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ORIGINAL PAPER

Assessment of the biomass hydrolysis potential in bacterial isolates from a volcanic environment: biosynthesis of the corresponding activities

Panagiota M. Stathopoulou · Anastasia P. Galanopoulou · George E. Anasontzis · Amalia D. Karagouni · Dimitris G. Hatzinikolaou

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Abstract The biomass degrading enzymatic potential of 101 thermophilic bacterial strains isolated from a volcanic environment (Santorini, Aegean Sea, Greece) was assessed. 80 % of the strains showed xylanolytic activity in Congo Red plates, while only eight could simultaneously hydrolyze cellulose. Fifteen isolates were selected on the basis of their increased enzyme production, the majority of which was identified as *Geobacilli* through 16S rDNA analysis. In addition, the enzymatic profile was evaluated in liquid cultures using various carbon sources, a procedure that revealed lack of correlation on xylanase levels between the two cultivation modes and the inability of solid CMC cultures to fully unravel the cellulose degrading potential of the isolates. Strain SP24, showing more than 99 % 16S DNA similarity with *Geobacillus* sp. was further studied for its unique ability to simultaneously exhibit cellulase, xylanase, β-glucosidase and β-xylosidase activities. The first two enzymes were produced mainly extracellularly, while the β-glycosidic activities were primarily detected in the cytosol. Maximum enzyme production by this strain was attained using a combination of wheat bran and xylan in the growth medium. Bioreactor cultures showed that aeration was necessary for both enhanced growth and enzyme production. Aeration had a strong positive effect on cellulase production while it negatively affected expression of β-glucosidase. Xylanase and β-xylosidase production was practically unaffected by aeration levels.

Keywords Biochemical screening · Volcanic · *Geobacillus* · Cellulase · Xylanase · Aeration

P. M. Stathopoulou · A. P. Galanopoulou ·
G. E. Anasontzis · A. D. Karagouni · D. G. Hatzinikolaou (✉)
Microbiology Group, Department of Botany, Faculty of Biology, National and Kapodistrian University of Athens, Zografou Campus, 15784 Athens, Greece
e-mail: dhatzini@biol.uoa.gr

Present Address:
G. E. Anasontzis
Industrial Biotechnology, Department of Chemical and Biological Engineering, Chalmers University of Technology, Kemivägen 10, 41296 Gothenburg, Sweden

Introduction

Thermophilic enzymes represent a widely used class of versatile biocatalysts. Their inherent thermal stability provides increased process flexibility during various industrial applications, offering advantages such as higher reaction and diffusion rates, higher solubility of the substrates and decreased contamination risks by opportunistic mesophilic microorganisms (Champdoré et al. 2007; Vieille and Zeikus 2001). Thermostable biomass degrading enzymes (mostly cellulases and xylanases) are increasingly gaining attention due to their potential use in the processing of lignocellulosic agro-industrial wastes for the production of fermentable monosaccharides that can be utilized as raw materials for commodity chemicals such as biofuels (Barnard et al. 2010; Turner et al. 2007). The technologies for the conversion of lignocellulosic raw materials at higher temperatures are quite complex and require advanced fermentation configurations and suitable novel enzymes capable to efficiently attack the highly ordered and insoluble structures of the corresponding substrates (Viikari et al. 2007). Since lignocellulose biodegradation is more likely to require a pretreatment step at elevated temperatures, the simultaneous use of efficient thermostable

cellulolytic and xylanolytic enzymes certainly provides an important process advantage (Barnard et al. 2010) and drives scientific research towards the isolation of novel thermostable biomass degrading microorganisms that are able to produce such enzymes.

Diverse thermophilic environments such as hot springs (Abdelnasser and El-diwany 2007; Kublanov et al. 2009), compost sites (Baharuddin et al. 2010; Ng et al. 2009), subsurface mines (Rastogi et al. 2009) and marine hydrothermal fields (Wu et al. 2006) are the habitats for bacteria that produce enzymes with the ability to degrade cellulose and/or xylan at high temperatures. Most of these studies deal with the isolation and characterization of the hydrolase of interest, either directly from the thermophilic microbial strain or following expression of the corresponding genes in a mesophilic host. Such work is fueled by research providing information about the enzymatic potential—not confined only towards biomass hydrolysis—of microorganisms that have been isolated from thermophilic habitats. Despite the obvious scientific and biotechnological significance of this approach, studies of this kind are rather limited and concern environments such as oil fields (da Cruz et al. 2010) and compost sites (Izquierdo et al. 2010; Tiago et al. 2004).

Volcanic areas represent environments of rich metabolic diversity especially among bacteria and archaea (Martinez et al. 2006; Pachiadaki et al. 2010; Prokofeva et al. 2005) and the isolation of many thermophilic microorganisms has been described from them. The vast majority of these studies though, are focused on the biodiversity and phylogenetic analysis of the isolates and we could identify only one recent report available in literature assessing the enzymatic activity potential of microbial strains isolated from hot-springs at the Uzon volcanic caldera in Kamchatka Peninsula, Russia (Kublanov et al. 2009).

Based on the above rational, in this work we examined the enzymatic biomass degrading potential of 101 bacterial strains that have been previously isolated from the volcanic habitat of the Santorini volcano, in South Aegean Sea (Meintanis et al. 2006). A thorough biochemical screening for xylanase and cellulase activities was performed both in solid and liquid media using various carbon sources in an attempt to establish benchmark criteria for such an analysis. The most active strains towards this direction were also characterized with respect to their 16S rDNA sequences that revealed high similarity with *Geobacillus* species. A strain selected for its ability to produce all four major biomass degrading enzymes, namely, endo-cellulase, endo-glucanase, β-glucosidase and β-xylosidase, was selected in order to study the physiology of enzyme expression with respect to medium composition and aeration levels in a series of bioreactor cultures.

Materials and methods

Materials

Carboxy methyl-cellulose (CMC—low viscosity), birchwood xylan, para-Nitro-Phenyl β-D-xylopyranoside (pNPX), dinitro-salicylic acid (DNS) and soluble starch were obtained from Sigma (St.Louis, MO, USA). Yeast extract, Casein, Tryptone and Nutrient Agar (NA) were obtained from Applichem (Darmstadt, Germany). All other reagents used, were of the highest available analytical grade. Fine grinded (<1 mm) corn cob [42 % cellulose, 38 % hemicelluloses, 8 % lignin, w/w] and wheat bran [12 % cellulose, 35 % hemicelluloses, 29 % starch, 11 % protein, 5 % lignin, w/w] were obtained from the Agricultural University of Athens and Loulis Mills S.A. (Pireas, Greece), respectively.

Bacterial strains

The 101 thermophilic bacterial strains used in this study have been previously isolated as pure cultures from the soil and sediment near the active volcano of Santorini island in Aegean Sea, Greece, based on their ability to grow at 60 °C on various rich solid media (Meintanis et al. 2006). Stock cultures of all isolates were maintained in 30 % (w/v) glycerol stocks at −80 °C in the Microbiology Laboratory culture collection at the Department of Biology of the University of Athens. Strain SP24 that was selected for further study during this work has been deposited (free access) in the Greek Coordinated Collections of Microorganisms—Agricultural College of Athens (http://ett.aua.gr/ergastiria/Egalaktokomias/microorganisms/collection.html), under the Catalog# ACA-DC 4059.

