

Preliminary culture conditions for *Aspergillus niger* GH1 tannase production in submerged culture

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Abstract

Nowadays, tannase is produced by submerged culture (SmC) being the enzyme obtained in an intracellular form resulting, as a consequence, in high production costs. To demonstrate that fungal tannase excretion is strongly influenced by the culture conditions, tannase production by *Aspergillus niger* GH1 on SmC using different bioreactors was evaluated. Two sets of experiments were conducted using in a first step a 1.5 L bioreactor and in a second step 100 mL erlenmeyer flasks. Modifications to culture conditions previously reported allowed to obtain positive results for tannase production in SmC, being the selected conditions: initial pH 5, temperature 35°C, initial substrate concentration 25g/L and agitation 200 rpm.

Resumo

A cultura submersa (SmC) é o processo empregado para produzir comercialmente a enzima tanasa, porém neste sistema da cultura, a tanasa é expressada principalmente de maneira intracelular o que representa custos de produção elevados. Para demonstrar que a excreção da tanasa do fungo está fortemente influenciada pelas condições da cultura, a produção da enzima tanase por *Aspergillus niger* GH1 em SmC foi avaliada. Dois conjuntos de experiências foram conduzidos usando em uma etapa um fermentador de 1.5 L e em uma segunda etapa matrizes (erlenmeyers) de 100 mL. As modificações às condições da cultura previamente descritas permitiram a obtenção de resultados positivos para a produção do tannase neste modo de produção, tendo sido definidas as seguintes condições de cultura: pH inicial 5, concentração inicial de substrato 25g/L, temperatura 35°C e agitação 200 rpm.

Introduction

Tannase hydrolyzes the molecule of tannins by its esterase activity, giving gallic acid and glucose as products [1]. Tannase can be obtained from animal, plant and microbial sources being this the most important, because the enzyme produced by the microbial route is more stable than the analogous obtained by other sources. Also, microorganisms can produce larger amounts using friendly techniques of fermentation that coupled with genetic manipulation result in an increment in the activity of the produced enzyme [2]. The production on commercial scale of tannase is through submerged fermentation – SmC - using filamentous fungi in which the enzyme is expressed in an intracellular form being located in cell periplasm [3]. The enzyme is retained in the cell wall of the fungus requiring, as a consequence, an efficient recovery and purification so that an enzyme extract with high activity is obtained [4]. Tannase has many applications in the food industry, in particular in the juice industry. The production system to obtain this enzyme has been

intensively studied and with the aim of developing an efficient processes new culture conditions, new reactors and new strains have been investigated. Solid state fermentation – SSC - has been reported as an attractive process alternative [5] as, in this system, tannase is excreted from the hyphae in higher levels than in SmC and has been reported as more stable in a wide range of temperatures and pH values.

Results on the effect of agitation, temperature, air flowrate and substrate concentration on tannase production by *Aspergillus niger* GH1 in submerged fermentation are presented.

Materials and Methods

Microorganism

The fungus obtained from a lyophilized stock was hydrated with sterile distilled water. The obtained sample was inoculated in a plate with potato dextrose agar (PDA) and incubated at room temperature during one week. In the next step, the fungus was inoculated in other PDA containing plate and the obtained pure culture was distributed in three parts - one to be stored, one to be used as a working culture and another for the fermentation experiments.

Submerged culture

The effect of temperature (25 and 35°C) on the *Aspergillus niger* GH1 biomass production was evaluated in a 1.5L bioreactor, at an agitation rate of 200 rpm. An initial tannic acid concentration of 25 g/L on a Czapek dox medium and a pH of 5.5 were used. The spore's concentration was 1×10^6 spores/mL. In the experiments using 100 mL Erlenmeyer flasks, the effect of pH on tannase production was evaluated for initial pH values of 3 and 5. The use of mycelia or spores as alternative inoculation processes was also considered.

Tannase assay

One unit of tannase was defined as the amount of substrate (methyl gallate) consumed per minute under assay conditions. The used protocol is as follows: the blank contained 100 μ L buffer acetate 50 mM pH 5 and 100 μ L of methyl gallate 0.1M; the sample was formed by 100 μ L of extract and 100 μ L of methyl gallate and the control by 100 μ L of buffer with 100 μ L of extract; solutions were incubated at 30°C for 30 minutes and the reaction stopped by the addition of 200 μ L of 2M HCl.

Analytical methods

The consumed substrate was evaluated using the phenol sulphuric acid method (Dubois 1954), the protein was determined with the Bradford method and the biomass concentration by dry weight. Intracellular extracts were obtained by disrupting the cells with liquid nitrogen and the proteins resuspended with acetate buffer.

