

Full Length Research Paper

Process parameters influencing tannase production by *Aspergillus niger* using mangrove (*Rhizophora apiculata*) bark in solid substrate fermentation

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***Rhizophora apiculata* bark is a tannin-rich waste material obtained from charcoal industry. This industrial waste was used as solid substrate in the study for the production of tannase and at the same time, help in minimizing the country's industrial wastes. This study was carried out to optimize the physical parameters for the maximal tannase production by *Aspergillus niger* via solid substrate fermentation using a shake flask system. The optimized physical parameters obtained were combination of 0.75 and 2.00 mm of substrate particles at the ratio of 1: 1, Czapek- Dox medium as moisturizing agent at pH 7, initial moisture content of 1: 1.5 (w/v), incubation temperature at 30°C, mixing at every 24 h and the size of inoculum at 1×10^6 spores/ml which finally, produced a maximum tannase of 4.97 U/g of fermented substrate on the 6th day of cultivation.**

Key words: Solid substrate fermentation, tannase, *Aspergillus niger*, *Rhizophora apiculata* barks.

INTRODUCTION

Tannase or tannin acyl hydrolase (EC 3. 1. 1. 20) is an inducible enzyme, produced mainly by fungi but also can be produced by bacteria and plants (Sabu et al., 2006). Tannase hydrolyses hydrolysable tannins and catalyses the hydrolysis of ester bonds in tannic acid releasing glucose and gallic acid (Mahapatra et al., 2005). Tannase is used for a variety of purposes, including the manufacturing of coffee, instant tea and flavored soft drinks and the clarification of beer and fruit juices (Batra and Saxena, 2005). Another potential use for tannase is in the treatment of wastewater contaminated with polyphenolic compounds, such as tannins (Aguilar et al., 2001). Tannins are widespread in the plant kingdom and can be found in seeds, fruits, leaves, bark and wood

(Vaquero et al., 2004). Substrates used for the production of tannase in solid substrate fermentations (SSF) include natural tannin-rich substrates, such as myrobalan fruits and gallo seed cover (Mahapatra et al., 2005), wheat bran (Sabu et al., 2005), jamun leaves, amla leaves and ber leaves (Kumar et al., 2007).

Rhizophora apiculata bark (mangrove bark), is a tannin-rich waste material generated by the charcoal industry. Debarb is a must prior to charcoal making process, as the high moisture content of bark will interfere with the burning process. Bark disposal is a serious problem because it is toxic and leads to environmental pollution. Here, for the first time, we report on the utilization of this charcoal industry waste as the solid substrate for the production of tannase by *Aspergillus niger*. This tannin- rich bark which initially has no market value can now be converted to tannase enzyme which has a big market value especially in various industries. In this study, we optimized process parameters influencing fungal growth and enzyme production to obtain the maximum yield of tannase.

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Abbreviations: SSF, Solid substrate fermentation; BSA, bovine serum albumin.

MATERIALS AND METHODS

Microorganism

Aspergillus niger, isolated from mangrove barks (*R. apiculata*) disposal area of charcoal manufacturing industry at Larut Matang, Perak, Malaysia was used. The fungus was isolated, identified and was available in the culture collection of our laboratory. The microorganism was grown on malt extract agar slant with the addition of 1% (w/v) tannic acid at 25°C for 5 days. This 5 day-old cultures were then used for further work or preserved at 4°C for short term storage.

Inoculum preparation

Ten milliliters (10 ml) of sterilized distilled water was added to the 5 day-old fully sporulated slant culture. The spores were scrapped off aseptically. Spore count was done by using haemocytometer and the spore suspension was adjusted to 10^4 , 10^5 , 10^6 , 10^7 or 10^8 spores/ml, as required for the experiment being conducted.

Solid substrate

Rhizophora apiculata bark was used as the solid substrate without any major pretreatment. The bark was dried at 50°C in an oven to remove free moisture and ground to the size of 0.50 to 2.00 mm particles with a grinder. Particles with the size of 0.50 and 0.75 mm were screened and grouped as fine particles, while 1.00 and 2.00 mm were treated as coarse particles. Fine and coarse particles were used either alone or in combination.

