Carbohydrate Polymers 78 (2009) 651–657

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

High level synthesis of levan by a novel *Halomonas* species growing on defined media

Annarita Poli [a], Hande Kazak [b], Bahar Gürleyendağ [c,1], Giuseppina Tommonaro [a], Giuseppina Pieretti [d], Ebru Toksoy Öner [b], Barbara Nicolaus [a,*]

[a] *Institute of Biomolecular Chemistry (ICB), Consiglio Nazionale delle Ricerche C.N.R., Via Campi Flegrei 34, 80078 Pozzuoli (Na), Italy*
[b] *Department of Bioengineering, Marmara University, Goztepe 34722, Istanbul, Turkey*
[c] *Department of Chemistry, Marmara University, Goztepe 34722, Istanbul, Turkey*
[d] *Department of Organic Chemistry and Biochemistry, Università Federico II, Via Cinthia 4, 80126 Napoli, Italy*

ARTICLE INFO

Article history:
Received 6 April 2009
Received in revised form 20 May 2009
Accepted 29 May 2009
Available online 7 June 2009

Keywords:
Exopolysaccharide (EPS)
Halomonas sp.
Levan
Polysaccharide thermostability
Anti-toxic activity

ABSTRACT

A novel halophilic isolate from soil samples taken from Çamaltı Saltern area in Turkey, *Halomonas* sp. AAD6 (JCM 15723) strain, was found to produce high levels of exopolysaccharides (EPS), in the presence of sucrose in defined media, by flasks and bioreactor condition yielded 1.073 g L^{-1} and 1.844 g L^{-1}, respectively. Sugar analysis, methylation studies and NMR analysis of EPS indicated the repeating unit of this polysaccharide was composed of β-(2,6)-D-fructofuranosyl residues. Hence with this work, *Halomonas* sp. has been described as a levan producer microorganism for the first time. Biocompatibility studies showed this EPS did not affect cellular viability and proliferation of osteoblasts and murine macrophages. The protective effect of the polymer against the toxic activity of avarol implied its additional use as an anti-cytotoxic agent. *Halomonas* sp. AAD6 could represent an alternative cheap source of levan polymer when grown on defined media hypothesizing its larger employment in industrial application being non pathogenic microorganism.

1. Introduction

Biopolymers are superior to petrochemical-derived polymers in several aspects that embrace biocompatibility, biodegradability, and both environmental and human compatibility. Due to their many interesting physicochemical and rheological properties with novel functionality, the microbial exopolysaccharides (EPS) act as new biomaterials and find wide range of applications in many industrial sectors like textiles, detergents, adhesives, microbial enhanced oil recovery (MEOR), wastewater treatment, dredging, brewing, downstream processing, cosmetology, pharmacology, and food additives (Iyer, Mody, & Jha, 2006; Martinez-Cánovas, Quesada, Martinez-Checa, & Béjar, 2004). Xanthan, dextran, pullulan and levan are examples of microbial polysaccharides with a considerable market due to their exceptional properties.

Biopolymers with properties much better than the commercial ones could not preserve their functions under industrial process conditions like extremes of temperature, salinity or pH (Lee, Park, Park, Lee, & Lee, 2003). Hence, most research is focused on the identification of EPS producing extremophiles with the idea that as these microorganisms survive environmental extremes of desiccation, temperature, pressure, salinity and acidity, it is to be expected that their biopolymers will also have some unique properties to adapt to such extreme conditions.

Since 1991, EPS producing extremophilic microorganisms have been isolated from deep-sea hydrothermal vents characterized by extreme pressure and temperature, high concentrations of H_2S and heavy metals. These include some *Bacteria* such as *Alteromonas macleodii* sub sp. *fijiensis*, *Vibrio diabolicus*, *Alteromonas infernos* (Raguénès et al., 1997; Roger, Kervarec, Ratiskol, Colliec-Jouault, & Chevolot, 2004) and *Thermotoga maritima* and *Archaea* such as *Thermococcus litoralis* (Rinker & Kelly, 2000). EPS producing bacteria from marine Mediterranean shallow vents have also been described (Maugeri et al., 2002; Schiano Moriello et al., 2003). Hypersaline environments are also known to harbor a variety of EPS producing strains such as moderately halophilic bacteria of the genus *Halomonas* (Bouchotroch, Quesada, Izquierdo, Rodriguez, & Bejar, 2000), *Halomonas maura* (Arias et al., 2003), *Halomonas alkaliphila*, strain CRSS (Poli et al., 2004), *Halomonas ventosae* and *Halomonas anticariensis* (Mata et al., 2006), *Halomonas cerina* (González-Domenech, Martínez-Checa, Quesada, & Béjar, 2008), *Aphanocapsa halophytica* (Matsunaga, Sudo, Takemasa, & Wachi, 1996), haloalkalophilic *Bacillus* sp. I-450 (Kumar, Joo, Choi, Koo, & Chang, 2004).

* Corresponding author. Tel.: +39 081 8675245; fax: +39 081 8041770.
E-mail address: bnicolaus@icb.cnr.it (B. Nicolaus).
[1] Present address: R&D (Pharma), Eczacıbaşı-Zentiva Health Products Co., Kırklareli, Turkey.

