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Purification and Properties of a Thermostable Pullulanase from a Newly Isolated Thermophilic Anaerobic Bacterium, *Fervidobacterium pennavorans* Ven5

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Extremely thermophilic anaerobic fermentative bacteria growing at temperatures between 50 and 80°C (optimum, 65 to 70°C) were isolated from mud samples collected at Abano Terme spa (Italy). The cells were gram-negative motile rods, about 1.8 μm in length and 0.6 μm in width, occurring singly and in pairs. Cells commonly formed spheroids at one end similar to *Fervidobacterium islandicum* and *Fervidobacterium nodosum*. The new isolate differs from *F. nodosum* by the 7% higher G+C content of its DNA (40.6 mol%) but is similar to *Fervidobacterium pennavorans* and *F. islandicum* in its G+C content and phenotypic properties. The phylogenetic dendrogram indicates that strain Ven5 belongs to the order *Thermotogales* and shows the highest 16S ribosomal DNA sequence similarity to *F. pennavorans*, *F. islandicum*, and *F. nodosum*, with similarities of 99.0, 98.6, and 96.0%, respectively. During growth on starch the strain produced a thermostable pullulanase of type I which preferentially hydrolyzed α-1,6 glucosidic linkages. The enzyme was purified 65-fold by anion-exchange, gel permeation, and hydrophobic chromatography. The native pullulanase has a molecular mass of 240,000 Da and is composed of three subunits, each with a molecular mass of 77,600 Da as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Optimal conditions for the activity and stability of the purified pullulanase were pH 6.0 and 85°C. At pH 6.0, the half-life of the enzyme was over 2 h at 80°C and 5 min at 90°C. This is the first report on the presence of pullulanase type I in an anaerobic bacterium.

Starch is found in many plants as a food reserve polysaccharide, and it represents a carbon as well as an energy source for various microorganisms. This macromolecule is composed of two high-molecular-weight compounds, namely, amylose (15 to 25%) and amylopectin (75–85%), but the size and the shape of starch granules are characteristic of the plant (25, 42). The importance of the starch industry is demonstrated by the large amount of sweeteners produced worldwide per year (5, 17, 38). Several successive steps involving different microbial enzymes are required during the manufacture of sugars (2, 3). The first step of the process is carried out at extremely high temperature (95 to 105°C) and at pH 6.0 to 6.5. In the second step all process conditions have to be changed to a pH of 4.5 and to a temperature of 60°C because of the absence of suitable enzymes. Finding more stable and specific enzymes would be significant for the improvement of the starch conversion process. Pullulanases that specifically attack the branching points of amylopectin are of special interest. The action of such enzymes would lead to the formation of linear oligosaccharides that can be attacked efficiently by other amylolytic enzymes, leading to high levels of glucose or maltose. The thermostability of enzymes is the main factor in more effective starch degradation, but the availability of new debranching enzymes would also enhance the saccharification process. So far, it has been found that the pullulanases of thermophilic microorganisms attack both α-1,6 and α-1,4 glycosidic linkages in amylopectin and malto-oligosaccharides (pullulanases of type II) (3). A number of thermostable pullulanases with dual specificities have been investigated, including pullulanases from thermophilic bacteria belonging to the genera *Clostridium*, *Thermoanaerobacter*, and *Thermobacteroides* (26, 27) and from the hyperthermophilic archaea *Pyrococcus furiosus* and *Thermococcus litoralis* (11, 26, 28, 40). The only enzymes which hydrolyze preferentially α-1,6 glycosidic linkages (pullulanases of type I) were found to be produced by *Klebsiella pneumoniae*, *Bacillus acidopullulyticus*, and *Bacillus stearothermophilus* (1, 22, 29, 34). In this paper we report on the purification and characterization of a thermostable pullulanase of type I from an anaerobic thermophilic bacterium which was isolated from a thermal spa in Italy and identified as a species of the genus *Fervidobacterium* (4). Until now, members of the genus *Fervidobacterium* had been isolated only from hot springs in New Zealand and Iceland (20), but it can be assumed that they might be present also in other terrestrial geothermally heated fields.

MATERIALS AND METHODS

Collection sites. Water and mud samples were collected from different thermal spas in the north of Italy (Abano Terme, Calzignano Terme, Montegrotto Terme, Battaglia Terme, and Agnano Terme). Samples were transferred into sterile 50-ml vials that were immediately sealed with butyl rubber stoppers and were transported to the laboratory at 4°C in thermic containers.

Isolation and culture conditions. One or two milliliters of liquid and 5 to 10 g of solid samples were inoculated into 45 ml of prereduced medium in 150-ml serum vials (Wheaton), and the enrichment cultures were incubated anaerobically at 60 and 70°C. The medium was prepared by following the anaerobic techniques described by Balch and Wolfe (6), with N_2 as the gas phase. The complex medium contained the following (per liter): K_2HPO_4, 1.6 g; $NaH_2PO_4 \cdot 2H_2O$, 1.0 g; NH_4Cl, 0.6 g; $(NH_4)_2SO_4$, 0.8 g; $MgSO_4 \cdot 7\ H_2O$, 0.4 g; $CaCl_2 \cdot 2\ H_2O$, 0.2 g; $NaHCO_3$, 1.0 g; $NaWO_4 \cdot H_2O$, 0.033 mg; $NaSeO_3 \cdot H_2O$, 0.026 mg; tryptone, 1.0 g; yeast extract, 1.0 g; peptone, 1.0 g; starch, 5.0 g; trace element solution (7), 1 ml; vitamin solution (7), 2 ml; resazurin, 0.001 g; and $Na_2S \cdot 9H_2O$, 0.5 g.

