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Production and characterization of a microbial glucan, synthesized by *Geobacillus tepidamans* V264 isolated from Bulgarian hot spring

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ABSTRACT

The thermophilic strain producing extracellular polysaccharide (EPS) was isolated from Velingrad hot spring, Bulgaria. StrainV264 showed 98% similarity in 16S rRNA gene sequence with *Geobacillus tepidamans*. Maltose was found to be the most appropriate carbon source for exopolysaccharide production. Maximum EPS production was reached in the early stationary phase. Highest EPS production of 111.4 mg L^{-1} was obtained in fermentor batch cultures at 300 rpm. The purified EPS expressed a high molecular weight and it was very stable at high temperature, showing a degradation at 280 °C. Chemical composition of the bio-polymer, determined by using a high pressure anion exchange-pulsed amperometric detector (HPAE-PAD), showed glucose as its major component. The polysaccharide was also investigated by spectroscopic methods (^{1}H and ^{13}C NMR) which demonstrated the structure complexity of this biopolymer.

1. Introduction

The restricted volume of plant polysaccharides, the increased consumption of microbial exopolysaccharides (EPS) in recent years and their fast recovery provide an opportunity for the successful replacement of plant with microbial polysaccharides in many industrial areas. The diversity in chemical composition of microbial polysaccharides results in a variety of properties that could not be found in plant polysaccharides (Poli et al., 2004; Raguenes et al., 1996). A few EPS like xanthan and gellan, are already utilized in the food industry as a gelling agent and thickener for salad dressings, desserts, sauces, syrups and ice cream (Kornmann, Duboc, Marison, & von Stockar, 2003). New areas for the application of microbial polysaccharides include improving the efficiency of liquid herbicides and insecticides, stabilization of emulsified pharmaceutical and cosmetic creams (Moonmangmee et al., 2002; Sutherland, 1998) as thickeners and stabilizers in shampoos, toothpaste and make-up, solidifier of microbiological and plant tissue culture media. In recent years there has been an increasing interest in their biological activities, like antitumor, antiviral, immunostimulatory (Arena et al., 2006; Weiner, Langille, & Quintero, 1995) and anti-inflammatory (De Stefano et al., 2007). Although EPS synthesis by certain bacterial strains has been recognized for more than a century and dozens of them have been reported. The accumulated knowledge on the structural and rheological properties of bacterial exopolysaccharides is poor.

Extreme environments are proving to be a precious source of microorganisms known as extremophiles that are able to produce interesting molecules, including exopolysaccharides. Thermophilic bacteria are extremophiles and the properties of their extracellular polysaccharides seem to offer numerous applications in various fields of industry. As the viscosity of the fermentation liquor progressively increases to a high level during fermentation, thermophiles suggest much more comfortable processes for polymer production with decreased viscosity at high temperature. Extremophiles offer a great diversity in chemical and physical properties of their EPS compared to anywhere else in the biosphere (Guezennec, 2002). Between thermophiles, representatives of genera *Thermus* (Silipo et al., 2004) and thermophilic bacilli (Manca et al., 1996; Nicolaus et al., 2002) were recently reported as EPS producers. Hyperthermophilic archaeon *Thermococcus litoralis* and bacterium *Thermotoga maritima* (Rinker & Kelly, 2000) were also established to form EPS. Moreover two sulfated EPSs containing mannose and glucose as major components have been found in *Bacillus thermantarcticus* (Manca et al., 1996). Also marine thermophilic microorganisms were described to produce mannan exopolysaccharides (Nicolaus et al., 2002; Nicolaus, Schiano Moriello, Maugeri, Gugliandolo, & Gambacorta, 2003). New isolated polysaccharide

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– producing microorganisms suggest novel chemical composition of those compounds and correspondingly many new applications in the near future.

This paper describes exopolysaccharide production from a thermophilic bacterium identified as *Geobacillus tepidamans* and chemical–physical characterization of the purified polymer.

