academicJournals

Vol. 7(10), pp. 197-202, November, 2013
DOI: 10.5897/AJBR12.073
ISSN 1996-0778 ©2013 AcademicJournals
http://www.academicjournals.org/AJBR

African Journal of Biochemistry Research

Full Length Research Paper

Production of tannase and gallic acid by *Aspergillus tamarii* in submerged and solid state cultures

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Accepted September 17, 2013

The hydrolytic enzyme, tannase and the antioxidant phenolic compound, gallic acid are useful in many biotechnological processes especially in food and pharmaceutical areas. The purpose of this study was to evaluate the production of tannase and gallic acid by *Aspergillus tamarii* developed in submerged and solid state cultures using tannic acid as substrate. In submerged cultures, maximal tannase activity (20,400 ± 2,900 U/L) was obtained after 2 days of cultivation using 2% tannic acid as substrate. In solid state cultivation using polyurethane foam as inert support, maximal tannase activity was obtained after 4 days of cultivation in 15% tannic acid cultures (25,470 ± 1,600 U/L). In both types of cultures, high accumulation of gallic acid was found in the two day-culture filtrates, 0.36±0.05 and 0.67±0.08 g of gallic acid per g of tannic acid, in submerged and solid state cultures, respectively. The accumulation of gallic acid in the cultures is, however, a transitory phenomenon, considering that the fungus slowly absorbs and metabolizes gallic acid.

Key words: *Aspergillus tamari,* gallic acid, inert support, solid-state cultures, submerged cultures, tannase.

INTRODUCTION

Tannin acyl hydrolase (EC 3.1.1.20), commonly called tannase, catalyzes the hydrolysis of ester bonds into hydrolysable tannins such as tannic acid, thereby releasing glucose and gallic acid (Aguilar et al., 2007; Bhat et al., 1998; Lekha and Lonsane, 1997). The chemical formula for commercial tannic acid is often given as $C_{76}H_{52}O_{46}$, which corresponds to decagalloyl glucose, but it is a mixture of polygalloyl glucoses or polygalloyl quinic acid esters with the number of galloyl moieties per molecule ranging from 2 to 12, depending on the plant source used to extract the tannic acid.

Tannase is an important enzyme used in the pharmaceutical and food industries and also for analytical and developmental purposes (Aguilar et al., 2007). Tannases contribute to removal of the undesirable effects of tannins in the elaboration of instantaneous tea. They also act as clarifying agents in some wines, juices of fruits and in refreshing drinks with coffee flavour (Boadi and Neufeld, 2001; Belmares et al., 2004). Other important application of tannase in the food industry is its use in the production of gallic acid, a substrate for the chemical synthesis of pyrogallol, which is used in a variety of industrial sector, such as a developer in photography, to make colloidal solutions of metals, as a mordant for wool, for staining leather, in process engraving, in the manufacture of various dyes, and in the dyeing of fur, hair, etc (Cruz-Hernandez et al., 2005). Gallic acid is also precursor in the enzymatic synthesis of

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propyl gallate, which is mainly used as antioxidant in fats and oils, as well as in beverages, and in an important intermediary compound in the synthesis of the antibacterial drug trimethropim, used in the pharmaceutical industry (Sharma and Gupta, 2003, Banerjee et al., 2005).

Although tannase is present in plants, animals and microorganisms, it is mainly produced by microorganisms. A recent review list the main microorganisms evaluated as tannase producers (Chávez-González et al., 2012). Filamentous fungi belonging to the genera *Aspergillus* and *Penicillium* have been widely used for tannase production (Batra and Saxena, 2005; Murugan et al., 2007; Sabu et al., 2005a, b; Seth and Chand, 2000). Production of tannase by *Aspergillus* sp. can occur in the absence of tannic acid, but some fungi tolerate tannic acid concentrations as high as 20% without having a deleterious effect on both growth and enzyme production. Depending on the strain and the culture conditions, the enzyme can be constitutive or inducible, showing different production patterns. Phenolic compounds such as gallic acid, pyrogallol, methyl gallate and tannic acid are usually considered as tannase inducers, although repression in the production of tannase by gallic acid has also been described (Costa et al., 2008, 2012).