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Before autoclaving, the pH was adjusted to 6.8 with NaOH, and sterile Na_2S was added just before inoculation.

The isolation of pure cultures from the enrichments was carried out by repeated subculturing at 70°C and by isolation of single colonies on agar plates; the above-described medium amended with 2% agar and 0.2% starch-azure was used. All plating and streaking procedures were carried out in an anaerobic glove box (InterMed). To further ensure the purity of the strains isolated, the strains were restreaked on agar plates and repeatedly isolated after microscopical observations of cell morphology and colony phenotype. For the specific cultivation of isolate Ven5, cultures were grown anaerobically with N_2 in a 10-liter glass fermentor (BCC, Göttingen, Germany) at 70°C. *Escherichia coli* SJ6, used as the host strain for the DNA recombination work and expressing the recombinant pullulanase from isolate Ven5, was grown aerobically at 37°C in 2-liter flasks containing 1 liter of medium with the following: tryptone, 10.0 g; yeast extract, 5.0 g; NaCl, 10.0 g; starch, 10.0 g; and chloramphenicol, 0.006 g.

Light and electron microscopy. A phase-contrast microscope (Zeiss Axioplan) was used for routine examinations and to obtain microphotographs of organisms. Electron microscopy (negative staining and ultrathin section) was performed with a Philips EM 301 transmission electron microscope at calibrated magnifications. Evaluations of electron micrographs were performed on enlarged prints at calibrated magnifications. Negative staining of cells was done with 2% uranyl acetate as described by Valentine et al. (43) and Beuscher et al. (9). The preparation of samples for ultrathin sectioning was done as previously described (15, 41); uranyl acetate and lead citrate were used for poststaining.

DNA isolation and base composition. The DNA was isolated by the method of Marmur (32). After the purified DNA was hydrolyzed with P1 nuclease and dephosphorylated with bovine alkaline phosphatase, the resulting deoxyribonucleosides were analyzed by high-performance liquid chromatography (HPLC) (33). The G+C content of the DNA was calculated from the ratio of deoxyguanosine to deoxythymidine as described by Mesbah et al. (33). Nonmethylated lambda phage DNA (Sigma, Munich, Germany) with a G+C content of 49.858 mol% served as an external standard.

DNA homology. Levels of DNA-DNA homology were determined spectrophotometrically from the renaturation rates as described by DeLey et al. (13) and Huss et al. (21) with a Gilford 2600 spectrophotometer equipped with a 2527 thermoprogrammer and plotter.

16S rDNA analysis. Isolation of genomic DNA, PCR-mediated amplification of 16S ribosomal DNA (rDNA), purification of PCR products, sequencing with the *Taq* Dye-Deoxy Terminator Cycle sequencing kit (Applied Biosystems, Foster City, Calif.), and electrophoresis were performed as described previously (36, 37). The alignment of the 16S rDNA sequence of strain Ven5 was made against representatives of the order *Thermotogales* and other deeply branching members of the domain *Bacteria* for which sequence data are available from the public databases.

Pairwise evolutionary distances were calculated with the correction of Jukes and Cantor (24). The phylogenetic dendrogram was reconstructed by using neighbor joining analysis as described by Saitou and Nei (39).

Characterization of isolate Ven5. The medium used for physiological and biochemical characterization was the same as that used for isolation with the following modifications: $(NH_4)_2SO_4$ was omitted; the amounts of $MgSO_4 \cdot 7H_2O$ and $CaCl_2 \cdot 2H_2O$ were reduced to 0.16 and 0.06 g/liter, respectively; and cysteine-HCl and $Na_2S \cdot 9H_2O$ (each 0.3 g/liter) were added as reducing agents. Substrates were added from separately sterilized, anaerobic stock solutions to a final concentration of 0.5% (wt/vol). Substrate utilization was tested at an incubation temperature of 65°C. Glucose was used as the substrate for ascertaining the growth temperature and sodium chloride requirements and in hydrogen inhibition studies. All tests were performed in 5 ml of medium in 15-ml Hungate tubes. Gelatin hydrolysis was tested on agar plates supplemented with 0.4% gelatin. After incubation for 4 days, plates were flooded with a saturated solution (at 55°C) of Na_2SO_4 in 1 N H_2SO_4.

Enzyme assays. Enzymatic tests were carried out with supernatants after centrifugation of cultures (12,000 × *g*, 40 min) and concentration up to 100-fold with the Amicon system (models 8400 and 8050; Amicon, Lexington, Mass.). Amylolytic and pullulanase activities were determined by measuring the amounts of reducing sugars released during incubations with starch and pullulan, respectively. A 50-μl volume of 1% soluble starch or 1% pullulan was added to 50 μl of 0.1 M sodium phosphate buffer (pH 6.0). A portion (25 or 50 μl) of enzyme solution was added, and the samples were incubated at 70 to 80°C for 30 to 60 min. The reactions were stopped by cooling on ice, and the amounts of reducing sugar released were determined by the dinitrosalicylic acid method (8). Sample blanks were used to correct for nonenzymatic release of reducing sugars. One unit of enzyme activity is defined as the amount of enzyme that releases 1 μmol of reducing sugars (with maltose as the standard) per min under the assay conditions specified. To determine the pH optimum for enzyme activity, the same protocol was used, except for the substitution of 0.12 M universal buffer (10) for sodium acetate buffer, to obtain values from pH 4.0 to 12.0; the enzyme assay was performed at 80°C. The effect of temperature on enzyme thermostability was investigated in supernatants and (for the recombinant *E. coli*) in cell extracts after incubation at different temperatures. In all cases, the incubations were carried out in closed Hungate tubes in order to prevent boiling of the solutions. After various time intervals, samples were withdrawn and clarified by centrifugation, and then enzymatic activities were determined. The protein contents were determined, with bovine serum albumin as the standard (31).

