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ORIGINAL RESEARCH ARTICLE

Isolation, Screening and Characterization of Bio-surfactant Produced by *Bacillus* sp. from Automobile Oil Contaminated Soil

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ABSTRACT

The biosurfactant producing strain was isolated from oil contaminated soil from an automobile workshop .The strain was identified as *Bacillus* spbased on Gram staining and biochemical test. From the screening tests i.e, (oil spreading test and blood haemolysis test) *Bacillus* sp. showed the capacity to produce biosurfactant. The biosurfactant showed a better emulsifying activity against a variety of substrate and achieved a maximum emulsion index (E_{24}) of 67% for coconut oil. Using FT-IR spectroscopy, the chemical structure of the purifiedfraction of crude biosurfactant was identified as lipopeptide. Based on our present study, glucose and yeast extract were identified as best carbon and nitrogen source respectively. The results from this study showed that the biosurfactant properties of *Bacillus* sp may have potential environmental.

Key words: Biosurfactant, Oil contaminated soil, Oil spread test, Emulsion index and Lipopeptide.

1. INTRODUCTION

Surfactants are amphiphilic compounds containing both hydrophobic and hydrophilic moieties, thus conferring them the ability to accumulate between fluid phases, such as oil/water or air/water, reducing the surface and interfacial tensions and forming emulsions [1]. Surfactants are classified according to their ionic properties in water (anionic, non-ionic, cationic and amphoteric) and are used in a great variety of applications for instance house- hold detergents, food processing and industrial cleaners, cosmetics and personal care products, microbial enhanced oil recovery, remediation and bioremediation [2].

Biosurfactants are a structurally diverse group of surface-active molecules mainly synthesized by microorganisms [3, 4]. Microbial biosurfactants include a wide variety of compounds, such as glycolipids, lipopeptides (LPs), polysaccharide-protein complexes, phospholipids, fatty acids, and neutral lipids. They are usually produced extracellularly or as part of cell membrane by bacteria, filamentous fungi and yeast [5]. Different kinds of bacteria have been employed by many researchers in producing biosurfactants using culture media. Most of such bacteria are isolated from contaminated sites usually containing petroleum hydrocarbon by products and/or industrial wastes [6].

Interest in microbial surfactants has been steadily increasing in recent years, as they have numerous advantages compared to chemical surfactants including a lower toxicity, higher biodegradability [7, 8], higher foaming, better environmental compatibility [9, 10] and effective properties at extreme temperature, pH levels and salinity [11, 12].

Bacteria are the main group of biosurfactant-producing microorganisms, although it is also produced by some yeast and filamentous fungi [13]. A number of studies have reported the potential of *Bacillus* species as biosurfactant producers and they produce lipopeptide type of biosurfactant [14, 15]. The objective of the present study is to screen the biosurfactant producing microorganism from oil contaminated soil, its identification, and screening, determining the effects of carbon and

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nitrogen sources on biosurfactant production and characterization of biosurfactant using FT-IR.

2. MATERIALS AND METHODS

Sampling area and Sampling

For isolation of biosurfactant producing bacteria, oil contaminated soil sample was collected from automobile workshop at Chidambaram, Tamilnadu, India. A total 250 g soil sample was collected and stored in sterile polythene bags.

Enrichment, isolation and identification of isolate

A few grams of the soil sample was transferred to 100ml of Mineral salt medium (MSM) in 250ml Erlenmeyer flask with 2% (vv^{-1}) of diesel oil as carbon source. The flasks were incubated at 30°C on a rotatory shaker at 200rpm for 4days. After 4days 1.0ml of culture was transferred to fresh media containing diesel oil and reincubated for another 4 days. After five cycles of such enrichment, 1 ml of the culture was diluted and plated on MSM agar plates containing diesel oil (2% vv^{-1}) as sole carbon source and incubated at 30°C. The single colony was streaked onto nutrient agar plates. The composition of the mineral medium used was as follows (g/L):5 g $NaNO_3$, 2 g KH_2PO_4, 1.5 g K_2HPO_4, 0.2 g $MgSO_4{\cdot}7H_2O$, 0.02 g $CaCl_2$, and 0.024 g $FeSO_4{\cdot}7H_2O$ and supplemented with 1 ml trace element solution containing (L-1): 0.75 g $ZnSO_4{\cdot}7H_2O$, 0.08 g $COCl_2{\cdot}6H_2O$, 0.075 g $CuSO_4{\cdot}5H_2O$, 0.5 g $MnSO_4{\cdot}H_2O$, 0.15 g H_3BO_3, and 0.06 g $NaMoO_4{\cdot}2H_2O$.Control and replica plates were maintained. The isolated microorganism was identified by Gram staining and Biochemical tests[16].

