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Optimization of Tannase Production by *Aureobasidium pullulans* DBS66

BANERJEE, DEBDULAL* AND BIKAS R. PATI

Department of Microbiology, Vidyasagar University, Midnapore-721102, West Bengal, India

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Abstract Tannase production by *Aureobasidium pullulans* DBS66 was optimized. The organism produced maximum tannase in the presence of 1% tannic acid after 36 h. Maximum gallic acid accumulation was observed within 36 h and tannic acid in the fermented broth was completely degraded after 42 h of growth. Glucose had a stimulatory effect on tannase synthesis at 0.1 % (w/v) concentration. The organism showed maximum tannase production with $(NH_4)_2HPO_4$ as nitrogen source. Shaking speed of 120 rpm and 50-ml broth volume have been found to be suitable for maximum tannase production.

Keywords: *Aureobasidium pullulans*, gallotannin, optimization, tannase, yeast

Tannin acyl hydrolase (EC 3.1.1.20), commonly called tannase, is an inducible enzyme produced in the presence of gallotannin by several microorganisms [2, 4, 7, 15]. This enzyme hydrolyzes the ester linkages of gallotannin and liberates gallic acid (3,4,5-trihydroxy benzoic acid) from glucose residues [4, 12]. Gallotannins are plant polyphenolic compounds that can easily precipitate any protein. Although these polyphenols display high antimicrobial activity, due to their complexation with proteins and enzymes, few fungal species are known to develop resistance against gallotannin by production of tannase [22]. This enzyme is used in different food and beverage processing, but it is extensively used in the manufacture of instant tea and in the production of gallic acid [2, 12].

Though tannase has been produced by a number of bacterial [8, 15, 17] and fungal [2, 4, 16] species, optimization of this enzyme production has not been worked out in all the cases. Report on tannase production from yeast cells is very scanty; only Aoki *et al.* [1] have reported tannase production from *Candida* sp. *Aureobasidium pullulans*, a yeast-like dimorphic fungi, was reported by several authors for its application in exopolysaccharide and different enzyme production [19–21, 23]. Tannase production from *Aureobasidium* sp. was not reported earlier. The present communication deals with the production of extracellular tannase by a newly isolated tannase-producing *A. pullulans* DBS66.

Aureobasidium pullulans DBS66, isolated from forest soil, was used in the present investigation. Identification of the organism was confirmed from the Microbial Type Culture Collection & Gene Bank (MTCC), Institute of Microbial Technology, India.

The fungal strain was grown in a 250-ml Erlenmeyer flask containing 50 ml of tannic acid broth containing (g/l) $(NH_4)_2HPO_4$, 3.0; $MgSO_4$, 1.0; KH_2PO_4, 0.5; $CaCl_2$, 0.3; tannic acid (filter sterilized), 10.0; at 30°C for 36 h. All the experiments were performed in triplicate and data are presented as mean±SD.

Tannase activity was determined by the method of Mondal *et al.* [14]. Enzyme solution (0.05 ml) was incubated with 0.3 ml of 1.0% (w/v) tannic acid, in 0.2 M acetate buffer (pH 5.0) at 40°C for 10 min and then the reaction was stopped by addition of 2.0 ml bovine serum albumin (BSA) (1 mg/ml), which precipitated the remaining tannic acid. A control reaction was done side by side with heat denatured enzyme. The tubes were then centrifuged (5,000 ×g, 10 min) and the precipitate was dissolved in 2.0 ml of SDS-triethanolamine (1% w/v SDS in 5% v/v triethanolamine) solution and the absorbency was measured at 550 nm after addition of 1.0 ml of $FeCl_3$ (0.13 M) (Systronics spectrophotometer 105).

One unit of tannase activity is defined as the amount of enzyme required to hydrolyze 1.0 μmol of ester linkage of tannic acid in 1 min under specified condition.

Gallic acid in the culture filtrate was estimated by the method of Bajpai and Patil [2]. Filtrate was diluted to 100-fold in 0.2 M acetate buffer at pH 5.0. The absorbency was recorded at two selective wavelengths of 254.6 and 293.8 nm. The concentration of gallic acid was measured using specific extinction coefficient by the following equation: Concentration of gallic acid (μg/ml)=21.77 ($A_{254.6}$)−17.17 ($A_{293.8}$).

