

Bacterial exopolysaccharides from extreme marine habitat of Southern Ocean: Production and partial characterization

Exopolisacáridos bacterianos obtenidos desde hábitat marino extremo del Océano Antártico: Producción y caracterización parcial

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ABSTRACT

Deep marine microorganisms survive under extreme ecological settings and harsh environmental conditions of low temperature, high salinity, and high atmospheric pressure making it significant of scientific interest. Southern Ocean (SO) is one such example of deep marine ecosystem and the microorganisms inhabiting in such hostile environment may produce different bioactive secondary metabolites. SO (Indian Sector) is relatively less documented in terms of microbial composition and community dynamics. The present study involves isolation of exopolysaccharides (EPSs) from three potent SO (Indian Sector) bacteria, optimization of the EPS production and partial characterization of them. Three different EPSs show varying structural conformation, that is from porous to strong flakes mimicking polymeric structure with C/N ratio ranging between 4 - 11. FTIR spectra have exhibited the presence of different active groups of carbohydrate moieties, water molecules and protein-associated amides. EPSs produced by marine microorganisms show high biotechnological promises such as drug carrier in pharmaceutical field, emulsifier and cryo-protectant in food-processing industry, detoxification of petrochemical oils and much more. The three bacterial isolates in this study showed potential of producing EPS biopolymer that can be further explored in terms of its proper biotechnological applications.

Keywords: Southern Ocean, marine bacteria, exopolysaccharides characterization

RESUMEN

Los microorganismos marinos de zonas profundas sobreviven en entornos ecológicos extremos y condiciones ambientales adversas de baja temperatura, alta salinidad y alta presión atmosférica, lo que los hace tener un interés científico particularmente significativo. El Océano Antártico, es uno de esos ejemplos de ecosistemas marino profundo, y los microorganismos que habitan en ese ambiente hostil pueden producir diferentes metabolitos secundarios bioactivos. El Océano Antártico (sector indio) está relativamente menos documentado en términos de composición microbiana y dinámica comunitaria. El presente estudio consiste en el aislamiento de los exopolisacáridos (EPS) de tres potentes bacterias del Océano Antártico (sector indio), la optimización de la producción de EPS y la caracterización parcial de los mismos. Tres EPS diferentes muestran una conformación estructural variable, es decir, de copos porosos a fuertes que imitan la estructura polimérica con una relación C/N que oscila entre 4 y 11. Los espectros FTIR han exhibido la presencia de diferentes grupos activos de macromoléculas de carbohidratos,

moléculas de agua y amidas asociadas a proteínas. Los EPS producidos por microorganismos marinos muestran un alto potencial biotecnológico, como transportador de drogas en el campo farmacéutico, emulsionante y crioprotector, todos ellos en la industria de procesamiento de alimentos, desintoxicación de aceites petroquímicos y mucho más. Los tres aislamientos bacterianos de este estudio mostraron potencial de producción de biopolímeros de EPS que pueden ser explorados más a fondo en términos de sus aplicaciones biotecnológicas adecuadas.

Palabras clave: Océano Antártico, bacterias marinas, caracterización de exopolisacáridos

INTRODUCTION

As the ocean is an important sink for the rapidly increasing anthropogenic CO₂ concentration, flux of biogenic carbon to deep ocean water must be monitored on a regular basis (Delille 2004). The Southern Ocean (SO) contributes around 15% of the primary production of the oceans and thus play an important role in sequestering atmospheric CO₂ through physical and biological processes (Huntley *et al.* 1991). Biodiversity of SO is least discovered element, making it a hub of unexplored biotic communities including Bacteria, Microalgae, Bryozoans, Diatom, Sponges, Coral, Tunicate or Fungi (Spolaore *et al.* 2006). Marine microorganisms are of particular interest because it survives under extreme ecological pressure and harsh environmental conditions; viz. low temperature, high salinity, high atmospheric pressure and exposure to radioactivity. Marine aggregates are also abundantly present in all the oceans around the world (Fowler & Knauer 1986). Under hostile marine conditions, microorganisms produce several secondary metabolites including exopolysaccharides (EPSs) as a part of survival strategy (Nichols *et al.* 2005, Ewert & Deming 2013). EPSs also provides a network that hold the bacterial structures together (Flemming & Wingender 2001). EPSs such as dextran, xanthan, cellulose, succinoglycan are of high economic importance with good industrial values (Freitas *et al.* 2011, Banerjee & Bandyopadhyay 2016, Halder *et al.* 2017). Dextran, specifically is the first commercialized bacterial EPS and is highly used in biomedical applications (Banerjee & Bandyopadhyay 2016).

