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Production and characterization of exopolysaccharides (EPS) from mangrove filamentous fungus, *Syncephalastrum* sp.

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In this study, the production and characterization of exopolysaccharide from *Syncephalastrum* sp. was carried out. This is the first report on the production of exopolysaccharides from *Syncephalastrum* sp. Totally, four different fungi (*Aspergillus niger, Aspergillus flavus, Penicillium expansum* and *Syncephalastrum* sp.) were screened for exopolysaccharide production. Among these, maximum polysaccharide producing species was selected for further large scale production. Different concentrations (6, 8 and 10%) of sucrose were also used to increase polysaccharide production. Overall results depicted that 8% of sucrose concentration exhibited higher amount of polysaccharide production. Further, structure of polysaccachride was confirmed by FT-IR and NMR spectroscopy. Based on this spectroscopy results, the polysaccharide produced by *Syncephalastrum* sp. was 3 linked β-D-galactopyronosyl units. The polysaccharide produced by this species composed of galactose as predominant monosccharides in the cell wall of *Syncephalastrum* sp. and their idealized structures were established.

Key words: Exopolysaccharide, mangroves, filamentous fungi, *Syncephalstrum*, β-D-galactopyronosyl.

INTRODUCTION

Polysaccharides are relatively complex carbohydrates consisting of multiple monosaccharides joined together and often branched. Polysaccharides have a characteristic which allows them to produce a material that makes them to stick each on other surfaces. Polysaccharides derived from plants and seaweeds have been in use for thousands of years. However, over the past 20 years, a new class of microbial products: the microbial polysaccharides have grown in industrial importance (Laroche and Michaud, 2007). They can function in foods as viscosifying agents, stabilizers, emulsifiers and gelling agents. These products can be used as alternatives to other synthetic or natural water-soluble polymers. Many bacteria and fungi often secrete

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polysaccharides as an evolutionary adaptation to help them adhere to surfaces and to prevent them from drying out (Mathur and Mathur, 2006).

The fungal kingdom estimated with 1.5 million species contains a diverse range of species presenting polysaccharides that possess various biological activities and chemical properties exploitable for commercial applications. Glucans, chitin and mannans are particularly abundant in the fungal cell wall. Well characterized immunoactive polysaccharides produced by fungus include alpha and beta glucans. For the production of microbial polysaccharides, the main interest is extracellular polysaccharides (EPS). EPS are widely secreted by various marine organisms, including plants, animals, diatoms, microalgae and bacteria (Decho, 1990; Gutierrez et al., 1996; De Philippis et al., 1998; Philippis and Vincenzini, 1998). The EPS produced by these organisms have been explored for various biotechnological applications, such as anti-tumor agents, anticoagulants (heparin analogues) and wound dressings for eye and joint surgery. Apart from that, EPS are also important as emulsion stabilizers (in food and thixotrophic paints), flocculants (in water clarification and ore extraction), foam stabilizers (in beer and fire-fighting fluids), gelling agents (in cell and enzyme technology and foods), hydrating agents (in cosmetics and pharma-ceuticals), and as inhibitors of crystal formation in frozen foods and sugar syrups (Colwell et al., 1986; Sogawa, 1998; Sutherland, 1985). It is likely that hitherto unexplored groups of marine microorganisms produce novel and useful EPS.

Secondary metabolites of marine microbes have been well investigated to discover a lot of natural products of fascinating biological and chemical interest and few reports dealing with the isolation and chemical characterization of polysaccharides of marine microbial origin could be found to date. Hence an attempt has been made to explore the possible potential of polysaccharide production and the structural characteri-zation of an extracellular polysaccharide isolated from *Syncephalastrum* sp.

MATERIALS AND METHODS

Isolation

The fungi were isolated from the mangrove rhizosphere soil of *Rhizopora annamalayana* Kathir, the only endemic species to India. They were sub-cultured in malt extract yeast extract agar medium containing (g/l): malt extract 20, yeast extract 4, agar 20, pH 5.3.

