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Review

Geobacillus thermantarcticus as source of biotechnological thermozymes and exopolysaccharides: A review

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ABSTRACT

The thermophilic bacterium, *Geobacillus thermantarcticus*, (DSM 9572) strain M1, isolated from geothermal soil, near the crater of Mount Melbourne (74° 22' S, 164° 40' E) in Antarctica, was screened for its capacity to produce enzymes with thermostable enzymatic activities. This microorganism produces an extracellular xylanase, β-xylosidase, an intracellular xylose/glucose isomerase and protease. Moreover, *Geobacillus thermantarcticus* produces two exocellular polysaccharides (called EPS 1, EPS 2) that give a typical mucous character to colonies. The exopolysaccharidic fraction was produced with all single carbon substrate assayed, although a higher yield of 400 mg/L was obtained with mannose used as sole carbon and energy source. NMR spectra confirmed that EPS 1 was a heteropolysaccharide where the repeating unit was constituted by four different *α*-D-mannoses and three different *β*-D-glucose residues. It seems to be close to some xanthan polymers. EPS 2 was a mannan: four different *α*-D-mannoses were found as the repeating unit. The scale-up of the batch process and the development of a fed-batch strategy to further improve polysaccharide production were performed, reaching an EPS yield of 3g/L.

KEYWORDS: *Geobacillus thermantarcticus*, thermophilic, thermozymes, polysaccharides

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INTRODUCTION

Thermophilic microorganisms including the thermophilic endospore-forming bacilli, can serve as excellent sources for new molecules and/or more thermostable biocatalysts than those presently available [1, 2]. Recent developments have clearly shown that thermophiles are good sources of novel catalysts of industrial interest. Some of these catalysts have been isolated and their genes successfully cloned and expressed in mesophilic hosts [1, 3].

The majority of the thermophilic bacteria investigated belong to the genus *Geobacillus*, with strains that have been isolated from mesophilic and thermophilic environments [1, 4]. The development of extremophiles from geothermal areas, as resource for novel thermostable enzymes, has received attention over the past decade and even in Antarctica, one of the most extreme environments on earth, the presence of thermophiles has been suggested by the presence of geothermal areas [5].

Thermophilic enzymes (also called thermozymes) are intrinsically stable and active at high temperatures, and offer major biotechnological advantages over mesophilic enzymes. Their thermostability is associated with a higher resistance to chemical denaturants, and performing enzymatic reactions at high temperatures allows higher substrate concentrations, and often higher reaction rates [6].

A large series of thermophilic microorganisms were investigated as source of industrial enzymes for biotechnological processes mainly hydrolytic

enzymes that degrade polysaccharides such as starch, cellulose, which are renewable resources. In view of this, a thermophilic bacterium, *Geobacillus thermantarcticus*, (DSM 9572) strain M1, [7] isolated from geothermal soil, near the crater of Mount Melbourne (74° 22' S, 164° 40' E) in Antarctica, was screened for its capacity to produce enzymes with thermostable enzymatic activities. This microorganism produces an extracellular xylanase, a β-xylosidase, an intracellular xylose/glucose isomerase and a protease.

Enzyme's catalytic properties such as substrate specificity, cofactor specificity, catalytic efficiency and temperature optimum will be described. The relationship between the physiological role of these enzymes and their potential applications will be discussed. Finally, the advantages of the use of these enzymes for bio-processing will be considered.

Only a few years ago, extremophiles were exotic organisms, explored by only few research groups through out the world. Now, although they still retain some of their eccentric status, they are often routinely used as sources of new molecules of biotechnological interest [8]. In fact, it is clear that some extremophiles, particularly those from Archaea, have novel metabolic pathways and so might serve as a source of metabolites with novel properties and applications [8, 9]. Most of the work has been devoted to thermophiles and hyperthermophiles, but other groups have received more attention recently because of their biotechnological potential [10]. Little information about polysaccharide production by extremophiles has been reported in the literature [7, 11-13]. Therefore, a wide search for microorganisms able to produce good yields of new polysaccharides with potentially useful properties has been undertaken, also involving extremophiles because of their well-known capability for biotechnological employment.