Rapid test for screening of biosurfactant

Simple and effective rapid test, used for screening of biosurfactant are haemolytic activity, drop collapse test, oil displacement test. These tests are very reliable for screening of biosurfactant.

Haemolytic activity (HA)

Bacterial cultures were streaked on blood agar plates supplemented with 5% fresh human blood and incubated at 28ºC for 48-72hrs. Observation was made for alpha, beta and gamma haemolysis. Haemolytic activity was correlated with the production of biosurfactant [17].

Drop collapse test

The drop collapse technique was carried out following indications described in the literature for qualitative and quantitative assays. A modified drop collapse method was carried out using 96 well microtiter plates containing 100 µl mineral oil which was equilibrated for an hour at room temperature. Five microliter bacterial culture was grown in MSM agar at 28ºC under 200rpm for 24hrs.The inoculum were added individually to the centre of the 96 well microtitre plates. The biosurfactant procedures were detected from the drop collapsing within a minute from the oil coated well [18].

Oil displacement test:

Twenty four hour old inoculum grown in nutrient broth was used for this analysis. Petriplate base was filled with 50ml of distilled water. On this water, twenty microliter of diesel oil was layered uniformly. Further, ten microliter of culture was added at different spots on the diesel oil which is coated on the water surface. Occurrence of the clear zone was indication of biosurfactant producers [19].

Emulsification index (E_{24})

E_{24} of culture samples was determined by adding 2 ml of a hydrocarbon (diesel oil) to the same amount of culture, mixing with a vortex for 2 min, and leaving to stand for 24 hours. The E_{24} index is given as percentage of height of emulsified layer (mm) divided by total height of the liquid column (mm) [20].

$$Emusificationindex\ (E_{24}) = \frac{h_{emulsion}}{h_{total}} \times 100$$

Foaming and emulsifying properties

The ability of the biosurfactant to emulsify some hydrocarbon and oil such as Ground nut oil, kerosene, sunflower oil and coconut oil was determined. The sterile biosurfactant (2 ml) was added into each test tube (in a set of three) containing the substrate (2 ml). The content of the tubes were vortexed at high speed for 2 min and left undisturbed for 24 h. The emulsion index (E_{24}) was determined as the height of the emulsion layer divided by the total height and multiplied by 100.

Effect of carbon and nitrogen sources on biosurfactant production

The carbon source and nitrogen source are some of the factors which influence the biosurfactant production. In order to increase the biosurfactant yield by the isolate, different carbon and nitrogen sources were evaluated for biosurfactant production The carbon sources used in the present study were glucose, sucrose, glycerol, mannitol and starch (1%) and yeast extract, peptone, ammonium chloride, urea, sodium nitrate (0.3%) were the nitrogen sources. The flasks were maintained at 30ºC in an incubator shaker at 150

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rpm. A control flask without carbon/ nitrogen source was also maintained.

Surface tension determination:

The culture was inoculated in the MSM and incubated for 24 hrs. The cells were centrifuged at 10000 rpm for 25 min and the cell free supernatant was used for surface tension measurement. The surface tension was measured using Du-nouy ring tensiometer. (Kruss, GmbH, Germany). The distilled water and medium was used as negative control and for positive control tween-20 was used. The value was repeatedly taken thrice and average value was used to express the surface tension of the sample

Extraction of biosurfactant:

Biosurfactant was extracted from the whole cell-free culture broth. The bacterial cells were removed by centrifugation at 5000rpm, 4ºC for 30 minutes. The supernatant was adjusted to pH 2 using 1M sulphuric acid, prior to biosurfactant extraction using equal volume of chloroform-methanol (2:1). The mixture was shaken for three hours at 30ºC and 200 rpm. The organic phase was separated using separating funnel. The crude biosurfactant was concentrated using rotary evaporator at 60-70ºC.