Microorganisms

Isolated colonies like *Aspergillus niger, Aspergillus flavus, Penicillium expansum* and *Syncephalastrum* sp. were grown on potato dextrose agar (PDA) plates at 28°C and incubated for 5 days. After that time, the plates were maintained at 4°C until used. They were transferred once every two weeks to maintain availability and stability for extra cellular polysaccharides production.

Screening for polysaccharide producing microorganism

The fungus was inoculated in a large test tube containing 15 ml of the screening medium (g/100 ml): sucrose, 6; $NaNO_3$, 0.3; KCl, 0.05; $MgSO_4.7H_2O$, 0.005; KH_2PO_4, 0.1; $FeSO_4.5H_2O$, 0.005 an initial pH 6 and incubated at 30°C as static liquid medium. Five days old cultures were heated at 80°C for 15 min. Then two volumes of ethanol were added to the supernatant and the resulting precipitate was collected by centrifugation (10,000 xg, 5 min). The precipitate was dissolved in water and the phenol H_2SO_4 method of Dubois et al. (1956) was used for quantitative estimation of the sugar contents.

Inoculum

Actively growing cells from a newly prepared PDA plate were inoculated in 250 ml flask containing 50 ml liquid medium. Liquid cultures were incubated for 72 h at 28°C in an incubator-shaker. After the incubation phase, 50 ml liquid cultures were used for the production medium.

Culture media and cultivation of *Syncephalastrum* sp.

Maximum polysaccharide producing fungus was grown in the 250 ml Erylinmeyer flask containing 100 ml of the medium. Various concentrations of sucrose: 6, 8 and 10% were added individually with the following composition of $NaNO_3$, 0.3; KCl, 0.05; $MgSO_4.7H_2O$, 0.005; KH_2PO_4, 0.1 and $FeSO_4.5H_2O$, 0.005 (g/100 ml). The media were adjusted to pH 6 and fermentation periods were maintained at 15 days. After that, flasks were autoclaved at 120°C for 15 min and then allowed to cool.

Estimation of mycelial growth

Mycelial growth of *Syncephalastrum* sp. was expressed as dry cell weight (DCM). The mycelia were harvested at the end of incubation periods and then filtered through pre weighed Whatman No.1 filter paper followed by drying at 80°C for approximately 48 h in an oven, after which DCW were measured.

Isolation and purification of polysaccharides

The culture broth was heated for 15 min at 60°C and then cell free solution was obtained by centrifugation (8900 xg, 15 min). The crude polysaccharide was separated from the supernatant by the addition of two volumes of ethanol and the precipitate around the stirrer. The precipitation procedure was repeated thrice and the final product was dried at 60°C and ground to a fine powder. In an old culture, the precipitate did not wound around the stirrer and had to be collected and washed by centrifugation (Leal and Ruperez, 1978).

Analytical techniques

Total sugars were determined by the phenol-sulfuric acid method of Dubois et al. (1956) and reducing sugars by the di-nitro salicylic

Table 1. Effect of different concentrations of sucrose on the production of exopolysaccharides.

Substrate concentration	6% of sucrose	8% of sucrose	10% of sucrose
Volume of filtrate (ml)	91	75	83
Weight of mycelium	1.61	2.12	1.86
Weight of polysaccharides (g/100ml)	0.69	1.2	0.97
Carbohydrate (g/100ml)	10.25	14.43	12.65
Reducing sugar (g/100ml)	1.76	2.82	1.94
Protein (U/ml)	0.83	2.30	1.16
Nitrogen (g/ml)	0.13	0.36	0.18

acid method of Miller (1972) with D-glucose as the standard. Protein was measured by the method of Lowry et al. (1951) using bovine serum albumin as standard. The nitrogen content was calculated from the protein values by dividing the factor 6.25 because 1 mg of nitrogen is equal to 6.25 mg of protein. So this is protein nitrogen (Ghai et al., 1981).