Growth studies

Pre-cultures of all strains were prepared by streaking the corresponding glycerol stocks on nutrient agar (NA) plates followed by incubation for 24 h at 60 °C. The initial screening of the cellulolytic and xylanolytic potential of all bacterial strains was performed in solid state agar plates using a basal medium consisting of (g L^{-1}): $NaNO_3$, 3; K_2HPO_4, 1; KCl, 0.5; $CaCl_2$, 0.1; $MgSO_4 \cdot 7H_2O$, 0.5; yeast extract, 1; agar 30; trace elements solution, 1 mL L^{-1}. The higher than usual agar concentration used, was employed in order to withstand the high incubation temperature. The above medium was supplemented with either 5 g L^{-1} CMC or birchwood xylan for endo-cellulase and xylanase detection, respectively. A 5 mm circular disc was removed with a sterile cork borer from the agar medium and the resulting well was filled with 100 μL of cell suspension of

known viable cell concentration (cfu mL^{-1}). In order to be able to fully assess the range of cellulose/xylan degrading potential of the strains we used multiple plates per strain, filling the inoculation well with viable cell concentrations ranging from 10^6 to 10^9 cfu mL^{-1}. The plates were incubated at 60 °C for 48 h, each plate was flooded with a 0.1 % (w/v) Congo Red solution and left at room temperature (15 min) for staining the β,1-4 glycosidic bonds of the substrate (CMC or xylan). Destaining of the plate was effected with 1 M NaCl at the same conditions (Sharrock 1988). Relative hydrolase activities were evaluated from the diameter of the destained areas (hydrolysis of the β,1–4 glycosidic bonds) around each well and were expressed in mm per 10^6 cfu.

The composition of the basal liquid medium for submerged cultures was the following (in g L^{-1}): $NaNO_3$, 5; KH_2PO_4, 1; K_2HPO_4 2; KCl, 0.1; $MgSO_4{\cdot}7H_2O$, 0.5; $CaCl_2$, 0.01; $FeSO_4{\cdot}7H_2O$, 0.02; yeast extract, 1; trace elements solution, 1 mL L^{-1}. This medium was supplemented with various pure or complex carbon sources at an initial concentration of 20 g L^{-1}. All media were sterilized prior to inoculation for 20 min at 121 °C. Liquid cultures took place in 100 mL Erlenmeyer flasks of 25 mL working volume and incubated in an orbital shaker at 60 °C and 200 rpm. Possible water evaporation was compensated by weighting the flasks on a daily basis and supplementation of the losses with sterile distilled water.

Bioreactor studies were conducted in a 2.5 L glass bioreactor (B. BRAUN, BIOSTAT® B) equipped with pH, T and aeration control, using a working volume of 2.3 L. The vessel was filled with 2.25 L of growth medium, sterilized (121 °C, 20 min) and inoculated directly with 50 mL of cell suspension (in basal salt medium) prepared by collecting the cells from fully grown (48 h) Petri dishes of the microorganism, properly diluted to yield an initial cell concentration in the bioreactor of 10^6 cfu mL^{-1}. Growth took place at 60 °C using a constant agitation speed (standard square blades) of 300 rpm. The pH was kept constant at 7 with the automatic addition of 2 N NaOH or HCl. Four batch experiments were conducted, each one at different aeration levels, namely, 0, 0.15, 0.37 and 0.60 vvm, by adjusting the air supply flow rate. Samples were withdrawn aseptically at different time intervals and were analyzed for biomass (cfu) concentration and enzyme activities (see below).

Physiological and molecular characterization of isolates

General morphology was determined by streaking a full loop from each bacterial strain on Nutrient Agar plates and incubation for 24 h at 60 °C prior to Gram staining. Oxidase and catalase tests (Mac Faddin 1980) were also performed in order to study the presence of these enzymes.

The phylogenies of the fifteen selected isolates were determined by 16S rDNA sequence analysis. Genomic DNA extraction from liquid cultures was performed following the protocol of Haught et al. (Haught et al. 1994). The quantity of the isolated DNA was determined photometrically (Sambrook et al. 1989). The 16S rDNA was amplified by polymerase chain reaction (PCR) using two universal primers (Edwards et al. 1989; Lane 1991): pA (5′-AGAGTTTGATCCTGGCT CAG-3′) and R1492 (5′-TACGGYTACCTTGTTACGA CTT-3′). Amplification reactions were performed in volumes of 50 µL containing 40 ng template DNA, 0.4 μM of each primer, 1X buffer with Mg^{2+}, 1 U KapaTaq DNA Polymerase (Kapa Biosystems, Woburn, MA, USA) and 0.2 mM dNTPs (Fermentas, Canada). Nucleases free water was used to bring the reaction volume to 50 µL. After initial denaturation at 95 °C for 2 min, samples were cycled for 30 PCR cycles using the following profile: 95 °C denaturation for 30 s, primer annealing at 53 °C for 30 s and primer extension at 72 °C for 2 min, plus a final 2 min elongation step at 72 °C. Amplified PCR products were separated by gel electrophoresis on 1.2 % (w/v) agarose gel and then purified using Nucleospin® Extract PCR kit (Macherey–Nagel, Germany). The 16S rDNA fragment, was partially sequenced commercially (http://www.macrogen.com). Gene sequences were compared with published ones in GenBank databases using the NCBI BLAST software and were assigned the following Accession numbers: JN692238 to JN692252 (http://www.ncbi.nlm.nih.gov/).

Different published sequences of thermostable cellulases and xylanases from annotated *Geobacillus* strains were aligned and two pairs of primers were designed in the 5′and the 3′ conserved ends of the genes. Primer pair GeoCel_F (5′-ATGGCGAAGTTGGACGAAACG-3′) and GeoCel_R (5′-TTATTCGTCAAACGTCAGTTGTTTC-3′) was used to amplify the cellulase gene. The open reading frame of the xylanase gene was amplified using the following primers GeoXyl_F (5′-ATGAACAGCTCCCTCCC CTCCC-3′) and GeoXyl_R (5′-TCAGACACTCAC TGCCCGCCAAA-3′). Amplification reactions were performed in volumes of 50 µL containing 50 ng template DNA, 0.5 μM of each primer, 1X Phusion™ HF buffer, 1 U Phusion™ DNA Polymerase (FINNZYMES, Finland) and 0.2 mM dNTPs (Fermentas, Canada). Nucleases free water was used to bring the reaction volume to 50 µL. The cycling conditions used were: an initial denaturation step at 98 °C for 30 s, 30 cycles at 98 °C for 10 s, annealing at 63 °C for cellulase gene or 69 °C for xylanase gene for 30 s and extension at 72 °C for 35 s except for the final extension for 5 min. Several efforts have been made in order to optimize the annealing temperature for each template—primer pair combination.

