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Isolation, Preliminary Screening and Process Optimization for Production of Surface Active Agent from Chlorella Pyrenoidosa by Non-Disruptive Method

Microbiology

KEYWORDS : Biosurfactant; surface tension; oil spreading; emulsification index

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ABSTRACT

Biosurfactants are structurally diverse group of surface active molecules, synthesized by microorganisms. In this research work algae are isolated from different natural aquatic habitats and identified for biosurfactant production. Genus Chlorella was one of the most promising identified algae. Pure strain of Chlorella pyrenoidosa NCIM No. 2738 was collected and ability of biosurfactant production was identified by performing different primary confirmation tests. Optimization of biosurfactant production was done which shown that carbon, nitrogen sources and PH affects on biosurfactant production. Partial extraction and purification was done by Silica gel Chromatography. Product was chemically analyzed by thin layer chromatography where presence of carbohydrates and lipids were confirmed by Rf value 0.75 and 0.7 for lipids after brown spot development when exposed to iodine. Thus, primary structure of biosurfactant was confirmed in the form of glycolipids but it is necessary to characterize this product on different levels of identification.

Introduction:

Many organic surfactants are released from algae and plants, and decomposed when they die. More recently, algae have received wide attention and popularity as they have the potential of high yield of oil that is used as a feedstock for synthesis of biodiesel but not on role of biosurfactant production for their different class of lipids. Lipids obtained from microalgae can be categorized in three parts such as crude lipids, neutral lipids and total lipids. Crude lipids include neutral lipids and pigments. Neutral lipids are comprised of triglycerides, free fatty acids, hydrocarbons, sterols, wax and sterol esters and free alcohols. Total lipids comprises of pigments, phospholipids, glycolipids and the neutral lipids (Bhaskar, Yogesh Sharma, & John Korstad, 2011).

Biosurfactants are active compounds produced by microorganisms. These molecules reduce surface tension between aqueous solution and hydrocarbons mixture, stabilizing emulsions, promoting foaming and are generally non toxic and biodegradable. These biosurfactants are amphiphilic molecules consisting of hydrophobic and hydrophilic domains that find in a wide variety of industrial processes. They are used in the industries of cosmetics, food, pharmaceutics, agriculture, enhanced oil recovery and bioremediation of oil contaminated site. Basically, biosurfactants are mainly five types, glycolipids, lipopeptides or lipoproteins, polymeric surfactants, & Particulate surfactants (Desai & Patel, 1997).

Glycolipids constitute an important class of membrane lipids that are synthesized by both prokaryotic and eukaryotic organisms. A variety of methods for the screening of biosurfactant producing microbes has been developed and successfully applied. An efficient screening strategy is the key to success in isolating new and interesting microbes or their variants, because a large number of strains need to be characterized. With this all it is also true that the number of factors influences the biosurfactant production (Desai & Banat, 1997) are carbon source, nitrogen source & other environmental factors like temperature, pH, etc.

The aim of present work is to isolation and identification of biosurfactant producing algae and characterization of class of biosurfactant with process optimization to get more surfactant from algae.

Materials and Method:

1. Collection and Isolation of Algal species for biosurfactant production

1.1 Field Collection

Four different algal samples were collected from natural aquatic habitats and after collection samples were stored in 150 ml plastic bottles and all samples were examined microscopically. Filaments of such material were taken on slide, teased it and separated them under stereo binocular dissecting microscope with the help of platinum needle (Padovan, 1992). Fragments or filaments are cleaned by sterile distilled water. With the help of another sterile platinum needle, cells are picked and then transferred to the sterile 500 ml algal medium and placed in incubator.

1.2 Separation and identification of algae

All samples were filtered through a 200 micrometer net to remove zooplanktons and large foreign particles again filtered through the series of the net to remove phytoplanktons. Cells retained at each stage were back washed with the same medium. The samples were allowed to stand overnight in a 100 ml measuring flask and the buoyant cells were gently skimmed off the surface before passing the sample through the net series. To ensure algae from each fraction 1ml aliquot were transferred to the sterile 250 ml flask containing 50 ml algal medium. All fractions examined microscopically and suitable used for streaking on the agar and plates were incubated in the presence of light source for week by taking green colony transformed on the slide and by taking drop of water and using cover slip it was observed under compound microscope. It was showing some bacterial contamination so to avoid that the culture is again restricted to reduce bacterial contamination (Padovan, 1992). Finally thallus was identified (Sharma, 1996) for four different cultures of algae and each of were transferred in the sterile liquid medium. All samples were grown in sterile algal broth aseptically providing 10 Klux light intensity for 24 hours. After 15 days of incubation period, biomass was measured by optical density and cell count was done.

