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Short Communication: Plant Leaves as Alternative Substrates for Tannase Production by *Aspergillus ochraceus*, *Aspergillus phoenicis* and *Emericela nidulans* Under Solid-State Fermentation

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Abstract: Maximal production of tannases under SSF by *Aspergillus ochraceus*, *Aspergillus phoenicis* and *Emericela nidulans* was obtained using *Eucaliptus*, *Psidium guajava* and *Punica granatum* leaves as substrate, respectively, for 6-10 days, using Khanna or Vogel salt solutions as moistening agent. Maximal activities were observed from 40-65°C and pH 4.0-5.5.

Key words: Tannin acyl hydrolase · Tannase · Filamentous fungi · *Aspergillus* · Solid-state fermentation

INTRODUCTION

Tannases (tannin acyl hydrolase; EC 3.1.1.20) are enzymes that are able to hydrolyze ester and depsidic linkages from molecules of hydrolyzable tannins releasing gallic acid (from gallotannins) or ellagic acid (from ellagitannins) and glucose. These enzymes have attracted the attention of different industrial sectors because of their biotechnological potential. For example, tannases can be used in the beverage industries as wine, beer and juice [1], in feed industries; in the treatment of effluent from the leather industries; and for obtainment of gallic acid that can be used for pyrogallate and gallate esters synthesis [2]. In addition, some tannases are able to catalyze the transesterification reaction in organic medium, as for example in the presence of 1-propanol, producing propyl gallate which is used as antioxidant [2].

Microorganisms, especially filamentous fungi, are the main sources of tannases with biotechnological potential. The genus *Aspergillus* has been mentioned as an important producer of enzymes for industrial application. Then, *Emericela* is a teleomorphic genus associated with the genus *Aspergillus*. The production of tannases by filamentous fungi can be achieved using both Submerged Fermentation (SbmF) as demonstrated for *A. flavus* [3] and Solid-State Fermentation (SSF) as observed for *A. niger* ATCC 16620 [4] and *A. ruber* [5], among others. Despite the SSF advantages when compared to the SbmF, most works describe the tannase production using the submerged condition. The filamentous fungi *A. ochraceus* [6], *A. phoenicis* [7] and *E. nidulans* [8] are able to produce extracellular tannases with biotechnological properties under SbmF. However, the production of tannases under SSF using these fungal strains was not described until this moment, especially using alternative substrates. In this paper, the production of extracellular tannases by *A. ochraceus*, *A. phoenicis* and *E. nidulans* using plant leaves as alternative substrates under SSF is described.

MATERIALS AND METHODS

The fungal strains were isolated from Brazilian soil and maintained on PDA slants stored at 4°C. Spores were obtained from 30-days old culture and used to inoculate new PDA slants, maintained at 30°C for 6 days and after

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that stored at 4°C as mentioned above. The SSF cultures were obtained in 125 mL Erlenmeyer flasks by the addition of 1 mL of aqueous spore suspension (10^5 spores/mL) on 1/5 (w/v) of agro industrial residues/ products and different plant leaves as substrates, humidified with distilled water, tap water, Khanna salt solution [9], Vogel salt solution [10] and SR salt solution [11], previously autoclaved at 120°C, 1.5 atm for 30 min. Before cultivation, the plant leaves were submitted to drying at 40°C for 5 days using a stove until the obtainment of constant mass and then disrupted using a porcelain graal.

The SSF cultures were maintained at 30°C for different periods (3-16 days) in a stove with 60% of humidity controlled by a thermo hygrometer. After incubation, the cultures were added by 25-30 mL of cold distilled water and disrupted by agitation using a magnetic stirrer for 20 min at 4°C and then harvested by vacuum filtration using gauze and Whatman n°1 filter paper. The free cell filtrate was dialyzed overnight at 4°C against distilled water and used for quantification of tannase activity. The tannase activity was determined using 0.2% methyl gallate as substrate in 100 mmol/L sodium acetate buffer, pH 5.0. The gallic acid released was quantified using methanolic rhodanine (0.667% w/v) according to Sharma *et al.* [12]. The reaction was conducted for different temperatures (30-90 °C) and periods. One unit of enzymatic activity (U) was defined as the amount of enzyme necessary to produce 1 µmol of gallic acid per gram of substrate under the assay condition.