FT-IR spectroscopy

FT-IR was used to investigate the vibrations of molecules and polar bonds between the different atoms. Structures of polysaccharides, such as monosaccharides types, glucosidic bonds and functional groups can be analyzed using FT-IR spectroscopy. Samples were mixed with KBr and pressed into pellets of 13 mm size and infrared spectrum was recorded using Perkin-Elmer IR spectrophotometer (Model IR 577).

NMR analysis

The proton spectra at 400 MHZ and proton decoupled ^{13}C NMR spectra at 100 MHZ were recorded at room temperature on DR x 400 NMR spectrometer using 10 mm sample tube. Sample were prepared by dissolving about 10 mg of the sample in 0.5 ml of ethanol-d containing 1% TMS for ^{1}H and 0.5 ml of sample in 2.5 ml of ethanol-d and a few drop as TMS for ^{13}C. The solvent ethanol-d also provided the integral field frequency lock signal.

RESULTS

Screening for the polysaccharides producing microorganisms

In this study, 4 different fungal strains such as *A. flavus*, *A. niger*, *P. expansum* and *Syncephalastrum* sp. were used for the production of extracellular polysaccharides. Among these, *Syncephalastrum* sp. produced maximum amount of polysaccharides. Hence this strain was selected for further studies.

Effect of sucrose on the production of extracellular polysaccharides

Three different concentrations of sucrose *viz*., 6, 8 and 10% were used for the production of extracellular polysaccharides from *Syncephalastrum* sp. Among these concentrations of sucrose tested, 8% gave maximum production of polysaccharides (1.2 g/100 ml) and biomass (2.12 g/100 ml) which was achieved within the 15 days of incubation periods (Table 1). Ten percent of sucrose also gave satisfactory amount of polysaccharides (0.97 g/100 ml) and biomass production (1.86 g/ 100 ml).

Chemical composition of culture filtrate

Total carbohydrates, reducing sugar, total protein and nitrogen of the culture filtrates were estimated and the results are presented in Table 1. Higher level of carbohydrates (14.43 g/ 100 ml), reducing sugar (2.82 g/100 ml), protein (2.30 U/ml) and nitrogen (0.36 g/ml) were recorded in 8% sucrose concentration at 15 day of incubation periods. At 10% sucrose concentration, the levels of carbohydrates, reducing sugar, protein and nitrogen declined.

FT-IR spectroscopy

Configuration of the polysaccharide fraction was ascertained using FT-IR spectroscopy. The sharp peak at 1067 cm^{-1} was assigned to C-O-C mode. Cyclic C-H stretching vibration appeared at 2934 cm^{-1} and assigned to the stretching vibration of the OCH_3 (methoxy) observed at 1383 cm^{-1}. The obvious absorption peaks at 825 cm^{-1} revealed the existence of β-galatopyranosyl linked IR spectrum of polysaccharide units as shown in Figure 1.

^{1}H NMR spectroscopy

In the ^{1}H-NMR spectrum of polysaccharide unit, methoxy carbon was observed in the region, 3.45 to 3.60 ppm integrals. The signals around 3.38 to 3.42 ppm were assigned to methylene proton of β-D-galactopyronosyl units. Anomeric porton H-1 was observed at 5.11 ppm, and it was attached with carbon atom C-1. The remaining methane protons were observed at 4.53, 3.14 and 3.12 ppm, respectively. ^{1}H- NMR spectrum of polysaccharide is shown in Figure 2.

