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ORIGINAL ARTICLE

Characterization of a native cellulase activity from an anaerobic thermophilic hydrogen-producing bacterium *Thermosipho* sp. strain 3

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Abstract A bacterial strain, designated as strain 3 and identified as a member of the *Thermosipho* species on the basis of its phenotypic and genotypic characteristics, was isolated from a deep sea hydrothermal vent. Sequence analysis of the 16S rRNA gene revealed that its closest neighbor was *Thermosipho africanus* (99.5 %). This isolate *Thermosipho* sp. strain 3 (DSM 27729), a thermophilic, anaerobic, fermentative hydrogen-producing bacterium, produced a thermostable endocellulase that hydrolyzes carboxymethylcellulose (CMC) and β-glucan. The cellulase was purified and its activity characterized. The estimated molecular weight of the protein was about 40 kDa as determined by gel-filtration chromatography, SDS-PAGE and zymogram analyses. The optimal cellulase activity was at pH 5.5 and at a temperature of 80 °C. The enzyme was thermostable with about 50 % residual activity after 48 h and 4 h at 60 °C and 70 °C, respectively. Interestingly, endocellulase activity was increased about 2-fold by 5 mM $MnCl_2$. MALDI-TOF PMF and the N-terminal amino acid sequence analyses of the purified enzyme revealed the extensive homology of the protein with a glycoside hydrolase family protein from *Thermosipho africanus* (NCBI protein accession number: 419759359; UniProt: K2PFP0).

Keywords Hydrogen · Cellulase · Carboxymethylcellulose · Thermophilic · Hydrothermal vent

Introduction

Nowadays, over 80 % of the energy consumed in the world is derived from fossil fuels, which will eventually become depleted in the not too distant future. In addition, burning of fossil fuels contributes severely to climate change, environmental deterioration and threatens public health. For this reason there is a considerable tendency worldwide to increase the use of renewable sources of energy and, in this context, hydrogen is believed to be an ideal energy vector because of its high conversion efficiency, recyclability and non-polluting nature (Zhang et al. 2007). A promising way to produce hydrogen sustainably is bacterial fermentation of organic substrates in the absence of light—defined as dark fermentation (Levin et al. 2004). Thermophilic bacteria are considered more promising than mesophilic microorganisms for fermentative hydrogen production. Several thermostable enzymes have been purified from such bacteria, e.g., enzymes that catalyze the hydrolysis of complex polysaccharides such as cellulose—the major component of the plant cell wall and the largest organic carbon reservoir on Earth (Festucci-Buselli et al. 2007). Due to their complexity and variety, the degradation of cellulose-based polysaccharides into monosaccharides (like glucose and xylose) requires several synergistically operating enzymes (Lynd et al. 2002), including endo-β-glucanases (4-β-D-glucan 4-glucanohydrolase [EC 3.2.1.4]), exoglucanases (4-β-D-glucan cellobiohydrolase [EC 3.2.1.91]), glucan glucosidases (4-β-D-glucan glucohydrolase [EC 3.2.1.74]) and β-glucosidases (β-D-glucoside

16S rRNA gene sequence of *Thermosipho* sp. strain 3 is available in the GenBank/EMBL/DDBJ databases under the accession number FM876224.

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glucohydrolase [EC 3.2.1.21]) (Bok et al. 1998). Endoglucanases hydrolyze internal glycosidic linkages randomly, which results in a rapid decrease in polymer length and a gradual increase in reducing sugar concentration (Wood and Bhat 1988; Béguin and Aubert 1994). Exoglucanases hydrolyze cellulose chains by removing cellobiose either from the reducing or the nonreducing ends of these molecules (Teeri 1997), which results in rapid release of reducing sugars, but little change in polymer length. Glucose is produced primarily by the action of glucan glucosidases on cellooligomers and by the action of β-glucosidases on cellobiose (Rixon et al. 1992; Goyal and Eveleigh 1996).

Endoglucanases (or cellulases) are key enzymes since they are involved in the first step of the hydrolysis process and, more generally, are exploited for several industrial applications. Cellulases are used widely in the textile industry for biostoning and biofinishing of cellulosic fibers (Andreaus et al. 2000; Miettinen-Oinonen et al. 2004); and in the food industry to increase the yield of fruit juices, to improve beer filtration, oil extraction and nutritive quality of bakery products and animal feed (Bedford 1995; Bhat 2000; Haros et al. 2002). Moreover, cellulases and hemicellulases can be used for the saccharification of agricultural wastes for their subsequent fermentation to alternative fuels (Gray et al. 2006; Lin and Tanaka 2006).

Many cellulolytic enzymes have been found in several fungal species and in various thermophilic bacteria. To the best of our knowledge, with the exceptions of the *Thermotoga* genus and *Fervidobacterium nodosum* (Zheng et al. 2009, 2011, 2012), there have been no studies to date on cellulase activities produced by other members of the Thermotogales order, which includes the *Thermosipho* genus.

Members of the *Thermosipho* genus are thermophilic and rod-shaped anaerobes that grow heterotrophically on a variety of carbohydrates as energy and carbon source. So far, seven species belonging to this genus have been described: *T. africanus* (Huber et al. 1989; Ravot et al. 1996), *T. melanesiensis* (Antoine et al. 1997), *T. japonicus* (Takai and Horikoshi 2000), *T. geolei* (L'Haridon et al. 2001), *T. atlanticus* (Urios et al. 2004), *T. globiformans* (Kuwabara et al. 2011) and *T. affectus* (Podosokorskaya et al. 2011).

Herein, we report the isolation and characterization of a thermophilic, anaerobic, fermentative hydrogen-producing bacterium, designated strain 3, isolated from a deep sea hydrothermal vent. According to its phenotypic and genotypic characteristics, it should be placed in the *Thermosipho* genus. Moreover, we describe for the first time the purification and characterization of a native endocellulase produced by a member of the *Thermosipho* genus that is involved in the breakdown of carboxymethylcellulose (CMC).

