Polish Journal of Microbiology
2009, Vol. 58, No 4, 289–294

ORIGINAL PAPER

Highly Thermostable Exopolysaccharide Produced by the Moderately Halophilic Bacterium Isolated from a Man-Made Young Salt Lake in Romania

ROXANA COJOC[1], SIMONA MERCIU[1], PETRUTA OANCEA[2], ELENA PINCU[2], LUCIA DUMITRU[1], MĂDĂLIN ENACHE[1*]

[1] Institute of Biology Bucharest of the Romanian Academy, Bucharest, Romania
[2] Department of Physical Chemistry, Faculty of Chemistry, University of Bucharest, Bucharest, Romania

Received 24 April 2009, revised 10 October 2009, accepted 15 October 2009

Abstract

Halophilic bacterial strains isolated from a man-made salt lake in Romania produce a specific exopolysaccharide. This product is synthesized under both static and stirring conditions, and the yield of this exoploysaccharide depends on the composition of the culture medium. The highest amount of polymer was obtained in the presence of casamino acids and yeast extract, under stirring conditions. This polymer has high thermostability, with a melting point at 207°C. The melting process is associated with the thermal degradation of the compound. This polymer is characterized by maximum absorbtion at 260 nm and fluorescence emission at 530 nm. The FT-IR analysis of the pdymer revealed the presence of a saccharide structure and of amine and sulfate groups throughout the sugar backbone.

Key words: halophilic bacteria, halophilic exopolysaccharides, salt lakes microflora, thermostable polymer

Introduction

Microorganisms capable to produce exopolysaccharides (EPSs) inhabit various environments. Therefore, a broad range of bacterial polysaccharides are present in nature and suitable for biotechnological applications. Although the great number of isolated and characterized polymers, only a few are compatible with industrial applications due to their characteristics and the requirements for industrial processes (Sutherland, 1998; 2001). The microbial EPS are used in several biotechnological applications counting food, textile, pharmaceutical, agricultural, paint and petroleum industries. Their composition and structure are very diverse, consisting of homo- or heteropolysaccharides that may contain a number of different organic and inorganic substituents. Many functions have been assigned to microbial exopolysaccharides, such as providing self-protection against antimicrobial compounds, antibodies and bacteriophages, and allowing the adherence to other bacteria or inert surfaces (Sutherland, 2001).

Because of their putative range of applications in medicine, pharmacy, cosmetic and oil industry, the microbial EPSs present a real interest among biotechnologists. The preference for using microbial EPS's instead of vegetable or synthetic polymers in these fields is given by their biodegradation capacity and the improved versatility and efficiency (Mata *et. al*., 2006; Quesada *et. al*., 1993; Sutherland, 1999).

Moderate halophilic bacteria include a wide range of microorganisms of different taxonomy and physiology. Their common feature is their optimum growth in the presence of salt concentrations from 0.5 M to 2.5 M (Kushner and Kamekura, 1988), although they can adapt to large salinity ranges in diverse hypersaline habitats, such as salt lakes, solar salterns, deserts, hypersaline soils. These hypersaline environments are a putative reservoir for unusual microorganisms of biotechnological interest adapted to extreme conditions (Margesin and Schinner, 2001) such us the oil deposits generally characterized by high salt concentrations, where the use of salt-resistant surfactants might constitute a real advantage for the process of

* Corresponding author: M. Enache, Institute of Biology of the Romanian Academy, Splaiul Independentei 296, P.O Box 56-53, Sector 6, Bucharest 060031, Romania; phone: (+40) 21 221 9202; fax: (+40) 21 221 9071; **e-mail: madalin.enache@ibiol.ro; madalin_enache@yahoo.com**

oil recovery. Exopolysaccharide-producing moderately halophilic bacteria can be used for oil recovery if they present specific characteristics such us high viscosity, pseudoplasticity, and resistance to thermal degradation, high salt concentrations and osmotic stress (Arias *et al*., 2003; Bouchotroch *et al*., 2001).

This study reports the isolation and characterization of highly thermostable exopolysaccharides produced by moderately halophilic bacteria isolated from man-made young (around 200 years old) salt lake in Romania resulted after the exploitation of Neogen evaporates.

