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Full Length Research Paper

Culture conditions for the production of a tannase of *Aspergillus tamarii* IMI388810 (B)

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***Aspergillus tamarii* IMI388810 (B) a tannic acid degrading fungus was isolated from soil inundated by effluent of a tannery at Oji River local Government Area of Enugu State, Nigeria. It was identified by CABI Bioscience, United Kingdom as *A. tamarii* with accession number of IMI 388810 (B). This fungus produced tannase in a fermentation medium M containing tannic acid as the only carbon source. Time course of enzyme synthesis by the fungus showed that the enzyme production followed logarithmic growth phase with maximum enzyme yield being obtained after 6 days corresponding to the culture pH of 3.8.**

Key words: Tannin, tannase, tannic acid, methyl gallate, *Aspergillus tamari* IMI388810 (B), Oji River L.G.A., Enugu, Nigeria.

INTRODUCTION

Tannins are naturally occurring plant polyphenolic compounds that have wide-ranging effects on animals and microbes (Waterman and Mole, 1994). They are polyphenolic secondary metabolites of plants, which form hydrogen bonds in solutions (Spencer et al*.,* 1988) resulting in the formation of tannin-protein complexes (Hagerman, 1989).

A large number of tree leaves and grasses that form a substantial portion of livestock diet are known to be rich in tannins. Ingestions of these complex phenol rich polymers by animals especially ruminants, leads to decrease in feed intake, lower nutrient utilization, toxicity and even death at higher levels of intake (Garg et al*.,* 1992; Makkar and Becker, 1994). Animals normally consuming these feeds may develop defense mechanisms against tannins. Toxicity generally results from the remaining tannins or its degradation products which are not detoxified by the detoxification processes of the animal. Other consequences of these complexes of tannin include environmental problems in clean-up requirements for effluents of the leather industry (Suseela and Nandy, 1985) and haze formation in chilled beverages (Siebert et al., 1996).

Tannins have been long considered toxic to microorganisms and this toxicity is mainly due to enzyme inhibition and substrate deprivation action on membranes and metal ion deprivation (Reed, 1995). Nevertheless, some fungi, yeasts, and bacteria are quite resistant to tannins and can also degrade them (Scalbert, 1991; Saxena et al*.,* 1995). Osawa (1992) demonstrated the presence of tannin-protein degrading microbes in the faeces of the koala, which feeds on eucalyptus leaves with high tannin content. These microbes play an important role in koala's ability to obtain dietary proteins from tannin-rich eucalyptus leaves. Therefore, another mechanism of adaptation by ruminants could be degradation of tannins to innocuous compounds by rumen microbes.

Tannase has been isolated from strains of *Aspergillus* (Adachi et al., 1968; Barthomeuf et al*.,* 1994; Bhardwaj et al., 2003; Yamada et al*.,* 1968a), *Penicillium* (Ganga et al., 1978; Rajakumar and Nandy, 1983), *Candida species* (Aoki et al., 1976a) and bacterial species such as *Bacillus licheniformis* (Mondal et al., 2000) and *Citrobacter freundii* etc (Kumar et al., 1999).

There is a constant search for tannase with more desirable properties for commercial applications (Sharma et al., 2000). Although other species of *Aspergillus* have been reported to be capable of degrading tannins there is none on *Aspergillus tamarii*.

MATERIALS AND METHODS

Fungal strain

The *A. tamarii* IMI388810 (B) used in this study was isolated from

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soil inundated by effluent from a tannery at Oji River L.G.A. of Enugu State, Nigeria.

Reagents and media

Tannic acid (E. Merck), methyl gallate (Sigma), gallic acid (Sigma), rhodanine (Aldrich), dipotassium hydrogen phosphate (M and B), potassium dihydrogen phosphate (M and B), magnesium sulphate (BDH), calcium chloride (Hopkin and William), ammonium chloride (Hopkin and William), Agar (Oxoid), potassium hydroxide (Arondole Lab. Ltd, England), Methanol (BDH)), citric acid (Fisher Chemical/ Scientific), sodium citrate (BDH), filter paper, sabouraud dextrose agar (Lab.M) were used in this study.

Tannin degrading fungi

Fungal species that utilize tannic acid as the sole carbon source were screened for and isolated from soil on the effluent pathway of a tannery at Oji River Local Government Area in Enugu State, Nigeria. 10 g of the soil were suspended in 90 ml of sterile deionized water in a beaker. From the slurry inoculation was made on plates of a basal medium containing tannic acid as the sole carbon source according to Mondal et al. (2000). The constituted medium contained (g/l): K_2HP0_4, 0.5; KH_2P0_4, 0.5; $MgS0_4$, 2.0; $CaCl_2$, 1.0; NH_4Cl, 3.0; tannic acid, 10.0; Agar, 20.0 and deionized water, 1000 ml. The medium also contained 0.05 mg/ml chloramphenicol. The inoculated plates were incubated at room temperature, (approximately 28°C) for10 days. Utilization of tannic acid was confirmed by growth of fungal colonies.