Materials and methods

Collection site

Strain 3 was isolated from samples collected from deep sea hydrothermal vents located in Lucrino (Pozzuoli, Naples, Italy, 40°49′N 14°5′E), at a depth of 12 m. At the site of sampling, the temperature was 30 °C and the pH value was about 5. The samples—a mixture of sediment and water—were collected in sterile plastic bottles and stored at 4 °C until their use as inoculum into the enrichment medium.

Enrichment, isolation and growth conditions

Several aliquots (10 mL) of enrichment medium were inoculated with sediment and water samples (1 mL) and incubated at different temperatures ranging between 50 °C– and 80 °C, under anaerobic conditions and without agitation. Visible growth was observed by using a culture medium (*Ta*) containing the following components (g/L): NaCl 10; KCl 0.1; $MgCl_2$ 6 H_2O 0.2; NH_4Cl 1; K_2HPO_4 0.3; KH_2PO_4 0.3; $CaCl_2$ 2 H_2O 0.1; cysteine-HCl 1; yeast extract 2; tryptone 2; glucose 5; resazurin 0.001; before sterilization, the pH was adjusted at 7.5 with 1 M NaOH at room temperature. After sterilization, the medium was also supplemented with 10 mL/L each of filter-sterilized vitamins and trace element solutions from DSM medium 141. Aliquots of growth medium and isolation of hydrogen-producing cultures were set up according to Romano et al. (2010). All transfers and culture samplings were performed using sterile syringes and needles.

Several anaerobic hydrogen-producing strains were selected by means of the technique described by Romano et al. (2010); culture purity was checked by uniformity of colony morphology and by the examination of single cells in phase contrast microscopy. Taxonomic properties of only one of these microorganisms, strain 3, will be presented in this paper.

Physiological, morphological and biochemical tests

The optimum parameters for growth and hydrogen production were determined in the culture medium *Ta* by varying the temperature from 50 °C to 80 °C, the pH from 4 to 9 (determined at room temperature) and NaCl concentration from 0 to 80 g/L. Bacterial growth, colony morphology, cell morphology, Gram reaction, motility study, spore formation test, heat-resistance of cells, effects on growth of various electron acceptors (cysteine 20 mM, elemental sulfur 1 g/L, sodium sulfide 20 mM, sodium thiosulfate 20 mM or sodium hydrosulfite 20 mM), H_2S formation, ability to utilize different carbon sources, ethanol tolerance, gelatin hydrolysis, nitrate and nitrite reductions and gaseous end products (H_2 and CO_2) were determined according to Romano et al. (2010).

To test antibiotic sensitivity, Sensi-discs (6 mm, Oxoid) of erythromycin (5 and 30 μg), fusidic acid (10 μg), chloramphenicol (10 and 50 μg), lincomycin (15 μg), streptomycin (25 μg), ampicillin (25 μg), vancomycin (30 μg), novobiocin (30 μg), neomycin (30 μg), gentamicin (30 μg), kanamycin (30 μg), tetracycline (30 and 50 μg), penicillin G (2 and 10 units), bacitracin (10 units) and nystatin (100 units) were used in 1 % (w/v) gelrite plate cultures on medium *Ta* (Romano et al. 1993). Plates were incubated in a stainless steel anaerobic jar for 72 h.

The amounts of ethanol, lactic and acetic acids produced during growth on glucose (5 g/L) were quantified by ^{1}H-nuclear magnetic resonance (NMR, 400 MHz) by using a Bruker Avance 400 spectrometer and 14 % (v/v) of a solution of D_2O containing 0.05 % (w/w) 3-(trimethylsilyl)propionic-2,2,3,3-d_4 acid (Sigma-Aldrich, St. Louis, MO) as standard (Figueiredo et al. 2006).

If not otherwise indicated, all tests were performed in triplicate by using standard culture medium *Ta* and incubating the samples at the optimal growth temperature (70 °C) for the required time.

Phylogenetic analyses and G+C-DNA content

Genomic DNA extraction, PCR amplification and sequencing of the 16S rRNA gene were carried out as described previously (Romano et al. 2007, 2010). Multiple sequence alignment was obtained using CLUSTAL_X (Thompson et al. 1997) and then inspected visually to identify positions of uncertain alignment, usually at the ends of the sequences. Phylogenetic relationships were analyzed using the program MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance and Maximum Parsimony Methods (Tamura et al. 2011). Neighbour-joining (NJ) analysis, with Kimura's two-parameter correction, was computed using a pairwise deletion method for gaps and missing sites. Bootstrap analysis was performed using 1,000 replications. The values for pairwise 16S rRNA gene sequence similarity among *Thermosipho* species were determined using the EzTaxon server (http://eztaxon-e.ezbiocloud.net/) (Kim et al. 2012). DNA purification and G+C-DNA content were performed as previously published (Romano et al. 2010).

Standard enzyme assay and protein determination

Cellulase activity was measured by the increase of reducing sugars using the dinitrosalicylic acid method (Bernfeld 1955). A reaction mixture (0.5 mL) contained 250 μL 0.4 % (w/v) CMC in 50 mM sodium acetate buffer pH 5.5 and 250 μL of appropriately diluted enzyme sample. After 30 min at 80 °C the reaction was stopped in ice and 500 μL 1 % (w/v) dinitrosalicylic acid solution was added. The samples were boiled at 100 °C for 5 min and then cooled immediately in ice. The absorbance was measured at 546 nm against 50 mM sodium acetate buffer pH 5.5. The dinitrosalicylic acid color reaction was calibrated under the assay conditions by using a glucose solution (2 g/L). One unit of cellulase activity was defined as the amount of enzyme that released 1 μmol glucose equivalents per minute under the standard assay conditions.