*Corresponding author
Phone: 91-03222-276554; Fax: 91-03222-275329;
E-mail: debu33@yahoo.com

Growth of the organism was estimated after drying the biomass at 60°C for 24 h.

The remaining tannin content in the fermented broth was estimated by the modified method of Hagerman and Butler [11]. The tannin content of the broth was precipitated by addition of BSA and kept at room temperature for 15 min. After centrifugation (5,000 ×*g*, 5 min), the precipitate was dissolved in SDS-triethanolamine solution. Then, 1.0 ml of $FeCl_3$ reagent was added to it and the mixture stood for 30 min to stabilize the color. The colored solution was diluted with water and absorbency was measured at 530 nm. The tannic acid concentration in the fermented broth was measured from a standard curve.

Enzyme and gallic acid production in relation to growth of *Aureobasidium pullulans* DBS66 was studied in tannic acid basal medium for 60 h and is presented in Fig. 1A and Fig. 1B, respectively. The organism started enzyme production at 6 h of incubation and produced maximum enzyme at 36 h, and the organism attained its stationary phase of growth after 36 h (Fig 1A). The amount of tannic acid present in the medium was completely degraded at 42 h growth of *A. pullulans* DBS66 (Fig 1B). Maximum gallic acid accumulation was observed within 36 h, and after that it declined.

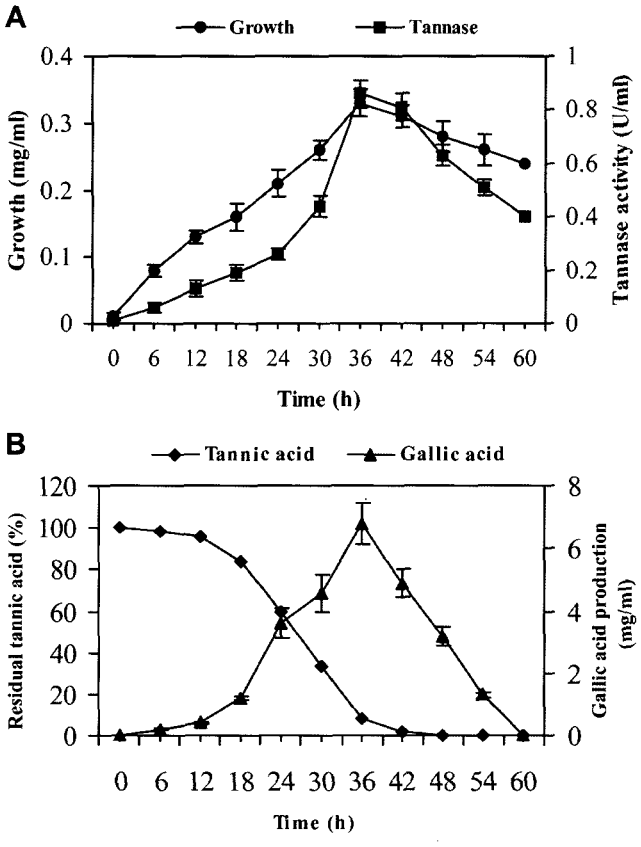

Fig. 1. A. Growth and tannase production by *Aureobasidium pullulans* at different culture times. **B.** Degradation of tannic acid and accumulation of gallic acid during fermentation (the initial tannic acid content [100%] indicates 1 g % concentration).

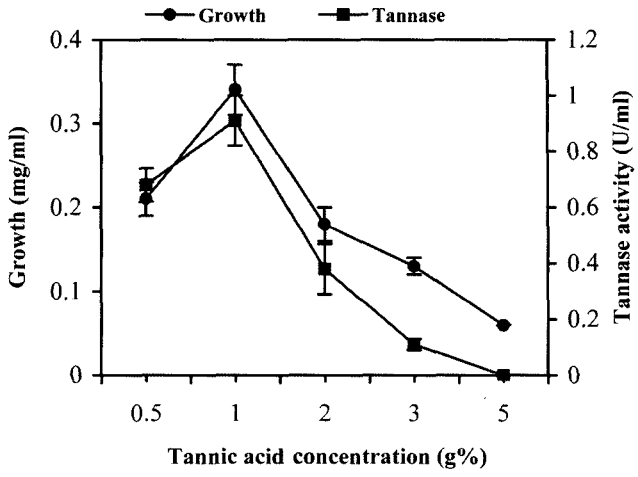

Fig. 2. Effect of tannic acid concentration on growth and tannase production (growth condition: basal medium containing tannic acid, incubated at 30°C for 36 h).