Pullulanase purification. The culture broth (60 liters) containing extracellular enzyme was centrifuged at 12,000 × *g* for 40 min. After concentration of the supernatant (to 1 liter, by a Sartocon II system [Sartorius] equipped with an Ultrasart model [20,000-molecular-weight cutoff {MWCO}] to 200 ml by a 500-ml Amicon chamber with a 10,000-MWCO filter), the sample was dialyzed overnight at 4°C against 20 mM sodium phosphate buffer (pH 7.5). The sample was then applied to a Q-Sepharose Fast Flow column (5 by 40 cm; Pharmacia, Uppsala, Sweden) that was equilibrated with 20 mM sodium phosphate buffer, pH 7.5, at 4°C. Proteins were eluted by a NaCl gradient (0 to 500 mM in 2,000 ml of 20 mM sodium phosphate buffer, pH 7.5) at a flow rate of 5 ml/min, and fractions with a volume of 12.5 ml were collected. The column was then washed until no absorbance at 280 nm was detectable. Fractions were tested for the presence of both amylolytic and pullulanase activity, and samples showing the highest pullulanase activity were concentrated to a final volume of 5.5 ml under nitrogen pressure in a 10-ml Amicon chamber with a 10,000-MWCO membrane. After dialysis against sodium phosphate buffer, up to 200 μl of concentrated protein solution (0.4 mg of protein) was applied to a Superose-12 column (1 by 30 cm) equilibrated with 150 mM NaCl in 20 mM sodium phosphate buffer (pH 7.5). Elution was carried out with the same buffer at a flow rate of 0.2 to 0.6 ml/min. Fractions showing pullulanase activity were concentrated to a final volume of 10 ml and dialyzed against 1.5 M $(NH)_2SO_4$ in 20 mM sodium phosphate buffer (pH 7.5). Up to 2 ml of sample (0.6 mg of protein) was applied to a Phenylsuperose column (HR 5/5; 0.5 by 5 cm) which was equilibrated with 1.5 M $(NH)_2SO_4$. Proteins were eluted with an $(NH)_2SO_4$ gradient (1.5 to 0 M) at a flow rate of 0.3 ml/min. After the enzyme activity was measured, the positive fractions were mixed and concentrated to a final volume of 2.6 ml. This sample was further dialyzed against sodium phosphate buffer before it was subjected to preparative gel electrophoresis. Final purification of the enzyme was performed by preparative gel electrophoresis. The concentrated Phenylsuperose column fraction (2 ml; 0.35 mg of protein/ml) was mixed with 200 μl of bromophenol blue and crystals of sucrose and was applied to an acrylamide gradient gel (5 to 10%) in continuous-elution electrophoresis using a Prep Cell system model 491 (Bio-Rad Laboratories, Hercules, Calif.). Elution of the proteins was carried out in Tris-glycine buffer (pH 8.0) at a constant voltage of 150 V at 4°C for 24 h.

For the determination of the molecular masses of proteins the following standard proteins were used: thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), lactate dehydrogenase (140 kDa), albumin (67 kDa), and ovalbumin (45 kDa).

Characterization of hydrolysis products. Enzyme samples were dialyzed overnight at 4°C in 50 mM sodium acetate buffer (pH 5.5) and were incubated at 70°C with 0.5% (wt/vol) polysaccharides (starch, pullulan, glycogen, amylose, amylopectin, and dextran) or with a mixture of oligosaccharides for 0, 2, 4, 12, 24, 48, or 72 h. After incubation, samples were clarified by centrifugation and the sugars were analyzed by separation on an HPLC Aminex HPX-42A column (Bio-Rad) (27, 40).

Gel electrophoresis. The patterns of extra- and intracellular proteins were determined by sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis after boiling the samples for 3 to 5 min as described by Laemmli (30). Silver staining of protein bands was carried out as described by Heukeshoven and Dernik (18). Protein bands possessing amylolytic and/or pullulanase activities were detected on native gels (23) and polyacrylamide gradient gels (5 to 27.5%) as described by Koch et al. (28).

RESULTS

Isolation of bacteria. After enrichment of the water and mud samples in the complex medium amended with starch and several subculturing steps at 70°C, a number of anaerobic strains were selected for further characterization. After serial dilutions and streaking on agar plates containing starch-azure, numerous positive colonies were isolated and restreaked to ensure the purity of the isolates. Ten strains were then screened for the presence of amylolytic and pullulanase activity.