The protein concentration was determined according to Bradford's method (Bradford 1976) with bovine serum albumin (1 g/L) as standard protein.

Purification of enzymatic activity

Preliminary tests were carried out in batches in 25 mL standard medium *Ta* with and without CMC 1 g/L and in medium *Ta* without glucose, but supplemented with cellobiose 5 g/L or CMC 2 g/L and 4 g/L. Cultures were incubated for 48 h at 70 °C. Cells were then collected by centrifugation at 10,000 rpm for 30 min at 4 °C, suspended in lysis buffer [50 mM Tris–HCl pH 7, 1 mM phenylmethylsulfonyl fluoride (PMSF)] and broken by three-fold passage through a French pressure cell (Thermo Electron Corporation, Waltham, MA) at 1,500 psi for 5 min. Crude extract was centrifuged at 3,000 rpm for 10 min at 4 °C to remove unbroken cells; then, the opalescent supernatant was clarified by centrifugation at 20,000 rpm for 1 h at 4 °C. The resulting supernatant was dialyzed extensively against 50 mM Tris–HCl pH 7 at 4 °C. The protein concentration of each sample was determined and cellulase activity measured under the assay conditions.

For cellulase enzymatic activity purification, strain 3 was grown in a glass bioreactor (working volume=2 L) by using the standard culture medium *Ta* plus CMC 1 g/L. The growth was conducted at 70 °C under anoxic condition by sparging N_2 and stirring at 250 rpm; the pH was controlled at 7. The cells were collected by centrifugation at 10,000 rpm for 30 min at 4 °C after 20 h growth. Wet cells (≈ 3.5 g) were suspended in lysis buffer and broken as described above. After dialysis, the resulting soluble fraction was used for the following purification steps.

The sample was loaded onto a Q-Sepharose Fast Flow column (1.6 cm×27 cm, Pharmacia, Uppsala, Sweden) equilibrated with 50 mM Tris–HCl pH 8 (buffer A) at a constant flow rate of 3 mL/min. Proteins were eluted by applying a linear NaCl gradient (0–1 M) in buffer A and collected in 12 mL fractions. The active fractions were pooled, concentrated and dialyzed against buffer A by ultrafiltration using Amicon, YM 10 filters (Millipore, Billerica, MA).

Measurement of the molecular mass and zymogram

The apparent molecular mass of the native enzyme was determined by gel filtration chromatography on a Sephadex G-100

(Pharmacia) using a standard gel filtration calibration kit (75–6.5 kDa). The column was equilibrated with 50 mM sodium acetate buffer pH 5.5, 0.15 M NaCl at a flow rate of 1 mL/min; the elution was performed with the same buffer, but with a flow rate of 0.5 mL/min and fractions (1 mL each) were assayed for cellulase activity.

The apparent molecular mass of the enzyme was confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using a Mini Protean II apparatus (Bio-Rad, Hercules, CA). The electrophoresis was carried out as described by Laemmli (1970) using 10 % acrylamide in the resolving gel, 5 % acrylamide in the stacking gel and Tris-glycine buffer, pH 8.3 containing SDS 1 g/L as running buffer. Markers for molecular mass determination were perfect protein markers (LMW, 97.4–20.1 kDa, http://www.emdmillipore.com). Protein bands were stained with 0.1 % (w/v) Coomassie brilliant blue R-250 and destained with a mixture of distilled water/methanol/acetic acid (5:4:1 v/v/v).

For zymogram analysis samples were loaded in a standard 10 % SDS-polyacrylamide gel containing CMC 1 g/L as substrate, which copolymerized with resolving gel; the samples were not heated prior to electrophoresis. At the end of electrophoretic run, the gel was washed with isopropanol 20 % (v/v) in 50 mM sodium acetate buffer pH 5.5 for 30 min and only with the buffer for the following 30 min. Then it was incubated overnight in 50 mM sodium acetate buffer pH 5.5 at 70 °C. Finally, the gel was stained with Congo Red 1 g/L for 30 min and then destained in NaCl 1 M for a few minutes, until clear bands, resulting from substrate hydrolysis, were visible against a red background.

In-gel trypsin digestion of SDS-PAGE separated protein

Protein bands were excised manually from the 10 % SDS-PAGE gel, rinsed with 100 μL ultrapure water and completely destained by repeated washing with 25 mM NH_4HCO_3 in 50 % (v/v) aqueous acetonitrile. Protein bands were in-gel Cys-reduced with 10 mM dithiothreitol in 25 mM NH_4HCO_3 for 45 min at 55 °C and then *S*-alkylated with 55 mM iodoacetamide in 25 mM NH_4HCO_3 for 30 min at room temperature in the dark. The gel pieces were washed, immersed in pure acetonitrile and dried in a speed-vac centrifuge. Afterwards, protein bands were re-hydrated with 15 μL water solution of 12.5 ng/μL sequencing grade modified trypsin (Promega, Madison, WI) in an ice-cold bath. After 45 min, the gel pieces were covered with 30 μL 50 mM NH_4HCO_3 and incubated overnight at 37 °C. The tryptic digests were extracted three times with 40 μL acetonitrile/5 % (v/v) formic acid solution (1:1 v/v) and the recovered solutions were pooled. Peptides were concentrated in a speed-vac centrifuge, lyophilized and finally re-dissolved in 15 μL 0.5 % (v/v) formic acid. Before mass spectrometric analysis, peptides were desalted by C_{18} reversed-phase Zip-Tip® microcolumns (Millipore, Bedford, MA), washed with 0.1 % (v/v) trifluoroacetic acid (TFA) and eluted with 50 % (v/v) acetonitrile/0.1 % (v/v) TFA.

