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Isolation, Screening and Production of Biosurfactant by *Bacillus clausii* 5B

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Biosurfactant-producing bacteria were isolated from soil samples contaminated with petroleum hydrocarbons. Isolates were screened for biosurfactant production using Cetyl Tri Ammonium Bromide (CTAB)-Methylene blue agar selection medium. A candidate bacterial strain was selected for further studies based on surface tension reduction capacity and rapid drop collapse activity. Biochemical characteristics and partially sequenced 16S rRNA gene of the isolate, 5B, identified the bacterium as *Bacillus clausii*. Different carbon and nitrogen sources were evaluated for their effect on biosurfactant production. Maximum biosurfactant synthesis (2.11 g/L) was observed at 96 hours when the cells were grown on minimal medium containing 1 % (w/v) glucose as carbon source. Among nitrogen sources tested, ammonium chloride showed maximum biosurfactant production of 2.41 g/L. The biosurfactant produced by the bacterial isolate reduced the surface tension of the cell free broth from 53.56 mN/m to 29.48 mN/m. Compositional analysis of the biosurfactant revealed that it was of lipopetide type, composed of high percentage of lipid (~56 %, w/w) and protein (~39 %, w/w) content.

Keywords: Biosurfactant; *Bacillus clausii*; surface tension; carbon and nitrogen sources; compositional analysis

Surfactants constitute a diverse group of specialty chemicals that are derived from non-renewable petroleum products. The chemical surfactants are costly and due to their recalcitrant nature, they pose serious threat to the environment causing environmental pollution. Therefore, in the recent past, attentions have been paid to alternative, environmental friendly, surface active products synthesized by microorganisms known as biosurfactants. The main reason is that biosurfactants take advantages over their synthetic counterparts include lower toxicity, biodegradability, better environmental compatibility, higher foaming, high selectivity, specific activity at extreme temperatures, pH and salinity (Desai and Banat, 1997, Illori et al., 2005, Raza et al., 2007).

Biosurfactants possess both hydrophobic and hydrophilic moieties, they are able to reduce surface tension and interfacial tension between two fluids at the

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surface and interface respectively. On the basis of the relative molecular mass, biosurfactants are generally classified into two groups: (1) low-molecular mass biosurfactants, such as glycolipids, lipopeptides and phospholipids; and (2) high-molecular mass biosurfactants, such as emuslan, alasan, liposan, polysaccharides and protein complexes (Rosenberg and Ron, 1999). From a biotechnology perspective, the production of biosurfactants is important owing to their vast applications in food, cosmetics, pharmaceuticals, agricultural and the petrochemical industries (Nguyen et al., 2008, Pruthi and Cameotra, 2003, Robert et al., 1989).

Bacteria are the main group of biosurfactant-producing microorganisms, although it is also produced by some yeasts and filamentous fungi (Desai and Banat, 1997). A number of studies have reported the potential of *Bacillus* species as biosurfactant producers and they produce lipopeptide type of biosurfactant (Nakano and Zuber, 1989, Nitschke and Pastore, 2004). Lipopeptides represent a class of microbial surfactant with remarkable surface properties and biological activities, such as surplus crude oil recovery, food-processing, de-emulsification, antimicrobial and antitumor, antiviral, antiadhesive activities, etc. (Banat, 1995, Peypoux et al., 1999, Vater, 1986). Production of an effective lipopeptide type biosurfactant, surfactin, was first reported for a strain of *Bacillus subtilis* (Arima et al., 1968).

Even though interest in biosurfactants is increasing, these surface active compounds do not compete economically with synthetic surfactants. To reduce production costs, different routes could be investigated such as the increase of yields and product accumulation; the development of economical engineering processes and the use of cost-free or cost-credit feedstock for microorganism growth and surfactant production (Mercade and Manresa, 1994). Due to the increasing demand of microbial biosurfactants in the recent years, discovery of new biosurfactant producing strains and finding the optimum conditions for biosurfactant production is of utmost importance. In the present investigation, we report the isolation of a biosurfactant producing bacterial strain, effect of carbon and nitrogen sources on growth and biosynthesis of the surfactant and compositional analysis of the biosurfactant.

