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STUDY OF BIOSURFACTANT PRODUCING BACTERIA AND PRELIMINARY CHARACTERIZATION OF BIOSURFACTNAT PRODUCED BY *BACILLUS* species ISOLATED FROM PETROLEUM CONTAMINATED SOIL

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ABSTRACT

Chemically synthesized amphiphilic molecules like surfactants are used for variety of purposes, but they are costly and less or non-degradable and may be toxic once released in the environment. Microbially produced biosurfactants are better alternatives of chemical surfactants as they are stable as well as easily biodegradable and also due to structural and functional similarities with chemical surfactants. The aim of this study was to isolate and characterize biosurfactant producing bacteria from oil contaminated sample (garage soil, Anand, Gujarat). Thirty bacterial cultures were isolated on the basis of differences in their colony characters. To confirm and compare the efficiency of biosurfactant production, hemolytic activity and reduction in surface tension was analyzed. Biochemical and morphological tests identified bacterial isolate C5C (Gram positive rods arranged in short chains) as *Bacillus* species. Optimum pH and temperature for biosurfactant production were 5 and 30°C, respectively. It produced 3.12 g/l of biosurfactant in MSM medium with 1% diesel as carbon source. Biosurfactant produced by C5C contains lipid. No carbohydrates or amino acid component was detected on TLC plate.

Keywords: *Bacillus* species, Biosurfactant, Biochemical characterization, TLC, Mass of Drop

INTRODUCTION

Reduction in surface tension is aided by variety of surfactants. Commercially used surfactants are produced chemically as derivatives from petroleum products **(Ali *et al.*, 2013)**. Such surfactants are used in variety of applications like foaming agent, detergents, emulsifiers, solubilizer, in wetting and spreading etc. because of their amphiphilc nature **(Ali *et al.*, 2013)**. They lower interfacial tension by accumulating at interfaces and micelles formation **(Rufino *et al.*, 2014)**. Major drawback of using the chemical surfactants is their cost and undesirable residues which are difficult to dispose off and degrade leading to environmental pollution **(Batista *et al.*, 2006; Aparna *et al.*, 2012; Marchant and Banath, 2012; Ali *et al.*, 2013)**. Therefore there is continuous search for eco-friendly biosurfactants to replace chemical surfactants.
Biosurfactants are better alternatives because they are easily biodegradable **(Rufino *et al.*, 2014)**. They are structurally and functionally similar to chemical surfactants and synthesized both by bacteria and fungi including yeast **(Satpute *et al.*, 2010; Marchant and Banath, 2012; Ali *et al.*, 2013)**. They are having hydrophobic and hydrophilic parts in their structure. They are produced extra-cellularly or as part of cell structure **(Sneha *et al.*, 2012)**. In recent years, development of biosurfactant technology has taken paramount importance due to their biocompatibility, low toxicity, higher biodegradability and higher stability under extreme conditions like pH and temperature **(Sadat *et al.*, 2013; Kim *et al.*, 2004; Rufino *et al.*, 2014)**. Biosurfactants are so diverse chemically that they can be used in medical field, cosmetic industries, for environmental applications as well as other processes like enhanced oil recovery process. Despite such advantages and applications, biosurfactants are not exploited much because of limited knowledge regarding production condition and extent to which organism produces this substances. Present study was undertaken to screen bacteria isolated from oil spilled region (garage soil) for biosurfactant production and their characterization.

MATERIAL AND METHODS

Sampling area

For isolation of biosurfactant producing bacteria soil samples were collected from different garages around Anand (Gujarat, India). The soil samples from contaminated sites were collected in sterile containers and brought to the laboratory for further studies.

