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Characterization of extracellular polymeric substances (EPS) produced by marine *Micromonospora* sp.

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ABSTRACT

The extracellular polymeric substances (EPS) were extracted from Micromonospora sp. strains isolated of the Gulf of California. Different spectrophotometric, chromatographic and microscopic techniques were used to determine the composition of the EPS. The extraction methods employed are fast and simple and allowed an efficient extraction with a minimum degree of cell lysis. The Fourier Transformed Infrared (FT-IR) spectra showed coupling attributed to polysaccharides, proteins and lipids. Nuclear Magnetic Resonance (NMR) reveals the presence of protons of anomeric carbons and protons of primary amine, aromatic-compounds, halides, aliphatic and sulfide. Mass Spectrometry (MS) we corroborate the composition of polysaccharide hydrolysates in EPS samples. High Performance Liquid Chromatography (HPLC) and Thin-Layer Chromatography (TLC) revealed the presence of majority sugars as xylose, arabinose, mannose and melibiose, and the presence of oligosaccharides such as nystose. Scanning Electron Microscopy (SEM) was performed to observe the morphology of the EPS, the Energy Dispersive Spectrometer (EDS) showed the presence of elements such as carbon, calcium, potassium, sodium, sulphur and phosphorous.

Keywords: *EPS* characterization, marine *Micromonospora* sp., carbohydrate profile

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INTRODUCTION

The marine ecosystem currently represents a source of microorganism that generate substances with potential applications, largely due to the conditions under which they develop such as low temperatures, high pressures, high salt and low nutrient concentrations, these ecological conditions lead to the excretion of secondary metabolites such as EPS by several microorganisms, among these actinobacteria. The importance of EPS has been noticed in recent

publications [1, 2], because of its physicochemical composition, structural diversity and rheological properties [3] as well as the role they play in microbial ecology, bioremediation [4]. The application of EPS is growing in different areas: Environmental applications and bioleaching industries, such as water treatment, wastewater flocculation and settling, color removal from wastewater. In pharmaceutical and biomedical industries have been showed anti-inflammatory properties and other therapeutic functions like development of cell therapy and regenerative medicine, as inhibitor of pulmonary metastasis in humans and a preventer of adhesion and proliferation of tumor cells among other applications; by which study of EPS extracted of marine microorganisms brings new expectations to environmental and biomedical fields [5, 6].

Many marine bacteria of different genus, specially *Pseudoalteromonas*, *Alteromonas*, *Thermococcus*, *Geobacillus* and *Bacillus* produce EPS to endure environmental stress, and therefore survive adverse conditions [7]; no reports have been found regarding EPS production by marine *Micromonospora* sp. strains, thus being relevant to our investigation.

Recent publications about EPS mention the composition of a wide range of organic substances such as polysaccharides, proteins, nucleic acids and phospholipids, excreted by eukaryotes and prokaryotes. These exopolysaccharides are metabolic products accumulated in the bacterial cell wall, provide protection to cells and serve as carbon and energy source [8]. It has been shown that EPS plays a wide variety of biological functions for the cell, included preventing the desiccation, protection against environmental stress, toxins and antibiotics, adhesion to surfaces, pathogenesis and symbiosis [9]. These EPS can absorb nutrients from the surrounding environment, as part of a survival strategy [10]. During the colonization process on a particular surface, a bacterium produces excess of EPS that aid in biofilm formation.

The phase of bacterial growth, the medium composition (nitrogen and carbon source), pH and temperature affect EPS production, being the medium composition the most important [11, 12]. Biosynthesis of EPS has been associated with the primary metabolism of carbohydrates in the cells; its production takes place during sugar consumption, which requires a lot of energy.

There have been reports about several EPS extraction techniques, of which results have been controversial because of cell lysis, for this reason there have been efforts to find an efficient extraction technique. Most describe use of physical and chemical technics or a combination of both. Among the extraction methodologies, it can be mentioned the application of centrifugation [13], ion exchange resins, EDTA extraction [14], using ultrasound [15], ethanol and using NaOH [16]. The efficiency of the physical extraction methods is lower than chemical methods [17], however, chemical methods are associated with problems during the extraction and analysis, largely due to cell lysis. In the present research EPS obtained from four *Micromonospora* sp. strains isolated from the Gulf of California, México was characterized; furthermore EPS were compared, regarding three different extraction methods: direct centrifugation (DC), EDTA and NaOH, they are the most efficient technique with a minimum degree of cell lysis [18], such extraction techniques were compared using spectrophotometric, chromatographic and microscopic techniques.

