0273-2289/92/37(3)—0227$03.00

Acid Pullulanase from *Bacillus polymyxa* MIR-23

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Received August 5, 1992; Accepted September 14, 1992

ABSTRACT

Within the frame of a screening program aimed at the isolation of amylolytic sporeformers, one strain with high amylolytic activity designated MIR-23 was selected. The microbial characterization was carried out by morphological and biochemical tests and, by means of statistical treatment, was identified as *Bacillus polymyxa*. The organism could grow in acidic conditions (pH 5.0) on a starch medium and produce α-amylase, pullulanase, and α-glucosidase. Batch cultures showed the highest enzyme activities in the stationary phase. Pullulanase activity exhibited an optimal temperature of 52–57°C at pH 4.5–5.5. These properties would allow its use in the saccharification processes in the starch industries.

Index Entries: *Bacillus polymyxa;* pullulanase, α-amylase; α-glucosidase; starch degradation; batch culture; acidophilic microorganism.

INTRODUCTION

Starch degradation for the production of sweeteners is developed in two stages: The first stage involves the liquefaction of the substrate with α-amylase, and the second stage or saccharification stage is performed by

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β-amylase with pullulanase to obtain better yields (*1*). However, few debranching enzymes were able to operate under the industrial conditions prevailing in the saccharification stage: pH 4.5–5.5 and 55–60°C (*2*).

In previous work, an α-1,6 debranching enzyme has been described in *B. polymyxa*, but this enzymatic activity was not identified (*3*). In another work, it has been reported that 91% of 20 strains analyzed by plate technique belonging to the *polymyxa* species were able to degrade pullulan. The authors suggested that the enzyme was probably a pullulanase rather than an isoamylase (*4*). However, to our knowledge, there are no reports on the production of pullulanase by this species.

The aim of this work was to study the production of acid-resistant pullulanase by an aciduric-thermoresistant strain of *Bacillus polymyxa*, designated MIR-23, which we have isolated from natural sources.

MATERIALS AND METHODS

Strain

The strain MIR-23 was selected in a previous screening program of amylolytic microorganisms (*5*). The characterization was done following international criteria (*6,7*). The statistical treatment was carried out by the Jacquard coefficient (S_J) (*8*). The matrix frequencies were derived from the data of previous workers (*6,7*).

Medium

Batch cultures were carried out in a medium (DM) described by Darland and Brock (*9*) with some modifications. It contained in g/L: $(NH_4)_2SO_4$, 0.4; $MgSO_4 \cdot 7H_2O$, 0.2; $ZnSO_4 \cdot 7H_2O$, 0.001; $MnSO_4 \cdot 7H_2O$, 0.05; KH_2PO_4, 2.0; $CaCl_2 \cdot 2H_2O$, 0.2; $FeSO_4 \cdot 7H_2O$, 0.01; $NaMoO_4 \cdot 2H_2O$, 0.01; $CuSO_4$, 0.001; yeast extract, 2.0, starch, 8.0. The pH was adjusted to 5.0 with 100 m*M* H_3PO_4. The reagents were of bacteriological or analytical grade (Merck).

Bacterial Cultures

The strain MIR-23 was grown on a shaker (180 rpm) in Erlenmeyer flasks (500 mL) with 125 mL of DM medium. Growth was measured at 560 nm, diluting with 145 m*M* NaCl when necessary.

Analytical Procedures

Proteins, starch, reducing sugars, glucose, and α-amylase activities were assayed as previously described (*10*). One α-amylase unit is defined as the amount of enzyme needed to debranch 10 mg of starch in 30 min at 45°C.

Pullulanase was assayed with 3,5 dinitrosalicylic acid using pullulan (5.0 g/L) in 50 m*M* citrate buffer, pH 5.0, incubated at 45°C for 30 min. A pullulanase unit is defined as the amount of enzyme required to produce 1 μmol of reducing sugars (as maltose)/min. Extracellular activities were determined by centrifuging the cultures for 20 min at 10,000×*g* in the cold (2–4°C).

Optimal Conditions for Enzyme Activities

The effect of temperature and of pH on the different enzyme activities was assayed in optimal conditions for the other parameters. The determinations were performed with the crude enzymes.