Enzymes: Methods, purification and applications

Microorganism

Phylogenetic analyses placed *Bacillus thermantarcticus*, strain M1, as a peripheral member of the thermophilic *Bacillus* rRNA group 5, phylogenetically and phenotypically coherent group of thermophilic bacilli [7, 14]. This group comprises of the established species of thermophilic bacilli, *B. stearothermophilus*, *B. thermocatenulatus*, *B. thermoleovorans*, *B. kaustophilus*, *B. thermoglucosidasius*, *B. thermodenitrificans*, *B. thermantarcticus*, *Saccharococcus thermophilus*, and some species not yet validly published. Some of them were transferred to a new genus *Geobacillus*, according to Nazina *et al.* [15]. *Bacillus thermantarcticus*, proposed at first with the name of the genus "*Bacillus*" by Nicolaus *et al.* 1996 [16], was recently transferred to genus *Geobacillus* as *G. thermantarcticus* [17].

Strain M1, Gram-positive sporulating rod, grew optimally at 60°C at pH 6.0 using a standard growth media composed of 0.8% yeast extract and 0.3% NaCl [16]. Production of enzymes was investigated on media containing 0.1% yeast extract, 0.3% NaCl and 0.6% of different carbon sources such as glucose, xylose, xylan, galactose, arabinose, milk-agar [18].

Xylanase and β-Xylosidase

β-1,4-Xylan, is the major hemicellulose component of lignocellulose biomass [19]. Several enzymes are required for complete hydrolysis and assimilation of xylans, including β-1,4-xylanase (1,4-β-D-xylan xylanohydrolase; E.C 3.2.1.8) and β-xylosidase (1,4-β-D-xylan xylohydrolase; EC 3.2.1.37) [20].

One of the major potential applications of xylanases involves the pulp and paper industry, since the hydrolysis of xylan facilitates the release of lignin from paper pulp and reduces the use of chlorine as bleaching agent [21-23].

The antarctic isolate, *Geobacillus thermantarticus*, produced extracellular xylanase (1,4-β-D-xylan xylanohydrolase; E.C 3.2.1.8) and β-xylosidase (1,4-β-D-xylan xylohydrolase; EC 3.2.1.37), that can be induced by growth conditions. Both enzymatic activities were expressed in a late exponential growth phase. Among carbon sources tested, the best inducer for both enzymes expression was xylan, a cheaper medium, that enhanced xylanase and β-xylosidase activities 11 and 21 fold, respectively [24].

Each extracellular enzyme was separated by gel filtration with Sephacryl S-200 and further purified to homogeneity (141 fold for xylanase

and 160 fold for β-xylosidase). The isoelectric points and molecular masses were 4.8 and 40 kDa for xylanase and 4.2 and 150 kDa for β-xylosidase, respectively. The optimum temperatures were 80°C for xylanase at pH 5.6 and 70°C for β-xylosidase at pH 6.0. At pH 8.0 xylanase retained 43% of activity found at the optimal pH, while β-xylosidase retained 34% of the maximum activity at pH 4.0 and 8.0. The xylanase was stable for 24 h at 60°C whereas at 70°C about half of the initial activity remained after 24 h. The half life of the xylanase activity was 50 min at 80°C. Heat treatment at 60°C for 1 h did not cause inhibition of the activity of β-xylosidase, that retained only 2.5% of initial activity at 80°C [24].

The K_M of xylanase for xylan was calculated to be 1.6 mg/ml while the K_M of β-xylosidase for the substrate *p*-nitrophenyl- β-D-xylopiranoside was 0.5 mM and for *o*-nitrophenyl-β-D-xylopiranoside was 1.28 mM [24].

The purified xylanase digested xylan to form mainly xylobiose and xylotriose while purified β-xylosidase did not digest xylan. The analysis of hydrolytic products was carried out at different temperatures using crude extract as enzyme source. The behavior of two enzymes, in mixture, with respect to the reaction products, was different with respect to the incubation temperature (Fig. 1). In fact at 70°C after 24 h of incubation the major product of hydrolysis was xylose. These results indicated that, xylanase cleaved the substrate to liberate xylo-oligosaccharides and the resulting oligosaccharides were cleaved to form xylose by β-xylosidase action.

At temperature of 80°C, the relative amounts of xylobiose and xylotriose increased with respect to xylose after 24 h, probably due to lower thermostability of β-xylosidase compared with xylanase (Fig. 1).

G. thermantarcticus, growing on xylan as the sole carbon source, had a characteristic xylan digestion system in two steps that is advantageous for recovery of hydrolysis products by modulating growth conditions and physico-chemical parameters [24].