Localization of enzyme activities

Extracellular enzyme activities were determined directly in the culture supernatants following clarification by centrifugation (15 min, 10,000×*g*, 10 °C). Fractionation of the cell bound enzyme activity, was conducted according to Prathumbai et al. (Prathumpai et al. 2004) adjusted as follows. A certain volume of culture broth was filtered through a sieve (250 μm) to remove most of the insoluble carbon source particles and the filtrate was centrifuged (10,000×*g*). The pellet was resuspended in sodium phosphate buffer (50 mM, pH 7) containing 1 mM EDTA and 20 μM PMSF and was subjected to sonication (Vibra Cell™, Sonics & Materials Inc., USA) for 3 min at 30 s intervals with intermediate cooling in an ice bath. Following centrifugation, the supernatant was collected and served as the enzyme source for the cytoplasmic cell-bound fraction while the pellet was washed once with extraction buffer containing 1 % (w/v) Triton X-100. Following centrifugation, the collected supernatant was designated as the loosely cell-bound fraction. A second washing step was done with an extraction buffer containing 1 % (w/v) SDS in order to determine any tightly associated with the pellet fraction. Enzyme activities were determined in all three cell-bound fractions and determined activities were back calculated in nkat per mL culture volume.

Enzyme assays

Endo-cellulase and *endo-xylanase* activities were determined using CMC (Sharrock 1988) and birchwood xylan (Bailey et al. 1992) as substrates, respectively. Enzyme samples (50–250 μL, depending on the anticipated enzyme concentration and prepared as described in Localization of enzyme activities—see above) were added to the corresponding substrate solutions (in 100 mM potassium phosphate buffer pH 7) to yield a total assay volume of 500 μL. The concentration of substrate solutions was always adjusted in order to yield an initial substrate concentration in the assay mixture equal to 2 and 1 % (w/v) for cellulase and xylanase, respectively. Assay mixtures were incubated at 800 rpm and 60 °C in a thermoshaker (BOECO, model TS-100, Germany). Following 15 or 30 min incubation, 200 μL aliquots were removed from the assay mixture in order to determine the concentration of the reducing sugars produced, through the dinitro-salicylic acid (DNS) method (Miller 1959). Appropriate calibration curves prepared with the DNS procedure using either glucose (cellulase) or xylose (xylanase) were employed for this purpose. Activities were expressed in SI units, nkat, being the amount of enzyme required for the liberation of 1 nmole of reducing sugars—expressed as glucose equivalent for endo-cellulase and as xylose equivalent for endo-xylanase—per second.

β-glucosidase activity was determined using cellobiose as substrate (Chauve et al. 2010) at a final concentration of 2 % (w/v). The reaction mixture consisted of 500 μL of 4 % (w/v) cellobiose in 100 mM potassium phosphate buffer, pH 7, 400 μL of this buffer and 100 μL of enzyme sample (diluted if necessary). An aliquot of 50 μL was removed from the assay mixture, following 15–30 min incubation at 60 °C, in order to determine the concentration of glucose released, using a Glucose Oxidase/Peroxidase kit (BIOSIS™, Ag. Dimitrios—Athens, Greece) according to the manufacturers' recommendation. The amount of enzyme required for the release of 1 nmole of glucose per second (nkat) from cellobiose was used as activity unit.

β-xylosidase was assayed in a similar manner using *p*-NitroPhenyl-*β*-D-Xylopyranoside (pNPX) at a concentration of 1 mM (Lama et al. 2004) in 100 mM phosphate buffer, pH 7 (1 mL total volume). The reaction was terminated by the addition of 1 mL of 1 M Na_2CO_3 solution and the concentration of released para Nitro-Phenol (pNP) was determined at 410 nm using a pNP calibration curve prepared at the above assay conditions.

For all enzyme assays, an autoclaved (30 min, 121 °C) enzyme sample, undergone the same procedure was used as a blank.

Determination of biomass concentration

Biomass concentration throughout this study was expressed as colony forming units (cfu) per unit volume. The procedure involved serial dilutions of the sample in Ringer solution, spreading—100 μL—on NA plates, incubation at 60 °C and counting of cfu after 24 h. At least three plates, not necessarily from the same decimal dilution, were used for each determination.

Results

Solid state screening of the isolates

The initial screening of the cellulose and xylan degrading potential of all strains was performed in solid state cultures using CMC and birchwood xylan as sole carbon sources, respectively, in combination with plate staining with Congo Red. Potential cellulase or xylanase activity would eventually produce a decolorized circular area around the inoculation well upon destaining. In order to clearly distinguish strains with high cellulolytic or xylanolytic potential we compared the isolates performance on the basis of the ratio of decolorization zone diameter over the viable inoculum size (cfu). The corresponding relative activities are compared in Table 1 for the total of 101

Table 1 Comparison of endo-glucanase and xylanase activity[a] profile in solid state cultures[b]

Strain	Enzyme		Strain	Enzyme		Strain	Enzyme	
	Xylanase	CMCase		Xylanase	CMCase		Xylanase	CMCase
SP1	0.00	0.00	SP35	0.00	0.00	**SP69**	**21.25**	**0.00**
SP2	6.33	0.00	SP36	0.00	0.00	SP70	1.71	0.00
SP3	0.00	0.00	**SP37**	**3.71**	**0.81**	SP71	0.00	0.00
SP4	0.30	0.00	**SP38**	**0.66**	**0.15**	SP72	0.67	0.00
SP5	0.64	0.00	SP39	0.43	0.00	SP73	0.58	0.00
SP6	0.00	0.00	SP40	0.52	0.00	**SP74**	**9.50**	**0.00**
SP7	5.36	0.00	SP41	0.00	0.00	SP75	0.00	0.00
SP8	**10.40**	**0.00**	SP42	6.94	0.00	SP76	2.29	0.00
SP9	0.00	0.00	SP43	0.18	0.00	SP77	2.09	0.00
SP10	1.46	0.00	SP44	0.11	0.00	SP78	4.56	0.00
SP11	**11.43**	**0.00**	**SP45**	**0.14**	**0.18**	SP79	2.80	0.00
SP12	7.57	0.00	**SP46**	**0.14**	**0.60**	SP80	2.19	0.00
SP13	0.33	0.00	**SP47**	**0.08**	**0.23**	SP81	1.81	0.00
SP14	0.00	0.00	SP48	0.29	0.00	SP82	2.50	0.00
SP15	6.25	0.00	SP49	1.28	0.00	SP83	0.21	0.00
SP16	0.75	0.00	**SP50**	**0.93**	**0.37**	SP84	0.00	0.00
SP17	**0.17**	**0.09**	SP51	2.16	0.00	SP85	0.00	0.00
SP18	0.40	0.00	SP52	0.00	0.00	SP86	0.00	0.00
SP19	1.25	0.00	SP53	1.24	0.00	**SP87**	**8.53**	**0.00**
SP20	1.34	0.00	SP54	1.02	0.00	SP88	3.21	0.00
SP21	0.94	0.00	SP55	3.21	0.00	SP89	0.00	0.00
SP22	0.44	0.00	SP56	3.91	0.00	SP90	4.10	0.00
SP23	0.15	0.00	SP57	0.58	0.00	SP91	0.54	0.00
SP24	**0.29**	**0.12**	SP58	0.40	0.00	SP92	2.50	0.00
SP25	0.32	0.00	**SP59**	**10.08**	**0.00**	SP93	7.50	0.00
SP26	0.00	0.00	SP60	1.29	0.00	SP94	1.19	0.00
SP27	0.23	0.00	SP61	0.92	0.00	SP95	0.47	0.00
SP28	1.04	0.00	SP62	0.41	0.00	SP96	1.78	0.00
SP29	0.00	0.00	SP63	1.91	0.00	SP97	0.00	0.00
SP30	0.34	0.00	SP64	5.19	0.00	SP98	0.00	0.00
SP31	0.58	0.00	SP65	0.79	0.00	SP99	0.00	0.00
SP32	4.03	0.00	SP66	8.10	0.00	SP100	0.65	0.00
SP33	1.53	0.00	SP67	7.73	0.00	SP101	0.46	0.00
SP34	1.56	0.00	**SP68**	**27.78**	**0.00**			

