POLYSACCHARIDES FROM EXTREMOPHILIC MICROORGANISMS

B. NICOLAUS*, V. SCHIANO MORIELLO, L. LAMA, A. POLI and
A. GAMBACORTA
Istituto di Chimica Biomolecolare CNR, via Campi Flegrei 34, 80078 Pozzuoli (Na), Italy
(* author for correspondence, e-mail: bnicolaus@icmib.na.cnr.it, phone: 0039 081 8675245,
fax: 0039 081 8041770)

Abstract. Several marine thermophilic strains were analyzed for exopolysaccharide production. The screening process revealed that a significant number of thermophilic microorganisms were able to produce biopolymers, and some of them also revealed interesting chemical compositions. We have identified four new polysaccharides from thermophilic marine bacteria, with complex primary structures and with different repetitive units: a galacto-mannane type from strain number 4004 and mannane type for the other strains. The thermophilic *Bacillus thermantarcticus* produces two exocellular polysaccharides (EPS 1, EPS 2) that give the colonies a typical mucous character. The exopolysaccharide fraction was produced with all substrates assayed, although a higher yield 400 mg liter^{-1} was obtained with mannose as carbon and energy source. NMR spectra confirmed that EPS 1 was a heteropolysaccharide of which the repeating unit was constituted by four different α-D-mannoses and three different β-D-glucoses. It seems to be close to some xantan polymers. EPS 2 was a mannan. Four different α-D-mannoses were found as the repeating unit. Production and chemical studies of biopolymers produced by halophilic archaea, *Haloarcula* species were also reported.

Keywords: extremophiles, halophiles, thermophiles, exopolysaccharides, chemical studies, production

1. Introduction

Many parts of the world are considered extreme, such as geothermal environments, terrestrial and marine, polar region, acid and alkaline springs and the cold pressurised depths of the oceans. It is now recognised that extreme environments, once thought to be too hostile to permit survival of living organisms, are the natural habitat of certain microorganims known as extremophiles.

The discovery and characterisation of a wide array of prokaryotic species that thrive under extreme values of temperature, acidity, alkalinity, salt concentration, and pressure have led not only to major improvements in our understanding of the environmental conditions limiting life, but also in our descriptions of early stages of cellular evolution (Hough and Danson, 1999). It is very likely that higher organisms are unable to survive under extreme conditions because of their cellular complexity and compartimentalization. The realization that extreme environments

harbour different kinds of prokaryote lineage has resulted in a complete reassessment of our concept of microbial evolution and has given considerable impetus to extremophiles research (Bertoldo and Antranikian, 2002).

Extremophiles are organisms that have evolved to exist in a variety of extreme environments and fall into a number of different classes and domini, belonging to Archaea as well as Bacteria. They include thermophiles, acidophiles, alkaliphiles, psychrophiles, barophiles, radiophiles, etc. (Segerer *et al.*, 1993).

Thermophiles, which grow optimally between 60 °C and 80 °C are widely distributed among the genera of the Domain Bacteria (*Bacillus, Clostridium, Thermoanaerobacters, Thermus, Fervidobacter, Thermotoga and Aquifex*), (Nicolaus *et al.*, 1999a). Hyperthermophiles, capable of growing optimally between 80 °C and 110 °C, were generally positioned into Archaea, consisting of two major kingdoms and short phylogenetic branches that indicate a rather slow clock of evolution.

Backward extrapolation of the basal position of hyperthermophiles in rooted molecular phylogenies has led not only to the hypothesis of a heat-loving last common ancestor of all living beings, but also to a high-temperature origin of life, which according to some took place in extreme environments such as those found today in deep sea vents or other sites in which mineral surfaces may have fuelled the appearance of primordial chemoautolithotrophic biological systems (Lazcano, 2002).

Only few years ago, extremophiles were exotic organisms, explored by only a few research groups through the world. Now, although they still retain some of their eccentric status, they are often routinely used as sources of new molecules of biotechnological interest (Demerjian *et al.*, 2001). In fact, it is clear that some extremophiles, particularly those from Archaea, have novel metabolic pathways and so might serve as a source of metabolites with novel properties and applications (Demerjian *et al.*, 2001; Hough and Danson, 1999).

Most of the work has been devoted to thermophiles and hyperthermophiles, but other groups have received more attention recently because of their biotechnological potential (Maugeri *et al.*, 2002).