2. Experimental

2.1. Screening for EPS producers

Water, soil, and algobacterial mat samples were collected from different springs in Velingrad region, Central Bulgaria. The temperature measured at the sampling sites varied between 54 and 87 °C and pH varied between 6.8 and 9.8. One litre of water from each place was filtered through a sterile filter (Millipore, 0.22 μm), the filter was kept for 2 h on a Petri dish and the growth on Petri dish determined after overnight cultivation. The colonies grown were collected with sterile medium and used for inoculation of 20 mL medium in 100 mL Erlenmeyer flasks. Soil and algobacterial mats (about 1 g each) from the spring areas were used also for inoculation of the liquid medium. The cultures were enriched in PY medium, containing peptone (2 g L^{-1}) and yeast extract (1 g L^{-1}). pH of the medium was adjusted before autoclaving to 7.0 or 10.0 using 1 M NaOH. Cultivation was performed at 60 °C with shaking at 240 rpm. Pure cultures were obtained in Petri dishes in a medium containing peptone (2 g L^{-1}), yeast extract (1 g L^{-1}) and agar (Difco) (20 g L^{-1}). Cultures expressing some carbohydrate degrading activity were further purified by streaking samples on agar for at least three times. Single colonies were then subcultured until pure cultures were obtained. The subcultures were considered pure after microscopic observation of one type bacterium per culture. The isolated strains were screened for exopolysaccharide production on complex agar medium. The mucoid consistency of colonies was used as a selection characteristic.

The growth of the selected microorganisms was tested in the following medium MSM (%): Sucrose (0.6); $(NH_4)_2HPO_4$ (0.1); KCl (0.02); $MgSO_4$ (0.01), yeast extract (0.01), thyamine hydrochloride (0.00001). Different sugars in a concentration of 0.6% were tested as a carbon source by replacement of sucrose in MSM: Ribose, lactose, galactose, rhamnose, xylose, maltose, fructose, mannose, trehalose, glucose, and cellobiose. The concentration of the chosen sugar was varied between 0.3 and 4.8% (0.3% step). The effect of pH on growth of the selected strains was determined in the area 5.0–9.0 with 0.5 pH steps. The influence of temperature on growth was determined at pH 7.0 at different temperature with 5 °C steps under shaking at 240 rpm.

2.2. Taxonomic characteristics

Growth experiments were done in 16 mL tubes with 3 mL minimal salt medium. Methods described by Smibert and Krieg (1981) were used for physiological characterization of strain *Geobacillus tepidamans* V264. Anaerobic growth was tested in anaerobic agar medium. Catalase activity was assayed by mixing a pellet of freshly centrifuged 6 h culture with a drop of 6% hydrogen peroxide. Growth was determined by measuring OD_{600}. One unit OD was established to correspond on 1 mg mL^{-1} cells. The proportionality between turbidity and cell density was established to be constant. 16S rRNA gene of the isolate was amplified directly from cell suspension of the isolate (containing 1–50 ng of bulk DNA) by polymerase chain reaction (PCR). The supernatant was used to amplify the small-subunit rRNA genes with two primers, corresponding to positions 8–28 and 1498–1509 of the *Escherichia coli* small-subunit rRNA sequence. The amplified DNA molecule was 1.5 kb long. Sequencing was performed in Macrogen Service Center (Korea).

2.3. Polymer production

Polymer production was investigated at 60 °C and pH 7.0 in flasks and in 0.75 L bioreactor (Bioflo, New Brunswick, Co Inc., USA) with a working volume of 0.35 L. Culture liquid was inoculated with 1.5% (v/v) suspension of cells in exponential phase. The agitation rates tested in fermentor were 100, 200, 300, and 400 rpm. Air flow rate was fixed to 1:1 (v/v).

2.4. Isolation of EPS

Cells were collected by centrifugation in a stationary phase of growth. The supernatant was treated with an equal volume of cold absolute ethanol added drop wise under stirring in ice bath, held at −18 °C overnight and then centrifuged at 13,000g for 30 min. The pellets were washed two times with ethanol and dissolved in hot water, dialyzed against distilled water and lyophilized. The samples were tested for carbohydrate, protein and nucleic acid content.

2.5. Purification of EPS

Purification of the polysaccharide was performed by gel filtration on a Sepharose DEAE CL-6B column eluted with 0.1 L of H_2O and 0.4 L of a gradient of NaCl from 0 to 1 M at flow rate of 0.3 mL min^{-1}, and fractions of 5 mL were collected. All fractions were tested for spot on thin-layer chromatography (TLC) sprayed by α-naphtol reagent to identify fractions containing carbohydrates.

