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ORIGINAL ARTICLE

Brevibacillus themoruber: a promising microbial cell factory for exopolysaccharide production

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Abstract

Aims: This study aims to identify a high level exopolysaccharide (EPS) producer thermophile that in turn could be used as a model organism to study the biological mechanisms and whole genome organization of EPS-producing thermophilic bacteria.

Methods and Results: Thermophilic isolates were screened, and then growth and EPS production of the best producer *Brevibacillus thermoruber* strain 423 were investigated under different carbon and nitrogen sources, temperature, pH and agitation rates. Rheological characterization revealed that the EPS behaved like a typical Newtonian fluid and viscosity of the EPS solution increased with increasing Ca^{2+} ion concentration. Chemical characterization by TLC, GC-MS, FT-IR and NMR suggested a heteropolymer structure with glucose as major monomer unit. High biocompatibility of pure EPS fractions suggested their potential use in biomedical applications.

Conclusions: This study reports on the comprehensive description of microbial production conditions as well as chemical, rheological and biological characterization of the EPS produced by *B. thermoruber* strain 423. The bioreactor cultures were found to reach two times higher yields and three times higher productivities when compared with literature.

Significance and Impact of the Study: *Brevibacillus thermoruber* strain 423 combined the advantages of its nonpathogenicity with the advantages of fast productivity and hence proved to be a very promising model organism and cell factory for microbial EPS production.

Introduction

Polysaccharides are natural, nontoxic and biodegradable polymers that have numerous applications in health, bionanotechnology, food, cosmetic and environmental sectors. These biopolymers are either extracted from biomass resources like algae and higher order plants or recovered from the fermentation broth of bacterial or fungal cultures. For their sustainable and economical production, rather than plants and algae, microbial sources are preferred since they enable fast and high yielding production processes under fully controlled fermentation conditions. However, the global hydrocolloid market is still dominated by algal and plant polysaccharides like starch, galactomannans, pectin, carrageenan and alginate and superseding these traditionally used gums by their microbial counterparts requires innovative approaches (Toksoy Oner 2013). One such approach is the use of extremophiles as microbial resources for bioactive polysaccharides (Nicolaus *et al.* 2010). During the last decade, significant improvements have been made in discovering and developing new microbial extracellular polysaccharides [exopolysaccharides (EPSs)] from extremophiles that possess novel industrial significance (Nicolaus *et al.* 2010; Freitas *et al.* 2011). From these, thermophiles constitute the least explored group despite their obvious advantages like high EPS production rates due to their fast metabolic activities or lower viscosity of the fermentation medium (Turner *et al.* 2007; Kambourova *et al.* 2009). Hence thermophiles can be considered good cell factories for industrially important EPSs. As part of an ongoing research on understanding the biological mechanisms and whole genome organization of EPS-producing thermophilic bacteria, within the scope of this study, thermophiles isolated from hot spring water samples taken from Turkey and Bulgaria were screened for their EPS production capabilities. Consequently, *Brevibacillus thermoruber* strain 423 was selected as best producer and studies were conducted to optimize important fermentation parameters, EPS and biomass profiles under both shaking flask and controlled bioreactor conditions. The pure EPS fractions obtained from anion exchange chromatography were chemically and biologically characterized.

Materials and methods

All chemicals and solutions used in this study were supplied by Merck (Darmstadt, Germany) or Sigma Aldrich (St Louis, MO, USA).

Isolation of thermophilic bacteria from hot springs

Water samples taken from Kaynarca (82°C, pH 6·8) and Karamustafapasa (58°C, pH 6·6) hot springs located in Bursa (Turkey) were serially diluted on ATCC 697 Medium agar plates (pH 7) and after incubating at 60–80°C, four colonies exhibiting high mucoidity were selected for further screening. On the other hand, water samples taken from hot springs in the regions of Blagoevgrad and Sofia (Bulgaria) were filtered through a sterile filter (0·22 μm; Millipore, Darmstadt, Germany), the filter was kept for 2 h on MSM agar (Kambourova *et al.* 2009) containing maltose as carbon source and after overnight cultivation, ten mucoid colonies were selected for further screening. The best EPS producer *B. thermoruber* strain 423 was isolated from a hot spring close to the village Gradechnitsa (N 41·683°; E 23·183°), Blagoevgrad region, South-West Bulgaria (temperature of spring water – 59°C, pH 6·5).

Taxonomic characterization of bacteria

Wizard® Genomic DNA Purification Kit (Promega, Madison, WI, USA) was used for genomic DNA isolation. 16S rDNA genes were amplified from the extracted DNAs using primers specific to bacterial 16S rDNA gene, 8F and 1513R. Their sequences were determined with Applied Biosystems model 310 DNA sequencer (Foster City, CA, USA) using the ABI PRISM cycle sequencing kit. After BLAST search using the BLAST network service (Altschul *et al.* 1990), the sequences were aligned with sequences from the NCBI database to identify the closest phylogenetic relatives.

Growth media

Previously reported growth medium was slightly modified and then used as basal medium in this study (Kambourova *et al.* 2009; Radchenkova *et al.* 2011). Screening of the thermophilic isolates was performed in the following basal medium (g l^{-1}): 6 maltose; 1 peptone from casein; 0·1 $MgSO_4$; 0·2 KCl; 0·4 yeast extract and 0·0001 thiamine, pH 7. For the EPS production and optimization studies, the basal medium was supplemented with trace elements to give a semi-defined (SD) medium with the following composition (g l^{-1}): six maltose; one peptone from casein; 0·1 $MgSO_4$; 0·2 KCl; 0·4 yeast extract; 0·00044 $ZnSO_4{\cdot}7H_2O$; 0·00005 $CuSO_4{\cdot}5H_2O$; 0·00036 $MnCl_2{\cdot}4H_2O$; 0·00005 $CoSO_4{\cdot}5H_2O$; 0·0023 $FeSO_4{\cdot}7H_2O$; 0·0001 thiamine, pH 7. Composition of the optimized medium (OM) was (g l^{-1}): 18 maltose; 1 peptone from casein; 0·1 $MgSO_4$; 0·2 KCl; 0·4 yeast extract; 0·00044 $ZnSO_4{\cdot}7H_2O$; 0·00005 $CuSO_4{\cdot}5H_2O$; 0·00036 $MnCl_2{\cdot}4H_2O$; 0·00005 $CoSO_4{\cdot}5H_2O$; 0·0023 $FeSO_4{\cdot}7H_2O$; 0·0001 thiamine, pH 6·5.

