Isolation of Amylolytic Strains of
Thermoactinomyces vulgaris and
Production of Thermophilic
Actinomycete Amylases

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ABSTRACT

KUO, M. J. (Iowa State University, Ames), AND P. A. HARTMAN. Isolation of amylolytic strains of Thermoactinomyces vulgaris and production of thermophilic actinomycete amylases. J. Bacteriol. 92:723–726. 1966.—Of 759 isolates obtained from dung, compost, and soil samples, a culture of Thermoactinomyces vulgaris (strain 5) was selected for further study on the basis of quantities of amylase produced in synthetic and nonsynthetic media, rapid growth and sporulation, culture stability upon prolonged storage at 5 C, and growth temperature range. Inoculum preparation, temperature optimum for amylase formation, and the effects of various kinds and levels of carbon and nitrogen sources on amylase production were studied with T. vulgaris strain 5. An optimal procedure for production of T. vulgaris amylases is proposed.

Although amylases produced by mesophilic actinomycetes have been studied (4, 9; P. Hyslop and B. P. Sleeper, Bacteriol. Proc., p. 89, 1964) and thermophilic actinomycetes are known to hydrolyze starch, no study of amylase production by thermophilic actinomycetes has been published. This report presents details regarding the isolation of thermophilic, amylolytic actinomycetes and production of amylases by a selected strain of Thermoactinomyces vulgaris.

MATERIALS AND METHODS

Selection of production strain. Dung, compost, and soil samples were collected from various locations in Iowa. Additional soil samples were obtained from the Iowa State University Soil Testing Laboratory. Each sample was plated on a partially dried plate (14) of Tendler and Burkholder’s medium Ia (13), supplemented with 0.2% soluble starch. After incubation at 55 C for 48 hr on the Trypticase-yeast extract-dung extract-salts medium Ia, colonies were picked and streaked on the same medium containing 1.0 μg/ml of penicillin G (see also 16). Amylase production was observed after flooding the plates with Lugol’s iodide. Trypticase Soy Agar (14), modified by addition of 0.2% starch, also was used satisfactorily for some samples. A few samples were plated on arginine-glycerol-salt (AGS) medium (2), modified Benedict’s medium (8), 0.5% starch-agar, and glycerol-asparagine-agar II (15); however, use of these media was discontinued early in the study because colony yields were low.

Purified cultures were examined for rapidity of growth. Each isolate was inoculated onto slants of starch medium Ia which were incubated at 55 C. The rate of growth was observed visually. Slants of rapidly growing cultures were stored at 5 C for 3 months; survivors were selected for further studies. A loopful from a slant culture of starch medium Ia, grown at 55 C for 72 hr, was inoculated into a 250-ml flask containing 50 ml of asparagine-yeast extract-salts medium VII (13), supplemented with 0.2% soluble starch. The flasks were shaken at 120 rev/min on a rotary shaker (model V; New Brunswick Scientific Co., New Brunswick, N.J.) for 42 hr at 55 C; the culture was filtered through Whatman no. 5 paper; the final volume of the filtrate was brought to 30 ml; and the amylase activity was determined by the Nelson-Somogyi (7, 11) procedure. Similarly, growth on nitrate-salts medium II (13), modified by replacement of glucose with 0.5% soluble starch, was estimated. Cultures surviving initial screening procedures

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were examined for growth after incubation at 32, 37, 45, 55, 60, and 63 C for 5 days on starch medium Ia.

Production of thermophilic actinomycete amylases. *T. vulgaris* strain 5 was maintained on slants and plates of A-agar, which had the following composition: 2.0% soluble starch, 1.0% N-Z Case, 0.1% yeast extract, 0.1% KH₂PO₄, 0.05% MgSO₄·2H₂O, and 2.0% agar. The pH was adjusted to 6.3 with 1 N HCl prior to sterilization. After incubation for 3 or 4 days at 55 C, the cultures were stored at 4 C. Strain 5 also was lyophilized successfully.