Experimental

Materials and Methods

Strains and media. The investigated bacterial strains were isolated from salt lake Sheperd Bath located in Slanic, Prahova county, Romania, using MH medium with the following composition (Ventosa *et. al*., 1982) (g/l): NaCl (100), $MgCl_2 \times 6H_2O$ (7), $MgSO_4 \times 7H_2O$ (9.6), $CaCl_2 \times 2H_2O$ (0.36), KCl (2), $NaHCO_3$ (0.06), NaBr (0.026), glucose (1), proteose peptone (5), yeast extract (10). The pH of the medium was 7.0–7.2. When necessary, the medium was solidified with 10 g/l agar. For this study, ten bacterial strains were randomly selected and tested for synthesis of exopolysaccharides.

The preliminary characterization of the strains follow the standard microbiological methods, including Gram staining, NaCl concentration range for growth, sodium deoxycholate treatment and antibiotic resistance in the presence of 50 μg/ml of chloramphenicol, ampicillin, penicillin, and neomycin.

The effect of the growth medium composition on the synthesis of exopolysaccharides by the selected bacterial strains was tested in the presence of either (1) casamino acids and yeast extract, (2) glucose, or (3) sucrose. The strains were cultivated under static or stirring at 250 rpm conditions.

Total genomic DNA was extracted using NucleoSpin$^{(R)}$ kit (Macherey-Nagel) and the 16S rRNA genes were amplified using the PCR method (Wilson *et al*., 1990), using the universal bacterial forward and reverse primers 27 F (AGAGTTTGATCCMTGGCT CAG) and 1492R (TACGGYTACCTTGTTACGA CTT), respectively (Polz and Cavanaugh, 1998).

The ability to produce exopolymer was determined in the case of the isolated halophilic bacterial strains.

Exopolymer isolation. The bacterial cultures were incubated at 28°C for 144 hours under stirring conditions. The cell growth was determined spectrophotometrically at 660 nm. Ten-ml samples were taken every 24 hours and centrifuged at 9500 × g for 30 minutes. The presence of exopolysaccharides in the supernatant was determined by precipitation with 3 volumes of cold ethanol. The resulted polymer was then filtered, dried and quantitated gravimetrically.

Structural analysis of the polymer was performed by UV and fluorescence spectral characterization, thermal stability and FT-IR investigations.

Spectral characterization of the polymer was performed using a HP α-Helios UV-VIS spectrophotometer for absorption, and by fluoresence using a Jasco FP emission spectrofluorimeter.

Thermal stability. The thermal stability of the exopolymer was determined using a differential scanning calorimeter (Perkin-Elmer, DSC-2) with a heating rate of 10 Kmin-1. The instrument was calibrated for temperature and enthalpy by melting high purity indium, and flushed with argon. Samples of 2–4 mg were placed into aluminium crucibles which were sealed and weighed with the Partner XA balance with a 10-μg precision.

FT-IR analysis. IR transmission spectra were obtained in transmission mode using potassium bromide (KBr)-based pellets in the 4000–400 cm – 1 range using a FT-IR spectrometer (BRUKER, Vertex 70) and OPUS software.

Results and Discussions

The samples collected from the Shepherd Bath salt lake in Romania (Slanic, Prahova) were cultivated using MH medium, as described in Material and Methods. The lake is characterized by 97.4 gram per litter salinity. Under these conditions, 16 distinct colonies were isolated. A preliminary microbiological characterization indicated that all these strains represented Gram-positive rods. These strains were able to grow on medium supplemented with sodium deoxycholate, but they could not grow in the presence of chloramphenicol or other antibiotics such as neomycin and penicillin. The strains showed positive growth on medium containing with NaCl up to 4M. The presence of bacterial 16S rRNA genes was revealed in the case of all the investigated strains by using PCR amplification with bacterial specific primers as described in Material and Methods section (data not shown). Consequently, these strains belong to moderately halophilic bacteria.

