European J Appl Microbiol Biotechnol (1981) 12:113–117

European Journal of Applied Microbiology and Biotechnology

Comparative Study of Amylolytic Enzymes Production by *Aspergillus niger* in Liquid and Solid-State Cultivation

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Summary. Amylolytic enzymes produced by a strain of *Aspergillus niger* cultivated on cassava starch in liquid or solid culture were found to be mainly glucoamylases. For the same initial amount of substrate, the glucoamylase activitiy increased even after 60 h of culture on solid medium whereas it decreased in liquid culture. Some characteristics of the amylases produced in both culture conditions were compared. The pH optima for enzymes produced in solid and liquid cultures were 4.5 and 5.0 respectively. Glucoamylase synthetized in solid cultures was significantly more thermostable than that from liquid culture and was maximally active at 70 °C compared to 50 °C for the enzyme from liquid cultures. The Km values expressed as mg soluble starch/100 ml were 0.1% for crude enzyme from solid culture and 0.057% for crude enzyme from liquid culture.

Introduction

Glucoamylases are produced by many species of filamentous fungi. Purified enzymes from numerous cultures have already been studied (Pazur and Ando 1959; Lineback et al. 1969; Freedberg et al. 1975). However, despite much work, few data are available concerning the time course of hydrolase production during liquid or solid culture (Kundu and Das 1970; Barton et al. 1972; Ramachandran et al. 1978; Nishio and Nagai 1979; Nishio et al. 1979). Furthermore, many of the properties of glucoamylases isolated from *Aspergillus niger* strains are still in dispute (Pazur et al. 1971; Lineback and Aira 1972; Freedberg et al. 1975).

Using an original culture device for solid state fermentation (Raimbault and Alazard 1980), it was possible to compare the kinetics of production and some properties of glucoamylases synthetized by *Aspergillus niger* on cassava meal in liquid and solid culture conditions.

Materials and Methods

Fungal Strain

A strain of *Aspergillus niger* isolated in our laboratory from rotting cassava tubers was used throughout the present investigation. This isolate was determined by the Centraalbureau voor Schimmelcultures at Baarn (Netherlands) as *Aspergillus hennebergi* Blochwitz. Raper and Fennell (1977) described this species as a natural mutant of *Aspergillus niger*. The mould was maintained on cassava meal agar slants at 4 °C.

Media Composition and Cultivations

The medium for liquid culture consisted of 10 g of cooked cassava meal, 0.97 g $(NH_4)_2SO_4$; 0.24 g urea; 0.5 g KH_2PO_4 and 1 l tap water. Solid state culture medium contained 10 g of cooked cassava meal; 0.97 g $(NH_4)_2SO_4$; 0.24 g urea; 0.5 g KH_2PO_4 and 10 ml tap water. The cooked cassava meal used was a coarse powder and had a moisture content of 9.5%. Liquid cultures (1 l) were grown in a Biolafitte fermentor at 35 °C, with agitation at 500 rpm and an air-flow of 40 l/h. The pH was adjusted to 3.5 after autoclaving and controlled automatically during growth with 0.5 N NaOH.

Solid state cultivations were grown at 35 °C in glass incubators by the method of Raimbault and Alazard (1980). Aeration was at 6 l/h with water saturated air. The pH of the liquid containing spores and mineral salts was adjusted to 2.7 before mixing with cassava meal and this resulted in a final pH of about 4.5. Both cultures were inoculated with a spore suspension prepared according to the method described previously using 2×10^7 spores per gram of cassava meal.

Analytical Methods

Duplicate aliquots (5 ml) of broth were removed from liquid cultures homogenized in a Potter homogenizer and diluted (1 : 10) before analysis.