Presently, applied uses exist or have been proposed for a variety of extremal materials. The extremely chemically stable lipids of archaeal membranes represent a novel drug delivery (Patel and Sprott, 1999). Self-assembling archaeal components such as the S-layer glycoprotein and bacteriozoolopsin have drawn interest for their nanotechnological potential (Sleytr *et al.*, 1997). Endo- and exopolymers synthesised from halophilic archaea and bacteria could find use in oil exploration efforts or/and raw material for biodegradable plastics (Fèrnandez-Castillo *et al.*, 1986; Rodriguez-Valera, 1992; Romano *et al.*, 1996; Nicolaus *et al.*, 1999b). Although a variety of external-related products carry significant commercial value, biotechnologically useful archaeal enzymes represent the main focus of industrial interest (Hough and Danson, 1999; Demerjian *et al.*, 2001; Eichler, 2001; Bertoldo and Antranikian, 2002).

Little information about polysaccharide production by extremophiles has been reported in the literature (Manca *et al.*, 1996; Nicolaus *et al.*, 2000; Nicolaus *et al.*, 2002). Therefore a wide search for microrganisms able to produce good yields of new polysaccharides with potentially useful properties has been undertaken, also involving extremophiles because of their well-known capability for biotechnological applications.

2.   **Materials and Methods**

2.1. MICROORGANISMS

Thermophilic strains 4001 to 4014 Mast project (MAS 3-CT95595-0034) were isolated from shallow hydrothermal vents and marine hot springs, near Lucrino area (gulf of Pozzuoli, Naples, Italy), and around Ischia Island (Flegrean areas, Italy). The strains were selected for their ability to show a mucoid phenotype on solid medium and to produce viscous broth. The strains were tested for polysaccharide production in shake flasks at 65 °C at pH 7.0, by using a sugar-containing medium (g/l): salt compositions of BMB 2216 medium (Difco), and yeast extract 0.2; peptone 0.1; and the tested sugar 10 (glucose, mannose, galactose, fructose, xylose, cellobiose, trehalose, sucrose; Nicolaus *et al.*, 2002).

Bacillus thermantarcticus, a thermophilic bacterium, was isolated near the crater of Mount Melbourne (74°22′ S, 164°40′ E) and grew optimally at 60 °C, at pH 6.0 in the following medium (g/l): yeast extract 8.0; NaCl 3.0. For exopolysaccharide production the culture medium contained (g/l): NaCl 3.0; glucose 6.0; and yeast extract 1.0. The glucose medium supplemented with 2% agar was used for agar plate preparations. The colonies on plates, observed with a Leica Wild M 8 stereomicroscope, showed the presence of a mucous layer (Nicolaus *et al.*, 1996; Manca *et al.*, 1996).

Halophilic archaea were enriched from 50 samples collected at 1 meter intervals in the last ponds of a marine saltern located close to Monastir in Tunisia. From these samples were isolated three new strains of *Haloarcula japonica* (T5, T6, T7) that usually grow at 37 °C at pH 7.5 with NaCl 3.5 M on medium containing (g/1) yeast extract 10; casamino acids 7.5; trisodium citrate 3.0; KCl 2.0; MgSO$_4$ · 7H$_2$O 20; NaCl 200; MnCl$_2$ · 4H$_2$O (mg/l) 0.36; FeSO$_4$ · 7H$_2$O 50 (Nicolaus *et al.*, 1999a). The ability to utilize different carbon sources was tested by reducing the yeast extract concentration to 1.0 (g/l), replaced by the tested compound at a concentration of 6.0 (g/l) in the saline solution above described.

2.2. POLYSACCHARIDE PRODUCTION

The EPS production from strains was studied by using batch fermentation using all minimal media before reported. The temperature was maintained at optimal value of growth, the pH was adjusted to optimal value at the beginning of the culture and

measured but not controlled during the experiment. Samples (20 ml) were removed at regular intervals for growth measurement (A 540), and EPS production.

For exopolysaccharide recovery, cells were harvested in a stationary phase of growth by centrifugation (1 litre, 9800 g, 20 min). The supernatant phases were treated with 1 volume of cold absolute ethanol added drop-wise under stirring. Alcoholic solutions were kept at −18 °C overnight and then centrifuged at 15000 g for 30 min. The pellets were dissolved in hot distilled water. The same procedure was repeated again. The final water solutions were dialysed against tap water (48 h) and distilled water (20 h), then freeze-dried and weighted. The samples were tested for carbohydrate, protein, and nucleic acid contents (Manca *et al.*, 1996). EPS production was tested on cell free cultural broth with phenol-sulphuric acid method using glucose as standard. The polysaccharide fractions were purified by Gel Chromatography as reported in Manca *et al.* (1996).

