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Characterization of Novel Neopullulanase from *Bacillus polymyxa*

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ABSTRACT

Bacillus polymyxa CECT 155 produces an extracellular neopullulanase activity that degrades pullulan to panose. This activity was stimulated by the presence of pullulan in the culture, and repressed by glucose. The apparent mol wt determined for the enzyme was 58 kDa. The optimum pH and temperature for neopullulanase activity were pH 6.0 and 50°C, respectively. The enzyme was stable in a pH range of 4.0–8.0, and temperatures up to 60°C. These properties make it suitable for the saccharification processes in the starch industries.

Index Entries: Extracellular enzyme; neopullulanase; panose; pullulan; *Bacillus polymyxa*.

INTRODUCTION

The name of neopullulanase refers to that enzyme that hydrolyzes α-1,4-glycosidic linkages in pullulan to produce panose *(1)*. There are two other related types of enzymes described that hydrolyze pullulan: pullulanase (E. C. 3.2.1.41) hydrolyzes α-1,6-glycosidic bonds to release maltotriose *(2,3)*, and isopullulanase (E. C 3.2.1.57), which attacks α-1, 4-glycosidic bonds and produces isopanose *(4)*. Neopullulanase activity was originally described in a strain of *Bacillus stearothermophilus (5)*. A similar enzyme, also from *B. stearothermophilus*, hydrolyzes not only α-1, 4-glycosidic bonds, but also specific α-1,6-glycosidic bonds of several branched oligosaccharides *(6)*. In addition to thermophilic *Bacillus* strains,

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neopullulanase activities have also been identified in microorganisms including *Thermo-actinomyces vulgaris* and *B. megaterium (7)*. A strain of *B. polymyxa* has been identified as producer of a debranching enzyme, but this enzymatic activity was not identified *(8)*. It has been reported *(9)* that most of the *B. polymyxa* species, assayed by plate technique, were able to degrade pullulan. Nevertheless, it is not clear if the hydrolysis was caused by pullulanase-type enzymes, as the authors suggested, or was caused by other amylolytic activities present. Recently, another strain of *B. polymyxa* has been isolated that exhibited α-amylase, pullulanase, and α-glucosidase activities *(10)*, but the hydrolysis product from pullulan was not determined for the pullulanase.

The simultaneous use of debranching enzymes and amylase has been shown to be a method of rapidly increasing rates and yields of hydrolysis products in the starch saccharification process *(11–13)*. A combination of pullulanase and bacterial α-amylase has also been described as an antistaling agent in the baking industry *(14)*. This biotechnological process required enzymes active at acidic pH and an optimum temperature range of 65–75°C *(15,16)*. Although some pullulan-hydrolyzing enzymes obey one of those characteristics, only very few of them attain both *(7)*. The characterization of novel debranching enzymes could lead to optimization of those industrial processes that involve starch bioconversion. Here we described the characterization of a neopullulanase activity in *B. polymyxa* CECT 155 that produces panose as the final product of its action on pullulan.

MATERIALS AND METHODS

Growth of Microorganism

B. polymyxa CECT 155 was obtained from the Spanish Type Culture Collection, (Valencia, Spain), and was cultivated in a rich medium (1% peptone, 0.5% yeast extract, 0.5% NaCl), supplemented only with 0.5% pullulan or 0.5% pullulan and 2% glucose. The pH of the medium was adjusted to 7.0 with NaOH. The incubation of the microorganism was performed with shaking (200 rpm) at 37°C. Agar plates were prepared containing the same rich medium supplemented with 0.1% of red pullulan (Megazyme, Sydney, Australia).

Preparation of Enzymatic Extract and Pullulanase Assay

Cultures of *B. polymyxa* were centrifuged at 6000*g* for 10 min to remove the cells. The resulting cell-free supernatant was used as crude enzyme preparation. When the cultures were grown in the presence of glucose, this sugar was removed from the cell-free supernatant by ultrafiltration using Microsep Microconcentrators (Hucoa-Erlöss, Northborough, MA). The pullulanase activity was determined by measuring the amount of reducing sug-

ars liberated into the reaction mixture at 37°C. A standard reaction mixture (0.4 mL) contained 0.1% pullulan and 0.1 mL of crude extract in 50 m*M* sodium phosphate buffer, pH 6.0. The reducing sugars released from pullulan were determined by the dinitrosalicylic acid method *(17)*. One unit of pullulanase catalyzes the formation of 1 μmol of reducing sugars per min under the defined conditions; panose was used as a standard.