2.6. Chemical–physical analyses of EPS

Carbohydrate content was determined according to Dubois's method using glucose as standard (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). Total protein, nucleic acid and uronic acids contents were detected as previously described (Nicolaus, Lama, Manca, & Gambacorta, 1999). The molecular weight of the sample (at a concentration of 1 mg mL^{-1} in H_2O) was estimated by two methods. The first method was gel filtration on a Sepharose CL-6B column (1 × 80 cm) using H_2O as the eluent with a flow rate of 0.3 mL min^{-1}. Fractions of 1 mL each were collected. All fractions were tested by TLC. The second method was density gradient centrifugation (Nicolaus et al., 1999) with a sucrose gradient from 0% to 50% (w/v) at 130,000 g for 16 h. The centrifuge tubes were fractionated in 0.2 mL fractions diluted with water, dialyzed against distilled water for 72 h, and then tested for the presence of carbohydrate. In both experiments, 10 mg of polysaccharide and a mixture of dextrans for the calibration curves (2 mg each of the next dextran standards: 150,000 Da; 670,000 Da; and 2,000,000 Da) were used. Hydrolysis of the polysaccharide was performed with 2 M trifluoroacetic acid (TFA) at 120 °C for 2 h. The sugar components were identified both by TLC technique, using as standards: Glucose, fructose, galactose, fucose, mannose, xylose and by using a high pressure anion exchange-pulsed amperometric detector (HPAE-PAD DIONEX) equipped with a Carbopac PA1 column, and the sugars were eluted isocratically with 16 mM NaOH. The optical rotation value was obtained on a Perkin-Elmer 243 B polarimeter at 25 °C in water. The NMR spectra (^1H, ^{13}C) of exopolysaccharide (15 mg mL^{-1} D_2O) were recorded on a Bruker AMX-500 MHz spectrometer at 70 °C. For ^1H analysis, sample was exchanged twice with D_2O with an intermediate lyophilizing step and then dissolved in 500 μL D_2O. Thermogravimetrical analysis was performed by using a Mettler TGA apparatus. 5 mg of the sample

were heated from 30 to 400 °C at a heating rate of 20 °C/min under nitrogen.

2.7. Biological activity – Brine shrimp test

The brine shrimp (*Artemia salina*) assay was performed in triplicate with the appropriate amounts of exopolysaccharide dissolved in DMSO (1% final volume) at concentrations of 500, 50, and 5 ppm, in the presence of avarol at 10 ppm, using 10 freshly hatched larvae suspended in 5 mL of artificial seawater. Briefly, for each dose tested, the surviving shrimps were counted after 24 h, and the data were statistically analyzed by using the Finney program, which affords LD_{50} values with 95% confidence intervals (Finney, 1971).

3. Results and discussion

3.1. Screening for effective EPS producer

Between 377 tested strains, twelve were chosen as exopolysaccharide producers on PY medium due to mucoid consistence of their colonies. Nine of them were able to grow in a minimal salt medium with sucrose as a sole carbon source and three of them (V264, R275, and R279 showing EPS production more than 5 mg L^{-1}) were chosen for further work.

The phylogenetic analysis of 16SrRNA gene sequence of strain V264, R275, and R279 referred them correspondingly to *Geobacillus tepidamans* (98% similarity), *Brevibacillus thermoruber* (99.5% similarity) and *Bacillus flavithermus* (100% similarity), respectively. Strain V264 showed highest quantity of EPS in MSM medium with maltose (19.4 mg L^{-1}), ribose (16 mg L^{-1}), and sucrose (9 mg L^{-1}); strain R275 synthesized EPS most efficiently in presence of mannose (12 mg L^{-1}), maltose (9 mg L^{-1}), and cellobiose (8 mg L^{-1}). The best carbohydrate sources for strain R279 were maltose (14 mg L^{-1}), fructose (7 mg L^{-1}), and glucose (7 mg L^{-1}). Maltose is an efficient carbon source for EPS by the three strains.

3.2. Phenotypic and metabolic characteristics of the isolate V264

The highest EPS production reached by the strain V264 offered its selection for further work. It was isolated from spring water sample with temperature 79 °C and pH 7.8. Strain V264 appeared as a non motile, facultative anaerobic, Gram variable rod 0.6–1.0 by 1.8–3.9 μm in size. Colonies on minimal salt medium were mucoid, glistening, 2–4 mm in diameters after over night cultivation.