1.3 Selection of biosurfactant producing algae

Thus, for each sample the growth was detected and these cultures were studied for biosurfactant production by using various preliminary identification methods. Chlorella was one of the most promising algae out of those four samples of algae for biosurfactant production.

2. Collection of pure culture and maintenance

Chlorella pyrenoidosa NCIM No. 2738 collected from National Chemical Laboratory, Pune. Algal cells were inoculated on agar slants of defined Fog's media and placed at room temperature under 10 Klux light intensity for 15 days at the P^H 7.5, providing light for 24 hours. Thereafter, the well grown slant cultures were inoculated in to the liquid medium.

2.1 Determination of algal growth

A fixed volume of cell medium was taken for both cultures in a haematocrit tubes and centrifuged at 10,000 rpm for 15 minutes. Cell free broth (Supernatant) was collected and used for further studies (Desai & Banat, 1997).

3. Primary confirmation test for biosurfactant production-

3.1Surface/interfacial activity
The majority of screening methods for biosurfactant producing microorganisms are based on the interfacial or surface activity. Various methods have been developed for measuring this property but the method selected for screening of biosurfactant producing microbes is Stalagmometric method and Du-ring surface tensiometer.

3.1.1 Stalagmometric method
Surface tension was measured by Stalagmometric method (Aparna, Smitha & Srinikethan, 2011), where stalagmometer was washed two times with distilled water and acetone to remove impurities. Distilled water was sucked and tightens the screw. Number of drops was adjusted in such a way that 10-15 drops per minute. The level A to B of stalagmometer was adjusted for distilled water and the number of drops was recorded from level A to B. Similarly, the number of drops were taken for the samples of 1, 2, 4, 6 , 8 and 10 % of concentration respectively, from the level A to B. Density was measured in gm/ml and the surface tension was measured by using following formula,

Measurement of surface tension in dyne/cm[5,]

$y_1 / y_2 = \sigma_1 n_2 / \sigma_2 n_1$ Where, y_1 = Surface tension of water, y_2 = Surface tension of sample, σ_1 = Density of water, σ_2 = Density of sample, n_1 = Number of drops of water, n_2 = Number of drops of sample. The surface tension can be determined on the basis of the number of drops which fall per volume, the density of the sample and the surface tension of a reference liquid, e.g., water. The surface tension is given by following formula,

$$y_2 = \frac{y_1 \times \sigma_2 \times n_1}{\sigma_1 \times n_2}$$

Whereas, σ_1 is the density of water and σ_2 is the density of sample. The surface tension of water is denoted by y_1 and y_2 indicate the surface tension of sample. Here n_1 is the number of drops of water and n_2 indicate number of drops of sample respectively.

3.1.2 Du- ring surface tensiometer
Surface tension was measured according to the Du Nouy ring (Desai et al., 1997) method using surface tensiometer (Jenson Company, India). The tensiometer was calibrated first and ring was cleaned with benzene at low heating for each measurement. The ring was introduced in 50 ml of cell free culture broth applying an ascending force until the ring was pulled out from the culture broth, and the surface tension was recorded from the graduated dial.

3.2 Drop collapse assay
The algal culture was checked for surfactant production by drop collapse method. In this assay the supernatant (cell free broth) was used. Drop of 25 µl was placed on a hydrophobic surface of Para film paper and the shape of drop on the surface was inspected after 1 minute. The diameter of droplets was evaluated. Distilled water used as a negative control and sodium lauryl sulfate used as a positive control, respectively (Noha & Youssef, 2004).