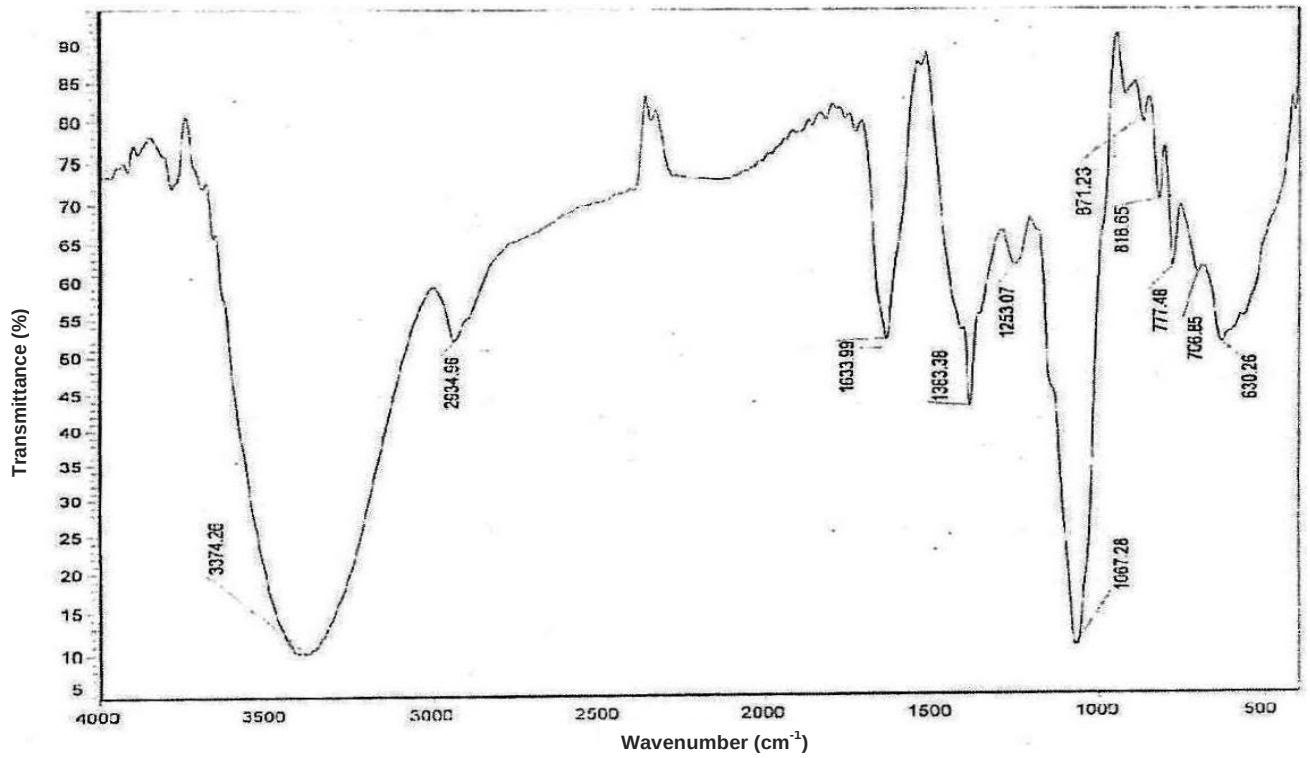

Figure 1. FT-IR spectrum of exopolysaccharides from *Syncephalastrum* sp.