MATERIALS AND METHODS

Materials

All the chemicals used in the present study were of analytical grade.

Isolation of microorganisms

The bacterial strains were isolated using enrichment technique from hydrocarbon contaminated soil samples in Karnataka, India. The soil samples were suspended in Bushnell Hass medium (1941) containing 1% crude oil and incubated at 30^{o}C, 150 rpm for 3 days.

Screening of biosurfactant producers

The potential biosurfactant producers were initially screened on Cetyl Tri Ammonium Bromide (CTAB)-Methylene blue agar medium (Siegmund and Wagner, 1991). The colonies showing halo on the selective CTAB-Methylene blue agar medium were further screened by drop collapsing test (Jain et al., 1991) and surface tension measurement (Bodour and Maier, 1998).

Among eighteen bacterial strains isolated, an efficient biosurfactant-producing bacterial strain, designated as 5B, was used for further studies based on rapid drop collapse reaction and surface tension reduction. The isolated bacterial culture was maintained on nutrient agar medium and stored at 4^{o}C.

Identification of the bacterium by biochemical and 16S rRNA sequence analysis

The bacterial strain 5B was identified based on its morphological and biochemical characteristics according to Bergey's Manual of Determinative Bacteriology. The gene sequence coding for 16S rRNA was identified at Agharkar Research Institute, Pune, India.

Medium and culture conditions for biosurfactant production

The production of biosurfactant was carried out in 250 ml Erlenmeyer flasks containing 50 ml of modified mineral medium with the following composition: KNO_3 (0.3 %), Na_2HPO_4 (0.22 %), KH_2PO_4 (0.014 %), NaCl (0.001 %), $MgSO_4$ (0.06 %), $CaCl_2$ (0.004 %), $FeSO_4$ (0.002 %) and 0.1 ml of trace element solution containing (g/L): 2.32 g $ZnSO_4 \cdot 7H_2O$, 1.78 g $MnSO_4 \cdot 4H2O$, 0.56 g H_3BO_3, 1.0 g $CuSO_4 \cdot 5H_2O$, 0.39 g $Na_2MoO_4 \cdot 2H_2O$, 0.42 g $CoCl_2 \cdot 6H_2O$, 1.0 g EDTA, 0.004 g $NiCl_2 \cdot 6H_2O$ and 0.66 g KI (Makkar and Cameotra, 1998) and 1% crude oil as carbon source. The medium was inoculated with 2% of 12 hours seed culture grown on nutrient broth. A control flask without carbon source was also maintained. Incubation was carried out at 30°C in an incubator shaker at 150 rpm. Time course samples of culture medium were drawn in appropriate time intervals and monitored for biosurfactant production, biomass estimation and surface tension. Biosurfactant concentration in the culture broth was estimated according to the method of Samadi et al. (2007).

Effect of carbon and nitrogen sources on biosurfactant production

The effect of carbon and nitrogen sources on biosurfactant production were determined under submerged conditions. The carbon sources evaluated in the study were glucose, sucrose, glycerol, molasses and sodium acetate (1%). Yeast extract, peptone, ammonium chloride, urea, ammonium nitrate (0.3%) were the nitrogen sources tested in the present study. A control flask without carbon/ nitrogen source was also maintained. The flasks were maintained at 30°C in an incubator shaker at 150 rpm.

Analytical methods

Biomass estimation

Bacterial cell growth was monitored by measuring the dry cell weight method. It was determined by centrifugation (10,000 rpm for 30 minutes) of a 1 ml culture broth, the cell pellet was washed with distilled water twice and dried by heating at 50°C until constant weight was attained.