3.3 Oil spreading test
The selected strain was compared by measuring of the diameter of clear zones occurred when a drop of a surfactant containing solution (cell free broth) is placed on an oil-water surface. The 50 ml of distilled water was added to a large Petri dish (9.7 cm diameter) followed by the addition of 20 µl of crude oil to the surface of water, 10 µl of supernatant of culture broth (Leelapornpisid, Lumyong, Santiarwan, & Techaoei, 2007) and the diameter of zones of triplicate assays from the same sample was determined.

3.4 Emulsification index (E_{24})
This is the process in which emulsifying capacity was evaluated by an emulsification index (E_{24}). The emulsification index of culture sample was determined by adding 2 ml of kerosene and 2 ml of cell free broth in test tube (Leelapornpisid et al., 2007). The test tube vortexed at high speed for 2 minutes and allowed to stand for 24 hours. The E_{24} index is given as percentage of the height of emulsified layer (cm). The percentage of emulsification index calculated by using following formula,

$$E_{24} = \frac{\text{Height of Emulsion formed}}{\text{Total height of solution}} \times 100$$

3.5 CTAB methylene blue agar plate assay
Mineral salt Cetyl trimethyl ammonium bromide (CTAB) methylene agar assay plate was prepared (CTAB 0.2 mg/ml and MB 5 µg/ml) and shallow wells were cut in to the surface of indicator plate. 10 µl samples were placed into the well & a plate was incubated at 30°C for overnight for the formation of dark blue halos around the sample (Siegmund & Wagner, 1991).

4 Optimization of biosurfactant production
Many factors can affect on biosurfactant production by microorganisms including P^H of culture medium, carbon and nitrogen sources (Seghal Kiran, Anto Thomas, Seghal Kiran, Joseph Selvin, Sabarathnam, & Lipton, 2010).

4.1 Cultures grown at different P^H
By using Fog's liquid medium culture was grown at different P^H such as 6, 7, 7.5, 8 & 9, respectively in the presence of light arrangement at room temperature for 15 days. After incubation period cell free broth were obtained by centrifuging the culture grown at different P^H and at 4°C using 10,000 rpm for 10 to 15 minutes. Again Surface tension reduction was measured by Du ring surface tensiometer.

4.2 Cultures grown at different carbon, nitrogen sources
Sample was grown by using same liquid culture medium at same growth conditions in the presence of glucose, sucrose, glycerol, paraffin, xylene and vegetative oils such as sunflower oil and soya bean oil, separately. All were studied for growth phases after 5, 10 and 15 days of interval of growth period by measuring optical density using colorimetric assay at 600nm and each time centrifugation was done at 10, 000rpm for 10-15 minutes at 4 degree Celsius. Cell free broth was used for the study of surface tension reduction making 10% concentration of sample for each one.

5. Partial extraction and purification of biosurfactant
Surface active agent was partially extracted by using ethanol extraction and precipitation method. After extraction purification was done by Silica gel column chromatography (Arunrattiyakorn, Chotelresak, Kanzaki, Nitoda, Thanomsub, & Watcharachaipong, 2004).

6. Chemical analysis of biosurfactant
Carbohydrates and lipids are determined for selected algal sample. Presence of carbohydrates and lipids were detected by thin layer chromatography.

6.1 Separation and detection of sugars
By using silica gel thin layer chromatography technique presence of sugar content was analyzed by butanol; ethanol: water (5:3:2) and compound developed by using DPA reagent {Diphenyl amine reagent- Aniline: DPA: Acetone: Phosphoric acid (4:4:200:15)}, (Chopade, Jagtap, Pardesi, & Yavankar, 2010).

6.2 Separation and detection of lipids
The solvent system used for lipid consisted of Benzene: Diethyl ether: Ethyl acetate: Acetic acid (80:10:10:1) and Hexane: Ethyl ether: Formic acid (80:20:2) and the spot was developed by iodine vapors (Chopade et al., 2010).

6.3 Thin layer chromatography analysis for glycolipids
Cell free broth of biosurfactant producing algae was analyzed by thin layer chromatography analysis by using chloroform: methanol: water = 65:15:2 (V/V/V) solutions as developing solvent. The chromogenic reagent contained phenol-sulfuric acid reagent in which 3 g phenol and 5 ml sulfuric acid were dissolved into 95 ml ethanol (Li, Shim, Xu, Yang, & Zhang, 2012).