Fermentation conditions

For the shake flask cultures, Certomat BS-1 orbital shaker (B. Braun. Germany) set at 55°C and 180 rev min^{-1} agitation rate was used and cultivation time was 24 h.

To study the influence of various carbon sources on EPS production by *B. thermoruber* strain 423, maltose in the SD medium was replaced with 6 g l^{-1} glucose, lactose, sucrose, arabinose, xylose, raffinose, fructose, galactose, mannose, glycerol or mannitol. For studying the effects of different nitrogen sources, SD medium was supplemented with peptone, urea or ammonium chloride, each at 1 g l^{-1} concentration. In studies aimed for optimization of carbon and nitrogen source contents, cultures were grown in SD media with varying maltose (6, 12, 18 and 24 g l^{-1}) and peptone (0·3, 0·5, 1 and 2 g l^{-1}) concentrations. To determine the effect of initial pH of cultivation medium on growth and the yield of EPS produced, pH of the OM medium was adjusted to 6·0, 6·5, 7·0, 7·5 or 8·0. Cultivation temperature was determined in the range of 50–60°C using OM medium and at 180 rev min^{-1} agitation rate. Finally, to determine the effect of agitation rate on growth and EPS yield, *B. thermoruber* 423 cultures were grown in OM medium in orbital shaker set to 60, 120, 180 or 240 rev min^{-1} agitation rate. In all these experiments, shaking cultures were incubated for 24 h and then samples were taken and centrifuged to remove cells. EPS in the supernatants was precipitated with one volume of ethanol, re-dissolved in distilled water and then dialysed (Poli *et al.* 2009).

Bioreactor cultivations were performed in a 1L BIOSTAT Q multi-fermenter with well-controlled environment of pH and temperature. In each run, the working volume was 300 ml, the fermentation temperature and pH were kept constant at 55°C and pH 6·5, respectively. Aeration was provided at a rate of 0·15 vvm (volume air/volume medium/min), agitation was set to 180 rev $\min^{-1}$ and total fermentation time was 24 h. OM medium was used for the cultivations. Samples were taken at certain time intervals and EPS was recovered by alcohol precipitation and dialysis as described above.

Reported results are averages of three different runs.

Isolation and purification of EPS

For the isolation of EPS, cells were harvested by centrifugation at 4602 g for 10 min and the supernatant phases were treated with an equal volume of ethanol at 4°C added dropwise under stirring, held at −18°C overnight, and then centrifuged at 13523 g at 4°C for 30 min using a refrigerated centrifuge. The pellets were dissolved in hot distilled water, dialysed against distilled water for 24 hours and then lyophilized using a Lyovac GT2 (Steris, Cologne, Germany) freeze dryer set at −60°C and at a low pressure (*c.* 60 mTorr).

Purification of EPS was performed using an anion exchange chromatography (DEAE-Sepharose CL-6B, 1·5 × 40 cm) eluted with 0·1 l H_2O and 0·5 l NaCl gradient from 0 to 1 mol l^{-1} with a flux of 0·3 ml $\min^{-1}$. The volume of each fraction was 10 ml. Fractions were tested for carbohydrate, protein and nucleic acid contents.

Analytical methods

Cell growth was monitored by measuring the optical densities at 660 nm using Lambda35 UV/Vis spectrophotometer (PerkinElmer, Waltham, MA, USA). To convert optical density (OD) values to biomass concentration in terms of grams dry cell weight per litre, a calibration chart was prepared by a gravimetric method as described in Poli *et al.* (2009). Carbohydrate content was determined using phenol/sulfuric acid method using glucose as standard (Dubois *et al.* 1956). Protein concentration was determined by the Bradford test using Bovine Serum Albumin (BSA) as standard (Bradford 1976). Nucleic acid content of the samples was determined by absorbance of UV light at 260 nm.

Chemical characterization of EPS

The monomer composition was determined after acid hydrolysis: lyophilized samples (1–2 mg) were treated with 2 mol l^{-1} trifluoroacetic acid (TFA) at 120°C for 2 h. The resulting mixture was analysed by TLC using standards (Manca *et al.* 1996). TLC was developed with the following solvent system: (i) acetone/ButOH/$H_2$0 (8/2/2, by vol.) for neutral sugars; (ii) ButOH/H_2O/AcOH (3/1/1, by vol.) for acidic sugars. The monomer composition was also investigated by GC-MS analysis of acetylated methyl glycosides. Methanolysis of purified EPS was performed in 1·25 mol l^{-1} HCl in methanol. Briefly, sample (1·5 mg) was solved in 1·25 mol l^{-1} HCl/MeOH (1 ml) and then heated at 80°C overnight. Subsequently, air-dried samples were acetylated with acetic anhydride (50 μl) and pyridine (50 μl) at 100°C for 30 min. Then, samples were dried and washed with methanol to evaporate pyridine. Samples were then partitioned with $CHCl_3$ (500 μl) and H_2O (500 μl) by vortexing and centrifugation at 251 g for 3 min. The samples were additionally washed three times with H_2O, and the final organic phase was dried. Samples were successively dissolved in acetone (400 μl) and 1 μl was used. GC-MS analysis was performed with an Agilent Technologies Gas Chromatograph 6850A equipped with a Mass Selective Detector 5973N and a Zebron ZB-5 capillary column (Phenomenex, 30 m × 0·25 mm i.d.) with a temperature program set as follows: 150°C for 3 min. 150–280°C at 3°C $\min^{-1}$. Gas carrier was used at a constant flow rate of 1 ml $\min^{-1}$.

Rheological characterization of EPS

To study rheological properties of EPS, lyophilized samples were dissolved in distilled water at 1, 5 and 10 g l^{-1} concentrations and the shear viscosity measurements were recorded using Bohlin Gemini 2 Rheometer (Malvern, Worcestershire, UK) at 25 and 55°C. The shear rate used was in the range of 0·1–12 000 s^{-1}. To investigate effect of $CaCl_2$ (0, 0·2, 0·4, 0·6, 0·8 and 1 mol l^{-1}) on the viscosity of 10 g l^{-1} EPS solution, viscosities were measured between shear rates 10–1200 s^{-1} at both 25 and 55°C.

