Production of Amylase in Liquid Culture by a Strain of *Aspergillus oryzae*

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The effect of different media and pH on the formation of amylase by *Aspergillus oryzae* EI 212 is described. Depending upon the composition of the medium and growth conditions, the fungus was found to secrete α- or β-amylase, or both. Some of the properties of the partially purified α-amylase were found to be different from α-amylases from other sources.

Various reports have appeared on the microbial synthesis of amylase on solid substrates (8, 13, 22, 23, 33) and in liquid media under stationary (27) and submerged conditions (2, 3, 5, 11, 12, 17, 20, 24-26, 30-32). It appears from all these reports that the production of amylase is dependent on the strains, composition of media and methods of cultivation.

*Aspergillus oryzae* strain EI 212 differs from other strains in the elaboration and the type of extracellular proteinases (15). It also produces high amounts of amylase when grown on a solid substrate (16). In view of these properties, it was considered worthwhile to study the extracellular amylolytic enzyme system of the organism in greater detail. The present communication describes the influence of different media upon the production of extracellular amylase by this fungus, the development of a suitable medium for maximum production of extracellular amylase in liquid culture fermentation, and some of the properties of the enzyme.

**MATERIALS AND METHODS**

**Production of fungal amylase.** Fresh cultures were prepared by inoculating the asparagine-starch agar slants with the stock culture of *A. oryzae* EI 212 (21). For static culture, 100-ml amounts of liquid media of different composition in 250-ml Erlenmeyer flasks were inoculated with the spore suspension obtained by adding 5 ml of sterile distilled water to a 48-hr agar slant prepared from fresh culture. For submerged culture, 100 ml of media in 250-ml Erlenmeyer flasks was inoculated with 10 ml of seed. This seed was prepared by adding 5 ml of spore suspension from fresh culture to a 100 ml containing NH₄NO₃ (0.6%), KH₂PO₄ (0.2%), MgSO₄ (0.2%), soluble starch (2%), and FeSO₄·7H₂O (trace). The seed medium and the submerged fermentation media were incubated in a Kahn-type shaker and shaken at 90 3-inch strokes per min, at 30 C for 72 hr. The enzyme activity was determined in the clear solution obtained by centrifuging the fermented liquids at 650 × g for 10 min.

**Preparation of partially purified enzyme.** Broth obtained by submerged fermentation of Roy's (30) modified medium containing 2% Pharmamedia was centrifuged at 650 × g for 10 min, and the clear supernatant fraction was decanted off, cooled to 0 C, and brought to 30% ethanol concentration. The precipitate formed was centrifuged off and discarded. The clear supernatant fraction was then brought to 70% ethanol concentration. The precipitate which was formed (650 × g, 20 min) was made to the original volume of the fermented liquid with distilled water and dialyzed against several changes of distilled water for 72 hr at 5 C. This solution (0.9 mg of protein/ml) was used in the study of the properties of the enzyme.

**Assay of amylase activities.** Saccharolytic amylase activity was assayed by the modified method of Bernfeld (7), and the amylase unit was defined as that amount of activity which produces 1 mg of maltose. Dextrinizing amylase activity was assayed by the modified method of Yamaguchi et al. (34). The assays were carried out at pH 5.0 and 50 C for 10 min.

**Molecular weight determination.** Molecular weight was determined by the method of Andrews (1) by comparing the elution volume of α-amylase with that of known proteins through Sephadex G-75 (Pharmacia Fine Chemical, Uppsala, Sweden).

**Estimation of protein.** Protein was estimated according to the method of Lowry et al. (19).

**Chemicals.** Pharmamedia was obtained as a gift from Traders Protein Division of Traders Oil Mill Co., Fort Worth, Tex., and hydroprotein, a pharmaceutical waste protein hydrolysate manufactured by Bengal Immunity Co., India, was purchased from a local market.

**RESULTS AND DISCUSSION**

Influence of different media upon the formation of amylase. A small quantity of amylase was formed in all the media under the stated pH con-
ditions. Some media, however, favored the stationary liquid culture fermentation at pH 7.5 and submerged culture fermentation at pH 7.0 in presence of 0.5% CaCO₃. Ohara's medium (25) containing NaNO₃ (0.3%) and urea (0.3%) as nitrogen sources was found to be suitable at pH 7.5 in submerged fermentation but Feniksova and Dvadsatova’s medium (12), containing NaNO₃ (0.9%) and malt (1%) as nitrogen sources, gave highest amylase yields (Table 1).

**Medium for maximum production of amylase.** Media which showed higher amylase production (Table 1) were modified further, and additional ones were also examined. The media which gave highest production of amylase are shown in Table 2. About 50% increase of amylase yield was made possible by the use of these media.