Enrichment and isolation of bacterial cultures

5.0 gm of soil samples were suspended by thorough mixing in 100 ml phosphate buffer saline (PBS). After settling of soil debris 5 ml supernatant was transferred to 50 ml of Bushnell-Haas (BH) Medium [$MgSO_4$ 0.2 g/L, $CaCl_2$ 0.02 g/L, KH_2PO_4 1.0 g/L, K_2HPO_4 1.0 g/L, NH_4NO_3 1.0 g/L, $FeCl_3$ 0.05 g/L] with 1% diesel as sole source of carbon and incubated at 25°C, 200 rpm for 4 days **(Batista *et al.*, 2006)**. After incubation 1ml of sample was transferred to fresh BH medium (with 1% diesel) and incubated again as stated above. After three cycle of enrichment, loop full of culture was streaked on BH agar medium with 1% diesel and incubated at 37°C for 1week. Colonies with different morphology were selected and transferred on nutrient agar slant (pH 7.0 ±0.2) and stored at 4°C.

Hemolytic activity of isolates

Hemolytic activity is considered to be good and easy criteria to screen out biosurfactant producers. For that fresh single colonies of isolated cultures were taken and streaked on blood agar plates. Plates were incubated for 24 to 48 hrs at 37°C. The plates were visually inspected for zone of clearance (hemolysis) around colonies. Hemolysis was designated as alpha (α), beta (β) and gamma (γ) hemolytic activity and was used as qualitative method for selection of better producer of biosurfactant **(Satpute *et al.*, 2010; Ali *et al.*, 2013; Varjani *et al.*, 2014)**. Five cultures were selected on the basis of the hemolytic activity for further study.

Identification of bacterial culture

The selected bacterial isolates were characterized by morphological/microscopic and biochemical tests such as Gram staining, Methyl Red (MR test), Voges Proskauer (VP test), Citrate utilization test, Nitrate reduction test, H_2S production test, Gelatine hydrolysis test, Glucose and Sucrose fermentation test and catalase test. Cultures were also subjected to Triple Sugar Ion (TSI) test. Results from biochemical analysis were used to find the closest match with known bacterial genus and to assign the bacterial signature according to Bergey's manual **(Nasr *et al.*, 2009; Anandraj and Thivkaran, 2010; Ali *et al.*, 2013)**.

Quantitative measurement of reduction in surface tension

Reduction in surface tension was analyzed from broth medium. For that, bacterial cultures were activated in LB broth for 24 hrs at 37°C. After activation1 ml inoculums with 1.00 $O.D._{600nm}$ cells were transferred to the 100 ml BH medium with 2% diesel as carbon source. Broth was agitated at 200 rpm for one week for growth and biosurfactant production. Surface tension was measured by drop weight method described by **William Burns Tucker (1938)**. It is second most precise and easy method for determination of surface tension. Cells were removed by centrifugation at 5000 rpm for 15 min and supernatant was collected. Cell free supernatant was poured in a burette which was attached to a rubber tube on one side and glass tube of 3 mm diameter at another side. An empty pre-weighed beaker was placed under burette and supernatant was released slowly drop by drop. 50 drops were poured in to the beaker and it was weighed to determine the weight of 50 drops. Mass of one drop was calculated by using following formula **(Ramesh *et al.*, 2011).**

$$Mass\ of\ Drop\ (M) = \frac{(Sample + Beaker\ weight) - (Weight\ of\ empty\ Beaker)}{Number\ of\ drops}$$

Surface tension and surface activity was measured by following formula.

$$Surface\ tension = \frac{Mg \times 10^{-3} \times nM^{-1}}{\pi r}$$

Where, "M" is mass of one drop, "g" is gravity and "r" is radius of glass tube. Value of Surface tension was used to calculate surface activity.

$$\text{Surface activity} = Surface\ tension\ of\ Uninoculated\ medium - Surface\ tension\ of\ Supernatant$$

Optimization of growth medium

pH optimization

The initial pH value of them MSM medium with 1% diesel was adjusted over a range of 2 to 9 and media were sterilized in triplicates. Media were inoculated with 1% v/v inoculum and incubated for seven days at 120 rpm. After incubation period, cell free supernatants (crude biosurfactant) were obtained by centrifugation (8000 rpm for 15 min) and E24 % was determined for samples of each pH according to method suggested by **Chandran and Das (2011)**. For E24% 1ml of cell free supernatant was added in test tube containing 1ml diesel followed by through mixing for 1min by vortex. After 24 hrs emulsification index was calculated as

$$E24\% = \frac{Hight\ of\ Emulsuion\ Formed}{Total\ Hight\ of\ Solution} \times 100$$