[a] Activities are expressed as the ratio of the destained area diameter (mm) per 10^6 cfu's inoculated in each well. Data represent the mean of three plates. For all strains and enzyme activities the standard deviation (SD) ranged from 1.3 to 17.7 %

[b] Strains selected for further evaluation are bolded

bacterial strains examined. Eighty-one isolates (80 %) revealed a clear xylanolytic activity halo. The expression levels though, varied a lot among strains, ranging from as low as 0.11 mm 10^{-6} cfu for strain SP44 to as high as 27.8 mm 10^{-6} cfu for isolate SP68. Among these isolates, eight were also able to simultaneously reveal clear cellulose degrading activity. Cellulase expression on agar plates was on average at much lower intensities not exceeding 1 mm 10^{-6} cfu. It is noteworthy, that we could not identify any strain showing xylanase free cellulolytic activity in solid state cultures.

Characterization of selected isolates and molecular analysis

Based on the results of the solid state screening, fifteen isolates were selected for further characterization; eight that exhibited both xylanase and cellulase activity (bolded

Table 2 Percentage 16S rDNA similarity among the selected isolates based on the partial 16S rDNA sequence obtained with the pA and R1492 primers[a]

Strain (NCBI Acc. #)	Percentage of 16S DNA similarity														
	SP08	SP11	SP17	SP24	SP37	SP38	SP45	SP46	SP47	SP50	SP59	SP68	SP69	SP74	SP87
SP08 (JN692238)	100														
SP11 (JN692239)	98.1	100													
SP17 (JN692240)	**98.0**	**99.1**	**100**												
SP24 (JN692241)	**98.4**	**99.2**	**98.8**	**100**											
SP37 (JN692242)	**98.3**	**98.8**	**98.8**	**98.8**	**100**										
SP38 (JN692243)	**98.4**	**99.4**	**98.8**	**99.0**	**98.6**	**100**									
SP45 (JN692244)	**97.8**	**98.7**	**98.3**	**97.8**	**98.4**	**98.3**	**100**								
SP46 (JN692245)	**97.9**	**98.7**	**99.3**	**98.8**	**98.3**	**98.8**	**98.5**	**100**							
SP47 (JN692246)	**97.7**	**98.4**	**98.5**	**97.3**	**97.7**	**97.7**	**97.3**	**98.4**	**100**						
SP50 (JN692247)	**97.7**	**98.4**	**98.3**	**96.7**	**97.6**	**96.6**	**96.9**	**98.4**	**96.0**	**100**					
SP59 (JN692248)	89.1	89.0	89.0	89.5	89.4	89.3	89.0	88.8	89.3	88.6	100				
SP68 (JN692249)	98.3	99.3	99.1	99.1	99.2	99.4	99.0	99.0	98.1	98.7	88.9	100			
SP69 (JN692250)	98.0	98.8	98.5	99.3	99.4	99.4	99.0	98.8	98.3	98.3	89.4	99.0	100		
SP74 (JN692251)	98.2	98.8	98.9	98.6	99.1	98.7	98.2	99.0	97.0	97.1	89.2	98.9	99.5	100	
SP87 (JN692252)	89.2	89.7	89.3	88.9	88.7	88.2	88.7	89.7	87.8	88.3	95.5	89.1	89.4	88.5	100

Strain (NCBI Acc. #)	Closest phylogenetic relative[b]			PCR analysis[c]	
	Strain	NCBI accession #	% Identity	Xase	Case
SP08 (JN692238)	*Geobacillus* sp. KTL04	JF276453.1	98.7	++	++
SP11 (JN692239)	*Geobacillus* sp. W2-10	HM059717.1	99.3	++	++
SP17 (JN692240)	***Geobacillus sp. ABRII9***	**JN315635.1**	**99.1**	**++**	**++**
SP24 (JN692241)	***Geobacillus sp. WBI***	**JN088182.1**	**99.1**	**++**	**++**
SP37 (JN692242)	***Geobacillus sp W2-10***	**HM059717.1**	**99.0**	**−−**	**++**
SP38 (JN692243)	***Geobacillus sp. W2-10***	**HM059717.1**	**99.4**	**++**	**++**
SP45 (JN692244)	***Geobacillus sp. Y1-2 clone 9-2***	**EF555516.1**	**98.4**	**++**	**++**
SP46 (JN692245)	***Geobacillus sp. ABRII9***	**JN315635.1**	**99.3**	**++**	**++**
SP47 (JN692246)	***Bacillus sp. MH-2***	**DQ839488.1**	**97.5**	**−−**	**++**
SP50 (JN692247)	***Bacillus sp. MH-2***	**DQ839488.1**	**96.4**	**−−**	**−−**
SP59 (JN692248)	*Geobacillus* sp. W2-5	HM059722.1	95.5	−−	−−
SP68 (JN692249)	*Geobacillus* sp. 71	JN021318.1	99.5	++	++
SP69 (JN692250)	*Geobacillus* sp. XT15	HQ891030.1	99.5	−−	++
SP74 (JN692251)	*Bacillus* sp. MH-2	DQ839488.1	98.6	−−	−−
SP87 (JN692252)	*Geobacillus* sp.W2-5	HM059722.1	95.0	−−	−−

[a] Bolded rows correspond to the sub-group of isolates that revealed both xylanolytic and cellulolytic activity in solid agar plates

[b] Closest phylogenetic relative analysis for each isolate based on *blastn* with NCBI sequences

[c] Detection (++) or not (−−) of the PCR product against *Geobacillus* xylanase and cellulase primers

in all Tables) and seven additional strains that revealed the highest xylanolytic potential. A primary biochemical characterization revealed that all isolates were Gram positive, oxidase and catalase positive bacilli. The 16S rDNA sequence analysis, summarized in Table 2, revealed that with the exceptions of isolates SP47, SP50 and SP74, the selected strains had the highest phylogenetic similarities with members of the genus *Geobacillus*. The percentage of similarity to known *Geobacillus* sequences was between 95.0 and 99.0 % for eight isolates, while the remaining seven revealed very high similarity (>99 %) to annotated geobacilli (Table 2). The 16S rDNA percent similarities among the isolates that are also given in Table 2 revealed a clear phylogenetic differentiation for strains SP59 and SP87. These two isolates, had only 89.1 and 88.9 % average similarity, respectively, with the rest thirteen strains and revealed the lowest (95.5 and 95.0 %, respectively) 16S rDNA identities, with *Geobacillus* sp.

clone W2-5. It is noteworthy, that both strains had almost the same enzyme activity profile in solid state cultures, since they produced similar xylanase activities and no detectable cellullase levels. The average percentage of 16S rDNA similarity among the rest of the strains was high ranging from 97.5 to 99 %.