RESULTS AND DISCUSSION

One of the isolates of a screening program was designated MIR-23 and showed higher amylolytic activity than *Bacillus subtilis* NCIB 8565 used as positive control in the cultures. In the experiments, a clear zone appeared around the selected colonies grown on starch plates and stained with iodine. This result implied that the strain MIR-23 produced amylase/s capable of hydrolyzing starch, but the test could not determine what amylolytic enzymes were present, and whether these activities were cell bound or extracellular.

The microbiological characterization of the strain MIR-23 involved 78 biochemical and morphological tests. However, these assays did not identify the selected strain as any recognized species of the genus *Bacillus*. The fact is common for natural isolates belonging to the genus *Bacillus* (*6*). Therefore, we analyzed the data statistically by means of the Jacquard coefficient, which allowed its identification in the *circulans* cluster and suggested that the organism was *Bacillus polymyxa* ($S_J = 0.790$) (data not shown).

The results obtained in batch-culture experiments in starch acidic medium are shown in Fig. 1. The pullulanase activity was detected in the stationary phase with a maximum activity of 42 U/L (Fig. 1A); extracellular activity was not detected. This pullulanase temporal pattern and location have been observed in other microorganisms (*11,12*). After the maximum activity was reached, the enzymatic activity decreased probably because of protease action (results not shown) (*13*).

The total activity of α-amylase showed a maximum of 7.4 U/L in the late stationary phase (results not shown); however, approx 30% was extracellular, and this percentage remained more or less constant in the stationary phase (Fig. 1B), although it is commonly accepted that the activity of α-amylase is extracellular in the species of the genus *Bacillus*.

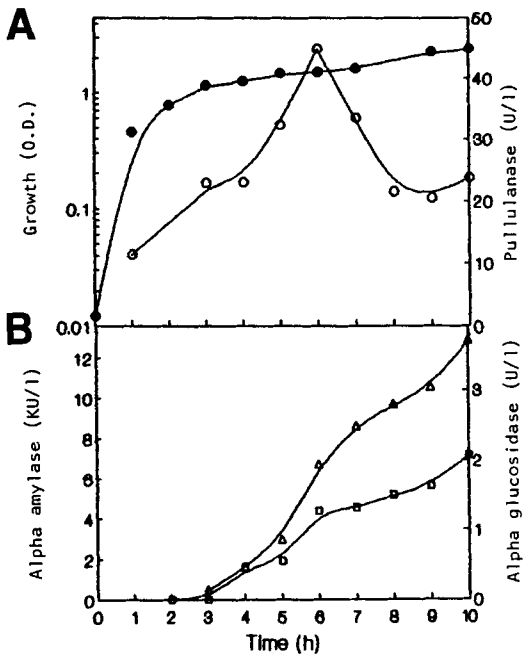

Fig. 1. (A) Kinetics of growth (●) and pullulanase activity (○); (B) production of enzymatic extracellular activities: α-glucosidase (△) and α-amylase (□).

The activity of α-glucosidase was found in total extract and cell-free supernatants. The maximum activity was obtained in the late-stationary phase (Fig. 1B). The activity profiles of these fractions were parallel and resembled the α-glucosidase pattern of *B. circulans* F-2 (*14*). However, the extracellular-total activity ratio increased with time, and it was found that 60–70% of the activity was extracellular in the late-stationary phase, in contrast to a previous report where the extracellular activity remained at 10% in a *circulans* species (*14*) belonging to the same cluster. In this case, the enzyme secretion was not affected by the acidic pH of the medium, and it could be attributed to a different mechanism of enzyme export and/or different membrane binding with respect to the α-amylase. Variable location and properties of these amylolytic enzymes reflect the heterogeneous characteristics of this genus.