0144-8617/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carbpol.2009.05.031

With the ultimate goal of finding EPS with novel and valuable properties, halophilic microorganisms isolated from Çamaltı Saltern Area in Turkey were screened for their polysaccharide-producing abilities. The best EPS producer, a new *Halomonas* sp. AAD6 (JCM 15723), (DQ131909 Accession No. for 16S rRNA gene sequence available in the GenBank database, http://www.ncbi.nlm.nih.gov/Genbank/index/.html), was found to excrete high levels of levan polysaccharide. Carbon source requirements for biomass and EPS production and EPS yields under controlled bioreactor conditions are reported along with the chemical and rheological characterization of the polymer.

2. Experimental

2.1. Microorganism and growth conditions

Soil samples taken from Çamaltı Saltern area in Turkey were serially diluted in Ringers solution and plated on agar medium containing 5 g L^{-1} yeast extract, 3 g L^{-1} sodium citrate, 20 g L^{-1} $MgSO_4 \cdot 7H_2O$, 2 g L^{-1} KCl and 250 g L^{-1} NaCl. After ten days of incubation at 39 °C, colonies were isolated by repeated serial dilution technique (Nicolaus et al., 2000). Ten microorganisms requiring at least 30 g L^{-1} NaCl for growth were selected. Since they all showed growth between 30 and 200 g L^{-1} NaCl concentration, they were classified as moderately halophilic microorganisms. Genomic DNA of the isolates were extracted and when the sequences of their 16S rDNA regions were aligned using Clustal W program (Higgins, Bleasby, & Fuchs, 1992), they were found to belong to *Halomonas* genus. The ten *Halomonas* sp. isolates were screened for exopolysaccharide production on the following chemical medium (g L^{-1}): 7 K_2HPO_4; 2 KH_2PO_4; 0.1 $MgSO_4 \cdot 7H_2O$; 1 $(NH_4)_2SO_4$; 0.5 Peptone; 100 NaCl and 10 carbon sources, pH 7.0. Between 10 tested strains, one of them (strain AAD6, JCM 15723), was able to produce exopolysaccharide and was chosen for further work.

To study the influence of various carbon sources on EPS production by *Halomonas* sp. AAD6, glucose, lactose, sucrose, arabinose, xylose, maltose, raffinose, fructose, galactose, mannose, acetate, glycerol, trisodiumcitrate, rhamnose were added as sole carbon source to chemical medium at concentration of 10 g L^{-1}. For the shake flask cultures, Certomat BS-1 orbital shaker (B. Braun, Germany) set at 37 °C and 180 rpm agitation rate was used. Bioreactor cultivations were performed with BIOSTAT Q multi-bioreactor system. In each run, the working volume was 500 ml, the temperature and pH were kept constant at 37 °C and pH 7, respectively. Aeration was provided at a rate of 0.1 vvm and agitation was set to 200 rpm. The composition of medium used for the bioreactor cultivations, was (g L^{-1}): 137.2 NaCl; 50 Sucrose; 7 K_2HPO_4; 2 KH_2PO_4; 0.1 $MgSO_4 \cdot 7H_2O$; 1 $(NH_4)_2SO_4$ and 0.5 Peptone. The maximum specific growth rate (μ_{max}) of the culture was determined from the slope of the lnx versus time plots in the exponential phase using linear regression as 0.3162 ± 0.005 h^{-1}. To determine the EPS yields, samples were taken at different phases of the growth and EPS was purified from the culture supernatants by alcohol precipitation and dialysis. Results are averages of four different runs.

2.2. Isolation and purification of EPS

For the purification of EPS, cells were harvested at early stationary phase by centrifugation at 13000*g* for 20 min and the supernatant were treated with an equal volume of ethanol as previously described (Poli et al., 2004). Alcoholic solutions were kept at −18 °C overnight, centrifuged at 15000 g for 30 min and then the pellets were dissolved in hot distilled water. The same procedure was repeated again. The final water solutions were dialyzed against tap water (48 h) and distilled water (20 h), then freeze-dried and weighted. The EPS samples were analyzed for total carbohydrate, protein, nucleic acid and uronic acid contents. Purification of EPS was performed using a DEAE-Sepharose CL-6B, (1.5 × 40 cm) eluted with 0.1 l H_2O and 0.4 l NaCl gradient from 0 to 1 M with a flux of 0.3 ml min^{-1}. Fractions (5 mL each) were tested for carbohydrate presence and content. Each step of purification was estimated for protein content, reading the absorbance at 280 nm, and for nucleic acid content reading the absorbance at 260 nm.

2.3. Analytical methods

The growth of the cells was monitored by measuring off-line the optical densities at 660 nm using Lambda35 UV/vis spectrophotometer (Perkin-Elmer, USA). To convert optical density (OD) values to dry cell weight (DCW), a calibration chart was prepared. For this, known volumes of culture samples at varying OD values were centrifuged at 8000*g* for 15 min; harvested cells were washed twice with distilled water and then dried at 100 °C until constant cell dry weight was achieved. Carbohydrate presence was analyzed by spot test on TLC sprayed with α-naphthol and carbohydrate content was determined using phenol/sulfuric acid method using glucose as standard (Dubois, Gilles, Hamilton, Robers, & Smith, 1956). Protein concentration was determined by the Bradford test using Bovine Serum Albumin (BSA) as standard (Bradford, 1976). Uronic acid content was determined according to Blumenkrantz and Asboe-Hansen (1973).

2.4. Chemical characterization of EPS

For the sugar analysis, lyophilized samples (3–4 mg) were hydrolyzed with 0.5 M trifluoroacetic acid (TFA) at 120 °C for 1.5 h. The sugar composition of the EPS was analyzed by TLC and HPAE-PAD using standards for identification and calibration curves (Manca et al., 1996).