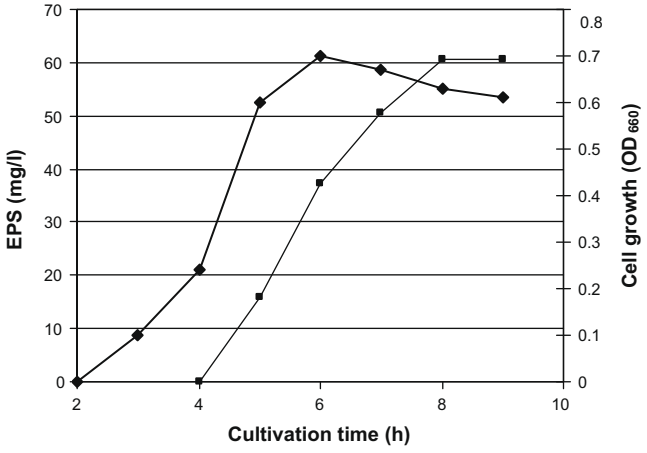

Fig. 1. Time course of EPS production from a strain V 264 at 60 °C, pH 7.0, 240 rpm in a defined medium. Samples were taken in 1 h interval and assayed for growth (▬◆▬) and polysaccharide content. (▬■▬).

The strain grew in pH range between 6 and 9 with an optimum at pH 7.0 and in temperature range between 40 and 65 °C with an optimum at 60 °C. Positive reactions were observed for H_2S and catalase. Neither citrate nor urease utilization was observed. Negative reactions were observed for reduction of nitrates, indol formation, casein degradation, methyl rot and Voges–Proskauer reaction. It grew at 1% NaCl. Different carbohydrates were used as sole carbon source at 1% of concentration: Dextrin, cellobiose, dulcite, glycogen, mannose, ribose, xylose, maltose, fructose, trehalose, glucose, and sucrose. It didn't grow in the presence of lactose, adonitol, galactose, rhamnose, and inulin.

3.3. EPS synthesis

Among twelve different sugars tested, the strain was able to grow in MSM with nine of them. The best growth was observed for maltose, ribose, and sucrose with highest quantity of polysaccharide for maltose as a carbon source (19.4 mg L^{-1}). As a result of batch experiments in flasks with different concentration of carbon and nitrogen, the most suitable concentration of maltose was established to be 30 g L^{-1} with 3 g L^{-1} $(NH_4)_2HPO_4$ and a crude

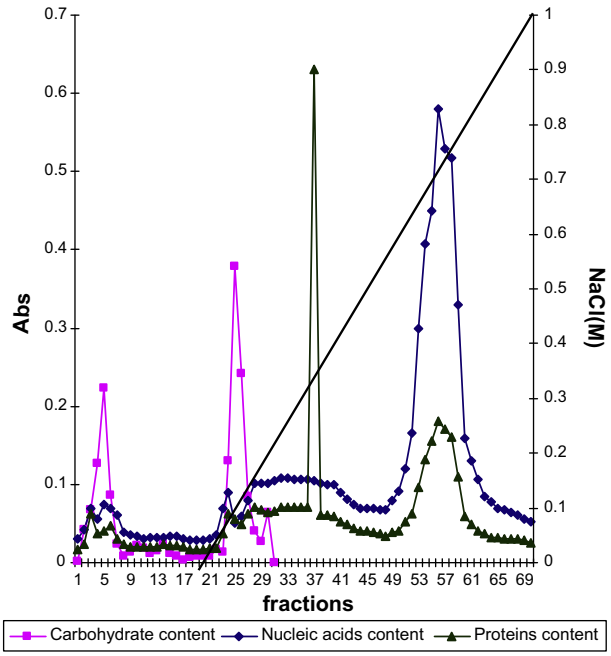

Fig. 2. Purification of EPS on a Sepharose DEAE CL-6B column. Purification of the exopolysaccharide was performed by gel filtration on a Sepharose DEAE CL-6B column eluted with 0.1 L of H_2O and 0.4 L of a gradient of NaCl from 0 to 1 M at flow rate of 0.3 mL min^{-1}. (■ carbohydrate content by Dubois method λ = 490 nm; ♦ nucleic acid content Optical Density λ = 260 nm; ▲ protein content Optical Density λ = 280 nm).

Table 1
Physicochemical properties of exopolysaccharide from *Geobacillus tepidamans* V264 strain.