EXPERIMENTAL SECTION

Process for the production and extraction of EPS

Four *Micromonospora* sp. strains were selected, isolated and purified from sediment of the Gulf of California, coded as M007, M009, M010 and M012. These were grown in solid GYM medium (glucose 4 g/L, yeast extract 4 g/L, malt extract 10 g/L, $CaCO_3$ 2 g/L, agar 12 g/L), at 30°C for 15 days.

In an Erlenmeyer flask of 1000 mL containing 700 mL of liquid GYM medium (glucose 4 g/L, yeast extract 4 g/L, malt extract 10 g/L, $CaCO_3$ 2 g/L), the strains were inoculated and maintained at 150 rpm, 30°C for 25 days. When fermentation time was completed, the extraction methods described below were use:

1. Extraction by DC: Fermentation media of the studied strains (M007, M009, M010 and M012), were directly centrifuged (without pre-concentration or wash) at 14,000 rpm (20,817 g) for 20 min at 4°C [19]. The supernatant was discard and the cells were filtrated through a 0.22 µm Millipore filter. Biomass was dialyzed (to remove

metabolites and low molecular weight salts) using a cellulose dialysis membrane (33 mm, typical molecular weight cut-off = 14 kDa) in a volume of 80 mL distilled water, for 24 h at 4°C [20].
2. Extraction with EDTA: 60 g of EDTA disodium salt was added to the fermentation medium [14], and allowed to stand for 3 h at 4°C [21].
3. Extraction with NaOH: 140 mL of 1M NaOH were added to fermentation medium, and allowed to stand for 3 h at 4°C [16].

Methods 2 y 3 were centrifuge, filtered and dialyzed in the same conditions as in DC.

EPS characterization
Several techniques were used to determine the composition and morphology of EPS extracted.

The total protein content was determined by the Bradford method in microplate [22]. For determination of functional groups FT-IR was performed (Perkin Elmer Spectrum 1000 FT-IR, cm^{-1} resolution, 4400-650), for this analysis, samples were re-suspended and precipitated using 3 volumes of cold ethanol in ice for 2 h, followed by centrifugation at 14,000 rpm for 20 min at 4°C, the precipitate was dried in an oven at 50°C overnight. FT-IR spectra were obtained on a wavelength range from 600 to 4000 cm^{-1}[23]. NMR spectra were obtained in an Agilent DD2 600MHz One-probe 343 K (25^oC) and 35 kHz decoupling field strength were conducted. To obtain ^1H NMR and single quantum correlation heteronuclear (HSQC) spectra, the dialyzed EPS sample was lyophilized and dissolved in 0.7 mL of D_2O and added 5 mg of NaOH and 5 µL of DMSO to increase solubility. A solution of TMS and were used as reference internal (deuterated solvents were purchased from Cambridge isotope Laboratories, Inc. USA).

Once removed the EPS by different extraction methods, hydrolysis of the samples with 1M H_2SO_4 at 90°C for 3 hours, neutralized with 1M NaOH to pH 7 and filtered on Whatman 0.45 mm. A scanning of compounds present, was performed by direct injection with ESI-MS in a Varian 500MS; with the following conditions: ion trap in positive mode, needle at 5000 volts 2.6 µAmps, nitrogen was used as drying gas at 350ºC, 15 psi, housing at 50ºC, nebulizer 25 psi, capillary 70 volts, spray shield 600 volts, data acquisition was recorded for 10 minutes in each sample.

For determination of EPS carbohydrates with HPLC and TLC, hydrolyzed samples were used; ten standards were used for HPLC and TLC for carbohydrate assignment such as monomers, dimers, trimers, tetramers and a pentamer [7]. With HPLC, a method was developed employing a Phenomenex Luna aminated (NH_2) column (5µ, 150 x 3 mm), using as mobile phase acetonitrile:water (80:20) and refractive index detector (RID) at 40°C, flow of 0.8 mL/min [24], and 5 µL injection for standard and 30 µL for EPS samples. For TLC a mixture of buthanol:acetic acid:water (2:1:1) were used as mobile phase, the plate was dried at 60°C and revealed with a solution of α-naphtol 2.4% (w/v) and heated at 120°C for 3-5 minutes [25, 26].