Tannic acid is the sole carbon source as well as the main substrate for tannase synthesis in the fungal strain. Enzyme production by the organism was studied in the medium containing various concentrations of tannic acid and is presented in Fig. 2. Maximum growth and tannase production by *A. pullulans* was noticed in the medium containing 1.0% tannic acid.

A number of carbohydrates were tested in presence of tannic acid for tannase production by the organism (Fig. 3). No additional carbon sources in the tannic acid medium

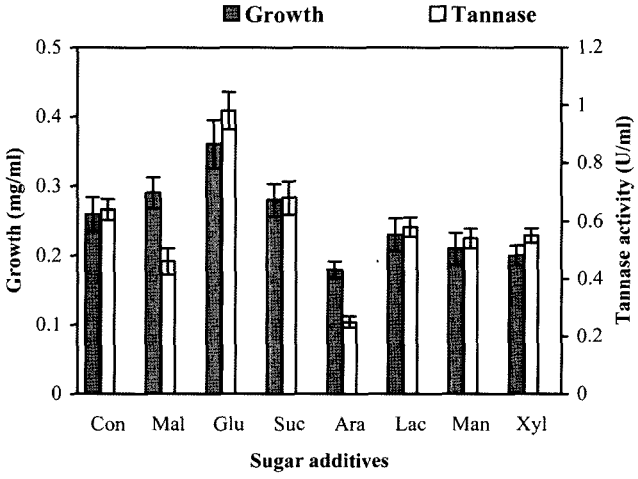

Fig. 3. Effect of different sugar additives (0.1%, w/v) on growth and tannase production by *Aureobasidium pullulans* (Con, Control [without sugar]; Mal, maltose; Glu, Glucose; Suc, Sucrose; Ara, Arabinose; Lac, Lactose; Man, Mannitol; Xyl, Xylose).
Basal carbon source used as 1% tannic acid, pH 5.5, incubated at 30°C for 36 h.

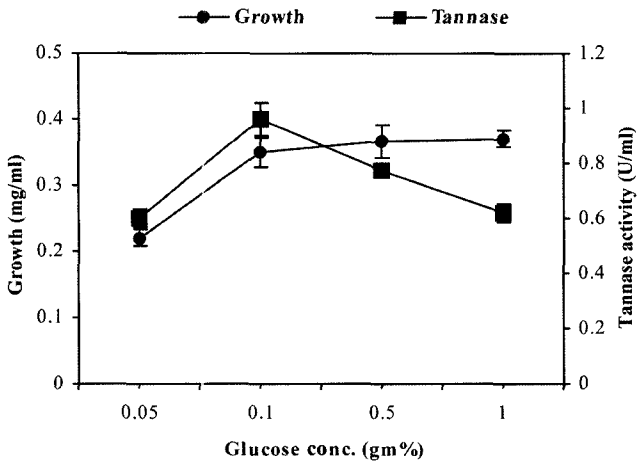

Fig. 4. Growth and tannase production by *Aureobasidium pullulans* in the presence of different concentrations of glucose as additive. Basal carbon source used as 1% tannic acid, pH 5.5, incubated at 30°C for 36 h.

had a positive effect on tannase formation by the organism, except glucose. However, maximum growth of *A. pullulans* was observed in the medium with maltose, glucose, and sucrose. Glucose at 0.1% (w/v) concentration was most effective for tannase production and beyond that concentration it was inhibitory (Fig. 4).

The specificity of nitrogen sources on tannase production and growth of the organism was determined and is presented in Fig. 5. Most of the used nitrogen sources were effective for enzyme production. *A. pullulans* showed its highest growth and maximum tannase production in the presence of $(NH_4)_2HPO_4$. The increasing order of enzyme production in the organism in relation to nitrogen sources can be arranged in the following order: $(NH_4)_2HPO_4$>$NH_4H_2PO_4$>NH_4Cl>NH_4NO_3.