Solid Substrate fermentation

Solid substrate fermentation (SSF) was conducted in 250 ml Erlenmeyer flasks. Five grams (5 g) of *R. apiculata* bark particles between 0.5 and 2 mm either single or in combination were sterilized by autoclaving at 121°C for 20 min. Moisturizing agents were sterilized by filter sterilization through a 0.20 µm sterile membrane filter. After cooling to room temperature, the solid substrates were moistened to a desired quantity using a moisturizing agent. Each flask was inoculated with 1 ml of prepared inoculum (20% w/w), mixed well and incubated statically for variable periods of time (0 to 10 days).

Enzyme extraction

Flasks were harvested by the addition of 30 ml of sodium acetate buffer (50 mM, pH 5.5) and agitated at 200 rpm ($30 \pm 2^\circ\text{C}$) for 1 h on a rotary orbital shaker. Crude enzyme was extracted by direct filtration using filter paper Whatman No. 1. The extractant was collected in screw-capped bottles and kept at 4°C for future analysis.

Enzyme assay

Tannase activity was determined colorimetrically by the method reported by Mondal et al. (2001). The crude enzyme sample measuring 0.5 ml was added to 0.1 ml of tannic acid (0.5% w/v, dissolved in 0.2 M sodium acetate buffer, pH 5.5) and incubated at 60°C for 10 min. Three milliliters (3 ml) of 1.0 mg/ml bovine serum albumin (BSA) was used to stop the reaction. The BSA solution was prepared in 0.17 M sodium chloride in 0.2 M sodium acetate buffer (pH 5.0). The tubes were centrifuged at 5000 rpm for 10 min

to pellet down the tannic acid residues. Pellets were dissolved in 3 ml of SDS-triethanolamine. Absorbance was measured at 530 nm against a blank after the addition of 1 ml of FeCl₃ reagent. A control reaction was done side-by-side with a heat-denatured enzyme. One unit of tannase activity was defined as the amount of enzyme that hydrolyzed 1 mM of substrate tannic acid in one minute under the assay conditions. Enzyme activity was expressed as units/gram of fermented substrate (U/g of fermented substrate).

Determination of fungal growth

Fungal growth was determined by the estimation of glucosamine content present in the cell wall, as suggested by Swift (1973). Growth was expressed as milligrams/gram of fermented substrate (mg/g).

Optimization of process parameters

The protocol adopted for optimizing the process parameters influencing tannase yield was to optimize one particular parameter at a time. The effect of individual parameter was evaluated, while keeping other parameters constant. Then, the evaluated parameter was incorporated at its optimized level in the subsequent optimization experiment (Suresh et al., 2005). The parameters analyzed are given in the following sections.

Effect of substrate particle size

Different sizes of *R. apiculata* bark either singly or in combination was used as solid substrate. Bark size used singly were 0.1, 0.75, 1 and 2 mm. Bark combinations were made by mixing fine and coarse bark particles in a 1: 1 (w/w) ratio. Bark combinations used were 0.5: 1, 0.5: 2, 0.75: 1 and 0.75: 2 mm.

Effect of the type of moisturizing agent and initial moisture content

Three different moisturizing agents were evaluated: tap water, distilled water and modified Czapek dox medium. The modified Czapek dox medium contained (g/l): 0.4% NaNO₃, 0.15% KH₂PO₄, 0.075% MgSO₄·7H₂O, 0.075% KCl and 3% tannic acid. The substrate to initial moisture content ratios (w/v) evaluated were: 1: 0.5, 1: 1, 1: 1.5, 1: 2 and 1: 2.5.

Effect of initial pH of moisturizing agent

Initial pH values tested were: pH 4.0, 5.0, 6.0, 7.0, 8.0 and 9.0. The pH of the moisturizing agent was adjusted with 0.1 M NaOH or 0.1 M HCl and then filters sterilized.

Effect of incubation temperature

The flasks were incubated at temperatures ranging from 25°C, room temperature ($27 \pm 3^\circ\text{C}$), 30, 40 and 50°C for 8 days.

Effect of mixing frequency

Inoculated flasks were mixed at different intervals: every 12, 24, 48 and 72 h, during the incubation period.