The SEM and EDS were performed using JEOL JSM Thermo Scientific 7500 and 5600-LV, with a voltage accelerated from 10 to 15 kV to observe the morphology and elemental composition of EPS. Previously, the samples were lyophilized and homogenized to a fine powder, this is adhered to a small aluminum sample holder by dispersion under vacuum, carbon was used as conductive element [27].

RESULTS AND DISCUSSION

Culture growth and EPS extraction
Since the factors affecting the formation of exopolymers are related to changes in the cultivation medium; the production of EPS is higher in glucose, fructose, sucrose and starch rich medium; where carbon and nitrogen availability can also affect actinobacteria growth [28], therefore in GYM medium, the growth of marine *Micromonospora* sp. strains was favorable compared to glucose-yeast extract medium (GYEA).

The growth of M007, M009, M010 and M012 strains was monitor in GYM solid medium observing the growth of the strains, having sporulation stage and production of EPS at day 25. Approximately 5 g of EPS was obtain with the

tested extraction techniques and presented the same organoleptic properties: black color, mucoid consistence and sulphured smell. The extraction methods required minimal steps and were easy to follow. DC method was use as a control method to have a reference in composition and morphology of EPS obtained. The technique with EDTA was select because it chelates divalent cations responsible for the interconnection of charged compounds in the matrix of the EPS. The extraction with NaOH induces ionization of carboxyl groups, resulting in repulsion between the exopolymer and the cell, causing the separation of the EPS [29].

Total protein by Bradford method

Protein analysis reveals important information about the extraction method showing cell lysis [30], obtaining values between 0.13 and 0.16 µg/mL of total protein. A one-way ANOVA analysis was performed and no significant differences ($p<0.05$) in protein concentration were found between the extraction methods and the strains.

FT-IR spectrometry

IR analysis of EPS extracted by the three methods of extraction of the four strains was perform to determine functional groups associated with macromolecules, such as lipids, proteins, polysaccharides and nucleic acids as it can be seen in **Figure 1** and **Table 1** for EPS extracted by DC, similar results were found on the other extractions methods (data not shown). The FT-IR spectrum of EPS confirms the presence of primary amine, aromatic compound, aliphatic and polysaccharides [23, 31, 32].

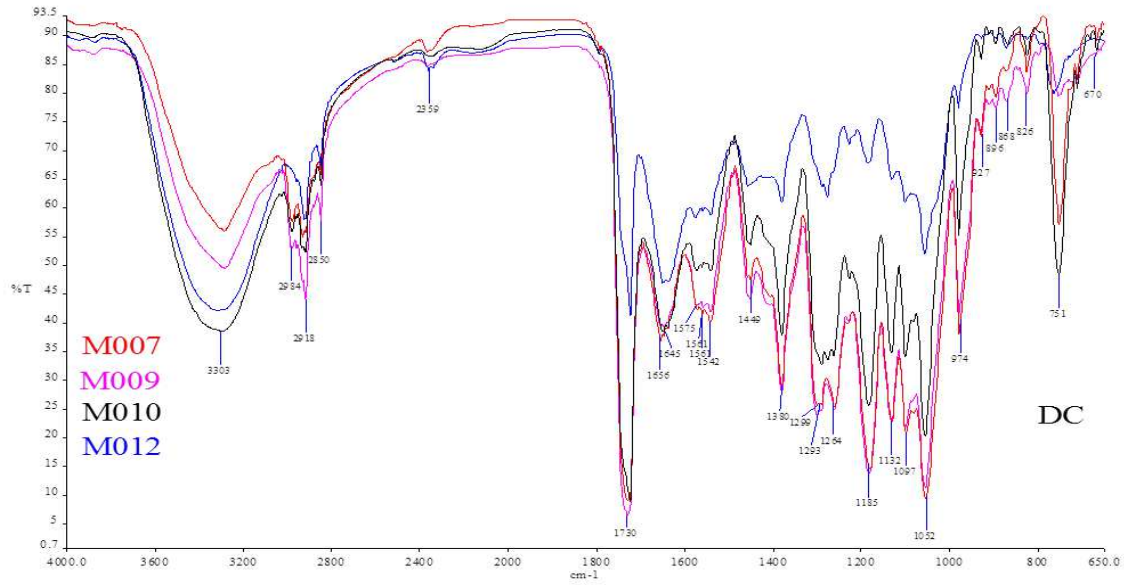

Figure 1. FT-IR of *Micromonospora* sp. strains: M007, M009, M010, M012 in DC

Table 1. Assignment of functional groups by FT-IR

Signals (cm^{-1})	Stretching vibration	Allocation
3291-3287	N-H and O-H	Polysaccharides and proteins
1200-900	C-O-C and C-O	polysaccharides
2924-2850	$-CH_3$ and $-CH_2$	Lipids
1740-1680	C=O	lipidic esters
1695-1655	NH_2 and -COO	amide I and α-helix of protein
1550-1520	C=O, -COO and NH_2	amide II bonds of proteins
1415-1400	$-COOCH_3$, $-CH_2-C=C-$ and $-CH_2-CO-$	amino acids and fatty acids
1240 to 1236	P=O	nucleic acid and phospholipids