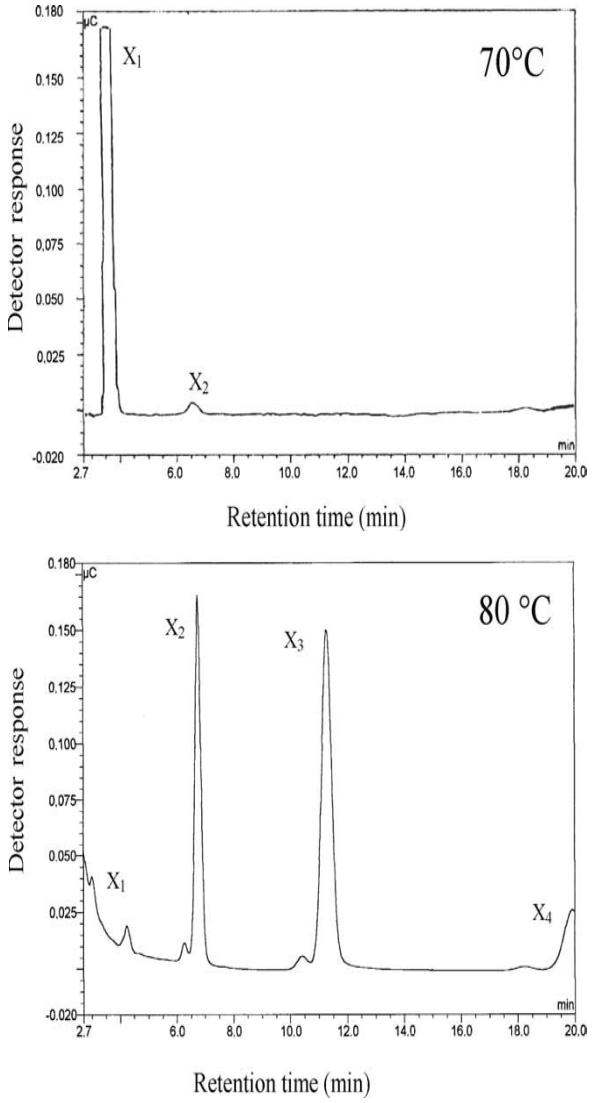

Fig. 1. HPAE-PAD analysis of hydrolysis of birchwood xylan by crude extract of *G. thermantarcticus*. The reactions were performed at 70°C and 80°C and stopped after 24 h incubation (x1, xylose; x2, xylobiose; x3, xylotriose).

Xylose(glucose) isomerase

This enzyme has been used commercially because of its capacity to produce a high-fructose corn enriched syrup (HFCS) by converting D-glucose into fructose [25, 26]. D-Xylose isomerase is also of industrial interest for the isomerization of D-Xylose to D-Xylulose, which can be ultimately fermented to ethanol by conventional yeasts [27].

Geobacillus thermantarcticus produced cytoplasmatic D-Xylose (glucose) isomerase at the late exponential growth phase. The enzyme is able to convert both xylose and glucose into their isomers, xylulose and fructose, respectively. Production of the enzyme was investigated on growth media containing

different carbon sources and the best enzyme yield was obtained in the media containing xylose, both for xylose isomerase and glucose-isomerase. The growth on xylan, a cheap medium, caused an increase in xylose isomerase expression of 23 fold, but did not induce glucose isomerase [28].

The enzyme, purified 73 fold, was a homotetramer with a native molecular mass of 200 kDa and a subunit molecular mass of 47 kDa, with an isoelectric point at 4.8. D-glucose isomerase activity was co-purified exactly with D-Xylose isomerase activity. The enzyme preference for xylose as substrate over glucose was illustrated by lower K_M and higher V_{MAX} toward this substrate. In fact, for xylose isomerase, K_M and V_{MAX} were 33 mM and 57 U/mg, respectively, while for glucose isomerase they were 167 mM and 6.3 U/mg, respectively. This reflected the presumed physiological function of the enzyme, which acted in the organism to produce xylulose, which was subsequently metabolized via either the pentose phosphate pathway or phosphoketolase pathway [28].

The enzyme activity was measured at various temperatures utilizing D-Xylose and D-glucose as substrates. In both cases the optimum temperature was 90°C and the Arrhenius plots of the enzyme activity of xylose isomerase were linear up to 85°C. The optimum pH of the enzyme with both substrates, was around 7.0, showing 80% of its maximum activity at pH 6.0. Glucose isomerase and xylose isomerase were stable in the broad pH range 5.5-10.0 [28].