Molecular size analyses were carried out by Sepharose CL-6B column (Pharmacia) using H_2O as eluant, with a flux of 0.3 mL min^{-1}, and by density gradient centrifugation method, using a sucrose gradient from 0 to 50% w v^{-1} at 13000*g* for 16 h (Pazur, 1994). In both methods 10 mg of EPS and a mixture of dextran for calibration curves (150,000, 670,000 and 1,800,000 Da, Fluka) were used.

Fourier transform-infrared (FT-IR) spectroscopy spectra of EPS were obtained with Nicolet 6700 FT-IR Spectrometer between 400 and 4000 wave numbers (cm^{-1}). Thermogravimetric analysis of EPS was obtained with Mettler TGA apparatus where a known amount of EPS sample (5 mg in 2 ml H_2O) was heated from 30 to 400 °C at a rate of 20 °C min^{-1} under a constant flow of nitrogen.

For glycosyl analysis, the sample (1 mg) was mildly hydrolyzed with 1% AcOH at 100 °C for 4 h. The product was reduced with $NaBD_4$ and acetylated using Ac_2O and pyridine at 100 °C for 30 min. The presence of glucitol and mannitol was detected by using GC–MS. The linkage positions of the monosaccharides were determined by methylation analysis. Briefly, the sample (1 mg) was methylated with CH_3I in dimethyl sulfoxide and NaOH (2.5 h). The product was hydrolyzed using TFA 1 M at 70 °C for 45 min. After reduction with $NaBD_4$ the sample was acetylated and finally analyzed by GC–MS. The sugar derivatives were analyzed on a Agilent Technologies gas chromatograph 6850 A equipped with a mass-selective detector 5973N and a Zebron ZB-5 capillary column (Phenomenex; 30 m × 0.25 mm i.d.; flow rate: 1 ml min^{-1}; carrier gas: He). The temperature program for alditol acetates was: 150 °C for 5 min, from 150 °C to 310 °C at 3 °C min^{-1}. The partially methylated alditol acetates were analyzed with the following temperature program: 90 °C for 1 min, from 90 to

140 °C at 25 °C min^{-1}, from 140 to 200 °C at 5 °C min^{-1}, from 200 to 280 °C at 10 °C min^{-1}, 280 °C for 10 min.

Optical rotation value was obtained on a Perkin-Elmer 243 B polarimeter at 25 °C. NMR spectra of polysaccharide (15 mg mL^{-1} D_2O) were performed on a Bruker AMX-300 MHz (^1H NMR) and 400 MHz (^{13}C NMR) at 70 °C and samples were prepared according to Nicolaus et al. (2000). Measurements of specific viscosity as a function of concentration of aqueous solutions of EPS were carried out using Cannon-Ubbelohde 75 suspended level viscometers at 25 °C.

2.5. Biocompatibility studies

Brine Shrimp Test. The inhibition of avarol toxic activity on brine shrimp (Artemia salina) test was performed in triplicate using 10 naupli, for each dose, in artificial sea water (Meyer et al., 1982). Briefly, 10 ppm of avarol dissolved in DMSO (1% of final volume) were added to each vial containing the polysaccharide in three different doses (500, 50 and 5 ppm) and for each dose survivor shrimps were counted after 24 h and data were statistically analyzed by the Finney program which yields LD_{50} values (Finney, 1971).

Cellular viability and proliferation on osteoblasts and on murine macrophagy. The cell culture preparation was performed according to Rocha et al. (2005). The cells were seeded into 10 ml tissue culture flasks, and grown in a controlled 5% CO_2 incubator at 37 °C. Osteoblasts were then plated 1×10^5 in 24-well plates, and after 2 h, cultures were stimulated by changing the medium with sterile 1% (w v^{-1}) EPS solution that was prepared by suspending EPS in RPMI culture medium. After 48 h incubation, osteoblasts morphology, viability and secretion capability (alkaline phosphatase) were tested. Whereas osteoblast viability was evaluated by MTT assay, the cell morphology was analyzed by inverted optical microscopy and the alkaline phosphatase production was evaluated by BCIP-NBT assay. Cell cultures without EPS were used as control. Results represent mean ± SD of triplicates from three separate experiments ($P > 0.05$). The mouse monocyte/macrophage cell line J774 were plated in 96 culture wells (3.5×10^4 cells/per well) and allowed to adhere for 2 h, at 37 °C in the presence of CO_2 at 5%. The medium was then replaced with fresh medium containing various concentrations of EPS (1–10–100 μg ml^{-1}) and the cells were kept for 72 h. Cell viability was determined according to Mossman (1983). Daunorubicin, an anti-cancer agent that inhibits DNA and RNA synthesis, at concentration of 0.1 μM, was used as positive control.

