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Culture Conditions Dictate Protease and Tannase Production in Submerged and Solid-State Cultures of *Aspergillus niger* Aa-20

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Abstract

Undesirable protease production by *Aspergillus niger* Aa-20 in submerged culture and solid-state culture was evaluated using different concentrations of tannic acid as sole carbon source in a model system designed for tannase production. Protease production was found to be dependent on the culture system used (submerged culture or solid-state culture) and on the initial tannic acid concentration. Expression of protease activity in submerged culture was higher (up to 10 times) than activity obtained in solid-state culture, using identical culture medium composition. In submerged culture, the lowest final protease activity (0.13 IU) was obtained with the highest tannic acid concentration, while in solid-state culture protease activity was not affected by changes in initial substrate concentration. Absence of detectable proteolytic activity in solid-state culture is related to high production of tannase enzyme. Hence, the use of solid-state culture for fungal enzyme production may allow for higher and more stable enzyme titers present in culture extracts.

Index Entries: Protease; solid-state fermentation; tannase; tannin acyl hydrolase; liquid fermentation; submerged fermentation; *Aspergillus niger*; tannic acid.

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Introduction

Submerged culture and solid-state culture systems have been used to compare activity titers of several industrial enzymes, such as invertases *(1)*, amylases *(2)*, pectinases *(3,4)*, tannases *(5)*, and proteases *(6)*. In all cases, titers for enzymes produced by solid-state culture were higher than those obtained by submerged culture. In addition, enzymes produced by solid-state culture exhibited activity over a wider pH range and were more thermotolerant than the same enzymes produced by submerged culture *(7)*. Such differences have been attributed to several factors: water activity *(2,8,9)*, cell membrane composition *(4)*, and substrate diffusion *(10)*. However, the same enzyme produced by submerged culture and solid-state culture shows different induction and production patterns *(11)*, which are highly influenced by culture conditions *(12)*.

In addition to high levels and stability of enzymes resulting from solid-state culture, production of microbial enzymes is an attractive process, because of low-technology requirements and prevention of bacterial contamination during culture at low water activity values, characteristic of solid-state culture processes.

Protease production has been extensively studied by submerged culture *(11–15)* and by solid-state culture *(16–21)*. Proteases produced by solid-state culture are used in foods, fermentation processes, and detergent additives or as depilatory agents in the leather industry *(22)*. Godfrey and Reichelt (23) reported that proteases are probably the most important class of industrial enzymes in worldwide use. Until recently, most studies focused on screening proteases with a criterion set only to increase activity levels *(24)*. There are, however, an increasing number of reports on proteases having specific properties such as substrate specificity, regioselectivity, chiral selectivity, thermostability, and solvent stability *(25)*. However, when the target enzymes are not proteases (e.g., tannases), then proteolytic activity becomes unsuitable since it considerably reduces final titers and the stability of desired enzymes, reducing their potential in industrial applications. Thus, millions of dollars are spent in trying to eliminate this proteolytic activity, such as by selecting protease-deficient strains or by using protease inhibitors.

Comparative studies of protease production in submerged culture and solid-state culture are scarce *(26,27)*. Yang and Wang *(27)* reported higher protease production in solid-state culture than submerged culture. In addition, the enzyme activity produced in solid-state culture was more stable toward pH and temperature changes than those produced in submerged culture. Generally, most protease production studies by solid-state culture involve the use of proteinaceous substrates with the objective of inducing such an enzymatic activity *(18,19)*. However, recently it was reported that protease production in solid-state culture using nonproteinaceous substrates employed as supports to produce hydrolytic enzymes (i.e., polygalacturonase, cellulase, and xylanase) was slightly associated with the stabilization of the enzymatic extract *(28)*.

In the present study, protease and tannase productions were monitored, in a model system, using tannic acid as inducer of tannase activity in both submerged culture and solid-state culture. Differences in protease production by submerged culture and solid-state culture were evaluated using a model system primarily designed for tannase production but with the aim of minimizing unwanted proteolytic activity by the appropriate choice of culture conditions.