Identification of the isolated fungal colonies

The chosen fungal strain that grew faster was identified by CABI Bioscience, United Kingdom as *A. tamarii* with an accession number of IMI 388810 (B).

Time course of tannase production

The time course of tannase production by *A. tamarii* IMI388810 (B) was carried out in shake flask cultures. Erlenmeyer flasks (250 ml) each containing 50 ml of fermentation medium hereafter called medium M of the following composition (g/l): K_2HP0_4, 0.5; KH_2P0_4, 0.5; $MgS0_4$, 2.0; $CaCl_2$, 1.0; NH_4Cl, 3.0; tannic acid 1% (w/v); deionized water, 1000 ml pH 5.9 were inoculated with two agar plugs (1.4 cm in diameter) of *A. tamarii* grown on SDA medium. Fermentation was carried at room temperature (approximately at 28°C) at 160 rpm. Following the termination of the fermentation at 48 h intervals, the culture broth was filtered through filter paper (Whatman No.1) and the tannase activity of the filtrate, biomass (gramme dry weight) and pH measured.

Tannase assay

Tannin hydrolysis was determined spectrophotometrically using rhodanine according to the method described by Sharma et al. (2000). The substrate solution (0.10 M methyl gallate in 0.05M citrate buffer, pH 5.0), 10.0 ml; enzyme sample, 1.0 ml; and buffer (0.05 M citrate buffer, pH 5.0), 1.0 ml were separately preincubated at 40°C for 10 min before the enzyme reaction was started. The reaction mixture in the blank, test and control tubes contained 0.5 ml of substrate solution to which 0.5 ml of the buffer and 0.5 ml of the enzyme sample were added to the blank and test, respectively. The tubes were incubated at 40°C for 5 min and 0.6 ml of methanolic rhodanine (0.667%, w/v) was added to all the tubes and further incubated at 40°C for 5 min. Thereafter, 0.4 ml of 0.5 M potassium hydroxide was added to each tube followed by further incubation at 40°C for 5 min. This was followed by addition of the enzyme sample (0.5 ml) to the reaction mixture in the control tube only. Finally, each tube was diluted with 8 ml deionized water and incubated at 40°C for 10 min and the absorbance was recorded against water at 520 nm using a Spectronic21 (Milton Roy Company) spectrophotometer. The enzyme activity was calculated from the change in absorbance:

$$\Delta_{A520} = (A_{test} - A_{blank}) - (A_{control} - A_{blank})$$

One unit (U) of the enzyme activity was defined as micromole of gallic acid formed per minute.

RESULTS AND DISCUSSION

Isolation and choice of working culture

Two isolates, A and B of tannin degrading fungi were isolated. Isolate B identified by CABI Bioscience, U.K. as *A. tamarii* with accession number IMI 388810(B) was chosen for the study because it grew faster than isolate A on a basal medium containing tannic acid as the only source of carbon (Mondal et al*.,* 2000).

Time course of tannase production

The time course of tannase production by *A. tamarii* IMI388810 (B) is shown in Figure 1. Tannase production followed the logarithmic growth phase of the fungus with maximum yield occurring at 144 h during the stationary phase of growth and corresponding pH of 3.8 (Figure 2).

A. tamarii IMI388810 (B) produced extracellular tannase when grown in medium M. A number of protocols have been developed for the production of tannase by various fermentation procedures (Yamada, 1967). These include surface culture of *Aspergillus niger* (Doi et al*.,* 1973; Barthomeuf et al*.,* 1994), solid-state process for economic production of tannase by *A. niger* (Lekha and Lonsane, 1994), and solid-state fermentation of *Rhizopus oryzae* (Hadi et al*.,* 1994; Chattergee et al*.,* 1996). The use of liquid medium M with continuous shaking must have created enough medium-enzyme interfaces for the secreted tannase to act. Suseela and Nandy (1985) found out that the decomposition of tannic acid and gallic acid by *Penicillium chrysogenum* was maximally in shake cultures at 28°C.

There was a fall in pH from initial pH of 5.9 of the fermentation medium M to pH 3.5 on day 4 (Figure 2) and then rose gradually to 3.8 on day 6 on which highest tannase activity was recorded. This is indicative that gallic acid molecules were liberated into the medium M thereby lowering the pH of the system. Probably, as the fungus utilized part of the products of hydrolysis, gallic acid and glucose, the pH of the system gradually rose to 4.0.