Primers were constructed based on *Geobacillus* sp. endo-xylanase and endo-cellulase sequences (see "Materials and methods"). The results of the PCR analysis on the genomic DNA of the strains are also presented in Table 2. Despite the fact that all selected strains produced xylanolytic activity, we were able to identify the corresponding PCR product in only eight of them. For cellulase, the PCR product was detected in eleven isolates. To our surprise, four out of the seven selected isolates that did not reveal any cellulolytic activity on solid agar growth, yielded a clear cellulase PCR product with the *Geobacillus* primers we constructed. In general though, the high or low 16S rDNA similarity with known *Geobacillus* strains did not seem to correlate with the existence or not of the xylanase/cellulase PCR product.

Liquid culture activities

The capacity of the fifteen selected isolates to produce cellulase and xylanase was also evaluated in liquid cultures. The strains were grown on basal liquid medium supplemented with 2 % (w/v) of either birchwood xylan, CMC, cellobiose, corn cob and wheat bran. Aliquots were withdrawn daily and assayed for endo-xylanase and cellulase activities. The results concerning the maximum enzyme titers (usually obtained between the 3rd and 5th day of growth) in the corresponding culture supernatants are summarized in Table 3. The enzymatic profile revealed in liquid cultures was significantly different to that observed on solid agar growth using xylan or CMC. Concerning xylanase production, all strains were able to grow and produce xylanase activity with xylan as carbon source. In fact, xylan was on average the best xylanase inducer among the carbon sources used, although there were isolates with maximum xylanase activities obtained with cellobiose (SP8, SP59, SP69 and SP87), wheat bran (SP24 and SP47) or corn cob (SP37). It is important to stress though that there was no correlation between xylanase expression intensities in solid media and the maximum enzyme activities determined in liquid culture supernatants. Concerning cellulase production, the results obtained from liquid cultures proved that the use of CMC for screening microbial strains has also only qualitative value. Although all eight isolates that produced cellulase in CMC agar plates also revealed cellulolytic activity in liquid cultures, all seven strains that did not exhibit any detectable CMC hydrolysis on CMC agar plates, were able to produce cellulolytic activity in liquid media with at least one of the carbon sources. In addition, CMC was proved to be on average the poorest cellulase inducer in liquid cultures. The highest cellulase activities in liquid cultures were obtained using cellobiose a sole carbon source. In addition to cellobiose, our study revealed that wheat bran was also a very good inducer for both cellulase and xylanase production by the thermophilic bacilli evaluated in our study.

Enzyme biosynthesis by isolate SP24

Strain SP24, showing a 99.1 % 16S rDNA similarity to *Geobacillus* sp. strain WBI (NCBI Accession # JN088182.1) was chosen as a representative strain in order to study in more detail the production physiology of the biomass degrading enzymes. The choice was based on the fact that this strain produced the highest extracellular activities of both cellulase and xylanase when grown on wheat bran. Initially, we examined the effect of additional carbon/nitrogen sources as a supplement to wheat bran on growth and enzyme production in a series of shake flask experiments, in which case the basic liquid medium was supplemented with 20 g L^{-1} of wheat bran (control) plus an additional 5 g L^{-1} of a second carbon or nitrogen source. In this experimental set we additionally determined the activity of the corresponding disaccharide hydrolases, namely β-glucosidase and β-xylosidase. Since there is adequate verification in literature on the cell-bound nature of the latter enzymes, all enzyme activities were additionally assayed in the cell extracts. Figure 1 summarizes the results of the corresponding experimental set. The addition of nitrogen sources (casein, tryptone or ammonium phosphate) as a supplement to the yeast extract and $NaNO_3$ (that already exist in the basal medium) did not seem to markedly affect the enzyme production in the wheat bran cultures. Tryptone and ammonium phosphate significantly decreased the biosynthesis of three out of the four enzymes of interest. On the contrary, supplementation with starch and xylan proved quite beneficial for enzyme production, especially for CMCase where the corresponding activities were 3 and 3.5 fold higher than in the cultures with only wheat bran as carbon source. Nevertheless, both polymeric carbon sources, repressed to a certain extent the glycosidic activity that was not related to their corresponding monomers i.e. starch—β-xylosidase and xylan—β-glucosidase (Fig. 1).

Using the combination of wheat bran and xylan as optimum carbon sources we conducted a series of bioreactor experiments in order to study the effect of aeration conditions on microorganism growth and enzyme production. Four bioreactor runs were conducted at 0, 0.15, 0.37 and 0.6 vvm as described in Materials and Methods. Figure 2 shows the time course of biomass concentration expressed as viable cells (cfu mL^{-1}). Very little growth

Table 3 Production of endo-xylanase (Xase) and cellulase (Case) activities (nkat/mL) by the fifteen selected isolates in liquid media[a] supplemented with 2 % w/v of the indicated carbon source

	Xylane		Cellobiose		CM-cellulose		Corn Cob		Wheat bran	
	Xase	Case	Xase	Case	Xase	Case	Xase	Case	Xase	Case
SP8	1.12	0.47	12.69	7.80	nd	0.32	nd	nd	3.45	0.91
SP11	7.55	4.98	7.11	14.68	0.85	0.35	0.78	0.41	1.89	5.50
SP17	**6.57**	**0.83**	**2.57**	**20.27**	**1.95**	**0.18**	**1.43**	**0.23**	**4.86**	**1.08**
SP24	**2.00**	**4.43**	**2.09**	**1.81**	**0.52**	**1.45**	**3.12**	**2.45**	**12.62**	**10.08**
SP37	**1.31**	**0.45**	**nd**	**0.32**	**0.68**	**0.63**	**7.92**	**2.48**	**4.94**	**5.99**
SP38	**16.04**	**3.26**	**4.41**	**13.54**	**1.92**	**1.16**	**0.36**	**0.36**	**0.69**	**11.04**
SP45	**18.77**	**4.17**	**8.16**	**8.79**	**1.06**	**0.19**	**0.90**	**0.98**	**nd**	**5.80**
SP46	**7.33**	**1.15**	**1.91**	**8.99**	**0.81**	**1.32**	**1.68**	**1.88**	**0.49**	**1.33**
SP47	**4.72**	**9.41**	**6.75**	**3.73**	**0.96**	**0.15**	**0.77**	**1.17**	**9.66**	**0.91**
SP50	**10.78**	**0.45**	**8.01**	**12.06**	**0.57**	**1.10**	**0.41**	**0.28**	**nd**	**1.77**
SP59	7.71	0.63	14.76	3.34	nd	nd	2.84	0.25	nd	3.43
SP68	11.92	2.43	2.13	20.40	0.12	nd	1.05	0.90	1.46	3.64
SP69	0.59	nd	4.07	4.95	0.54	nd	1.49	0.84	2.03	11.37
SP74	4.03	0.27	1.35	4.33	nd	nd	0.36	0.32	3.22	10.98
SP87	0.55	nd	6.75	nd	0.40	nd	0.58	nd	4.51	1.77
Mean	6.73	2.53	5.91	8.93	0.86	0.69	1.69	0.97	4.15	5.04
SD	5.46	2.58	3.96	6.24	0.54	0.49	1.91	0.78	3.50	3.89

nd result below the detection limit of the assay system

[a] Values represent the maximum obtained titer e.g. the maximum enzyme activity in the culture supernatant that determined during the course of 7 growth days. All cultures were performed in triplicates (SD ranged from 2.7 to 14.4 %). Bolded rows correspond to the sub-group of isolates that revealed both xylanolytic and cellulolytic activity in solid agar plates