Nuclear magnetic resonance (NMR)

The ^{1}H NMR spectra of EPS reveal characteristic chemical shifts (ppm), corresponding to functional groups of carbohydrates and proteins, in **Figure 2** shows the spectra obtained of EPS with DC (control method). The ppm (t) of δ 4.70 to 5.13 (d, *J* = 1.2 Hz) and δ 4.56 to 4.31 range (d, *J* = 1.2 Hz), are attributed to α-anomerics carbons for some hexoses, having from two to three anomeric carbons for each strain analyzed (M007, M009, M010 and M012), and extraction method studied (DC, EDTA and NaOH), giving a coupling constant (*J*) approximately 3.6 Hz. Carbons α- and β- anomeric are diastereoisomers have differences observed in the NMR spectra [33, 34]. We also observe a series of overlapping peaks in the region of the ring carbohydrates into δ range 3.0 to 4.0 ppm assigned to δ proton of the carbon that forms the glycosidic bond. A relaxation of the N-H was observe at δ 1.3 ppm associated with the coupling of primary amides and some additional signals, not associated with carbohydrate between δ 0.8 and 3.0 ppm due mainly amino acids. With this signal the presence of carbohydrates and protein is confirmed [35]**.**

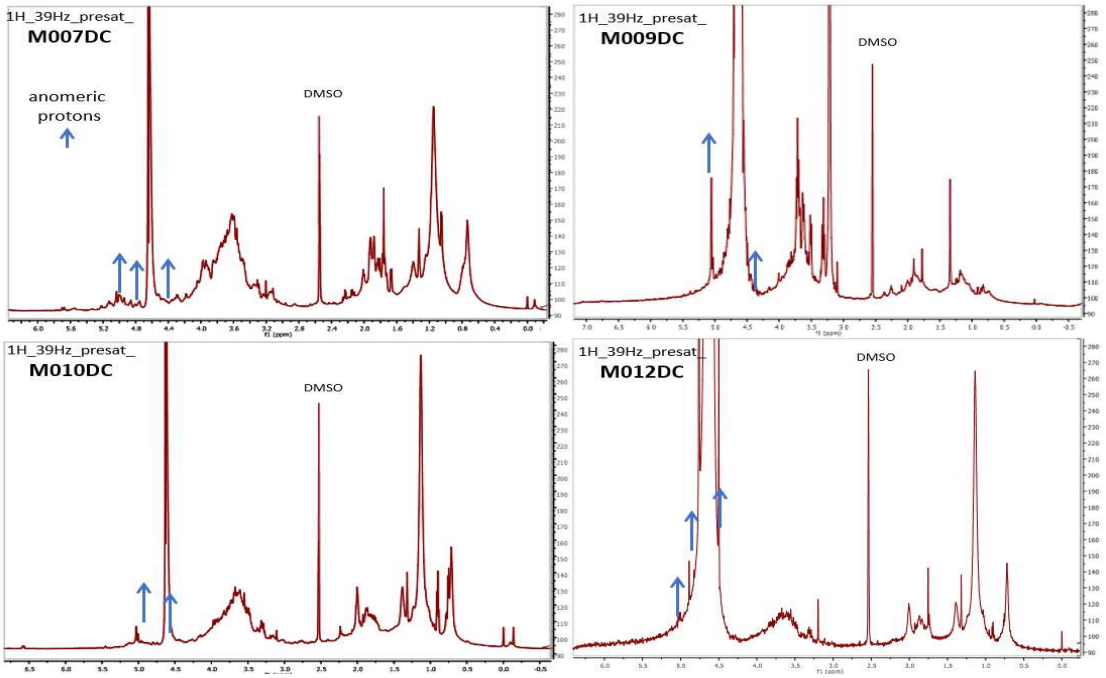

Figure 2. Proton nuclear magnetic resonance (^{1}H NMR) spectra of EPSs extracted from *Micromonospora* sp. strain (M007, M009, M010 and M012 by DC)