Characterization of isolate Ven5. Morphological analysis of the new isolate showed that strain Ven5 was a straight rod with pointed ends, occurring singly or in pairs or sometimes in short chains (Fig. 1). Occasionally, swellings in the middle of elongated cells were observed in older cultures, but spores were never detected. Cells measured 0.6 to 1.1 μm in width and 2 to 5 μm in length and were generally larger in sugar-containing media and smaller in sugar-free yeast extract medium. They were motile in young cultures and stained gram negative. The majority of the cells in liquid cultures possessed terminal spheroids of different sizes easily detectable by phase-contrast and

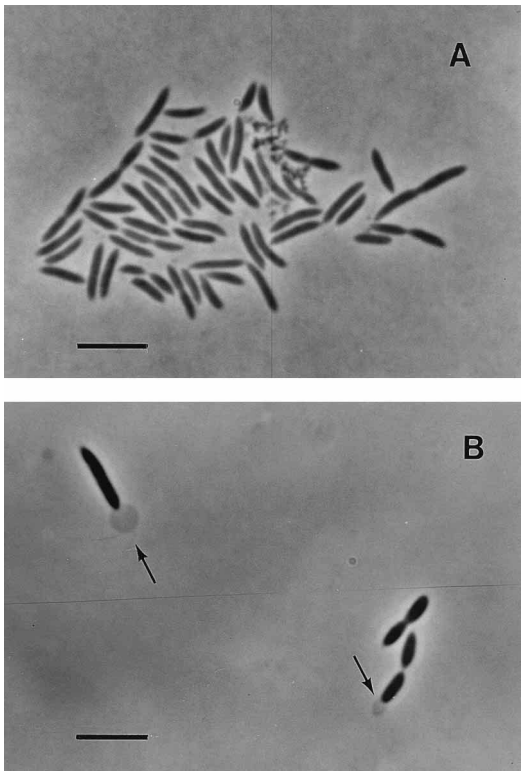

FIG. 1. Phase-contrast micrographs of strain Ven5. (A) Cells grown on sugar-free medium for 10 h. Spheroids are not visible because of refraction changes caused by the agar-covered microslides used. (B) Cells after 33 h of growth in glucose-containing medium showing spheroids (arrows). Bar, 5 μm.

electron microscopy (Fig. 1 and 2). At the end of exponential growth, free spheroids as well as spheroids enveloping one or two cells were observed. The addition of lysozyme (5 mg/ml) caused the cells to round up within a few minutes, indicating the presence of a murein cell wall.

Strain Ven5 grew within a temperature range of 55 to 80°C with an optimum at 65 to 70°C. The optimum pH for growth was at around 7.0. Best growth was observed at low NaCl concentrations of 0.1 to 0.6%. Growth was strongly inhibited by 2.1% NaCl, and no growth occurred at 4.1% NaCl.

Under optimal conditions (65°C; pH 6.8) and continuous gassing of the culture with 80% N_2–20% CO_2 in starch-containing medium, the strain grew with a doubling time of 200 min and a maximum optical density at 528 nm of 1.2 was reached. During starch fermentation the pH of the medium decreased from 6.8 to 5.4. The fermentation products formed after growth on glucose were acetate (3.1 mM), lactate (1.5 mM), succinate (0.4 mM), H_2, and CO_2. Neither ethanol nor any other organic product was detected.

Strain Ven5 was saccharolytic and fermented cellobiose, esculin, galactose, glucose, glycogen, lactose, maltose, mannose, ribose, salicin, starch, sucrose, trehalose, and pullulan. Amygdalin, arabinose, fructose, inositol, inulin, mannitol, melezitose, melibiose, raffinose, rhamnose, xylose, cellulose, glycerol, and pyruvate were not utilized. Gelatin was hydrolyzed, but casein and milk were not attacked. Tests for sulfate and nitrate reduction were negative. Yeast extract was required for growth with carbohydrates. Little growth occurred with 0.3% peptone or Casamino Acids.

The growth of strain Ven5 was stimulated by elemental sulfur and L-cystine but was totally inhibited by sulfite (0.2 g of sodium sulfite added per liter of medium). After 22 h of growth, a twofold-higher cell density was reached in media supplemented with 0.5% (wt/vol) sulfur or 3.7 mg of L-cystine per ml. Growth which was inhibited by hydrogen was restored by the addition of sulfur to the medium, but cystine was less effective (Fig. 3).

According to the gas chromatographic analysis, long-chain, C_{30} to C_{34} α,ω-dicarboxylic acids, characteristic of members of the order *Thermotogales*, with 81.7% 15,16-triacontane-1,30-dioic fatty acid and 10.4% 15-methylhentriacontane-1,31-dioic fatty acid as the main components, were detected in cells of strain Ven5 (20).

The DNA base composition of strain Ven5 was 40.6 mol% G+C, as determined by the HPLC method (33). By DNA-DNA hybridization, strain Ven5 exhibited a low level of DNA homology with *Fervidobacterium nodosum* DSM 5306 (31% ± 2%) and *Fervidobacterium islandicum* DSM 5733 (36% ± 3%). Almost the complete 16S rDNA sequence (>95% of the *E. coli* 16S rDNA sequence) of strain Ven5 was determined. The phylogenetic dendrogram (data not shown) indicates that strain Ven5 belongs to the order *Thermotogales* and shows highest 16S rDNA sequence similarity to *Fervidobacterium pennavorans*, *F. islandicum*, and *F. nodosum*, with similarities of 99.0, 98.6, and 96.0%, respectively (16).

Enzyme characterization. After cultivation of the selected strains in 1-liter batch cultures, the supernatant was collected by centrifugation and concentrated up to 100-fold. Samples were then investigated for the presence of amylolytic as well as pullulanase activity; the influence of pH and temperature on enzymatic activities was also assessed. All strains produced pullulanase in the range of 10 to 90 U/liter except for one isolate (Ven4) that showed a much higher yield (280 U/liter). The pH optimum for activity ranged usually from 5.5 to 6.0, and temperature optimum ranged from 55 to 85°C.