Temperature optimization

100 ml of MSM medium with 1% Diesel (pH 7) sterilized at 121 psi for 15 min. After sterilization, 1ml of activated cells with 1.00 $O.D._{600nm}$ were transferred to medium under aseptic condition and incubated at 120 rpm for seven days at different temperatures; 30, 40, 50, 60, 70, 80, 90 and 100°C. Experiment was carried out in triplicates. After incubation E24% was calculated from cell free broth for each flask.

Inoculum development:

For inoculum preparation, single colony of C5C isolate was aseptically transferred to fresh sterile Luria-Bretani broth and incubated at 37°C until its $O.D._{600}$ reaches to 1.00.

Production and extraction of biosurfactant:

The production of biosurfactant was carried out in MSM medium with 1% diesel. 1.00 $O.D._{600nm}$ cells were aseptically transferred to production medium with optimized pH. After inoculation medium was incubated for seven days at optimized temperature. Biosurfactant was extracted by solvent extraction method **(Chander *et al.*, 2012)**. In order to precipitate lipids and proteins, 6N HCl was added in supernatant to bring pH 2 and kept overnight at 4°C. White colour precipitates were collected by centrifugation at 10,000 rpm for 20 min. Further extraction was carried out by adding 10 ml chloroform: methanol (2:1 v/v) to the precipitated pellet which was dissolve in 10 ml of distilled water. Phases were allowed to separate. Organic phase was collected in pre-weighted petriplate and kept at room temperature for evaporation. After complete evaporation, petriplate was weighted and produced biosurfactant was calculated as

$$Biosurfactant\ Production = Weight\ of\ petriplate\ after\ drying - Weight\ of\ empty\ petriplate$$

Characterization of biosurfactant by Thin Layer Chromatography [TLC]

To identify the component parts of biosurfactant silica gel plates were used. A spot of crude biosurfactant was placed on silica gel plate. The biosurfactant was separated on plate using following four systems. System 1 Petroleum ether-diethyl ether-acetic acid (80:20:1) for neutral lipid. System 2 Chloroform-methanol-water (65:52:4) for polar lipid. System 3 *n* butanol-acetic acid-water (4:1:1) for amino acid. System 4 ethyl acetate-acetic acid-methanol-water (12:3:3:2) for carbohydrates. After development visualization was carried out for separated components. Lipid was visualized by placing the plates in closed jar saturated with iodine vapour which gave yellow colour spots. Amino acids were visualized by using ninhydrin reagent followed by heating at 90°C for 15 min which gives red or purple colour. Carbohydrate was visualized by detecting red colour spot on spraying with alpha-naphthol-sulphuric acid solution **(Yin *et al.*, 2009)**.

RESULTS AND DISCUSSION

Isolation and screening of bacteria

Thirty bacteria with different colony characteristics were isolated from three soil sample each from various location of a Garage by following three successful cycles of enrichment. Twenty one isolates were found to be Gram positive and rest of nine were Gram negative in gram reaction.

Hemolytic activity and biochemical characterization of bacteria

All isolates were streaked on the blood agar plates. The hemolytic activity was observed in all thirty isolated strain, results showed alpha hemolytic activity by strain SD2-B, SO2-A ,C1C, C6C, C3N, C6N and M-B, beta hemolytic activity by SD1-A, SD1-B, SD1-C, SD1-D, SO1-A, SO1-B, SO2-B, M-A, H-A, H-B, H-C, C2C, C3C, C4C, C5C, C7C, C2N and C4N and hemolytic activity gamma was indicated by SO2-A, H-D, C1N, C5N, C6N and C7N (Table-1). Hemolytic activity is qualitative measurement of biosurfactant ability of cultures **(Satpute *et al.*, 2010)**. Hemolytic zone on blood agar plate have linear relation with the concentration of biosurfactant produced by bacteria **(Youssef *et al.*, 2014)**. Therefore out of thirty bacterial cultures five isolates SD1, SO1-B, SD2-A, SO2-B and C5C were selected for further study on the basis of hemolytic zone on blood agar plates and characterized biochemically (Table-1).