Being one of the relatively unknown habitats of the earth with several polyextremophilic factors (De Broyer & Danis 2011) and secondary metabolites or bioactive compounds produced by the SO microorganisms, it just might be a potent source of novel bioproduct with promising applications. EPSs also have an important role in Antarctic marine environment possibly acting as a ligand to the circling trace nutrients and cryo-protection for microbial growth at low temperature-high salinity environment (Nichols *et al.* 2005). Our present study

is a first step to understand the biological potential of the SO bacteria and the role of bacterial EPSs in relatively less studied SO (Indian sector) environment. This study involves, isolation of EPSs from three potent SO (Indian Sector) bacteria, production optimization for maximum yield and partial characterization of the EPSs to get an idea about the structural composition of the constituting polysaccharides.

MATERIALS AND METHODS

BACTERIAL ISOLATES

Three different marine bacteria *Zunongwangia profunda* MB-24 (KF019669), *Marinobacter algicola* MB-44 (KF019649) and *Halomonas hydrothermalis* MB-45 (KF019650) have been isolated from water sample taken from the Southern Ocean (Indian Sector) at a depth of 30 m, 500 m and 500 m respectively. Image of the study site has been adapted from Google Earth Pro. Ink. and is represented in Fig. 1. The methods of sampling and isolating the strains are described earlier (Gupta *et al.* 2015). Throughout the study, the strains are maintained at 20 °C and routinely grown in Zobell Marine Broth (Himedia®).

MICROSCOPIC STUDY

Morphological characterizations of the EPS producing marine isolates were done by fluorescence microscopy. Cultures were grown overnight on Zobell Marine Broth at 20 °C and cell pellet were formed by centrifugation at 500 rpm for 5 min. They were then washed with 1× PBS buffer (pH 7.4) for several times and incubated at room temperature with zinc acetate for 30 min. Excess zinc acetate was washed with 1× PBS buffer (pH 7.4). Each sample was smeared over a clean grease free slide. Over this smear one drop 1 mM zinc binding SALTm ligand (Kumari *et al.* 2015) was placed and covered with cover slip to observe under fluorescence microscope at 450 nm emission (Leica, DM 1000) by 100× objective lens.