2.3. CHEMICAL CHARACTERISATION OF EPS

Sugar analysis was performed by hydrolysis of EPS with 2 M trifluoroacetic acid (TFA) at 120 °C for 2 h. Sugar mixture was identified by TLC (thin layer chromatography) and HPAE-PAD (high-pressure anion-exchange pulsed amperometric detection) using standards for identification and calibration curves (Manca *et al.*, 1996). HPAE-PAD Dionex equipped with Carbopac PA 1 column was eluted isocratically with (a) 15 mM NaOH for neutral sugars; (b) buffer 100 mM NaOH and 150 mM NaOAc for acidic sugars.

Molecular size analyses were determined as reported in Manca *et al.* (1996). Methylation analysis of the polysaccharides was carried out as described in Manca *et al.* (1996). Identification of sugars was obtained by GLC (gas chromatography) and GC-MS (gas chromatography combined with mass spectrometry) using standards. GLC runs were performed on a Hewlett-Packard 5890A instrument, fitted with a FID detector and equipped with a HP-5-V column and N_2 flux of 100 ml/min. The temperature program was: 170 °C 1 min, from 170 to 180 °C at 1 °C/min, 180 °C 1 min, from 180 to 210 °C at 4 °C/min. GC-MS was performed on a Hewlett-Packard 5890–5970 instrument, equipped with a HP-5-MS column and with a N_2 flux of 50 ml/min; 170 °C 1 min, from 170 to 250 °C at 3 °C/min was used as temperature program.

Optical rotation value was obtained on a Perkin-Elmer 243 B polarimeter at 25 °C in water.

NMR spectra were obtained on a Bruker AMX-500 (500,13 MHz for ^{1}H). The EPS spectra were run in D_2O at 70 °C (Manca *et al.*, 1996; Pazur, 1994; Perlin and Casu, 1982).

3. Results

The supernatants of the strains were examined for exopolysaccharide (EPS) content. The maximum yield of EPS for all isolates was reached in a stationary phase of growth. After centrifugation the supernatants formed stringy precipitates with ethanol showing the presence of exopolysaccharides. Production of EPSs was conducted in 1 liter batch culture. The wet cell yield was for all isolates in the range of 0.9–1.0 g/liter.

The highest concentrations of EPSs harvested after 48 h of culture were approximately 60 and 50 EPS mg/cell gram for isolates 4001 and 4004 respectively (Mast-strains).

The yield of EPSs, using different carbohydrates in growth medium, for all marine thermophilic isolates were analysed (Figure 1). Trehalose, saccharose, cellobiose and galactose, added in a minimal medium, produced the best EPS formation. In particular galactose and saccharose caused a strong increase of EPS production for 4001 strain and trehalose and saccharose for 4004 strain. In addition trehalose, galactose and glucose induced EPS production in 4008 strain. The biosynthesis of the exopolysaccaride was induced by using trehalose medium for 4009 strain, reaching 60 EPS mg/cell gram at the end of stationary phase (Figure 2). In the absence of trehalose, or cellobiose, or mannose in the medium, the strain 4009 did not release any material in the culture broth. The other strains produced exopolysaccharide in few amounts, less than 10 EPS mg/cell gram in the presence of some sugars (Nicolaus *et al.*, 2002).

The main features of polysaccharides produced by the strains are reported in Table I. The optical rotation for EPS-4001 was $[\alpha]_D^{20} = +0.04$ (c 5 mg ml $^{-1}$ H$_2$O) and the molecular size, estimated using a calibration curve of standard dextrans obtained by gel filtration on Sepharose CL-6B and also by density gradient centrifugation, was about 380.000 Da. The UV spectra of EPS did not indicate any strong absorption peaks in the range of 350–210 nm. The GC/MS of the permethylated 4001-EPS showed the presence of mannose/glucose/galactose/mannosamine in a relative proportion of 1/0.1/tr/tr (trace amount) (Table I).

The ^{1}H spectrum of EPS-4001 was indicative of a complex structure and showed, inter alia, six anomeric signals with α conformation, a doublet at δ 5.35, with a coupling constant of 3.5 Hz, representative of a big amount of an α-gluco/galacto residue conformation, more than 30%, at δ 5.19 (s), at δ 5.38 (s), at δ 5.45 (s), at δ 5.49 (s), and at δ 5.55 (s); one with β conformation at δ 4.80 (d) (Table II), (Nicolaus *et al.*, 2002). Nuclear magnetic resonance spectrum of the 4001-EPS confirmed the presence of a repetitive unity formed by seven monosaccharides, six with α gluco/galacto configuration and one residue with β conformation.

