**Note**

**Cell-bound Pullulanase from *Streptomyces* sp. No. 27**

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Received June 25, 1992

Pullulanase is an enzyme that hydrolyzes α-1,6-glucosidic linkages of pullulan to produce maltotriose. Pullulanase has many applications such as use with glucoamylase to improve the yield of glucose from starch, with β-amylase to obtain higher yields of maltose from starch, and with maltotriosaccharide-producing amylases to improve the yield of maltotriosaccharides such as maltotriose, maltotetraose, and maltotriose. Because the optimum pHs of these amylases are from pH 4.5 to 8.0, the most useful pullulanase should be active in this wide pH range, and also have at least an optimum temperature of 60°C to prevent microbial infection during the industrial use.

Pullulanases have been reported to be produced by many microorganisms such as *Aerobacter aerogenes* (Klebsiella pneumoniae), *Streptococcus mitis*, *Escherichia intermedia*, *Streptomyces* sp., and *Bacillus* sp. However, most of the enzymes reported so far are rather thermo-labile with optimum temperatures of 40 to 50°C or thermo-stable but with an acidic or neutral optimum pH and not effective in the wide pH range.

Ueda et al. reported that *Streptomyces* sp. produces a pullulanase extracellularly. This enzyme is rather thermostable but was acid-labile to use with glucoamylase for the production of glucose. It was found that a cell-bound pullulanase produced by a *Streptomyces* sp. No. 27 newly isolated from soil produces maltotriose from pullulan as the main product, is active from around pH 4 to 7 and stable, especially on the acidic side, compared to the pullulanase from *Streptomyces* sp.7 (Fig. 2).

The strain was cultivated in a medium (40 ml, pH 6.5) containing: polypeptone, 2%; soluble starch, 1.0%; K₂HPO₄, 0.25%; MgSO₄·7H₂O, 0.15%; (NH₄)₂MoO₄·4H₂O, 7.4 × 10⁻³ M, and MnSO₄, 7.5 × 10⁻² M in a 200-ml Erlenmeyer flask. The cultivation was done for 42 h at 30°C on a rotary shaker at 225 rpm.

The culture broth was centrifuged for 5 min at 10,000 rpm and the extracellular pullulanase activity was measured in the supernatant. The cells were washed, suspended in distilled water, sonicated (one min at 20 KC) and then centrifuged (10,000 rpm, 5 min). The intracellular pullulanase activity was measured for these cell extracts. The cell debris was suspended in distilled water and then the cell-bound pullulanase activity was measured. The cell-bound pullulanase can mostly be extracted by incubating the cell debris for 1~2 h at 30°C in the presence of 3.5% sodium chloride.

The pullulanase activity was measured in 1.0 ml reaction mixture containing 0.5 ml of 2% pullulan in 5 × 10⁻² M acetate buffer (pH 5.0) and the enzyme. The reaction was done for 30 min at 60°C and the reducing sugar formed was measured by the Somogyi and Nelson method. One unit of the enzyme was defined as the amount of enzyme that produced reducing sugar corresponding to one μmol of glucose from pullulan in one min under the assay conditions.

To each enzyme solution obtained above, ammonium sulfate was added to 70% saturation. The precipitates formed were collected, dissolved in distilled water, dialyzed against distilled water, and used for the study of the enzymes.

Table I shows the production of pullulanase by *Streptomyces* sp. No. 27 and the effects of addition of sodium chloride to the culture medium. As can be seen from Table I, most of the pullulanase was cell-bound. This is different from the pullulanase of *Streptomyces* sp. reported by Ueda et al. However, the addition of sodium chloride to the culture medium increased the ratio of extracellular pullulanase.

Figure 1 shows the effects of pH on activity of these three pullulanases.

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**Table 1.** Production of Pullulanase by *Streptomyces* sp. No. 27 and Effect of Addition of Sodium Chloride

<table>
<thead>
<tr>
<th>NaCl (%)</th>
<th>pH</th>
<th>Cell growth</th>
<th>Pullulanase (units/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>E.P.</td>
<td>I.P.</td>
</tr>
<tr>
<td>0</td>
<td>5.9</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>0.50</td>
<td>5.9</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>0.75</td>
<td>5.8</td>
<td>+</td>
<td>+</td>
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<tr>
<td>1.00</td>
<td>5.7</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>1.25</td>
<td>5.7</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>1.50</td>
<td>5.7</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Basic medium: K₂HPO₄, 0.2%; MgSO₄·7H₂O, 0.15%; soy bean meal, 2%; soluble starch, 1%; (NH₄)₂MoO₄·4H₂O, 7.4 × 10⁻³ M; MnSO₄, 7.5 × 10⁻² M; pH 6.0.