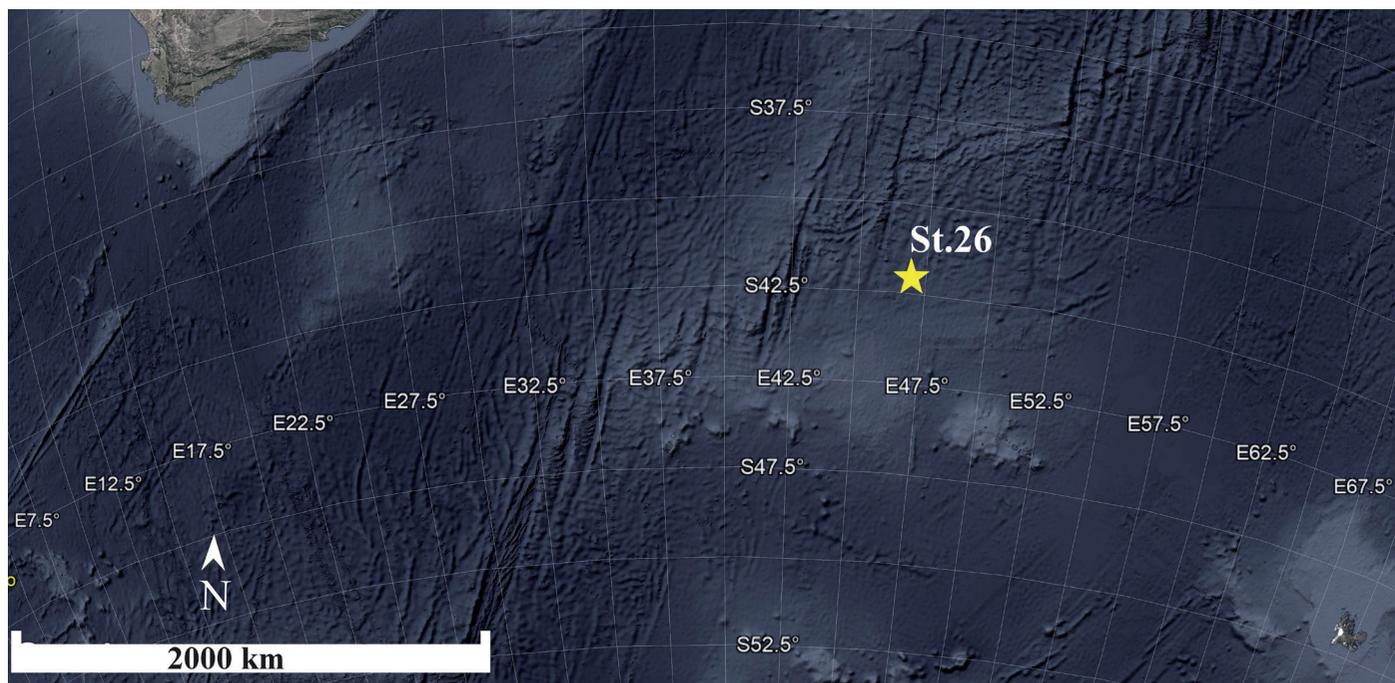


FIGURE 1. Study map representing the water sample collection point of station 26 in Southern Ocean (Indian sector). / El Mapa de estudio representa el punto de toma de muestras de agua de la estación 26, en el Océano Antártico (sector indio).

EPS PRODUCTION AND OPTIMIZATION

Production and purification of the EPSs were done following the previously reported procedures (Banerjee *et al.* 2018, Sengupta *et al.* 2019). Zobell Marine Broth (Himedia®) was used for the growth of *Z. profunda* MB-24, *M. algicola* MB-44 and *H. hydrothermalis* MB-45. The cultures were maintained at 20 °C with 150 rpm shaking for 7 days. The production of EPS was done through centrifuging the culture medium in order to separate the cells from the supernatant at 15,000 ×g for 20 min. The cell-free supernatant was collected for extraction of released EPSs. EPSs were extracted through a vacuum evaporating to half volume. To the solution, equal volumes of chilled ethanol was added, mixed and kept in at -4 °C for 24 h for precipitation of polysaccharides. Precipitates of formed EPSs were collected by centrifugation at 15,000 ×g and 20 min, washed with same chilled solvent for few times. After that, it was centrifuged at 4 °C, 15,000 ×g for 20 min in order to remove the proteins denatured by trichloroacetic acid. Finally, the purified EPS was obtained after dialysis followed by lyophilization. The dry weight of the EPS was calculated and stored for further analysis. Subsequently, the entire process was repeated with methanol, acetone and isopropanol as solvent to observe the change in EPS precipitation.

Media for the best EPS production was optimized by the addition of different carbon sources to the media (Glucose

and Sucrose) followed by variation of the carbon source percentage supplemented with Zobell marine Broth as the standard (pH 7.5). This media was further optimized by varying the nitrogen source content (Peptone and NH₄Cl). The inoculated flasks were incubated for 7 days at 20 °C with 150 rpm shaking followed by same EPS extraction protocol as mentioned above.

SURFACE MORPHOLOGY CHARACTERIZATION

The surface morphology of isolated bacterial EPSs was analysed through scanning electron microscopy (SEM) (S530 Hitachi) study. The lyophilized EPSs were placed on an adhesive carbon tape. Gold coating (300 Å) of the samples were performed using IB2 ion coater to make it conductive. The observations were made on surface morphological similarities of the different EPSs at different magnifications.