Materials and Methods

Organism and Culture Medium

Inoculum was prepared by transferring *Aspergillus niger* Aa-20 spores to potato dextrose agar medium followed by incubation at 30°C for 5 d. The spores were then scraped into a 0.01% Tween-80 solution and counted in a Neubauer chamber.

Medium for enzyme production was the same as reported previously by Aguilar et al. *(12)*. Salt-containing medium was autoclaved at 121°C for 15 min. Tannic acid (Sigma, St. Louis, MO) solution was filter-sterilized and added to a final concentration of 12.5, 25.0, 50.0, or 100.0 g/L, respectively. Unless stated, for all experiments, a C/N ratio of 11.1 was used.

Submerged Culture

Erlenmeyer flasks (250 mL) with 50 mL of sterile liquid medium at an initial pH of 5.5 were inoculated with 2.5 mL of the spore suspension (2×10^7 spores/mL of suspension). Incubation was carried out at 30°C on a rotary shaker (220 rpm) for 48 h. Cultures were monitored over a 48-h period. Biomass was separated by filtration through Whatman No. 41 filter paper, and quantified by dry weight. The cell-free culture broth was assayed for tannase and protease activities.

Solid-State Culture

Solid-state culture involved the use of polyurethane foam (Expomex, México) as a support to absorb the liquid medium. Polyurethane foam was washed as reported by Zhu et al. *(29)* and then pulverized in a plastic mill. Column reactors (25×180 mm) were packed with 10 g of inoculated medium (2×10^7 spores/g of dry inert support). Culture conditions were as follows: temperature, 30°C; aeration rate, 20 mL of air/(g of support · min); initial pH, 5.5; initial moisture content, 65%; incubation time, 48 h. Solid-state culture was monitored over a period of 48 h. Enzyme leaches were obtained by mixing the content of each reactor with distilled water (1:10 [w/v]) and vortexing for 1 min. Solid particles were filtered (Whatman No. 41) and the clear filtrate was assayed for tannase and protease activities.

Enzyme Production in Presence of Protease Inhibitor

For tannase and protease production by submerged culture in the presence of a protease inhibitor, cultures were grown as already described,

with an initial tannic acid concentration of 12.5 g/L. A 10-µL vol of a protease inhibitor cocktail specific for fungi (P-8215; Sigma) was added at 10 h of culture. The inhibitor effect on fungal biomass production was then evaluated. Cultures were carried out in duplicate over a 48-h period.

Use of Different C/N Ratios

To evaluate the effect of C/N ratio on tannase and protease production in solid-state culture, two C/N ratios (4.5 and 11.1) were tested. The initial concentration of tannic acid (25 g/L) was kept constant in both cases. All experiments were performed in triplicate, and samples were collected for analysis after 40 h of growth.

Enzyme Assays

Protease activity was assayed using the modified spectrophotometric method of Dosoretz et al. *(30)*. One unit of protease was defined as the amount of enzyme that catalyzes the release of 1 µmol of the azo dye molecule that produces a change in absorbance (λ = 520 nm) of 0.001/(mL · min). Tannase activity was assayed using the high-performance liquid chromatography methodology proposed by Beverini and Metche *(31)*. One unit of enzyme was defined as the amount of enzyme releasing 1 µmol of gallic acid/(mL · min). Each point represents the mean of the experimental values obtained from triplicate samples.

Results and Discussion

Protease activity produced by *A. niger* Aa-20 was inversely related to initial tannic acid concentration in the submerged culture system (Fig. 1A). However, in solid-state culture, protease production was independent of the initial tannic acid concentration and at least six times lower than the minimal proteolytic activity obtained in submerged culture (Fig. 1B). Protease activity was more than 10 times higher in submerged culture than in solid-state culture using the same culture medium. The results presented in Fig. 1A show that the lowest final protease activity in submerged culture (0.13 IU) was obtained in the culture medium with the highest initial tannic acid concentration.