Table 1 Microscopic and biochemical characterization of selected cultures

Test	Cultures				
	C5C	**SD1-A**	**SO1-B**	**SD2-A**	**SO2-B**
Gram Reaction	Positive	**Positive**	Negative	**Positive**	**Negative**
Blood Hemolysis	B	β	β	γ	β
Zone of hemolysis*	++++	+++	+++	+++	+++
Methyl Red test	Negative	**Positive**	Negative	**Positive**	**Positive**
Voges Proskauer Test	**Positive**	**Positive**	**Positive**	Negative	Negative
Citrate Utilization	**Positive**	Negative	Negative	Negative	**Positive**
Catalase test	Negative	**Positive**	**Positive**	**Positive**	**Positive**
Gelatine liquefaction	Negative	Negative	Negative	**Positive**	**Positive**
Nitrate reduction	**Positive**	**Positive**	**Positive**	**Positive**	**Positive**
H_2S Production	Negative	Negative	**Positive**	Negative	**Positive**
Starch Utilization	**Positive**	Negative	Negative	**Positive**	**Positive**
Sucrose fermentation	Negative	**Positive**	**Positive**	**Positive**	**Positive**
Glucose fermentation	**Positive**	**Positive**	**Positive**	**Positive**	**Positive**
TSI**	**A/Ak/-/+**	**A/Ak/-/-**	**Ak/A/+/+**	**Ak/Ak/-/-**	**Ak/A/+/+**

Legends: * (-) no zone ;(+) poor activity; (++) Moderate activity; (+++) Good activity; (++++) Excellent activity, **** - Reaction in slant/Reaction in butt/

H_2Sproduction/Gas production

Results showed that C5C was positive for Voges Proskauer test (VP test), citrate utilization test, nitrate reduction test, starch hydrolysis test and glucose fermentation test (Table-1). These data are quit comparable with results of *Bacillus* **(Aparna *et al.*, 2012)**. Figure 1 showed the morphology of C5C by Gram's staining. SD2-A was able to ferment glucose and sucrose and showed starch hydrolysis. It gave positive result for methyl red test (MR test), but negative for VP test and citrate utilization test. All other positive tests included catalase test, gelatine liquification, nitrate reduction and H_2S production. Triple Sugar Ion slant (TSI) gave alkaline reaction at both slant and butt region with neither H_2S nor gas production (Table-1). Result shows similarities with the results reported for *Pseudomonas* **(Sneha *et al.*, 2012; Sadat *et al.*, 2013)**. SO2-B culture showed similar results as SD2-A except citrate utilization test and TSI reactions. Cultures SD1-A and SO1-B were able to reduce nitrate, ferment glucose and sucrose. They showed positive results for catalase test and VP test. More confirmatory tests are needed to identify the isolate.

