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Some Properties of a Thermostable β-Xylosidase from *Rhodothermus marinus*

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ABSTRACT

The extracellular β-xylosidase (EC 3.2.1.37) excreted by the thermophilic eubacterium *Rhodothermus marinus* when grown on xylan has been investigated. The enzyme has been partially purified by ultrafiltration and gel filtration, and some of its characteristics are presented. *Rhodothermus marinus* grew on xylan with $\mu max = 0.4\ h^{-1}$ and the β-xylosidase activity was 50 nkat/mL after 24 h in a batch fermentation. The β-xylosidase activity had a half-life of more than 1 h at 90°C and of 14 h at 85°C. At 80°C, 80% of the initial activity remained after 24 h. The initial activity increased with increasing temperature, showing maximal activity at 90°C. The β-xylosidase had a pH-optimum of 6 and was stable in the range between pH 5 and 9. At pH 10 and 11, 82 and 66%, respectively, of the initial activity remained after 24 h when incubated at 65°C. The molecular weight was estimated to be 169,000 dalton by gelfiltration.

Index Entries: *Rhodothermus marinus*; β-xylosidase; xylan degradation; thermophilic microorganism; thermostable enzyme.

INTRODUCTION

Xylan is the second most abundant renewable polysaccharide in nature and is one of the major components of hardwood hemicelluloses. Hemicellulosic compounds of wood are the most abundant next to cellulose and lignin. Xylan appears to be a major interface between lignin and other carbohydrates. Xylan consists of both linear 1,4-β-linked chains of D-xylose and of branched heteropolysaccharides with 4-*o*-methyl-D-glucoronic acid

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and L-arabinosylic side chains. β-Xylans have a varying degree of polymerization and the frequency and composition of branches are dependent on their source and the method of their isolation. For the complete degradation of xylan to xylose residues, several groups of enzymes are required: endo 1,4-β-xylanase, β-xylosidase, α-L-arabinofuranosidase, α-glucoronisidase, and acetylesterase (*1–3*). The xylanases randomly break internal xylosidic linkages in the backbone of polymeric xylan producing xylooligosaccharides of varying chainlengths. The β-xylosidase release xylosylmonomeres by endwise attack of these xylooligosaccharides, and are thus necessary in the production of monomeric xylose frrom xylan.

Enzymes produced by thermophiles are generally thermostable and also have a general robustness and resistance to denaturing agents and organic solvents. Stability is a highly desirable characteristic of industrial enzymes because it permits enzyme recycling and simpler handling and storage conditions. Therefore thermophilic organisms are advantageous as sources of industrial enzymes (*4–8*).

Xylanolytic enzymes are used in the pulp industry. By hydrolysing the xylose–xylose bonds and arabinose–xylose bonds a greater release of lignin occurs during bleaching and the amount of chlorine needed would decrease. Bachman and Mc Carthy (*9*) have reported that the enzymatic degradation of xylan becomes more efficient when a mixture of xylanolytic enzymes, including the β-xylosidase, is used. The use of thermostable and alkaline tolerant enzymes are advantageous in the pulp and paper industry (*10*). One way of dealing with agricultural and wood residues is the bioconversion of lignocellulosic materials to fermentable sugars. Converting lignocellulose to fermentative products probably requires maximum hydrolysis of complex substrates to yield monomeric residues. This requires the action of β-xylosidase. Selected fermentative organisms may convert hydrolyzed products from lignocellulose into liquid fuel, single cell proteins, solvents, and other chemical products. Other products of industrial interest derived from xylose are xylitol, polyurethane, nylon, resins, polymers, and polyacrylate (*1*).

Several β-xylosidases from different organisms have been characterized and found thermostable (Table 1). We have found that the β-xylosidase from *Rhodothermus marinus* is very thermostable and it is probably the most thermostable found so far. The temperature stability, temperature optimum, pH-stability, and pH-optimum of this extracellular β-xylosidase are reported in this work.