Studies on tannase production by *Aspergillus* sp. can be done by various methods such as liquid surface, submerged, modified solid-state cultures and solid state cultures (Belmares et al., 2004; Murugan et al., 2007; Costa et al., 2008, 2012; Rana and Bhat, 2005; Trevino et al., 2007; Trevino-Cueto et al., 2007; van de Lagemaat and Pyle, 2004; Viniegra-González et al., 2003). Submerged cultures (SC) have advantages in process control and easiness of recovery of the extracellular enzymes, mycelia or spores, while the main advantages of solid-state cultures (SSC) include simplicity, lower production costs, high enzyme yield and low wastewater output (Pandey et al., 1999; Sandhya et al., 2005). A new tannase producer, *Aspergillus tamarii*, able to produce tannases in submerged cultures using tannic acid, methyl galatte and gallic acid as substrates was recently described (Costa et al., 2008, 2012). The purpose of the present study was to further extend these studies by comparing the production of tannase and gallic acid by *A. tamarii* in submerged and solid state cultures using tannic acid as substrate.

MATERIALS AND METHODS

Microorganism and maintenance of culture

Spores of *A. tamarii* belonging to laboratory collection (Costa et al., 2008) were inoculated on potato dextrose agar using 250 mL Erlenmeyer flasks and incubated at 28°C for five days. The fungal spore inoculum was prepared by adding 20 mL of sterile distilled water containing 0.01% Tween 80 to a fully sporulated culture. The spores were dislodged using a sterile inoculation loop under strict aseptic conditions and the number of viable spores in the suspension was determined using the plate count method in Newbauer chamber.

Chemicals

Tannic acid, gallic acid, methyl gallate and rhodanine were were obtained from Sigma Chemical Co. Other chemicals were analytical degree.

Cultivation of *A. tamarii* submerged conditions

The submerged cultures were developed in 250 mL Erlenmeyer flasks containing 50 mL of sterilized mineral medium Vogel 1956) plus 0.1% yeast extract. Filter-sterilized tannic acid at 1-5% concentration was added to the sterilized medium. Spore suspensions (5×10^9 spores) were inoculated in each flask. Cultures were then developed for up to 5 days at 120 rpm in shaker at 28°C. The mycelial biomasses were separated by filtration through Whatman no 1 filter paper. For calculation of fungal growth, the mycelial biomasses were dry until constant weight at 40°C. The cell-free culture broths were assayed for tannase activity and gallic acid contents.

Cultivation of *A. tamarii* in solid-state conditions

Commercially available polyurethane foam (PUF) with bulk density of 40 kg/m^3 was used as inert carrier. The foam was cut into 4.0 mm cubes, washed three times with warm water, dried for 4 days at 65°C and pre-weighed. Subsequently, it was sterilized at 121°C for 15 min and dried at 65°C for 1 day (Van de Lagemaat and Pyle, 2004). Three grams of sterilized PUF were placed into a 250 mL Erlenmeyer flask containing 50 ml of mineral solution described above enriched with filter-sterilized tannic acid at 1-20% concentration. Cultures were then developed for up to 6 days at 28°C. For extraction of tannase and gallic acid, a volume of 20 mL was added in each culture. After agitation for 15 min at 10°C, the mixtures were filtered through Whatman no 1 filter paper. The mixtures of PUF plus fungal biomasses were dry at 40°C until constant weight. For calculation of fungal biomasses, the weight of PUF was considered. The cell-free culture broths were assayed for tannase activity and gallic acid contents.

Tannase assay

Tannase activity was estimated by the method of rhodanine (Sharma et al., 2000). The method is based on the formation of a chromogen between gallic acid (released by the action of tannase on methyl gallate) and rhodanine (2 thio-4-ketothiazolidine). The pink color developed was read at 520 nm using a spectrophotometer (Shimadzu UV-1600A, Japan). The tannase activity was expressed in international units. One unit of tannase activity was defined as the amount of enzyme required to liberate one micromole of gallic acid per minute under defined reaction conditions.

Partial characterization of crude tannases

Crude tannases from the two day submerged culture filtrates and four day-solid state culture filtrates were precipitated by the drop-wise addition of cooled acetone at 4°C to a saturation level of 75% (v/v). After 1 h contact time, the precipitates were removed by centrifugation at 5,000 rpm (4°C) for 30 min, freed of acetone by

vacuum drying and dissolved in a minimal amount of destilled water. The acetone precipitated materials were used in the biochemical characterization of tannase. The optimum pH was determined using the substrate in McIlvaine's buffer (pH 3.5 to 8.5). The stability of the crude enzyme was examined at different pH by incubating the enzyme at different pH by incubating the enzyme with the buffers of different pH ranging from 3.5 to 8.5 for 24 h. The residual activity was estimated after incubation under the standard assay conditions and expressed as the percentage of the initial activity. For the determination of the temperature optimum, the enzyme assays were carried out at temperature ranging from 25 to 70°C. The thermal stability was investigated by incubating the enzyme at temperatures ranging from 30 to 60°C for 1 h. Afterwards the reaction mixtures were immersed in an ice bath and then the residual activities were tested under standard conditions and expressed as the percentage of the initial activity.