The 2-D spectra showed signs related to anomeric protons, making the allocation of protons associated with displacement ^{1}H / ^{13}C NMR (600MHz, D_2O), ppm: δ 5.05 / 93.44 , 3.69 / 71.99, 29.03 / 69.54, 3.71 / 60.35, 3.61 / 60.33, 2.53 / 38.56, 2.55 / 38.05, 2.53 / 37.94, and ppm lower in rank from δ 28.4 to 13.32; confirming the presence of monomers, dimers, trimers and tetramers, without the possibility of distinguishing between any of them (spectra not shown). The usefulness of these experiments is based on the fact that despite the structural similarity carbohydrate, present a characteristic HSQC spectra.

Mass spectroscopy (MS)

It is a widely used technique for the determination of polymers, since it has proved to be fast and sensitive method for structural analysis of oligosaccharides [36]. Analysis of carbohydrates by mass spectrometry (MS) is required not only for the complex nature of these molecules [37]. In **Figure 3**, we can see the MS spectra of EPS samples previously hydrolyzed in the analysis of the M007 strain by the three extraction methods, and the spectrum denotes the presence of hexoses, obtaining a typical spectrum of a carbohydrate chain. The mass 202.9 m/z positive ion mode, corresponding to a hexose with adduct of sodium. Besides, the ion mass 365.1 m/z was also observed in the

positive ion mode, corresponding to the masses of disaccharides of hexose-hexose with adduct of sodium. Peaks of mass 527.2 m/z correspond to a trisaccharide and a signal of mass 689.4 m/z corresponding to a tetrasaccharide.

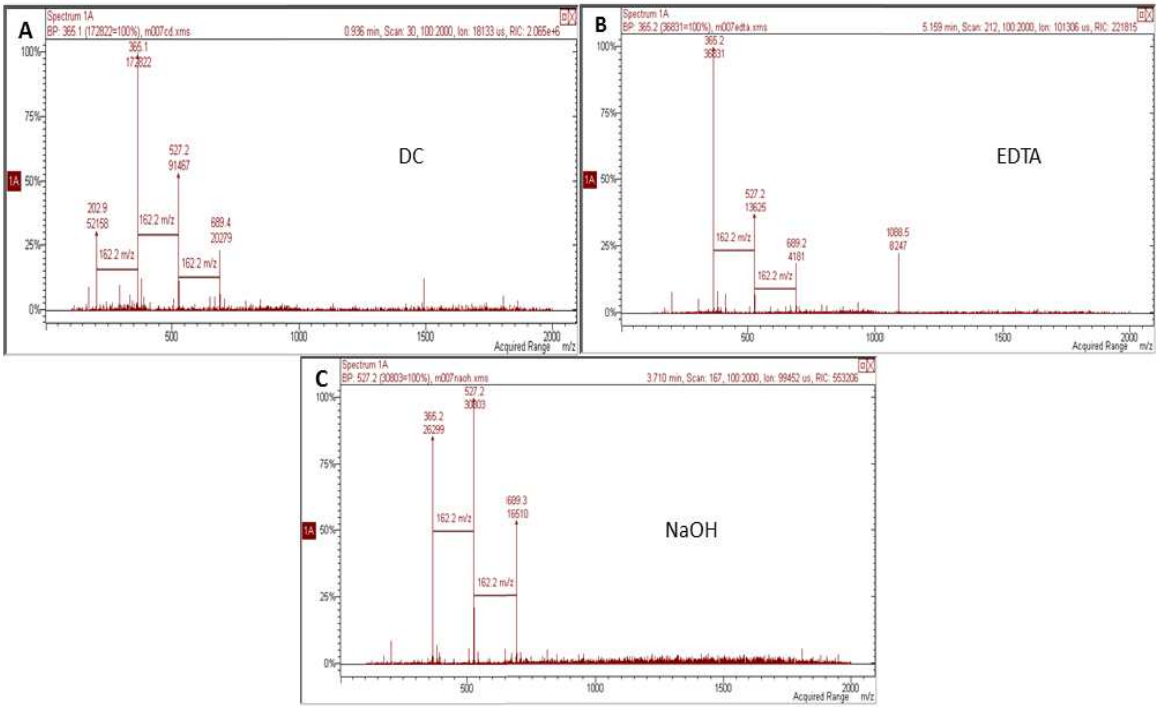

Figure 3. MS spectra of EPS extracted by A) DC (direct centrifugation), B) EDTA and C) NaOH of M007 *Micromonospora* sp. strain

Considering that the use of electrospray ionization in positive ion mode leads to the formation of adducts, such as $[M + NH_4]^+$, $[M + Na]^+$, $[M + K]^+$ or $[M + M + Na]^+$, the molecular ion $[M + H]^+$ is not in all cases the most abundant ion present in the MS spectra. In this respect our spectrum reveals the presence of adduct Na^+ ions (as detected by spectroscopic analysis and EDS) by the difference of molecular weights [35, 38].