MALDI-TOF analysis and protein identification by peptide mass fingerprinting

Tryptic peptides were analyzed by matrix assisted laser desorption ionization (MALDI-TOF) mass spectrometry (MS). Mass spectra were acquired on a Voyager DE-Pro spectrometer (PerSeptive BioSystems, Framingham, MA) equipped with a N_2 laser (λ=337 nm), using α-cyano-4-hydroxycinnamic acid [10 mg/mL in 50 % (v/v) acetonitrile/0.1 % (v/v) TFA]. The instrument operated with an accelerating voltage of 20 kV in the reflector mode using Delay Extraction (DE) technology. External mass calibration was performed with a separate acquisition of a low mass peptide standard (Sigma). Raw data were elaborated using the Data Explorer 4.1 software provided by the manufacturer.

Peptide mass fingerprinting-based identifications were carried out interrogating the non redundant National Center for Biotechnology Information (nrNCBI) and Swiss-Prot/TrEMBL databases with Mascot (Matrix Science, London, UK) and Protein Prospector MS-FIT (http://prospector.ucsf.edu/) search engines. Mass tolerance of 0.3 Da, fixed carbamidomethylation of cysteines, variable pyro-glutamic acid formation at N-terminal Gln and possible methionine oxidation were set as search parameters. Up to one missed tryptic cleavage was accepted. Searches were taxonomically restricted to Bacteria.

N-terminal amino acid sequencing

For N-terminal amino acid sequencing, the blotting condition was that indicated in the Bio-Rad instructions for protein sequencing by Sequi-Blot PVDF membrane (Matsudaira 1987). Sequencing was performed using an Applied Biosystems Procise 494 automatic sequencer (Applied Biosystems, Foster City, CA), equipped with on-line detection of phenylthiohydantoin amino acids. Protein sequence databases were searched using the BLAST software at the NCBI server.

Effects of pH and temperature on cellulase activity

The dependence of cellulase activity on pH was assessed at 80 °C in the range of 3–10 with the following buffers (50 mM): glycine-HCl (pH 3), acetate buffer (pH 3.5–5.5), Na_2HPO_4–NaH_2PO_4 (pH 6–7.5), Tris–HCl (pH 8–8.5) and glycine-NaOH (pH 9–10).

The influence of temperature on CMC hydrolysis was studied over the range of 40 °C–90 °C in 50 mM sodium acetate buffer pH 5.5. In both cases, the relative activity was measured by the standard assay conditions.

Thermal stability

Thermal stability studies were carried out by incubating purified enzyme solutions of ~5 μg for varying time intervals at different batches of temperatures in 50 mM sodium acetate buffer pH 5.5. The residual activity on CMC was determined under standard assay conditions.

Effects of metal ions on cellulase activity

To determine the influence of metal ions, cellulase activity was assayed in the standard conditions in the presence of 5 mM of several metal ions and referred to the activity in metal ion-free conditions (100 %).

Substrate specificity

Enzymatic activity was assayed in the standard conditions measuring the increase of reducing groups with the dinitrosalicylic acid method using the following substrates (0.5 % w/v): CMC, β-glucan, xylan from birchwood, xylan from oat spelt, avicel, α-cellulose, filter paper, laminarin and curdlan. One unit of enzyme activity was defined as the amount of enzyme that releases 1 μmol/min reducing sugars.

Hydrolysis of *p*-nitrophenyl-β-D-cellobioside (*p*NPC) was measured at 80 °C in 50 mM sodium acetate buffer (pH 5.5) and one unit of activity was defined as the amount of enzyme liberating 1 μmol/min *p*-nitrophenol.

Sugar analysis

The hydrolysis of CMC and β-glucan after 24 h of incubation under standard conditions was monitored by thin layer chromatography (TLC) on 0.2-mm silica-gel-coated aluminium sheet (type 60; Merck, Darmstadt, Germany) developed with butanol/acetic acid/H_2O (6:2:2 v/v/v). Spots were detected with α-naphtol, which is specific for carbohydrates.

Results

Isolation

Anaerobic thermophilic hydrogen-producing cultures were obtained by inoculating culture medium *Ta* with samples recovered from a deep sea hydrothermal vent. Cell growth was observed after incubation at 70 °C for 48 h: microscopic examination revealed bacterial populations composed of small rods with a characteristic outer sheath-like structure. Strain 3, the predominant strain in the enrichment culture medium and the only colony-forming organism at the highest dilutions, was studied in detail, also because it exhibited a cellulase activity.

Physiological, morphological and biochemical tests

Strain 3 was an obligate anaerobe Gram-negative bacterium. Its optimal temperature for growth and hydrogen production was 70 °C, while no growth was observed at a temperature lower than 50 °C or higher than 80 °C; moreover, optimum growth and hydrogen production occurred at pH 7 in the presence of 5–70 g/L NaCl with an optimum at 10 g/L.

After 3 days of incubation at 70 °C on solid medium *Ta*, the colony area appeared colorless, uniformly round, with a glossy surface and a diameter of about 3 mm. In liquid medium, the strain formed straight, non-motile, rod-shaped cells, both singly and in pairs, which were the predominant forms in the early exponential growth phase. In contrast, in the late exponential and in the stationary growth phases, rods tended to assume spherical shapes.

In the culture medium plus Mn^{2+}, no spores were observed under light microscopy examination after 24 h, 48 h and 72 h of incubation. When strain 3 cultures were heated at 100 °C for 30 min and then sub-cultured into fresh standard medium, growth and hydrogen production were observed after 3 days of incubation at 70 °C, thus suggesting the presence of heat-resistant forms.

Strain 3 was able to reduce cysteine, elemental sulfur, sodium sulfide, sodium thiosulfate and sodium hydrosulfite to hydrogen sulfide. Bacterial growth was improved strongly in the presence of these electron acceptors compared to non-supplemented control cultures.