Partial purification of pullulanases. All the strains investigated produced both pullulanase and amylolytic activities. For this reason, samples were examined by anion-exchange chromatography in order to detect the presence of specific pullulanases. After NaCl gradient elution of samples, both amylolytic and pullulanase activities were always detected in the same fractions, indicating that pullulanase of type II (active on both α-1,6 and α-1,4 linkages) was involved. In the supernatant of only one strain (Ven5) was the pullulanase activity 10-fold higher than the amylolytic activity. Based on these results, isolate Ven5 was selected further studies.

Purification of pullulanase specific to α-1,6 glycosidic linkages. After repeated cultivation of isolate Ven5 in the 10-liter glass fermentor, 60 liters of culture broth was concentrated to a final volume of 200 ml. By applying anion-exchange chromatography, gel permeation chromatography, and hydrophobic chromatography, a specific pullulanase was purified 65-fold with a yield of 22% and a specific activity of 110 U/mg (Table 1 and Fig. 4). In some cases, the purified enzyme was also subjected to preparative gel electrophoresis (Fig. 4). The native enzyme has a molecular mass of 240,000 Da. After denaturation and sodium dodecyl sulfate-gel electrophoresis, it was found that the enzyme appeared to be composed of three identical subunits, each with a molecular mass of 77,600 Da.

Characterization of pullulanase. The enzyme was active between 45 and 100°C and from pH 4.0 to 8.0. Maximal relative activity was detected at pH 6.0 and 85°C (Fig. 5), and at 90°C, 70% activity was still detectable. The thermostability of the enzyme was tested at 70, 80, 90, and 100°C in the absence of any substrate and at 80°C in the presence of starch, pullulan, or

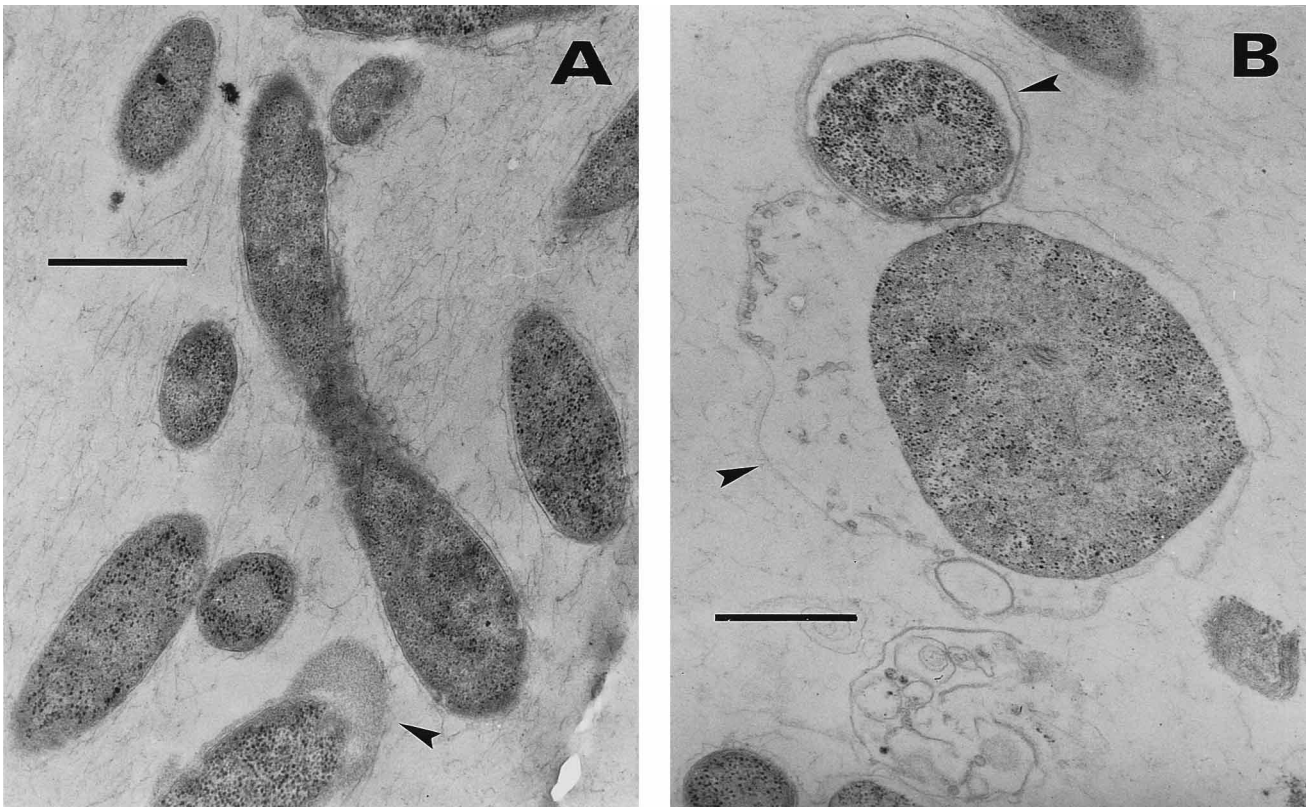

FIG. 2. Electron micrographs of *Fervidobacterium* sp. strain Ven5 showing a dividing cell (A) and a thin section of a cell surrounded by an outer sheath-like structure (B). Arrowheads show spheroid structures. Bar, 0.5 μm (A) or 0.2 μm (B).

an oligosaccharide mixture. The half-life of pullulanase was around 5 min at 90°C and 2 h at 80°C (Fig. 6a). In the presence of starch, pullulan, or oligosaccharides, the enzyme thermostability at 80°C was significantly enhanced; the half-life was over 50 h with starch and around 10 to 15 h with pullulan or other oligosaccharides (Fig. 6b).

