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Improvement of tannase production under submerged fermentation by *Aspergillus niger* FBT1 isolated from a mangrove forest

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Abstract

Aspergillus niger FBT1, a local extracellular strain for tannase production, was isolated from soil collected from Matang Mangrove Forest Reserve in Perak, Malaysia. This fungus strain was cultivated in an Erlenmeyer flask under a submerged fermentation system. Medium compositions play a very important role in enhancing enzyme production during fermentation. The production of tannase by *A. niger* FBT1 increased significantly (95%) when the medium compositions and various process parameters were optimized. Incubation for 72 hours (30°C, pH 7) in medium complemented with sodium nitrate was found optimal. Additional supplementation with tannic acid (2% w/v) as the sole carbon source strongly increased the yield of the enzyme.

Key words: tannase, submerged fermentation, *Aspergillus niger*, Mangrove

Introduction

Tannin acyl hydrolase (EC 3.1.1.20), commonly referred to as tannase, is an important enzyme with various industrial applications. It is an extracellular hydrolase enzyme that is induced in the presence of tannic acid (Belmares et al., 2004; Ramirez-Coronel et al., 2003). It catalyzes the hydrolysis of ester and depside bonds in hydrolysable tannins such as tannic acid and gallic acid esters (Belmares et al., 2004), releasing glucose and gallic acid (Banerjee, 2005).

Tannase is extensively used in the preparation of instant tea, to improve the flavour of grape wine, for the clarification of beer and fruit juices, in coffee-flavoured soft drinks, and in the production of gallic acid (Aguilar et al., 2001). Gallic acid can be used in the manufacture of ordinary writing inks and dyes, as a photographic developer, in the enzymatic synthesis of propyl gallate, in the tannery industry for homogenization of tannins, and for the production of pyrogallol and gallic acid esters (Banerjee et al., 2005). However, currently, the most commercial application of tannase is in the manufacture of instant tea, where it is used to eliminate water-insoluble precipitates (called "tea cream") (Aguilar et al., 2001).

Tannase can be obtained from plant, animal and microbial sources. The most important method to obtain the enzyme is via a microbial process, because the produced enzymes are more stable than similar ones obtained from other organisms (Murugan et al., 2007). Of all microbes, filamentous fungi of the *Aspergillus* and *Penicillium* genus, however, constitute the major source. These organisms are also used in the majority of research work (Belur et al., 2011). The fungal species of *Aspergillus* and *Penicillum* are the most active microorganisms known, capable of producing tannase through submerged and solid state fermentation (Abdel-Nabey et al., 2011). The use of submerged fermentation (SmF) is advantageous due to its better process control, and simple sterilization method (Mahapatra and Banerjee, 2009; Selwal et al., 2012).

Most of the literature on fungal tannase has reported that the selection of fungal isolates may be obtained from culture collection centers. Though many different exotic environments have been explored and exploited for tannase-producing microbes, the mangrove ecosystem belongs to those the least studied. The mangrove ecosystem in Matang Mangrove Forest Reserve, Perak, Malaysia is very particular in that it harbors several tannin-rich plant species, such as *Rhizophora apiculata* and *R. mucronata*. Because of this specific nature of the ecosystem, mangrove microbial flora have been found to be

worth exploitation for the production of tannase. Therefore, we have attempted to isolate tannase-producing fungi strains from the soil of Matang Mangrove Forest Reserve. In this paper, we report on the production of tannase using *Aspergillus niger* FBT1, a newly isolated strain from a mangrove area which could, potentially, be a source of tannase. Several medium compositions were optimised in order to increase tannase production under a submerged culture.

Materials and methods

Micro-organisms

Tannase-producing fungi were isolated by plate dilution and a spread technique from different soils of Matang Mangrove Forest Reserve in Perak using a selective tannic acid agar medium consisting of (w/v): 1% tannic acid, 0.05% K_2HPO_4, 0.05% KH_2PO_4, 0.05% $MgSO_4$, 0.3% NH_4NO_3, 2.5% agar, pH 5.0. The diluted soil samples were spread on a tannic acid agar medium and incubated at 30 °C for 5 days. Fungal growth was examined daily. The formation of a clear zone around the colony confirmed tannase production and this was subsequently grown on liquid medium of the same composition as the isolation medium, but without agar. The isolated *Aspergillus niger* FBT1 strain showing the highest tannase activity in a liquid medium was selected for further studies of tannase production. The fungus was preserved at 4 °C on an agar slant and subculturing was performed every month to assure its viability.

The fungal strain was grown at 30 °C for 3 days on an agar slant containing an isolation medium until sporulation was completed and then used for inoculum preparation.