Spectroscopic analysis of EPS

The infrared spectra of EPS (100-mg KBr tablet) were recorded at room temperature with a Fourier transform infrared Bio-Rad spectrometer (Hercules, CA, USA). ^1H NMR spectra were obtained on a Bruker AMX-400 instrument at 70°C. Before the analysis, EPS samples were exchanged twice in D_2O with intermediate lyophilization and then dissolved in 500 ml of D_2O to a final concentration of 40 mg ml^{-1}. Chemical shifts were reported in parts per million relative to sodium 2.2.3.3-*d*4-(trimethylsilyl)propanoate.

Biocompatibility studies

To assess the biocompatibility of EPS from *B. thermoruber* strain 423, WST-1 assay (Roche Applied Science, Penzberg, Germany) was performed with the monkey kidney fibroblast cell line Cos-7. WST-1 assay measures the increase in metabolic activity and is an index of expansion in the number of viable cells (Berridge *et al.* 1996). Briefly, the cells were seeded into a 96-well plate at a density of $1{\cdot}5 \times 10^3$ cells well^{-1} in a 100 μl Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin (100 units ml^{-1}) and streptomycin (100 μg ml^{-1}) and cultured overnight in an atmosphere of 5% CO_2 at 37°C. After 24 h incubation, the culture medium was removed and replaced with a 100 μl fresh medium containing varying concentrations of EPS (dissolved in serum- and antibiotic- free medium). After 24 h of EPS treatment, WST-1 reagent (10 μl) was added directly to culture wells and incubated for 2 h. The absorbance was measured at 450 nm with a GloMax Multi+ Microplate Multimode Reader (Promega). Untreated cells were used as control. The control cells were considered 100% viable. Statistical analyses were performed by one-way ANOVA followed by the Tukey test for multiple comparisons using the Prism analysis program (V 5.0; Graphpad, La Jolla, CA, USA).

Results

In order to identify a high EPS producer thermophilic strain, samples taken from hot springs located in Bursa (Turkey) as well as from hot springs in the regions of Blagoevgrad and Sofia (Bulgaria) were subjected to preliminary screening and colonies exhibiting high mucoidity were selected for further screening. Based on the EPS production capabilities of the suspension cultures of these thermophilic micro-organisms, strain 423, which was isolated from a hot spring close to the village Gradechnitsa, Blagoevgrad region, South-West Bulgaria, was selected as the best producer.

Identification of strain 423

Based on the 16S rDNA sequence analysis (Genbank accession number KF192950), the selected strain belonged to the phylum Firmicutes and it was closely related with other strains from the species *B. thermoruber* (99% sequence similarity). In 1985, *B. thermoruber* was identified as Gram-positive, spore forming, rod shaped micro-organism that is 0·8 to 1·0 by 2·5 to 4·8 μm in size, neutrophilic, growing in the range 34–58°C and a strict aerobe (Manachini *et al.* 1985). Hence, the strain 423 was preliminarily identified as belonging to the species *B. thermoruber*.

Optimization of fermentation conditions

The production of EPSs is sensitive to many factors such as temperature, pH, incubation periods, carbon sources and nitrogen sources (Mahmoud *et al.* 2004). Hence, an increase in the yield and quality of polymer production is expected by manipulating the culture conditions. In order to achieve this, the growth and EPS production abilities of thermophilic *B. thermoruber* 423 cultures were analysed using different carbon (glucose, lactose, sucrose, arabinose, xylose, maltose, raffinose, fructose, galactose, mannose, glycerol and mannitol) and nitrogen sources (peptone, urea and NH_4Cl), temperatures, pH values and agitation rates.

As shown in Table 1, highest EPS production levels were reached in the presence of maltose. Although cultures reached high biomass concentrations when lactose, sucrose and arabinose were used as carbon sources, their EPS production levels were very low when compared with maltose. Specific product yield (Yp/x) of the cultures was calculated by dividing the net produced EPS amounts by the biomass concentration and highest Yp/x value was observed in the presence of maltose (Table 1). In the light of these results, maltose was chosen as the best carbon source. From the three different nitrogen sources, namely, peptone from casein, urea and NH_4Cl, lack of growth pointed to the fact that urea was most probably not utilized by the micro-organism. While cultures were found to grow in the presence of peptone and NH_4Cl, EPS production (0·195 g l^{-1}) could only be detected in cultures growing on peptone. Considering these results, peptone was chosen as the best nitrogen source for both biomass and EPS production (Table 2).

Table 1 The effect of carbon sources on growth and EPS production by *Brevibacillus thermoruber* strain 423 shaking cultures (24 h, SD medium, 55°C, 180 rev min^{-1})

C – source	Biomass (mg l^{-1})	EPS production (mg l^{-1})	Yp/x (g g^{-1})
Glucose	168	144	0·855
Lactose	594	34	0·057
Sucrose	597	28	0·047
Arabinose	602	36	0·060
Xylose	204	195	0·955
Maltose	208	202	0·968
Raffinose	540	43	0·079
Fructose	383	121	0·315
Galactose	567	145	0·255
Mannose	461	35	0·076
Glycerol	578	66	0·114
Mannitol	470	134	0·284

EPSs, exopolysaccharide.

Table 2 Effect of nitrogen sources on growth and EPS production by *Brevibacillus thermoruber* 423 shaking cultures (24 h, SD medium, 55°C, 180 rev min^{-1})

N – source	Biomass (mg l^{-1})	EPS production (mg l^{-1})	Yp/x (g g^{-1})
Peptone	765	195	0·254
Urea	0	0	0
NH_4Cl	403	0	0

EPS, exopolysaccharide.

In the process of quantitative optimization of the medium composition, biomass yields were found to decrease with increasing maltose concentrations, highest EPS yields could be recovered in the presence of 18 g l^{-1} maltose concentration (Fig. 1a). Similar results were also reported for EPS production by *B. thermoruber* strain 438 (Radchenkova *et al.* 2011).