Results and Discussion

Fig. 1 shows the main fermentation results for the experiments carried in the 1.5 L agitated tank, for two temperatures – 25 and 35 °C. Samples were taken each 8 h in a total operation time of 120 and 90 hours. The results presented in Fig. 1A show that biomass concentration decreases during the first 48 h while, for the higher temperature (Fig. 1B) this decrease is prolonged till 60 hours of fermentation.

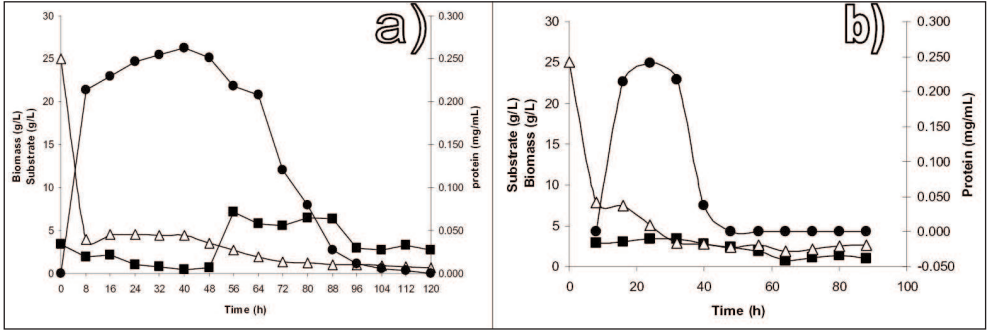


Figure 1. Substrate degradation (Δ), protein production (\bullet) and biomass production (\blacksquare) at 25°C (A) and 35°C (B) for the fermentation experiments in a 1.5 L bioreactor

Following the results obtained in the 1.5 L bioreactor, it was necessary to have a more detailed evaluation of the fermentation behaviour due to the fast uptake of substrate that occurs during the first 12 hours. The same trend has not been observed for biomass production and has been different according to the used temperature.

More detailed information concerning biomass formation was obtained by carrying fermentation experiments in shake flasks. Experiments were done using two types of inoculum – spores and homogenous fungal mycelium - as enzyme production might reduce the lag phase for microbial growth when mycelium is used. These experiments also aim at giving relevant information for the establishment of the best fermentation parameters. Figure 2A shows the amount of biomass produced in the fermentation process mentioned above during the first growth stages. The production of biomass during this analysis presented a constant performance in the experiments with spores and mycelium.

Concerning substrate degradation, (Figure 2B) no major change was observed due to the short fermentation time. The fungus had not enough time for developing remaining in the lag phase, although a small reduction in substrate concentration was observed. Protein content in the extracellular space increased slightly as the fungus is still starting the biosynthesis of the enzymes production system after 12h of fermentation. Although protein concentration is a reference parameter for tannase production, no tannase was measured in the extract after 12 hours of fermentation. Figure 2D shows a similar performance concerning protein in the culture medium when different types of inoculums are used. The intracellular tannase activity was also measured for these fermentations, being observed that the inoculum properties affect the intracellular enzyme production. After 12 hours, a higher tannase concentration was obtained for the experiment done using spores as inoculum. The amount of enzyme was clearly smaller for the other types of inoculum (Figure 2C). The obtained results require that longer fermentations are done to have a correct evaluation of the expression of tannase, both intracellular and extracellular.

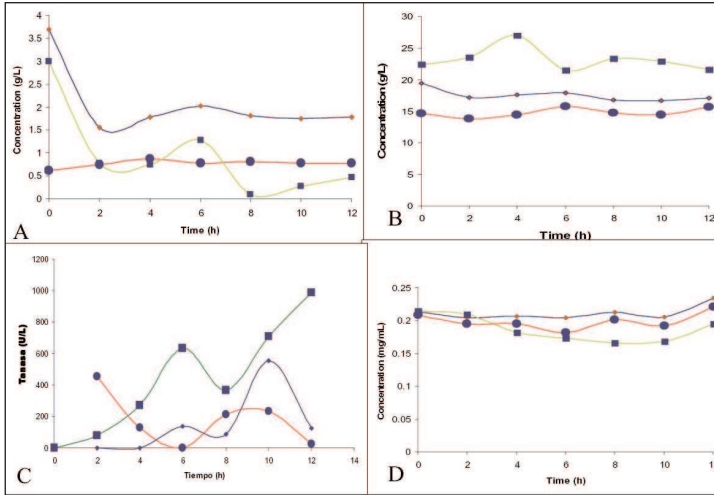


Figure 2. A) Biomass production, B) Substrate degradation, C) Tannase production D) Protein production using different types of inoculum.