Characteristic	Results
Carbohydrate content (%)	98%
Protein content (%)	1.8%
Uronic acids (%)	0.2 %
Molecular weight	>1.000.000 Da
Optical rotation $[\alpha]^{25\,°C}{}_D$1 mg mL^{-1}	−244,45
Sugar analysis (molar ratio)	Glu/Gal/Fuc/Fru1/0.07/0.04/0.02
Thermogravimetric analysis (T°C)	280 °C
Configuration	α-Glucogalacto

exopolysaccharide production of 47.5 mg L^{-1} was achieved after 12 h of cultivation. Optimal conditions for EPS synthesis was established to be the same as those for growth – temperature 60 °C and pH 7.0.

Investigation of agitation performed in fermentor tank showed the highest productivity of the cells at 300 rpm – 111.4 mg L^{-1} (106.6 mg L^{-1} at 200 rpm, 60.5 mg L^{-1} at 400 rpm and 58.0 mg L^{-1} at 100 rpm). Time course of growth and EPS production at 300 rpm are presented in Fig. 1. The polysaccharide synthesis begun in the middle of the exponential phase of growth and maximum quantity was reached at stationary phase after 8 h of cultivation. The shortest generation time of 31 min and μ_{max} of 1.335 h^{-1} were established at 300 rpm. The specific polysaccharide production was estimated to be 111.4 μg mg^{-1} cells. Together with other representatives of *Bacillus* and *Geobacillus*, already described as EPS producers (Manca et al., 1996; Nicolaus et al., 2000), the isolated strain V264 clearly confirm the conclusion that thermophilic bacilli could be of commercial value in synthesis of that important class of compounds of growing interest for many sectors of industry. Although EPS production is less than that reported for *Bacillus thermantarcticus* – 400 mg L^{-1} max production (Manca et al., 1996), the unusual short fermentation process (8 h) in comparison with other described (more than 24 h) unambiguously referred the strain as a perspective EPS producer.

3.4. Chemical–physical characterization

The chemical–physical analyses of the EPS revealed 98% carbohydrate in crude exopolysaccharide and low presence of protein (1.8%) and nucleic acid (0.9%). Presence of uronic acid (0.2%) was also registered. Elution profile of EPS purification performed by gel filtration on a Sepharose DEAE CL-6B column is showed in Fig. 2. Fractions containing carbohydrates were identified by TLC and two fractions with highest carbohydrate content were recovered. The first was eluted in neutral condition and the second at 0.2 M of NaCl, but the yield was low; than all following analyses were performed on crude exopolysaccharide. The EPS properties determined are summarized in Table 1. Molecular weight of exo-

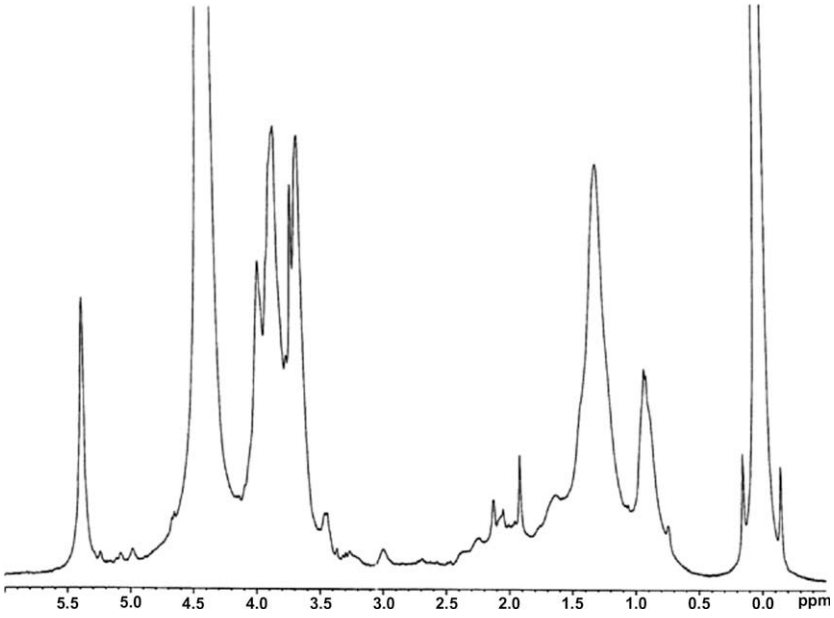

Fig. 3. ^{1}H spectrum of EPS. ^{1}H NMR spectrum of EPS (15 mg mL^{-1} D_2O) were recorded on a Bruker AMX-500 MHz spectrometer at 70 °C. Sample was exchanged twice with D_2O with an intermediate lyophilizing step and then dissolved in 500 μL D_2O.