Surface tension determination

For the determination of surface tension, the cell free broth was subjected to measurement of surface tension according to the ring method at room temperature using a tensiometer (Surface tension meter, DST 30 series, Surface and Electro Optics Corporation, Korea).

Biocompositional analysis of the biosurfactant

The biosurfactant obtained from *Bacillus clausii* 5B was submitted to protein (Lowry et al., 1951) and lipid (Nitschke and Pastore, 2002) determinations. Further, the biosurfactant was separated by Thin Layer Chromatography (TLC) on silica gel plates using $CHCl_3$: CH_3OH: H_2O (65: 15: 1) as development system. Ninhydrin reagent (0.5 g ninhydrin in 100 ml of anhydrous acetone) was used to detect lipopeptide biosurfactant.

RESULTS AND DISCUSSION

Isolation and screening of biosurfactant producers

Eighteen different bacteria were isolated from the soil sample contaminated with petroleum hydrocarbons. Based on the

screening tests performed, four isolates showed halos around the colonies on CTAB-Methylene blue agar medium. Among these, one of the bacterial isolates, designated as 5B, was grouped as potential biosurfactant producer. The isolate 5B showed rapid drop collapsing reaction and showed surface tension value of 32.78 mN/m. In the present study, we report the biosurfactant production potential of 5B.

Identification of the bacterium by biochemical and 16S rRNA sequence analysis

Based on the morphological and biochemical characteristics, the bacterial isolate 5B was identified as *Bacillus sp.* The strain *Bacillus sp.* was motile, Gram-positive and rod-shaped. Colonies of *Bacillus sp.* formed filamentous margins that appeared cream-white in color. The biochemical characteristics of the isolate are presented in Table 1. Alignment of the 16S rRNA gene sequence of the bacterium with sequence obtained by doing a Blast search revealed 98% similarity to *Bacillus clausii*. In the present paper, the bacterial strain 5B is designated as *Bacillus clausii* 5B.

Table 1: Biochemical characteristics of *Bacillus sp.*

Sl no.	Test	Result
1	Indole Test	Negative
2	Methyl Red Test	Negative
3	Voges Proskauer Test	Negative
4	Citrate Utilization	Negative
5	Starch Hydrolysis	Positive
6	Casein Hydrolysis	Positive
7	Catalase Test	Positive
8	Oxidase Test	Negative
9	H_2S Production	Positive
10	Nitrate Reduction	Negative
11	Glucose fermentation	Positive
12	Fructose fermentation	Positive

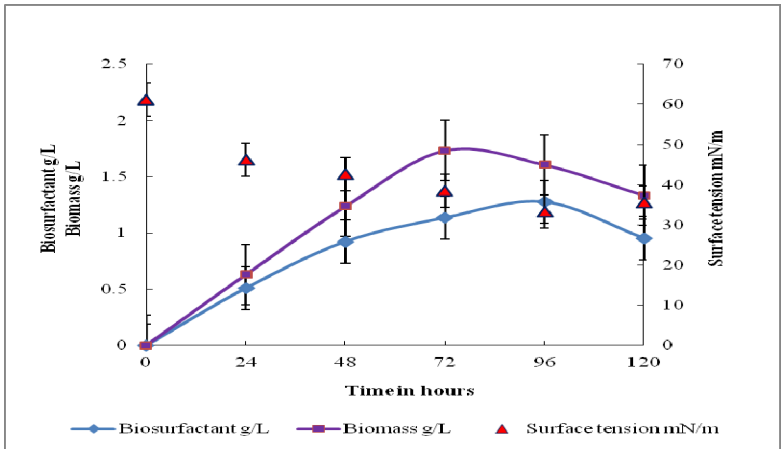

Fig. 1: Time course profile of extracellular biosurfactant synthesis, cell growth, surface tension reduction by *Bacillus clausii* 5B in 1% (v/v) crude oil containing modified PPGAS medium. Results are represented as Mean ± SEM (n=3)