3. Results

3.1. Production of EPS

Ten microorganisms requiring at least 30 g L^{-1} NaCl for growth were selected from soil samples taken from Çamaltı Saltern area in Turkey. They were classified as moderately halophilic microorganisms in that the growth was observed between 30 and 200 g L^{-1} NaCl concentration. All the isolates were Gram-negative. Genomic DNAs of the isolates were extracted and the gene sequences of their 16S rDNA genes were aligned using Clustal W program (Higgins et al., 1992). They were found to belong to the genus *Halomonas*. Further identification of the isolates are still in progress (E. Toksoy Oner, personal communication). In this study, the ability of halophilic *Halomonas* sp. AAD6 cells to use different carbon sources for growth and EPS production was analyzed by growing cells in chemical medium containing 14 different carbon sources. Samples were taken from shake flask cultures at certain times, analyzed for biomass and EPS production and maximum yields are summarized in Table 1. *Halomonas* sp. AAD6 cells grown in the presence of sucrose were found to reach highest EPS production levels of 1.073 g L^{-1}. Although higher biomass concentrations were observed when glucose, maltose, fructose and galactose were used as carbon source, their EPS production levels were very low when compared with sucrose. Acetate, trisodium citrate, rhamnose and glycerol were most probably not utilized by the microorganisms' metabolism resulting in very poor growth and biopolymer production levels. Arabinose reached almost the same biomass concentrations like xylose but the cells' biopolymer production yields were about 10-fold lower than xylose. In the light of these results, sucrose was chosen as the best carbon source for both biomass and EPS production. Fig. 1 shows the time course of the growth and EPS production of *Halomonas* sp. AAD6 cells cultivated under controlled bioreactor conditions. Biomass and net carbohydrate concentrations reached by the bioreactor cultures after 95 h of fermentation period were 1.141 g L^{-1} and 4.502 g L^{-1}, respectively. The net carbohydrate concentration was calculated by means Dubois method. The maximum specific growth rate (μ_{max}) of the culture was determined from the slope of the lnx versus time plots in the exponential phase using linear regression as 0.3162 ± 0.005 h^{-1}. To determine the EPS yields, samples were

Table 1
The effect of carbon sources on growth and EPS production using chemical media in shake flask cultures.

C source	Biomass (g L^{-1})	EPS (g L^{-1})[a]
Glucose	1.108	0
Lactose	0.103	0.060
Sucrose	0.863	1.073
Arabinose	0.104	0.027
Xylose	0.096	0.267
Maltose	1.374	0.189
Raffinose	0.135	0.206
Fructose	1.548	0
Galactose	1.199	0
Mannose	0.607	0
Rhamnose	0.085	0
Acetate	0.051	0
Glycerol	0.001	0
Trisodium citrate	0.003	0

[a] To determine the EPS yields, EPS was purified from the culture supernatants by alcohol precipitation and dialysis. Results are averages of four different runs.

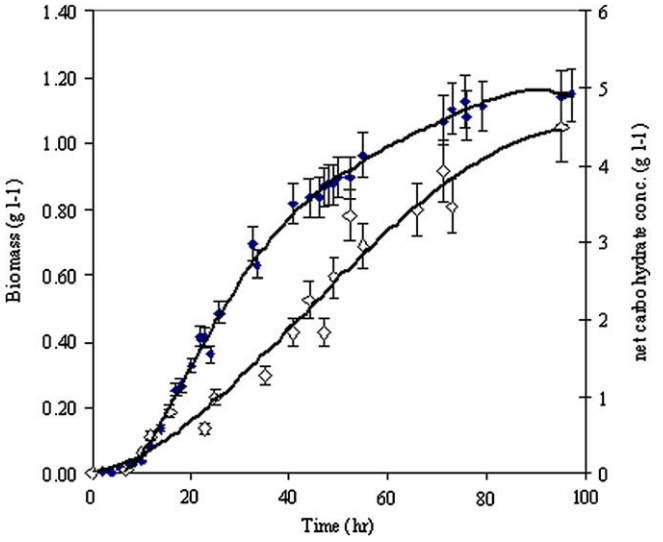

Fig. 1. Time course of biomass (full rumbles) and net carbohydrate concentration (empty rumbles) of *Halomonas* sp. AAD6 bioreactor cultures.

Table 2
Biomass and EPS yields at different phases of growth.

Growth phase	Pre-exponential	Exponential	Pre-stationary	Stationary
Biomass ($g\,L^{-1}$)	0.395	0.492	0.911	1.069
EPS yield ($g\,L^{-1}$)[a]	0.368	0.502	1.844	1.718
EPS yield on biomass ($g\,g^{-1}$)	0.931	1.021	2.024	1.608

[a] To determine the EPS yields, samples were taken at different phases of the growth and EPS was purified from the culture supernatants by alcohol precipitation and dialysis. Results are averages of four different runs.

taken at different phases of the growth and EPS was purified from the culture supernatants by alcohol precipitation and dialysis. The dry cell mass and EPS yields at the pre-exponential, exponential, pre-stationary and stationary phases of growth are summarized in Table 2. The EPS concentration and yield increased with biomass concentration suggesting a growth-associated production, which is also in good agreement with the concomitant increase of net carbohydrate concentration with cell growth (Fig. 1). Maximum EPS concentration and yield was obtained at the pre-stationary phase of growth.

3.2. Chemical characterization of EPS

EPS of bioreactor cultures, when purified on Sepharose DEAE CL-6B column, revealed a first major neutral fraction with highest carbohydrate content (92%) and a second neutral fraction (carbohydrate content 50%) eluted with water; a third and a fourth acidic fractions (carbohydrate content 41% and 13%, respectively) were eluted at 0.2 M and 0.25 M of NaCl, respectively. For further analysis, the first fraction was used. EPS presented low content of proteins (0.51%) and nucleic acids (1.8%) and uronic acid concentration was found as 2.4%. The UV spectra of EPS did not indicate any strong absorption peaks in the range of 210–350 nm. The molecular size of EPS, estimated using a calibration curve of standard dextrans obtained by gel filtration on Sepharose CL-6B and also by density gradient centrifugation, was greater than 1,000,000 Da. The optical rotation value of EPS solution (2.5 mg mL^{-1}), obtained on a Perkin-Elmer 243 B polarimeter at 25 °C, was −117.24.