Results:
After screening and isolation of four different algal samples from different natural aquatic sources, blue green algae Chlorella was shown satisfied results for biosurfactant production which was first detected by using different primary confirmation tests. On the basis of these results and after literature survey pure culture of Chlorella pyrenoidosa NCIM No. 2738 was collected from National Chemical Laboratory, Pune. Pure culture was cultivated on sterile Fog's agar medium slants by providing required light arrangements at room temperature (Figure-1) and enriched in Fog's liquid broth medium for further process. The surface tension of liquid was measured by a Stalagmometeric method (Aparna et al., 2011) in which the surface tension was determined on the basis of the number of drops which fall per volume, the density of the sample and the surface tension of a reference liquid, e.g., distilled water. Surface tension of distilled water was approximately reduced from 72.6 to 68 dyne/cm and the surface tension reduction of distilled water was shown by the same algal sample was in the range of 52.6 dyne/cm by using Du ring surface tensiometer at 10 % of concentration.

By performing drop collapse assay it was observed that the drop of cell free broth of organism was collapsed with the average of 4-5 mm. Oil spreading test for detection of biosurfactant production showing the oil displacement area was displaced at 2.5 cm^2 to 2.0 cm^2 with clear zone. Emulsification activity of the biosurfactant from the cell free broth was measured with kerosene and cell free broth. E_{24} ranged from 13.04 EA%.

Another confirmation test to detect biosurfactant production was performed in which detection of surfactant was studied by CTAB methylene blue agar plate assay where the formation of a purple-blue haze with Sharpe defined edge around the culture well was observed after overnight incubation (Figure -2 & 3). All results are interpreted by observations and calculations.

After primary confirmation tests for biosurfactant production, cultivation practice was developed for getting maximum production of biosurfactant from the same sample. Culture was grown at different P^H conditions such as 6, 7, 7.5, 8, and 9, and also in the presence of different carbon and nitrogen sources. Culture was also grown in the presence of vegetative oil samples by using proper light arrangement. When culture was grown at different P^H conditions and studied for surface tension reduction using distilled water for different concentrations of sample such as 1, 2, 4, 6, 8, 10%, it was found that the culture grown at 7-7.5 and 9 P^H range was showing good surface activity as compare to other P^H values (Graph- 1).

Glucose and sucrose was taken as a carbon source. Glycerol, paraffin, xylene, kerosene and petrol indicate hydrocarbons. Sunflower, soya bean oil was used as vegetative oils respectively. It was found that the culture shown good surface tension reduction of distilled water in the presence of Soybean oil, paraffin, kerosene and petrol after 5, 10 and 15 days of incubation period at 10% concentration of sample. Culture growth enrichment was studied by taking optical density at 600nm using spectrophotometer for each 5 days interval within 15 days incubation period. Optical density reading was constantly increased for each culture at fifth days, tenth days & fifteenth days of incubation period. In presence of xylene and glycerol the color of algal broth was fade (Figure- 4). Surface tension reduction of distilled water shown by paraffin is 72.6 to 63.8 dyne/cm and by kerosene is up to 41.8 dyne/cm by using Du- ring surface tensiometer. Soya bean oil and sunflower oil shown surface tension reduction of distilled water on 5^{th} and 10^{th} days of incubation period but it was increased on 15^{th} days of incubation (Graph-2).

Surface active agent was partially extracted by using ethanol extraction and precipitation method. After extraction purification was done by Silica gel column chromatography method as Arunrattiyakorn et al. (2004). The surface active agents were qualitatively analyzed by using thin layer chromatography for sugar and lipid detection. It revealed the presence of carbohydrates was confirmed by R_f values 0.75 and the brown dot blot show existence of glycolipids as Li et al. (2012) demonstrated by thin layer chromatography. Thin layer chromatogram with iodine showed brown spot development with R_f values 0.7 for lipid.