Bacillus thermantarcticus, a new species of group five of *Bacillus* genus (Int. J. Syst. Evol. Microbiol, 2002), produced two different sulphated polysaccharides named EPS1 and EPS2. Their production was done in 3 litre fermenters with aera-
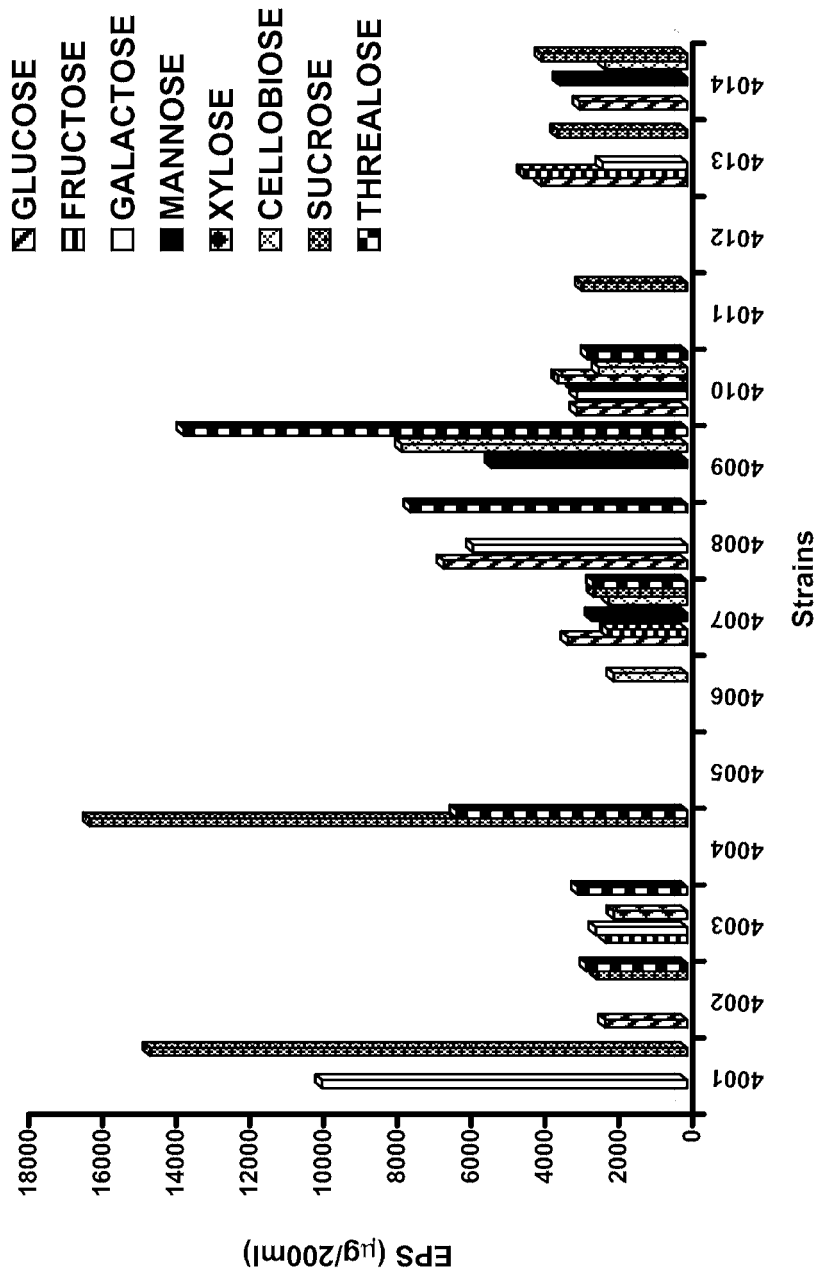
Figure 1. Production of exopolysaccharides from Mast strains grown on minimal media.
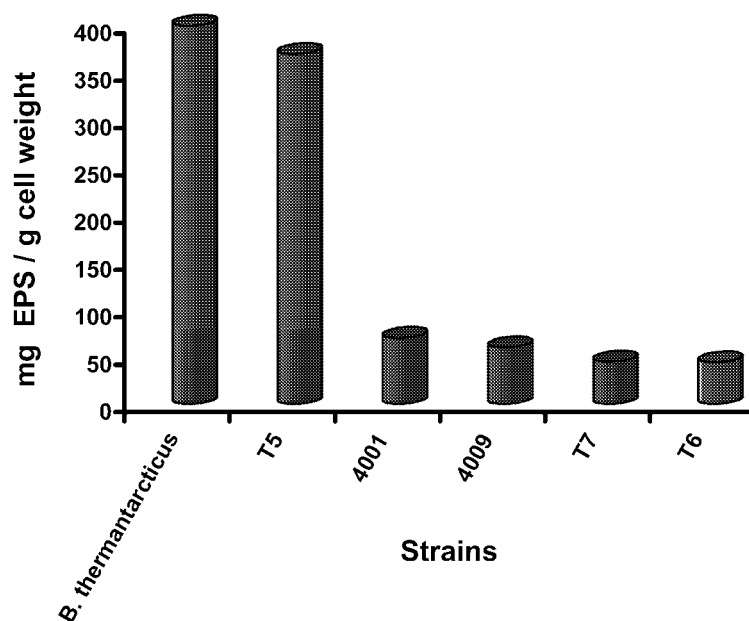
Figure 2. Optimal yield of polysaccharides from *Bacillus thermantarcticus*, *Haloarcula* strains (T5, T6, T7), and Mast strains (4001, 4009) grown in fermenter.