The spore inoculum was prepared by adding 10 ml of Tween 80 (0.1%, v/v) to the agar slants and then shaking vigorously. The obtained spore suspension was adjusted to 2×10^7 spores/ml using a hemocytometer slide chamber (Neubauer, Germany) and then used as the inoculum.

Optimization of tannase production under submerged fermentation

The fungus culture was grown in 250-ml Erlenmeyer flasks containing 50 ml of the isolation medium with tannic acid as a sole carbon source, pH 5.5. The initial study was conducted to determine the optimal incubation or cultivation time that secured the highest tannase production. Therefore, 1 ml of the fresh inoculum (2×10^7 spores/ml) was transferred into a liquid medium and incubated at 30 °C on an orbital shaker (200 rpm) for up to 5 days. Optimization of tannase enzyme production was conducted with regards to the incubation time, the incubation temperature, the initial pH, concentrations of tannic acid, nitrogen sources, and concentrations of nitrogen source. The production was performed in the liquid isolation medium. The culture filtrates (through Whatman No. 1 paper) were assayed periodically for tannase activity. The growth of the organism was estimated on the basis of biomass dry weight (g/ml).

Tannase assay

Tannase activity was assayed according to the method described by Libuchi et al., (1967). Enzyme solution (0.5 ml) was added to 2 ml of 0.35% (w/v) tannic acid in 0.05 M citrate buffer (pH 6.0). The substrate solution was pre-incubated at 40 °C for 5 minutes in a test tube before the enzyme solution was added. The reaction mixture (0.05 ml) was withdrawn and added into 5 ml of 95% ethanol to stop the enzyme reaction after 10 minutes of incubation. The absorbance at 310 nm was measured immediately (t_1). After 40 minutes of incubation, the next sample was collected and measured in a similar manner (t_2). The differences between the absorbencies at t_1 and t_2 were determined. Tannase activity (IU/ml) was calculated using the following equation: $1\ \text{IU} = 114 \times (t_2 - t_1)/30$. One unit of tannase activity was defined as the amount of enzyme which was able to hydrolyze one micromole of ester bond per minute.

All the experiments were performed in triplicate and the mean values were reported together with standard errors.

Results and discussion

Identification of Tannase-Producing Fungus

A. niger (FBT1) produced darkly pigmented roughened spores but the hyphae showed septate hyaline, and not dematiaceous. This fungus produced mature colonies within 2 to 5 days. Growth begins initially as a yellow colony that soon develops a black dotted surface, as conidia are produced (Fig. 1A). With age, the colony becomes jet black and powdery, while the reverse of the agar medium remains buff or cream in colour and this occurs on any culture medium (Fig. 1A). Microscopic-

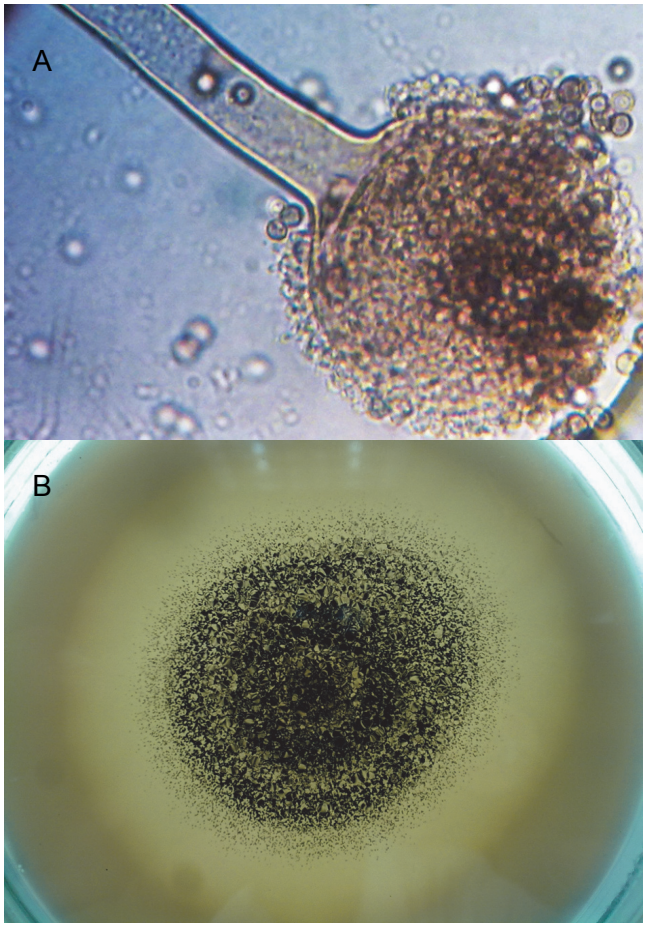

Fig. 1. Morphological characteristics of *Aspergillus niger* FBT1: A) under a light microscope (40 ×), B) growth on a petri dish

ally, FBT1 exhibits septate hyphae and long conidiophores that support spherical vesicles that give rise to large metulae and smaller phialides (biseriare), from which long chains of brown to black, rough-walled conidia are produced (Fig. 1B). The entire surface of the vesicle is involved in sporulation. Based on the above characteristics, the organism FBT1 has been identified as *Aspergillus niger* (Forbes et al., 2007).