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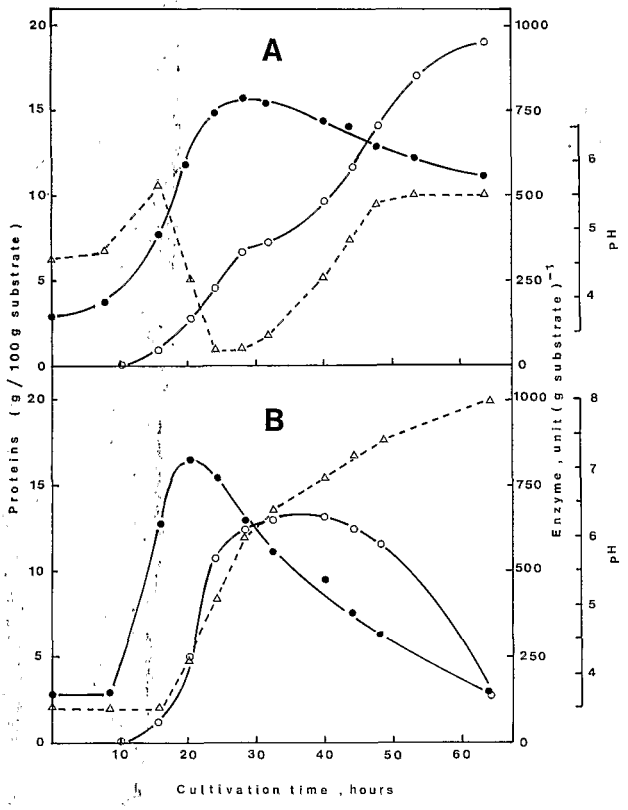

Fig. 1. Time course of pH, protein and amylolytic enzyme contents of the media. *A*, Solid state culture; *B*, Liquid culture. ●——●, protein content; o——o, Amylase activities; △---△, pH

Duplicate samples of three incubator contents from solid cultures were blended in distilled water (1:10) then diluted to about 1 mg dry weight per ml and analyzed.

A standard Technicon Auto-Analyser was used for automated determination of protein and reducing sugar. After an alkaline digestion as described previously (Raimbault and Alazard 1980), proteins were measured by the method of Lowry et al. (1951) using bovine serum albumin as standard (Industrial method n° 249-78-A, Technicon Instruments Corp. Tarrytown, N.Y. USA). Reducing sugars were determined by a modified ferricyanide method (Ind. method n°142-71-A, Technicon Instr. Corp.) adapted from Park and Johnson (1949). Glucose was determined by the Glucostat method (Worthington Biochemical Corp., Freehold, NJ, USA).

Hydrolysis products of soluble starch (Prolabo) were identified in the reaction mixture in a n-butanol, pyridine, water (6:4:3 V/V) solvent system described by French and Wild (1953). Reducing sugars were detected with the silver-dip method of Mayer and Larner (1959).

Enzyme Assay

Amylase activities were determined by measuring the amount of reducing sugars produced from soluble starch (Prolabo). The reaction mixture, containing 4.5 ml of 0.6% soluble starch in 0.05 M citrate buffer at the optimum pH for each enzyme and 0.5 ml of a suitable dilution of the enzyme solution, was incubated at various temperatures for 15 min. The reaction was stopped by heating the test tubes in a boiling water bath for 5 min. Control tubes containing the same fractions were immersed directly in the boiling water bath. Reducing sugars were estimated either by the ferricyanide method and expressed as glucose equivalents (i.e., the amount of glucose giving the same colour intensity) or, by the Glucostat method. The optimal conditions for enzymes from solid state cultivation were pH 4.5 and 65 °C and for enzymes from liquid culture, pH 5.0 and 50 °C.

In the assay conditions, liberation of reducing sugars was linear with respect to both time and enzyme concentration. One unit of amylase activity was defined as the amount of enzyme required to relaese one micromole of glucose per min under the conditions of the assay.

Results

Amylolytic Enzyme Production with Respect to Mycelial Growth

The time course of the changes in pH, protein content and amylase activity during solid and liquid cultures are shown in Fig. 1A, B respectively. In order to compare growth in liquid and solid media, proteins and amylase activities were calculated as percentages of the initial substrate present in the media (dry weight of cassava meal). In comparing amylase synthesis during culture, it was apparent that in both liquid and solid culture most of amylolytic activities were synthetized in the post exponential growth phase. In liquid culture, autolysis of mycelium after 24 h resulted in a decrease of the protein content and amylase activity. On the contrary, only a slight decrease of proteins and an increase of amylase activity were observed in solid culture even after 48 h of incubation. Therefore, maximum amylase production was reached after 40 h in liquid culture (650 units/g substrate) and after 64 h in solid culture (950 units/g substrate). In the latter case, it was observed that amylase synthesis was maintained even during sporulation which occured between 70 and 100 h of cultivation.