ELEMENTAL ANALYSIS

The total nitrogen, carbon, hydrogen and sulfur content of the EPS samples determined using a CHNS analyzer (Vario EL III, M/s Elementar, Germany). For CHNS analysis, lyophilized and powdered EPS samples were weighed (5-10 mg) and mixed with an oxidizer (vanadium pentoxide) in a tin capsule, which was then combusted in a reactor at 1000 °C. The EPSs were run along in order to find out the total content of CHNS present in samples with the C/N ratios.

INFRA-RED SPECTROSCOPIC ANALYSIS

Spectrums of bacterial EPSs were tested using a fourier transform infrared spectrophotometer (IR-Prestige 21, Shimadzu Corporation, Japan). The spectrums of EPSs were obtained by KBr pellet method where the EPSs were pressed into dry Potassium bromide (KBr) at a sample: KBr ratio of 1:90 to form a disc of around 3 mm diameter. These spectra were further analyzed for interpretation of the major functional groups present in the EPSs after subjecting to FTIR- spectra measurement in the frequency range of 400 and 4000 cm^{-1} .

RESULTS

MICROSCOPIC STUDY

Fluorescence microscopic observation revealed that the EPSs producing marine isolates were small branched rod or coccoid shaped bacteria (Figs. 2A, 2B and 2C). All the three marine

isolates; viz. *Z. profunda* MB-24, *M. algicola* MB-44 and *H. hydrothermalis* MB-45 have shown one common unique feature of closely attached clumps of bacterial cells which are probably an indication of EPS production for survival at extreme temperature by staying together under the sheath of EPS.

EPS PRODUCTION AND OPTIMIZATION

Highest EPS production was observed by *H. hydrothermalis* MB-45, which was 5.26 g/l with 1% glucose and sucrose combination added to the media as carbon source and isopropanol as solvent for precipitation. While both *Z. profunda* MB-24 and *M. algicola* MB-44 produced EPSs maximum 2.4 g/l and 4.4 g/l respectively when precipitated using ethanol as a solvent and 2% Glucose as added carbon source in production medium. Methanol was comparatively proved to be a poor solvent for EPS precipitation, (Figs. 3A and 3B) for all the three different Southern Ocean isolates.

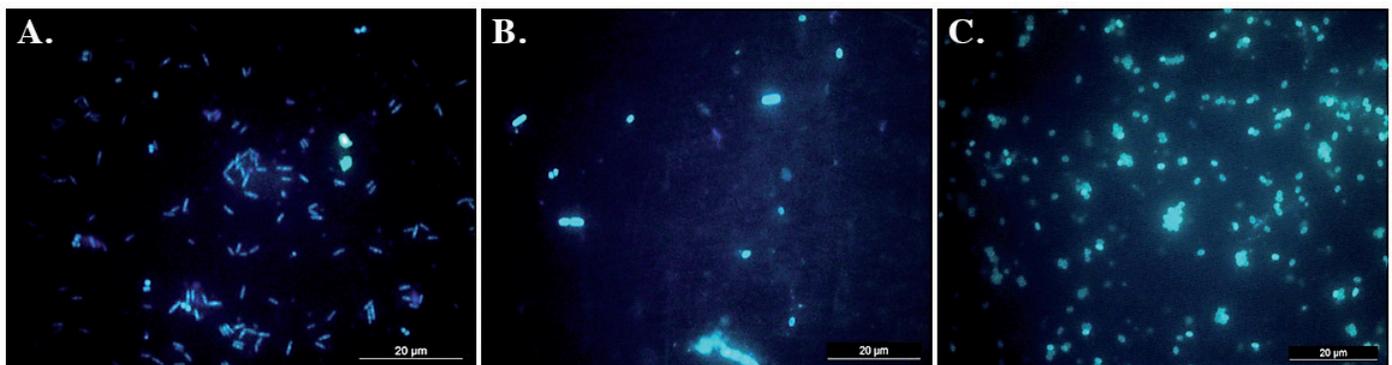


FIGURE 2. Fluorescence microscopy images of the EPS producing Southern Ocean bacteria; where A. *Zunongwangia profunda* MB-24, B. *Marinobacter algicola* MB-44 and C. *Halomonas hydrothermalis* MB-45 respectively. / Imágenes de microscopía de fluorescencia de EPS producido por bacterias del Océano Antártico; donde A. *Zunongwangia profunda* MB-24, B. *Marinobacter algicola* MB-44 y C. *Halomonas hydrothermalis* MB-45 respectivamente.