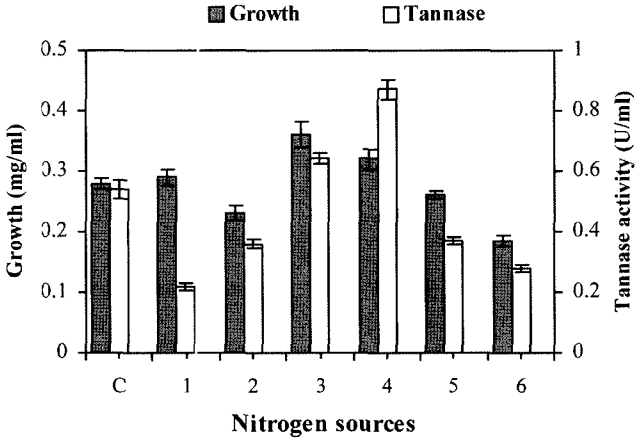

Fig. 5. Growth and tannase production by *Aureobasidium pullulans* in the presence of different nitrogen sources (C, control [NH_4Cl]; 1, $NaNO_3$; 2, NH_4NO_3; 3, $NH_4H_2PO_4$; 4, $(NH_4)_2HPO_4$; 5, KNO_3; 6, $(NH_4)_2SO_4$).
Nitrogen sources were used at the concentration of 0.3% (w/v).

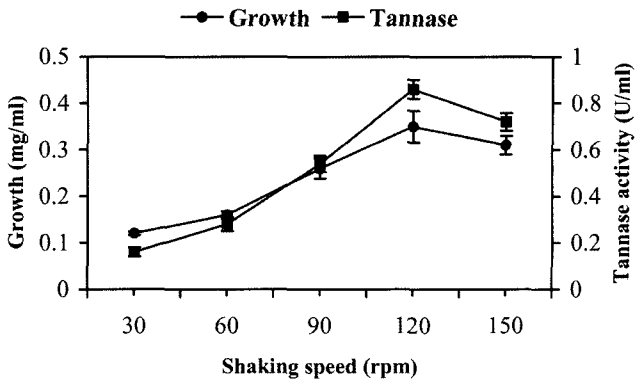

Fig. 6. Effect of shaking speed on growth and tannase production.

The enzyme production of the organism was studied under shaken and still culture conditions (Fig. 6). It was found that the shaking condition was suitable for growth and tannase production. In *A. pullulans*, a shaking speed of 120 rpm was found to be suitable for both growth as well as tannase production. A negligible enzyme activity was found in the still culture of the organism (data not shown).

The enzyme production of the organism was studied with different broth volumes (Fig. 7). It was found that 50 ml of broth in a 250-ml Erlenmeyer flask gives the highest growth and tannase production.

Tannins are in fact antimicrobial agents, and most of the microorganisms cannot tolerate its polyphenolic nature. Only a few microorganisms can degrade tannic acid and utilize it as a nutrient [12]. Owing to the presence of tannase, soil microbes play an active role in the decomposition and recycling of plant materials containing tannin [13]. The present newly isolated soil fungi *A. pullulans* DBS66 is also a tannase producer. Tannase production by this strain and its growth were studied for 60 h in tannic acid broth and it was found that synthesis of the enzyme by this

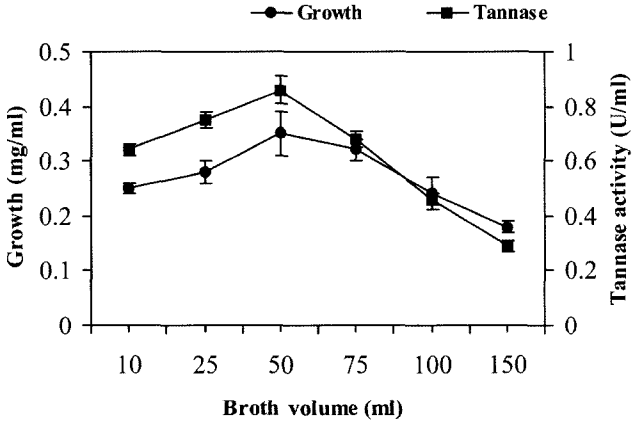

Fig. 7. Effect of broth volume on tannase production.
Different broth volumes were taken in a 250-ml Erlenmeyer flask with 2% (v/v) inoculums.

organism was directly proportional to its growth. Production of enzyme started to decrease when the organism entered the stationary phase of growth. The present observations are comparable with the finding of Vermeire and Vandamme [22] in *Aspergillus flavus*, but contradict with the observation of Ganga *et al.* [9] where maximum tannase production by different species of *Aspergillus* was observed at the stationary phase of growth.