After examination of several methods of inoculum preparation, the following method was found to be the most satisfactory. Moistened cotton swabs were used to remove spores and aerial mycelia from stock culture plates. Fresh plates were inoculated and incubated for 48 hr at 55 C. An 11-mm diameter cork borer was used to cut discs from plates containing thick and uniform aerial mycelia and spores. Five discs, selected at random from different plates, were placed into each 250-ml Erlenmeyer flask containing 50 ml of A-broth. A-broth had the same composition as A-agar, except that the agar was omitted and 0.05% glucose was added. Inoculated flasks were shaken at 120 rev/min. Incubation for 12 hr at 55 C was better than shorter (6 hr) or longer (18 hr) incubation periods. These 12-hr cultures were used to inoculate flasks containing 50 ml of media similar in composition to A-broth; the primary carbohydrate and nitrogen sources were varied. Five flasks were prepared for each treatment. Inorganic nitrogen sources were used at nitrogen levels equivalent to those of the organic nitrogen sources.

Amylase activities were estimated by a method similar to that of Smith and Roe (10), modified by Manning and Campbell (6). One amylase unit was defined as the quantity of enzyme hydrolyzing 1 mg of starch per minute at 60 C and pH 5.9.

**RESULTS AND DISCUSSION**

**Selection of production strain.** A total of 759 isolates were obtained from 22 dung, compost, and soil samples positive for thermophilic actinomycetes. Of these isolates, 218 produced substantial quantities of amylase, as determined by the size of zones of hydrolysis on the starch-containing isolation media. Many soil samples did not contain detectable thermophilic actinomycetes. In other samples, 5 to 100% of the isolates examined produced amylases. Probable duplicates (cultures isolated from the same sample and possessing the same colony morphology) and low-yielding strains were discarded; thus, 178 "different" cultures that possessed considerable amylolytic activity remained. Of these cultures, 142 survived the rapid growth (spore formation) and storage stability (3 months, 5 C) tests. Most isolates grew on medium II; 60 grew on starch medium VII. Among the 109 cultures that produced amylases in starch media II or VII, 10 were superior to all others. Three of these isolates (strains 5, 92, and 116) grew at temperatures of 37 to 60 C and had an optimal growth temperature of about 55 C. We were interested in these three cultures that had a wider growth range than the other amylolytic cultures examined because future plans included a comparison of amylases produced at widely divergent temperatures.

The three cultures were examined quantitatively for amylase production in starch media II and VII (Table 1); pH changes also were followed. Cultures 5 and 116 were superior in amylase production to culture 92 in starch media VII and II. Culture 5 produced more amylase than culture 116 in starch medium II; this culture was arbitrarily selected for further studies because future studies on amylase production in synthetic media were anticipated. However, the choice of culture 5 might have been unfortunate because control of culture pH by medium constituents probably would have been facilitated by selection of culture 116 (Table 1). Furthermore, culture 5 produced large quantities of proteinases, and the presence of these enzymes was a disadvantage during amylase purification.

Characteristics of culture 5 were compared with descriptions of thermophilic actinomycetes

<table>
<thead>
<tr>
<th>Strain no.</th>
<th>Medium VII</th>
<th>Medium II, 48 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>12 hr</td>
<td>24 hr</td>
</tr>
<tr>
<td></td>
<td>pH</td>
<td>per cent</td>
</tr>
<tr>
<td>5</td>
<td>7.4</td>
<td>22</td>
</tr>
<tr>
<td>92</td>
<td>6.6</td>
<td>19</td>
</tr>
<tr>
<td>116</td>
<td>6.9</td>
<td>21</td>
</tr>
</tbody>
</table>

* The initial pH values of media VII and II were 6.9 and 6.6, respectively.

* Percentage of starch in reaction mixture hydrolyzed by a 1:5 dilution of the culture filtrate.
given in Bergey's Manual and in other reports (1, 3, 5). The isolate closely resembled T. vulgaris; John Lacey (Rothamsted Experimental Station, Harpenden, Hertfordshire, England) kindly confirmed this identification. A transfer has been deposited as strain NRRL B-3196 in the collection of the Northern Utilization Research and Development Division, U. S. Department of Agriculture, Peoria, Ill.

Production of thermophilic actinomycete amylases. As shown in Table 2, inorganic nitrogen sources did not support substantial amylase production; growth was scant. The data in Tables 1 and 2 indicated that yields in synthetic media would be too low for these media to be useful in production of amylase for later purification procedures. On the other hand, growth was good in all media containing organic nitrogen sources, except the medium containing Thi tone (Table 2). N-Z Case and Trypticase were better than the other four primary nitrogen sources examined for amylase production.