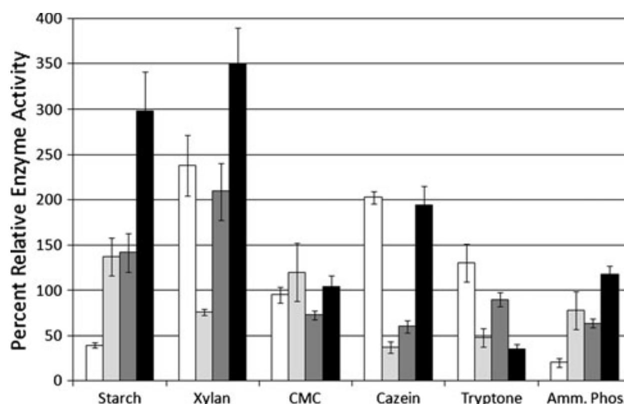

Fig. 1 Percent relative maximum total (extracellular plus cell bound) enzyme activities upon supplemental addition of different carbon or nitrogen sources (0.5 % w/v) on the basal mineral medium supplemented with 2 % w/v wheat bran. 100 % corresponds to the activity of each individual enzyme that was obtained in the control culture which contained only wheat bran. *Black bars* cellulase, *Dark grey bars* xylanase, *Light gray bars* β-glucosidase, *White bars* β-xylosidase. Data represent the mean of triplicate flasks ± SD (*error bars*)

and only traces of enzyme activity (<1 nkat mL^{-1}) were observed when there was no aeration supplied into the bioreactor. Aeration had a very profound positive effect on viable growth since it affected, both the maximum determined biomass concentration as well as the growth rate. A maximum of almost 3×10^9 cfu mL^{-1} was achieved only at 6 h post-inoculation under the highest aeration levels employed (0.6 vvm). For the two highest aeration rates, the viable biomass concentration started to gradually decrease from its maximum value and reached back to the inoculation levels after approximately 60 h of growth. At the lower aeration level though (0.15 vvm) a two phase growth was observed with two clear maxima at 24 and 90 h.

Total enzyme production (cell bound plus extracellular) for the three aerated bioreactors is given in Fig. 3. The production of all biomass degrading enzymes was growth associated since it followed more or less the viable cells' concentration. Aeration level though, did not uniformly affect enzyme production. Concerning the cellulose degrading enzymes, maximum cellulase concentration increased rapidly from 7.6 to 25 nkat mL^{-1} following the increase in aeration from 0.15 to 0.37 vvm but practically leveled off after that level since at 0.6 vvm the total enzyme concentration dropped only slightly to 21 nkat mL^{-1}. β-glucosidase activity levels revealed a strong negative correlation with aeration, dropping from more than 3 nkat mL^{-1} at the low aeration to less than 0.6 nkat mL^{-1} at the high aeration levels. The maximum total xylanase and β-xylosidase activities revealed a much weaker dependence on culture aeration since there was less than 30 % difference

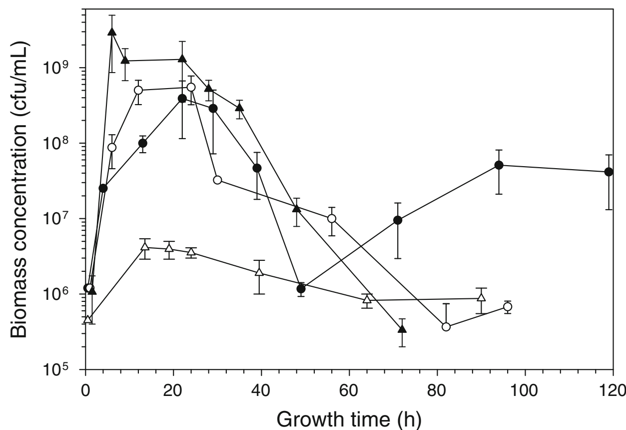

Fig. 2 Biomass concentration time profile, expressed as cfu/mL, for the four bioreactor experiments conducted at different aeration levels. *Open triangles* 0 vvm, *Filled circles* 0.15 vvm, *Open circles* 0.37 vvm, *Filled triangles* 0.6 vvm. *Error bars* represent the SD of at least three plates (not necessarily of the same decimal dilution)

between the maximum enzyme levels at 0.6 and 0.15 vvm, but the production of both enzymes was positively affected by aeration. The two phase growth observed with viable biomass at 0.15 vvm, was closely followed only by β-glucosidase and β-xylosidase while cellulase and xylanase levels followed the general trend observed with the other two aeration levels that involved a gradual reduction in their activity following a maximum between the 15th and 25th hour of growth. This rate of reduction of enzyme activity was increased with aeration levels resulting in no detection of enzyme activity after 60 h of growth at 0.6 vvm.

Localization of all enzyme activities was finally examined in all SP24 cultures. Figure 4 summarizes the corresponding results for two time points during bioreactor growth; one at the mid-log phase (approx. at the 24th hour of growth) and one at the late stationary phase (approx. after 50 h of growth). Cellulase and xylanase revealed clear extracellular enzyme profiles, since at the beginning of the fermentation more than 50 % of their total activity is detected in the culture supernatant, with the rest of the activity being detected at the cytosolic cell-bound fraction. At this stage, the extracellular portion gradually increased with aeration level, following the faster microorganism growth. As a result, at 0.6 vvm almost 100 % of the cellulase and xylanase activities were extracellularly detected. On the contrary, β-glucosidase and β-xylosidase activities revealed a cell-bound profile with most of the activity being detected at the cytosolic cell-bound fraction during the early stages of growth. For β-glucosidase, significant activity was additionally detected at the loosely and tightly cell-bound portions, especially at the higher aeration levels (Fig. 4). At the latter stages of growth, the activity of both glycosidases was mostly detected in the culture supernatant, probably as a result of partial autolysis of the cells.

Discussion

The biomass degrading enzymatic profile of isolates from a volcanic environment was performed for the first time. For this purpose, we exploited a collection of 101 thermophilic