Previous results showed that tannase was produced up to six times more in solid-state culture than in submerged culture and was associated with a reduction in tannase activity owing to proteolytic activity *(21)*. Studies carried out by Ramesh and Lonsane *(2)* also demonstrated more α-amylase production by *Bacillus licheniformis* in solid-state culture than in submerged culture; differences were associated with low levels of enzyme end products around the cells. Maldonado and Strasser de Saad *(4)* demonstrated differences in membrane composition of cells growing under solid-state culture and submerged culture conditions. Although the low activity titers obtained in submerged culture might be related to at least one of these

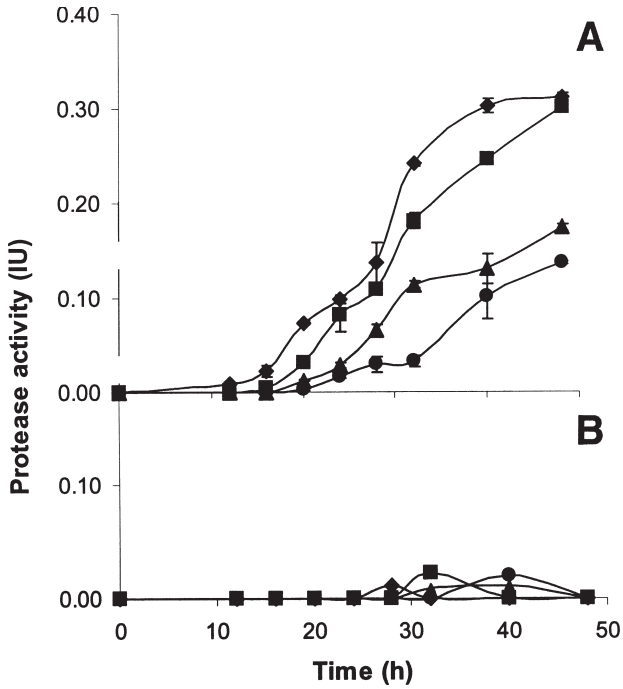

Fig. 1. Protease production by *A. niger* Aa-20 in **(A)** submerged culture and **(B)** solid-state culture at initial tannic acid concentrations of (◆) 12.5, (■) 25, (▲) 50, and (●) 100 g/L.

factors, the results obtained in our work demonstrate that proteolytic activity participates in the depletion of tannase activity in submerged culture.

On the other hand, in solid-state culture, a decrease in the C/N ratio significantly affected protease levels. Proteases were undetectable at a C/N of 4.5. However, at a C/N of 11.1, 0.24 ± 0.01 IU of proteases was measured. An increase in the nitrogen source (corresponding to a C/N ratio of 4.5) reduced protease production as compared to that obtained at a C/N ratio of 11.1. In addition, tannase titers were higher when the nitrogen content was increased (4.9 ± 0.3 IU at a C/N of 4.5 and 3.5 ± 0.1 IU at a C/N of 11.1). Low protease activity at high tannic acid concentration *(22)* might also be related to quenching of extracellular protease by tannic acid, which is a well-known phenomenon, tannic acid being an effective protein precipitant. Nevertheless, even when lower values of protease were found in the culture medium with high tannic acid concentration (100 g/L), protease activity was present at all tannic acid concentrations tested in submerged culture.

To demonstrate the effect of protease activity on the production of a nonproteolytic enzyme (tannase), experiments were conducted in submerged culture using a cocktail of protease activity inhibitors, with an initial tannic acid concentration of 12.5 g/L (at which the highest proteolytic activity was found in submerged culture). The addition of such a cocktail considerably reduced protease activity (Fig. 2B). Nevertheless, without pro-