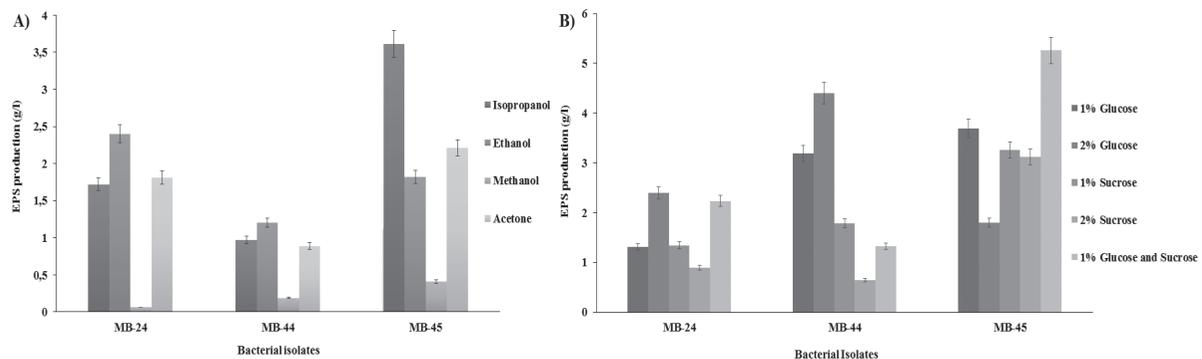


FIGURE 3. Production and optimization of EPS by the Southern Ocean bacteria; where A. Optimization of EPS production by solvent variation and B. Optimization of EPS production by varying the carbon source using the best solvent for EPS precipitation. The error bars represent 95% confidence interval for both cases. / Producción y optimización de EPS por bacterias del Océano Antártico; donde A. Optimización de la producción de EPS mediante variación del solvente y B. Optimización de la producción de EPS mediante la variación de la fuente de carbono usando el mejor solvente para la precipitación de EPS. Las barras de error representan un intervalo de confianza del 95% para ambos casos.

SURFACE MORPHOLOGY CHARACTERIZATION

The lyophilized EPS were found to be a creamish powder, which was water soluble in nature. Surface morphology of the EPSs characterized by SEM, revealed its structural similarity with earlier reported EPSs of Southern Ocean (Indian Sector)-

origin marine resources (Sengupta *et al.* 2019). While *Z. profunda* MB-24 showed porous structure (Figs. 4A and 4B); *M. algicola* MB-44 (Figs. 4C and 4D) and *H. hydrothermalis* MB-45 (Figs. 4E and 4F) exhibited compact flakes-like structural EPS units.