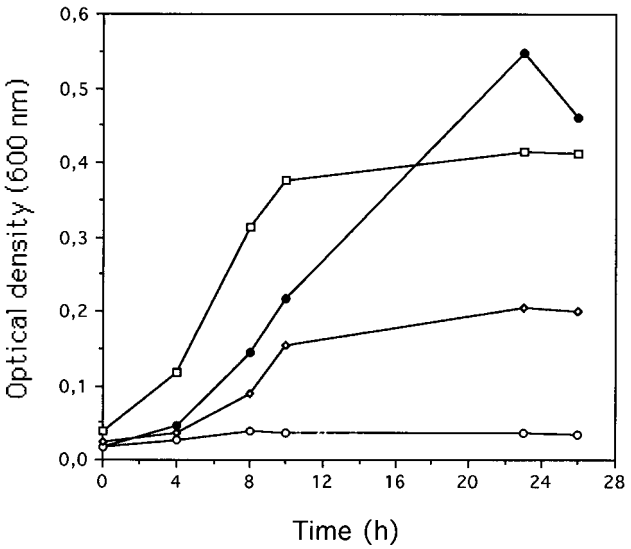

FIG. 3. Overcoming of the hydrogen-induced growth inhibition of strain Ven5 by elemental sulfur and L-cystine. ●, no sulfur, N_2 (control); ○, no sulfur, H_2; □, 0.25 g of sulfur, H_2; ◇, 12 mg of L-cystine, H_2. The inoculated culture tubes (Hungate tubes with 5 ml of medium) were pressurized to 2×10^5 Pa of hydrogen overpressure, except the control tube. Optical densities were measured at 600 nm directly in the culture tubes with an Ultrospec II spectrophotometer (LKB Pharmacia, Uppsala, Sweden). Each point represents the average of measurements for two tubes.

Enzyme specificity and hydrolysis products. The incubation of pullulanase from isolate Ven5 with pullulan, glycogen, starch, amylose, amylopectin, dextran, or a mixture of oligosaccharides (0.5% [wt/vol] each in 20 mM Na phosphate buffer [pH 6.0]) at 85°C for different times showed that maximal relative activity was obtained with pullulan (100%) and that 41% activity was obtained with a mixture of oligosaccharides. The incubation of pullulan with the purified enzyme caused its conversion to DP_3, DP_6, DP_9 (DP, degree of polymerization), and long-chain oligosaccharides. The final product after 4 h of incubation was DP_3 exclusively and not panose or isopanose. This was confirmed by treatment of the hydrolysis product with α-glucosidase from yeast, which attacks exclusively α-1,4 glycosidic linkages in oligosaccharides. By HPLC analysis it was

TABLE 1. Purification of pullulanase from *Fervidobacterium* sp. strain Ven5[a]

Step[b]	Fraction vol (ml)	Total protein (mg)	Total activity (U)	Sp act (U/mg)	Yield (%)	Purification (fold)
Concentrated culture filtrate	200	130	226	1.7	100	
Q-Sepharose	5.5	13.3	223	16.7	99	10
Superose-12	10.5	2.7	176	65.1	78	38
Phenylsuperose	2.6	0.45	49.4	109.7	22	65

[a] Cells were cultivated at 70°C in a 10-liter fermentor, and a total of 60 liters of culture broth was concentrated to a final volume of 200 ml.

[b] Q-Sepharose, anion-exchange chromatography; Superose-12, gel permeation chromatography; Phenylsuperose, hydrophobic chromatography.