Investigation of biomass and EPS production capacities of *B. thermoruber* strain 423 on different concentrations of peptone revealed that 1 g l^{-1} was the optimum concentration resulting in highest EPS yields (Fig. 1b). The established ratio between the carbon and nitrogen source (C : N) was 18 : 1 which in turn was in good agreement with previous reports on improved EPS yields at high C : N ratios (Nicolaus *et al.* 2010).

When the effect of initial pH was investigated, biomass concentration was found to vary at low levels between pH 6 and pH 7 and highest EPS production was obtained at pH 6·5 (Fig. 1c). Investigation of varying temperatures on the capacity of biomass and EPS synthesis (Fig. 1d) showed that highest growth and EPS production were obtained at 55°C.

Agitation speed is closely related to oxygen supply and some rheological properties during the cultivation

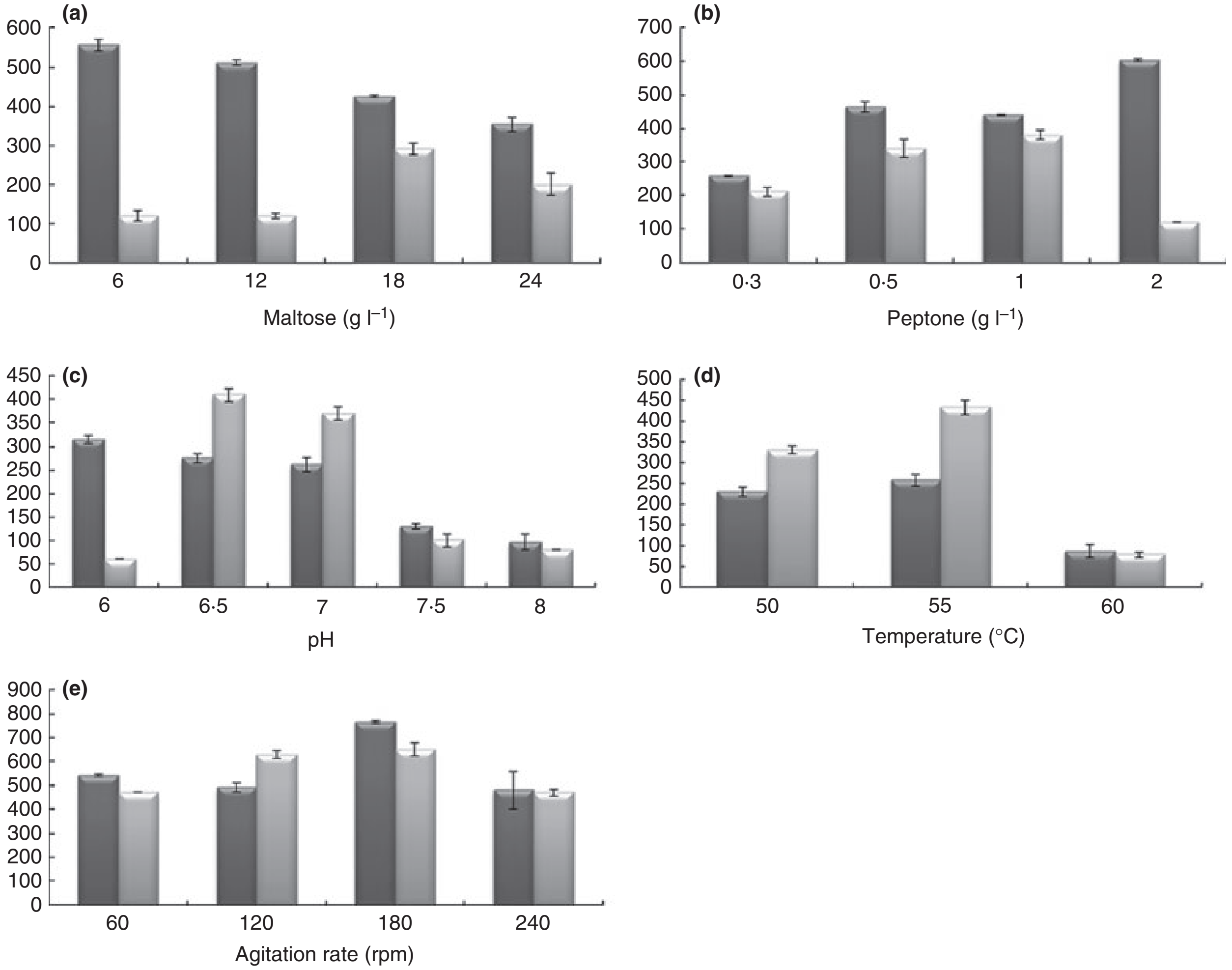

Figure 1 Optimization of fermentation conditions [▓ Biomass (mg l^{-1}), ░ exopolysaccharide (mg l^{-1})]. (a) The effect of maltose concentration (6–24 g l^{-1}). (b) The effect of peptone concentration (0·3–2 g l^{-1}). (c) The effect of pH. (d) The effect of temperature. (e) The effect of agitation rate.

processes. Oxygen supply can influence the formation and accumulation of bioactive metabolites in submerged cultivation. So, agitation rate might play an important role for the production of biomass and EPS. In this study, the effect of agitation speed was investigated in flasks and best agitation speeds for the strain was determined (Fig. 1e). Highest biomass and EPS production were obtained at 180 rev min^{-1}. Biomass and EPS concentrations for *B. thermoruber* strain 423 were 765 and 650 mg l^{-1} respectively.

EPS production in bioreactor

For bioreactor cultivations, a multi-fermenter with well-controlled environment of pH and temperature was used and cultivations were performed under optimized conditions. At certain times, samples were analysed to determine the profiles for biomass and EPS concentrations (Fig. 2). The maximum specific growth rate (μ_{max}) of the culture was determined from the slope of the lnx *vs* time plots in the exponential phase using linear regression as 0·2814 ± 0·0067 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. The dry cell mass and EPS yields were investigated at the pre-exponential, exponential, pre-stationary and stationary phases of growth that spanned *c.* 2–5, 5–12, 12–16 and 16–24 h of the fermentations respectively (Table 3).

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. 2).

Thermophilic bacteria show short fermentation processes due to the high growth rate for micro-organisms at elevated temperature. However, EPS production yield was significantly lower when compared with mesophilic EPS production. EPS production profile in fermenter showed highest EPS (897 mg l^{-1}) production at early stationary phase and Yp/x was 0·71.