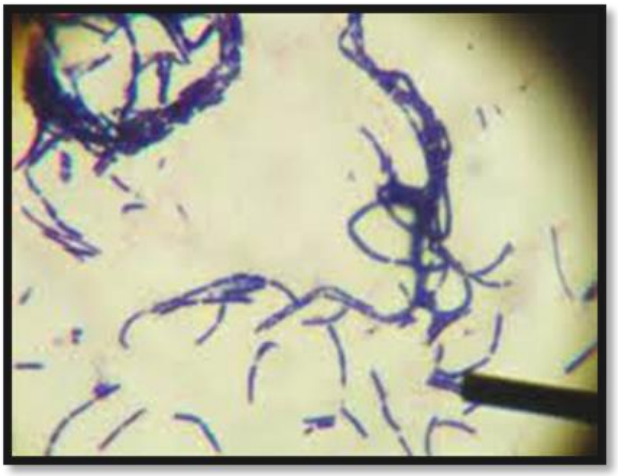

Figure 2 Gram staining of C5C

Quantitative measurement of surface tension of fermented broth

On the basis of hemolytic zone obtained on blood agar plate, cultures C5C, SD1-A, SO1-B, SO2-A and SO2-B which gave excellent or good hemolytic activity were selected for further studies. All five selected cultures were studied further for the reduction of surface tension. Results are shown in Table 2. Culture C5C reduced surface tension of broth by 40.56 mN/m which was lowest compare to other four cultures. **Mathiyzhagan *et al.*, (2011)** reported the surface tension reduction of cell free cultures broth ranging from 64.0 mN/m to 28 mN/m using organisms isolated from oil contaminated soil. *Bacillus subtilis* isolated from Kanchipuram reported to have surface activity 51.38 nm^{-1} **(Ramesh *et al.*, 2011).** Similar results were also observed in the present study.

Table 2 Quantitative measurement of surface tension and surface activity of selected culture broth

Cultures	Surface tension [nm^{-1}]	Surface activity[nm^{-1}]
Control	92.8 x $10^{(-3)}$	00
SD1-A	51.11 x $10^{(-3)}$	41.69
SO1-B	91.24 x $10^{(-3)}$	1.56
SO2-A	78.49 x $10^{(-3)}$	14.31
SO2-B	56.72 x $10^{(-3)}$	36.08
C5C	40.56 x $10^{(-3)}$	51.78

Cultures C5C, SO2-A and SD1-A were found to be good biosurfactant producers according to criteria demonstrated by **Mulligan (2005).** Amongst the isolates selected C5C showed highest surface activity and thus it was further studied for detail study.

Production and extraction of biosurfactant from C5C (*Bacillus* species)

Isolate C5C was used for further studies on biosurfactant production by inoculating it in MSM medium (with 1% diesel as carbon source) at 37°C at 121 rpm for seven days. Isolate C5C produced 3.12 g/l dry weight of biosurfactant. *Bacillus clausii* 5B produced 2.41 g/l of biosurfactant in minimal medium with 1% w/w glucose as carbon source and ammonium chloride as nitrogen source **(Aparna *et al.*, 2012)**. *Bacillus subtilis* SPB1 produced 2.93 (±0.32) g/l of biosurfactant when media was incorporated with 15g/l of glucose, 6 g/l of urea and 1 g/l of K_2HPO_4 **(Abdel-Mawgoud *et al.*, 2008)**.

pH and Temperature optimization

Biosurfactant produced by C5C gave maximum E24% at pH 5 and 30°C temperature in MSM medium with 1% diesel (Fig 2 and 3). pH optimization results showed that there was significant increase in emulsification in pH range 2 to 5 and as further increase in pH emulsification reduced. Similarly maximum E24% was observed at 30°C. After that with E24% is positively decreases. Because of easy evaporation of diesel at higher temperature biosurfactant production might be decrease due to lack of carbon source under batch fermentation operate under shaking flask condition. Surfactin production from *Bacillus subtilis* DSM 3256 was reported to be highest at 6.75 pH and 37.4°C temperature **(Sen and Swaminathan, 1997)**. Iturine-A was produced maximally by *Bacillus subtilis* at pH 5.9-6.3 and 25°C temperature **(Mizumoto and Shoda, 2007)**. *Bacillus megaterium* produced maximum glycolipid at pH 8 and 37°C under solid state fermentation **(Thavasi *et al.*, 2007)**.Similarly optimum condition for surfactin production form *Bacillus subtilis* BS5 was 6.5-6.7 pH and 30°C as reported by **Abdel-Mawgoud *et al.*, (2008)**. *Penicillum chrysogenum* SNP5 which showed increase in E24% between 2 to 8 pH range, while E24% decreased between pH 8 to 12 **(Gautam *et al.*, 2014).**