### Table 1. Influence of different media upon the formation of amylases by Aspergillus oryzae

| Fermentation medium | Units of saccharifying amylase activity/ml | Units of dextrinizing amylase activity/ml |
|---------------------|------------------------------------------|----------------------------------------|
|                     | Submerged culture | Stationary culture | Submerged culture | Stationary culture |
| Roy's medium        | 16.0             | 6.0               | 40.0             | Nil               |
| Mihasi and Tatsumi’s medium | 11.0           | 7.5               | Nil              | Nil               |
| Asai and Minoda’s medium | 10.8          | 9.3               | 7.9              | 5.8               |
| Ohara’s medium      | 17.6             | 16.0              | 44.0             | 35.5              |
| Feniksova and Dvadsatova’s medium | 18.5        | 19.5              | 78.0             | 63.0              |
| LeMense et al. media | 22.5           | 12.5              | 45.5             | 38.5              |
| Thin stillage + glucose | 16.5           | 11.5              | 43.5             | 33.5              |
| Thin stillage + corn meal | 17.8          | 12.6              | 44.5             | 43.5              |
| Thin stillage + lactose | 16.5           | 11.5              | 42.5             | 44.5              |
| Thin stillage + sucrose | 22.8           | 12.0              | 43.5             | 42.0              |

* For stationary culture, initial pH of the medium was 7.5 and for submerged culture, pH was 7.0 in presence of 0.5% CaCO₃ except in Ohara’s medium (pH 7.5). Fermentation temperature was 30°C.

* (NH₄)₂SO₄ (0.1%), MgSO₄ (0.05%), NaCl (0.2%), KH₂PO₄ (0.25%), NH₄NO₃ (0.5%), (NH₄)₂SO₄ (0.5%), MgSO₄ (0.5%), CaCO₃ (0.5%), corn starch (5%).

* NaCl (0.25%), KH₂PO₄ (0.25%), NH₄NO₃ (0.5%), (NH₄)₂SO₄ (0.5%), MgSO₄ (0.5%), CaCO₃ (0.5%), corn starch (5%).

* (NH₄)₂SO₄ (0.3%), KH₂PO₄ (0.1%), MgSO₄ (0.05%), soluble starch (3%).

* Peptone (2%), soluble starch (3%).

* NaNO₃ (0.3%), urea (0.3%), KCl (0.05%), KH₂PO₄ (0.1%), MgSO₄ (0.05%), FeSO₄·7H₂O (trace), tapioca starch (1%).

* NaNO₃ (0.9%), malt (1%), KCl (0.05%), MgSO₄ (0.05%), K₂HPO₄ (0.1%), FeSO₄·7H₂O (trace), Thin stillage (3%), carbohydrates sources (20%).

### Table 2. Development of media for maximum production of amylase by Aspergillus oryzae

| Fermentation medium | Units of saccharifying amylase activity/ml | Units of dextrinizing amylase activity/ml |
|---------------------|------------------------------------------|----------------------------------------|
|                     | Submerged culture | Stationary culture | Submerged culture | Stationary culture |
| Roy's medium        | 17.5             | 22.5              | 70.0             | 78.0              |
| Hydroprotein         | 18.5             | 8.5               | 115.0            | 46.0              |
| Pharmamedia         | 17.5             | 6.5               | 75.0             | 25.0              |
| Soybean meal        | 40.0             | 106.0             | 44.0             | 176.0             |
| Seed medium         | 22.5             | 23.0              | 45.5             | 38.5              |
| Hydroprotein         | 10.8             | 9.3               | 7.9              | 5.8               |

* Average of triplicate trials.

* The respective protein added in 2% concentration.

* Seed medium contains NH₄NO₃ (0.6%), KH₂PO₄ (0.2%), MgSO₄ (0.2%), soluble starch (2%), FeSO₄·7H₂O (trace).
over Feniksova and Dvadsatova's medium (12) was obtained in submerged fermentation by supplementing Roy's medium (30) with 2% Pharmamedia, and about 125% higher yield was obtained in static condition by adding 2% Hydroprotein as an additional nitrogen source in the seed medium developed in this laboratory (see above). Trace element and vitamin requirements of A. oryzae for amylase production were satisfied by these media.

Early commercial amylase fermentation processes employing A. oryzae used wheat bran as solid substrate. The submerged fermentation processes for amylase production have recently become economically feasible, and A. niger growing in starch-salt medium has been used for large-scale production of the enzyme (9). By careful manipulation of the composition of the media, pH, and the incubation period, the production of amylase by A. oryzae strain El 212 on solid (16) and in liquid culture fermentations can be achieved. Generally fungi (17) secrete α-amylase (dextrinizing enzyme), although a few fungi have been known to secrete α-amylase and β-amylase (saccharifying enzyme). A. oryzae El 212 secretes α- or β-amylase, or both enzymes, depending upon the composition of the medium and the fermentation conditions (Table 1). It appears that α-amylase but not β-amylase is formed in Roy's medium (30) supplemented with 2% Pharmamedia. Small saccharifying value associated with this amylase activity may be associated with pure α-amylase of similar dextrinizing activity (6).
Optimal temperature and thermostability of the enzyme. Optimal temperature for enzyme activity was 50 to 55 °C at pH 5.0 (Fig. 1). The enzyme was stable at pH 7.0 for 60 min at 25 to 60 °C.