3.3. Sugar analysis

In our study, sugar analysis was performed by hydrolysis of EPS with 0.5 M trifluoroacetic acid (TFA) at 120 °C for 1.5 h and was identified by HPAE-PAD using standards for identification and calibration curves. Hydrolyzed EPS presented a peak attributable to fructose (RT 7.8 min).

The presence of fructose was clearly showed by GC–MS analysis. After a mild acid hydrolysis of the EPS the product was reduced and acetylated. The reduction of the prochiral keto group lead to the formation of diastereoisomers mannitol and glucitol, which, after acetylation, were revealed in the GC–MS chromatogram.

The linkage positions of the monosaccharides were determined by methylation analysis. The chromatogram showed the presence of 1,3,4,6-tetra-*O*-methyl(2,5-di-*O*-acetyl)-mannitol and – glucitol, 1,3,4-tri-*O*-methyl(2,5,6-tri-*O*-acetyl)-mannitol and – glucitol and 3,4-di-*O*-methyl(1,2,5,6-tetra-*O*-acetyl)-mannitol and – glucitol, which corresponded to t-Fruf, 6-Fruf and 1,6-Fruf, with mole percentages of partially methylated alditol acetates as 1%, 10% and 1%, respectively. The reduction at C-2 of fructose was performed by using $NaBD_4$. This allowed to discriminate between 6-Fruf and 1-Fruf (Hellerqvist & Sweetman, 1990).

The ^1H NMR spectrum of polysaccharide did not exhibited any peaks in the anomeric region. The ^{13}C NMR spectrum of polysaccharide (Fig. 2a) showed the presence of 6-well resolved peaks. The carbon chemical shifts were attributed to β-configurated fructofuranose units, by comparison with the carbon chemical shifts of the standard methyl glycoside (Bock & Pedersen, 1983). Moreover the presence of a downfield shifted signal at 65.9 ppm confirmed a β-(2 → 6) backbone structure (levan-type) for the EPS (Tomasic, Gennings, & Glaudemans, 1978). Thermogravimetric analysis of EPS indicated a degradation temperature (Td) of 253 °C (Fig. 2b). Moreover, the infrared spectrum of this biopolymer showed the characteristic peak signals of polysaccharides: broad stretching peak at around 3300 cm^{-1}, (OH stretching), a weak C—H band at around 2900 cm^{-1}, C=O stretching at 1660 cm^{-1} and several sharp peaks around 1000 cm^{-1} typical of carbohydrates (Fig. 2c). Chemical and spectroscopical data indicated the levan structure for the EPS of *Halomonas* sp. AAD6.

The specific viscosity (η) of aqueous solution of exopolysaccharide at different concentration values was determined at 25 °C. As concentration increases coils start to overlap and become entangled, with viscosity showing a more marked dependence on concentration reaching $\eta = 0.710$ at 2% (w v^{-1}) of concentration.

3.4. Biocompatibility studies of EPS

To asses the biocompatibility of EPS, basic in vitro tests were performed with osteoblast cells isolated from the calvaria of Wistar rats and mouse monocyte/macrophage cell line J774. Osteoblast cell cultures were stimulated with EPS and after 48 h incubation, osteoblasts morphology, viability and secretion capability (alkaline phosphatase) was tested. No evident change in cell morphology and no significant difference in viability and alkaline phosphatase production were detected when the osteoblasts were incubated in the presence of EPS. Moreover, EPS did not affect cell viability on murine macrophage up to 72 h (>95% vs untreated cells), while Daunorubicin caused a cellular death of about 70% at 0.1 μM as expected using a reference anti-neoplastic drug. The inhibitory effect of EPS on the toxic activity of the sesquiterpene hydroquinone avarol (LD_{50} 0.18 ppm) on brine shrimp (*Artemia salina*) was also tested. With decreasing doses of EPS solution (500 ppm, 50 ppm, 5 ppm, respectively), survivor shrimps increased (2, 13 and 16, respectively). The polysaccharide might be forming aggregates at high concentration. It's possible to point out the protective effect only to lower concentration. EPS isolated from *Halomonas* sp AAD6 was found to increase the LD_{50} value of avarol from 0.18 ppm up to 10 ppm.

4. Discussion

Considering the widespread use of microbial polysaccharides in various industrial applications, halophilic microorganisms isolated from Çamaltı Saltern Area in Turkey were screened for their EPS production abilities and the new *Halomonas* sp. AAD6 (JCM 15723) strain was identified as the best producer. Methylation studies and ^{13}C NMR analysis indicated that the nature of this EPS was a levan with a repeating unit composed of β-(2,6)-D-fructofuranosyl residues. Microbial levans are produced from sucrose-based substrates by a variety of microorganisms and are polymers of fructose residues (fructans) synthesized by levansucrase enzymes (E.C. 2.4.1.10). These enzymes cleave their substrate sucrose and couple the fructose residue to a growing fructan chain. Most common levan producer microorganisms are *Lactobacillus reuteri* (Van Geel-Schutten et al., 1999), *Streptococcus salivarius* SS2 (Simms, Boyko, & Edwards, 1990), *Zymomonas mobilis* (Chiang, Wang, Chen, & Chao, 2009; de Oliveira, da Silva, Buzato, & Colabone Celligoi, 2007), *Microbacterium laevaniformans* (Bae, Oh, Lee, Yoo, & Lee, 2008) and *Bacillus* sp. (Arvidson, Rinehart, & Gadala-Maria,