Determination of gallic acid concentration in culture filtrates

The accumulation of gallic acid in the culture filtrates was measured by the methanolic rhodanine spectrophotometric method (Sharma et al., 2000). To compare the accumulation of gallic acid in two types of cultivation, the amount of gallic acid (in gram) was referred to the amount of tannic acid (in gram) added to each culture.

Data analysis

All the experiments were carried out in triplicate. Results obtained were analyzed by the Graph Pad Prism Program ®.

RESULTS AND DISCUSSION

The ability of *A. tamarii* to produce tannase was evaluated in submerged and solid state cultures. The option for using an inert support such as PUF in solid state cultures instead of tannin rich solid residues frequently used for cultivation of tannase producing filamentous fungi, was to make easier comparisons with submerged cultures, the presence of fewer impurities when compared with the natural substrates and the facility to recover the products of interest (Ooijkaas et al., 2000). The aspect of the mycelium in both types of cultivation was quite different. In the submerged cultures, the fungus grew forming small pellets. In PUF-solid state cultures, the fungus grew as filamentous mycelia. These mycelia were found uniformly distributed over the media, without being concentrated in the aerial regions. Aerial mycelia occur when the fungus has difficulties in colonizing the substrate or when there are regions with inadequate oxygen supply (Holker et al., 2004).

The same mineral culture medium, supplemented with tannic acid, was used in both types of cultures. In submerged cultures, the maximal tannase activity (20,400 ± 2,900 U/L) was obtained in 2 day-cultures using tannic acid at 2% as substrate (Figure 1A). No significant improvement in the production of tannase was found using a higher amount of tannic acid. In solid state cultures, however, the levels of tannase activity were lower than 2,000 U/L in cultures where tannic acid was present at concentrations up to 5%. The highest tannase activity, on the other hand, was obtained in 15% tannic acid cultures (25,470 ± 1,600 U/L) after 4 days of cultivation (Figure 1B). The maximal growth of *A. tamarii* was obtained in submerged cultures after 2 days of cultivation using 5% tannic acid as a substrate (2.5±0.3 g/L) (Figure 2). Tannic acid at concentrations above 5% inhibited considerably the growth (data not shown). In solid state cultures, the highest value of biomass was 3.4±0.5 g/L after 4 days of cultivation in 15% tannic acid cultures. Apparently, thus, the fungus was more resistant to high tannic acid concentrations in solid state cultures. The most tannin-resistant fungi are able to grow at a high concentration of tannic acid. The minimum inhibitory concentration usually reaches 2-3% and a few strains show good growth even when the concentration of tannic acid is higher than 10%. The tolerance of tannic acid concentration is about 20% for a strain of *Aspergillus niger* (Cruz-Hernandez et al., 2005).

Several works have shown the advantages of using solid-state cultures over submerged cultures for the production of tannases (Aguilar et al., 2007; Belur and Mugeraya, 2011; Chávez-González et al., 2012; Viniegra-González et al., 2003). In all studies, the production of tannase were affected by many factors including the choice of the enzyme producer microorganism, the solid support and some culture conditions such as pH, humidity percentage and airing. Viniegra-González et al. (2003) indicated that the diffusion of substrates on a solid support is an important physical parameter that affects the expression of enzymes and it is seriously affected by the continuity of the solid support. These factors were not investigated in the present study and they clearly remain as an object of future studies.

Some properties of *A. tamarii* tannase obtained from submerged and solid state cultures were studied. No significant difference between both crude enzymes was observed. Both presented a recovery above 75% after acetone precipitation, optimum pH at 5.0-6.0, and optimum temperature at 30°C. Concerning thermostability, both enzymes were stable for 1 h at temperature up to 45°C. Most tannases have been reported to have optimal temperature activity between 30 and 40°C and optimum pH at acidic values (Chávez-González et al., 2012).