RESULTS AND DISCUSSION

The SSF is an important way of fermentation that permits the use of alternative and different low cost substrates as agro industrial residues/products and plant leaves as can be observed in Table 1. The influence of the substrate on tannase production by *A. ochraceus*, *A. phoenicis* and *E. nidulans* in SSF is presented. For *A. ochraceus* the best substrate for tannase production was *Eucaliptus* sp. leaves (0.48 U/ g of substrate), while *Anacardium occidentale* (2.24 U/ g of substrate) and *Punica granatum* (0.3 U/g of substrate) leaves were the best substrates for enzyme production by *A. phoenicis* and *E. nidulans*, respectively. Considering these substrates, the tannase production by *A. phoenicis* was around 5 and 7-fold higher than that observed for *A. ochraceus* and *E. nidulans*, respectively. It is important to cite that *Psidium guajava* (2 U/g of substrate) and *Mangifera indica* (1.41 U/g of substrate) leaves were also good substrates for tannase production by *A. phoenicis*. Considering the same substrates used for all strains, it is possible to observe different levels of enzyme production. These results show that the tannase production is not only related to the tannin composition of the leaves but also with the metabolic potential of the microorganisms. Agro industrial residues also have been used as substrates for fungal cultivation as rice straw and sawdust [13], fruit peel waste [14], sorghum steam and sugarcane trash [15], among others. On the other hand, the tannase production by fungal strains in presence of agro industrial residues was reduced and the best result using these substrates were obtained for *A. phoenicis* with 0.62 U/ g of substrate with sugar cane bagasse as substrate. Production of tannase using plant leaves was reported for *Aspergillus ruber* using jamun leaves as substrate under SSF [5]. Robleto *et al.* [16] demonstrated the tannase production by *A. niger* under SSF using promegranate residues.

Agro-industrial products and residues are alternative substrates for enzyme production, especially using SSF methodology, which promotes similar conditions found by the microorganism in natural environment. Because the fungal growth is improved and, consequently, the enzyme production. In addition, the risk of culture contamination by bacteria is reduced considering the low water activity observed for the substrate. This is the first time that *M. esculenta*, *A. occidentale* and *P. granatum* leaves are described as substrates for tannase production by fungi under SSF.

The influence of the moistening agent should be considered for the optimization of culture condition. For *A. ochraceus* and *A. phoenicis* the higher levels of tannase were obtained using Khanna salt solution (data not shown). On the other hand, there were no significant differences in the tannase production by *E. nidulans* considering all moistening agents used and, consequently, tap water was selected. The maximum tannase yield was obtained using 1:1 (w/v) substrate to moistening agent ratio for all fungal strains. The enzyme yield for *A. phoenicis* was also maintained at values above (1:2 and 1:4; w/v) while the yield was reduced for *A. ochraceus* and *E. nidulans*. Filamentous fungi are organisms that are able to grow on substrates with low water activity as, for example, dry leaves and bark of tress, among others [17]. The maximum tannase production by *A. ruber* was obtained using tap water as moistening agent at a ratio of 1:2 (w/v) with the substrate.

The period of cultivation is another important aspect that should be considered for enzyme production. The highest tannase production by *A. phoenicis* was

Table 1: Influence of substrates used in SSF on tannase production by *A. ochraceus*, *A. phoenicis* and *E. nidulans*

Substrates	Tannase Activity (U/g of substrate)		
Agro-industrial residues/products	*A. ochraceus*	*A. phoenicis*	*E. nidulans*
Crushed Corn	0.11 ± 0.01	0.10 ± 0.02	0.03 ± 0.03
Crushed Corncob	0.12 ± 0.02	0.11 ± 0.03	0.05 ± 0.03
Wheat bran	0.11 ± 0.01	0.24 ± 0.08	0.04 ± 0.06
Sugar cane bagasse	0.14 ± 0.02	0.62 ± 0.27	0.01 ± 0.01
Rice straw	0.11 ± 0.05	0.12 ± 0.02	0.02 ± 0.01
Rye flour	0.11 ± 0.04	0.07 ± 0.03	0.01 ± 0.01
Leaves from:			
Anacardium occidentale	0.03 ± 0.02	2.24 ± 0.51	0.13 ± 0.03
Eucaliptus sp.	0.48 ± 0.05	0.38 ± 0.13	0
Manihot esculenta	0.31 ± 0.12	0.45 ± 0.07	0
Syzygium jambolanum	0.15 ± 0.06	1.15 ± 0.28	0.11 ± 0.02
Musa paradisiaca	0.30 ± 0.08	0.23 ± 0.07	Nd
Zea mays	0.06 ± 0.01	0.33 ± 0.21	Nd
Punica granatum	0.11 ± 0.01	1.18 ± 0.36	0.30 ± 0.08
Mangifera indica	Nd	1.41 ± 0.39	0.09 ± 0.02
Psidium guajava	0.14 ± 0.02	2.04 ± 0.37	0.17 ± 0.06
Vitis vinifera	0.15 ± 0.02	0.22 ± 0.02	Nd
Artocarpus interglifoliam	Nd	0.27 ± 0.04	0.04 ± 0.02
Eugenia uniflora	Nd	0.42 ± 0.02	0.22 ± 0.07
Coffea arabica	0.04 ± 0.01	0.47 ± 0.18	Nd

Nd – not determined.