Effect of incubation time on tannase production

With an increase in incubation time, tannase production increased and maximum activity was achieved at 72 hours (Fig. 2). With a further increase in incubation time, there was a steep decline in tannase activity. However, the biomass increased continuously and started to drop after 96 hours.

Some authors have reported that maximum tannase production is attained at 72 hours by different fungi, such as *Rhizopus oryzae*, *Aspergillus foetidus* (Kar el al., 2000; Mukherjee et al., 2006), *Aspergillus aculaetus*

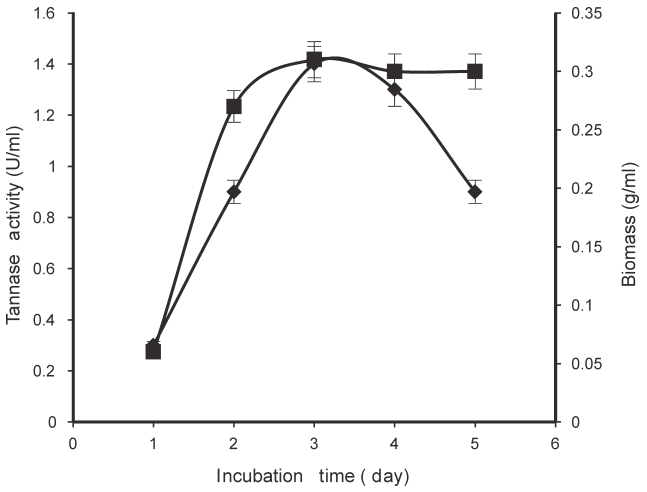

Fig. 2. Effect of incubation time on tannase production – activity (♦) and growth – biomass (■) by *A. niger* under submerged culture

DBF9 (Banerjee et al., 2007) and *Aspergillus niger* ITCC 6514.07 (Srivastava et al., 2009). Others also report that tannase is produced during the exponential phase of the growth of *Aspergillus japonicas* and *Paecilomyces variotii,* and thereafter enzyme production starts to decline while the growth continues (Bradoo et al., 1997; Raaman et al., 2010). The decrease in tannase production may be due to the accumulation of gallic acid, as tannase shows end-product repression with gallic acid (Hadi et al., 1994).

Effect of incubation temperature on tannase production

In the present investigation, maximal tannase production was achieved at the incubation temperature of

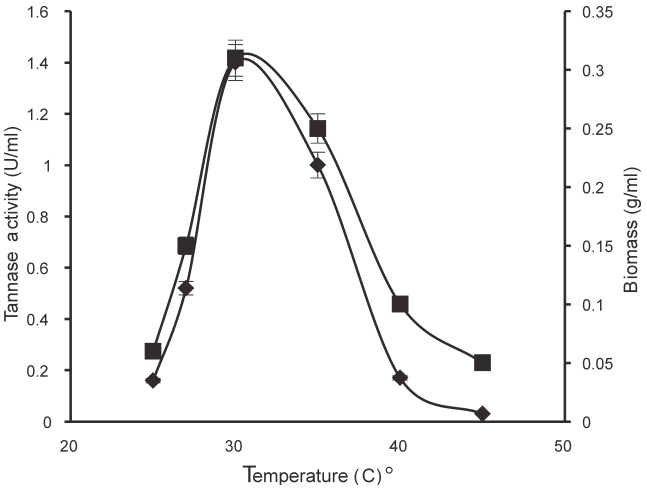

Fig. 3. Effect of different incubating temperatures on tannase production – activity (♦) and growth – biomass (■)

30 °C (Fig. 3), where the fungal growth also achieved its maximal level. However, tannase production and biomass decreased considerably at higher temperatures, i.e. above 30 °C. Similarly, many other researchers have reported an optimum temperature of 30 °C for maximal tannase production from various fungi, such as *R. oryzae* and *A. foetidus* (Batra el al., 2005; Mukherjee et al., 2006), *A. niger* (Cruz-Hernández et al., 2006; Treviño-Cueto et al., 2007), *A. aculeatus* DBF9 (Banerjee et al., 2007), *Aureobasidium pullulans* DBS66 (Banerjee and Pati, 2007), *A. tamarii* (Costa et al., 2008) and *Paecilomyces variotii* (Battestin and Macedo, 2007). The rapid growth of a fungal culture observed at temperatures of 30 °C also suggests that the fungus is mesophilic, and this may be explained by the fact that this fungus was isolated from a mangrove ecosystem.

