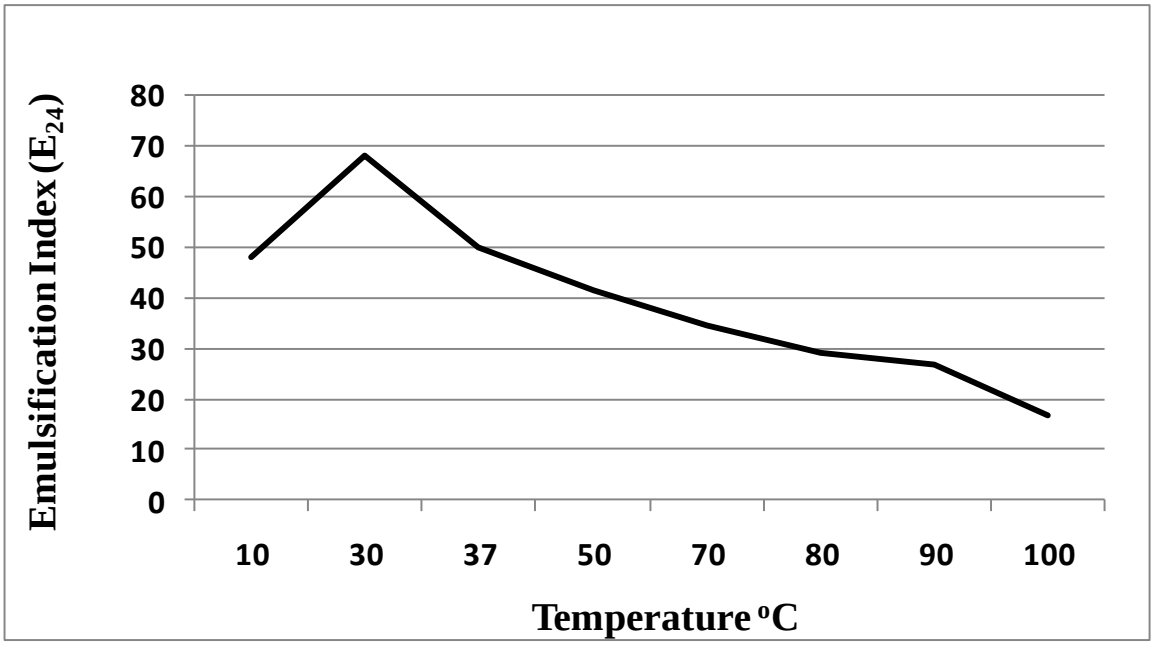

Fig.4 Effect of pH on the stability of biosurfactant from *Streptomyces* sp. V2

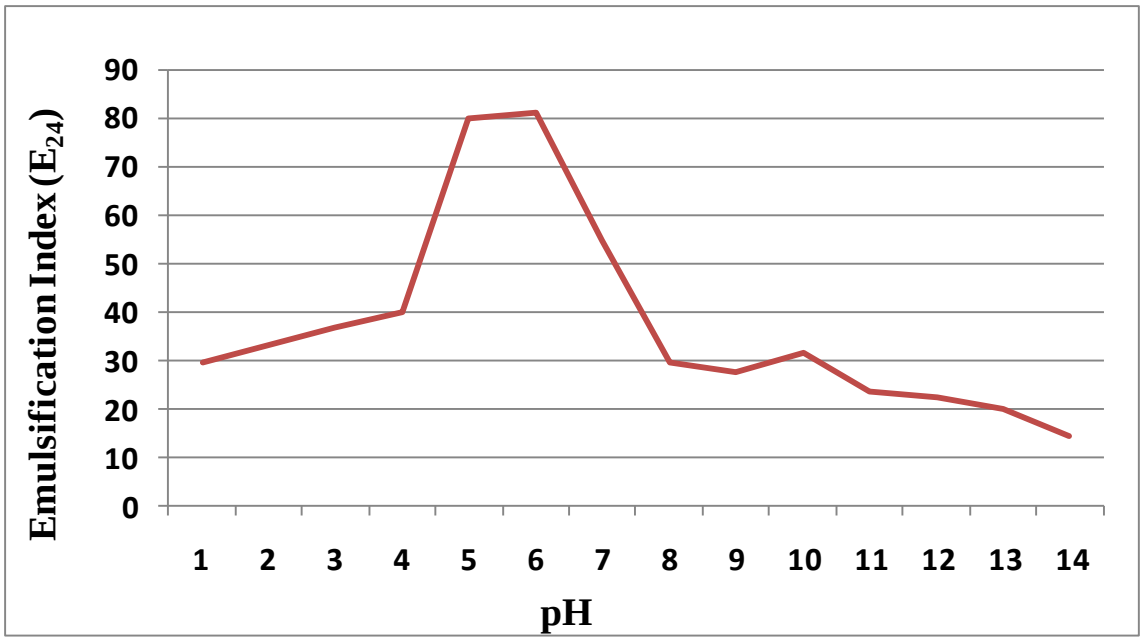

Fig.5 Effect of Effect of phenanthrene and biosurfactant supplementation on the vitality of *Triticum*

Fig.6 Effect of Effect of anthracene and biosurfactant supplementation on the vitality of *Triticum*

Fig.7 Effect of biosurfactant and phenanthrene supplementation on the vitality of *Trigonella foenum-graecum*

Fig.8 Effect of biosurfactant and anthracene supplementation on the vitality of *Trigonella foenum-graecum*

This indicates a great potential of the biosurfactant from *Streptomyces* sp. V2 in bioremediating PAH contaminated soils. Shoot and root lengths of both plants were significantly greater with biosurfactant amendment as compared to that of untreated control and soil amended with anthracene and phenanthrene. In a study conducted by

Pei *et al.* (2010) addition of rhamnolipid biosurfactant was shown to increase phenanthrene degradation by *Sphingomonas* sp. GF2B. Addition of biosurfactant gave an increase of 13% phenanthrene degradation over that without the biosurfactant alone. They suggest that preferential utilization of PHE as a carbon source and the enhanced solubility of PHE by the biosurfactant were likely responsible for higher biodegradation efficiency of PHE in the presence of biosurfactant. We suspect that a similar mechanism was responsible in exerting plant growth promoting activity by *Streptomyces* species strain V2 in presence of the biosurfactant.