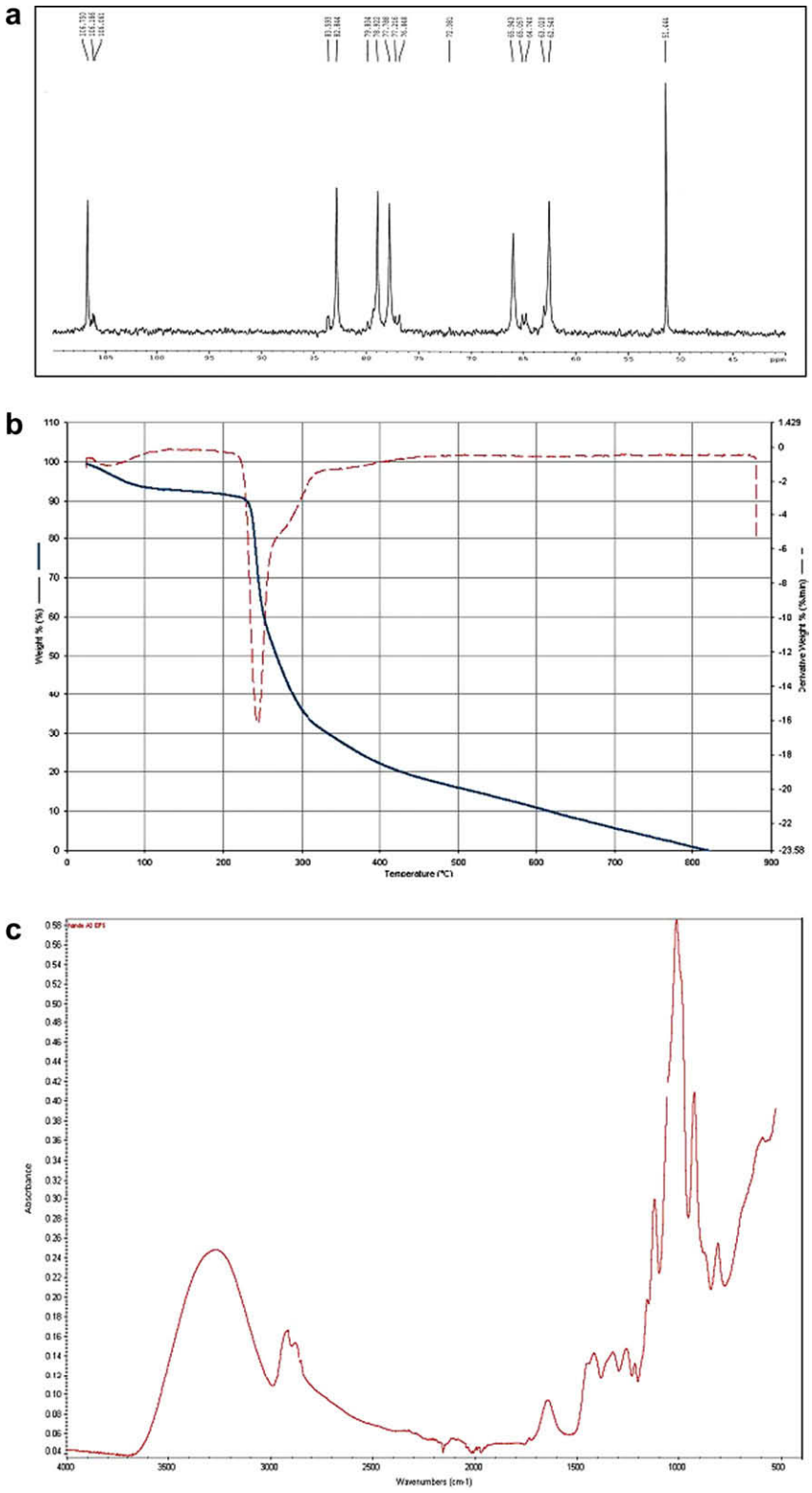

Fig. 2. ^{13}C NMR spectrum (a) TGA thermogram (b) and Fourier transform-infrared spectroscopy spectrum (c) of the EPS by *Halomonas* sp. AAD6.

2006; Shih, Yu, Shieh, & Hsieh, 2005). With this work, a new *Halomonas* sp. has also been described as a levan producer microorganism for the first time. In addition, 1.844 g L^{-1} levan yield from the stationary phase bioreactor cultures using a defined media containing sucrose as sole carbon source (Table 2), could be considered quite high and a good starting point for the on-going studies on the optimization of the cultivation conditions to improve the production yields.

The strain, culture conditions, and type of carbon source influence the amount and the composition of microbial EPS that is produced by a certain species. The production of microbial polysaccharides is sensitive to many factors such as temperature,

pH, incubation periods, carbon sources and nitrogen sources. Usually it involves growth using media with glucose or sucrose as the carbon and energy source and the biopolymer synthesis is often favored by high C:N ratios (Sutherland, 2002).

Finding of new levan producer species is of great interest considering its potential as a functional biopolymer in food, feed, cosmetic, pharmaceutical and chemical industries, overall when a bioproduct could be obtained at high level using a non pathogenic microorganism grown in defined media. Potential applications of levan as an industrial gum, a blood plasma extender, a sweetener, an emulsifier, a formulation aid, a stabilizer, a thickener, a surface-finishing agent, an encapsulating agent, and a carrier for flavor and fragrances have also been proposed (Beine et al., 2008; Shih et al., 2005). In addition, in vitro anti-tumor activity of levan produced from *Microbacterium laevaniformans*, *Rahnella aquatilis* and *Zymomonas mobilis*, has been shown against eight different tumor cell lines (Yoo, Yoon, Cha, & Lee, 2004). Levan produced by *Halomonas* sp. AAD6 did not affect cellular viability and proliferation in two different cellular system tested, osteoblasts and murine macrophages, supporting the high biocompatibility of this EPS. Moreover, in the Brine shrimp test, low concentrations of the polymer (5 ppm), showed a protective effect against the toxic activity of avarol, suggesting its potential use as an anti-cytotoxic agent.