The difference between mass signals found is 162.2 m/z, which corresponds to the glycosidic bond of the monosaccharide unit less the loss of a water molecule 180 -18 = 162 m/z, highlighting as the first MS spectra of EPS extracted from actinobacterial strains of the genus *Micromonospora* sp. reported.

Carbohydrates assignment in EPS using HPLC and TLC

Polysaccharides have several essential functions, in this case EPS formation, usually associated with adherence to surfaces and structural integrity maintenance. Their chemical nature can vary depending on the producing microorganisms. EPS are constituted by high molecular weight polysaccharides (10 to 30 kDa), these are generally heteropolymers, which contain monosaccharides like hexoses and pentoses, which unite to form the EPS [39]**.**

Carbohydrates standards were used for identification and carbohydrates assignment by HPLC present in EPS as it can be seen in **Figure 4**: xylose (1), arabinose (2), mannose (3), galactose (4), maltose (5), melibiose (6), trehalose (7), raffinose (8), nystose (9) and fructofuranosilnystose (10).

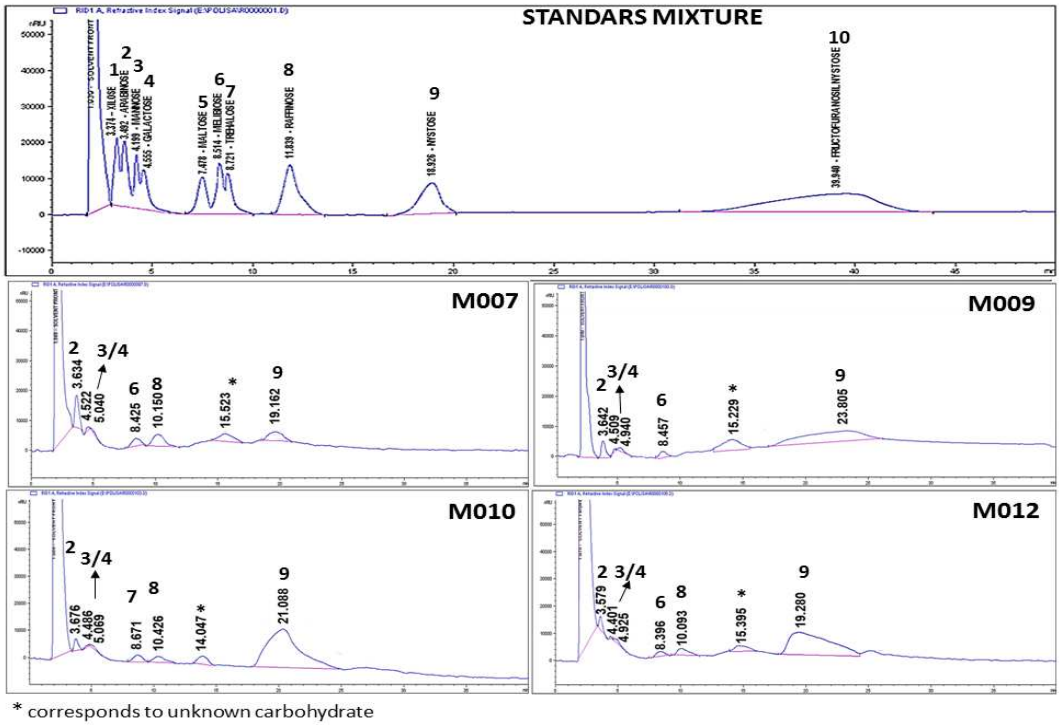

Figure 4. Chromatograms obtained by HPLC: Peak assignment in EPS hydrolysate obtained by DC in all strains

The carbohydrates assignment of the EPS based on retention time (t_r) was performed with a margin of ±0.5 min. Carbohydrate profile was affected by the EPS extraction method and the degree of hydrolysis. The carbohydrates found on EPS samples were xylose, arabinose, mannose, galactose (monosaccharides), melibiose, trehalose (disaccharides), raffinose (trisaccharide) and tetrasaccharides such as nystose, founding differences for each strain analyzed. Unknown carbohydrates were also detected during the HPLC analysis of EPS observed in the chromatogram as unassigned peaks (t_r of margin 14 to 15 minutes in all strains). Carbohydrate profile with EDTA and NaOH extractions was similar. Carbohydrates standards and EPS samples were analyzed at the same time by TLC and identified by their retention factor (Rf) see **Figure 5**.