Zymogram Analysis

Crude enzyme preparation was mixed 2:1 (v/v) with loading electrophoresis buffer (0.1% SDS, 62.5 m*M* DTT, 6.25 m*M* Tris-HCl, pH 6.8, 9.5% glycerol, and 0.001% bromophenol blue) and loaded on a 10% SDS-polyacrylamide gel containing 0.25% of red pullulan. After electrophoresis, the gel was soaked in a solution of 2% Triton X-100 for 1 h, washed several times with distilled water, and then incubated in 50 m*M* sodium phosphate buffer, pH 6.0, and 25 m*M* DTT at 50°C, until the protein bands with activity on pullulan appeared. To improve the visualization of the bands, the gel was immersed in ethanol.

Analysis of Hydrolysis Products

The reaction mixture (0.5 mL) contained 0.25 mL of cell-free supernatant enzyme preparation and 1% pullulan in 50 m*M* sodium phosphate buffer, pH 6.0. The reaction was incubated at 37°C overnight and then terminated by precipitation of the nondegraded pullulan with 2 vol of acetone. After 30 min on ice, the precipitate was discarted by centrifugation at 6000*g* for 15 min. The acetone was evaporated from the supernatant in a rotary evaporator, and the sample loaded on a Supelclean LC-18 SPE column (Supelco, Bellefonte, PA). After this step, the hydrolysis products of pullulan were analyzed by two methods: high performance liquid chromatography (HPLC) and thin-layer chromatography (TLC). In the first case, a Sugar-Pak I column (6.5 × 300 mm; Waters, Millipore, Milford, MA) was employed. The column was maintained at 85°C, and elution of sugars was performed with distilled water. The TLC was developed on silica gel plates (Kieselgel 60, Merck, Darmstadt, Germany) with acetonitrile–water (3:1 v/v), and the products were detected by spraying the gel with methanol–sulfuric acid (50:50 v/v) and heating it at 100°C. Glucose, maltose, maltotriose, panose, and maltotetraose were used as standards in both methods.

Determination of Optimum pH and Temperature

To determine the optimum pH, standard reaction mixtures (0.1% pullulan, 0.1 mL of crude extract in 0.4 mL of final volume) were incubated at 37°C for 30 min in the following buffers: sodium acetate, pH 4.0 and 5.0;

sodium phosphate, pH 6.0 and 7.0; and Tris-HCl, pH 8.0. To determine the optimum temperature, the samples were incubated at temperatures from 30°C to 70°C for 30 min in 50 m*M* sodium phosphate buffer, pH 6.0.

Effects of pH and Temperature on Enzyme Stability

To determine pH stability, the enzyme extract was maintained in 50 m*M* of each buffer solution described above at 37°C for 30 min. After appropriate dilution of the enzyme, the remaining activity was assayed at pH 6.0 in 50 m*M* of sodium phosphate buffer for 30 min at 37°C. To test the enzyme thermostability, the enzyme was kept in 50 m*M* of sodium phosphate buffer, pH 6.0, at temperatures from 30°C to 70°C for 30 min. The samples were cooled on ice and the residual activity was determined as previously described for pH stability.

RESULTS AND DISCUSSION

Detection of a Pullulan-Degrading Activity

The presence of a pullulan-hydrolyzing activity in *B. polymyxa* CECT 155 was first demonstrated with agar plates containing red pullulan. A clear halo of hydrolysis appeared around the colonies after incubation overnight (data not shown). Subsequently, we studied this activity using a zymogram analysis. This assay revealed only one band of activity, with apparent mol wt of 58 kDa (Fig. 1).

Production of the Enzyme in Presence of Pullulan and Glucose

We have investigated the growth and extracellular enzyme production of *B. polymyxa* CECT 155 in the presence of pullulan or in a mixture of pullulan and glucose. The pullulan-degrading activity was associated with the exponential growth phase in cells growing in the presence of pullulan, but not when glucose was added. In this case, the synthesis of the enzyme was repressed (Fig. 2), as reported recently in another species of *Bacillus* sp *(18)*. The activity decreased in the last part of the growth curve, probably because of protease degradation (Fig. 2). This enzymatic activity profile has been observed in other pullulan-degrading enzymes from the genus *Bacillus (10,18)*.