Effect of initial pH on tannase production

The effect of the initial pH of the medium on growth and tannase production by *A. niger* was tested in order to enhance the tannase yield. Optimal tannase production was observed at an initial pH of 7.0 (Fig. 4). A lower or higher initial pH of 7.0 resulted in low tannase production. Therefore, a pH of 7.0 was used in subsequent experiments.

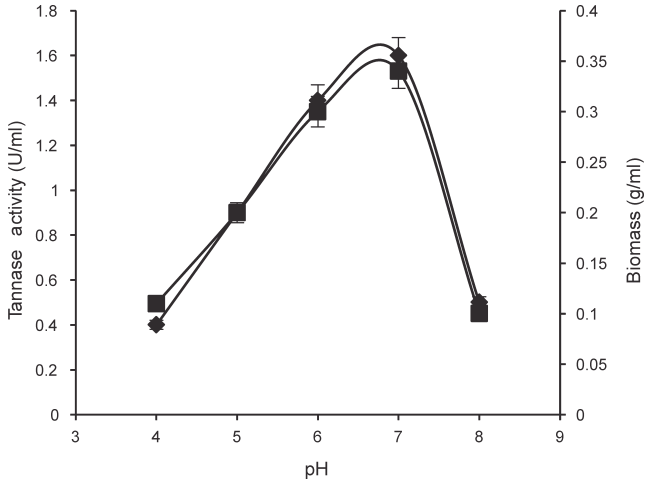

Fig. 4. Effect of pH on tannase production – activity (♦) and growth – biomass (■)

The majority of tannase productions reported to date have been optimally active in the neutral or acidic pH range. Similar to our observations, the optimum pH in a case of tannase produced from *Aspergillus japonicas* was 7.0 (Bradoo et al., 1997). Different results was reported by Lokeswari and Jaya Raju (2007) who found that the optimum pH value for tannase produced by *A. niger* was 5.5.

Effect of different tannic acid concentrations

It was observed that the presence of 2% (w/v) of tannic acid resulted in maximum tannase production and fungal growth (Fig. 5). The tannase production plunged after reaching its highest level.

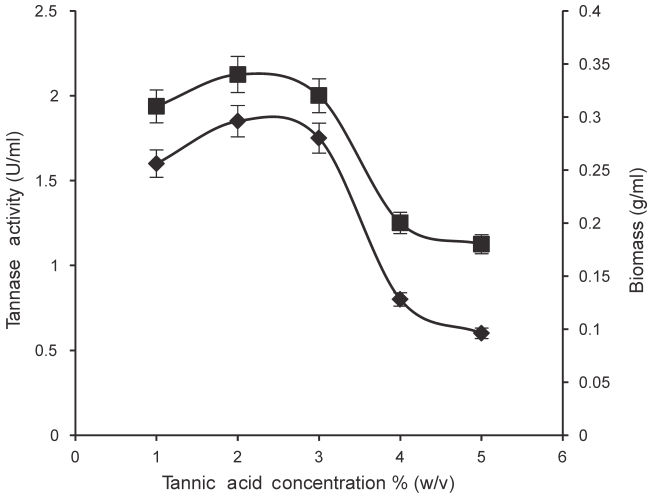

Fig. 5. Effect of various tannic acid concentrations on tannase production – activity (♦) and growth – biomass (■)

Banerjee et al. (2001) found maximum extracellular tannase after 36 hours of submerged fermentation by *A. aculeatus*, containing 2% (w/v) tannic acid. Tannic acid at higher concentrations produces complexes with membrane proteins of the organism; thereby, both the growth and enzyme production may be inhibited (Banerjee et al., 2007). In this case, tannic acid also served as a sole carbon and energy source for fungal growth, besides inducing the production of tannase.

Effect of nitrogen source on tannase production

Among the different nitrogen sources tested, it was found that the addition of sodium nitrate into the cultivation medium enabled maximum tannase production (Fig. 6). On basis of the results obtained, one can conclude that ammonium nitrate stimulates the synthesis of proteins and is a source of readily utilizable nitrogen (Sivashanmugam and Jayaraman, 2011).