Chang-Zheng *et al.* (2008) in a study to decode the degradation mechanism of anthracene found that rhamnolipid ehnaced degradation to a great extent. The authors evaluated the anthracene-degradtion ability of of *Sphingomonas* sp. 12A and *Pseudomonas* sp. 12B in presence of rhamnolipid biosurfactants produced by *Pseudomonas aeruginosa* W3.They found that rhamnolipids dramatically increased the solubility of anthracene and thereby its degradation; they also found out that in case of *Sphingomonas*, the rhamnolipids got degraded at a later phase which degreased anthracene degradation clearly indicating that rhamnolipids exhibit a degradation ability on PAH anthracene. This is similar to the effect observed by us on plantlets of *Triticum* and *Trigonella* where pots supplemented by biosurfactant exhibited a good vitality probability due to destruction of the PAH's by our biosurfactant. Similarly, Zhang *et al.* (2010) have shown a synergistic effect of mycorrhizal fungi, aromatic hydrocarbon bacteria and rhamnolipids in phytoremediation of PAH contaminated soils. Ławniczak *et al.* (2013) have recently reviewed in great detail about the contributions of biosurfactants in natural or induced bioremediation. Arun *et al.* (2011) have also compiled data pertaining to PAH's, their source, structure, microbes involved in their detoxification and the probable mechanisms.

With this study we present that the application of the biosurfactant to PAH contaminated soils could help in their utilization and aid in plant growth. Further field experiments will greatly reveal the applicability of this potent biosurfactant for alleviating environmental stress and thereby increasing plant productivity.

Acknowledgements

The authors wish to thank Prof. B. A. Chopade for providing tensiometer facilities. The authors whole heartedly acknowlege the support the college authorites for providing necessary infrastructure and financial assistance.

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