In conclusion, the EPS produced *Halomonas* sp. AAD6 can be considered quite promising for industrial exploitation and is worthy of further investigation to determine the most suitable fields of application. Studies are in progress to elucidate the mechanism of levan synthesis in *Halomonas* sp. AAD6. Considering the recent insight in genetic manipulation of microorganisms for production of new biopolymers with tailored properties suitable for biotechnology industries (Homann, Biedendieck, Götze, Jahn, & Seibel, 2007; Kang et al., 2009), the present paper could supply evidence for new perspectives and application of this novel levan producer bacteria.

Acknowledgments

This work was financed by Joint Project TBAG-U/122(104T263) under bilateral agreement CNR-TUBITAK. The authors thank Dr. Gianluca Anzelmo, PhD student for analytical assistance.

References

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

Arvidson, S., Rinehart, B. T., & Gadala-Maria, F. (2006). Concentration regimes of solutions of levan polysaccharide from *Bacillus* sp. *Carbohydrate Polymers, 65*, 144–149.

Bae, I. Y., Oh, I. K., Lee, S., Yoo, S. H., & Lee, H. G. (2008). Rheological characterization of levan polysaccharides from *Microbacterium laevaniformans*. *International Journal of Biological Macromolecules, 42*, 10–13.

Beine, R., Moraru, R., Nimtz, M., Na'amnieh, S., Pawlowski, A., Buchholz, K., et al. (2008). Synthesis of novel fructooligosaccharides by substrate and enzyme engineering. *Journal of Biotechnology, 138*, 33–41.

Blumenkrantz, N., & Asboe-Hansen, G. (1973). New method for quantitative determination of uronic acids. *Analytical Chemistry, 54*, 484–489.

Bock, K., & Pedersen, C. (1983). Carbon-13 nuclear magnetic resonance spectroscopy of monosaccharides. *Advances in Carbohydrate Chemistry & Biochemistry, 41*, 27–66.

Bouchotroch, S., Quesada, E., Izquierdo, I., Rodriguez, M., & Bejar, V. (2000). Bacterial exopolysaccharides produced by newly discovered bacteria belonging to the genus *Halomonas*, isolated from hypersaline habitats in Morocco. *Journal of Industrial Microbiology and Biotechnology, 24*, 374–378.

Bradford, M. M. (1976). A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Chemistry, 72*, 248–254.

Chiang, C. J., Wang, J. Y., Chen, P. T., & Chao, Y. P. (2009). Enhanced levan production using chitin-binding domain fused levansucrase immobilized on chitin beads. *Applied Microbiology Biotechnology, 82*(3), 445–451.

de Oliveira, M. R., da Silva, R. S. S. F., Buzato, J. B., & Colabone Celligoi, M. A. P. (2007). Study of levan production by *Zymomonas mobilis* using regional low-cost carbohydrate sources. *Biochemical Engineering Journal, 37*, 77–183.

Dubois, M. A., Gilles, K. A., Hamilton, J. K., Robers, P. A., & Smith, F. (1956). Colorimetric method for determination of sugars and related substances. *Analytical Chemistry, 28*, 350–356.

Finney, D. Y. (1971). *Probit analysis* (3rd ed.). Cambridge: Cambridge University Press.

González-Domenech, C. M., Martínez-Checa, F., Quesada, E., & Béjar, V. (2008). *Halomonas cerina* sp. nov., a moderately halophilic, denitrifying, exopolysaccharide-producing bacterium. *International Journal of Systematic and Evolutionary Microbiology, 58*, 803–809.

Hellerqvist, C. G., & Sweetman, B. J. (1990). Mass spectrometry of carbohydrates. In C. H. Smelter & J. Throck Watson (Eds.), *Methods of biochemical analysis* (pp. 91–143). Hoboken, NJ: Wiley-Interscience.

Higgins, D. G., Bleasby, A. T., & Fuchs, R. (1992). Clustal W: Improved software for multiple sequence alignment. *CABIOS, 8*, 189–191.

Homann, A., Biedendieck, R., Götze, S., Jahn, D., & Seibel, J. (2007). Insights into polymer versus oligosaccharide synthesis: Mutagenesis and mechanistic studies of a novel levansucrase from *Bacillus megaterium*. *Biochemical Journal, 407*, 189–198.

Iyer, A., Mody, K., & Jha, B. (2006). Emulsifying properties of a marine bacterial exopolysaccharide. *Enzyme and Microbial Technology, 38*, 220–222.

Kang, S. A., Jang, K-H., Seo, J-W., Kim, K. H., Kim, Y. H., Rairakhwada, D., et al. (2009). Levan: Applications and perspectives. In H. A. Bernd Rehm (Ed.), *Microbial production of biopolymers and polymer precursors*. Washington: Caister Academic Press [chap. 6].

Kumar, C. G., Joo, H., Choi, J., Koo, Y., & Chang, C. (2004). Purification and characterization of an extracellular polysaccharide from haloalkalophilic *Bacillus* sp. I-450. *Enzyme and Microbial Technology, 34*(67), 3–681.