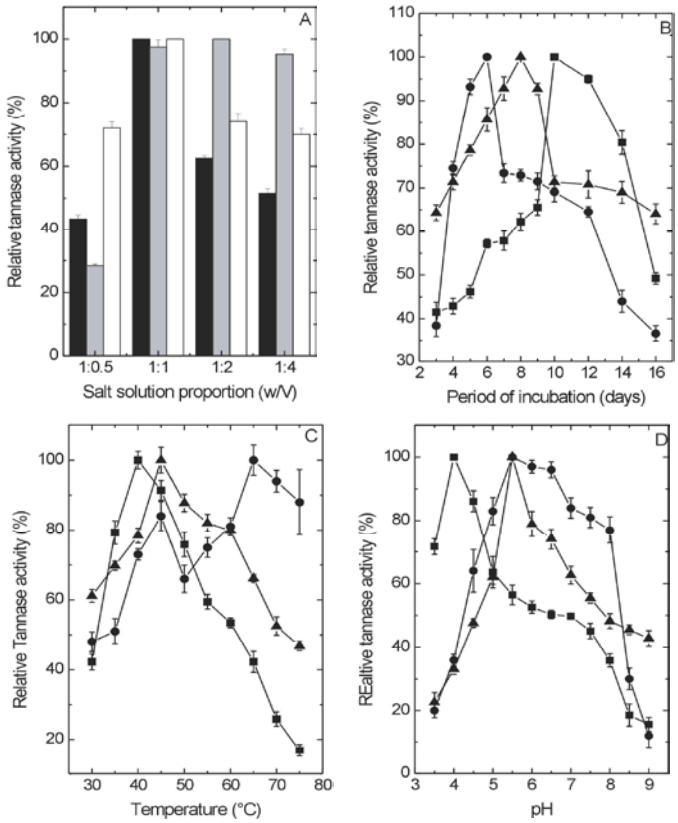

Fig. 1: Influence of salt solution proportion (A) added as moistening agent on the production of extracellular tannase by *A. ochraceus* (■), *A. phoenicis* (□) and *E. nidulans* (□). It was used Khanna salt solution for *A. ochraceus* and *A. phoenicis* and tap water for *E. nidulans* cultivations. Time course of tannase production (B) and optimal of temperature (C) and pH (D) of tannase activity from *A. ochraceus* (■), *A. phoenicis* (•) and *E. nidulans* (▲)

observed with 6 days of cultivation, while for *E. nidulans* and *A. ochraceus* the best production was achieved with 8 and 10 days, respectively (Figure 1B). According to Belur and Mugeraya [18], in general, the maximal production of fungal tannases under SSF has been reported from 72 to 86 hours. The production of tannase by *A. niger* Aa-20 using *Larrea tridentate* leaves was obtained with 43 hours [19] while a period of 48 hours was found to be the best for the tannase production by *A. oryzae* under SSF using cashew apple bagasse as substrate [20]. The period of cultivation necessary for tannase production by *A. phoenicis*, *E. nidulans* and *A. ochraceus* was higher than that reported for other fungi. This fact indicates that the full access to the tannin content of the leaves depends on the degradation of other compounds in the leaves that should occur previously.

Optimum of temperature for the activity was found to be 40°C for tannase from *A. ochraceus* and 45°C for tannase from *E. nidulans* (Figure 1C). However, two peaks of tannase activity, one at 45°C and another at 65°C, were observed for *A. phoenicis*, indicating the presence of two enzymatic forms. In addition, when the pH value of reaction was considered, both *A. phoenicis* and *E. nidulans* tannases showed optimal activities at pH 5.5, differing from that observed for *A. ochraceus* tannase with optimal activity at pH 4.0 (Figure 1D). The optimum of temperature and pH for fungal tannase activities have been reported to be 30-40°C and pH of 5.5-6.0 [1]. The temperature that corresponds to the second peak of tannase activity from *A. phoenicis* was higher than that observed for most of fungal tannases.

In conclusion, these three fungal strains were able to produce tannases using plant leaves as alternative and low cost substrates. The differences observed according to the substrate used reflect the physiological differences among these strains considering their phylogenetic proximity.

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