**TABLE 3. Effect of different chemicals upon the amylase activity**

| Treatment | Relative activity | Treatment | Relative activity |
|-----------|------------------|-----------|------------------|
|            | Chemical alone | +EDTA | +Histidine             |                |                |
| Cu²⁺      | 0                | 73     | 69                |                |
| Hg²⁺      | 36               | 94     | 86                |                |
| Fe²⁺      | 63               | 89     | 86                |                |
| Mn²⁺      | 89               | 98     | 96                |                |
| Zn²⁺      | 43               | 69     | 69                |                |
| Ca²⁺      | 75               | 94     | 94                |                |
| Mg²⁺      | 89               | 108    | 108               |                |

*Five-tenths milliliter of 10⁻⁴ M solution of each reagent (0.5 ml of 10⁻⁴ M mercuric salt) was added to 1.5 ml of reaction mixture separately.*

At higher temperatures, enzyme activity decreased sharply and a complete inactivation occurred at 65 °C (Fig. 2).

**Optimal pH and pH-stability of the enzyme.** Optimal activity of the enzyme in citrate-phosphate buffer at 50 °C was at pH 4.5 to 5.0 (Fig. 3). When the enzyme was kept at 30 °C for 60 min and at pH 5.0 to 11.0, the enzyme was very stable but lost its activity sharply on either side of this pH range (Fig. 4).

**Effect of chemicals on amylase activity.** Cu²⁺, Hg²⁺, Zn²⁺, Fe²⁺, and Mn²⁺ ions were inhibitory to α-amylase, but the inhibitory effect was partially reversed by ethylenediaminetetraacetic acid (EDTA) or histidine (Table 3). Ca²⁺ and Mg²⁺ ions had an inhibitory effect on amylase activity, which was completely reversed by EDTA. The inactivation by Cu²⁺ and Hg²⁺ is indicative of the presence of thiol groups, carboxyl groups, or histidine residue in the enzyme molecule (10).

The reversal of metal ion inhibition by histidine and absence of inhibition of enzyme activity by thiol reagents are suggestive of the presence of serine and imidazole group (4). This enzyme did not require Cl⁻, Br⁻, or I⁻ ions for its activity and, thus, differed from α-amylase of pancreas and saliva (6).

**Molecular weight.** Molecular weight of this α-amylase was about 56,000.

**Michaelis constant.** The effect of substrate concentration on amylase activity was determined in 0.05 M phosphate buffer of pH 5.0 and at 50 °C. The Michaelis constant, \( K_m \), calculated graphically, according to Lineweaver and Burk (18) was \( 7.70 \times 10^{-3} \) g/2 ml (Fig. 5).

Some of the properties of α-amylases from different sources are given in Table 4 for comparison. It appears that α-amylase from A. oryzae...
Table 4. Some of the properties of $\alpha$-amylase from different sources

| Source                  | Optimum $\phi$H | Temp | Stability $\phi$H | Temp | Molecular weight | $K_m$ value \(a\) | Effect of chemical                        |
|-------------------------|-----------------|------|------------------|------|------------------|-------------------|------------------------------------------|
| Human saliva (6)        | 6.9             | C    | 4.0-11.0         | C    | 60,000           | $>6.3 \times 10^{-1}$ | Requires Cl$^-$                         |
| Barley malt (6)         | 5.0-6.0         | C    | 5.0-9.0          | C    | 45,000           | $1.8 \times 10^{-4}$ | Requires Ca$^{2+}$, does not require Cl$^-$ |
| Pig pancreas (6)        | 6.9             | C    | 7.0-8.5          | C    | 60,000           | $6.3 \times 10^{-4}$ | Requires NH$_3$, SH groups, and Cl$^-$   |
| Bacillus subtilis (6)   | 5.3-6.9         | C    | 4.0-10.5         | C    | 45,000           | 7.7 \times 10^{-6} | No effect with salts                     |
| Aspergillus oryzae      | 4.5-5.8         | 40.0 | 5.4-8.6          | 45.0 | 45,000           | 7.7 \times 10^{-6} | Ca$^{2+}$ and some other metal ions have inhibitory effect; Cl$^-$ has no effect |
| A. oryzae (14)          | 5.5-5.9         | 40.0 | 5.5-8.5          | 45.0 | 45,000           | 7.7 \times 10^{-6} |                                           |
| A. oryzae EI212         | 4.5-5.0         | 50-55| 6.0-9.0          | 60.0 | 56,000           | 7.7 \times 10^{-6} |                                           |

\(a\) Expressed in grams per milliliter except where otherwise indicated.

\(b\) Expressed in grams per 2 ml.

oryzae is different from $\alpha$-amylases from other sources.

The high yield of enzyme in liquid culture fermentation (Table 2) and its stability over a wide range of $\phi$H (Fig. 4) and temperature (Fig. 2) suggest that the strain EI 212 amylase merits consideration as a desirable source of dextrinizing enzyme.

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