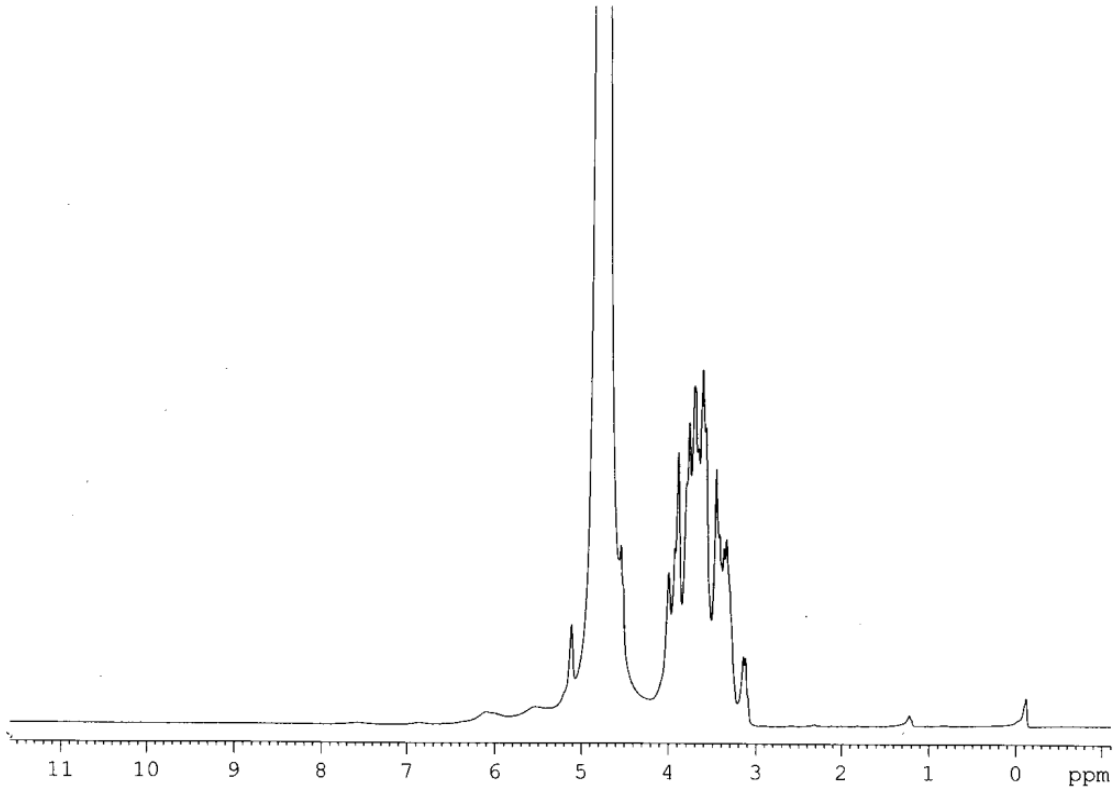

Figure 2. ^{1}H spectrum of exopolysaccharides from *Syncephalastrum* sp.

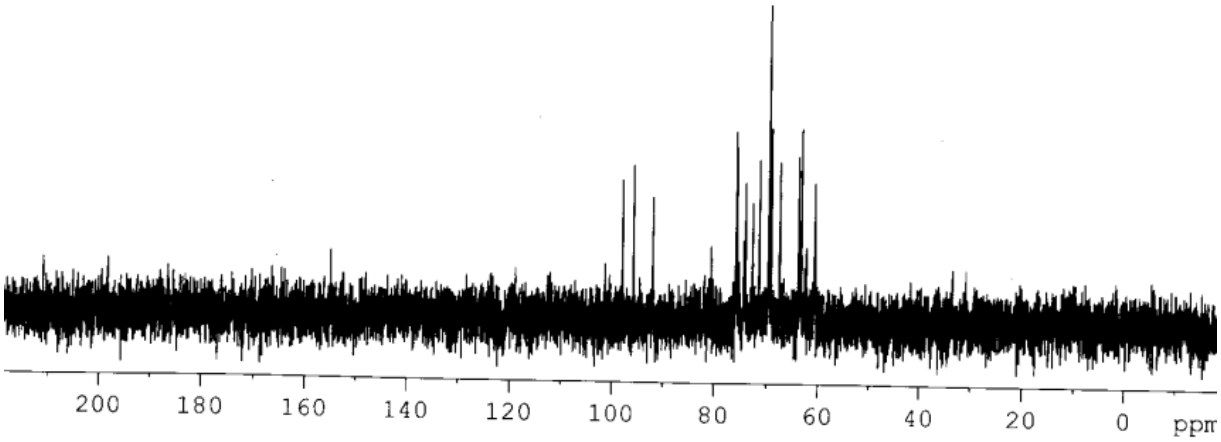

Figure 3. ^{13}C spectrum of exopolysaccharides from *Syncephalastrum* sp.