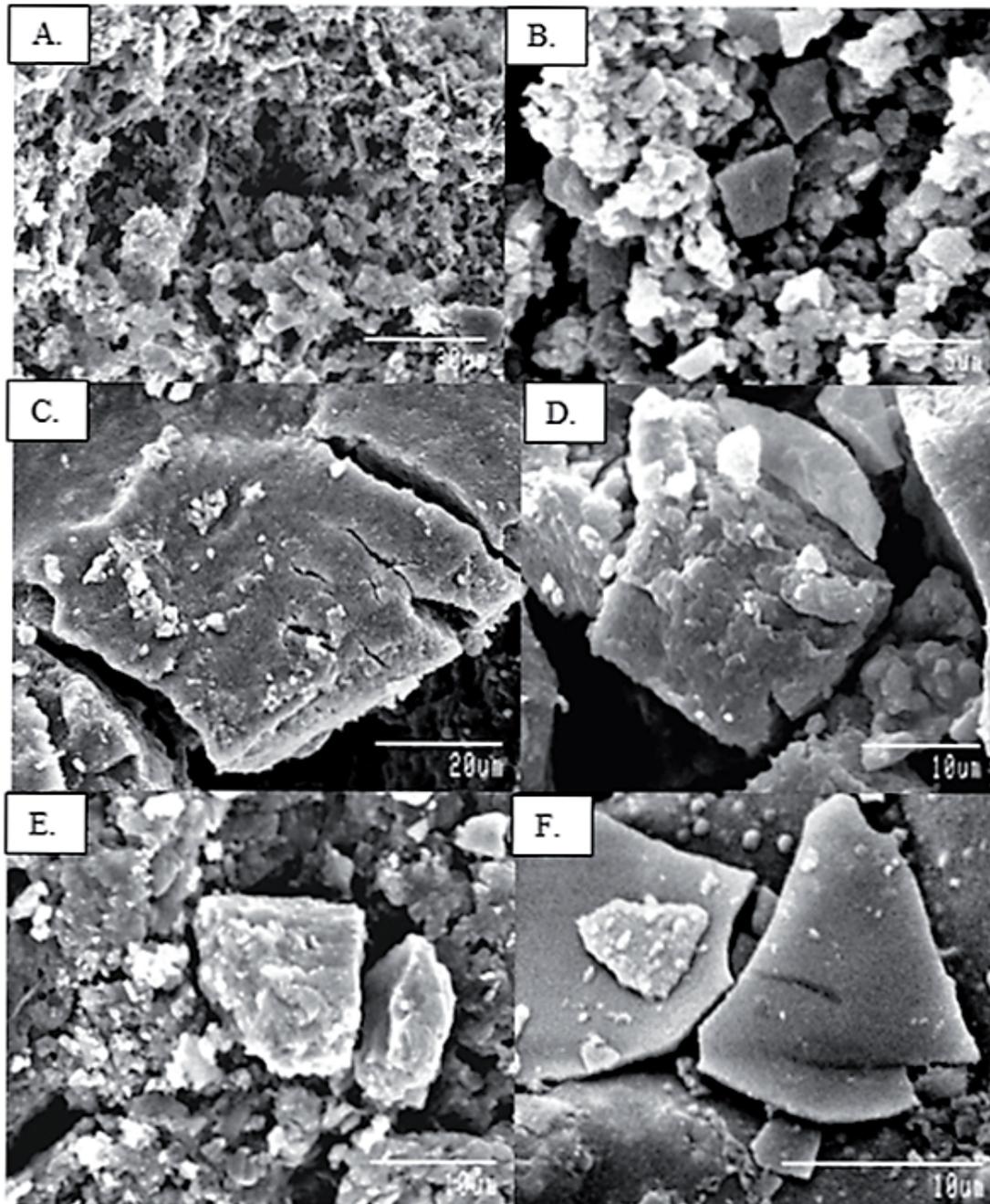


FIGURE 4. Surface morphology characteristics of the EPSs; where A. and B. *Zunongwangia profunda* MB-24 at 1500× and 6000×, C. and D. *Marinobacter algicola* MB-44 at 1500× and 3000×; and E. and F. *Halomonas hydrothermalis* MB-45 at 3000× and 5000× magnifications respectively. / Características morfológicas superficiales de los EPSs; donde A. y B. *Zunongwangia profunda* MB-24 a 1500× y 6000×, C. y D. *Marinobacter algicola* MB-44 a 1500× y 3000×; E. y F. *Halomonas hydrothermalis* MB-45 a 3000× y 5000× de magnificación respectivamente.

ELEMENTAL ANALYSIS

Elemental Analysis of EPSs revealed a C/N ratio range of fresh organic matter. Percentage of sulphur in trace amount also proved the isolated EPSs to be in pure polysaccharide form. Normally, polysaccharide-rich samples showed only small amounts of nitrogen ~1% (Synytsya *et al.* 2009). However, sometimes aminated polysaccharides contains

~5% nitrogen too (Du *et al.* 2017). Thus, from the results of elemental analysis EPS produced by *Z. profunda* MB-24 was polysaccharide rich; while *M. algicola* MB-44 and *H. hydrothermalis* MB-45 produced EPSs were probably aminated EPSs (Table 1). C/N ratio of *Z. profunda* MB-24, *M. algicola* MB-44 and *H. hydrothermalis* MB-45 are 11.59, 8.921 and 4.896 respectively.

