Bacillus stearothermophilus

I. Thermal and pH Stability of the Amylase

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Investigators are agreed (Knee and Sandstedt, 1946; Myrbäck and Neumüller, 1950) that bacterial amylases, except crystalline preparations (Meyer et al., 1947; Bernfeld, 1951), possess a greater thermostability than other alpha-amylases. Although many investigators have studied the heat stability of amylases from various sources, no study has been published comparing the thermal resistance of extracellular hydrolytic enzymes from thermophilic and mesophilic bacteria.

Conn, Johnson and Miller (1950) tested the temperature of inactivation of a group of alpha-amylases in the amylograph at 60 to 85 °C. These investigators found that a mesophilic bacterial amylase, Rhizyme-DX, was more heat stable than amylase preparations from certain other bacterial, malted flour, and fungal sources.

Stark and Tetrault (1951) described the isolation of cell-free, starch saccharifying enzymes from the culture medium of Bacillus stearothermophilus ATCC 7954 at 70 °C and demonstrated saccharogenic activity at 90 °C. Campbell (1952) compared the amylase preparations obtained from both B. stearothermophilus 1518 and B. coagulans 43P grown at 35 and 55 °C. He found that the amylases produced at 55 °C had a somewhat higher optimum activity temperature (60 to 70 °C) than those produced at 35 °C (45 to 55 °C). When exposed to a temperature of 90 °C, the amylases produced at 35 °C lost all detectable activity after 2 hours, but the amylases produced at 55 °C had retained 50 per cent of their activity for 24 hours.

Since thermostability of enzymes is important in some industrial processes and thermophilic bacterial amylases have been shown to be quite heat resistant, a comparative study was made of three commercially available bacterial amylases with a thermophilic bacterial amylase preparation. The pH stability of the thermostable amylase preparation at 60 °C was also observed.

Materials and Methods

SKB test. The reagents and procedure were the same as given by Sandstedt, Kneen, and Blish (SKB Test) (1939), except that: (a) 1 per cent soluble starch Merck, according to Lintner in m/15 Sörenson's buffer, pH 6.0, served as the substrate; (b) 52 °C, instead of 30 °C, was used for the reaction temperature; and (c) the end point was determined by comparison with an inorganic standard (Olson, et al., 1944), or spectrophotometrically. Since the SKB method was developed for the determination of the activity of cereal alpha-amylases, the above changes in procedure were made in order to adapt this method for the determination of the activity of bacterial amylases and to have conditions comparable to the disc-plate method.

Disc-plate test. The procedure was that of Wellerson et al. (1952, 1954) and Stark et al. (1953). Filter-paper discs, saturated with the amylase solution, were placed on the surface of a starch-agar substrate in pressed Pyrex Petri plates (4 discs/plate). The plates were allowed to incubate for 8 hours at 52 °C. The discs were removed, the plates were flooded with iodine, excess iodine was poured off, and the diameter of the zone of hydrolysis was read on a Fisher-Lilly zone reader. Preliminary assays have shown that a straight line relationship exists between the log of the enzyme concentration and the diameter of the zone of hydrolysis (figure 1).

Preparation of the thermostable enzyme. The amylase of B. stearothermophilus ATCC 7954 was produced by submerged culture. The medium consisted of 6 liters of 2 per cent trypticase, 0.4 per cent N-Z-Case, 0.6 per cent yeast extract, 0.75 per cent soluble starch and 0.01 per cent sodium citrate broth. The inoculum consisted of 500 ml of a 20 hour stationary culture grown on 2 per cent trypticase broth in a 2½ liter Fernbach flask at 60 °C. Dow-Corning Antifoam A3 (with emulsifier) was used to prevent excessive foaming. After an incubation period of 24 hours at 60 °C, the cells were removed from the medium by Sharples centrifugation. The enzyme was concentrated by precipitating it from undialyzed (pH 6.0) medium with 1.65 volumes of 100 per cent ethanol at 0 °C.

Heat stability tests. Each of the bacterial enzyme preparations was dissolved in 100 ml of a 0.0125 m CaCl2 solution in a concentration to give a zone of

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approximately 25 mm in diameter. This was necessary because of the different potencies per unit weight of the various preparations (table 1).

The enzyme solutions (100 ml per 250 ml beaker) were tested when placed in a water bath of the appropriate temperature, then further tests for enzyme activity were made at various intervals. The times required for the enzyme solutions to reach water-bath temperature were noted, but will not be included in the calculations because all preparations were treated in the same manner. The pH of each preparation was checked at the start and at the conclusion of each experiment; acid production was generally proportional to the degree of enzyme inactivation obtained.

pH stability tests. A thermostable enzyme preparation, prepared in a manner similar to that reported above, was suspended in 150 ml of 15 Sorensen’s phosphate buffer containing 0.0125 M CaCl₂ and 100 units per ml of streptomycin. Aliquots were adjusted to proper pH, the solutions were overlayed with toluene, tests were made and the solutions were incubated at 60°C. Further tests for enzyme activity were made after incubation periods of 12, 24, 48, and 90 hours. Final pH checks were also made (table 2).