Medium and culture conditions for biosurfactant production

In the preliminary experiment, biosurfactant was produced by *Bacillus clausii* 5B in modified mineral medium containing 1% crude oil as carbon source. As seen in Fig. 1, the biosurfactant production commenced within 24 hours and showed a maximum of 1.27 g/L at 96 hours. Maximum biomass (1.73 g/L) was observed at 72 hours. The maximum reduction in surface tension was achieved at 96 hours of fermentation (33.40mN/m). The production of biosurfactant in the presence of hydrocarbon

as carbon source in the present study indicates the potential of *Bacillus clausii* in hydrocarbon remediation. It is known that most of the bacteria utilize insoluble hydrocarbons by producing biosurfactants that promote substrate solubilization and/or emulsification, thus allowing the cells to get into direct contact with the oil phase (Rosenberg and Ron, 1999).

Effect of carbon and nitrogen sources on biosurfactant production

The quality and quantity of biosurfactant production are affected and influenced by the nature of the carbon substrate (Raza et al., 2007, Rahman and Gakpe, 2008). In order to increase the biosurfactant yield by the bacterial strain, different carbon substrates were evaluated for biosurfactant production. All the carbon sources tested favored extracellular production of surface active product by *Bacillus clausii* 5B, which was indicated by the reduction in surface tension of the broth as depicted in Table 2. Among the carbon substrates screened for the production of biosurfactant by *B. clausii* 5B, glucose increased the production (2.11 g/L) significantly compared to other carbon sources. Studies by various researchers have revealed that the presence of glucose in the production medium increased the biosurfactant production in the culture medium (Guerra-Santos et al., 1984, Wei et al., 2005). Least biosurfactant production (1.04 g/L) was observed when glycerol was used as the carbon source and the culture free broth showed surface tension value of 32.81 mN/m.

Table 2: Effect of different carbon sources on biosurfactant production by *Bacillus clausii* 5B. Results are represented as Mean ± SEM (n=3)

Carbon source	Biosurfactant g/L	Biomass g/L	Surface tension mN/m
Control	0.033±0.06	0.17±0.08	57.62±0.29
Glucose	2.11± 0.09	2.49±0.11	30.21±0.06
Sucrose	1.76±0.12	2.21±0.19	31.58±0.18
Molasses	1.59±0.28	2.03±0.07	31.76±0.10
Sodium acetate	1.34±0.10	1.46±0.12	31.97±0.33
Glycerol	1.04±0.04	1.94±0.03	32.81±0.25

The kinetic growth curve of *Bacillus clausii* 5B indicated a parallel relationship between biosurfactant production, bacterial growth, surface tension reduction and substrate utilization, suggesting a growth associated biosurfactant production when 1% glucose was used as the carbon source (Fig. 2). The exponential growth of the bacteria was observed at about 24 hours and after 72 hours of cultivation; the maximum biomass (2.42 g/L) was reached. Biosurfactant production commenced at about 24 hours, i.e., during the exponential phase, indicating its accumulation as primary metabolite during growth phase. Maximum biosurfactant production (2.17 g/L) occurred at 96 hours. The surface tension of the cell free broth showed lowest value, *i.e.* 30.23 mN/m. Therefore, it can be concluded that the biosurfactant produced by *Bacillus clausii* 5B is a primary metabolite, due to the production of growth-associated biosurfactant.