TABLE 1. CHNS percentage and C/N ratio present in the EPSs produced by the Southern Ocean bacteria. / Porcentaje de CHNS y relación C/N presente en los EPS producidos por bacterias del Océano Antártico.

Sample	C/N ratio	% of Carbon	% of Hydrogen	% of Nitrogen	% of Sulfur
<i>Zunongwangia profunda</i> MB-24	11.590	19.690	4.993	1.698	1.351
<i>Marinobacter algicola</i> MB-44	8.921	26.720	6.243	2.995	0.771
<i>Halomonas hydrothermalis</i> MB-45	4.896	28.940	6.292	5.911	0.698

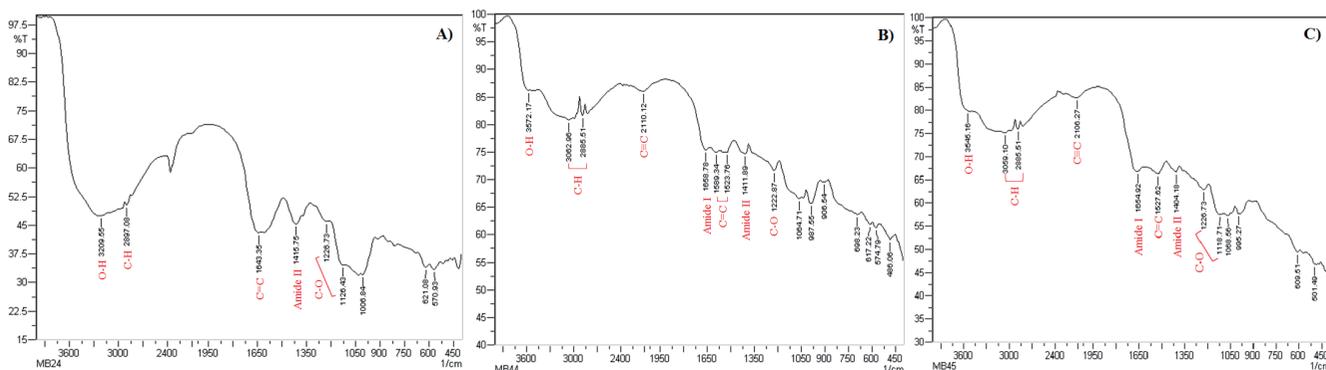


FIGURE 5. FTIR spectra of the EPSs produced by the Southern Ocean bacteria; where A. *Zunongwangia profunda* MB-24, B. *Marinobacter algicola* MB-44 and C. *Halomonas hydrothermalis* MB-45 respectively. / Espectros FTIR de los EPS producidos por bacterias del Océano Antártico; donde A. *Zunongwangia profunda* MB-24, B. *Marinobacter algicola* MB-44 y C. *Halomonas hydrothermalis* MB-45 respectivamente.

INFRA-RED SPECTROSCOPIC ANALYSIS

Infrared spectra of the EPSs extracted from all three marine isolates showed common specific absorbance of broad O-H stretching at 3200-3600 cm^{-1} , strong C-H stretching at 2850-3000 cm^{-1} , variable C=C stretching at ~1640 cm^{-1} , and strong C-O stretching at ~1200 cm^{-1} . Besides this, C=O of amide I at ~1680 cm^{-1} indicated the presence of protein-associated amides in the EPSs produced by *M. algicola* MB-44 and *H. hydrothermalis* MB-45 (Hellwig *et al.* 1998, Cao *et al.* 2011, Demirbaş *et al.* 2013). Presence of Amide II (N-H bending vibration and C-N stretching vibration) were also observed with the peaks ~1400 cm^{-1} . Overall, the results indicate the presence of water molecule of polysaccharide compound, alkane groups, alkenes, methylene group of alkanes respectively (Figs. 5A, 5B and 5C). *M. algicola*

MB-44 produced EPS is relatively complex in branching due to the presence of characteristic skeletal breathing of polysaccharide macromolecules (Fan *et al.* 2012).

