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Structural Characterization of Glycolipid Synthesized from *Chlorella Pyrenoidosa* NCIM 2738 by FT-IR and GC-MS and Its Effect on Fungal Spore Germination

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Abstract:
Unicellular microalgae Chlorella pyrenoidosa NCIM 2738 was able to produce glycolipid biosurfactant. Chemical nature of biosurfactant analyzed by Fourier transform infrared spectroscopy showed presence of functional groups for glycolipid was determined by peaks found at 1708 cm^{-1} confirmed for stretching of C=O primarily from lipids and at 1260-968 cm^{-1} was for C–O–C stretching of carbohydrates. Gas chromatography-mass spectroscopy analysis was confirmed after fatty acid and sugar analysis where the hydrophobic moiety of glycolipid envisaged being a C16 methyl ester and presence of carbohydrates glucose and galactose after alditol acetate derivatization. Application of glycolipid as antifungal agent was determined after the inhibition of fungal spore germination of Aspergillus niger NCIM 545.

Keywords: *Glycolipid, Biosurfactant, Antifungal, Algae, FT-IR, GC-MS*

1. Introduction

Glycolipids are low molecular weight biosurfactants mainly consist of four groups, rhamnolipids, trehaloslipids, sophorolipids and mannosylerythritol-lipids (Desai and Banat, 1997; Asselineau, 1978; Kitamoto et. al., 1993). In recent years biosurfactants have been investigated and studied as potential replacement for synthetic surfactants and for their various applications (Kosaric, 1990).
Glycolipids are components of carbohydrates joined to fatty acids or the chain of hydroxyl-fatty acids and they have many applications in environmental and biomedical field. Antagonistic properties of mannosylerythritol-lipids were reported by Kitamoto et. al., (1993) and many of them are known for their antifungal activity (Abalos et. al., 2001). Chemical and structural characterization of microbial glycolipids can be carried out using different analysis techniques involved HPTLC, FTIR, GC-MS and NMR. Mass spectroscopy provides essential information which provides identification and quantification information of glycolipid structure.
Recent days algae have received wide attention as they are abundance in nature and popularity. Unicellular microalgae *Chlorella pyrenoidosa* able to produce biosurfactant and we analyzed and reported biosurfactant activity of glycolipid biosurfactant produced from *Chlorella pyrenoidosa* NCIM 2738 with production optimization (Londhe et. al., 2014).
This research work is focused on structural elucidation of glycolipid biosurfactant using chromatographic (HPTLC) and spectroscopic techniques. Chemically analyzed glycolipid molecules investigated for antifungal activity.

2. Materials and Methods

2.1. Collection of Strain

Strain of microalgae *Chlorella pyrenoidosa* NCIM 2738 was collected from National Collection of Industrial Microorganisms (NCIM), Pune and cultivated by using sterile Fog's medium for 7 to 15 days at room temperature and under 1000-2000 lux light intensity. After screening of biosurfactant activity and optimized process of glycolipid production, glycolipid was extracted by chloroform/methanol 2:1 *(v/v)* at room temperature for 12 h and purified by silicic acid chromatography (Londhe et. al., 2014).

2.2. Structural Analysis of glycolipid biosurfactant

After analysis of glycolipid by thin layer chromatography (TLC) purified product separated and processed for further analysis by fourier transform infrared spectroscopy (FTIR) and Gas chromatography-mass spectroscopy (GC-MS).

2.2.1. FTIR

The Fourier transform infrared spectroscopy (Agilent technologies, Cary 630, FTIR) analysis for isolated purified fractions of biosurfactant was done in mid Infra Red region 4000-650 cm^{-1}. Sample was taken 10 µl and after spectra baseline correction principle components were analyzed.

2.2.2. GC-MS

Fatty acid analysis was done by acid hydrolysis where purified glycolipid (100 µl) was hydrolyzed with 6.0 M HCl at 100º C for 24 h. The mixture of solution was cooled at room temperature and fatty acids (FAs) extracted by using ether. The fatty acids esterified with 3.0 M HCl in methanol at 100º C for 1 h. After cooling these fatty acid methyl esters (FAMEs) extracted by using hexane and concentrated by evaporation at room temperature. The FAMEs analyzed by GC–MS apparatus (Shimazdu 2010 MS Engine) which equipped with integrated gas chromatograph with a RTZ column (60 m long, 0.25 mm i.d., nonpolar). Helium was used as carrier gas at a flow rate of 1 ml min-1. The injector temperature was maintained at 280 º C. The negative ion mode was used throughout and scan initiated over 50-1000 m/z range. The composition of sugar for biosurfactant glycolipid was determined by GC-MS.

