

Exopolysaccharides of Marine Cyanobacterium *Nostoc* Species as a Biosurfactant

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ABSTRACT: Offshore oil spills are a common occurrence in oceans. Approximately 706 million gallons of waste oil is spilled each year. Cyanobacterial cultures are available among which *Nostoc* sp. proves to fix more atmospheric nitrogen yielding more Exo Polysaccharides (EPS) and can serve as an effective biosurfactant. This economic-friendly cyanobacterium can be easily produced in large quantities. *Nostoc*, found in various environments forms colonies composed of filaments in a gelatinous sheath. Interestingly, biopolymers of marine cyanobacterium contain high concentrations of charged components, notably uronic acids useful for bioremediation purposes. FT-IR analysis of *Nostoc* exhibited presence of more C and O thereby confirmed the presence of glycolipids and therefore reducing the surface tension. In this paper, emulsification index of different oils were studied and the order of emulsification was found to be Gingelly oil > Crude oil > Neem oil > Coconut oil > Brake oil > Petrol > Diesel oil. Thus, the extracted EPS can serve as an efficient biosurfactant for oil-spill cleaning and are eco-friendly too which stands as a natural remediator better than the available commercial counterparts.

KEYWORDS: Cyanobacteria, biosurfactant, emulsification, FTIR, bioremediation, Exo Polysaccharides.

I. INTRODUCTION

In recent years, oil contamination and heavy metals pollution are found to be a serious environmental issue and various technologies are being discovered for their cleanup from environment. The use of biosurfactant for this purpose was found to be an eco-friendly approach and also an alternate to conventional complex remediation systems. Due to their diversity, biosurfactant is considered as a potential candidate for the environmental cleanup of pollutants. Therefore, a greater attention was paid on biosurfactant and identifying their potential applications. Biosurfactants (BS) are amphiphilic compounds produced on living surfaces, mostly microbial cell surfaces, or excreted extracellular and contain hydrophobic and hydrophilic moieties that reduce surface tension (ST) and interfacial tensions between individual molecules at the surface and interface, respectively. Many properties of microbial surface active compounds such as emulsification/de-emulsification, dispersion, foaming, wetting and coating make them useful in physico-chemical and biological remediation technologies of both organic and metal contaminants. Algal and microbial biosurfactant /bioemulsifier from marine and deep-sea environments are attracting major interest due to their structural and functional diversity as molecules used for food processing, pharmacology, and bioremediation of oil-polluted environments [16].

In this present study, the biosurfactant activity of cyanobacterial EPS was confirmed with the emulsification indices obtained for various oils and hydrocarbons.

II. LITERATURE STUDY

Biosurfactants, a group of surface active molecules are synthesized by diverse microorganisms, can reduce both surface and intersurface tension, making these molecules useful in emulsification processes. The use of biosurfactants has been found to enhance the degradation of crude oil and other hydrocarbons [2]. Biosurfactants are microbial surface active agents produced by certain microorganisms during their growth phase [28]. They may be extracellular or

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intracellular in nature [7]. Substrates for biosurfactant production are sugars, oils, alkanes and waste materials [25]. Biosurfactants are amphiphilic, non-toxic and biodegradable molecules with high specificity ([28], [8]) and are highly stable at extremities of temperature, pH and salt concentration ([11], [12]). These molecules have the ability to decrease the surface tension, critical micelle concentration and interfacial tension [1]. Biosurfactants have different chemical structures, compositions and a wide range of biotechnological applications in agriculture, health care [19] dairy, food, beverage, cosmetics, detergent, textile, paint, mining, petroleum, paper pulp and pharmaceutical industries ([23], [5], [24], [27]). Biosurfactants are thus used as an alternative for chemical surfactants.

Cyanobacteria as a potential candidate for exopolysaccharide production

Cyanobacteria (blue green algae) are a group of Gram-negative prokaryotes, photosynthetic organisms which are unicellular or filamentous and can fix their own nitrogen [6]. Owing to their ecological and biochemical diversity, cyanobacteria, as well as several species of microalgae, have been regarded as good candidates for various biotechnological applications [4]. They secrete complex polymers containing acetylated amino sugar moieties, as well as non-carbohydrate constituents. It has been reported that cyanobacterial EPS are not only composed of carbohydrates but also of other macromolecules such as polypeptides [17]. In general, the chemical composition, the type and the amount of the exopolysaccharides produced by a given cyanobacterial strain are stable features, mostly depending on the species and the cultivation conditions [21]. However, the sugar composition of the EPS produced by a certain strain may, qualitatively and quantitatively, vary slightly, especially with the age of the culture ([13], [10]).