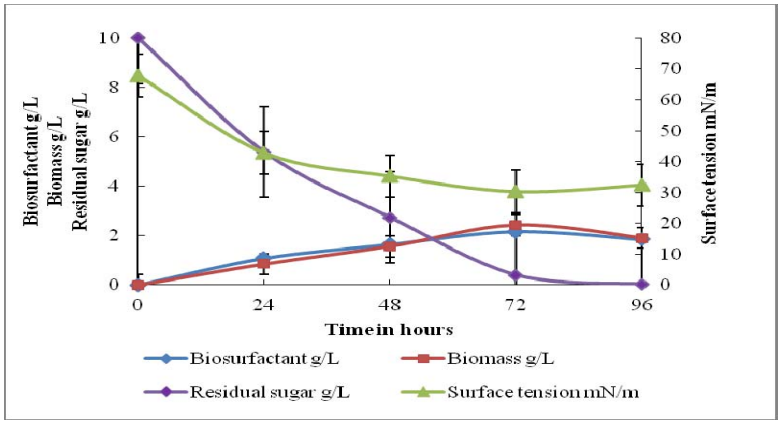

Fig. 2: Effect of 1% glucose on biosurfactant production, biomass, substrate utilization and surface tension reduction by *Bacillus clausii* 5B. Results are represented as Mean ± SEM

Table 3: Effect of different nitrogen sources on biosurfactant production, biomass and surface tension reduction by *Bacillus clausii* 5B. Results are represented as Mean ± SEM (n=3)

Nitrogen source	Biosurfactant g/L	Biomass g/L	Surface tension mN/m
Control	0.26±0.03	1.08±0.04	49.12±0.34
Ammonium chloride	2.41±0.36	2.04±0.06	29.48±0.09
Peptone	1.65±0.27	2.08±0.13	31.36±0.23
Yeast extract	1.39±0.12	1.82±0.09	31.83±0.41
Ammonium nitrate	1.21±0.15	1.26±0.09	32.89±0.23
Urea	1.19±0.09	1.23±0.21	32.55±0.16

Choice of nitrogen source affects the biosurfactant production as summarized in Table 3. Among the organic nitrogen sources, peptone and yeast extract, peptone enhanced biomass production (2.08 g/L) in the presence of glucose where as ammonium chloride resulted in maximum biosurfactant production (2.41 g/L). The cell free culture broth showed a surface tension value of 29.48 mN/m. These results are in agreement report of Fagade et al. (2009), in which ammonium salt was observed as a preferable nitrogen source for the biosurfactant production by *Arthrobacter paraffinus*. In media amended with urea, least biosurfactant production (1.19 g/L) by *Bacillus clausii* 5B was observed. Thus, optimization of carbon and nitrogen sources enhanced the biosurfactant production by *Bacillus clausii* 5B.

Biocompositional analysis of the biosurfactant

Preliminary biochemical characterization of the surfactant produced by the bacterial isolate showed that the product contained 56% (w/w) of lipids and 39% (w/w) of proteins. These results suggest that the surfactant was a lipopeptide type, probably related to the surfactin family of surface-active compounds which are characteristics of some *Bacillus* strains (Ohno et al., 1999, Sheppard and Cooper, 1991).

Biosurfactant from *Bacillus clausii* 5B was identified as red color spot on the silica gel plate. The R_f value of the biosurfactant was 0.51. Similar results have been observed by Priya and Usharani (2009) during lipopeptide biosurfactant production *by Bacillus subtilis*.

CONCLUSION

In the present study, eighteen bacterial cultures were isolated from petroleum hydrocarbon contaminated soil sample. Among them, four strains showed halos on CTAB-methylene agar medium, indicating their capacity to produce surface active substance. Based on rapid drop collapsing reaction and surface tension reduction capacity, *Bacillus clausii* 5B, was chosen for further studies. The effect of different carbon and nitrogen sources on growth and biosurfactant production was evaluated. Glucose as sole carbon source resulted in maximum growth as well as biosurfactant production. Among the nitrogen sources screened, peptone was found to be an essential component for bacterial growth, while ammonium chloride proved to be the most important inorganic nitrogen source for biosurfactant production. The biosurfactant production was improved in the shake flask studies by the optimization of carbon and nitrogen source, indicating *Bacillus clausii* 5B as a promising biosurfactant-producer.

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