Biosurfactant (1 ml) was hydrolyzed in a sealed tube with 150 µl of 2 M trifluoroacetic acid (CF3COOH) at 120º C for 4 h. The residue was washed twice with methanol after evaporation and the sample was then reduced with 1 molar aqueous solution of sodium borohydride (NaBH4, 100 µl) and these acetylated with a mixture of potassium acetate (100 µg) and acetic anhydride (100 µl) at 100º C for 2 h. The excess reagent was removed by evaporation and the sample washed with ethanol for several times. With ethyl acetate and water (1:1, *v/v*), the alditol acetates were extracted and analyzed by GC–MS apparatus (SHIMADZU, model QP 5050 A) equipped with a RTZ column using helium as carrier gas. Column temperature was increased from 100º C (1 min) to 300º C. Sugars were identified by comparing the relative retention times of sample peaks with standards.

2.3. Application

2.3.1. Effect on Spore Germination and Inhibition

Glycolipid produced by *Chlorella pyrenoidosa* NCIM 2738 was studied for their effect on fungal spore germination after addition of 0.5 ml solution of biosurfactant glycolipid to 0.5 ml of fungal spore suspension (containing 5×103/ml spores). In the control set biosurfactant solution was replaced by 0.5 ml of the potato dextrose broth medium and was incubated at 25º C for 18 h. The inhibition of hyphal extension or spore germination was compared with control set by microscopic observations using light microscope (40 X).

3. Results and Discussion

Glycolipid biosurfactant was produced by *Chlorella pyrenoidosa* NCIM 2738 and structural elucidation made after FTIR analysis showed distinct absorption bands over the wave number range 3400-700 cm^{-1} were observed but a broad peak at 3360 cm^{-1} and 3337 cm^{-1} assigned to O-H stretching. The band at 1708 cm^{-1} was confirmed for stretching of C=O primarily from lipids and the region from 1260-968 cm^{-1} associated with C–O–C stretching of carbohydrates (Fig.1.). Similar results were found by Andrew et. al., (2010) for the peak of interests.

Fatty acid and sugar analysis was done by GC-MS where the glycolipid biosurfactant was acid hydrolyzed first and after that analyzed by gas chromatography of FAMEs. Gas chromatography-mass spectroscopy analysis showed hydrophobic moiety of glycolipid envisaged to be a C16 methyl ester (Fig. 2.). Carbohydrates in glycolipid was determined by GC-MS after hydrolysis and alditol acetate derivatization.

The peaks characterized for carbohydrate moiety of glycolipid was being composed of glucose and galactose (Fig. 3.). The standard peak values of these sugars are mentioned by Jadhav et. al., (2011) in their reports.

Antifungal activity of glycolipid biosurfactant produced by *Chlorella pyrenoidosa* NCIM 2738 was showed satisfied inhibitory effect against the fungal starin *Aspergillus niger* NCIM 545. The fungal spores viability was detected by microscopic observation of germination. The fungal spores of *Aspergillus niger* showed total germination inhibition of fungal spores when they were incubated with biosurfactant glycolipid and fungi did not show mycelia growth but in contrast control set showing mycelia growth which indicating germination (Fig. 4. a. and b.). The inhibition of *Sclerotinia sclerotiorum* ascospore germination was observed from biosurfactant produced by *Bacillus* BNM 122 (Souto et. al., 2004).

Present study indicates characterization of glycolipid produced by *Chlorella pyrenoidosa* NCIM 2738 and application as an effective antifungal agent because glycolipids produced by *Chlorella pyrenoidosa* NCIM 2738 showed strong inhibitory effect on the starting stage of fungal life cycle that is the germination of fungal spores.