DISCUSSION

Microbial EPSs are ubiquitous in extreme marine environment where they are essential for bacterial survival. EPS is biopolymeric in nature and helps in biofilm formation (Di Donato *et al.* 2016). These biofilms provide a protection sheath to microorganisms by formation of complex association among cells and entrapment of nutrients (Di Donato *et al.* 2016). Thus, they assist the microbial communities to tolerate extreme temperature, salinity and allow nutrient availability

by creating a border between bacterial cells and its immediate environment (Kumar *et al.* 2018). Several EPSs produced by marine microorganisms show biotechnological promises (Dionisi *et al.* 2012). Marine prokaryotes are reported to produce biopolymers novel biomaterial properties and industrial suitability (Weiner 1997). Examining their structure and physio-chemical characteristics as focused in this present study is just a primary draft step to a large field of possibilities. Further, it is possible to gain insight into their commercial applications in pharmaceutical industry and food-processing fields or bioremediation of petrochemical spillage (Weiner 1997, Di Donato *et al.* 2016). All the three SO (Indian Sector) origin bacteria reported in this study were found to be good producers of EPSs but *H. hydrothermalis* MB-45 produced maximum EPS of 5.26 g/l. The C/N ratio of the EPSs were in the range of 4 – 11. C/N ratios in the range of 4-10 were usually reported to be from marine sources (Gray & Biddlestone 1973). In a previous report by Sengupta *et al.* (2019), a *Pseudoalteromonas* isolate of SO (Indian Sector) origin showed similar C/N ratio of the EPS. This supports the elemental analyses of this present study. Surface morphology of the EPS produced by *Z. profunda* MB-24 showed structural identities to earlier reported EPS from SO (Indian sector) origin *Pseudoalteromonas* sp. in terms of smooth and distinct porous regions in the EPS (Sengupta *et al.* 2019). Porous EPS produced by *Z. profunda* MB-24 was probably polysaccharide macromolecules joined each other with α -glycosidic bonds and were polysaccharide-rich. Compact EPSs produced by *M. algicola* MB-44 and *H. hydrothermalis* MB-45 were found to be amine-substituted. While compact structure of EPS indicated its potential to be used as viscosifier, thickener or stabilizing agent for use in food industry because of plasticized film like property (Sajna *et al.* 2013); porous EPS structure were proven to be a good candidate for heavy metal biosorption (Qiang *et al.* 2013), while also having the capillary force to hold more water (Rani *et al.* 2017). The fibrous pores of bacterial EPSs were already proved to help bacterial cells to stay attached to each other due to high affinity interactions (Rani *et al.* 2017). Thus the psychrophilic bacterial isolates from SO explored in this study may have produced EPSs as an extracellular response to the environmental constraints. Bacterial EPS production as a response to cold, sea environment was explained earlier by Ewert & Deming (2013). Infra-red spectra of the three EPSs showed distinctive functional groups normally present in polysaccharide macromolecules. *M. algicola* MB-44 produced EPS was particularly interesting as it shows highly complex aminated polysaccharide branching along with characteristic skeletal breathing (Fan *et al.* 2012) of polysaccharides. Presence of major amount of proteins in EPS produced by SO

bacteria is already earlier reported by Nichols *et al.* (2005). The frequency and types of functional groups present in a EPS macromolecule affect the tertiary structure and overall physicochemical characteristics of the biopolymer in response to the surrounding environment (Decho & Lopez 1993). SO origin bacterial EPSs were reported to have applications in food processing industry as thickening agent, anti-coagulant property, anti-HIV activity, biodegradation for waste water treatment (Nichols *et al.* 2005) and UV-protection capacity (Núñez-Pons *et al.* 2018). Considering that the microbial diversity of SO ecosystem is less documented and both the compact or porous nature of the EPS with substantial functional groups present in the biopolymer as documented in this study, it is reasonable to hypothesize that the isolation and identification of new marine microorganisms and identifying their different secondary metabolites; viz. EPS production will provide a deep insight into the wide opportunities for new industrial applications in future.

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