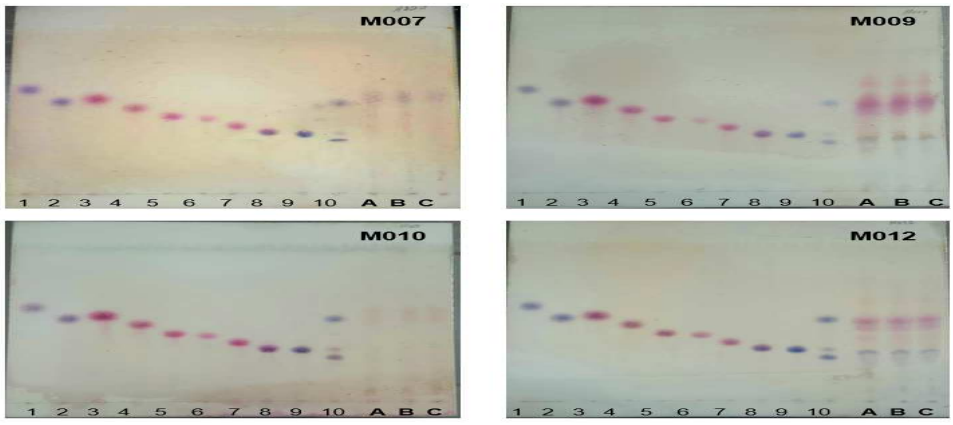

Figure 5. Carbohydrate TLC.1.-xylose, 2.-arabinose, 3.-mannose, 4.-galactose 5.-maltose, 6.-melibiose, 7.-trehalose, 8.-raffinose, 9.-nystose, 10.-fructofuranosilnystose. A.-DC, B.-EDTA, C.-NaOH

With these techniques the profile of carbohydrates present in the samples EPS produced by *Micromonospora* sp. strains was confirmed. Xylose, arabinose, mannose and galactose are sugars found in most terrestrial *Micromonospora* species [40, 41], while nystose and other carbohydrates yet to identify, were carbohydrates found in EPS from marine strains, being a possible differentiation with EPS from terrestrial *Micromonospora* species.

In addition the EPS containing polysaccharides and proteins have moieties non-sugar such as uronic acid, pyruvates, hexosamines, acetates, sulfate esters, and small amounts of lipids and nucleic acids, although these non-sugar components are present in small quantities, these components give unique characteristics to the EPS [42].

SEM and EDS analysis.

The EPS morphology of the strain M007 is seen on **Figure 6**, where it can be seen that the EPS extracted with EDTA and NaOH showed a higher agglomeration compared to direct centrifugation. The morphology can be observed in these organic samples as a matrix of polymer chains, is observed that the electron beam decomposed the samples, because no covering was used to avoid interference with elemental analysis (EDS).