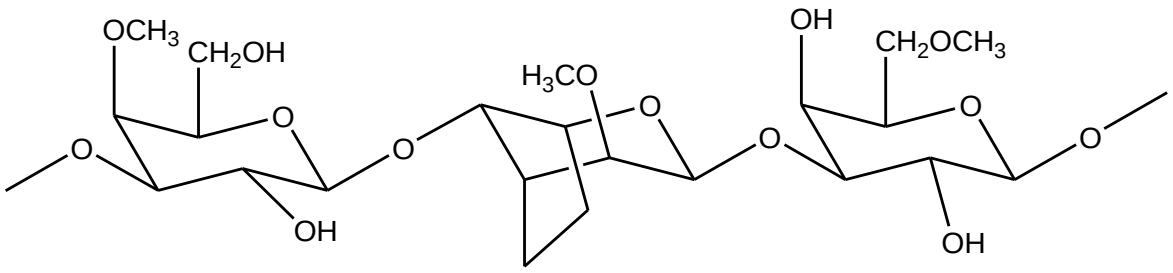

Figure 4. 3 linked β-D-galactopyronosyl units.

The ^{13}C NMR spectrum of *Syncephalastrum* polysaccharides samples showed that, 18 signals were assigned to carbon of polysaccharides unit (Figure 3). The signals at 102.00, 69.16, 80.63, 67.53, 74.11 and 60.69 ppm corresponded to the 3 linked β-D-galactopyronosyl units, while the signals at 98.03, 69.61, 75.90, 75.72 and 71.40 ppm are attributed to the 4-linked 3,6-anhydrno-α-L-galactopyranosyl units. Moreover, the additional signals (95.87, 80.63, 63.32 and 62.35 ppm) in the spectra revealed the presence of 4-Omethyl-α-L-galactopyrynosyl unit. The anomeric carbons were observed at 102.00 to 95.90 ppm which was attached with bridging oxygen atom in polysaccharide units. Based on the TLC, FT-IR and NMR spectroscopy revealed that cell wall of *Sycephalastrum* sp containing 3 linked β-D-galactopyronosyl units (Figure 4) and galactose as predominant monosccharides in polysaccharide units.

DISCUSSION

Most of the EPS produced by fungi are highly hygroscopic β-glucans, suggesting that its production could be related with tolerance to desiccation; similarly to that observed and described in bacteria. Recently, there has been a growing interest in studying the EPS production of a wider array of fungi, not only from the standpoint of comparative biology but also with the expectation of finding better EPS producing systems for use in various biological applications. Besides the health benefits, EPS represent a valid alternative to plant and algal products considering that their properties are almost identical to those employed in food, pharmaceutical and cosmetic industries (Sutherland, 2002).

In the present study, different levels of sucrose *viz*., 6, 8 and 10% were used to induce extracellular polysaccharide production in *Syncephalastrum* sp. Among the three different levels tested, 8% of sucrose gave maximum amount of polysaccharides (1.2 g/100 ml) and mycelia growth (2.12 g/100 ml) production on the 15th day of incubation. Similar results also observed by Leal-Ruperez (1978) who reported maximum production of 86.9 mg of polysaccharides/100 ml observed at 10% of sucrose concentration, at 15 days of incubation by *A. niger* in Egypt, 513 mg/ 100 ml. Because, this polysaccharide could be cell wall component, the microorganisms continue to produce when there is a little demand for cell wall synthesis. It could also be cytoplasmic

reserve that is produced in larger amounts than the fungus which is capable of storage (Leal et al., 1979).

Whereas, Leal-Serrano et al. (1980) recorded the maximum amount of polysaccharides isolated from *Aurobasidum pullulans* after the 15 days of incubation. Similar results were also observed by Senthilkumar and Murugesan (2010). The disparity in yield may be due to the difference in the strain.