The effect of pH on pullulanase activity at 55°C is shown in Fig. 2. Pullulanase activity has a broad pH profile; it retained maximal activity at pH 4.4–5.5 with different buffers of the same ionic strength. The optimum temperature at pH 5.0 was 52°C (Fig. 3), but it retained 97% of the maximal activity at 57°C for 30 min. These pH and temperature optima of pullulanase activity differ from a previous report on another strain of *Bacillus polymyxa* in which the maximum activity was shown at pH 7.0 (*13*). Other authors reported different pullulanases as shown in Table 1, but only one of these was able to operate in an industrial process, at acidic

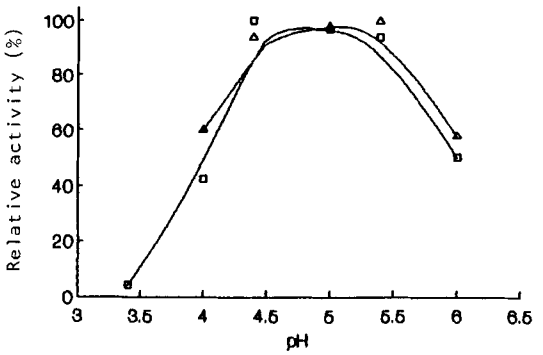

Fig. 2. Effect of pH on pullulanase activity. The enzyme was measured at 52°C for 30 min in 50 m*M* citrate (□) and acetate (△) buffers.

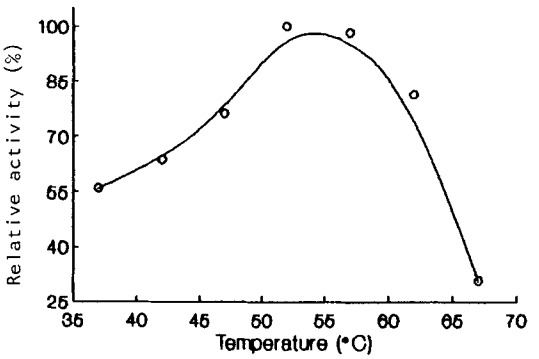

Fig. 3. Effect of temperature on pullulanase activity. The enzyme was determined in 50 m*M* acetate buffer (pH 5.0).

Table 1
Comparison of the Activity of Pullulanase Produced by *Bacillus polymyxa* MIR-23 with the Literature Data

Microorganisms	Optimal Temp., °C	Optimal pH	Rel. Act., % at pH 5.0	Rel. Act., % at pH 4.5	References
B. acidopullulyticcus	60	5.0	100	90	*2*
B. polymyxa MIR-23	52	4.5–5.5	98	97	Present
B. polymyxa	45	6.0–7.0	–	–	*13*
B. stearothermophilus	60–65	6.0	30	5	*15*
B. stearothermophilus	65	6.0	15	10	*16*
B. subtilis	60	7.0–7.5	50	20	*17*
C. thermohydrosulfuricum	85	5.5–6.0	90	70	*18*
Termus aquaticus	–	6.4	50	40	*19*
T. thalpophilus	70	7.0	–	–	*20*

conditions below pH 5.0 and high temperature (2); also, it is a commercial product. However, the enzyme activity at pH 4.5 shows 90% of its optimal activity. Under the same conditions, pullulanase activity of *B. polymyxa* MIR-23 showed 100–97% of its maximal activity.

The pH profile of α-amylase showed a maximal activity at a pH range of 7.0–7.3 with an optimum temperature at 47°C (results not shown). Maximum activity for α-glucosidase was obtained at 55°C and pH 5.0–5.5 (results not shown). The pH and temperature activity profiles of α-glucosidase are similar to pullulanase profiles, and the two enzymes could be used simultaneously in starch conversion to diminish the reaction time for oligomaltosaccharide or glucose syrup productions.

SUMMARY

A thermoresistant strain, designated MIR-23, was characterized in the *circulans* cluster as *B. polymyxa,* which grew in acidic medium and produced amylolytic enzymes. The amylolytic system comprises α-amylase, α-glucosidase, and pullulanase activities. Pullulanase activity showed an optimal temperature of 52–57°C between pH 4.5 and 5.5. These conditions would allow the enzymes to be used in the saccharification stage of production of a sweetener from corn starch.

ACKNOWLEDGMENTS

This work was supported by Instituto Antártico Argentino (Argentina) and SAREC (Sweden). We are indebted to Carmen Castro for technical assistance.

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