*(\square) first fermentation using spores, (\diamond) Fermentation using spores (\circ) Fermentation using fungal mycelium

Production of tannase can be done by various methods of fermentation as liquid surface, submerged, modified solid-state fermentation and solid-state fermentation. The use of submerged fermentation is advantageous because of the easy sterilization and the better process control process in these systems. Also, recovery of the products is easier.

Finally, several fermentations running for a longer time were done. The first variable evaluated was substrate degradation being observed that substrate concentration starts to diminish after 8 hours of fermentation achieving the lowest concentration at 48 h. At pH 5, almost complete substrate consumption is obtained. No relation could be established between substrate consumption and the production parameters. In what concerns biomass, a reduction in biomass concentration is observed, when most of the substrate consumption occurs, instead of the expected increase. The amount of protein also shows a decrease as fermentation proceeds and no protein is detected at the end of the process. Two different initial pH values were evaluated as formation of a precipitate was observed when 0.1 M NaOH was added to the fermentation medium in order to increase the initial pH. Figure 3A shows the fermentation behaviour at pH 3 and pH 5 - pH 3.0 is the standard value in the culture medium and the pH 5.0 is the adjusted pH. The results show that substrate degradation is higher when the medium pH is adjusted to 5, although a similar degradation profile is observed for both pH values. The rate of tannase production was slow being a significant increase in intracellular tannase concentration observed at 96 h of fermentation for pH 5.0. Results presented in Figures 3A and 3B clearly demonstrate the importance of adjusting the initial pH to 5 in order to obtain higher values of tannase concentration.

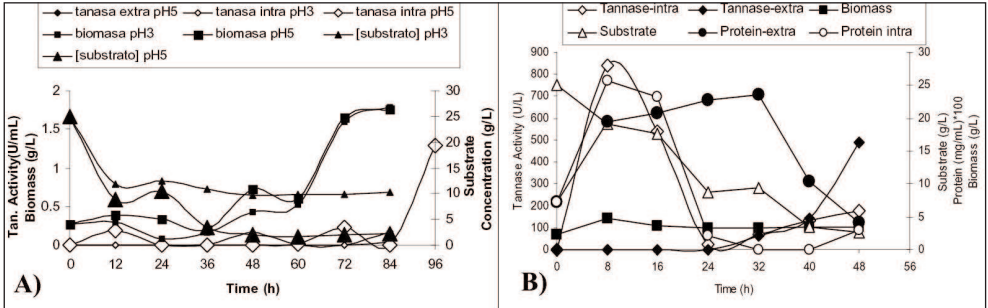


Figure 3. Time evolution of the fermentation parameters at pH 5 and pH 3 (a) and a detailed presentation of the fermentation parameters at pH 5 (b).

As the several fermentation runs were being carried and the obtained values for the fermentation parameters were not satisfactory, an optimization of the used analytical techniques was done and several improvements dealing with biomass, protein and tannase activity determination implemented. New procedures included dialysis of the samples, use of polyethylene glycol for samples concentration, cellular rupture using liquid nitrogen and measurement of tannase activity products by HPLC.

The application of these improved analytical procedures allowed for the obtention of the values represented in Figure 4. A clear biomass growth is observed in parallel with a decrease in substrate concentration and an increase in enzyme concentration.

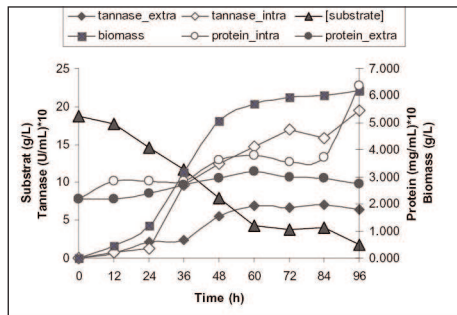


Figure 4. Time evolution of the fermentation parameters at pH 5, using the optimized analytical procedures

It is also shown that the intracellular tannase production is higher what may constitute a major disadvantage for this fermentation process as, usually, complicated recovery techniques are needed. Nevertheless, we must take in account that extracellular tannase production was also detected.

Conclusions

The best fermentation conditions for tannase production using submerged fermentation were: initial pH 5.0, temperature 35°C, initial substrate concentration 25g/L and agitation 200 rpm. In these conditions, most of the produce tannase was intracellular.

In order to correctly evaluate the evolution of the fermentation experiments, improvements in the used analytical techniques were developed and implemented.

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