Bradoo et al. (1997) reported maximum production of

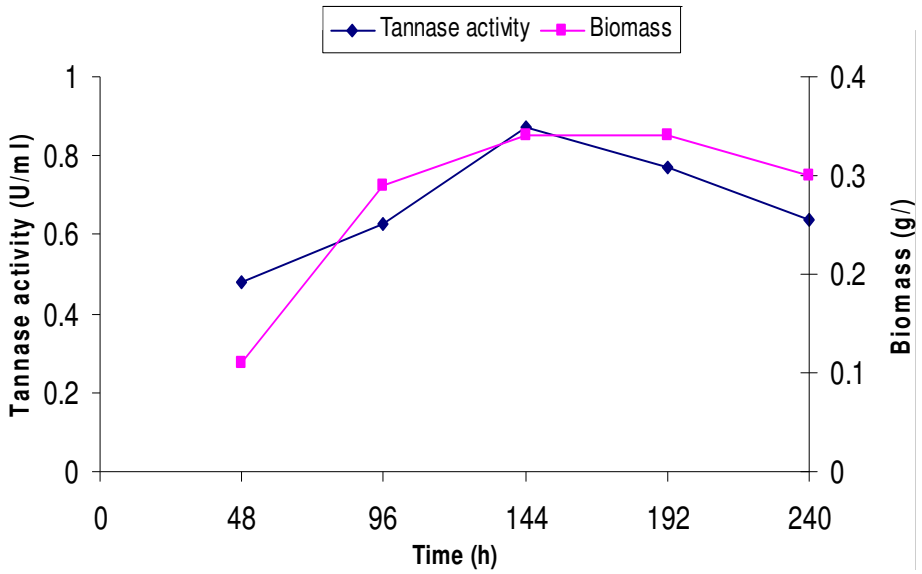

Figure 1. Time course of tannase production by *A. tamarii* IMI388810 (B) in relation to biomass.

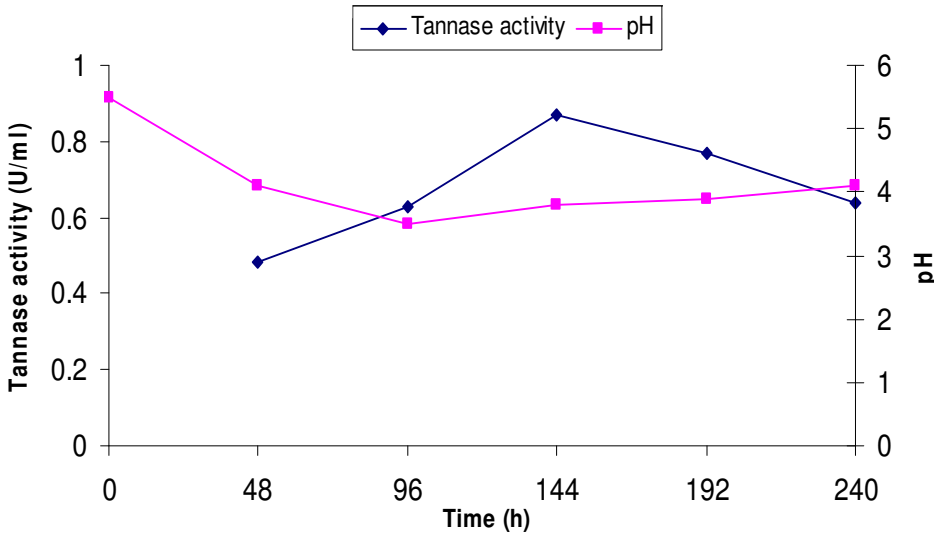

Figure 2. Time course of tannase production by *A .tamarii* IMI388810 (B) in relation to the pH changes in the medium.

tannase by *Andrias japonicus* after 24 h of incubation. Suseela and Nandy (1985) observed that both tannic acid and gallic acid were found to be completely decomposed in 3 days by *P. chrysogenum.* However, Bhat et al. (1997) harvested *A. niger* van Teighem tannase after 120 h incubation. For maximum tannase production by *A. tamarii* to reach 6 days it should be from interplay of other factors like the type and composition of the fermentation medium, the initial pH of the fermentation medium and from differences in the characteristics of the fungal species employed. Other workers (Sharma et al., 2000; 1999; Bhat et. al., 1997) adjusted their initial pH of the medium to 5.0. Although fungi are the major group of tannin degraders, they have the limitation of being comparatively slow in their growth.

The increase in biomass from day 2 to 6 (Figure 1) is suggestive of utilization of the liberated gallic acid and glucose by *A. tamarii* IMI388810 (B) for cell growth. The growth pattern followed a logarithmic phase from day 2 to 5, stationary phase from day 6 to 8, and a decline phase from day 8 to 10.

A fall in tannase activity from day 7 to 10 is suggestive

of a possible tannase inhibition probably by repression. This seems to be reflected in cell growth because there was no increase in biomass from day 6 to 8, and decrease was recorded from day 8 to 10 (Figure 1). Gupta et al. (1997) reported strong end-product inhibition with gallic acid which was of the competitive type with *A. japonicus* tannase production. Suseela and Nandy (1985) found that sugars present as additional carbon source at 3% level retarded the degradation of tannic acid and gallic acid by *P. chrysogenum*. On the contrary, it could be suggested that this effect occurred due to depletion of some nutrients in the fermentation medium.

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