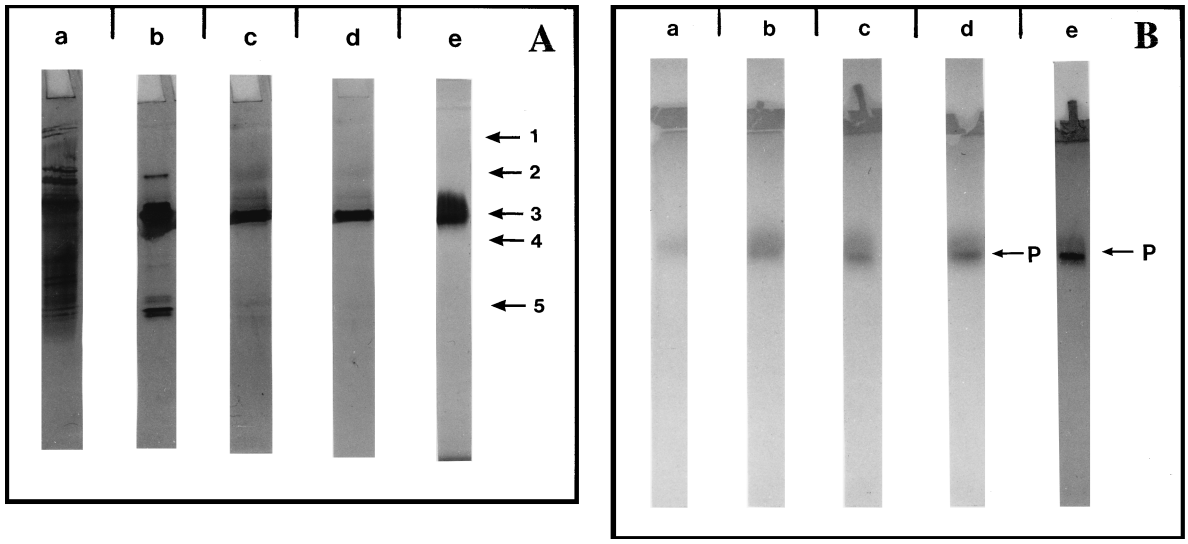

FIG. 4. Polyacrylamide gradient gel (5 to 27.5%) electrophoresis of purified and crude pullulanase from strain Ven5 after silver staining (A) and activity staining (B). Lanes: a, cell-free culture supernatant (29 μg); b, Q-Sepharose column fraction (28 μg); c, Superose-12 column fraction (16 μg); d, Phenylsuperose column fraction (8 μg); e, preparative gel electrophoresis fraction (2 μg). Molecular markers: 1, thyroglobulin (669 kDa); 2, ferritin (440 kDa); 3, catalase (232 kDa); 4, lactate dehydrogenase (140 kDa); 5, serum albumin (67 kDa); P, pullulanase activity.

evident that maltotriose and not panose or isopanose could be converted by α-glucosidase to glucose. The action of pullulanase on amylose was very slow, demonstrating its low affinity for α-1,4 glycosidic linkages. After incubation for 72 h, extremely low concentrations of DP_6, DP_5, and DP_2 were measured. The high degree of specificity of pullulanase for the α-1,6 linkages is showed in Fig. 7. Furthermore, the action of pullulanase from isolate Ven5 on an oligosaccharide mixture produced a pattern very similar to the one obtained with the pullulanase (pullulanase of type I) of *K. pneumoniae* on the same substrate (Fig. 7).

DISCUSSION

The alteration of different parameters during the manufacture of sugars from starch and the requirement for several time-consuming steps cause many handicaps in the starch industry. For this reason, numerous studies have been recently carried out to improve the starch conversion process and to lower the cost of sugar syrup production by finding new efficient and suitable enzymes (2, 3). The fact that amylases and pullulanases from thermophiles are all active at the same pH and temperature range makes these organisms suitable candidates for industrial applications. In this respect, the enzymes from anaerobic thermophilic bacteria and archaea are of special interest, as they are extremely thermostable and metal independent (3, 13, 27, 28, 29, 42).

For the replacement of the traditionally used enzymes it is necessary to find more thermostable enzymes with unique properties. The production of pullulanase of type II (also named amylopullulanase), which attacks simultaneously α-1,4 as well α-1,6 linkages, may certainly enhance the starch saccharification process, but finding highly thermostable type I pullulanases, which attack exclusively α-1,6 linkages, would be even more useful for industrial applications (38). The latter have been described only in a few microbes such as *K. pneumoniae*, *B. acidopullulyticus*, and *B. stearothermophilus* (1, 20, 31, 40).

This is the first report on the presence of a specific debranching enzyme (pullulanase type I) in an anaerobic bacterium. The purified enzyme produces exclusively maltotriose after incubation with pullulan and attacks preferentially the α-1,6 linkages of branched polysaccharides. The native enzyme was showed to be a homotrimer with maximal activity at 85°C and pH 6.0. Preliminary results have shown that the gene

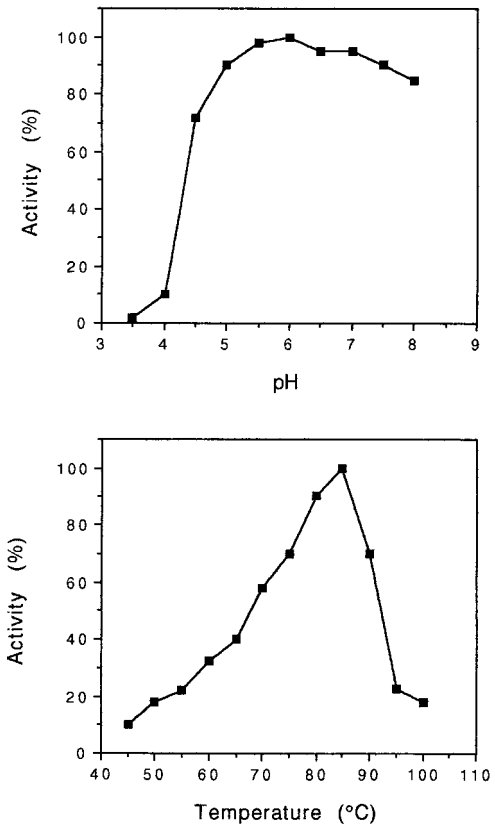

FIG. 5. Effect of temperature and pH on the relative pullulanase activity of isolate Ven5. The relative pullulanase activity of the purified enzyme was determined at various temperatures and pH values.