This enzyme required divalent cations for its activity and thermal stability, although the requirements were different, depending on the substrate used. Mn^{++}, Co^{++}, or Mg^{++} were of comparable efficiency for xylose isomerase reaction, while only Mg^{++} was necessary for glucose isomerase reaction. D-Xylose (glucose) isomerase was stable at high temperatures in the presence of bivalent ions; e.g. at 90°C glucose isomerase reaction showed 102% of residual activity after half an hour of incubation in the presence of Mg^{++} and Co^{++} [28]. Moreover, the enzyme was stable for several hours at room temperature and was stable to freezing and thawing with no loss of enzymatic activity. Treatment of purified enzyme with EDTA resulted in an almost complete loss of enzyme activity. However, the activity could be restored by the addition of metal ions. Increasing amounts of Mn^{++}, Mg^{++} or Co^{++} were able to restore only 60-80% of the original xylose isomerase activity, whereas for glucose isomerase 10mM Mg^{++} was required to restore 80% of the original activity. As reported for other isomerases [29], 10mM Mn^{++} in combination with 1mM Co^{++}, had the ability to restore full xylose isomerase activity, while 10mM Mg^{++} plus 1mM Co^{++} restored total glucose isomerase activity. D-Xylose (glucose) isomerase from *G. thermantarcticus* was remarkably stable to high temperatures. Therefore, this enzyme, stable at acidic pH values, is interesting from biotechnological point of view in that it may increase the efficiency of the process and may reduce the possibility of by-product formation [30].

In addition, the combination of saccharification and isomerization is an ideal development in the progress of HFCS production, and it is likely to be put into operation once an acid-stable enzyme was discovered. Xylose isomerase from *G. thermantarcticus* could be an ideal candidate for the production of high-fructose syrup and ethanol [31].

The high stability of the xylose isomerase is further confirmed by the fact that the enzyme could be immobilized by different techniques without severe losses of enzyme activity. In fact, immobilised cells of *G. thermantarcticus* in sodium alginate, using xylose as substrate, showed the capability to produce xylulose, reaching the same conversion of the free enzyme within 24 h. The same behavior was obtained by using glucose as substrate. The reaction products were identified by HPAE-PAD (Dionex) [32, 33].

Several thermophilic xylose isomerases were also described [34-37]. The molecular weight of about 200,000 Da for the native D-Xylose isomerase from *G. thermantarcticus* and of 47,000 Da for its subunits suggests that the enzyme was similar to those enzymes formed by *Thermotoga maritima, Thermotoga neapolitana, Thermus aquaticus and Thermus thermophilus* [34-36, 38], while the D-Xylose isomerase from *G. stearothermophilus* was described as a monomer with a MW of 127,000 Da [37] (Table 1). *G. thermantarcticus* enzyme showed a high thermostability and an optimum temperature of 90°C, similar to the isomerase from *Thermotoga maritima* that has an

optimum temperature at 100°C (Table 1) [34]. The optimum pH was 7.5-8.0 for the *G. stearothermophilus*, 6.5-7.5 for the *Thermotoga maritima*, 6.8 for the *Thermoanaerobacterium*, 6.0 for *Streptomyces* and 7.0 for the *G. thermantarcticus* (Table 1). Therefore, kinetic characteristics for xylose or glucose isomerization were similar to xylose isomerases from distantly related bacteria [29, 39-42] such as the ones from *Thermotoga* and *Thermus* (Table 1) [34, 36, 38, 43].

Protease

Proteases constitute one of the most important groups of enzymes produced commercially, in that they are widely applied in detergent, protein, waste treatment, etc. [14]. Microorganisms are the largest source for proteases and thermoactive proteases are advantageous in many industrial applications, because higher processing temperatures can be employed with accelerated reaction rates, increased solubility of reactants, and reduced incidence of microbial contamination from mesophilic organisms.

Moreover, *G. thermantarcticus* produced an extracellular proteolytic enzyme [44]. Proteolytic activity was found in the supernatant free-cells and its maximum yield was detected after 12 h, at the end of exponential growth phase, according to most extracellular proteases secreted by Bacilli [45].

The extracellular protease here described was strongly induced by growth conditions, such as the presence of skim milk in the growth medium. In fact, the highest protease production (ca.19 times more with respect to the medium YN) was obtained when the microorganism was grown on medium A containing skim milk 0.1%. Medium B, with and without skim-milk 0.1% gave a moderate increase (ca. 2 times) on protease production with respect to the medium YN (Table 2).

A single protease was purified from the culture supernatant of M1 by ammonium sulphate precipitation, Q-Sepharose F.F. and Superdex 200. The protease was purified about 169-fold with a specific activity of about 4326.5 U/mg and a final yield of 5%. The purified enzyme showed a molecular weight of 42 kDa, an optimal temperature at 70°C and an optimal pH at 7.0 [44].