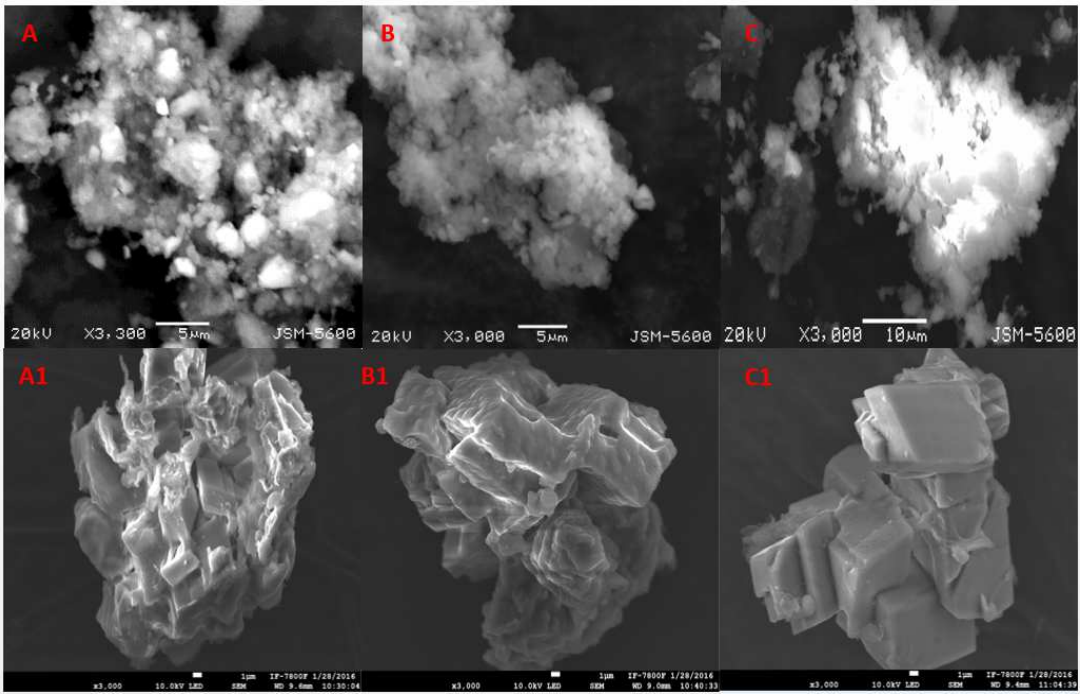

Figure 6. EPS observed by SEM, A) DC, B) EDTA, C) NaOH in JEOL5600 with thermionic emission with coal covering and SEM, A1) DC, B1) EDTA, C1) NaOH by JEOL F7800 with field emission, in strain M007

The analysis showed that microbial cells are embedded randomly within the microstructure of the EPS, there are also opened channels allowing the exchange of water present in the environment where the organism is developed, allowing nutrients access to deeper areas of the micro-colonies. The nutrients exchange provided by the EPS architecture; also allow these communities develop a considerable complexity, maintaining the optimal nutritional conditions [43].

The EDS analysis is shown in **Table 2,** a higher amount of carbon, oxygen, calcium, sulphur and phosphorous was observed with the chemical extractions techniques (EDTA and NaOH), compared to the physical method (DC).

Table 2. Elemental composition of EPS samples extracted by different methods (n=5)

%	Strain M007			Strain M009			Strain M010			Strain M012		
	Extraction Method			Extraction Method			Extraction Method			Extraction Method		
	A	B	C	A	B	C	A	B	C	A	B	C
C	39.0	64.0	55.8	54.3	58.7	43.9	19.6	54.4	53.8	55.6	56.3	54.3
N	19.4	14.4	-	-	11.3	11.0	-	13.9	17.0	-	15.2	9.4
O	23.7	20.8	34.0	34.9	28.7	37.8	33.1	28.3	26.0	42.2	22.7	33.8
Na	0.7	0.3	0.6	-	0.2	0.7	-	0.7	1.3	-	1.2	0.6
P	-	0.2	2.4	2.4	0.4	1.1	12.1	1.1	0.6	1.0	1.2	1.1
S	-	-	0.4	-	-	0.3	-	-	0.3	-	-	-
Cl	-	-	-	-	-	0.3	-	-	-	-	-	-
Ca	17.3	0.3	6.9	8.4	0.7	5.0	35.3	1.5	1.1	1.2	3.4	0.9

A: DC, B: EDTA, C: NaOH

Analyzing the extraction techniques, different amounts of calcium are observed (**Table 2**), attributed to the composition of GYM medium. The presence of Ca^{2+} increases the mechanical stability of the EPS because of the crosslinking of the biopolymers formed, this fact suggests that there are key bond between the components of the EPS with physicochemical interactions such as hydrogen bonds, Van der Waals force and electrostatic interactions [44]. Other elements such as sodium, sulphur and phosphorous attributed to proteins, amino acids, phospholipids and nucleic acids these elements are mostly in chemical extractions, not all strains, thus confirming the cell lysis provoked by these methods.

CONCLUSION

The presence of sugars commonly found in terrestrial *Micromonospora* strains was demonstrate, being these arabinose and xylose. Nystose, a different sugar from terrestrial *Micromonospora* strains was detected in marine *Micromonospora* sp. strains from the Gulf of California, This might be useful in the differentiation between these kind of strains. Sugar assignment was possible with HPLC, TLC, MS and NMR without purifying the mixture.

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