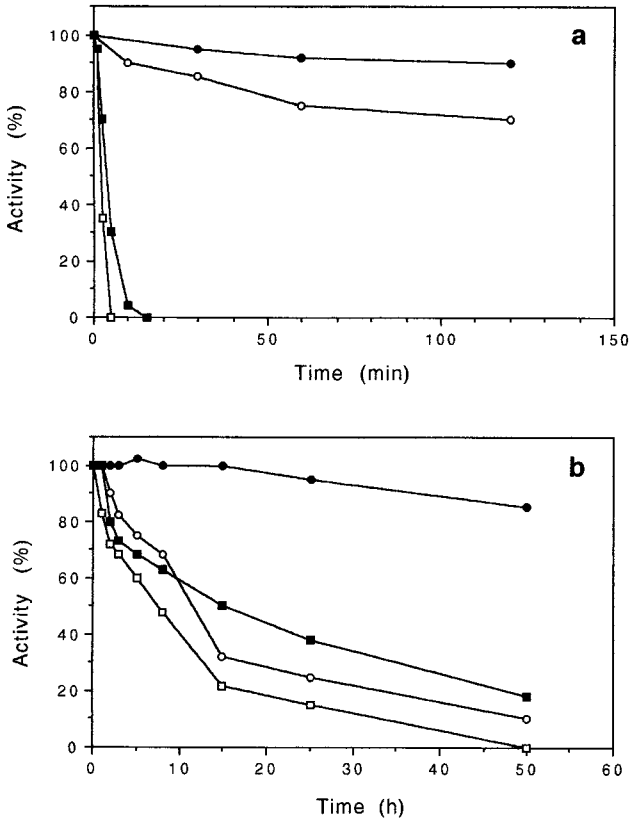

FIG. 6. Thermostability of the purified pullulanase (Phenylsuperose fraction) from isolate Ven5. (a) Relative activity determined after incubation at 70°C (●), 80°C (○), 90°C (■), and 100°C (□) for the indicated times; (b) relative activity after incubation at 80°C in the presence of starch (●), an oligosaccharide mixture (■), or pullulan (○) or with no addition (□). The enzyme thermostability was assayed in 20 mM sodium phosphate buffer, pH 6.0; the concentration of substrates was 1% (wt/vol).

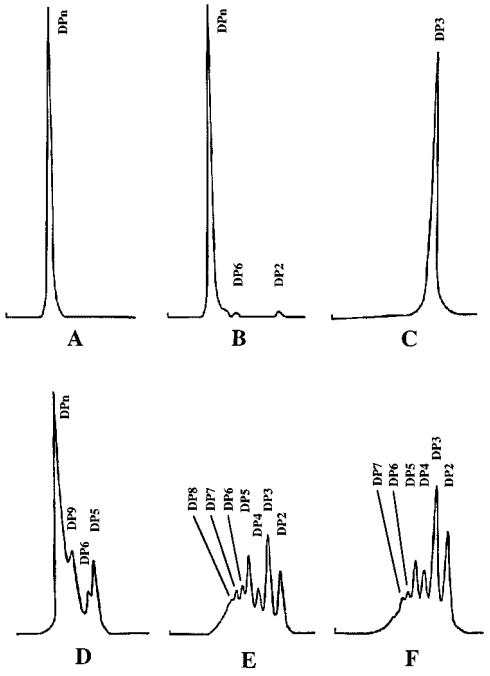

FIG. 7. HPLC analysis of hydrolysis products after incubation of the purified pullulanase from isolate Ven5 at 70°C in the presence of different substrates. (A) Amylose and pullulan not incubated (time zero); (B) incubation of pullulanase from isolate Ven5 for 4 h in the presence of amylose; (C) incubation of pullulanase from isolate Ven5 for 4 h in the presence of pullulan; (D) mixture of oligosaccharides not incubated (time zero); (E) incubation of pullulanase from isolate Ven5 for 10 h in the presence of an oligosaccharide mixture; (F) incubation of pullulanase from *K. pneumoniae* for 10 h in the presence of an oligosaccharide mixture.

encoding pullulanase was cloned and expressed in *E. coli*. Interestingly, the cloned pullulanase possesses properties similar to those of the native enzyme. Previously, the pullulanase gene of *K. pneumoniae* W70 was the only pullulanase type I gene reported to have been cloned into the chromosome of *E. coli* (14). The finding of this type of pullulanase in a thermophilic anaerobic bacterium and its successful introduction into *E. coli* suggest the possible application of this enzyme in the industrial saccharification process and its wider distribution in anaerobic thermophiles.

The strictly anaerobic thermophilic isolate strain Ven5 was assigned to the genus *Fervidobacterium* on the basis of morphological and physiological properties. 16S rDNA sequence comparisons confirmed this conclusion. Strain Ven5 showed 99.0% sequence homology with *F. pennavorans* and 98.0% homology with *F. islandicum* but only 96.0% similarity with *F. nodosum*. Its DNA moles percent G+C content (40.6%) was the same as that of *F. pennavorans* (16) and *F. islandicum* (40 to 41%) (19) but differed by 7% from that of *F. nodosum* (33.7%) (35). Similar to *F. pennavorans* and unlike *F. islandicum* and *F. nodosum*, the inhibition of the growth of Ven5 by hydrogen was overcome by elemental sulfur. Besides acetate and lactate, strain Ven5 produced succinate as a fermentation product but not ethanol, which is produced by the other three *Fervidobacterium* species. In contrast to *F. islandicum*, strain Ven5 was not able to ferment pyruvate, raffinose, or cellulose. The type strain, Ven5, isolated from mud samples at Abano Terme, Italy, was deposited as DSM 9078 in the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany, and is designated *F. pennavorans* Ven5.

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