On the basis of inhibition assays the protease from *G. thermantarcticus* was defined as a serine protease. In fact, enzyme activity was totally

Table 1. Physico-chemical properties of xylose/glucose isomerases from thermophilic microorganisms.

Microorganisms	Molecular weight (kDa)	Optimum Temperature (°C)	Optimum pH	Thermostability
Geobacillus thermantarcticus	200	90	7.0	90°C
Geobacillus stearothermophilus	130	80	7.5-8.0	75°C
Thermotoga maritima	200	105-110	6.5-7.5	100°C
Thermotoga neapolitana	200	95	7.1	Half life 95°C x 24’
Thermus thermophilus	200	95	7.0	85°C x 8h
Thermus aquaticus HB8	196	85	7.5	70°C x 1 month
Thermoanaerobacterium saccharolyticum B6AR1	200	80	7.0-7.5	85°C x 1h
Thermoanaerobacterium JW/SL-YS 489	200	80	6.8	80°C x 1h
Clostridium thermosulfurogenes	200	80	7.0-7.5	85°C x 1h
Streptomyces sp. (PLC)	183	80	7.0	53°C x 10 days
Streptomyces sp. SK	185	95	6.0	80°C x 5h

Table 2. Effect of different media on extracellular *G. thermantarcticus* protease production.

Media	Cell growth ($O.D._{540\ nm}$)	Protease total units	Protease production ($U/O.D._{540nm}$)*
YN	1.705	17.6	10.3
YN + milk 0.1%	1.915	18.4	9.6
Medium A	0.918	30.0	32.7
Medium A + milk 0.1%	1.334	253	190
Medium B	1.290	28.8	22.3
Medium B + milk 0.1%	1.660	35.2	21.2

*After 24 h of growth at 60°C, pH 6.5, the protease activity was assayed with 250 µl of extracellular broth in the standard assay conditions.
YN (standard medium): yeast extract 0.6 g/l; NaCl 0.3 g/l;
Medium A (g/l): yeast extract 1.0, tryptone-soya broth 0.8, $CaSO_4 \cdot 2H_2O$ 0.36, $MgCl_2 \cdot 6H_2O$ 1.6, iron citrate 0.01 M 4.0 ml, in distilled water and the pH of the medium was adjusted to 6.0 with NaOH or H_2SO_4;
Medium B: bactopeptone 15 g, K_2HPO_4 2.9 mM and $MgCl_2 \cdot 6H_2O$ 5 mM in 1 litre of distilled water.

inhibited by PMSF, a common inhibitor of this kind of enzyme [44, 46, 47]. The partial inhibition of protease by TPCK (chymotrypsin inhibitor) but not TLCK (trypsin inhibitor) suggests that the enzyme may have some preference for hydrophobic residues rather than positively charged residues adjacent to the peptide bond to be cleaved [44, 48]. Furthermore, as other *Bacillus* and *Geobacillus* serine proteases, this enzyme appears to be stabilized by Ca^{2+} [47] and shows a high degree of thermal stability in the presence of 2 mM $CaCl_2$. The role of the calcium ions seems to be linked to the stabilization of the enzyme more than needed for its activity [49].

At 70°C after 24 h of pre-incubation in the presence of Ca^{2+} there was also an increase of the proteolytic activity of about 4-fold. This can be due to a rearrangement of the protein structure, which would make the catalytic site more exposed to the substrate attack.

The partial inhibition of EDTA on protease may be attributed then to destabilization of the enzyme structure by removal of calcium ions and therefore the loss of activity was due to enzyme denaturation rather than inhibition [44].

The reversibility of the inhibition from EDTA confirms that the metallic ions have an important role in the maintenance of the active conformation of the protease. Generally, the serine proteases are not inhibited by metal chelating agents; however there are examples of serine proteases that are affected by EDTA [50-53]. Moreover, the resistance to urea and guanidine-HCl indicated that hydrogen bonds play only a very small role in protein stabilization; instead, the enzymatic sensitivity to β-mercaptoethanol and DTT suggested that disulfide-like bonds are involved in preserving the enzymatic structure. The protease produced by *G. thermantarcticus* was also resistant to surfactants, as SDS, DBS and Triton X-100. The increase of activity in the presence of SDS was unexpected; this could be attributed either to the sensitivity of the substrate to SDS, making it more susceptible to enzyme hydrolysis or to the exposure of the active site of the enzyme [54]. In addition, the protease isolated by *G. thermantarcticus* showed stability and compatibility with some commercial detergents in presence of $CaCl_2$ (Table 3).