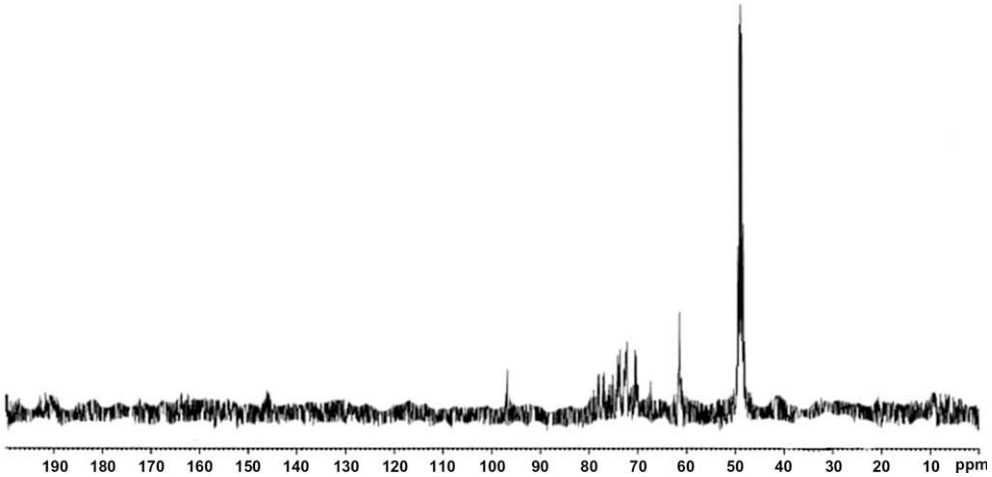

Fig. 4. ^{13}C spectrum of EPS. ^{13}C NMR spectrum of EPS (15 mg mL^{-1} D_2O) were recorded on a Bruker AMX-500 MHz spectrometer at 70 °C.

polysaccharide estimated from a calibration curve of standard dextrans and sucrose gradient centrifugation was approximately higher than 1×10^6 Da. The optical rotation of polysaccharide was $[\alpha]_D^{25\,°C} = -244.45$ (concentration of 1 mg mL of H_2O^{-1}). After hydrolysis of sample with 2 M TFA the sugar components, identified by both TLC and HPAE-PAD Dionex, were: Glucose, galactose, fucose, and fructose in the relative proportion of 1:0.07:0.04:0.02. This result was in a good agreement with the main spot on TLC, related to glucose. The exopolysaccharide synthesized by *Geobacillus tepidamans* V264 isolated from Bulgarian hot spring was a bio-polymer with a glucan like structure.

^{1}H NMR spectrum of polysaccharide showed an interesting profile (Fig. 3). It exhibited four well resolved peaks in region from $\delta = 3.5$ to $\delta = 5.6$ ppm; at $\delta = 5.4$ a signal of anomeric proton was observable. Chemical shifts values of this signal could indicated a probably α-glucogalacto configuration of glicosidic linkage. The signal in upfield region of spectrum was indicative of the probable presence of acetyl group ($\delta = 1.291$). ^{13}C NMR spectrum of biopolymer (Fig. 4) showed *interalia* a typical signal of anomeric carbon at $\delta = 97$, signal of carbons of repeating units and also a signal at $\delta = 61$ confirming the presence of acetyl group in the chain. Results about thermogravimetrical analysis are reported in Fig. 5. The exopolysaccharide showed a weight loss due to water presence, centred at 50–60 °C. From this temperature to 250 °C *ca* the biopolymer was very stable and started to decompose at about 280 °C.

We have studied the effect of exopolysaccharide by *Geobacillus tepidamans* V264 on the inhibition of cytotoxic effects produced by avarol. The anti-citotoxic activity of exopolysaccharide to induce inhibition of avarol (10 μg mL^{-1}) toxicity tested in brine shrimp (*Artemia salina*) bioassay was evaluated. Avarol (a natural toxic sesquiterpene hydroquinone isolated from *Dysidea avara* sponge) (Minale, Riccio, & Sodano, 1974) shows strong toxicity (LD_{50} 0.18 μg mL^{-1}) in brine shrimp bioassay, which gives results that correlate well with cytotoxicity in cancer cell lines such as KB, P388, L5178y, and L1210 (Crispino, De Giulio, De Rosa, & Strazzullo, 1989). For this assay we selected a concentration of avarol of 10 ppm in order to obtain the complete death of total brine shrimp larvae. The bio-polymer isolated from *Geobacillus tepidamans* was found to be an anti-cytotoxic compound in brine shrimp bioassay, increasing the value of LD_{50} of avarol, more than twelvefold, from 0.18 μg mL^{-1} up to 2.24 μg mL^{-1} (Table 2). A possible reason for their action as anti-cytotoxic agents could be the adhesion of toxic compounds to the surface of the polysaccharide.