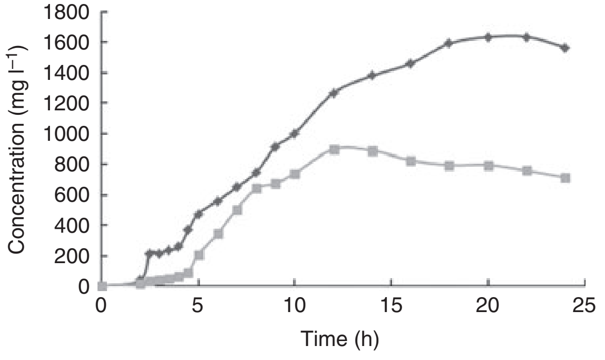

Figure 2 Time course of biomass formation (♦) and exopolysaccharide production (■) of *Brevibacillus thermoruber* strain 423 bioreactor cultures.

Table 3 Biomass and EPS yields at different growth phases of *Brevibacillus thermoruber* 423 bioreactor cultures (OM, 55°C, 0·15 vvm, 180 rev min^{-1})

Growth phase	Pre-exponential	Exponential	Prestationary	Stationary
Biomass (mg l^{-1})	39	651	1460	1633
EPS (mg l^{-1})	17	508	826	798
Yp/x (g g^{-1})	0·430	0·780	0·566	0·489

EPS, exopolysaccharide; OM, optimized medium.

Chemical characterization of EPS

Purification of EPS

Elution profile of EPS purification performed by gel filtration on a Sepharose DEAE CL-6B column is shown in Fig. 3. The first fraction (Peak 1) with highest carbohydrate content and eluted with water, the second and the third fractions were eluted at 0·3 and 0·5 mol l^{-1} of NaCl respectively (Table 4).

Monomer composition

TLC analysis of TFA hydrolysis mixture of EPS showed as a main monomer component glucose; nevertheless, the chemical composition was further investigated by GC-MS analysis that showed in addition to glucose and

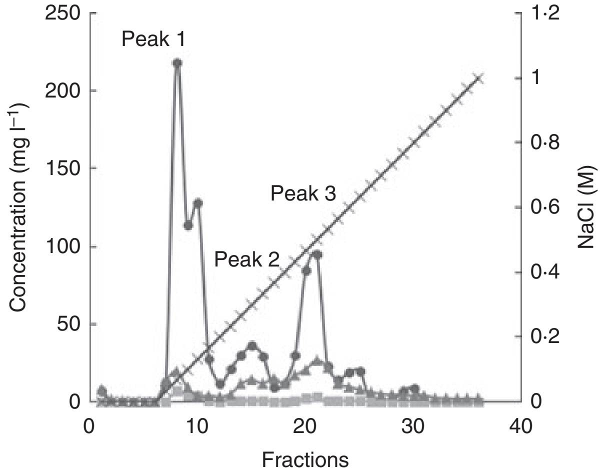

Figure 3 Anion exchange chromatography on DEAE Sepharose CL-6B of exopolysaccharide, (○) carbohydrates, (□) proteins, (△) nucleic acids, (x) NaCl.

Table 4 Chemical composition of EPS fractions recovered by ion exchange chromatography

	Fractions	NaCl (mol l^{-1})	Total volume (ml)	Yield (mg)	Carbohydrates (%)	Nucleic acids (%)	Proteins (%)
Peak 1	8–10	0·1	30	3·5	92	2·1	6
Peak 2	13–16	0·3	40	1·6	53	3·8	7·3
Peak 3	20–21	0·48	20	1·8	71	2·6	8·7

EPS, exopolysaccharide.

also the presence of mannose and galactose, with smaller amounts of galactosamine and mannosamine. According to the relative abundance of the assigned peaks identified in the GC-MS profile, the sugar composition of EPS resulted as follows: glucose/galactose/mannose/galactosamine/mannosamine (57·7/16·3/9·2/14·2/2·4). expressed as percentage of abundance for each monosaccharide (Fig. 4a).

The ^{1}H-NMR spectrum of the EPS showed the presence of three main anomeres at, respectively, 5·55. 5·25 and 5·15 ppm thus suggesting the presence of sugar monomers in an α-type configuration (Fig. 4b).