It was found that enzyme production by *A. pullulans* was directly related to tannic acid degradation and gallic acid formation. It was also observed that maximum tannic acid degradation by *A. pullulans* took place within 42 h of growth. Gallic acid was accumulated in the medium up to 36 h, and after that the amount of gallic acid declined. Because of its high molecular weight, tannic acid itself is impermeable to the cell membrane, but the tannase produced by the organism could break it into gallic acid and glucose. Initially, organisms assimilated glucose as a readily available carbon source for rapid growth and gallic acid remained in the culture broth. Gallic acid was ultimately utilized by the organism in the latter phase of its growth (after 36 h). After depletion of both the products like glucose and gallic acid, the organism entered into the stationary phase. In this regard, Lewis and Starkey [13] and Bhat *et al.* [6] mentioned that gallic acid could enter into the TCA cycle *via* pyruvic acid for energy production.

The concentration of tannic acid in the formulated medium was crucial for growth and tannase production. In this experiment, 1% tannic acid was found to produce maximum tannase in *A. pullulans*, and above this concentration, growth and enzyme production in the fungal strain was gradually decreased. Tannic acid is a polyphenolic compound, and in higher concentration, it makes an irreversible reaction with surface protein and impairs the growth of microorganism [13]. The optimum concentration of tannic acid was also necessary for fungal tannase production, which was reported by many workers [3, 7, 9, 10].

The presence of additional carbon sources like maltose, glucose, and sucrose at 0.1% (w/v) concentration in the tannic acid medium was found to be effective for growth of the organism, though tannase synthesis was inhibited in maltose and arabinose. Generally, the tannase activity by carbohydrate seems to depend on the cell growth. If the cell mass is high, the tannase activity is high, except with maltose and arabinose. It means that the general carbon source is not the inducer for tannase biosynthesis. Enhanced tannase production was reported in the presence of glucose. A similar result was also observed by Hadi *et al.* [10], where they reported that additional carbon source acted as a catabolic inducer for tannase synthesis. Glucose at higher concentration inhibits tannase synthesis. Bradoo *et al.* [7] pointed out that a higher concentration of additional carbon source (glucose) created an osmotic stress to depress enzyme synthesis of *A. japonicus*.

Nitrogen source is very essential for growth and enzyme production by the microorganism. Organic nitrogen sources are not used in the tannic acid medium as this forms complexes and becomes precipitated with tannic acid. Various inorganic nitrogen sources were used in this study, and it was observed that the organism produced maximum tannase in the presence of $(NH_4)_2HPO_4$. Earlier, Hadi *et al.* [10] and Bradoo *et al.* [7] observed maximum enzyme synthesis with $NaNO_3$ by *R. oryzae* and with $(NH_4)_2NO_3$ by *A. japonicus*, respectively. Fungal tannase production was studied in the presence of different inorganic nitrogen sources in the tannic acid medium by other workers, which included sodium nitrate [12], ammonium chloride [22], ammonium oxalate [12], and ammonium sulfate [12].

The aeration is an important determinant factor for growth and tannase synthesis. It was found that decreasing the shaking speed inhibited tannase formation, whereas with higher speed (upto 120 rpm), the growth rate of the fungi became faster and maximum enzyme accumulated in the media. With the increasing volume of broth in the Erlenmeyer flask, a decrease in tannase production was recorded. This also indicates the necessity of aeration for tannase production. With higher agitation speed (beyond 120 rpm) or lower broth volume (below 50 ml), however, huge aeration in the broth occurs, which may cause oxidation of the tannic acid that on the other hand slows down enzyme production. Under favorable conditions, the fungal mycelia simultaneously synthesized an antioxidant, which prevented tannin oxidation [5]. The production of a huge amount of enzyme resulted in rapid degradation of tannic acid in the broth, and thereby the end products like glucose and gallic acid were quickly assimilated by the organism and enhanced growth. From this result, it can be concluded that tannase production by *A. pullulans* is O_2-dependent, and aeration is necessary for both growth and enzyme production. This type of O_2-dependent tannase production by fungal strains was also observed by other workers [1, 5, 12, 18].

Newly isolated polymorphic fungal strain *A. pullulans* DBS66 produces a large amount of tannase in a short period of time. To find out its commercial viability, optimization of growth parameters in a bioreactor is needed.

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