Out of the ten different strains of moderately halophilic bacteria, three strains with best growth under the tested conditions were analyzed for their capacity to produce exopolysaccharides. These strains differ in salt concentration resistance, strain 1/1 being able to grow in the presence of 4 M NaCl, and strains 2A/6 and 6/49 at NaCl concentrations up to 3 M, respectively. The results (Table I) show that, under various growth conditions, only two strains showed the ability

Table I
The influence of growth conditions on bacterial cell growth and polysaccharide synthesis

Medium \ Growth		Strain 1/1				Strain 2A/6				Strain 6/49			
		stirring		*static*		*stirring*		*static*		*stirring*		*static*	
		OD	Pol	OD	Pol	OD	Pol	OD	Pol	OD	Pol	OD	Pol
CA+YE	24 h	2.05	–	0.51	–	1.56	0.014	0.84	–	1.90	–	0.41	–
	48 h	2.24	–	0.48	–	1.95	0.023	1,92	–	2.21	–	0.54	–
	72 h	2.33	–	0.48	–	2.12	0.026	2.29	0.024	2.24	–	0.83	–
	120 h	2.18	–	0.52	–	2.14	0.037	2.05	0.037	2.12	–	1.75	–
	144 h	2.14	–	0.57	–	2.09	0.048	1.68	0.037	2.07	–	1.91	–
G	24 h	0.75	–	0.37	–	0.59	–	0.42	–	0.99	–	0.39	–
	48 h	0.54	–	0.33	–	0.56	–	0.80	–	1.00	–	0.46	–
	72 h	0.48	–	0.29	–	0.51	–	0.91	–	1.01	–	0.57	–
	120 h	0.42	–	0.24	–	0.45	+	0.84	+	1.01	–	0.97	–
	144 h	0.40	–	0.29	–	0.42	+	0.89	+	0.95	–	1.01	–
S	24 h	1.49	–	0.35	–	0.64	–	0.33	–	0.45	–	0.38	–
	48 h	1.50	–	0.36	–	0.59	–	0.55	–	0.38	–	0.44	–
	72 h	1.42	–	0.32	–	0.55	+	0.65	–	0.38	–	0.48	–
	120 h	1.34	0.001	0.35	–	0.49	+	0.68	–	0.32	–	0.46	–
	144 h	1.11	0.003	0.28	–	0.43	+	0.67	–	0.30	–	0.38	–

The bacterial strains 1/1, 2A/6 and 6/49 were incubated in MH medium (Materials and Methods) containing different carbon source compounds up to 144 hours, under static or stirring conditions. CA: casamino acids; YE: yeast extract; G: glucose; S: sucrose; OD: optical density at 660 nm; Pol: exopolysaccharide quantity (g%); + = polysaccharide present in small amounts (<0.001 g%), (–): absence of polysaccharide

to synthesize exopolysaccharides. For all three bacterial strains analyzed, both the carbon source and the cultivation conditions influenced the maxim growth and the exopolymers synthesis. Bacterial strain 1/1 showed high maximum growth in the presence of casamino acids and yeast extract, but exopolysaccharides were synthesized only when using 2% sucrose under stirring conditions, even if the maximum growth was reduced (Table I). A different behavior was observed for the strain 6/49 that showed a high maximum growth on MH medium supplemented with 2% glucose or sucrose, but was unable to produce extracellular polysaccharides under these conditions (Tables I and II). The strain 2A/6 showed a capacity to synthesize exopolysaccharides using all the three carbon sources tested, under stirring conditions, but the highest yield of polysaccharide and highest growth were observed on MH medium containing casamino acids and yeast extract (Figure 1). Under these conditions, the polysaccharide production increased, reaching the maximum value (0,048 g/100 ml culture) after 144 hours of growth. These results show that, for these moderately halophilic bacterial strains, there is no strict correlation between the optimum conditions for the exopolysaccharides synthesis and optimum growth. Strain 1/1 preferred sucrose for producing extracellular polysaccharides and had a higher maximum growth in the presence of casamino acids and yeast extract. In most cases, exopolysaccharide synthesis starts in the exponential phase and intensifies during cellular growth.

The DSC analysis of the exopolysaccharide produced by strain 2A/6 (Figure 2) showed a high thermal stability of this polymer with a melting point at 207°C. The melting process is associated with the thermal degradation process of this compound.