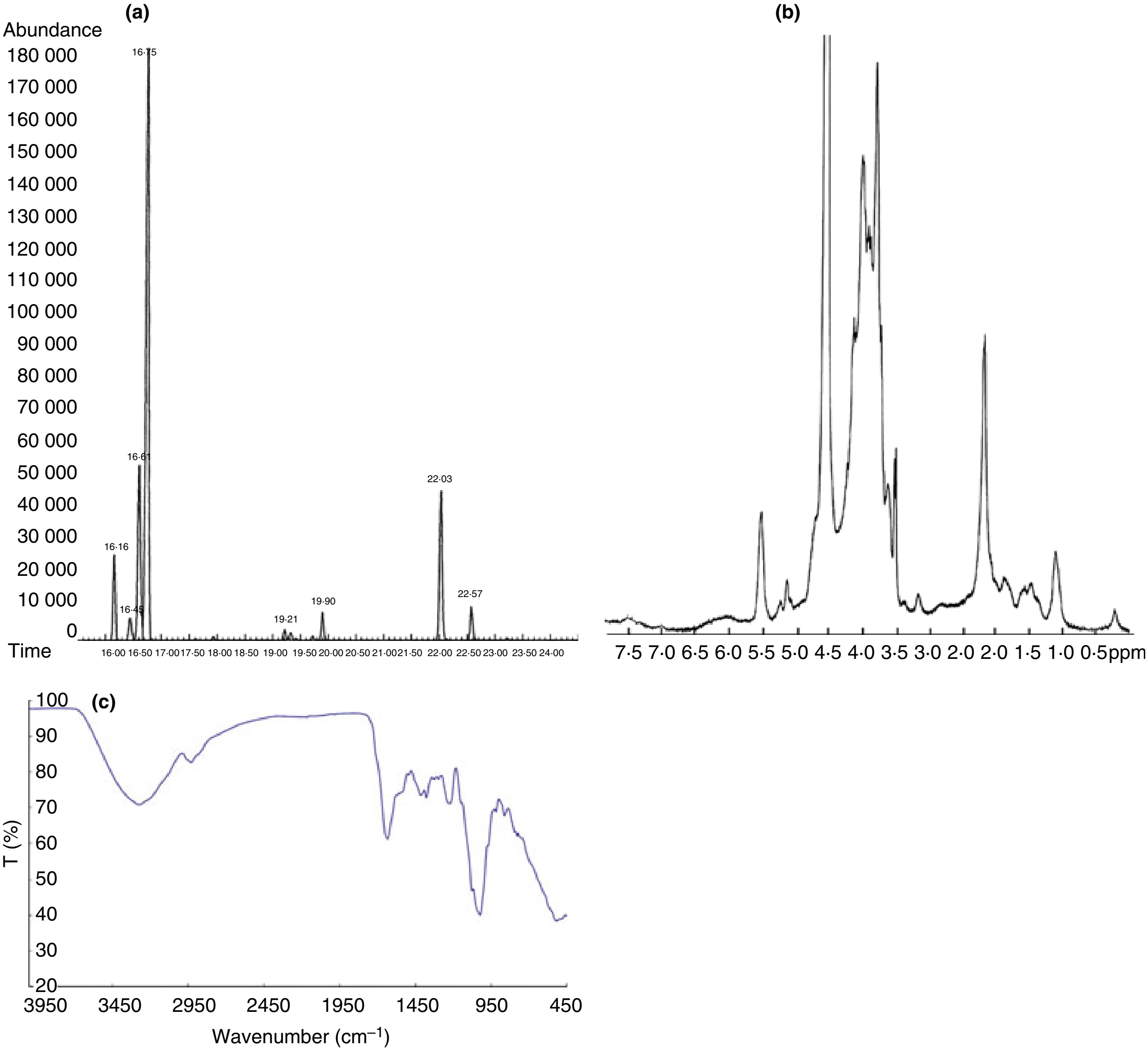

Figure 4 Chemical characterization of exopolysaccharide by *Brevibacillus thermoruber* 423. (a) GC-MS (b) 1H-NMR and (c) FT-IR spectra.

The FT-IR spectrum of the EPS was characterized by three main signals at about 1000, 1630 and 3200 cm^{-1}, respectively, corresponding to typical C-O bending, to N-H bending and to O-H stretching frequencies (Fig. 4c).

Overall, these data suggested a heteropolymer structure for this EPS composed by glucose as prevailing monomer unit.

Rheological studies

Shear viscosity measurements of EPS solutions at increasing concentrations were recorded under two different conditions, namely, at ambient temperature of 25°C and at optimum temperature of microbial production (55°C). Profiles revealed that *B. thermoruber* 423 synthesized EPS with characteristics of a typical Newtonian fluid and its shear stress increased linearly with increasing shear rate (Fig. 5). Moreover, the viscosity of the EPS solutions decreased with increasing temperatures and decreasing concentrations (Table 5).

EPSs are known to bind and accumulate cations from the bulk water phase (Nicolaus *et al.* 2010) which in turn may also alter their rheological properties (Calvo *et al.* 1998). In order to investigate such a possibility, rheological properties of 10 g l^{-1} EPS solution were investigated in the presence of increasing concentrations (0–1 mol l^{-1}) of $CaCl_2$ where Ca^{2+} served as a model divalent cation. Increasing viscosity with increasing concentrations of $CaCl_2$ was observed at all shear rates. At 1000 s^{-1} shear rate, viscosities were found to increase with increasing concentrations of Ca^{2+} by 13 and 14% reaching 10·7 and 8·5 mPa.s at 25 and 55°C respectively (Fig. 6).

Biocompatibility studies of EPS

To assess the biocompatibility of EPS from *B. thermoruber* strain 423, WST-1 assay (Roche Applied Science) was performed with the monkey kidney fibroblast cell line Cos-7. Untreated cells were used as control. The control cells were considered 100% viable. Statistical analyses were performed by one-way ANOVA followed by the Tukey test for multiple comparisons using the Prism analysis program (V 5.0; Graphpad). Results were given for two different samples. EPS purified as a flow through column and peak of salt elution (Fig. 7).

Table 5 Concentration- and temperature-dependent viscosity profile of EPS

EPS concentration (g l^{-1})	Temperature (°C)	Viscosity (mPa.s)
10	25	9·2
5	25	8·7
1	25	8·2
10	55	7·7
5	55	7
1	55	6·6

EPS, exopolysaccharide.

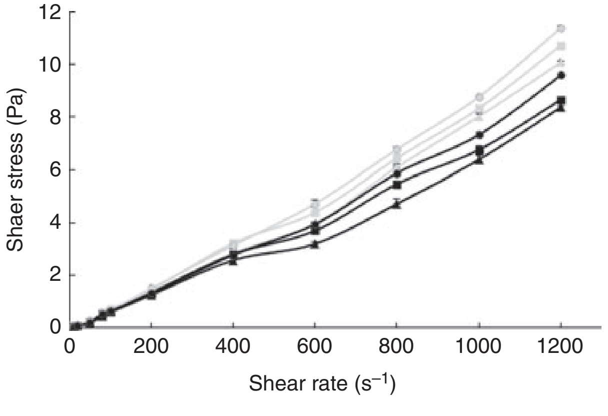

Figure 5 Shear stress–shear rate profile of exopolysaccharide (EPS) from *Brevibacillus thermoruber* 423, (△) 1 g l^{-1} EPS-25°C, (▲) 1 g l^{-1} EPS-55°C, (□) 5 g l^{-1} EPS-25°C, (■) 5 g l^{-1} EPS-55°C, (○) 10 g l^{-1} EPS-25°C and (●) 10 g l^{-1} EPS-55°C.

Considering the fact that biocompatibility is one of key factors for health-related applications. EPS purified as a flow through column and peak of salt elution were subjected to viability tests using monkey kidney fibroblast cell line COS-7. For this, cells were incubated for 24 h with EPS at a wide array of concentrations (10–500 μg ml^{-1}) and then subjected to WST-1 assay. As shown in Fig. 6, while the viability of COS-7 cells treated with 10–100 μg ml^{-1} of EPS were found to increase in the presence of very high concentrations of 500 μg ml^{-1} of EPS, cell viability decreased only by 6·78 and 20·18% with EPS FT and EPS P. respectively. When viabilities of EPS FT and P were compared, higher biocompatibility of EPS FT was observed, especially in the presence of very high concentrations of EPS.