Determination of Amylolytic Enzymes Produced in Different Cultures Modes

Glucose was the only hydrolytic product that could be detected either from solid or liquid cultures by paper chromatography of the degradation products of soluble starch after one hour.

When reducing sugars were analyzed in incubation mixtures both by the ferricyanide method and by the glucose-oxidase method, a very close agreement between glucose and total reducing sugars was observed for solid culture preparations (Fig. 2A). In liquid culture, glucose represented only 85% of the reducing sugars (Fig. 2B).

From these data, we suggest that degradation of starch by this *Aspergillus niger* strain was mainly catalyzed

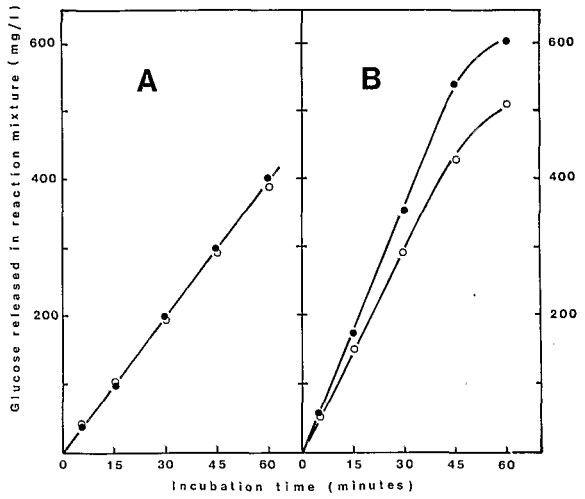

Fig. 2. Comparison of hydrolysis of soluble starch to glucose as measured by the ferricyanide method and by the glucose-oxydase method. *A*, Enzymes from a 40 h solid state culture. 0.11 mg protein was assayed at 60 °C and pH 4.5. *B*, Enzymes from a 24 h liquid culture. 0.09 mg protein was assayed at 50 °C and pH 5. ●—●, Ferricyanide method; ○—○, glucose-oxydase method

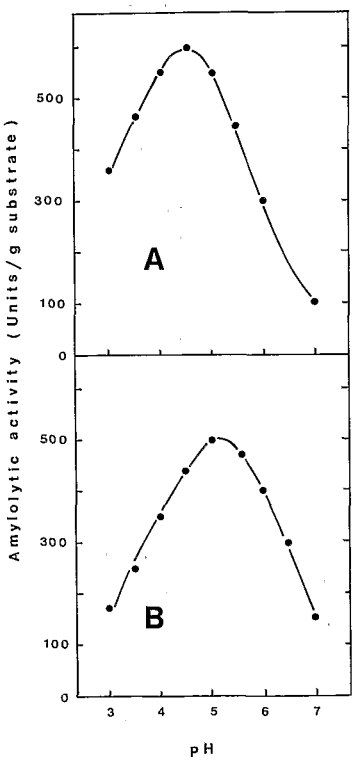

Fig. 3. Effect of pH on glucoamylase activities of: *A*, *Aspergillus niger* grown in solid culture. *B*, *Aspergillus niger* grown in liquid culture. Activities are expressed in units per gram of initial cassava meal

by glucoamylases in solid cultivation, but a low α-amylase activity was also evident in liquid culture.

From three successive washings with distilled water and centrifugation at 10,000 g for 15 min, 32% and 26% of the total amylolytic activities were recovered in the supernatant from solid and liquid samples respectively. These data suggest that glucoamylases are strongly bound to the mycelial cell walls.