Previous studies have studied the utilization of several carbon source viz., glucose and sucrose in 13 species of *Aspergillus* (Leal and Ruberze, 1978) as well as nitrogen sources namely L-alanine, L-aspargine, L-aspartic acid, L-glutamine, L-glutamic acid, L-leucine, L-phenlalanine, L-valine, potassium nitrate and di-ammonium tartarate in *Aspergillus nidulans* (Ruberez and Leal, 1979). Leal-Serrano et al. (1980) proved that among the carbon sources investigated for poly-saccharides production maltose (68.5 mg/100 ml) and mannose (66.5 mg/ 100 ml) proved to be the best, whereas mannitol (12.4 mg/ 100 ml) gave the lowest polysaccharide yield. Interestingly, in the present study, 6 to 10% sucrose as carbon source yielded fairly high level of extracellular polysaccharide (0.69 to 1.2 g/ 100 ml) in 15 days of incubation in *Syncephalastrum* sp. (Table 1). There was a clear trend that EPS have larger carbo-hydrates contents; in the present study, total carbo-hydrate contents was recorded (10.25 to 14.43 g/ 100 ml) in culture filtrate, but the reducing sugar (1.76 to 2.82 g/ 100 ml), protein (0.68 to 2.30 U/ml) and nitrogen (1.13 to 0.36 g/ 100 ml) content in culture filtrate was very low as compared to total carbohydrate content recorded in all the three different levels of sucrose. Similar results were also observed by Leal and Ruberez (1978) and Senthilkumar and Murugesan (2010) had less similar carbohydrate and protein values in *A. nidulans* and *A. niger*. Griffin (1996) investigated the proximate composition of fungus containing 16 to 85% carbohydrate, 0.2 to 87% lipids, 14 to 44% protein, 1 to 10% DNA, 0.15 to 0.3% RNA and 1 to 29% ash content.

Polysaccharide fraction could be ascertained with the help of IR-spectroscopy. Based on the TLC, FT-IR and NMR spectroscopy revealed that cell wall of *Syncephalastrum* sp. contain 3 linked β-D-galacto-pyronosyl units, and galactose as predominant mono-sccharides in polysaccharide units which was conformity with the observations of Archer et al. (1977) on *Monilinia frutigena*. The polysaccharide obtained from the three isolates of *Nectria cinnabarina* was similar to the polysaccharides found in species of *Gibberella* and *Fusarium* (Jikibara et al., 1992; Ahrazem et al., 2000) and *Penicillium vermoesenii* (*Gliocladium vermoesenii*) (Ahrazem et al., 1999) since they have similar residues, mainly 2,6-di-O-substituted galactofuranose (!2,6)- Gal*f*-(1!), and terminal glucopyranose (Glc*p*-(1!), and almost identical "H-NMR spectra. The differences found in the polysaccharide from *N. cinnabarina* and species of *Sesquicillium* and *Nectria* with *Sesquicillium* anamorphs were in agreement with the separation of these species according to their morphological characters (Samuels and Rossman, 1979) and support the creation of the genus *Bionectria* (Rossman et al., 1999).

Conclusion

The developments of microbial polysaccharides are best viewed in the context of their maximum utility in food and medical industry. The most promising developments seem possible in therapeutic and cosmetic applications as these compounds have been described as immunomodulators, antitumorogenic and antiviral (AIDS) agents for the treatment of hypercholesterolemia and agents for stabilization of glycemia. Moreover, specific action as food additives has been ascribed for glucan oligosaccharides. But, to the best of our knowledge, it is the first time that they have been described 3 linked β-D-galactopyrynosyl chain in *Syncephalastrum* sp. This species was able to produce a high level of EPS in production medium containing 8% sucrose concentration. This study has also confirmed that the concentration of EPS produced by *Syncephalastrum* sp. was dependent on the carbon sources. Further, immunomodulatory effects in animals are still under study.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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