Discussion

In this study, among the thermophiles isolated from hot spring water samples, *B. thermoruber* strain 423 stood out with its high EPS production capability.

Under optimized conditions, EPS and biomass profiles of bioreactor cultures showed that 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. Similar observation for growth-associated EPS synthesis was also reported for glucan (Kambourova *et al.* 2009), levan (Poli *et al.* 2009), xanthan (Kalogiannis *et al.* 2003) and scleroglucan (Survase *et al.* 2007) production. Typically for a thermophilic process, maximum EPS production by *B. thermoruber* strain 423 was registered for significantly

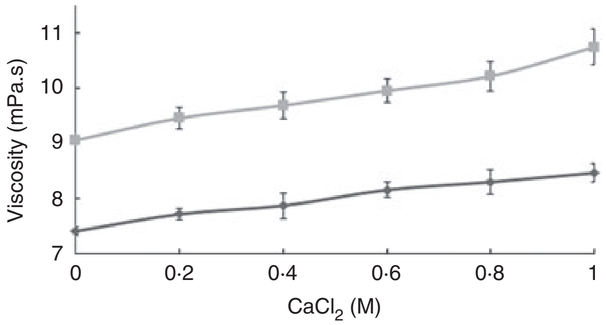

Figure 6 Viscosity profile of exopolysaccharide solutions with varying $CaCl_2$ concentrations at 1000 s^{-1} shear rate at (□) 25°C and (◆) 55°C.

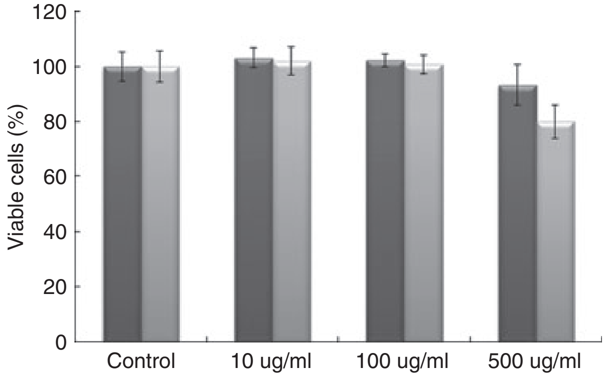

Figure 7 Effect of (▓) exopolysaccharide (EPS) flow through (FT) and (░) EPS peak (P) on monkey kidney fibroblast cell line Cos-7.

shorter period of less than a day when compared with mesophilic processes which may last from few days to several weeks.

EPS production by *B. thermoruber* strain 423 in terms of EPS yield (mg/ml), Yp/s and productivity [mg $(l.h)^{-1}$] was very high when compared with EPSs from thermophilic bacteria (Table 6). As a result, *B. thermoruber* strain 423 can be considered a potential microbial cell factory for EPS production.

In literature, viscosity of EPS produced by *Geobacillus thermodenitrificans* strain B3-72 was reported to be 330 mPa.s at 30°C (Arena *et al.* 2009). At the same temperature, specific viscosity values of 260, 360, 420 and 580 mPa.s were measured for 1, 2, 3, 4 and 5% aqueous solutions of EPS from *Bacillus licheniformis* respectively (Spano *et al.* 2013). In this study, rheological characterization revealed that the EPS produced by *B. thermoruber* had very low viscosity (6·6–9·2 mPa.s) and behaved like a typical Newtonian fluid. In the presence of increasing Ca^{2+} concentrations, viscosity was found to follow an increasing trend; however, <15% improvement could be obtained with $CaCl_2$. More pronounced changes in the rheological properties could be obtained by further studies with other monovalent and divalent cations and under varying acidity (Calvo *et al.* 1998).

Biocompatibility and nontoxic nature of some bacterial EPSs has prompted their uses in numerous medical applications; as scaffolds or matrices in tissue engineering, drug delivery and wound dressing, thus making them more attractive as compared to polysaccharides obtained from plants and microalgae (Nwodo *et al.* 2012). The bioactivity of polysaccharides in traditional medicine, particularly from medical plants, has been investigated for many years. Some polysaccharides have the ability to scavenge free radicals, induce differentiation of cancer cells and enhance animal or human antitumor ability via the activation of various immune responses in the host

Table 6 EPS production yields of thermophilic bacteria

Micro-organism	C-source	Conditions	Yield (mg l^{-1})	Yp/s (g g^{-1})	Productivity (mg l^{-1} h^{-1})	References
Brevibacillus thermoruber strain 423	Maltose	Fermenter, 24 h, 55°C, pH6·5, 180 rev min^{-1}	863	0·479	71·92	This study
Bacillus licheniformis	Sucrose	Fermenter, 48 h, 55°C, pH8, 300 rev min^{-1}	366	0·007	7·62	Spano *et al.* (2013)
Geobacillus stearothermophilus	Ribose	Shake flask, 72 h, 60°C, pH8, 240 rev min^{-1}	185	0·018	2·57	Gugliandolo *et al.* (2010)
Bacillus thermodenitrificans	Glucose/sucrose	Agar plate, 72 h, 65°C, pH7	70	0·117	0·97	Nicolaus *et al.* (2000)
Geobacillus thermantarcticus	Mannose	Fermenter, 24 h, 65°C, pH6, 100 rev min^{-1}	400	0·067	16·67	Manca *et al.* (1996)
Bacillus sp.	Trehalose	Agar plate, 48 h, 60°C, pH7·6	60	0·006	1·25	Nicolaus *et al.* (2002)
B. thermoruber 438	Maltose	Shake flask, 8 h, 55°C, pH8, 240 rev min^{-1}	78·1	0·043	9·76	Radchenkova *et al.* (2011)
Geobacillus tepidamans V264	Maltose	Fermenter, 8 h, 60°C, pH7, 300 rev min^{-1}	111·4	0·004	13·92	Kambourova *et al.* (2009)

EPS, exopolysaccharide.

(Liu *et al.* 2010). High biocompatibility of the pure EPS fractions recovered in this study suggested their potential use in biomedical applications.