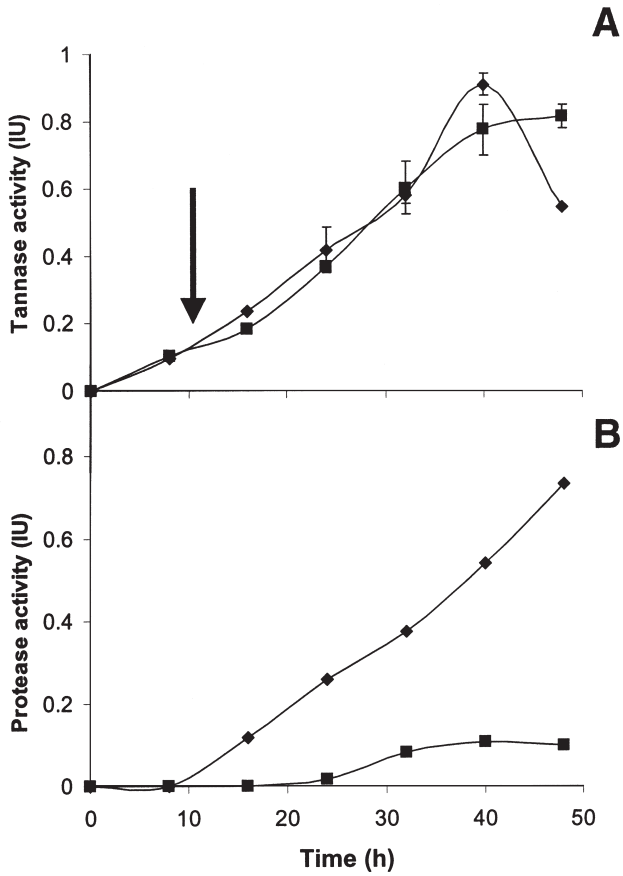

Fig. 2. Effect of addition of protease inhibitor on **(A)** tannase and **(B)** protease activities by *A. niger* Aa-20 in submerged culture: (■) with and (◆) without inhibitor. The arrow indicates the time at which the protease inhibitor cocktail was added.

tease inhibitors, proteolytic activity was eight times higher than in their presence. The effect of the addition of the protease inhibitor cocktail to the culture medium was also evaluated. Tannase activity decreased after 40 h of culture (Fig. 2A) without the addition of inhibitor; however, tannase activity remained constant up to 50 h of cultivation in its presence. Note that protease inhibitors did not affect fungal biomass production (Fig. 3).

Ours is the first report comparing the presence of protease in enzymatic extracts obtained from different fermentation systems (submerged culture and solid-state culture). It stresses the advantage of using solid-state culture to minimize unwanted proteolytic activities during the production of an inducible enzyme such as tannase.

Industrial enzyme production represents more than $600 million in sales, and most of it is produced by submerged cultivation *(32)*. Many multinationals spend enormous amounts of money trying to obtain strains deficient in extracellular proteases. In the present study, we have shown

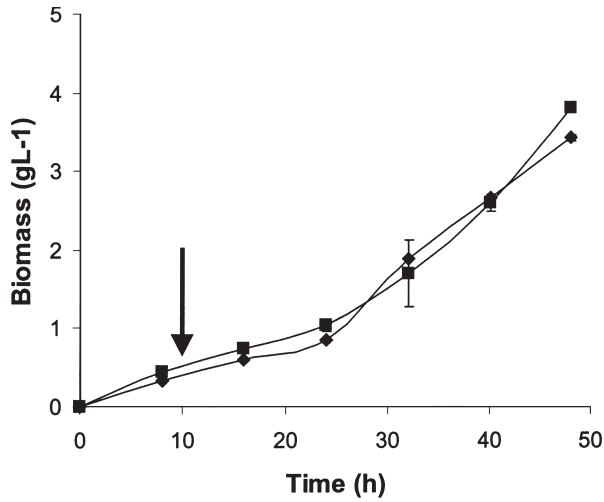

Fig. 3. Effect of addition of protease inhibitor cocktail on biomass production from *A. niger* Aa-20 in submerged culture: (■) with and (◆) without inhibitor. The arrow indicates the time at which the protease inhibitor cocktail was added.

that the use of solid-state fermentation could be an interesting alternative for the production of a given target enzyme but without the interference of unwanted proteolysis.

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