III. MATERIALS AND METHODS

1. Source of sample

The marine cyanobacterial strain *Nostoc* was obtained from the repository of National facility for Marine Cyanobacteria (NFMCC), Bharathidasan University, Tiruchirapalli, Tamilnadu, India for the present study. The organism was observed under microscope to identify the morphological features.

2. Media for maintenance and routine culturing

As the selected organism is capable of developing heterocystous forms, it was maintained in ASN III nitrogen free medium at $27 \pm 2^\circ\text{C}$ under white light of about $200 \mu\text{mol m}^{-2}\text{s}^{-1}$ light intensity. Routine subculturing was done once in 7 days. Nitrogen starvation has often been described as a condition that enhances EPS synthesis ([9], [22]), probably because this contributes to the increase in the C : N ratio, thus promoting the incorporation of carbon into polymers ([22], [20]).

3. Extraction of polysaccharides [18]

3a. Preparation of culture filtrate

30 days old culture of *Nostoc* was centrifuged at 7500rpm for 15 minutes and the supernatant was taken.

3b. Precipitation and purification of eps [18]

Cells were separated from the growth medium by centrifugation at 7000g at 4°C for 15 minutes. The supernatant was collected. The polysaccharide was extracted by precipitating it using 3 volumes of cold ethanol to the supernatant and kept at 4°C overnight. The precipitated EPS was further purified by dialysis against distilled water overnight at 4°C and freeze dried. The dried EPS is then lyophilised, weighed and stored at 4°C .

4. Fourier Transform Infrared Spectroscopy (FT-IR) analysis

The FT-IR spectra were measured using Perkin-Elmer, Spectrum RX1, Boston, USA instrument. The completely dried EPS of 30 days old potent culture was pressed into KBr pellets at a ratio of 1:100. The spectra were recorded in transmittance mode over the wave range of $4000\text{-}400\text{cm}^{-1}$.

5. Emulsification Assay

The emulsifying capacity of organism was evaluated by an emulsification index test. Emulsification index was determined by adding 1ml of oil, 1ml of water and 10 mg of EPS in test tube, and vortexed at high speed for 2 min and allowed to stand for 120 h. The emulsification index was measured in percentage which is calculated as, height of emulsified layer (cm) divided by the total height of the liquid column (cm).

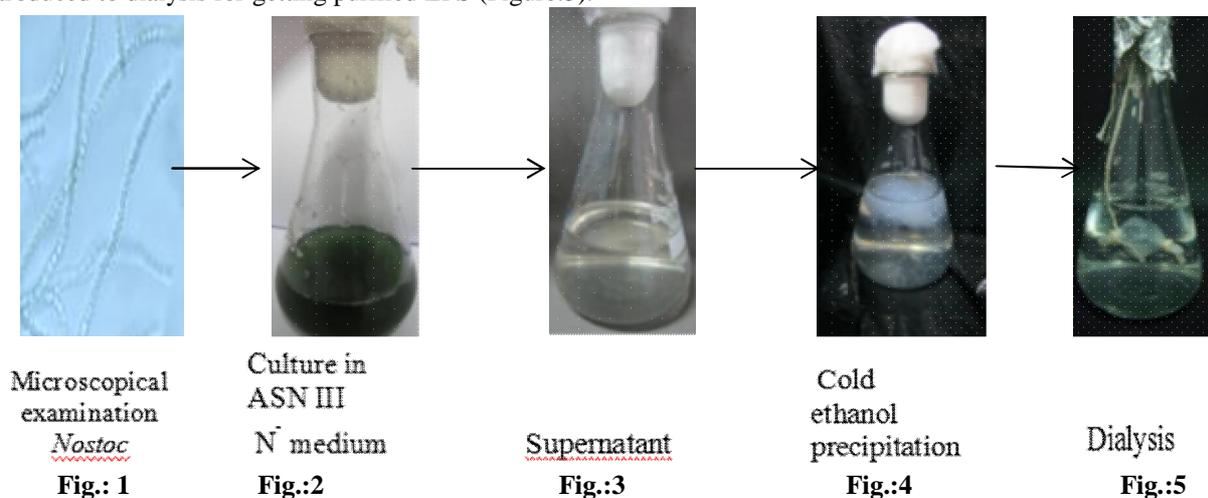
This assay was performed for the oils namely, coconut oil, gingelly oil, diesel oil, petrol, brake oil, neem oil and crude oil.

$$EI_{120} = \frac{\text{Height of emulsion formed}}{\text{Total height}} \times 100$$

Control used for emulsification index test was 1ml of water and 1ml of the all the test oils individually.