4. Acknowledgements

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5. Figures

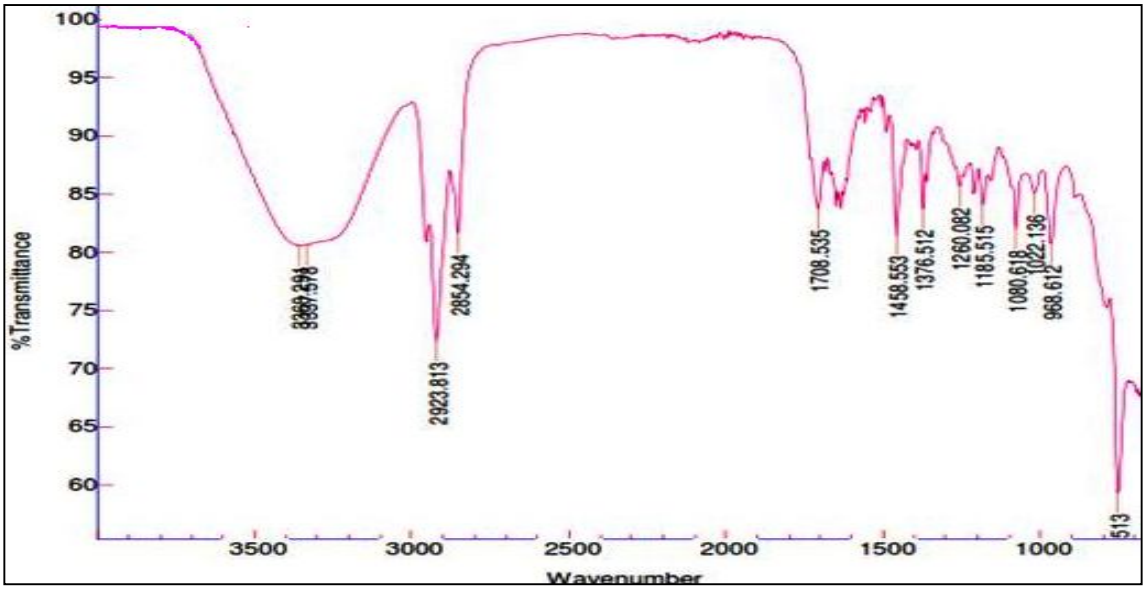

Figure 1: FT-IR spectrum of glycolipid biosurfactant produced by Chlorella pyrenoidosa NCIM 2738.

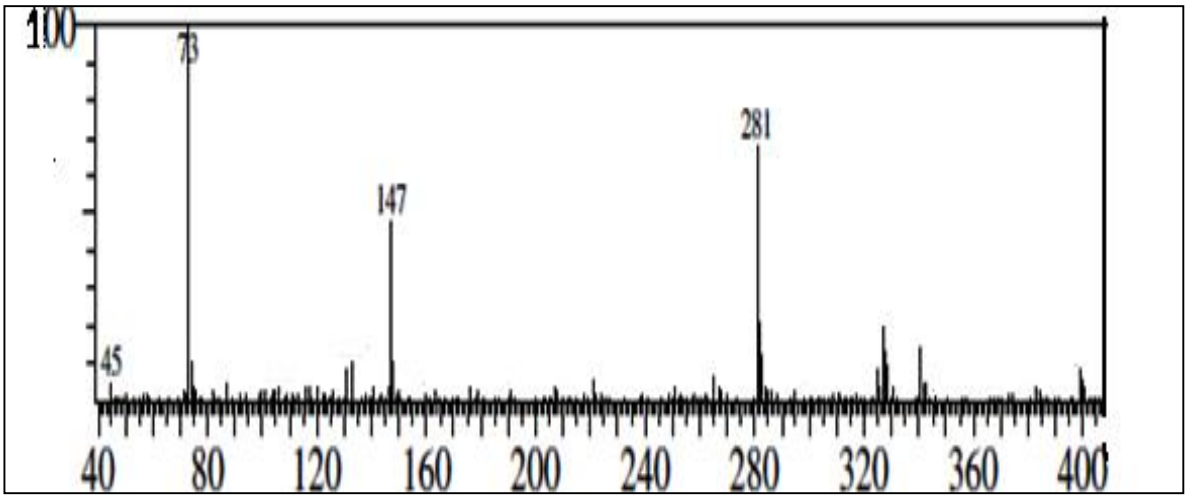

Figure 2: Gas Chromatography-Mass spectra of FAMEs analysis of glycolipid produced by Chlorella pyrenoidosa NCIM 2738 strain.