MATERIALS AND METHODS

Organism and Chemicals

Rhodothermus marinus is a thermophilic, reddish colored aerobic heterotrophic bacterium, isolated from submarine alkaline hot springs in Iceland,

Table 1
Properties of Xylosidases from Different Organisms

Organism	Temp. stab temp; $T_{1/2}$	pH opt	Mw, kDa	Reference
Thermoanaerobacter ethanolicus	86°C; 15 min	5	165	(*16*)
Thermomonospora fusca	65°C; 8 h 70°C; 1.5 h	5–9	168	(*17*)
Gene from *Caldocellum saccharolyticum* expr. in *E. coli*	80°C; 40 min			(*18*)
Bacillus stearothermophilus	70°C; 1 h	6	150	(*19*)
Gene from an anaerobic, thermophilic bacteria expr. in *E. coli*	65°C; 4.85 h 70°C; 40 min	5.7		(*20*)
Rhodothermus marinus	85°C; 14 h 90°C; >1 h	6	169	This report

collected from IceTec, Reykjavik, Iceland (ITI 376), also deposited at DSM (4252). *Rhodothermus marinus* has optimal growth at 65°C and pH 7. It requires NaCl for growth (*11*). Yeast extract (cat. no. Y4000), *o*-nitrophenyl-β-D-xylopyranoside, *o*-NPX (cat. no. N3629) and *o*-nitrophenol (cat. no. N9256) were purchased from Sigma Chemicals Co. (St. Louis, MO); birch xylan (no. 7500) from Roth, Karlsruhe, Germany; tryptone and Bacto agar from Difco (Detroit, MI); and High Molecular Gelfiltration Kit from Pharmacia LKB (Uppsala, Sweden). Other chemicals were of *pro analysi* quality.

Culture Conditions

The strain was grown in a complex, modified M 162 (*12*) growth medium consisting of (g/L): Tryptone 2.5; yeast extract 2.5. A base solution was added (100 mL/L) containing (g/L): nitriloacetic acid (titriplex I) 1.0; NaOH 0.2; $CaSO_4 \cdot 2\ H_2O$ 0.4; $MgCl_2 \cdot 6\ H_2O$ 2.0. Fe-citrate (0.05 *M*) 5 mL was also added. A buffer solution was added (100 mL/L) containing (g/L): KH_2PO_4 5.44; $Na_2HPO_4 \cdot 2\ H_2O$ 21.40. NaCl 2% (w/v) and birch xylan 0.5% (w/v) were added. The growth medium was sterilized at 121°C, 20 min (Fe-citrate solution was autoclaved separately) and the pH was adjusted to 7.1 with 1*M* NaOH. Difco agar 2.8% (w/v) was used to solidify the medium when required. In shake-flask cultivations, *Rhodothermus marinus* was grown at 65°C in 100 mL growth medium in 1 L baffled Erlehnmeyer flasks, in a glycerol bath with rotary shaking. In batch fermentations *Rhodothermus marinus* was cultivated at 65°C in a Chemoferm FLC-B-3 fermentor with a working volume of 2.5 L, inoculated from an o/n culture in a shake flask grown on the same substrate. The inoculum volume was 10%. The

pH was maintained at 7.1 by titration with 1*M* H_2SO_4 and 1*M* NaOH. The air supply was 1 vvm and the stirrer speed 300–400 rpm.

Analyses

Growth was followed by measuring the optical density at 620 nm. Cell dry weight determinations were carried out in precalibrated centrifuge tubes by weighing the pellets obtained from 5 mL culture samples, centrifuged in a Wifug laboratory centrifuge at 6000 rpm for 10 min, and drying for 3 h in 105°C. Samples taken during the cultivations were centrifuged as above and the supernatants filtered (cellulose acetate filter, 0.45 μm) for use as crude enzyme solutions in assays of enzymatic activities.

β-Xylosidase Assay

The enzyme activity was determined using *o*-nitrophenyl-β-D-xylopyranoside (*o*-NPX) as substrate. The substrate, 0.2% (w/v), was dissolved in 25 m*M* sodium phosphate buffer, pH 7.1. *o*-Nitrophenol was used as standard. The β-xylosidase activity was determined by measuring the amount of *o*-nitrophenol liberated from the substrate *o*-NPX. The substrate solution, 1.7 mL, was preheated to 65°C and incubated with 300 μL of the crude enzyme or standard solution (*o*-nitrophenol) at 65°C for 15 min. The reaction was stopped by adding 3 mL of 1*M* Na_2CO_3. Absorbance was read at 400 nm and the β-xylosidase activity expressed in katals. In every enzymatic assay a blank has been used to eliminate the substrate dependency on temperature and pH.

Protein Determination

The protein concentrations were determined according to Lowry (*13*) with bovine serum albumin as standard.