The spectral characterization of the polymer produced by strain 2A/6 showed absorption maximum

Table II
pH during bacterial growth

	Strain 1/1				Strain 2A/6				Strain 6/49			
	stirring		static		stirring		static		stirring		static	
	start	end	start	end	start	end	start	end	start	end	start	end
CA+YE	7.12	6.15	7.12	6.16	7.12	6.23	7.12	6.17	7.12	6.14	7.12	6.15
G	7.00	5.56	7.30	4.96	7.00	8.17	7.60	7.84	7.40	5.07	7.60	5.76
S	7.70	6.08	7.40	6.26	7.70	6.10	7.35	5.80	7.20	5.85	7.10	5.75

The optimal pH values of the growth medium were measured for the initial (start) and final (end) cultivation times under static and stirring conditions, and in the presence of various carbon sources, of the 1/1, 2A/6 and 6/49 strains. CA: casamino acids; YE: yeast extract; G: glucose; S: sucrose.

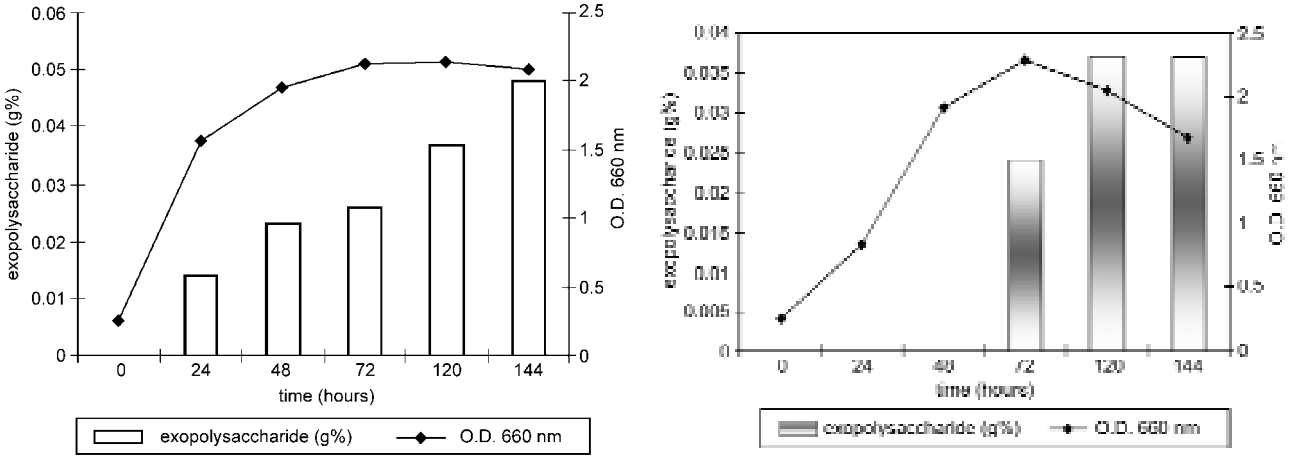

Fig. 1. Growth curve and exopolysaccharide synthesis of the strain 2A/6.

The 2A/6 strain was cultivated on MH medium supplemented with casaminoacids and yeast extract, under stirring (left) and static (right) conditions. The OD_{660nm} and the exopolysaccharide concentration (g%) were monitored up to 144 hours growth.

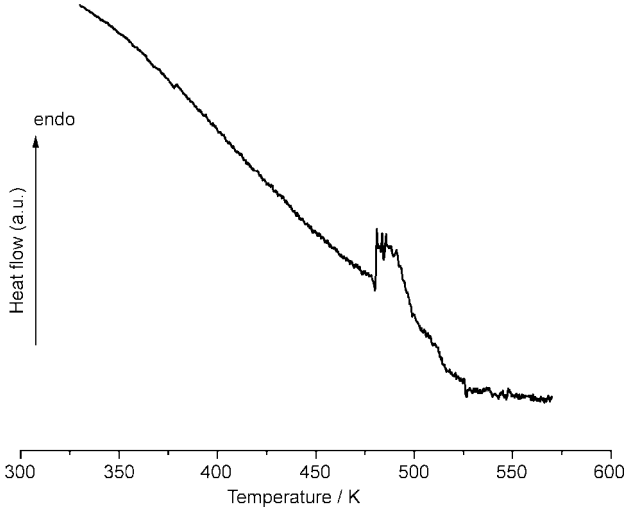

Fig. 2. DSC spectrum of the exopolysaccharide produced by strain 2A/6.