The accumulation of gallic acid in the culture filtrates was observed in both submerged and solid state cultures. In the submerged cultures, the accumulation was less accentuated than in the solid state cultures: a maximum of 0.67±0.08 g of gallic acid per each g of tannic acid was found in 2 day-solid state culture filtrates, while a maximum of 0.36±0.05 g of gallic acid per each g of tannic acid was found in 2 day-submerged culture filtrates (Figure 3). There are two reasons for this. First, the amount of tannic acid added in submerged cultures was 7.5 times lower than in solid state cultures. Second, the

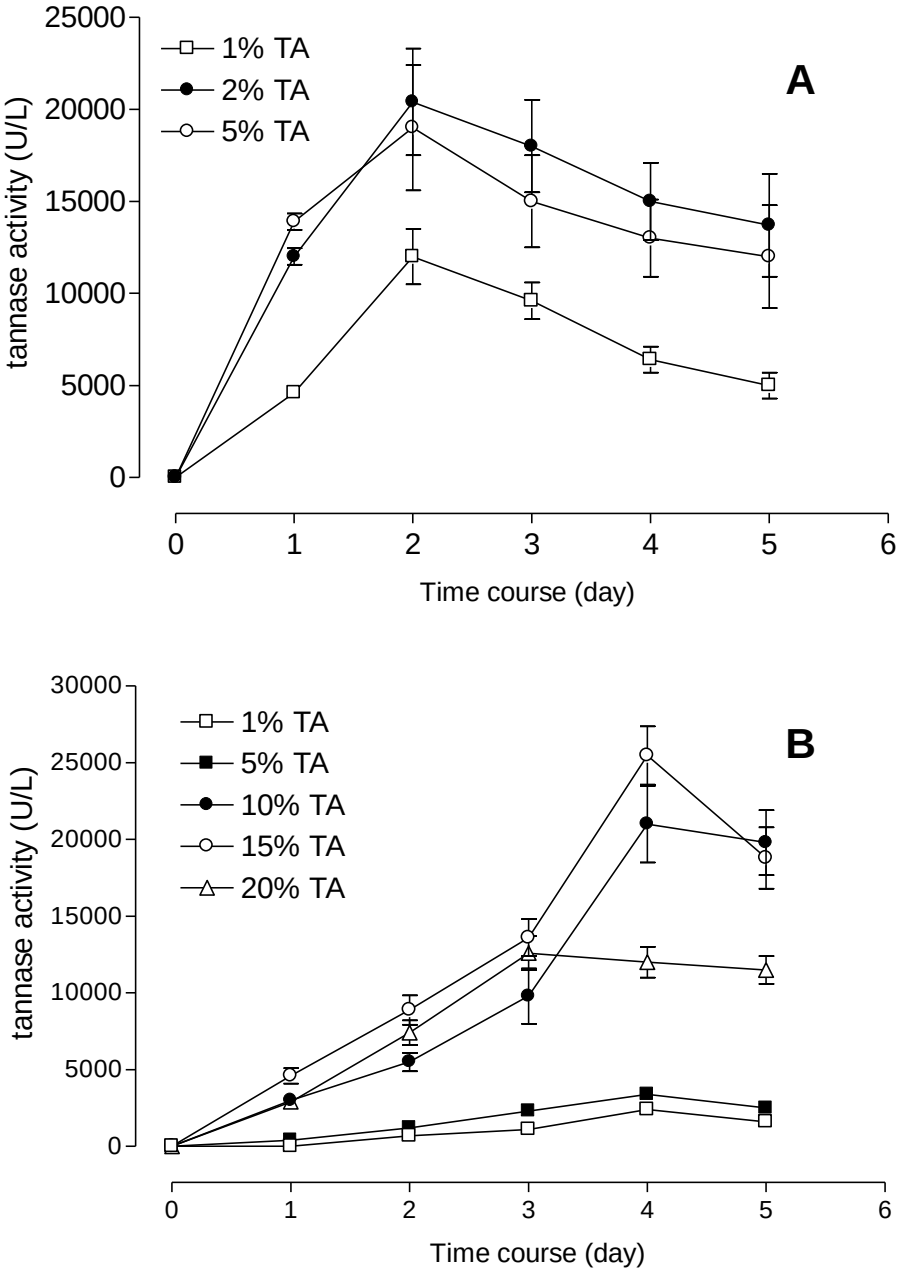

Figure 1. Production of tannase by *A. tamarii* in submerged (A) and solid state (B) cultures. The cultures were developed at 28°C. Submerged cultures were developed at 130 rpm. Values are the mean ± SD of triplicate cultures.

growth of the fungus was faster in submerged cultures than in solid state cultures, that is, in submerged cultures there were more cells for absorbing and metabolizing gallic acid. In both cases, accumulation was transitory. Such a transitory gallic acid accumulation has also been described in submerged cultures of *Aspergillus japonicus* (Bradoo et al., 1997) and *Penicillium glabrum* that did not consume significant amounts of gallic acid until 18 h when cultivated in SSC (van de Lagemaat and Pyle, 2001, 2005). In this period, the organism consumed glucose, and other metabolizable carbon source present in the medium.