Fourier transform infrared spectroscopy (FT-IR)

Fourier transform infrared spectroscopy (FT-IR) is most useful for identifying types of chemical bonds (functional groups), therefore can be used to elucidate some components of an unknown mixture. One milligram of freeze-dried partially purified biosurfactant was ground with 100mg of KBr and pressed with 7500 kg for 30 seconds to obtain translucent pellets. Infrared absorption spectra were recorded on a AVATAR-NICOLAT FTIR system with a spectral resolution and wave number accuracy of 4 and 0.01cm^{-1}, respectively. All measurements consisted of 500 scans, and a KBr pellet was used as background reference.

3. RESULTS AND DISCUSSION

The oil spill contaminated soil was used to isolate the biosurfactant producing microorganisms. A total fifteen colony were isolated. Among the obtained isolates, *Bacillus* sp. (Fig.1) was selected for further studies. The characteristic of the isolate showed an gram positive, rods and the biochemical tests (**Table 1**) was carried out according to Bergey's manual [16] clearly seemed to be *Bacillus* species.

Simple and rapid tests had been successfully used for the screening of potential biosurfactant producer. Bacillus sp was found to be the best candidate for the study of biosurfactant production. The strain showed β-hemolysis. The drop collapse test and oil displace test are indicative of the surface wetting activity[21]. Drop collapse and oil displacement test showed positive result. Emulsification index is one of the criteria to support the selection of potential biosurfactant producers. Emulsion index (E24) determine productivity of bioemulsifier. The emulsion activity was observed to be 76%.This observation is important to suggest that potent biosurfactant producing culture can be detected through such assays. The emulsification activity of the culture showing >30%, indicates high emulsion activity [29].Aqueous solutions of biosurfactant showed good foaming stability. Total disappearance of the foam was detected after 2 hours. The emulsification activity of biosurfactant against different substrate is shown in (**Table 2**). Coconut oil and kerosene were the best substrates and sunflower oil was least for emulsification activity.

Table - 1: Biochemical characteristics of *Bacillus* sp.

S. No	Test	Result
1	Indole test	Negative
2	Methyl red test	Negative
3	VogesProskauer Test	Negative
4	Citrate utilization	Negative
5	Casein Hydrolysis	Positive
6	Starch Hydrolysis	Positive
7	Glucose fermentation	Positive
8	Fructose fermentation	Positive
9	Nitrate Reduction	Positive
10	Catalase test	Positive

Table 2: Emulsification activity (E_{24}) of biosurfactant against different substrates

Substrate	Ground nut oil	Kerosene	Sunflower oil	Coconut oil
E_{24} (%)	50	55	45	67

Effect of carbon and nitrogen sources on biosurfactant production

Most of researchers reported that the quality and quantity of biosurfactant production are affected and influenced by the nature of the carbon substrate. The carbon source influences the biosurfactant synthesis either by induction or repression. Medium constituents other than carbon source also affect the production of biosurfactant. The carbon source generally used could be divided into three categories namely; carbohydrates, hydrocarbons and vegetable oils. Indeed the type, quality and quantity of biosurfactant production are affected and influenced by the nature of the carbon substrate [22, 23]. All the carbon sources tested favored extracellular production of surface active product by *Bacillus* sp, which was indicated by the