Cultivation, 30°C, 40 h.

E.P., extracellular pullulanase; I.P., intracellular pullulanase; C.P., cell-bound pullulanase; T.P., total pullulanase.

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**Fig. 1.** Effects of pH on Activity of Pullulanase.

The reaction was done in a mixture containing 1 × 10⁻³ M acetate or phosphate (Na₂HPO₄-KH₂PO₄) buffer at various pHs as indicated, with 1% pullulan and 6.0 × 10⁻³ M units of the pullulanase for 30 min at 50°C.

- ○—○—, cell-bound pullulanase (acetate buffer);
- □—□—, intracellular pullulanase (acetate buffer);
- ■—■—, extracellular pullulanase (acetate buffer);
- ▲—▲—, extracellular pullulanase (Na₂HPO₄-KH₂PO₄ buffer);
- ■—■—, intracellular pullulanase (phosphate buffer);
- ▲—▲—, extracellular pullulanase (phosphate buffer).

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**Fig. 2.** Comparison of pH–Activity Profiles of Cell-bound Pullulanase of *Streptomyces* sp. No. 27 and Pullulanase of *Streptomyces* sp.7

- ●—●—, from ref. 7; ○—○—, the reaction was done as described by S. Ueda et al. The reaction mixture containing 1 × 10⁻² M Malvavine-citrate-phosphate buffer at various pHs as indicated, 0.5% pullulan, and 8 × 10⁻³ units of the cell-bound pullulanase was incubated for 60 min at 40°C.
Fig. 3. Effects of Temperature on Activity of Pullulanase.

The reaction was done in a mixture containing $1 \times 10^{-2} \text{M}$ acetate buffer (pH 5.0), 1% pullulan and $7.5 \times 10^{-2} \text{units}$ of the pullulanase for 30 min at various temperatures as indicated. — , cell-bound pullulanase; — , intracellular pullulanase; — , extracellular pullulanase; — , cell-bound pullulanase in the presence of $1 \times 10^{-2} \text{M CaCl}_2$.

### Table II. Saccharification of Liquefied Starch with Glucoamylase and Pullulanase

<table>
<thead>
<tr>
<th>Reaction time (h)</th>
<th>Control 1)</th>
<th>+ Pullulanase 2)</th>
<th>+ Pullulanase 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>G1</td>
<td>G2</td>
<td>G3~</td>
</tr>
<tr>
<td>25</td>
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<td>31.5</td>
<td>82.1</td>
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<tr>
<td>95.5</td>
<td>89.7</td>
<td>2.6</td>
<td>7.7</td>
</tr>
</tbody>
</table>

Reaction mixture:
1) A mixture (1.0 ml) containing $4 \times 10^{-2} \text{M}$ acetate buffer (pH 4.5), 30% liquefied starch (DE 7.7) prepared as described in our previous paper, and glucoamylase (0.2 units/g substrate) was incubated at 60°C.
2) The reaction was done in the presence of pullulanase (0.5 units/g substrate) under the conditions of 1).
3) The reaction was done in the presence of pullulanase (0.5 units/g substrate) and $1 \times 10^{-2} \text{M CaCl}_2$ under the conditions of 1).

The optimum pHs of these three pullulanases were around 5.0—6.0. However, the intracellular and cell-bound pullulanases showed a broad pH-curve compared with those of the extracellular pullulanase.

Figure 2 shows the comparison of pH–activity profiles of the cell-bound pullulanase and the pullulanase of *Streptomyces sp.* 7) The former enzyme is more active and stable on the acidic side than the latter.

Figure 3 shows the effects of temperature on activity of the pullulanases of *Streptomyces sp. No. 27*. The optimum temperatures of the intracellular and cell-bound pullulanases were around 60°C and that of the extracellular pullulanase around 55°C. In the presence of Ca$^{2+}$, the optimum temperature of the cell-bound pullulanase was raised to about 65°C.

The cell-bound pullulanase was used for the production of glucose with glucoamylase from *Aspergillus niger*. Table II shows the courses of saccharification of liquefied starch with the glucoamylase in the presence or absence of the pullulanase. The sugar composition of hydrolysate of liquefied starch was measured by the high-performance liquid chromatography equipped with a column Shim-pack Ion KS-802 (Shimadzu Seisakusho Co., Ltd.). 8) The presence of the pullulanase promoted the saccharification and increased the yield of glucose. The maximum yield of glucose (96.7%) with this pullulanase was almost the same as those (96.5—97.0%, 9) 96.8% 10) of pullulanases from *Bacillus sp.*

### References