4. Conclusions

The results reported here gave evidence for good exopolysaccharide production by *Geobacillus tepidamans* V264 in a short fermentation process. The observed properties of the polymer are interesting for future application in industrial processes. Its molecular weight, one of the highest reported for microbial EPS (Nicolaus et al., 2003) could offer high viscosity in industrial products by comparatively low quantity of the polymer in them. The observed significant rigidity of its molecule proved by its decomposition at unusually high temperature is another advantage in its handling and storing. Unlike other agents with anti-cytotoxic activity, polysaccharides are much more neutral to normal metabolism of living organisms and their use in pharmacy could suggest significant priority in development of novel class of anti-cytotoxic drugs. Future investigation of the EPS from *Geobacillus tepidamans* V264 will

Table 2
Brine shrimp test – anti-cytotoxic assay. LD50 Avarol: 0.18 ppm.

	500 ppm[a]	50 ppm[a]	5 ppm[a]	LD_{50} (ppm)
EPS	28/3 0[b]	30/30	30/30	n.a.
EPS + Avarol 10 ppm	2/30	3/30	13/30	2.24

n.a, no activity.
[a] Concentration of samples.
[b] Survivals/total larvae of *Artemia salina*.

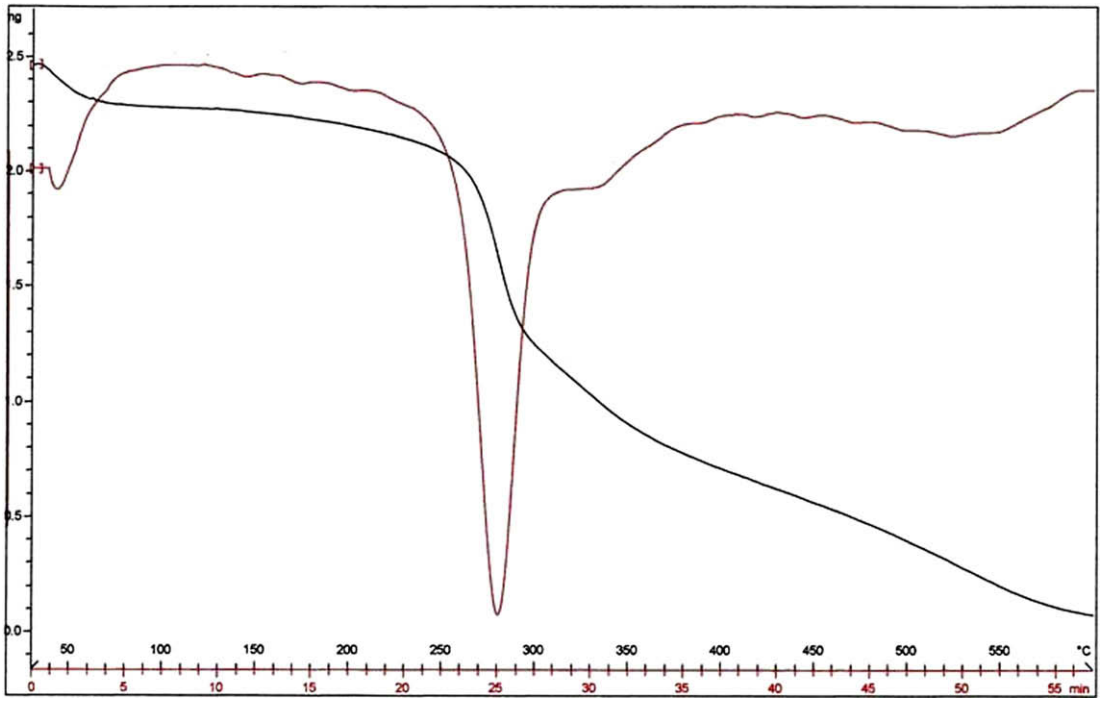

Fig. 5. Thermogravimetric analysis. Thermogravimetrical analysis was performed by using a Mettler TGA apparatus. Five milligrams of sample were heated from 30 to 400 °C at a heating rate of 20 °C/min under Nitrogen.

reveal its functional properties in regard to novel aspects of its biotechnological application.