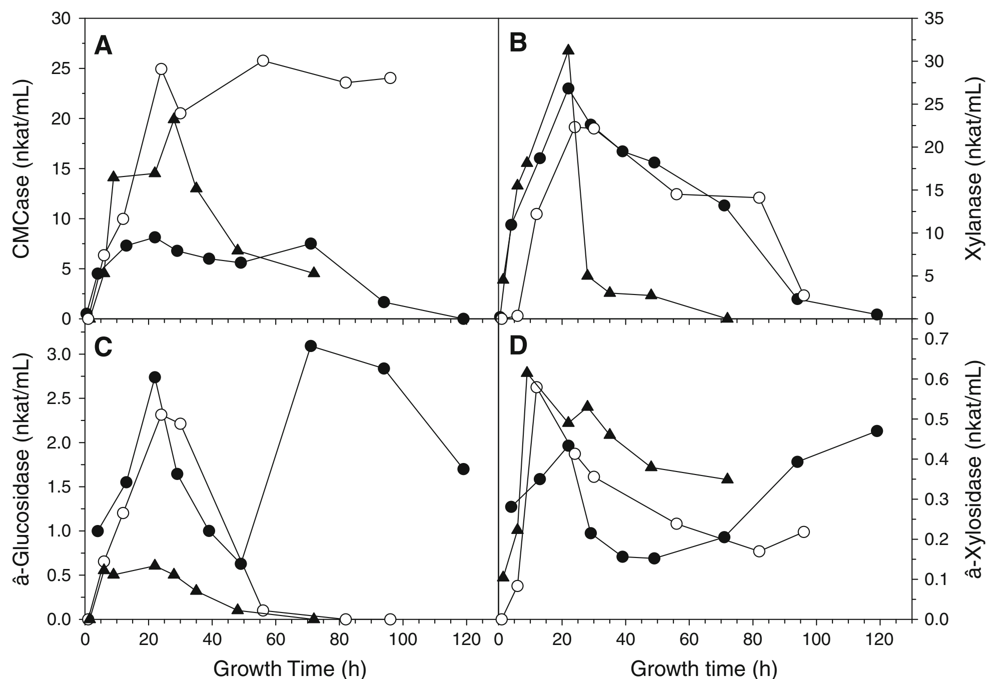

Fig. 3 Total enzyme production as a function of time in bioreactors at the three different aeration levels. The corresponding enzyme assays were performed in triplicates yielding very low errors (max. SD less than 5 %). *A* Cellulase, *B* Xylanase, *C* β-glucosidase, *D* β-xylosidase. *Filled circles* 0.15 vvm, *Open circles* 0.37 vvm, *Filled triangles* 0.6 vvm

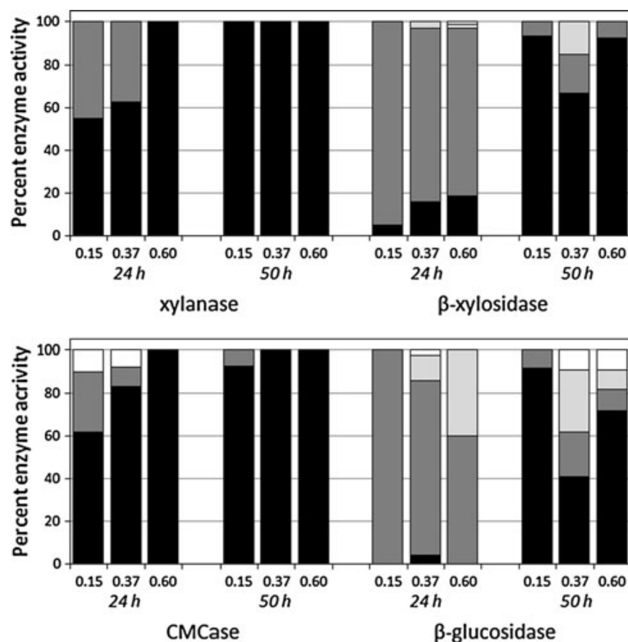

Fig. 4 Localization of enzyme activities at the bioreactor cultures for the three different aeration rates expressed as percentages of the total activity. *Black portion* extracellular fraction, *Dark grey portion* cytosolic intracellular fraction, *Light grey* loosely bound intracellular fraction, *White* tightly bound intracellular fraction

bacterial strains isolated from the Santorini volcano at the South Aegean Sea (Meintanis et al. 2006). Initial screening was performed in agar plates using either carboxy-methyl cellulose (CMC) or birchwood xylan as sole carbon sources. Excretion of the enzymes was visualized using the Congo Red indicator that binds to the β-1,4 glycosidic bonds of cellulose and xylan (Sharrock 1988; Teather and Wood 1982) and produces a decolorized halo in the presence of the corresponding hydrolases. The method has been used in literature for the initial screening of either cellulose (Abdelnasser and El-diwany 2007; Baharuddin et al. 2010; Percival Zhang et al. 2006; Singh et al. 2004) or xylan (Fontes et al. 2000; Kosugi et al. 2001) degrading microorganisms from various habitats. Xylanolytic activity appeared much more widespread among the isolates, since 80 % of them yielded clear halos of variable intensity upon growth on xylan. Among them, only eight strains also produced cellulase, although we could not identify in this plate screening any strains producing only this enzyme.

Fifteen strains were selected for their ability to produce both cellulase and xylanase, as well as elevated xylanolytic activities. The majority of these isolates showed high phylogenetic similarity to *Geobacillus* species as revealed from their 16S rDNA sequences although, only for seven among them the corresponding score was higher than 99 %. The genus *Geobacillus* has been relatively recently characterized (Nazina et al. 2001) and includes species with promising biotechnological potential (McMullan et al. 2004). Members of this genus have been reported to represent the majority of the species that inhabit a variety of diverse « hot » environments such as marine thermal vents (Maugeri et al. 2001, 2002), high temperature oil fields (Nazina et al. 2001), hot springs (Canakci et al. 2007) and sugar refinery wastewaters (Tai et al. 2004). In contrast to the above studies, the isolates from the Santorini volcanic habitat had relatively low percent phylogenetic similarity between each other, a fact that indicates a high interspecies microbial diversity in this environment. Due to the relatively recent characterization of this genus, studies reporting production and/or characterization of specific cellulases (Abdelnasser and El-diwany 2007; Ng et al. 2009; Rastogi et al. 2010; Tai et al. 2004; Zhang and Lynd 2005) or xylanases (Canakci et al. 2007; Sharma et al. 2007; Wu et al. 2006) by *Geobacilli* are limited. Based on conserved areas of published gene sequences for these enzymes we constructed PCR primers for *Geobacillus* endo-xylanase and endo-cellulase genes. Using these primers, we were able to identify the xylanase PCR product in the genomic DNA of only eight among the fifteen selected isolates despite the fact that all of them produced xylanase at the solid agar plates and although we performed an extensive optimization of the PCR program. This result most probably denotes that the corresponding strains express xylanase genes of primary structure that differ from those selected for primer construction or they do not represent members of the *Geobacillus* genus. The latter would most probably be the case for strains SP59 and SP87 that have less than 96 % 16S rDNA similarity with known *Geobacillus* strains and less than 90 % percent rDNA similarity with the other thirteen selected isolates (Table 2). All except one of the eight strains that exhibited cellulolytic activity in solid state cultures yielded the corresponding PCR product. It is most noteworthy, that four additional strains which did not grow on CMC agar plates proved to be positive on *Geobacillus* cellulase PCR product.