Lee, S. Y., Park, S. J., Park, J. P., Lee, Y., & Lee, S. H. (2003). Economic aspects of biopolymer production. In A. Steinbüchel (Ed.). *Biopolymers* (Vol. 10, pp. 307–337). Weinheim Germany: VCH-Wiley.

Manca, M. C., Lama, L., Improta, R., Esposito, E., Gambacorta, A., & Nicolaus, B. (1996). Chemical composition of two exopolysaccharides from *Bacillus thermoantarcticus*. *Applied and Environmental Microbiology, 62*, 3265–3269.

Martinez-Cánovas, M. J., Quesada, E., Martinez-Checa, F., & Béjar, V. (2004). A taxonomic study to establish the relationship between exopolysaccharide-producing bacterial strains living in diverse hypersaline habitats. *Current Microbiology, 48*, 348–353.

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

Matsunaga, T., Sudo, H., Takemasa, H., & Wachi, Y. (1996). Sulfated extracellular polysaccharide production by the halophilic cyanobacterium *Aphanocapsa halophytica* immobilized on light-diffusing optical fibers. *Applied Microbiology and Biotechnology, 45*, 24–27.

Maugeri, T. L., Gugliandolo, C., Caccamo, D., Panico, A., Lama, L., Gambacorta, A., et al. (2002). A halophilic thermotolerant *Bacillus* isolated from a marine hot spring able to produce a new exopolysaccharide. *Biotechnology Letters, 24*, 515–519.

Meyer, B. N., Ferrigni, N. R., Putnam, J. E., Jacobsen, L. B., Nichols, D. E., & McLaughlin, J. L. (1982). Brine shrimp: A convenient general bioassay for active plants constituents. *Planta Medica, 45*, 31–34.

Mossman, T. (1983). Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxic assays. *Journal Immunological Methods, 65*, 55–63.

Nicolaus, B., Panico, A., Manca, M. C., Lama, L., Gambacorta, A., Maugeri, T. L., et al. (2000). A thermophilic *Bacillus* isolated from an Eolian shallow hydrothermal vent, able to produce exopolysaccharides. *Systematic and Applied Microbiology, 23*, 426–432.

Pazur, J. H. (1994). Neutral polysaccharides. In M. F. Chaplin & J. F. Kennedy (Eds.). *Carbohydrate analysis* (2nd ed., pp. 73–124). Oxford, USA: IRL Press.

Poli, A., Schiano Moriello, V., Esposito, E., Lama, L., Gambacorta, A., & Nicolaus, B. (2004). Exopolysaccharide production by a new *Halomonas* strain CRSS isolated from saline lake Cape Russell in Antarctica growing on complex and defined media. *Biotechnology Letters, 26*, 1635–1638.

Raguénès, G. H. C., Peres, A., Ruimy, R., Pignet, P., Christen, R., Loaec, M., et al. (1997). *Alteromonas infernus* sp. nov., a new polysaccharide-producing bacterium isolated from a deep-sea hydrothermal vent. *Journal of Applied Microbiology, 82*, 422–430.

Rinker, K. D., & Kelly, R. M. (2000). Effect of carbon and nitrogen sources on growth dynamics and exopolysaccharide production of the hyperthermophilic archaeon *Thermococcus litoralis* and bacterium *Thermotoga maritima*. *Biotechnology and Bioengineering, 69*, 537–547.

Rocha, J. H. G., Lemos, A. F., Agathopoulos, S., Valeiro, P., Kannan, S., Oktar, F. N., et al. (2005). Scaffolds for bone restoration from cuttlefish. *Bone, 37*, 850–857.

Roger, O., Kervarec, N., Ratiskol, J., Colliec-Jouault, S., & Chevolot, L. (2004). Structural studies of the main exopolysaccharide produced by the deep-sea bacterium *Alteromonas infernus*. *Carbohydrate Research, 339*, 2371–2380.

Schiano Moriello, V., Lama, L., Poli, A., Gugliandolo, C., Maugeri, T. L., Gambacorta, A., et al. (2003). Production of exopolysaccharides from a thermophilic microorganism isolated from a marine hot spring in flegrean areas. *Journal of Industrial Microbiology & Biotechnology, 30*, 95–101.

Shih, I., Yu, Y., Shieh, C., & Hsieh, C. (2005). Selective production and characterization of levan by *Bacillus subtilis* (Natto) Takahashi. *Journal of Agricultural and Food Chemistry, 53*, 8211–8215.

Simms, P. J., Boyko, W. J., & Edwards, J. R. (1990). The structural analysis of a levan produced by *Streptococcus salivarius* SS2. *Carbohydrate Research, 208*, 193–198.

Sutherland, I. (2002). A sticky business. Microbial polysaccharides: Current products and future trends. *Microbiology Today, 29*, 70–71.

Tomasic, J., Gennings, H. J., & Glaudemans, C. P. J. (1978). Evidence for a single type of linkage in a fructofuranan from *Lolium perenne*. *Carbohydrate Research, 62*, 127–133.

Van Geel-Schutten, G. H., Faber, E. J., Smith, E., Bonting, K., Smith, M. R., Ten Brink, B., et al. (1999). Biochemical and structural characterization of the glucan and fructan exopolysaccharides synthesized by the *Lactobacillus reuteri* wild-type strain and by mutant strains. *Applied and Environmental Microbiology, 65*, 3008–3014.

Yoo, S. H., Yoon, E. J., Cha, J., & Lee, H. G. (2004). Antitumor activity of levan polysaccharides from selected microorganisms. *International Journal of Biological Macromolecules, 34*, 37–41.