The heat flow curve as a function of temperature was determined using 2–4 mg of polysaccharide synthesized by the strain 2A/6 cultivated in the presence of casamino acids and yeast extract

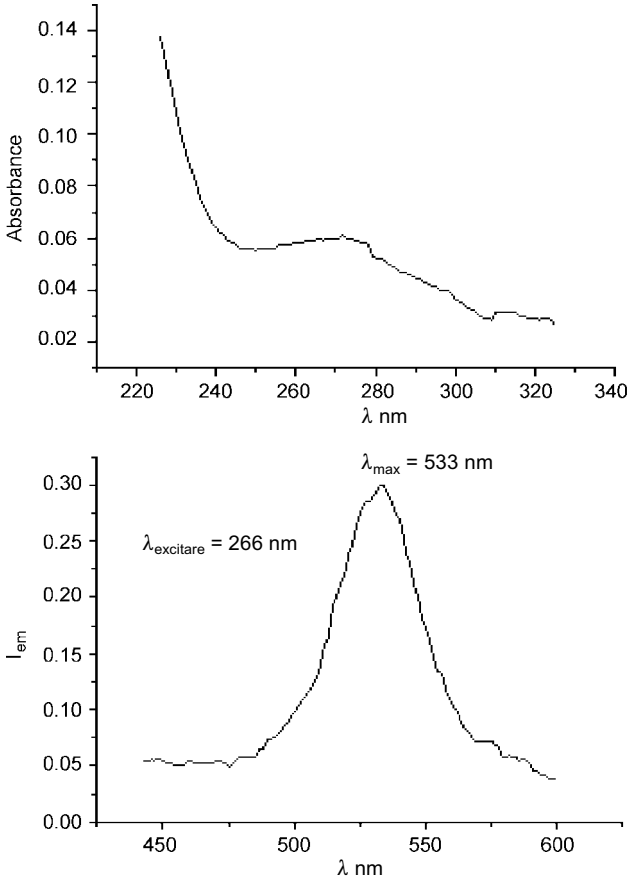

Fig. 3. Fluorescence analysis of the exopolysaccharide produced by strain 2A/6.

The emission (bottom) and absorption (top) fluorescence spectra were determined using x mg of the exopolysaccharide synthesized by strain 2A/6 cultivated in the presence of casamino acids and yeast extract.

at 260 nm and fluorescence emission at 530 nm (Figure 3).

The polysaccharide structure of the polymer was further analyzed by FT-IR spectrometry (Figure 4), which confirmed the presence of sugar residues having amino and sulfate groups bounded by double bridge to base sugar-backbone. The absorption at 3436 cm^{-1} confirmed the presence of OH groups and intermolecular hydrogen bridges. The presence of the double bond between carbon and nitrogen was indicated by the absorption peak at 1641.95 cm^{-1}. The bounds between carbon and hydrogen correspond to the absorption peak at 1642 cm^{-1} and those between carbon and oxygen, at 1060 cm^{-1}. The polysaccharide structures are indicated by the absorption at 876 cm^{-1}, and the presence of N-C = S and N = C-S bounds is indicated by the absorption peaks at 713 cm^{-1}.

Polysaccharides with a high sulfate content often form gels in the presence of metal ions and represent a great potential for bioremediation of high polluted environments and waste waters (Arias *et al.*, 2003; Philips and Vicenzi 1998). Therefore, the high con-