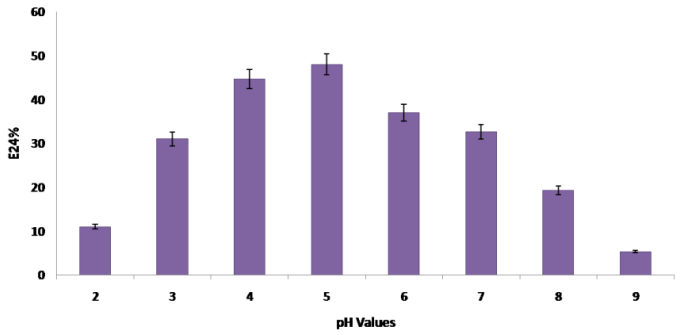

Figure 2 pH optimization

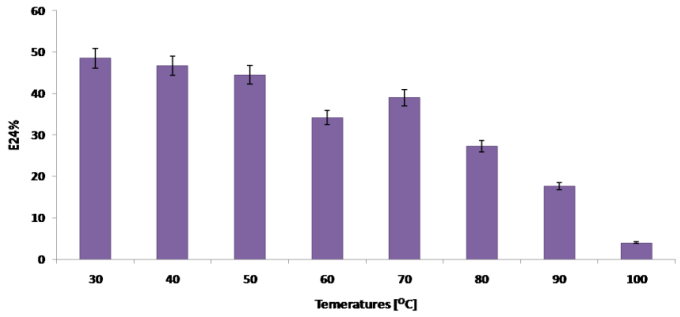

Figure 3 Temperature optimization

Characterization of biosurfactant by TLC

Biosurfactant produced from isolate C5C was analyzed by Thin Layer Chromatography (TLC). As shown in Fig 4, a purple colour spot appearing on TLC (*Rf* value 0.57) suggested presence of polar lipid content in biosurfactant. There was no colour development observed for amino acid or carbohydrate. Similar result was observed by **Aparna *et al.*, (2012)** that *Bacillus clausii* 5B biosurfactant produce red spot on silica gel plate. R_f value of biosurfactant was 0.51. **Nishanthin *et al.*, (2010)** have reported strains BPB7 and BPB13 isolated from petrochemical waste soil produced biosurfactant which is glycolipid in nature. Biosurfactant from *Arthrobacter* strain is reported to be lipopeptides and lipoprotein in nature **(Cipinvte *et al.*, 2011)**.

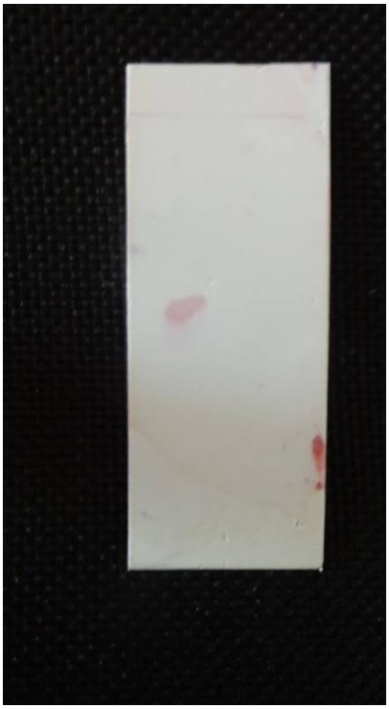

Figure 4 TLC analysis of biosurfactant

CONCLUSIONS

Thirty cultures were isolated from garage soil near Anand (Gujarat) region. They were characterized morphologically and biochemically. On the basis of screening techniques culture C5C was found to be potential isolate for production of biosurfactant among thirty isolates. It was identified as *Bacillus* sp biochemically as it gave positive response for Voges Proskauer test, citrate utilization test, nitrate test, starch utilization test and glucose fermentation test. Triple sugar ion test showed production of gas with acidic slant and alkaline butt reaction that confirm the *Bacillus* sp. Cultural conditions pH and temperature were also optimized for maximum production of biosurfactant. It produced 3.12 g/l of dry precipitate of biosurfactant in MSM medium (pH 5) with 1% diesel at 30°C 121 rpm. Further optimization of culture media and condition can be done. Structural characterization, indicate presence of polar lipid content in biosurfactant.

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