EPS: Methods, characterization and production

Geobacillus thermantarcticus, was grown for exopolysaccharide production in the culture medium contained (g/l): NaCl 3.0; glucose 6.0; and yeast

Table 3. Compatibility of protease activity from *G. thermantarcticus* with commercial laundry detergents in the presence of 2 mM $CaCl_2$.

Preincubation Time (h)	Residual Activity (%)						
	Control	Lenor	ACE	Mastro Lindo	Scala	Svelto	Soflan
0.5	100	103	59	99	76	70	99
1.5	100	86	52	79	44	29	66
3.0	100	60	49	49	35	18	53

The detergents - Lenor, ACE, Mastro Lindo (Procter & Gamble, Italy), Scala (Deco), Soflan (Colgate) and Svelto (Unilives, Italy) - were diluted in distilled water (0.5% w/v) and incubated with protease in Tris-HCl buffer, 20 mM pH 7.0, 2 mM $CaCl_2$ for 0.5, 1.5 and 3 h at 70°C. The residual protease activity was measured by standard assay conditions and the enzyme activity of a control sample without any detergent was taken as 100%. The average of three replicates was taken, with no statistically significant differences noted in the three replicate determinations.

extract 1.0. The glucose medium supplemented with 2% agar was used for agar plate preparations. The colonies on plates, observed with a Leica Wild M 8 stereomicroscope, showed the presence of a mucous layer [16, 11].

The EPS production was studied in batch fermentation using all minimal media reported above. The temperature was maintained at optimal value of growth, the pH was adjusted to optimal value at the beginning of the culture and measured but not controlled during the experiment. Samples (20 ml) were removed at regular intervals for growth measurement (Abs 540 nm), and EPS production. For exopolysaccharide recovery, cells were harvested in a stationary phase of growth by centrifugation at 9800 *g* for 20 min. The supernatant phases were treated with 1 volume of cold absolute ethanol added drop-wise under stirring.

Alcoholic solutions were kept at -18°C overnight and then centrifuged at 15000 *g* for 30 min. The pellets were dissolved in hot distilled water. The same procedure was repeated again. The final water solutions were dialysed against tap water (48 h) and then against distilled water (20 h), then freeze-dried and weighed. The samples were tested for carbohydrate, protein, and nucleic acid contents [11]. EPS production was tested on cell free cultural broth with phenol-sulphuric acid method using glucose as standard. The polysaccharide fractions were purified by Gel Chromatography as reported in [11]. Sugar analysis was performed by hydrolysis of EPS with 2 M trifluoroacetic acid (TFA) at 120°C for 2 h. Sugar mixture was identified by TLC (thin layer chromatography) and HPAE-PAD (high-pressure anion-exchange pulsed amperometric detection) using standards for identification and calibration curves [11]. HPAE-PAD Dionex equipped with Carbopac PA 1 column was eluted isocratically with (a) 15 mM NaOH for neutral sugars; (b) buffer 100 mM NaOH and 150 mM NaOAc for acidic sugars. Molecular size analyses were determined as reported in [11]. Methylation analysis of the polysaccharides was carried out as described in [11]. Identification of sugars was obtained by GLC (gas chromatography) and GC-MS (gas chromatography combined with mass spectrometry) using standards. GLC runs were performed on a Hewlett-Packard 5890A instrument, fitted with a FID detector and equipped with a HP-5-V column and N_2 flux of 100 ml/min. The temperature program was: 170°C 1 min, from 170 to 180°C at 1°C/min, 180°C 1 min, from 180 to 210°C at 4°C/min. GC-MS was performed on a Hewlett-Packard 5890-5970 instrument, equipped with a HP-5-MS column and with a N_2 flux of 50 ml/min; 170°C 1 min, from 170 to 250°C at 3°C/min was used as temperature program. NMR spectra were obtained on a Bruker AMX-500 (500,13 MHz for ^1H). The EPS spectra were run in D_2O at 70°C [11, 55, 56].

Geobacillus thermantarcticus, produced two different sulphated polysaccharides named EPS1 and EPS2. The production was done in 3 L fermenter