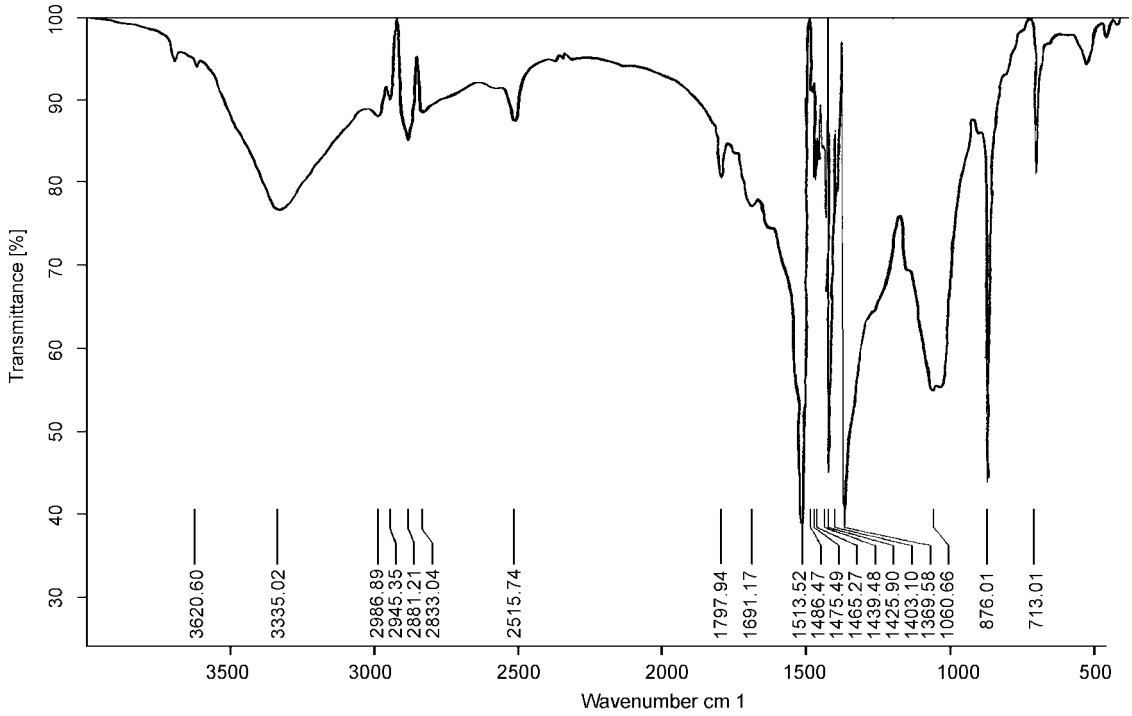

Fig. 4. FT-IR transmission spectra of the exopolysaccharide produced by strain 2A/6
Exopolysaccharide synthesized by strain 2A/6 were analyzed by FT-IR spectrometry in transmission mode using potassium bromide (KBr)-based pellets in the 4000–400 cm – 1 range.

tent of sulfate and amino groups in the exopolysaccharide from the 2A/6 halophilic bacterial strain is of high interest for biotechnological applications. Moreover, sulfated polysaccharides are known to inhibit some viruses and tumors (Hayashi *et al*., 1996; Hasui *et al*., 1995; Itoh *et al*., 1993; Riou *et al*., 1996; Witvrouw and Clerq, 1997). Such polysaccharides are produced by eukaryotic algae (Itoh *et al*., 1993) and are uncommon among prokaryotes. However, it has been isolated a halophilic exopolysaccharide with a high sulfate content (Bejar *et al*., 1998; Calvo *et al*., 1998) which is stable over a wide pH range between 3–8 (Arias *et al*., 2003) produced by *Halomonas maura*, but there are not any information available about its thermal stability. The present work revealed the isolation of a halophilic exopolymer, also rich in sulfate residues, which are highly thermostable with a melting point at 207°C.

The nutritional and environmental variables need to be optimized for each strain, in order to obtain maximum production, together with the best functional properties of the polymers. Both yield and chemical composition may be influenced by several parameters such as carbon source, limiting nutrients, and aeration and incubation temperature. Although microorganisms can use different carbohydrates and amino acids as carbon source for EPS production, glucose and sucrose are the most efficient (Bejar *et al*., 1998). Surprisingly, the 2A/6 strain favors casamino acids and yeast extract for the highest concentration of exopolysaccharides synthesized.

The results of this study revealed the identification of moderately halophilic bacteria that are capable of producing highly thermostable exopolysaccharides. This process is influenced by the composition of the culture medium and growth conditions. Moreover, a preliminary structural characterization of the exopolymer isolated from the moderately halophilic bacterial 2A/6 strain suggests the presence of valuable substituents for biotechnological applications.

Literature

Arias S., A. del Moral, M.R. Ferrer, R. Tallon, E. Quesada and V. Bejar. 2003. Mauran, an exopolysaccharide produced by the halophilic bacterium *Halomonas maura*, with a novel composition and interesting properties for biotechnology. *Extremophiles* 7: 319–326.

Bejar V., I. Llamas, C. Calvo and E. Quesada. 1998. Characterization of exopolysaccharides produced by 19 halophilic strains included in the species *Halomonas eurihalina*. *J. Biotechnol.* 61: 135–141.