TABLE I

Chemical shifts and coupling of anomeric signals in ^{1}H spectra of EPSs from *Bacillus thermantarcticus*, Mast-*4001* and *Haloarcula japonica* (T_5)

Residue	EPS *B. thermantarcticus*		EPS *4001*		EPS T_5	
	δ H-1	$^3J_{\text{H-1, H-2}}{}^a$	δ H-1	$^3J_{\text{H-1, H-2}}{}^a$	δ H-1	$^3J_{\text{H-1, H-2}}{}^a$
A	5.27	(bs)	5.55	(bs)	5.15	(bs)
B	5.10	(bs)	5.49	(bs)	5.01	(bs)
C	5.07	(bs)	5.38	(bs)	4.97	(bs)
D	4.92	(bs)	5.35	(d) 3.5	4.95	(bs)
E	4.73	(d) 8.0	5.19	(bs)	4.90	(d)
F	4.59	(d) 8.1	4.80	(d)		
G	4.52	(d) 8.0	2.30	(d)		
H			1.59	(d)		

^{a} Coupling constants are in hertz; bs, broadened singlet; d, doublet.

TABLE II

Mean features of exopolysaccharides from extremophilic microorganisms (Mast strains *4001* and *4004*; *Bacillus thermantarcticus*; *Haloarcula japonica* strain T_5)

Properties	*4001*	*4004*	*Bacillus thermantarcticus*	*Haloarcula japonica* strain T_5
Carbohydrate (%)	81%	65%	95%	70%
Protein content (%)	7%	2,6%	2%	4%
M.W.	380.000 Da	>1.000.000 Da	300.000 Da	/
Optical rotation	(+40,9)	(−199,7)	(−90)	/
Sugar analysis	Man/Glu/Gal 1/0,1/Tr	Gal/Man/GluN/Ara 1/0,8/0,4/0,2	Man/Glu 1/0,7	Man/Gal/Gluc.ac. 2/1/3
Repeating unit	Eptasaccharide	Pentasaccharide	Eptasaccharide	Pentasaccharide
Configuration	α and β	*Gluco-galacto* and *Manno*	*α-manno* and *β-gluco-galacto*	α and β

tion flux of 20 ml/min. The yield reached 400 EPS mg/cell gram in the presence of mannose as the carbon source (Figure 2). The production of EPSs increased with increasing cell density, reached a maximum at the beginning of the stationary phase and the EPS content was proportional to total biomass. On weight basis, EPS1 and EPS2 represented about 27% and 71% respectively of the total carbohydrate fraction. Analysis of hydrolysis products revealed the presence of a terminal glucose in EPS1; the chain sugars were 1,2-linked mannose, 1,4-linked glucose, 1,3-linked mannose, and 1,6-linked mannose, while 1,3,4-linked glucose and 1,2,6-linked mannose represented branch points in the molecule; in the relative proportion of 0,9/0,5/0,1/0,2/0,5/0,1/1,0, respectively. The same analysis revealed for EPS2, the presence of a terminal mannose; the chain sugars were 1,2-linked mannose, 1,4-linked mannose and 1,6-linked mannose, and the branch point was 1,2,6-linked mannose. Their relative proportions were 1,0/0,5/0,2/0,1/0,8 respectively. EPS2 showed a molecular weight of 3.0×10^5 Da and an optical rotation $[\alpha]_D^{20} = -90°$. Sulphate group and pyruvate presence were also detected (Table I).