Effect of pH on Glucoamylase Activities

The optimal pHs for amylolytic enzymes were determined in 0.05 M citrate buffer under the standard assay conditions. Enzymes produced from solid and liquid cultures had an optimum pH of 4.5 and 5.0 respectively (Fig. 3). Although a difference of 0.5 pH units was observed between the two culture conditions, optimum pHs were in the range of values quoted in the literature for amyloglucosidases produced by *Aspergillus niger* (Lineback et al. 1969; Freedberg et al. 1975).

Thermostabilities and Optimal Temperatures of the Enzymes

Temperature inactivation was studied by incubating the enzyme solutions for various times at temperatures between 45° and 75 °C and then cooling and carrying out the assay procedure. From solid state culture, the enzyme was stable at pH 7,0 for 30 min between 45° and 65 °C. At higher temperatures, enzyme activity decreased sharply and a complete inactivation was observed after 15 min at 75 °C (Fig. 4A).

From liquid culture, inactivation occured at 55 °C and the activity was completely lost in 15 min above 65 °C (Fig. 4B).

Optimum temperatures, determined by carrying out the standard assay procedure at temperature between 30° and 85 °C, were 65° to 70 °C and 50 °C for solid and liquid cultures respectively (Fig. 5).

The results show that the enzyme prepared from solid state culture were significantly more resistant towards thermal denaturation than those from liquid cultures. As might be predicted from this, the enzyme from solid cultures had a higher optimal temperature than the enzyme prepared from liquid cultures.

Effect of Soluble Starch Concentration on Enzymes Activities

The effect of the substrate concentration on amylase activities was determined in 0.05 M citrate buffer pH 4.5

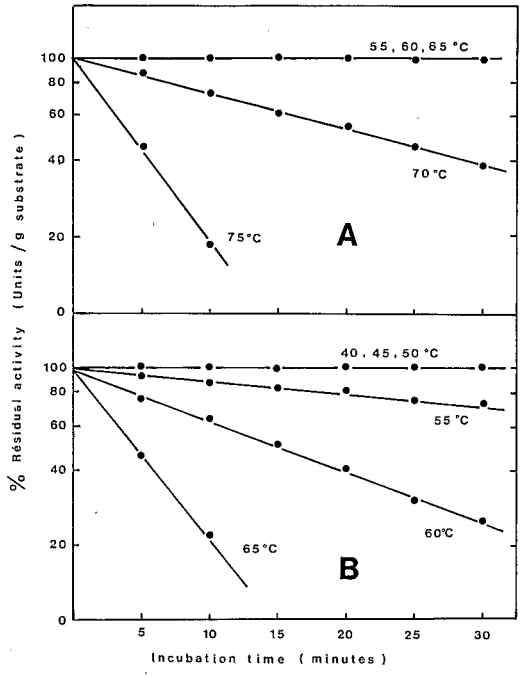

Fig. 4. Influence of temperature on the stability of amylolytic enzymes produced in solid state culture (*A*) and in liquid culture (*B*). Incubation conditions are described in text

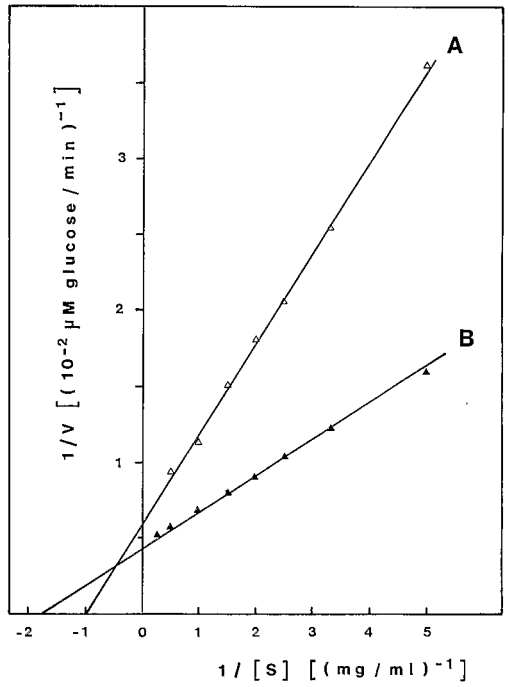

Fig. 6. Lineweaver-Burk plots of soluble starch hydrolysis by amylolytic enzymes of *Aspergillus niger*. The reaction mixture containing 4.5 ml of substrate solution and 0.5 ml of the enzyme solutions (85.7 and 88 µg proteins for solid and liquid culture preparations respectively) was incubated for 10 min at constant temperature. *A*, Solid culture crude preparation. *B*, Liquid culture crude preparation