Analysis of Hydrolysis Products from Pullulan

The hydrolysis pattern after the action of the *B. polymyxa* CECT 155 crude enzyme preparation on pullulan is shown in Fig. 3. The detected products were panose (retention time = 7.08 min) and several unidentified saccharides that eluted earlier. Analysis by TLC of the eluted products from

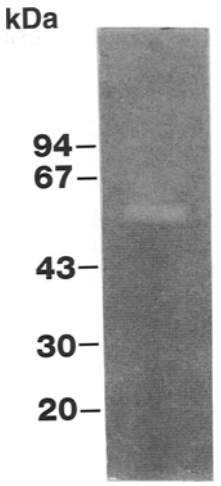

Fig. 1. Zymogram analysis of neopullulanase on red pullulan. Experiments were carried out on 10% SDS-PAGE containing 0.25% of substrate. Positions of the mol wt markers are indicated on the left side of the gel.

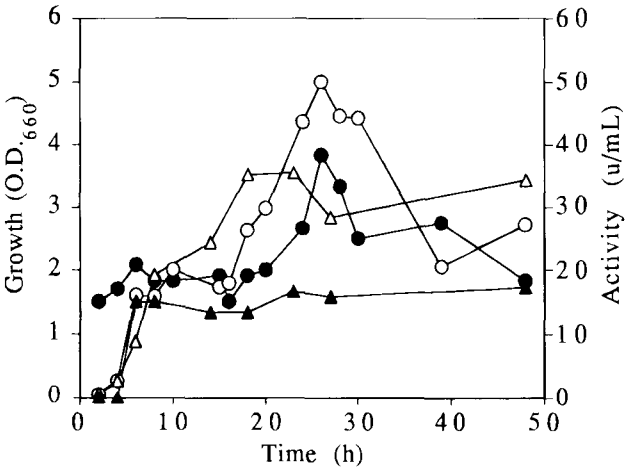

Fig. 2. Kinetics of growth and extracellular neopullulanase activity by *B. polymyxa* CECT 155. Enzyme activity was measured using cell-free culture supernatant and standard assay conditions. (○) Growth and (●) neopullulanase activity determined when the bacterium was cultivated in a rich medium supplemented with pullulan. (△) Growth and (▲) neopullulanase activity terminated when the medium was supplemented with pullulan and glucose.

the Sugar-Pack column confirmed that the products appearing at 5.36 and 5.82 min correspond to oligossacharides of variable chain length (data not shown). Imanaka and Kuriki *(6)* have shown that the neopullulanase activity from *B. stearothermophilus* TRS40 releases panose simultaneously with

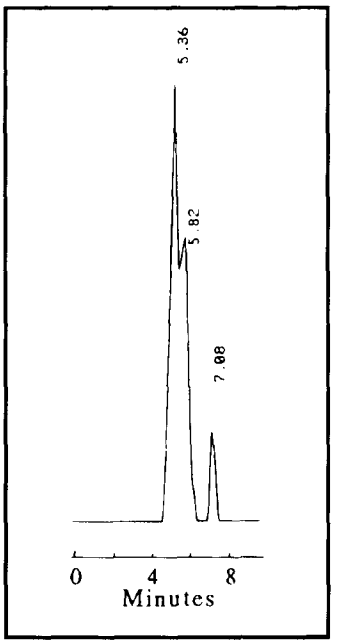

Fig. 3. HPLC analysis of pullulan hydrolysis products by crude neopullulanase preparation from *B. polymyxa* CECT 155. The reaction mixture (0.5 mL), incubated at 37°C overnight, contained 1% pullulan and 0.25 mL of enzyme preparation in 50 m*M* sodium phosphate buffer, pH 6.0. Retention time of 5.36 and 5.82 min corresponds to oligosaccharides, and 7.08 min to panose.

maltose and glucose from pullulan, and produces several intermediate products composed of some panose units. Unlike this enzyme, the neopullulanase of *B. polymyxa* CECT 155 studied here only produced panose as final product of pullulan hydrolysis. According to the model suggested for the enzyme of *B. stearothermophillus*, the oligosaccharides resulting from the action of the neopullulanase from *B. polymyxa* CECT 155 on pullulan should be formed by panose units. Therefore, the action pattern of the *B. polymyxa* CECT 155 enzyme on pullulan resembles random endo-attack.

Effects of pH and Temperature on Enzyme Activity and Stability

The neopullulanase displayed activity over a broad pH range, with more than 50% activity between pH 5.0 and pH 8.0. The optimum pH was at 6.0 (Fig. 4). The effects of pH on enzyme stability were also examined (Fig. 4). The enzyme was stable in the pH range assayed for at least 30 min at 37°C.