Molecular Weight Determination of β-Xylosidase from *R. marinus*

Approximately 1 L of the crude enzyme solution was concentrated by ultrafiltration in a 5 kDa Filtron Omega Membrane to a volume of 200 mL. The proteins in the concentrated enzyme solution were then separated by gelfiltration. The separation was carried out on a Sephadex G200 (Pharmacia LKB) column (diameter 1.1 cm, height 42 cm, with a total bed volume of 39.9 mL) equilibrated with 25 m*M* phosphate buffer, pH 7.1. The proteins in the High Molecular Gelfiltration Kit (Pharmacia LKB) were used as standards. The flow rate through the column was 3 mL/h (3.15 mL/cm² h) using 25 m*M* sodium phosphate buffer, pH 7.1, as eluant.

Characterization of β-Xylosidase from *R. marinus*

pH-Relationships

Crude enzyme solutions from a shake flask cultivation were used in the following experiments. The pH optimum was determined by diluting the crude enzyme samples and standards in different buffer solutions with pH ranging from 4 to 11 (citrate-phosphate pH 4–7, phosphate pH 6–8, Tris-HCl pH 7–9, and glycin-NaOH pH 9–11). The substrate was dissolved in the same buffers and the β-xylosidase assay was carried out as previously described. Both 100 m*M* and 25 m*M* buffer solutions were used. The pH-stabilities were determined by adding 1 mL of the crude enzyme to 9 mL of 25 m*M* buffer solutions, preheated to 65°C, with pH ranging from 4 to 11. The enzyme solutions were incubated at 65°C. Samples withdrawn were assayed for β-xylosidase activity.

Temperature Relationships

Crude enzyme solutions from a shake flask cultivation were used in studying the effect of temperature on β-xylosidase activity. The optimum temperature was determined by running the xylosidase assay at pH 6 at different temperatures (60–100°C). The temperature stabilities were determined by incubating the crude enzyme solutions at different temperatures (60–90°C). Samples withdrawn were assayed for β-xylosidase activity at 65°C and pH 7.1.

RESULTS AND DISCUSSION

Growth Conditions and Enzyme Production

When *Rhodothermus marinus* was grown on xylan in a batch fermentation, μ_{max} was determined to be 0.4 h^{-1}. The cell dry weight was 5.7 g/L after 10 h of cultivation and 4.3 g/L after 24 h. The optical densities did not show a corresponding decrease during the stationary phase. The β-xylosidase from *Rhodothermus marinus* is an extracellular enzyme and the enzyme production is coupled to growth. The β-xylosidase activity reached its maximum value after 24 h: 50 nkat/mL (Fig. 1). The enzyme activity increased slightly during stationary phase. This might be owing to a release of enzymes loosely attached to the cells (*14*). The bacteria tend to form aggregates when the cell density increased, this might be due to a slime capsule formed in carbohydrate rich medium (*11*).

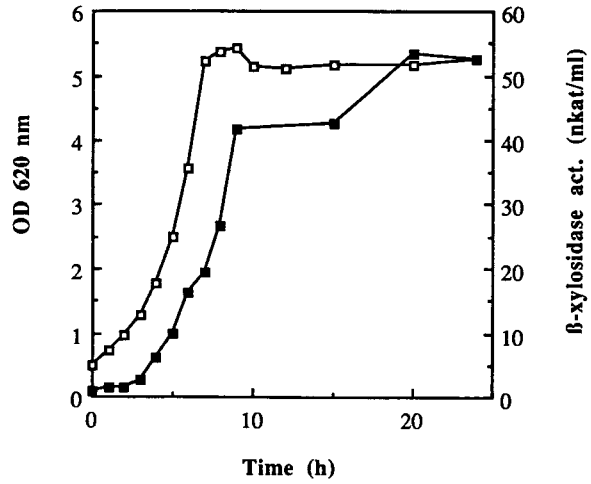

Fig. 1. Growth curve and β-xylosidase activity during a batch fermentation of *R. marinus* grown on xylan. (□) OD (620 nm), (■) β-xylosidase activity (nkat/mL).