IV. EXPERIMENTAL RESULTS AND DISCUSSION

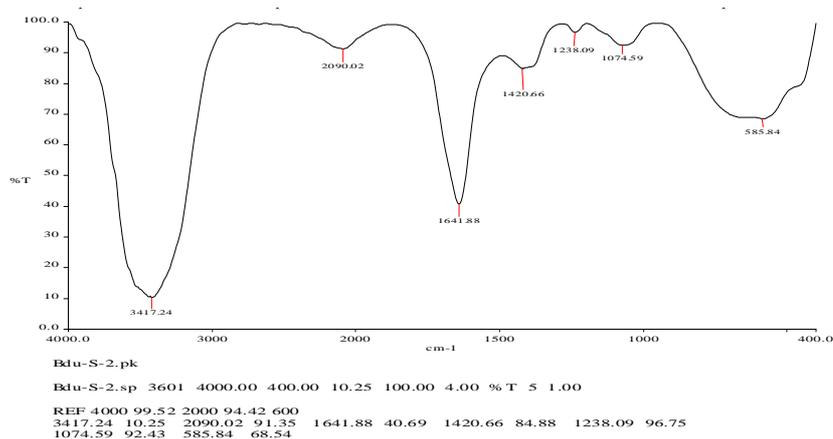
Cyanobacteria was obtained from NFMC, Bharathidasan University, Tiruchirapalli, TamilNadu. This organism was grown in ASN III N- medium and was then undergone microscopically examination (Figure:1). After thirty days of incubation (Figure:2), the culture was centrifuged and its supernatant was collected (Figure:3). The collected supernatant was poured in cold ethanol. EPS was then obtained as a precipitate (Figure:4). The EPS obtained was then introduced to dialysis for getting purified EPS (Figure:5).



Overall steps' starting from microscopical examination till purified EPS is obtained

A) Characterization of biosurfactant using FT-IR

The spectrum obtained (Graph 1) is based on the variation in stretching and bending modes of variation with single functional group that is normally coupled with the variation of adjacent group. This leads to the shifting or overlapping of the peaks of two or more functional group in the same region of IR spectrum [26].



Graph 1 : FT-IR spectrum of EPS

The IR spectrum of EPS obtained from *Nostoc* is shown in Graph 1. The spectrum predicted that the peak centered in the range of 3400-3900 cm^{-1} picturize the strong O-H stretch with weak NH^+ groups. A deeper stretch was observed at 1642 cm^{-1} which definitely denotes for the presence of C=O group. Besides this, an additional absorption at 1131.25 cm^{-1} indicates to the presence of dialkyl/ arylsulfones/ sulfate group. The other moderate stretch at 1392 cm^{-1} and 615.37 cm^{-1} correspond to C-H bending and C-N Stretching respectively. All these spectral observations clearly characterize the functional groups (O-H, C-O, N-H, S=O, C-H and C-N) of EPS. The percentage of carbon and oxygen is higher leads to the understanding that EPS contains glycolipids[15].described the production of crude biosurfactant by *S. thermophilus* B as mixtures of various components, with a glycolipid-like component being the most surface active.

B) Emulsification activity of biosurfactant

Hydrophobic pollutants present in petroleum hydrocarbons, and soil and water environment require solubilization before being degraded by microbial cells. Mineralization is governed by desorption of hydrocarbons from soil. Biosurfactant can increase the surface area of hydrophobic materials, such as pesticides in soil and water environment, thereby increasing their water solubility. Hence, the presence of surfactants may increase microbial degradation of pollutants. Use of biosurfactant for degradation of pesticides in soil and water environment has gained importance only recently. Here the Biosurfactant that is being extracted from Cyanobacteria Is being tested on various oils namely Coconut oil, Brake oil, Petrol, Diesel oil, Gingelly oil, crude oil and neem oil. After 5 days of incubation we were able to observe a stable, clear emulsified layer as viewed in Fig.6- Fig.12 . Some commercial emulsifiers, particularly those with fatty acid components, tend to form clumpy solution that limits their applications [14]. From the EPS analysis we were able to find the uronic acid was 9.38 micro gram/mg of EPS, and the presence of sulphate groups and C=O groups that are evident from FTIR analysis in the *Nostoc* Species Extra cellular polysaccharide played a very vital role of the Biosurfactant activity apart from functional groups(acetyl) present in EPS, which also provide hydrophobicity, imparting enhanced emulsifying activity [3]. The extracted biosurfactants has sulphate, acetyl and carboxyl functional groups and presence of these in the biopolymer proved its anionic nature which is useful for binding of cations and thus for its remediation. The extracted biosurfactant efficiently emulsified aliphatic, aromatic hydrocarbons and oils. It had a vital property of dissolving readily in water.