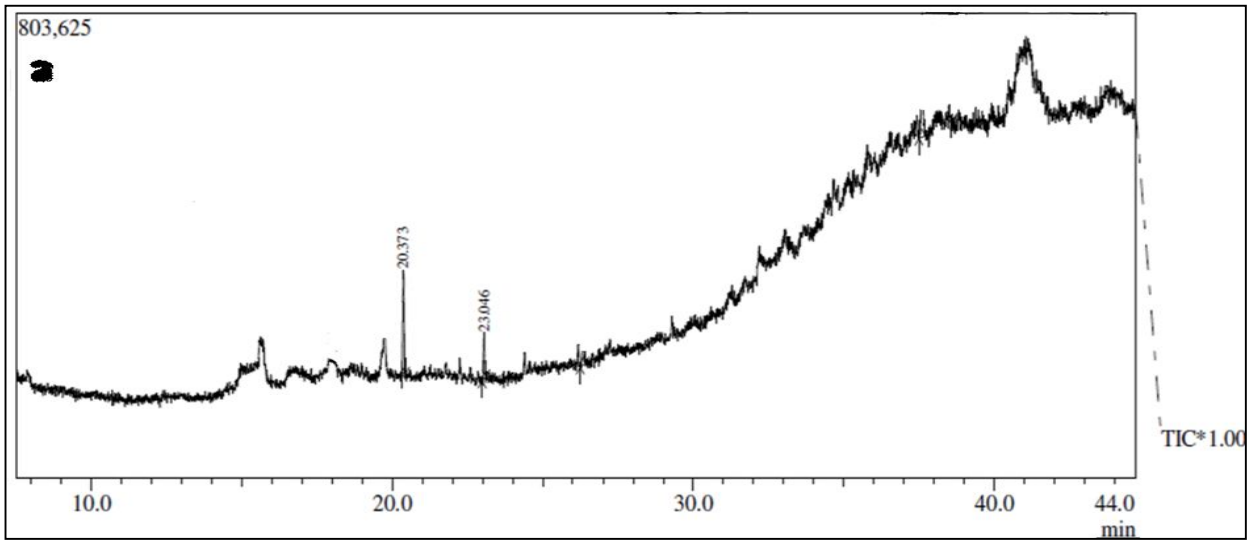

Figure 3: Gas chromatographic spectra of sugars of glycolipid produced by Chlorella pyrenoidosa NCIM 2738.

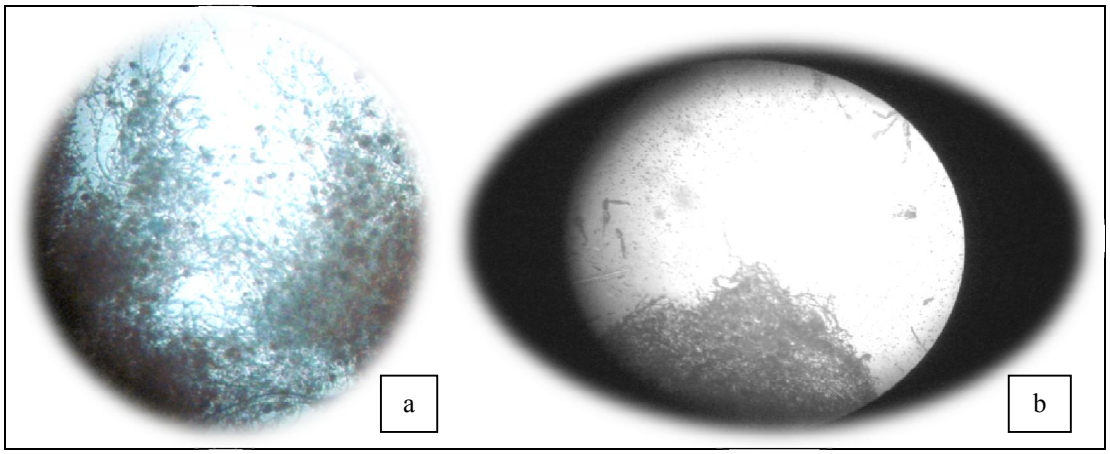

Figure 4: Spore germination of Aspergillus niger NCIM 545 (a) and spore germination inhibition (b) by glycolipid.

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