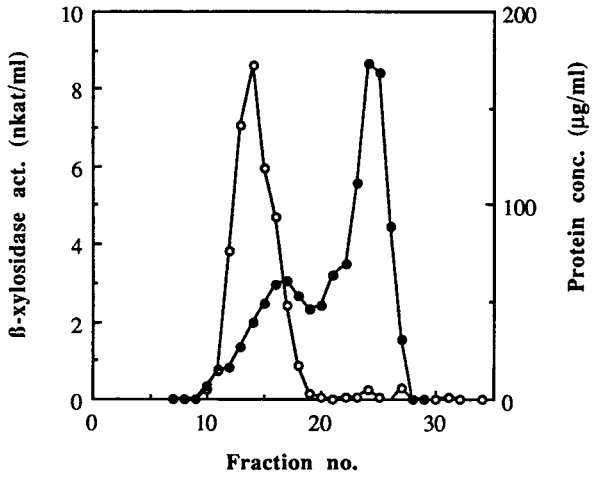

Fig. 2. β-Xylosidase activity (nkat/mL) and protein concentration (μg/mL) in the gel filtration (Sephadex G200) fractions. Flowrate 3 mL/h, each fraction having a volume of 1.35 mL. (○) β-Xylosidase activity (nkat/mL), (●) Protein concentration (μg/mL).

Molecular Weight Determination of β-Xylosidase from *R. marinus*

After ultrafiltration, the four-fold concentration enzyme solution was separated by gelfiltration on a Sephadex G200 column (Fig. 2). The molecular weight was estimated to be about 169,000 dalton and is similar of those reported for β-xylosidases from other organisms (Table 1).

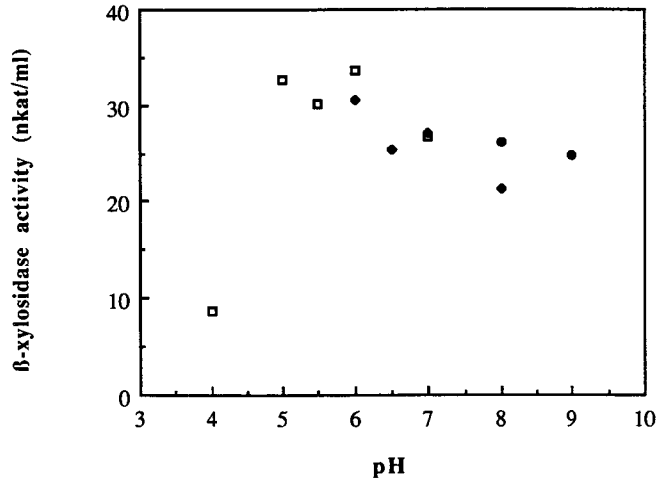

Fig. 3. Initial activity of β-xylosidase from *Rhodothermus marinus* as a function of pH in 25 m*M* buffers. (□) citrate-phosphate, (◆) phosphate, (●) Tris-HCl.

Characteristics of β-Xylosidase from *R. marinus*

pH-Relationships

The β-xylosidase activity exhibited maximal activity at pH 6 in 25 m*M* buffer (Fig. 3). At a buffer ionic strength of 100 m*M* the maximum β-xylosidase activity was obtained at pH 5 (data not shown). The β-xylosidase activity might vary with varying phosphate concentrations (*15*). With increasing pH, the β-xylosidase activity decreased, still showing 70% of its maximal activity at pH 9.0 (Fig. 3). The β-xylosidase from *Rhodothermus marinus* was stable for 24 h in the pH range 5–9 (Fig. 4). At pH 10 the enzyme was stable for 10 h; the activity then decreased to 82% of its initial value after 24 h. At pH 11 the activity decreased to 66% of its initial value after 24 h. The results obtained with Tris-HCl buffer were slightly lower than those obtained when the other buffer solutions were used. Kersters-Hilderson et al. (*15*) have reported inhibitory effects of Tris on the β-xylosidase activity.

Temperature Relationships

The initial β-xylosidase activity increased as the assay temperature increased, not showing any optimum at all (Fig. 5). At 100°C the substrate decomposes to fast and the β-xylosidase activity could not be measured. At 90°C the initial activity was three times higher than the activity at 65°C. β-Xylosidase from *Rhodothermus marinus* showed good temperature stability (Fig. 6), $T_{1/2}$ at 90°C was more than 1 h and $T_{1/2}$ at 85°C was 14 h. At 80°C, 80% of the initial β-xylosidase activity remained even after 24 h. At 75°C, no decrease in activity was found after 24 h.