Most bacterial EPS producers are pathogenic, which in turn limits the application areas of the EPSs produced by these strains. From this point of view, the nonpathogenic EPS producer *B. thermoruber* strain 423 of this study will have an important advantage over the other microbial systems. The strain is among the limited number of reported thermophilic EPS producers. Although the isolation of another producer belonging to the species *B. thermoruber* was earlier reported (Kambourova *et al.* 2009) as well as the influence of some factors on polymer production (Radchenkova *et al.* 2011), this work reports for the first time a detailed description of cultivation conditions for EPS production by *B. thermoruber*. Moreover, it is the first report on the detailed characterization of EPS synthesized by this species. The strain also exceeds other thermophilic producers in light of the high level of polymer synthesis. The main hypothesis of this research is that the *B. thermoruber* strain 423 will combine the advantages associated to its nonpathogenicity with the advantages due to its fast productivity as a thermophile and prove to be a very promising cell factory for microbial EPS production. Current studies are focused on understanding the polysaccharide production mechanisms in *B. thermoruber* strain 423 and their possible improvement.

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Conflict of Interest

No conflict of interest declared.

References

Altschul, S.F., Gish, W., Miller, W., Myers, E.W. and Lipman, D.J. (1990) Basic 400 local alignment search tool. *J Mol Biol* **215**, 403–410.

Arena, A., Gugliandolo, C., Stassi, G., Pavone, B., Iannello, D., Bisignano, G. and Maugeri, T.L. (2009) An exopolysaccharide produced by *Geobacillus thermodenitrificans* strain B3-72: antiviral activity on immunocompetent cells. *Immunol Lett* **123**, 132–137.

Berridge, M.V., Tan, A.S., McCoy, K.D. and Wang, R. (1996) The biochemical and cellular basis of cell proliferation assays that use tetrazolium salts. *Biochemica* **4**, 14–19.

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

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

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

Freitas, F., Alves, V.D. and Reis, M.A.M. (2011) Advances in bacterial exopolysaccharides: from production to biotechnological applications. *Trends Biotechnol* **29**, 388–398.

Gugliandolo, C., Lentini, V., Spano, A. and Maugeri, T.L. (2010) New bacilli from shallow hydrothermal vents of Panarea Island (Italy) and their biotechnological potential. *J Appl Microbiol* **112**, 1102–1112.

Kalogiannis, S., Iakovidou, G., Kyriakides, M.L., Kyriakidis, D.A. and Skaracis, G.N. (2003) Optimization of xanthan gum production by *Xanthomonas campestris* grown in molasses. *Process Biochem* **39**, 249–256.

Kambourova, M., Mandeva, R., Dimova, D., Poli, A., Nicolaus, B. and Tommonaro, G. (2009) Production and characterization of a microbial glucan, synthesized by *Geobacillus tepidamans* V264 isolated from Bulgarian hot spring. *Carbohyd Polym* **77**, 338–343.

Liu, C., Lu, J., Lu, L., Liu, Y., Wang, F. and Xiao, M. (2010) Isolation, structural characterization and immunological activity of an exopolysaccharide produced by *Bacillus licheniformis* 8-37-0-1. *Bioresour Technol* **101**, 5528–5533.

Mahmoud, M.N.E., Amira, A.E., EL-Shahat, A.T. and Faten, E.H. (2004) Exopolysaccharides production by *Pleurotus pulmonarius*: factors affecting formation and their structures. *Pakistan J Biol Sci* **7**, 1078–1084.

Manachini, P.L., Fortina, M.G., Parini, C. and Craveri, R. (1985) *Bacillus thermoruber* sp. nov. nom. rev. a red-pigmented thermophilic bacterium. *Int J Syst Bacteriol* **35**, 493–496.

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

Nicolaus, B., Panico, A., Manca, M.C., Lama, L., Gambacorta, A., Maugeri, T., Gugliandolo, C. and Caccamo, D.A. (2000) Thermophilic *Bacillus* isolated from an Eolian shallow hydrothermal vent, able to produce exopolysaccharides. *Syst Appl Microbiol* **23**, 426–432.

Nicolaus, B., Lama, L., Panico, A., Moriello, V.S., Romano, I. and Gambacorta, A. (2002) Production and characterization of exopolysaccharides excreted by thermophilic bacteria from shallow, marine hydrothermal vents of Flegrean Ares (Italy). *Syst Appl Microbiol* **25**, 319–325.

Nicolaus, B., Kambourova, M. and Toksoy Oner, E. (2010) Exopolysaccharides from extremophiles: from fundamentals to biotechnology. *Environ Technol* **31**, 1145–1158.

Nwodo, U.U., Green, E. and Okoh, A.I. (2012) Bacterial exopolysaccharides: functionality and prospects. *Int J Mol Sci* **13**, 14002–14015.

Poli, A., Kazak, H., Gurleyendag, B., Tommonaro, G., Pieretti, G., Toksoy Oner, E. and Nicolaus, B. (2009) High level synthesis of levan by a novel *Halomonas* species growing on defined media. *Carbohydr Polym* **78**, 651.

Radchenkova, N., Tomova, A. and Kambourova, M. (2011) Biosynthesis of an exopolysaccharide produced by *Brevibacillus thermoruber* 438. *Biotechnol Biotechnol Eq* **25**, 77–79.

Spano, A., Gugliandolo, C., Lentini, V., Maugeri, T.L., Anzelmo, G., Poli, A. and Nicolaus, B. (2013) A novel EPS-producing strain of *Bacillus licheniformis* isolated from a shallow vent of Panarea Island (Italy). *Curr Microbiol* **67**, 21–29.

Survase, S.A., Saudagar, P.S. and Singhal, R.S. (2007) Use of complex media for the production of scleroglucan by *Sclerotium rolfsii* MTCC 2156. *Bioresour Technol* **98**, 1509–1512.

Toksoy Oner, E. (2013) Pretreatment techniques for biofuels and biorefineries. In *The Microbial Production of Extracellular Polysaccharides from Biomass* ed. Fang, Z. pp. 35–56. Berlin, Heidelberg: Springer.

Turner, P., Gashow, M. and Karlsson, E.N. (2007) Potential and utilization of thermophiles and thermostable enzymes in biorefining. *Microb Cell Fact* **6**, 9.