Bouchotroch S., E. Quesada, A. del Moral, I. Llamas and V. Béjar. 2001. *Halomonas maura* sp. nov., a novel moderately halophilic, exopolysaccharide-producing bacterium. *Intl. J. Syst. Evol. Microbiol*. 51: 1625–1632.

Calvo C., F. Martinez-Checa, A. Mota, V. Bejar and E. Quesada. 1998. Effect of cations, pH and sulphate content on the viscosity and emulsifying activity of the *Halomonas eurihalina* exopolysaccharide. *J. Ind. Microbiol. Biotechnol*. 20: 205–209.

Hasui M., M. Matsuda, K. Okutani and S. Shigeta. 1995. *In vitro* antiviral activities of sulfated polysaccharides from marine microalga (*Cochlodinium polykrikoides*) against human immunodeficiency virus and other enveloped virus. *J. Biol. Macromol*. 17: 293–297.

Hayashi K., T. Hayashi and I. Kojima. 1996. A natural sulfated polysaccharide calcium spirulan, isolated from *Spirulina platensis*: *in vitro* and anti-human immunodeficiency virus activities. *Aids Res. Hum. Retroviruses*. 12: 463–471.

Itoh H., H. Noda, H. Amano, C. Zhuaug, T. Mizuno and H. Ito. 1993. Antitumor activity and immunological properties of marine algal polysaccharides, especially fucoidan, prepared from *Sargassum thunbergii* of Phaeophyceae. *Anticancer Res.* 13: 2045–2052.

Kushner D.J. and M. Kamekura. 1988. Physiology of halophilic Eubacteria. In: Rodriguez-Valera F (ed) Halophilic Bacteria, Vol. I. CRC Press, Inc. Boca Raton, Florida, pp. 109–138.

Margesin R. and F. Schinner. 2001. Potential of halotolerant and halophilic microorganisms for biotechnology. *Extremophiles* 5: 73–83.

Mata J.A., V. Béjar, I. Llamas, S. Arias, P. Bressollier, R. Tallon *et al*. 2006. Exopolysaccharides produced by the recently described halophilic bacteria *Halomonas ventosae* and *Halomonas anticariensis*. *Research in Microbiol*. 157: 827–835.

Philips R. de. and M. Vicenzini. 1998. Exocellular polysaccharides from cyanobacteria and their possible application. *FEMS Microbiol. Rev*. 22: 151–157.

Polz M.F. and M.C. Cavanaugh. 1998. Bias in Template-to-Product Ratios in Multitemplate PCR. *Appl. Environ. Microbiol*. 64: 3724–3730

Quesada E., V. Béjar and C. Calvo. 1993. Exopolysaccharide production by *Volcaniella eurihalina*. *Experientia*, 49: 1037–1041.

Riou D., S. Colliec-Jouault, D. Pinczon du Sel, S. Bosch, S. Siavoshian, V. Le Bert *et al*., 1996. Antitumor and antiproliferative effects of a fucan extracted from *Ascophyllum nodosum* against a non-small-cell bronchopulmonary carcinoma line. *Anticancer Res.* 16: 1213–1218.

Sutherland I.W. 1998. Novel and established applications of microbial polysaccharides. *TIBTECH* 16: 41–46.

Sutherland I.W. 1999. Microbial polysaccharide products. *Biotechnol. Genet. Env. Rev*. 16: 217–229.

Sutherland I.W. 2001. Microbial polysaccharides from Gram-negative bacteria. *Intl. Dairy J*. 11: 663–674.

Ventosa A., E. Quesada, F. Rodriguez-Valera, F. Ruiz-Berraquero and A. Ramos-Cormenzana. 1982. Numerical taxonomy of moderately halophilic Gram-negative rods. *J. Gen. Microbiol.* 128: 1959–1968.

Wilson K.H., R.B. Blitchington and R.C. Greene. 1990. Amplification of bacterial 16S ribosomal DNA with polymerase chain reaction. *J. Clin. Microbiol*. 28: 1942–1946.

Witvrouw M. and E. de Clerq. 1997. Sulfated polysaccharides extracted from sea algae as potential antiviral drugs. *Gen. Pharmacol*. 29: 497–511.