The stable emulsifying activity makes this biosurfactant as an alternative to the commercially available chemical surfactants/emulsifiers thus making this biosurfactant a promising candidate for biotechnological and industrial applications. The height of emulsified layer was noted on the fifth day after incubation. The emulsification activity was calculated and the results obtained were shown below in Table 1. The order of emulsifying activity was observed to be

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Gingelly oil > Crude oil > Neem oil > Coconut oil > Brake oil > Petrol > Diesel oil

Oils	Emulsification index (5 th day) in %
Coconut oil	28.57
Brake oil	26.7
Petrol	25
Diesel	21.4
Gingelly oil	37.5
Crude oil	36.8
Neem oil	31.25

Table 1: Emulsification activity of biosurfactant (10 mg EPS / ml oil / ml water) with various hydrocarbons and oils

The EPS added has a property of forming an emulsified layer between the oil and water interface and this can be viewed in all the figures.

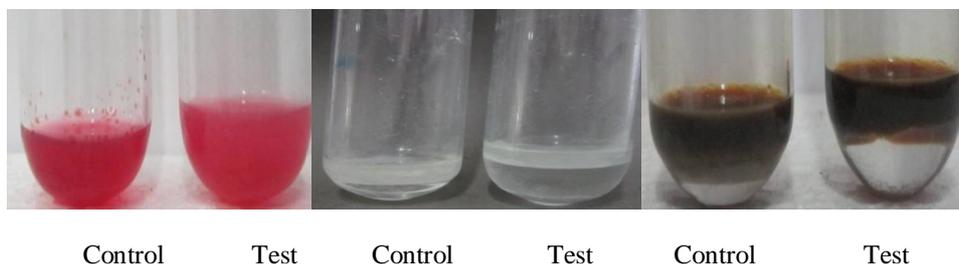


Fig. 6: BRAKE OIL Fig.: 7 COCONUT OIL Fig. 8: CRUDE OIL

Fig. 6 shows the control and test in which the test had 10 mg EPS mixed with 1 ml water and 1 ml brake oil. After 5 days of incubation, the emulsification index was calculated to be 26.7 %. Similarly, fig. 7 and fig. 8 shows the test tubes containing coconut oil and crude oil and the emulsification index was found to be 28.57% and 36.8% .

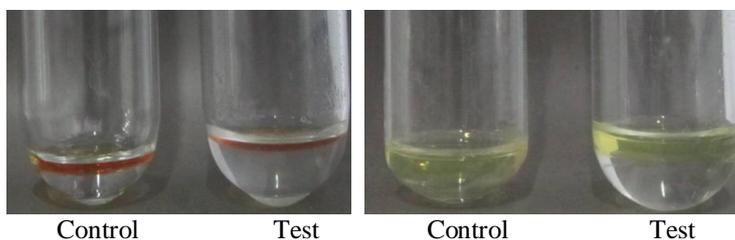


Fig. :9 PETROL Fig.:10 DIESEL

Fig. 9 and fig. 10 shows the control and test in which the test had 10 mg EPS mixed with 1 ml water and 1 ml petrol and 1 ml diesel respectively. After 5 days of incubation, the emulsification index was calculated and found to be 25% and 21.4 % respectively.

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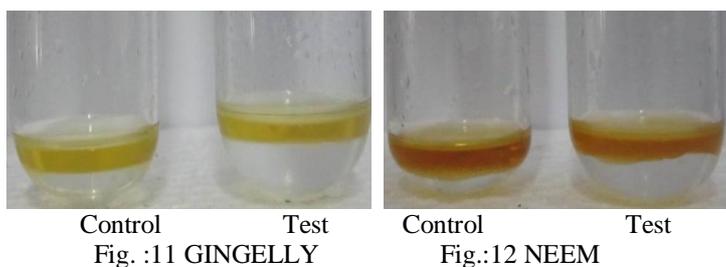


Fig. 11 and fig. 12 shows the control and test in which the test had 10 mg EPS mixed with 1 ml water and 1 ml gingelly and 1 ml neem oil respectively. Upon 5 days of incubation, the emulsification index was calculated and found to be 37.5% and 31.25 % respectively.

Figure 6, 7, 8, 9, 10, 11, 12 shows the emulsification layer of different oils and hydrocarbons

These results prove that the gingelly oil can be broken up by microbes after solubilization by these surfactants than the diesel oil. The structure of diesel oil being more complicated doesn't get solubilised easily and hence does require more amount of surfactant activity before the biological degradation could happen. In addition, we found that strains that produce high levels of biosurfactant can better degrade crude oil.

V. CONCLUSION

The exopolysaccharide extracted from the cyanobacterium *Nostoc* has sulphate as observed from FTIR analysis and so can be effectively used as biosurfactant and emulsifier thereby replacing the commercially available compounds. As *Nostoc* doesn't require much of controlled environment and needs less labour, they can be cheaper means of cultivation and harvesting EPS.

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