One of the most important reasons for studying tannase producing fungi is their potential use to produce gallic acid. Gallic acid has a large range of industrial applications (Hadi et al., 1994). In spite of the accumulation of gallic acid to be transitory, it was possible to obtain the high value of 0.67 ± 0.08 g of gallic acid per each gram of tannic acid added in solid state cultures under non-optimized conditions (Figure 3). High accumu-

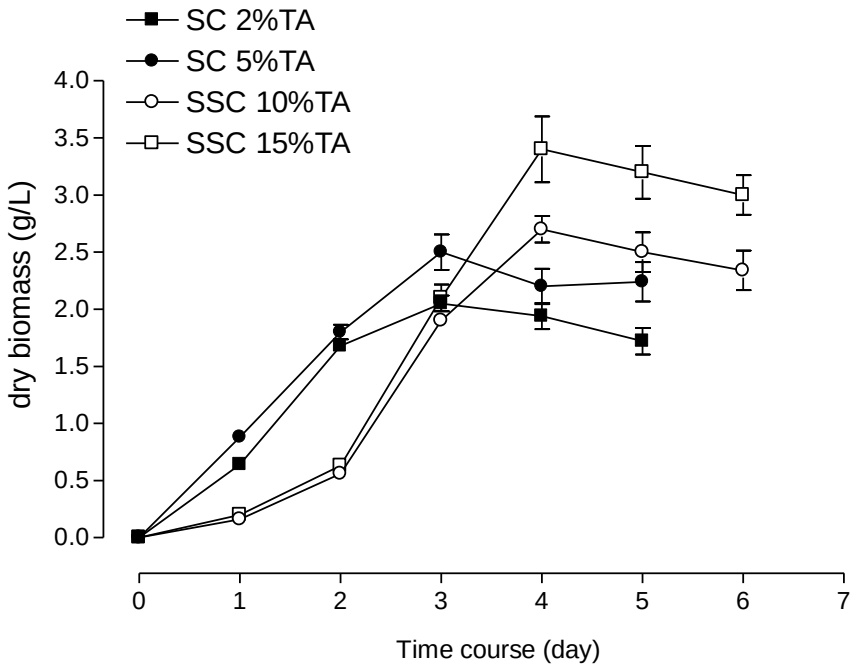

Figure 2. Dry biomass produced by by *A. tamarii* in submerged (SC) and solid state (SSC) cultures. The cultures were developed at 28°C. Submerged cultures were developed at 130 rpm. Values are the means ± SD of triplicate cultures.

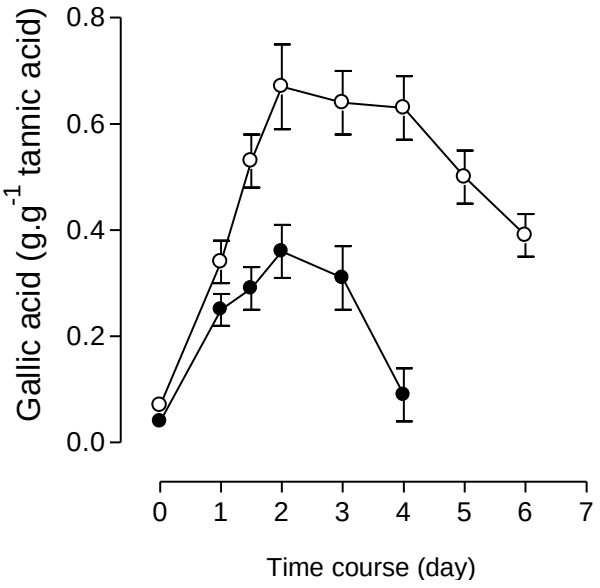

Figure 3. Gallic acid accumulation *in A. tamarii* culture filtrates. The fungus was cultivated in submerged cultures using 2% tannic acid as substrate (•) and in PUF-solid state cultures using 15% tannic acid as substrate (○).The accumulation of gallic acid is expressed in gram of gallic acid by gram of tannic acid. Values are the mean ± SD of triplicate cultures.

lation of gallic acid has been described in cultures of several filamentous fungi such as *Rhizopus oryzae* (Kar et al., 1999), *Aspergillus niger* (Treviño-Cueto et al., 2007) and *Aspergillus fischeri* (Bajpai and Patil, 2008).

Conclusion

The results obtained in this study show that high tannase activities were produced by *A. tamarii* in both submerged and solid state cultures. On the other hand, the PUF solid state cultures present a high potential for the production of gallic acid under non-optimized conditions.

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