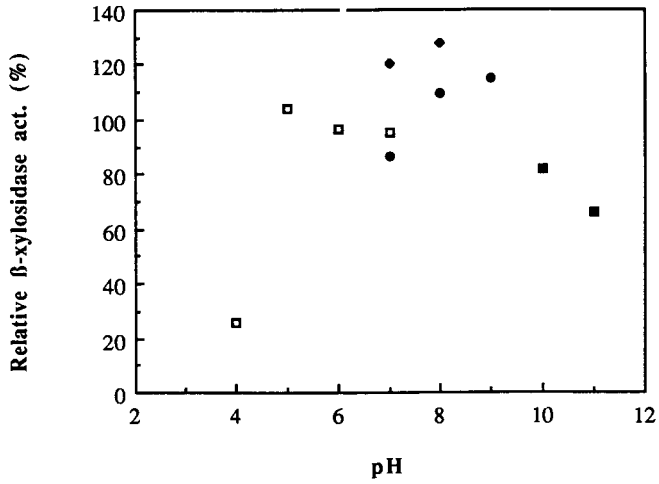

Fig. 4. Stability of β-xylosidase from *Rhodothermus marinus* as a function of pH, incubated for 24 h in 25 m*M* buffers at 65°C. Relative β-xylosidase activity in percent of initial activity at each pH. (□) citrate-phosphate, (◆) phosphate, (●) Tris-HCl, (■) glycin-NaOH.

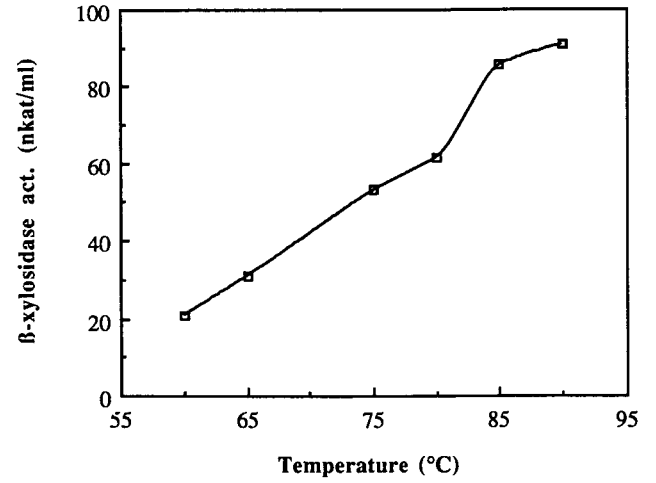

Fig. 5. Initial activity of β-xylosidase from *Rhodothermus marinus* as a function of temperature in 25 m*M* phosphate buffer.

The β-xylosidase produced by *Rhodothermus marinus* is the most thermostable reported so far (Table 1). The β-xylosidase is also stable and has high initial activity at pH values in the alkaline range. This enzyme should be suitable for industrial applications. The β-xylosidase from *Rhodothermus marinus* might be useful in bioorganic synthesis since it may be expected to have good stability in organic solvents. The β-xylosidase activity at temperatures above 90°C and at pH above 10 could not be measured because of instability of the substrate (*o*-NPX) used in the β-xylosidase

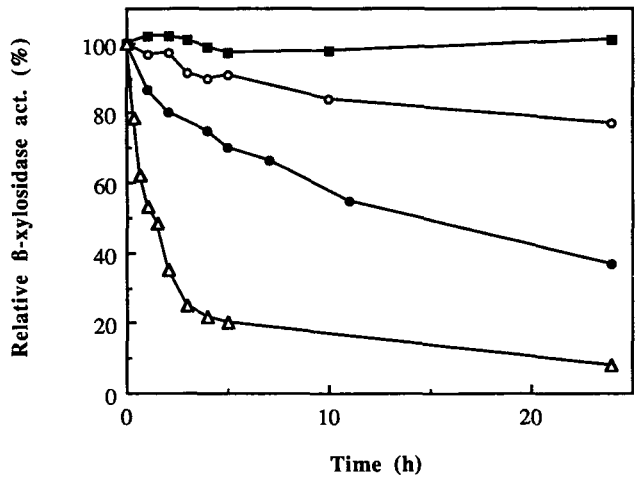

Fig. 6. Stability of β-xylosidase from *Rhodothermus marinus* as a function of temperature in 25 m*M* phosphate buffer. Relative β-xylosidase activity in percent of initial activity at different temperatures. (■) 75°C, (○) 80°C, (●) 85°C, (Δ) 90°C.

assay. It is also worth notifying that the enzyme investigated is from a crude enzyme solution. The β-xylosidase might show somewhat different properties if purified. Table 1 shows the properties of β-xylosidases from different organisms, but although the temperature stabilities are different, the pH optima and their sizes are similar. It would be of interest to compare the genes coding for the heat stable enzymes to try to reveal the structural differences responsible for the heat stability, of the proteins.

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