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References

Arena, A., Maugeri, T. L., Pavone, B., Iannello, D., Gugliandolo, C., & Bisignano, G. (2006). Antiviral and immunoregulatory effect of a novel exopolysaccharide from a marine thermotolerant *Bacillus licheniformis*. *International Immunopharmacology, 6*(1), 8–13.

Crispino, A., De Giulio, A., De Rosa, S., & Strazzullo, G. (1989). A new bioactive derivative of avarol from the marine sponge *Dysidea avara*. *Journal of Naural Products, 52*, 646–648.

De Stefano, D., Tommonaro, G., Simeon, V., Poli, A., Nicolaus, B., & Carnuccio, R. (2007). A polysaccharide from tomato (Lycopersicon esculentum) peels affects NF-κB activation in LPS-stimulated J774 macrophages. *Journal of Natural Products, 70*, 1636–1639.

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

Finney, D. J. (1971). *Probit analysis*. Cambridge: Cambridge University Press.

Guezennec, J. (2002). Deep-sea hydrothermal vents: A new source of innovative bacterial exopolysaccharides of biotechnological interest. *Journal of Industrial Microbiology and Biotechnology, 29*, 204–208.

Kornmann, H., Duboc, P., Marison, J., & von Stockar, U. (2003). Influence of nutritional factors on the nature, yield, and composition of exopolysaccharides produced by Gluconacetobacter xylinus I-2281. *Applied and Environmental Microbiology, 69*, 6091–6098.

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.

Minale, L., Riccio, R., & Sodano, G. (1974). Avarol a novel sesquiterpenoid hydroquinone with a rearranged drimane skeleton from the sponge *Dysidea avara*. *Tetrahedron Letters, 15*, 3401–3404.

Moonmangmee, S., Toyama, H., Adachi, O., Theeragool, G., Lotong, N., & Matsushit, K. (2002). Purification and characterization of a novel polysaccharide involved in the pellicle produced by a thermotolerant Acetobacter strain. *Biosciences Biotechnology Biochemistry, 66*, 777–783.

Nicolaus, B., Lama, L., Manca, M. C., & Gambacorta, A. (1999). Extremophiles: Polysaccharides and enzymes degrading polysaccharides. *Recent Research Development Biotechnology and Bioengineering, 2*, 37–64.

Nicolaus, B., Lama, L., Panico, A., Schiano Moriello, V., Romano, I., & Gambacorta, A. (2002). Production and characterization of exopolysaccharides excreted by thermophilic bacteria from shallow, marine hydrothermal vents of flegrean areas (Italy). *Systematic and Applied Microbiology, 25*, 319–325.

Nicolaus, B., Schiano Moriello, V., Maugeri, T., Gugliandolo, C., & Gambacorta, A. (2003). Bacilli from shallow mediterranean marine vents producers of exopolysaccharides. *Recent Research Development in Microbiology, 7*, 197–203.

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

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 medium. *Biotechnology Letters, 26*, 1635–1638.

Raguenes, G., Pignet, P., Gauthier, G., Peres, A., Christen, R., Rougeaux, H., et al. (1996). Description of a new polymer-secreting bacterium from a deep-sea hydrothermal vent, Alteromonas macleodii subsp. Fijiensis, and preliminary characterization of the polymer. *Applied and Environmental Microbiology, 62*, 67–73.

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

Silipo, A., Molinaro, A., De Castro, C., Ferrara, R., Romano, I., Nicolaus, B., et al. (2004). Structural analysis of a novel polysaccharide of the lipopolysaccharide-deficient extremophile Gram-negative bacterium *Thermus thermophilus* HB8. *European Journal of Organic Chemistry, 24*, 5047–5054.

Smibert, R., & Krieg, N. (1981). General characteristics. In P. Gerhardt, R. Murray, R. Costilow, E. Nester, W. Wood, & G. Phillips (Eds.). *Manual of methods for general bacteriology* (3rd ed, pp. 7–243). Washington, DC: American Society for Microbiology.

Sutherland, I. W. (1998). Novel and established applications of microbial polysaccharides. *Trends in Biotechnology, 16*, 41–46.

Weiner, R., Langille, S., & Quintero, E. (1995). Structure, function and immunochemistry of bacterial exopolysaccharides. *Journal of Industrial Microbiology, 15*, 339–346.