Strain 3 was a chemorganotrophic bacterium able to utilize various substrates for growth and hydrogen production (data not shown). Little growth was observed either on a yeast extract-tryptone medium without a fermentable carbon source or on glucose medium without yeast extract and tryptone. These results indicated that, for optimal growth, the presence of yeast extract, tryptone and a fermentable carbohydrate was required. During growth on glucose or other fermentable sugars, the pH of the medium decreased from the initial optimum pH to about 4.5. This pH reduction was attributable to the accumulation of organic acids as fermentation progresses. The relative content of the metabolic products of fermentation was affected by substrate type and concentration as well as by pH: however, when strain 3 grew on the standard culture medium supplemented with glucose (5 g/L) and in the presence of cysteine (1 g/L), the main fermentation products were H_2 (8.7 mM), CO_2 (2.2 mM), H_2S (7.5 mM), ethanol (0.9 mM), acetate (9.4 mM) and lactate (0.1 mM).

Isolate 3 was also able to grow in the presence of 2 % (v/v) of exogenous ethanol. Moreover, it had the capacity to hydrolyze gelatin at a concentration of 60 g/L, while reduction of neither nitrate nor nitrite was observed. In particular, the presence of $NaNO_2$ at 0.01 and 0.005 g/L completely inhibited bacterial growth.

Strain 3 was completely sensitive to erythromycin, chloramphenicol, lincomycin, streptomycin, ampicillin, vancomycin, novobiocin, tetracycline, penicillin G, bacitracin and nystatin, because no growth was observed around Sensi-discs. In contrast, its growth was inhibited only partially by fusidic acid, neomycin, gentamicin and kanamycin.

Phylogenetic analyses and G+C DNA content

A partial 16S rRNA gene sequence (915 nt) of strain 3 was determined. As shown in the NJ phylogenetic tree (Fig. 1), strain 3 was affiliated to the *Thermosipho* genus and, as can also be seen from the phylogenetic tree, this strain is placed in the same cluster with *T. africanus*, *T. globiformans* and *T. japonicus.* According to pairwise sequence comparisons, strain 3 showed 99.5 %, 98.7 %, 98.4 %, 95.3 %, 95.1 %, 94.5 % and 93 % gene sequence homology to *T. africanus*, *T. globiformans*, *T. japonicus*, *T. atlanticus, T. geolei*, *T. melanesiensis* and *T. affectus*, respectively. Also, the phenotypic features of strain 3 were consistent with those of other *Thermosipho* species.

The G+C content of strain 3-DNA was 31.3±0.3 mol% as evaluated on the basis of T_M determination. This value was comparable to G+C content described in the literature for the closest phylogenetic relatives of strain 3.

Purification of cellulase activity

The highest cellulase activity was expressed when cells were grown on standard medium *Ta* with CMC 1 g/L. The enzyme with cellulase activity was purified from the crude intracellular extract of strain 3 culture in a bioreactor by means of anionic exchange and gel filtration chromatographic steps. Even cellular debris was assayed for the presence of cellulase activity, but this appeared to be present to a lesser extent compared with that in the intracellular sample (data not shown).

The purification and total recovery of CMCase is summarized in Table 1. The specific activity of the enzyme increased by about 41-fold to 120 U/mg protein and recovery was 21 %.

Molecular properties, MALDI-TOF analysis, protein identification by peptide mass fingerprinting and N-terminal amino acid sequencing

SDS-PAGE analysis, carried out under denaturating and reducing conditions, of the purified cellulase resulted in a single protein band with estimated molecular mass of about 40 kDa (Fig. 2a). Also the zymogram (Fig. 2b), carried out under native conditions, showed a single band with intense enzymatic activity.

MALDI MS-based PMF analysis (Fig. 3) revealed extensive homology of the protein with a glycoside hydrolase family protein from *T. africanus* (NCBI protein accession number: 419759359; UniProt: K2PFP0). The predicted molecular mass of the protein was 39,384 Da (335 aa), in agreement with the apparent SDS-PAGE and gel filtration chromatography MW (~40,000 Da). The identification was considered successful as all of the following criteria were met: score higher than the identity threeshold ($P<0.05$), mascot score 134 (>84 corresponded to identity or extensive homology under the current search conditions), more than ten major signal matching tryptic peptides and sequence coverage of 60 %.

BLAST analysis of the glycoside hydrolase family protein from *T. africanus* (NCBI protein accession number: 419759359) revealed high sequence homology (ranging between 73 % and 49 %) with several endoglucanases from members of the Thermotogales order. In particular, amino acid sequence identities with a glycoside hydrolase family protein from *Fervidobacterium nodosum* (NCBI protein accession number: 154250235), an endoglucanase from *Fervidobacterium pennivorans* (NCBI protein accession number: 383787442) and a Cel5A from *Fervidobacterium gondwanense* (NCBI protein accession number: 428131024) of 73 %, 68 % and 49 % , respectively, were observed.

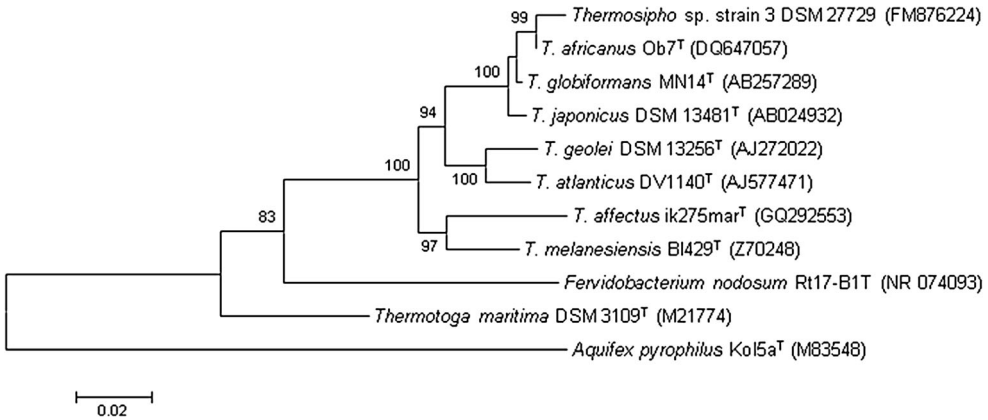


Fig. 1 Neighbor-joining (NJ) tree showing the phylogenetic position of *Thermosipho* sp. strain 3 and some related *Thermosipho* species based on partial 16S rRNA gene sequences (915 nt). Bootstrap values greater than 75 % confidence are shown at branching points (percentage of 1,000 resamplings). Sequence accession numbers are given in parentheses. *Bar* 0.02 % expected nucleotide substitution per site