The optimum temperature of the enzyme was 50°C. At 30°C and 70°C, the neopullulanase retained approx 70 and 50% of the maximal activity, respectively (Fig. 5). After incubation at 50°C for one-half hour at pH 6.0, the residual activity was 96%. A complete loss of activity was observed

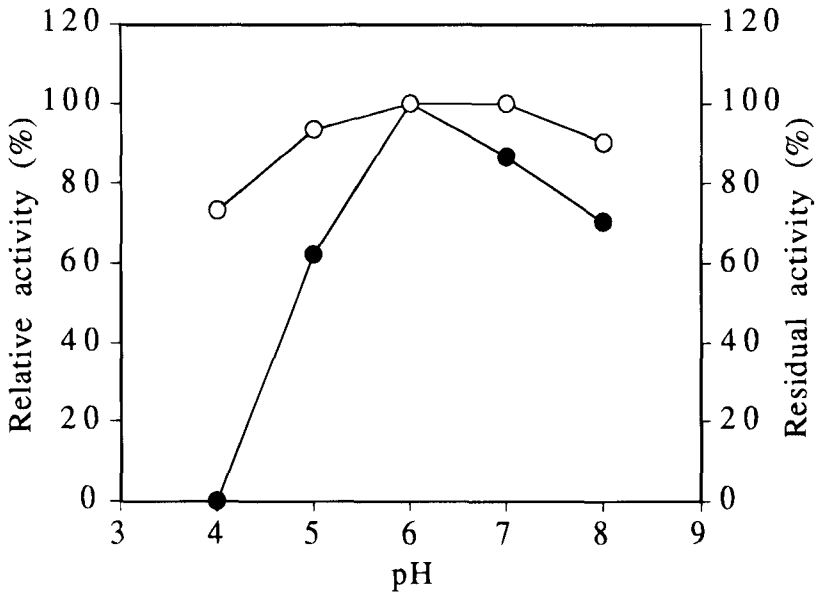

Fig. 4. Influence of pH on the activity and stability of the neopullulanase of *B. polymyxa* CECT 155. The neopullulanase activity was determined at 37°C for each pH (●). To determine the effect of the pH on stability (○), the enzyme extract was preincubated for 30 min at the indicated pH. One hundred percent of activity corresponds to 12.3 U/mL.

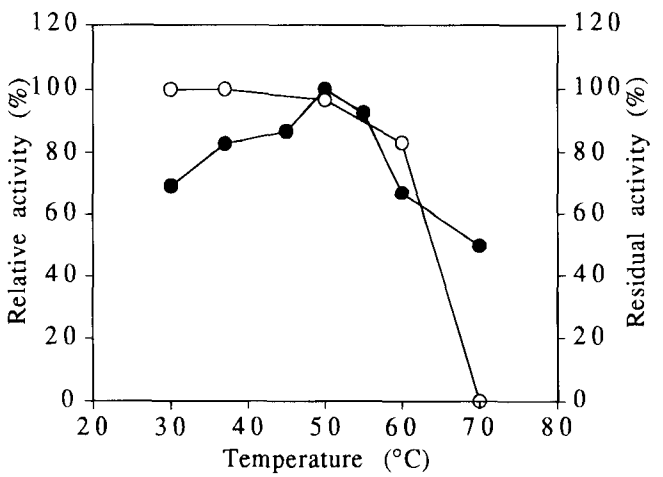

Fig. 5. Influence of the temperature on the activity and stability of the neopullulanase of *B. polymyxa* CECT 155. To determine the optimum temperature (●), the enzyme was incubated in sodium phosphate buffer, pH 6.0, for 30 min at various temperatures (30°C to 70°C). The effect of the temperature on enzyme stability (○) was assayed by preincubating the enzyme for 30 min at the indicated temperatures. One hundred percent of activity corresponds to 17.3 U/mL.

in the same conditions at 70°C (Fig. 5). Because the enzyme at the same temperature still had 50% of relative activity, that result can be explained by the decreased enzyme stability in the absence of substrate. Similar results have been described for other enzymes *(18,19)*.

The processes of liquefaction and saccharification involved in the starch processing industries require enzymes that have optimum pH between 4.0 and 6.5, and temperatures between 50°C and 100°C *(20,21)*. However, few debranching enzymes are active and stable at these conditions *(7)*. The enzyme of *B. polymyxa* CECT 155 studied here operates under a pH and temperature range that make it suitable for these industrial processes.

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