The enzyme activity profile of the fifteen selected isolates was more thoroughly examined in liquid cultures using a variety of carbon sources (Table 3). All strains were able to produce significant amounts of xylanase upon growth on xylan, which was proven to be the best inducer for the enzyme. Despite this fact though, we were not able to identify any correlation between the maximum volumetric activity in liquid cultures and the induction intensities observed in xylan agar plates. This may very well be attributed to the intrinsic differences between the two cultivation modes mainly in oxygen and nutrient availability. This result also suggests that the solid state activity screening may not be sufficient for the identification of the best xylanase producer, although this is an approach that has often been used by many researchers also for the

identification of thermophilic bacterial xylanases (Martins Cordeiro et al. 2002; Wu et al. 2006). High xylanase activities were also produced in liquid cultures on cellobiose, a situation that has been reported for several bacteria such as *Clostridium cellulovorans* (Kosugi et al. 2001), *Nocardiopsis* sp. (Saratale and Oh 2011) and *Bacillus circulans* (Bocchini et al. 2008) while this is the first report concerning xylanase production by thermophilic bacteria grown on cellobiose. The latter carbon source proved also to be the best cellulase inducer for most of the strains, a widely encountered phenomenon for a variety of bacteria such as *Clostridium thermocellum* (Zhang and Lynd 2005), *Nocardiopsis* sp. (Saratale and Oh 2011) and *Clostridium acetobutylicum* (Lopez-Contreras et al. 2004). Relevant studies concerning *Bacillus* and *Geobacillus* species are though very limited (Chan and Au 1987; Robson and Chambliss 1984). Carboxy-methyl cellulose proved to be the poorest substrate as far as enzyme production in liquid cultures is concerned. Of more importance was the fact that all seven strains that did not produce any cellulolytic activity in solid state CMC cultures, secreted CMCase activity in liquid media in at least one of the carbon sources used. These results suggest that CMC screening on agar plates although a widely used, due to its simplicity, screening method for cellulolytic microorganisms, may not fully unravel the cellulose degrading metabolic potential of the microorganisms under investigation. In addition, our results clearly show that liquid culture screening on various substrates yields more reliable results and should be preferred, especially today where several high throughput liquid enzymatic assay screening methods are being developed (da Cruz et al. 2010; Reymond and Babiak 2007).

Our study also revealed that wheat bran was on average a very good inducing carbon source for both xylanase and cellulase. This low cost agricultural by-product has been successfully used for enhanced production of biomass degrading enzymes by various microbial strains including mesophilic fungi and bacteria (de Souza et al. 2006; Qinnghe et al. 2004; Sun et al. 2008) as well as thermophilic *Bacillus* (Asha Poorna and Prema 2007; Subramaniyan et al. 1997) and *Geobacillus* strains (Sharma et al. 2007). With wheat bran as sole carbon source, strain SP24 that revealed 99.1 % 16S rDNA similarity to *Geobacillus* sp. produced significantly high cellulase and xylanase activities and consequently, was chosen as a representative strain in order to study in more detail the physiology of the biosynthesis of the full array of biomass degrading enzymes by thermophilic geobacilli. Supplementation of the basal medium with additional nitrogen sources such as casein, tryptone or ammonium phosphate generally suppressed enzyme production indicating that there was no nitrogen limitation in the basal medium that contains nitrate ions and yeast extract. Only casein addition yielded a double maximum concentration for β-xylosidase and CMCase but simultaneously reduced the production of the other two enzymes. On the other hand, starch and especially xylan had a strong positive effect on enzyme production when supplemented wheat bran in the culture medium, suggesting a more complex and multi-level induction scheme by the carbon source ingredients.

There is very limited information in literature concerning aeration effects on enzyme production by even mesophilic *Bacillus* species (Huang et al. 2001; Yuguo et al. 2001) while we were not able to identify any relevant studies concerning thermophilic *Bacillus* or *Geobacillus*. Although geobacilli are referred as facultative anaerobic species (Feng et al. 2007; Fong et al. 2006; Sharma et al. 2009) very little growth was observed when isolate SP24 was grown in non aerated bioreactor cultures. This is most probably associated with the complexity of the carbon source used (wheat bran and xylan) that lacks simple sugars and the fact that the biomass degrading enzyme production (cellulase and xylanase) was shown to be growth associated and positively influenced by aeration.

The selected strain SP24 was able to produce the four major biomass degrading enzyme activities in an aeration dependent manner. Production and isolation of these enzymes by geobacilli has been reported individually for xylanases (Canakci et al. 2007; Lama et al. 2004; Sharma et al. 2007; Wu et al. 2006), β-xylosidases (Lama et al. 2004; Quintero et al. 2007; Wagschal et al. 2009) and cellulases (Ng et al. 2009; Rastogi et al. 2010) but we were not able to identify any studies concerning β-glucosidase identification or isolation in this genus. Cellulase was extracellularly produced from the first stages of growth showing a strong dependence on aeration only up to 0.37 vvm. Similarly, Rastogi et al. (Rastogi et al. 2010) have reported that CMCase activity produced under micro-aerophilic conditions by two *Geobacillus* species isolated from a compost sample were similar to those produced under fully aerobic conditions, while the growth associated nature of the enzyme expression has been also verified by other researchers (Li et al. 2008; Rastogi et al. 2010; Tai et al. 2004). In all these studies the enzyme was always determined in the culture supernatant without specific referral on cell-bound activities. In the related *Anoxybacillus flavithermus* species though, the absence of cell bound cellulase activity was explicitly reported (Abdelnasser and El-diwany 2007). We could not identify any studies concerning β-glucosidase production from *Geobacillus* species despite the fact that several relevant reports exist for mesophilic bacilli (Chan and Au 1987; Kim et al. 2005; Robson and Chambliss 1984; Singh et al. 2004; Taechapoempol et al. 2011). Strain SP24, despite its ability to grow well on cellobiose, was able to yield significant β-

glucosidase activity during bioreactor growth on wheat bran and xylan which was mainly cell associated. Interestingly enough, aeration had a negative effect on the production suggesting a different control mechanism of the corresponding gene compared to cellulase. This finding combined with the cell-bound nature of the enzyme and relatively low expression levels may well explain the aforementioned absence of the relevant literature. We could not also identify a similar aeration effect on β-glucosidase levels for bacterial strains but such behavior has been reported for the β-glucosidase of *Aspergillus niger* (García-Kirchner et al. 2005) although the intensity of the phenomenon was relatively milder. Aeration was necessary for the expression of the xylan related activities in strain SP24 but its levels had a relatively weak effect on the maximum xylanase and β-xylosidase levels. Both enzymes were strongly growth associated in contrast to the findings of Lama et al. (Lama et al. 2004) who reported that both xylanase and β-xylosidase of *B. thermantarcticus* were produced at the late exponential growth phase. The xylanase of strain SP24 was an extracellular enzyme as is the case for other *Geobacillus* species (Canakci et al. 2007; Nanmori et al. 1990; Saratale and Oh 2011) but the β-xylosidase was mainly produced as a cell-bound enzyme. For the latter enzyme there are reports for its isolation and purification from the extracellular medium of *G. pallidus* (Quintero et al. 2007) and *B. thermantarcticus* (Nanmori et al. 1990) cultures, but taking into account the results from our work concerning its localization, these reported extracellular amounts would most probably be the result of cell lysis.

In conclusion, our work has revealed that volcanic sediments represent a relatively rich environmental niche for thermophilic bacteria with enzymatic potential towards biomass hydrolysis. The dominant genus with the relevant characteristics was the geobacilli, although for few among the strains selected for their high biomass degrading capacity, the phylogenetic similarity with this genus was relatively low (<97 %). The screening procedure proved to be an important parameter for the identification of the best producers since the expression of the genes encoding for the corresponding xylanolytic and cellulolytic activities was greatly influenced both by the cultivation mode as well as the nature of the carbon source. Certain complex and low cost carbon sources such as wheat bran can be used in this procedure for the identification of strains, such as SP24 (which has more than 99 % 16S DNA similarity with *Geobacillus* sp.) with the ability to produce elevated levels of cellulase, xylanase, β-glucosidase and β-xylosidase activities. The production levels of these enzymes can be further increased in bioreactor cultures, since they revealed significant dependencies on aeration levels and yield enzyme mixtures suitable for various biotechnological and research applications.

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