with aeration flux of 20 ml/min. The yield reached 400 EPS mg/cell gram in the presence of mannose as the carbon source. The production of EPSs increased with increasing cell density, reached a maximum at the beginning of the stationary phase and the EPS content was proportional to total biomass. On weight basis, EPS1 and EPS2 represented about 27% and 71% respectively of the total carbohydrate fraction. Analysis of hydrolysis products revealed the presence of a terminal glucose in EPS1; the chain sugars were 1,2-linked mannose, 1,4-linked glucose, 1,3-linked mannose, and 1,6-linked mannose, while 1,3,4-linked glucose and 1,2,6-linked mannose represented branch points in the molecule, in the relative proportion of 0.9/0.5/0.1/0.2/0.5/0.1/1.0, respectively. The same analysis revealed for EPS2, the presence of a terminal mannose; the chain sugars were 1,2-linked mannose, 1,4- linked mannose and 1,6-linked mannose, and the branch point was 1,2,6-linked mannose. Their relative proportions were 1.0/0.5/ 0.2/0.1/0.8, respectively. EPS2 showed a molecular weight of 3.0×10^5 Da and an optical rotation $[\alpha]^{20}_D$ = -90°. Sulphate group and pyruvate presence were also detected. ^{1}H and ^{13}C NMR spectroscopy were performed on both EPSs and showed that EPS1 was a heteropolysaccharide whose repeating unit consisted of four different α-D mannoses and three different β-D-glucoses. This structure was closely related to xanthan polymers. EPS2 was a mannan with four different α-D-mannoses and trace of pyruvic acid as the repeating unit.

Batch and fed-batch fermentations

The experiments were first carried out in a 1-L BiostatQ reactor (Chemup Swiss) with a working volume of 0.7 L. A constant pH of 6.0 was maintained via automated addition of 30% (v/v) NaOH and 30% (v/v) H_2SO_4; dissolved oxygen concentration (DO) was held constant by varying the air flow rate (1-1.5 L/min) and stirring (200-800 rpm) according to oxygen demand and at temperature of 63°C. A Biostat CT plus reactor (Chemup, Swiss), 2-L working volume provided with a digital control unit, was used for the scale-up of the batch process and for fed-batch experiments with the same process variables described above. Differently from the 0.7-L batch fermentations, the culture was spread with pure oxygen when the airflow increase coupled to a high agitation speed was not sufficient to meet the respiratory demand and keep the DO above 20% of saturation.

Fed-batch experiments started after approximately 8 h of growth; during the fed-batch phase, an exponential feeding strategy was applied. The concentrated feeding solution contained 60 g/L of glucose, and inorganic salts according to the medium recipe but 10-fold concentrated. Different feeding rates were used during the fed-batch phase in order to meet the strain's metabolic request.

For the duration of all cultivations, 5-mL samples were withdrawn from the reactors at regular time intervals for the determination of biomass, substrates, extracellular metabolites, and EPS. The basic defined medium containing 6 g/L of glucose and 3 g/L of NaCl was chosen for the scale-up of the process on 2-L bioreactors. The maximum concentration of polysaccharide reached in the processes was 400 mg/L after 24 h of fermentation with a final biomass yield of 0.45 g DCW/L on average [11].

A fed-batch process was next developed in order to provide a steady supply of glucose. In all experiments performed, the feed was provided following an exponential profile after glucose was exhausted in the medium. In Fig. 2 is reported the average of biomass, total EPS produced, and residual glucose concentration in the bioreactor (data obtained from four experiments). Cells on average reached a final concentration of 2.6 g DCW/L.

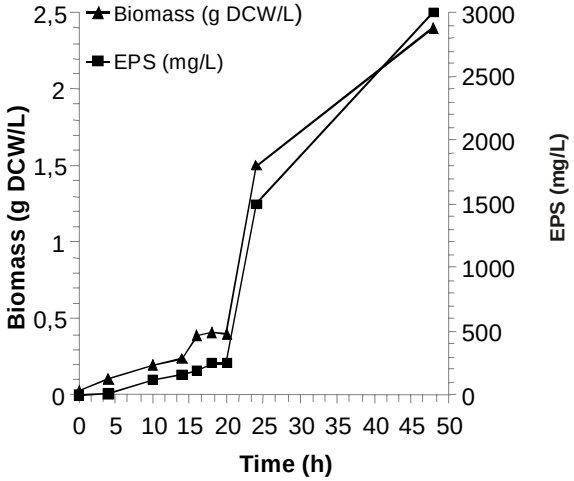

Fig. 2. Biomass yield and EPS production in fed-batch conditions of *G. thermantarcticus*.

Polysaccharide production continued until 48 h and reached a final concentration of 3.0 g/L. The Yx/s and Y EPS/x regarding the overall process were 0.25 and 0.091 g/g, respectively. The kinetics of EPS production were identical to a normal batch process up to 8 h, after which production increased, reaching a maximum after 48 h of growth when a slight decrease in the biomass concentration was constantly observed (Fig. 2).