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reduction in surface tension of the broth. In the present study, glucose, sucrose, glycerol, mannitol and starch was used as carbon source. Among these glucose increased the production (2.11 g/L) significantly compared to other carbon sources (Fig.3). Studies by various researchers have revealed that the presence of glucose in the production medium increased the biosurfactant production in the culture medium [24, 25]. Least biosurfactant production (1.04g/L) was observed when starch was used as the carbon source and the culture free broth showed surface tension value of 32.81 mN/m (Fig.4). Nitrogen can be an important key to the regulation of biosurfactant synthesis, and there is evidence that the nitrogen plays an important (definite) role in the production of surface-active compounds by microbes [26, 27]. Growth conditions such as temperature, agitation and oxygen availability, and environmental factors also affect biosurfactant production through their effect on cellular growth or activity [27, 28]. Yeast extract, peptone, ammonium chloride, urea, sodium nitrate (0.3%) were used as nitrogen sources and among these yeast extract (Fig.6) enhanced the biosurfactant production (2.41 g/L)The cell free culture broth showed a surface tension value of 29.48 mN/m(Fig.5). In media amended with urea, least biosurfactant production (1.19 g/L) by *Bacillus* sp.was observed. Thus, optimization of carbon and nitrogen sources enhanced the biosurfactant production by *Bacillus* sp.

Fig 1: The colony morphology of *Bacillus* on Nutrient agar

Fig 2: FT-IR of lipopeptide biosurfactant produced by *Bacillus* sp.

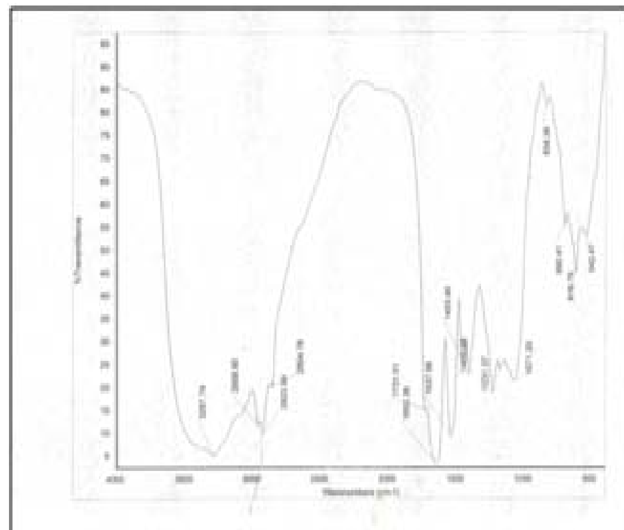

Fig 3: Effect of different carbon source on biosurfactant production by *Bacillus* sp.

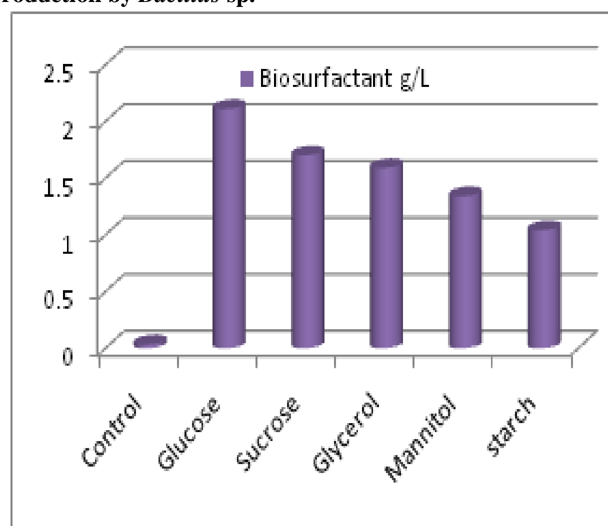

Fig 4: Effect of carbon source on surface tension of *Bacillus* sp.