^{1}H and ^{13}C NMR spectroscopy were performed on both EPSs and showed that EPS1 was a heteropolysaccharide whose repeating unit consisted of four different α-D mannoses and three different β-D-glucoses (Table II). This structure

was closely related to xanthan polymers. EPS2 was a mannan with four different α-D-mannoses and trace of pyruvic acid as the repeating unit.

Halophilic archaea (*Haloarcula* strains T5, T6, T7), grown on the minimal medium with glucose, were able to produce sulfated extracellular polysaccharide (EPS), that was isolated from cell free culture broth by precipitation with cold ethanol. The ethanolic precipitate, after dissolution in hot water, was centrifuged and the soluble fraction accounted for 80–90% of the total. This material was dialyzed, freeze-dried and weighed for each sample, 370 mg/cell gram (T5), 45 mg/cell gram (T6), 35 mg/cell gram (T7), respectively (Figure 2). A preliminary ^{1}H NMR spectrum of the crude EPS extract of the isolate T5 in D_2O showed the presence of five anomeric signals, two α and three β-linkages at δ 5.15, 5.01, 4.97, 4.95, and 4.90 (Table II).

The soluble fraction of T5 strain was chromatographed on DEAE-Sepharose CL-6B, with a yield of 80%. EPS fractions eluted at 0.5 M NaCl represented 50% of total carbohydrate fractions. Hydrolysis of EPS with 2M TFA yielded, as principal constituents: mannose, galactose and glucuronic acid in a relative proportions of 2/1/3, respectively (Table I). Sugar analysis of crude EPSs of strains T6 and T7 yielded, as principal constituents: mannose, galactose and glucose in a relative proportion of 1/0.2/0.2 respectively, glucuronic acid was also detected.

4. Discussion

Microbial exopolysaccharides have found a wide range of applications in industries, e.g. in the food industry, medicine, agriculture, and wastewater treatment. Via controlled fermentation processes several bacterial polysaccharides can now be tailor made produced starting from common cheep sugars. In fact, *Bacillus thermantarcticus* was strongly induced for the production of exopolysaccharide by using mannose as sole carbon source. In this growth condition the polysaccharide yield was 10 fold higher than that obtained in the growth in absence of the sugar. Because of the extraordinary diversity, marine organisms also represent an almost unexploited resource for biomolecules to be used in various processes. Among the various marine ecosystems that could provide promising microorganisms, marine hot springs both deep and shallow, have been used for obtaining new microorganisms able to produce new biopolymers. The screening studies have revealed that several marine thermophiles were able to produce biopolymers. We have identified four new polysaccharides from thermophilic marine bacteria, with complex primary structures and with different repetitive units: a galacto-mannane type from strain number 4004 and mannane type for the other strains.

It has been hypothesised that the synthesis of exo-cellular polysaccharides in microorganisms plays a major role in protecting cells from stress in extreme habitats (Nicolaus *et al.*, 1999a, b). Exopolysaccharide production, although not common among the archaea, is found in some species within the genera *Haloferax*

and *Haloarcula* (Nicolaus *et al.*, 1999a, b; Parolis *et al.*, 1996; Paramonov *et al.*, 1998; Parolis *et al.*, 1999). *Haloferax denitrificans* produced a linear acidic polysaccharide, *Haloferax gibbonsii* exuded into growth medium a neutral polysaccharide while *Haloferax mediterranei* as well as *Haloarcula japonica* strain T5, synthetised sulfated exopolysaccharide. The sulfated exopolysaccharide is known to interfere with the absorption and penetration of viruses into host cells and inhibited various retroviral reverse transcriptases (Hayashi *et al.*, 1996; Riccio *et al.*, 1996).

In recent years, significant advances in the understanding of the genetics and biochemistry of microbial exopolysaccharide (EPS) synthesis, both Gram-negative and Gram-positive bacteria, have been made. Many biosyntheses were elucidated, and several of the genes involved have been characterised. This knowledge can be applied to EPS engineering or to improve EPS production. These efforts are justified by the fact that the microbial EPS show over plants or marine macroalgal ones many advantages, i.e., its novel functionality, constant reproducible chemical and physical properties, and a stable cost and supply (Nicolaus *et al.*, 1996).

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