Discussion:
Production of biosurfacatant was checked for Chlorella pyrenoidosa NCIM No. 2738 strain by non-disruptive method, in which majority of screening methods are used for detection of biosurfactant producing microorganisms which are based on the interfacial or surface activity. Biosurfactants have ability to reduce surface tension of liquids and it is shown that the cell free broth (supernatant) of selected organisms were able to reduce surface tension of distilled water at some extent but most effective results were shown by Du-ring surface tensiometer. In drop collapse assay, drop of cell free broth for selected organism was collapsed with the some average values. This assay relies on the destabilization of liquid droplets by surfactants. Therefore, drops of a cell suspension or of culture supernatant was placed on an oil coated, solid surface. If the liquid does not contain surfactant, the polar water molecules are repelled from the hydrophobic surface and the drops remain stable. If the liquid contains surfactant, the drops spread or even collapse because the force or interfacial tension between the liquid drop and the hydrophobic surface is reduced. The stability of drops is dependent on surfactant concentration and correlates with surface and interfacial tension.

The oil spreading assay is the next method performed for detection of biosurfactant production. If biosurfactant is present in the cell free broth of sample, the oil is displaced and a clearing zone is formed. The diameter of this clearing zone on the oil surface correlates to surfactant activity, so it is referred as oil displacement activity. It is rapid and easy method and it does not require any specialized equipment. It can be applied when the activity and quantity of biosurfactant is low.

Another popular assay was carried out where the emulsion index E_{24} is calculated as the ratio of the height of the emulsion layer and the total height of liquid and actually E_{24} correlates to the surfactant concentration.

Biosurfactant production can be affected by many factors such as an environmental conditions and different carbon and nitrogen sources. Culture medium P^H can affect on biosurfactant production and it was studied here by growing the same culture at different P^H conditions and in the presence of different carbon and nitrogen sources.

After partial extraction and purification, surface active agent was analyzed by using different chemical assays where nature of characterized surfactant was in the form of glycolipid and it was confirmed after analysis of carbohydrates and lipid by thin layer chromatography. For the same selected algal sample presence of specific glycolipid type of biosurfactant can be characterized by using further chemical analysis methods.

Thus, different screening protocols proved to be a rapid and effective manner of studying surfactant producing organisms. Further studies are under way to scale up growth conditions for the production of biosurfactant by disruptive methods.

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blessings and wishes.

Figure-1

Growth of Chlorella pyrenoidosa on sterile slants of Fog's medium

Figure -2

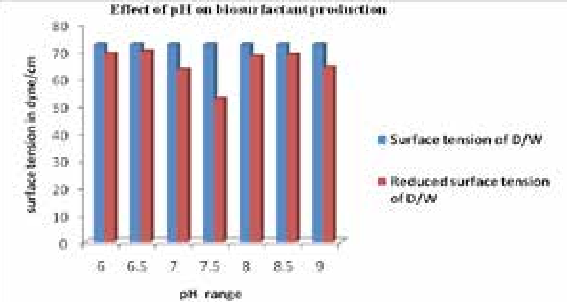
Figure -3

CTAB-methylene blue agar plate assay (Fig. 2 & 3)

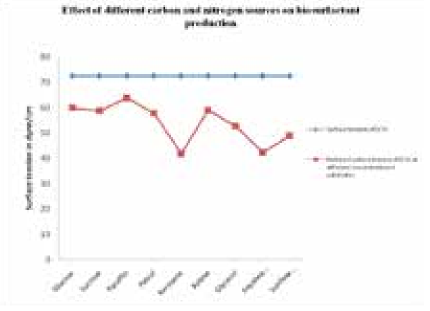
Figure- 4

Growth of Chlorella pyrenoidosa at different concentration of substrates

Graph- 1



Graph- 2



Abbreviations:

NCIM - National Collection of Industrial Microorganisms
P^H - Percentage of hydrogen ion concentration
Rf - Retardation fraction or ratio to front calculated by distance traveled by Compounds/ distance traveled by solvent
ml - Milliliter
rpm - Revolution per minutes
cm - Centimeter
µl - Microlitre
^{0}C - Degree Celsius
Nm - Nanometer
% - Percentage
V/V/V - Volume/ Volume/ Volume of solution
g - Gram
mm - Millimeter
EA % - Emulsification Activity in Percentage
E_{24} - Emulsification index at 24 hours
D/W - Distilled Water

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