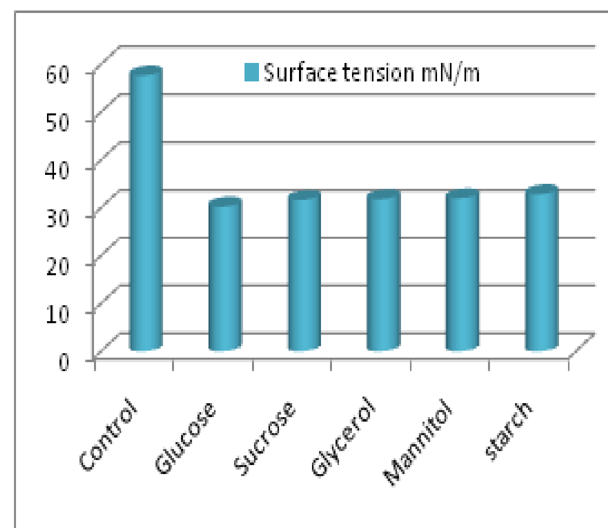

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Fig 5: Effect of different nitrogen source on surface tension of *Bacillus* sp.

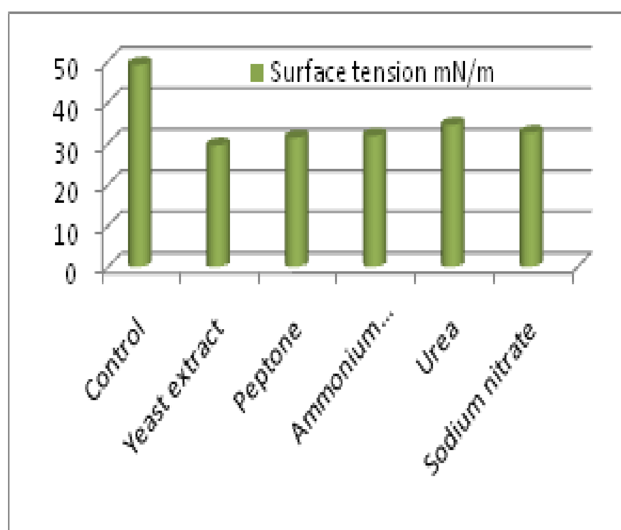
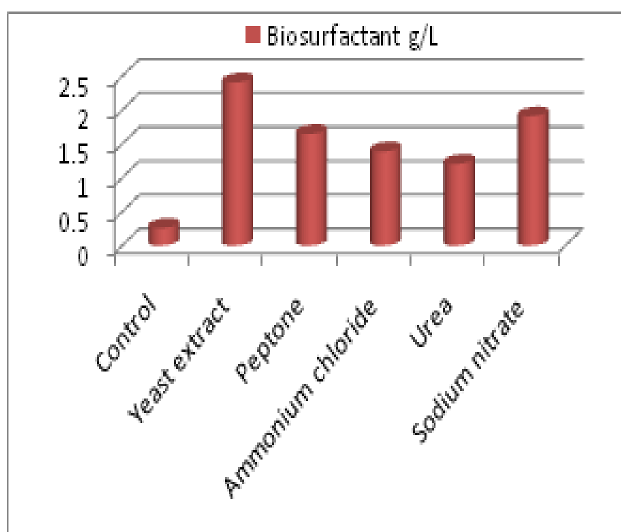


Fig 6: Effect of nitrogen source on production of biosurfactant by *Bacillus* sp.



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Fourier transform infrared spectroscopy (FT-IR)

The nature of biosurfactant as lipopeptide was further confirmed by FT-IRanalysis .The FT-IR spectrum (Fig.2) showed strong adsorption bands of peptide at 3287cm^{-1}, 1652 cm^{-1}and 1537 cm^{-1} These band resulted from the stretching mode of N-H ,stretching mode of the C=O bond and the deformation mode(combined C-N stretch mode) of the N-H bond, respectively. The band 2923cm^{-1},1453cm^{-1} reflect aliphatic chains(-CH_3,-CH_2)of the sample.1731 – 1652cm^{-1} due to lactone carbonyl adsorption.

4. CONCLUSION

The results indicated that *Bacillus* sp is one of the potent biosurfactant producer. 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, yeast extract 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. FT-IR spectrum confirmed the biosurfactant as lipopeptide. Biosurfactant produced by *Bacillus* sp were screened by various tests such as haemolytic activity, drop collapse, oil displacement test and emulsification index confirmed that the compound can be used in various fields such as environmental, agriculture, food and pharmaceutical industry.

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