Our results (Fig. 7) show that tannase production increased gradually as the concentration of sodium nitra-

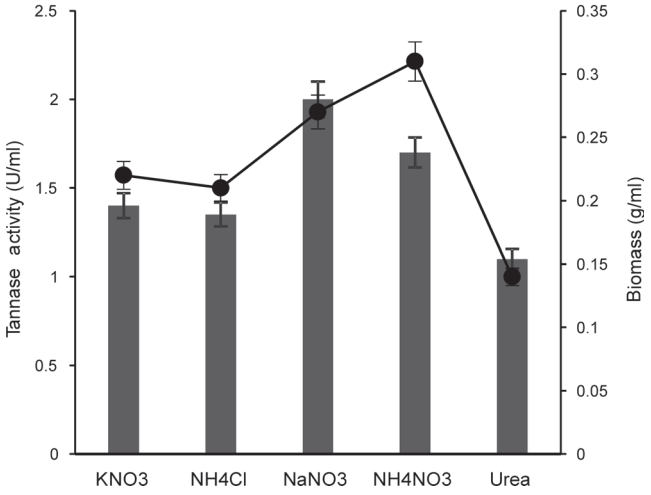

Fig. 6. Tannase production – activity (▬) and growth – biomass (●) with different nitrogen sources

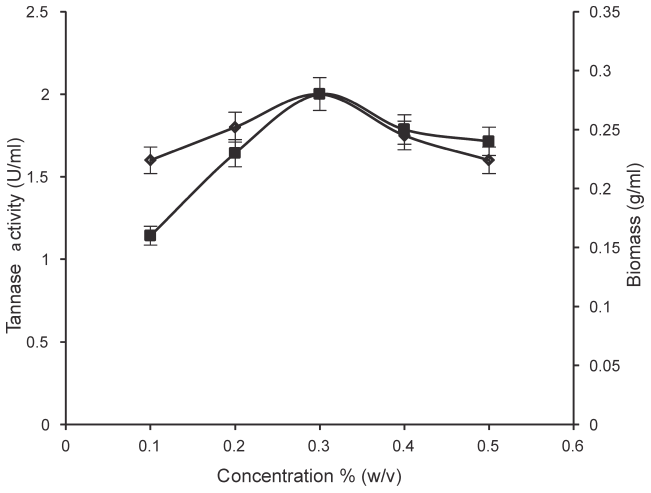

Fig. 7. Effect of various ammonium sulphate concentrations on tannase production – activity (♦) and growth – biomass (■)

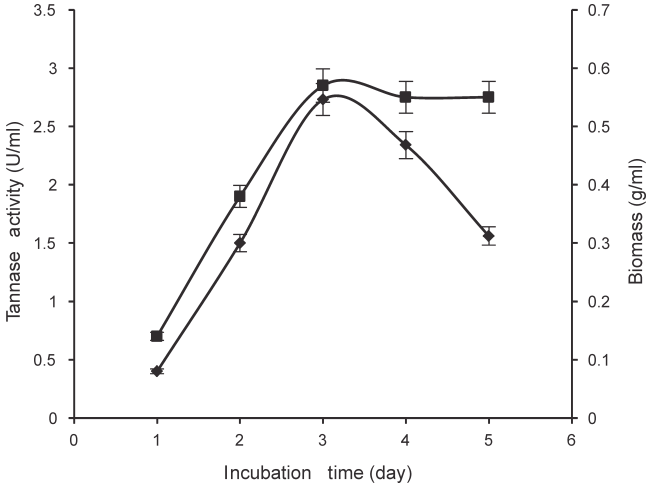

Fig. 8. Tannase production – activity (♦) and growth – biomass (■) by *A. niger* under submerged culture after the optimization of medium compositions

te increased and achieved the highest level at a concentration of 0.7% (w/v). Tannase production decreased slowly after achieving its highest level.

Among the different sources of inorganic nitrogen used, it was found that the addition of sodium nitrate into the cultivation medium produced the maximum tannase, and this has been reported with other species, such as *Aspergillus japonicas* (Bradoo et al., 1996) and *Rhizopus oryzae* (Hadi et al., 1994).

Enzyme production using improved physical parameters

It was found that the maximum values of tannase and biomass production were achieved on the third day of cultivation with about 2.73 U/ml (Fig. 8). A comparison of tannase production before and after optimization showed that optimization of the medium composition resulted as high as 95% increase in the enzyme yield.

Conclusions

This study revealed that the local isolate *Aspergillus niger* from mangrove forest shows a high potential in producing extracellular tannase in relatively high titre under submerged culture within 72 hours. This strain is able to produce tannase in a medium containing tannic acid as the sole carbon source.

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