Table 3 shows that maximal growth was obtained with fructose, maltose, mannitol, and soluble starch. As would be expected, maltose and soluble starch were the only two carbohydrates that induced substantial amylase production. Glucose, fructose, and mannitol were unique among the carbon sources examined; a pH of less than 6.0 was maintained after incubation of the cultures for 48 hr. The pH of cultures grown on maltose or starch also was relatively low, although high pH values were obtained when other carbon sources were used.

Levels of starch and N-Z Case were varied (Table 4) to discover the optimal concentration of each for amylase formation; 20% starch and

<table>
<thead>
<tr>
<th>Carbon source</th>
<th>Mycelial weight per flask (mg)</th>
<th>Final pH</th>
<th>Amylase units</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>104</td>
<td>8.1</td>
<td>3</td>
</tr>
<tr>
<td>Cellobiose</td>
<td>113</td>
<td>7.8</td>
<td>0</td>
</tr>
<tr>
<td>Fructose</td>
<td>240</td>
<td>5.9</td>
<td>2</td>
</tr>
<tr>
<td>Galactose</td>
<td>114</td>
<td>7.8</td>
<td>1</td>
</tr>
<tr>
<td>Glucose</td>
<td>163</td>
<td>5.6</td>
<td>4</td>
</tr>
<tr>
<td>Inulin</td>
<td>132</td>
<td>8.0</td>
<td>2</td>
</tr>
<tr>
<td>Lactose</td>
<td>121</td>
<td>8.1</td>
<td>4</td>
</tr>
<tr>
<td>Maltose</td>
<td>260</td>
<td>6.4</td>
<td>38</td>
</tr>
<tr>
<td>Mannitol</td>
<td>262</td>
<td>5.6</td>
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</tr>
<tr>
<td>Mannose</td>
<td>118</td>
<td>7.9</td>
<td>2</td>
</tr>
<tr>
<td>Sorbitol</td>
<td>138</td>
<td>8.2</td>
<td>2</td>
</tr>
<tr>
<td>Starch</td>
<td>196</td>
<td>6.9</td>
<td>54</td>
</tr>
<tr>
<td>Sucrose</td>
<td>149</td>
<td>8.2</td>
<td>2</td>
</tr>
</tbody>
</table>

1.0% N-Z Case were better than any of the other combinations examined.

Experiments utilizing 2.0% maltose or 20% starch showed that maltose was a better carbon source than starch for initial growth and later pH control of the culture. Starch was slightly better than maltose as an inducer of amylase formation. Since Suzuki and Tanabe (12) were able to increase amylase yields from Aspergillus oryzae by the addition of different carbohydrates at different stages of the fermentation, 35 combinations of concentration and time of addition of maltose and starch to T. vulgaris fermentations were examined. The optimal procedure (Fig. 1) was to start with an initial level of 1% maltose, and then, after incubation had proceeded for 24 hr, to add soluble starch to a concentration of 1%. Greatest quantities of amylase were present when the hyphae were beginning to lyse (Fig. 1) and the pH was rapidly becoming
alkaline. Additional studies showed that amylase was synthesized most rapidly at pH values between 6.5 and 7.5 and that amylase inactivation took place rapidly if the pH rose above pH 7.5. An initial pH of 5.9 was better than 5.5 or 6.5 because growth was slow at low pH values, and at high pH values, although growth was rapid, lysis of the mycelium occurred before substantial quantities of amylase accumulated in the medium.

Optimal procedures developed for amylase production by T. vulgaris strain 5 were: growth of the organism on A-agar plates for 48 hr at 55 C; inoculation of five agar discs, 11 mm in diameter, into 50 ml of A-broth; incubation for 12 hr at 55 C on a rotary shaker (Model V, New Brunswick Scientific Co.) set at 120 rev/min; utilization of 1 volume of the shaken culture to inoculate 9 volumes of A-broth containing 1% maltose; supplementation of the A-broth with 1% starch after incubation for 12 hr at 55 C; and harvest after continuation of incubation for 12 to 16 hr.

ACKNOWLEDGMENT
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