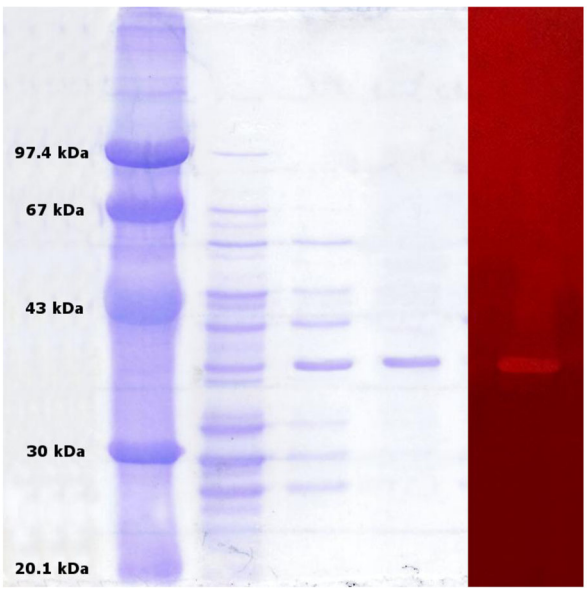
Table 1 Purification steps of cellulase from *Thermosipho* sp. strain 3

Purification step	Volume (mL)	Total protein (mg)	U/mL	Total activity (U)	Specific activity (U/mg)	Purification factor (-fold)	Yield (%)
Crude extract	11	38.5	10.3	113.3	2.9	1	100
Q-Sepharose F.F.	10	4.2	7.8	78	18.6	6.4	68.8
Sephadex G-100	5	0.2	4.8	24	120	41.4	21.2

The N-terminal sequence of the purified enzyme was ENKKLQAFDYNKMIG. The results of a BLAST search indicated that this partial amino acid sequence is conserved with respect to the glycoside hydrolase family protein (endoglucanase) from *T. africanus*, confirming results obtained by MALDI-TOF-MS analysis. The two sequences differed only in a puntiform ^{6}T→Q amino acid substitution.

Effects of pH and temperature on cellulase activity

Enzymatic activity assayed in standard conditions over a broad pH range from pH 3 to 10 showed an optimum at pH 5.5 in 50 mM sodium acetate buffer and about 70 % of the maximum activity was measured between pH 4.5 and pH 7 (Fig. 4a). Moreover, the cellulase exhibited the highest activity at 80 °C under the standard assay conditions at pH 5.5 (Fig. 4b).



a) b)

Fig. 2a,b Gel electrophoretic analyses of cellulase produced by *Thermosipho* sp. strain 3. **a** SDS-PAGE. Lanes: *1* low molecular weight standards (phosphorylase b 97.4 kDa, bovine serum albumin 67 kDa, ovalbumin 43 kDa, carbonic anhydrase 30 kDa, soybean trypsin inhibitor 20.1 kDa); *2* crude extract from *Thermosipho* sp. strain 3 (5 μg); *3* sample partially purified after Q-Sepharose F. F. column (5 μg); *4*, purified cellulase after Sephadex G-100 column (5 μg). **b** Zymogram: *clear band* resulting from substrate hydrolysis from purified cellulase (2 μg), visible against a *red* background

Thermal stability

CMCase of *Thermosipho* sp. strain 3 was optimally stable at 60 °C for about 24 h without any loss of activity, while about 50 % activity was retained after incubation for 48 h. At 70 °C, almost 50 % of activity was lost after 4 h of incubation (Table 2).

Effects of metal ions on cellulase activity

Among ion salts at 5 mM, Mn^{2+} and Fe^{2+} enhanced the relative enzyme activity about 1.5- and 0.5-fold, respectively, when compared with the control (100 %). Various metal ions (Mg^{2+}, Na^{+}, Co^{2+}, Sr^{2+}, Ca^{2+}) had no stimulatory or light inhibitory effects on enzyme activity, whereas the addition of 5 mM $CuSO_4$, $Pb(NO_3)_2$, $HgCl_2$, $ZnSO_4$ and $NiSO_4$ inhibited enzyme activity (Table 2).

Substrate specificity and sugar analysis

Substrates such as xylan, avicel, α-cellulose, filter paper, laminarin (β-1,3-1,6 linkages), and curdlan (β-1,3 linkages) were resistant to CMCase. The cellulase showed very high activities on CMC (100 %) and β-glucan (400 %) and very low activity on *p*NPC (0.16 %) (Table 2).

TLC and liquid assay analyses revealed that the cellulase of strain 3 liberated oligomeric intermediates from CMC and β-glucan, probably indicating an endo-type action (data not shown).