CONCLUSION

Bacillus thermantarcticus, proposed at first with the name of the genus "*Bacillus*" by Nicolaus *et al.* 1996 [16], has been recently transferred to genus *Geobacillus* as *G. thermantarcticus* [17].

Our attention was focused on *Geobacillus thermantarcticus*, among those isolated from Antarctica, that belongs to a group of thermophilic microorganism that produce interesting enzymes from a biotechnological point of view [2, 14, 15, 24, 28].

Industrial enzymes have become important components of numerous processes. The large numbers of novel microorganisms, with fascinating new properties, offer the prospect of exciting new molecules, especially enzymes for industrial application. New industrial enzymes may be derived from thermophiles and hyperthermophiles because of their inherent physical-chemical stability. Our research approach was to isolate target industrial thermozymes and then to study the enzyme's catalytic properties (i.e., substrate specificity, co-factor specificity, catalytic efficiency and temperature optimum).

A comparison of some properties of thermozymes isolated from related microorganisms is presented in Table 4. Enzymes from *G. stearothermophilus* DSM21 and *G. thermantarcticus* were isolated and characterised and enzymatic activities studied. Generally, the activities of enzymes from the antarctic thermophile were more thermophilic and more thermostable than those isolated from *G. stereathermophilus. G. thermantarcticus* produced four biotechnological enzymes as summarized in

Table 4. Comparison of physico-chemical properties of xylanase, β-xylosidase, xylose isomerase and protease from *G. thermantarticus* and *G. stearothermophilus*.

Enzymes	*G. thermantarcticus* DSM 9572	*G. stearothermophilus* DSM 21
Xylanase	+	+
Optimum T. °C	80	75
Optimum pH	5.6	6.5-7.0
Thermostability	60°C x 24 h	60°C x 1 h
β-Xylosidase	+	+
Optimum T. °C	70	70
Optimum pH	5.6	6.0
Thermostability	60°C x 1 h	60°C x 1 h
Xylose/glucose isomerase	+	+
Optimum T. °C	90	80
Optimum pH	7.0	7.5-8.0
Thermostability	90°C x 30 min	75°C x 10 min
Proteases	+	+
Optimum T. °C	80	75
Optimum pH	7.0	7.5
Thermostability	70°C x 24 h	60°C x 18 h

Table 5. Characteristics of thermozymes from *G. thermantarcticus*.

Enzyme	Localization	Carbon source for best yield	Molecular weight (kDa)	Optimal T. (°C)	Optimal pH
Xylanase	extracellular	xylan	40	80	5.6
β-Xylosidase	extracellular	xylan	150	70	5.6
Xylose/glucose Isomerase	cytoplasmatic	xylose	200	90	7.0
Protease	extracellular	Skim milk	42	80	8.0

Table 5. With the sole exception of xylose/glucose isomerase that is cytoplasmatic, the others are extracellular and all activities were best produced by the presence in the medium of an appropriate substrate: xylan for xylanase and β-xylosidase, xylose for the cytoplasmatic one, skim milk for protease. The enzymes are all thermophilic, and they show optimal temperatures for their activity in the range 70-90°C, while optimal temperature growth of *G. thermantarcticus* is 60°C. The optimal pH of the enzymes, as reported in Table 5, were of interest for biotechnological applications of the enzymes.

The high thermoactivity and thermostability of these enzymes and stability over a wide range of pH values, suggest that the enzymes from *Geobacillus thermantarcticus*, could be a good candidate for various biotechnological applications. In fact considering the great importance in industrial and commercial field of the heat-stable enzymes, the search for new microbial sources of these enzymes is continuously growing because the demand for the development of an industry more eco-compatible is becoming stronger. *Geobacillus thermantarcticus* is able to produce two different sulphated polysaccharides (EPS-1 and EPS-2) that confer a typical mucous character to the colonies. NMR spectra confirmed that EPS-1 was a heteropolysaccharide, whose repeating unit is constituted by four different α-D-mannose and three different β-D-glucose. It seems to be close to some xanthan polymers. EPS-2 displayed a *mannan* configuration with four different α-D-mannoses in the repeating unit. The EPS production reached, under fed-batch conditions, 3.0 g/L.

By examining their structure and chemical-physical characteristics it is possible to gain insight into their commercial applications, and they are employed in several industries. Indeed EPSs produced by microorganisms from extreme habitats show biotechnological promise ranging from pharmaceutical industries, for their immunomodulatory and antiviral effects, bone regeneration and cicatrizing capacity, to food-processing industries for their peculiar gelling and thickening properties. Moreover, some EPSs are employed as biosurfactants and in detoxification mechanisms of petrochemical oil-polluted areas.

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