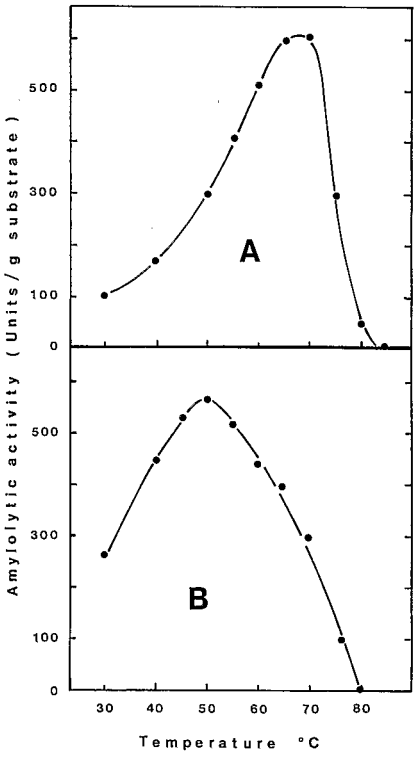

Fig. 5. Effect of reaction temperature on activities of glucoamylases (*A*) from solid state culture and (*B*) from liquid culture. Experimental conditions are described in text

at 65 °C for crude enzymes from solid cultures and pH 5.0 and 50 °C for crude enzymes from liquid cultures.

The Michaelis constants for crude amylolytic enzyme systems calculated graphically according to Lineweaver and Burk (1934) are shown in figure 6. Km values expressed as mg soluble starch/100 ml were 0.1% and 0.057% for solid and liquid cultures respectively [as the actual molecular weight of soluble starch (Prolabo) is not known].

Discussion

Using the culture device described in a previous paper (Raimbault and Alazard 1980) it was possible to compare the characteristics of amylases produced by *Aspergillus niger* strain in liquid or solid culture on the same substrate (cassava meal).

An important difference was the two phase production of amylase activity in solid culture. The first phase took place during active growth and protein synthesis and the pH decreased from about 5.0 to 3.5; the second phase corresponded to the secondary metabolism occuring at submaximal growth rates when the pH increased to 5.5. For the same initial amount of substrate, in liquid cul-

ture (corresponding to a 50 fold dilution of substrate and fungus cells) autolysis of mycelium occured more rapidly and the amylase concentration decreased after 40 h.

We suggest that the solid culture conditions protected cells from lysis, thus allowing production of amylases during the non growing phase.

It should be noted that α-amylase could only be detected in liquid cultures, therefore amylolytic enzyme synthesis might be greatly influenced by the water content of the medium.

An other important feature was the significant differences of some properties of the amylolytic activities associated with the conditions of growth. The affinity of crude enzyme preparations from mycelia grown in liquid culture for soluble starch was found to be about twice that of mycelia from solid culture. However, amyloglucosidases synthetized during solid state culture exhited a higher thermotolerance, an advantageous property with respect to industrial use of amylolytic enzymes (Ramachandran et al. 1978). As the influence of temperature on amylolytic activities was measured by incubating crude preparations without substrate (see text), it can be assummed that differences in the enzyme structure itself account for the different thermostabilities we observed in enzyme prparations from liquid and solid cultures.

We are presently purifying the enzyme fractions isolated from solid and liquid cultures in order to compare the characteristics of amylolytic enzymes produced by the same organism on the same substrate under the two conditions of growth.

Acknowledgements. This work was supported by the French 'Direction Générale de la Recherche Scientifique et Technique', grant N° 78.7.0420. The authors thank Dr. J. Baldensperger for discussing the manuscript and Professor Jacques C. Senez for scientific assistance.

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Received November 6, 1980