Results and Discussion

90°C. After 3 hours at 90°C the thermophilic bacterial amylase (T) demonstrated dextrinizing power greater than that possessed by any of the other three amylases after only 30 minutes (figures 2 and 3). 80°C. As would be expected, a decrease of 10°C resulted in a less rapid loss of enzyme activity (figures 4 and 5). However, only the thermophilic bacterial amylase retained measurable dextrinizing activity after 23 hours.

70°C. The dextrinizing activity of all but Takamine HT-44 (F) remained relatively constant for about 3 hours at 70°C. Thereafter, the other two mesophilic enzyme preparations slowly decreased in activity while the thermophilic amylase activity remained at a high level (figures 6 and 7).

60°C. The results shown in figure 8 indicate that the amylase activity was very stable over a pH range of 5.75 to 7.0 at 60°C. Fair enzyme stability was observed within a range of pH 5.25 to 7.5 at 60°C.

These results confirm the results of Stark and Tetrault (1951) and Campbell (1952), who found that bacterial alpha-amylases elaborated at high temperatures were more heat stable than the corresponding amylases elaborated at mesophilic temperatures. Similar findings have been reported by Militzer and co-workers (1949, 1950, 1951, 1952a, 1952b) for certain bacterial respiratory enzymes.

The results indicate that it may be very desirable to use thermophilic bacterial amylase preparations in

Table 1. Source and concentration of the amylase preparations used

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Source</th>
<th>mg/100 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>NB</td>
<td>Bacterial, Nutritional Biochemicals Corp.*</td>
<td>150</td>
</tr>
<tr>
<td>F</td>
<td>HT-44, Lot #F4975 Takamine Laboratories, Inc.†</td>
<td>1360</td>
</tr>
<tr>
<td>DX</td>
<td>Rhozyme-DX #297, Rohm and Haas Co.‡</td>
<td>1000</td>
</tr>
<tr>
<td>T</td>
<td>Bacillus stearothermophilus ATCC 7954</td>
<td>1300</td>
</tr>
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</table>

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Table 2. Initial and final pH values obtained in the pH stability test

<table>
<thead>
<tr>
<th>Initial pH</th>
<th>Final pH</th>
<th>Enzyme Stability†</th>
</tr>
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<tbody>
<tr>
<td>4.50</td>
<td>4.70</td>
<td>Unstable</td>
</tr>
<tr>
<td>5.00</td>
<td>5.10</td>
<td>Fair stability</td>
</tr>
<tr>
<td>5.25</td>
<td>5.30</td>
<td>Fair stability</td>
</tr>
<tr>
<td>5.50</td>
<td>5.50</td>
<td></td>
</tr>
<tr>
<td>5.75</td>
<td>5.75</td>
<td>Range of good pH stability</td>
</tr>
<tr>
<td>6.00</td>
<td>5.95</td>
<td></td>
</tr>
<tr>
<td>6.25</td>
<td>6.25</td>
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<tr>
<td>7.50</td>
<td>7.10</td>
<td>Fair stability</td>
</tr>
<tr>
<td>8.00</td>
<td>7.30</td>
<td></td>
</tr>
<tr>
<td>8.50</td>
<td>7.30</td>
<td></td>
</tr>
<tr>
<td>9.00</td>
<td>7.30</td>
<td></td>
</tr>
</tbody>
</table>

* Incubated at 60°C.
† Enzyme activity tested after 12, 24, 48, and 90 hr.
Figs. 2 through 7. The effect of temperature on the stability of four bacterial amylases.

any industrial process where high dextrinizing temperatures are either convenient or required.

The pH stability tests yielded results similar to those found by Stark (1951) and Campbell (1952) for amylases produced at 55 to 60 C.

**Summary**

The thermostabilities of three commercially available amylase preparations from mesophilic bacteria were compared with the heat resistance of an amylase preparation obtained from a thermophilic species.
Enzyme activity was followed by two methods (Sandstedt, Kneen, and Blish test [SKB] and Disc-Plate) at three temperatures (70, 80, and 90 °C). Under the conditions used, the thermophilic bacterial amylase preparation was shown to be more heat stable in every instance than any of the mesophilic bacterial amylases tested.

The thermophilic bacterial amylase activity was found to be reasonably stable within a pH range of from 5.25 to about 7.5 at 60 °C.

REFERENCES

Stark, E., and Tetrault, P. A. 1951 Isolation of bacterial, cell-free, starch saccharifying enzymes from the medium at 70 °C. J. Bact., 62, 247-249.