Discussion

Deep-sea hydrothermal vent ecosystems represent a primordial environment with respect to their physicochemical conditions (high pressures, high temperatures, low pH, high concentrations of metals, high concentrations of dissolved gases) and where primary production is based totally on chemosynthesis. Previous microbiological studies of such environments have led to the identification of several thermophilic microorganisms. In the case of strain 3 (DSM 27729), isolated from a deep sea hydrothermal vent located in Lucrino (Pozzuoli, Naples, Italy), 16S rRNA gene sequence analysis revealed that its closest relatives were the validated microorganisms

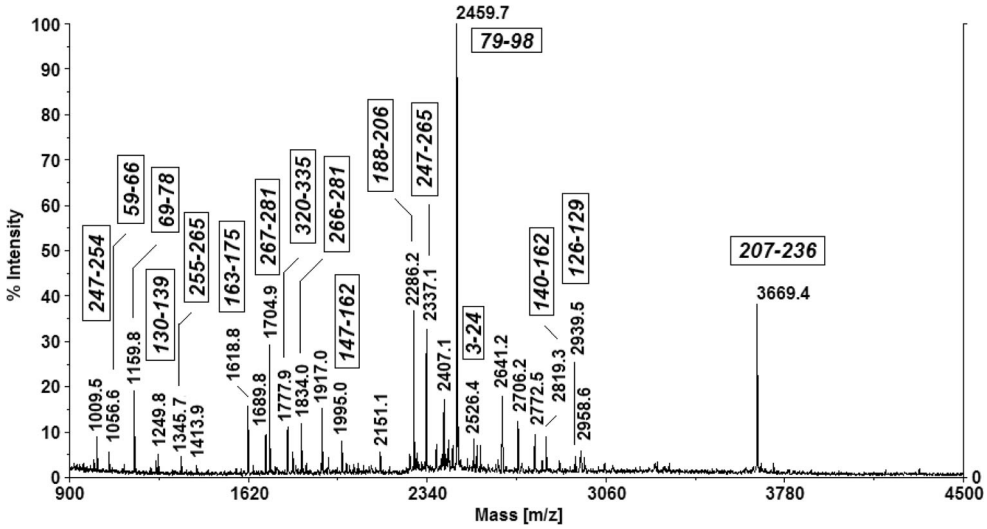


Fig. 3 Matrix assisted laser desorption ionization–mass spectrometry (MALDI-TOF MS) peptide mass mapping of the cellulase protein from strain 3. The position of the tryptic peptides within the protein sequence is indicated in the *boxed labels*

classified in the *Thermosipho* genus. Also, G+C DNA content (31.3 mol%) was in the range of values for members of this genus. Strain 3 growth was improved strongly in the presence of electron acceptors compared to a non-supplemented control culture. These results indicated the sensitivity of the strain to the produced hydrogen, in accordance with other *Thermosipho* species (Ravot et al. 1996; Antoine et al. 1997; Takai and Horikoshi 2000; L'Haridon et al. 2001; Urios et al. 2004; Kuwabara et al. 2011; Podosokorskaya et al. 2011). Strain 3 also had other features in common with *T. africanus* and *T. japonicus*, its closest phylogenetic congeners: these are fermentative thermophiles with an outer sheath-like structure and have the ability to form chains in the late exponential and in the stationary growth phases, a similar optimal temperature

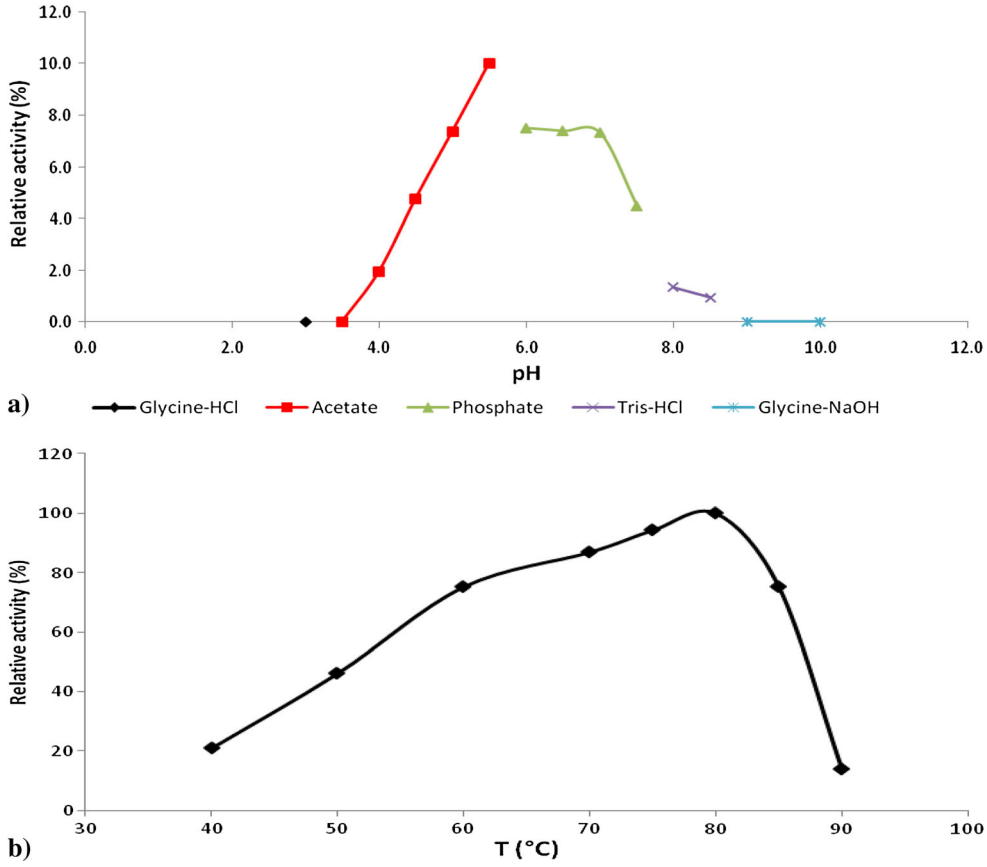


Fig. 4 Dependence on **a** pH and **b** temperature for cellulase activity from *Thermosipho* sp. strain 3

Table 2 Relative activity data about thermal stability assay, effects of metal ions on cellulase activity and substrate specificity assay. *CMC* Carboxymethylcellulose

Relative activity (%)													
Thermal stability	60 °C									70 °C			
	24 h					48 h				4 h			
	100.1±9.3					50.3±2.1				49.9±1.9			
Relative activity (%)													
Metal ions (5 mM)	None	Mn^{2+}	Fe^{2+}	Mg^{2+}	Na^{+}	Co^{2+}	Sr^{2+}	Ca^{2+}	Cu^{2+}	Pb^{2+}	Hg^{2+}	Zn^{2+}	Ni^{2+}
	100.4±5.9	239.7±6.8	148.7±5.5	100.3±5.2	92.4±3.9	87.8±3.5	81.0±3.0	76.3±3.1	11.8±2.4	9.1±0.3	0	0	0
Relative activity (%)													
Substrate specificity	CMC	β-glucan	Xylan	Avicel	α-cellulose	Filter paper		Laminarin		Curdlan		*p*NPC	
	100.3±6.5	400.3±14.6	0	0	0	0		0		0		0.16±0.03	

and pH, the absence of motility and H_2S production in the presence of electron acceptors. On the other hand, strain 3 also exhibited distinctive phenotipic characteristics with respect to *Thermosipho* genus members: e.g., minimal temperature required for growth, optimal NaCl concentration in culture medium, a wider spectrum of substrates used for growth and hydrogen production.

The potential industrial use of strain 3 is also related to its high tolerance to exogenous ethanol (2 % v/v) without previous adaptation. Indeed the general low tolerance to ethanol of thermophilic bacteria (generally below 1 % v/v) may be an impediment to the application of this group of organisms for industrial ethanol production due to the high cost of distillation at low ethanol concentrations (Lynd 1989).

An endocellulase was isolated from strain 3 and identified by MALDI-TOF PMF and N-terminal amino acid sequence analyses. The enzyme was purified to a single SDS-PAGE protein band and had an estimated molecular mass of about 40 kDa, which was within the range of the previously reported endoglucanases (23–43 kDa) expressed by *Bacillus* sp. (Au and Chan 1987; Kim and Pack 1988; Ozaki and Ito 1991; Hakamada et al. 2002), but lower than that produced by *B. pumilus* (Christakopoulos et al. 1999).

The cellulase showed optimal enzyme activity at 80 °C and was active over a broad temperature range with 70 % of residual activity at 60 °C and 85 °C. A similar range of optimal temperature has been reported for cellulases from *Acidothermus cellulolyticus* (Sakon et al. 1996; Skopec et al. 2003; Ransom et al. 2007) and *Fervidobacterium nodosum* (Wang et al. 2010).

Thermostability of an enzyme is a very important factor from the perspective of its potential industrial usage. We found that endoglucanase from strain 3 was a thermostable enzyme as it retained all of its original activity at 60 °C for 24 h and a 50 % decay was observed at 60 °C and 70 °C after 48 h and 4 h, respectively. A literature search revealed that, although several other thermophilic strains such as *Paenibacillus*, *Brevibacillus*, *Thermobifida* and *Cellulomonas* spp. have been shown to produce thermostable cellulases, these enzymes did not retain activity at higher temperatures (60 °C) for prolonged periods of time.

Cellulase was highly active in the presence of 5 mM Mn^{2+}, while Hg^{2+} completely inhibited enzyme activity, suggesting the presence of thiolic groups involved in the active site or important for the enzyme structure (Naumoff 2001). Similar results were reported for *Geobacillus* sp. 70PC53 (Ng et al. 2009), *Bacillus amyloliquefaciens* DL-3 (Lee et al. 2008) and *B. subtilis* YJ1 (Yin et al. 2010).

Substrates for cellulase activity assays can be divided into two classes, based on their solubility in water (Zhang et al. 2006). Strain 3 was tested for the hydrolysis of several soluble and insoluble polysaccharides. This enzyme showed detectable levels of activity only on soluble glucan-based substrates containing a β-1,4 linkage, including CMC and β-glucan. That the activity on β-glucan from barley was markedly higher than that towards CMC may be due to its good solubility in water. No activity was detected on the soluble polysaccharides laminarin (β-1,3 only glucose) and xylan or insoluble polysaccharides, such as avicel, α-cellulose and curdlan. In our case, CMC and β-glucan were the best substrates: this behavior was similar to that reported for cellulase from *Thermotoga neapolitana* (Bok et al. 1998), *T. maritima* (Chhabra and Kelly 2002), *Fervidobacterium nodosum* (Wang et al. 2010) and other strains (Zverlov et al. 1998; Ye et al. 2001; Arai et al. 2003).

Conventionally, hydrolysis of CMC has been used as an indicator of endoglucanase activity (Teather and Wood 1982) while true exoglucanases exhibit very low levels of terminal activity with CMC. On the basis of its high activity towards the substituted cellulose derivative CMC, cellulase from *Thermosipho* strain 3 may be classified as endo-β-1,4-glucanase. Moreover, cellulase from strain 3 also had low activity on *p*NPC: this aryl-β-D-cellobiosidase activity has also been described for other purified endoglucanases (Robson and Chambliss 1989).

Conclusion

In recent years there has been considerable interest in renewable sources of energy. In this context, hydrogen is believed to be an ideal energy vector because of its high conversion efficiency, recyclability and non-polluting nature, and utilization of plant materials could also be a renewable source of fermentable sugars. As a result of this interest, this paper described a thermophilic, hydrogen-producing bacterium able to synthesize a cellulase enzyme characterized by specific activities on CMC and β-glucan. We believe that this is a unique enzyme potentially useful in, but not limited to, applications in biomass conversion, detergent enhancement, paper pulping, textile manufacturing and juice clarification.

Moreover, to the best of our knowledge, this work is the first report on the purification and characterization of a native cellulase activity from a member of *Thermosipho* species.

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