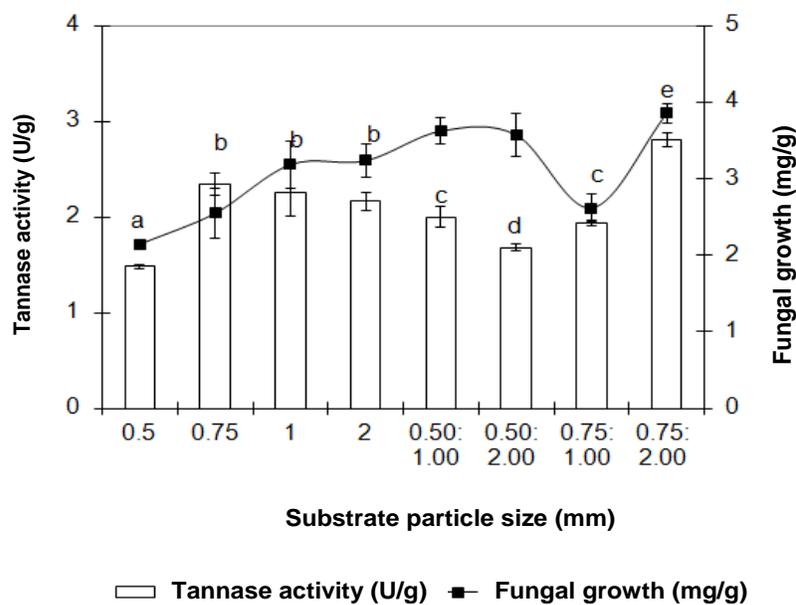


Figure 1. Effects of substrate particle size on tannase production and fungal growth.

Effect of inoculum size

Inoculum concentrations tested were: 1×10^4 , 1×10^5 , 110^6 , 1×10^7 and 1×10^8 spores/ml.

Effect of cultivation time

A time course study (0 to 10 days) in which all of the optimized parameters were used was conducted to determine when the enzyme yield was at the highest. The flasks were harvested at regular intervals of 48 h and analyzed for tannase activity.

All experiments were carried out in triplicates and the mean values with standard deviation were reported. One-way ANOVA was performed to analyse the results statistically. All statistical tests were done in SPSS version 11.5 and Microsoft office excel 2003. Letters in the figures represent mean values of tannase activity. Means with different letters shown in figures are significantly different (Duncan, $p < 0.05$). Error bars in the figures indicate the standard deviation values of the triplicates.

RESULTS

Initial profile

The initial profile (result not shown) conducted prior to the optimization studies showed that, 8 days was the optimal incubation period for the production of tannase, when 2.85 U/g of fermented substrate was obtained. Fungal growth achieved was 3.90 mg/g of fermented substrate.

Effect of substrate particle size

Different sizes of substrate particles either singly or in

combination were used in the fermentation (Figure 1). Combination of 0.75 and 2 mm gave the highest yield of enzyme, which is 2.81 U/g of fermented substrate. Maximal fungal growth (3.86 mg/g of fermented substrate) was observed from the combination of 0.75 and 2.00 mm as well.

Effect of the type of moisturizing agent and initial moisture content

Among the three different agents tested, tap water, distilled water and modified Czapek dox medium, tannase activity (3.75 U/g of fermented substrate) and fungal growth (5.44 mg/g of fermented substrate) were found to be highest from solid substrate fermentation (SSF) flasks using modified Czapek dox medium as moisturizing agent, followed by distilled water and tap water. The ratio of 1: 1.5 (w/v) was the best ratio of initial moisture content for the production of tannase (4.49 U/g of fermented substrate) with fungal growth, 4.10 mg/g of fermented substrate (Figure 2).

Effect of initial pH of moisturizing agent

Modified Czapek dox medium with pH 7.0 was found to be the optimum for the yield of tannase (3.45 U/g of fermented substrate) as well as fungal growth (4.70 mg/g of fermented substrate). Acidic Czapek dox media (pH 4.0 and 6.0) were found to be favoring the growth of *A. niger* but not high yield of the enzyme. Basic Czapek dox medium (pH 8.0 and 9.0) also supported higher yield of

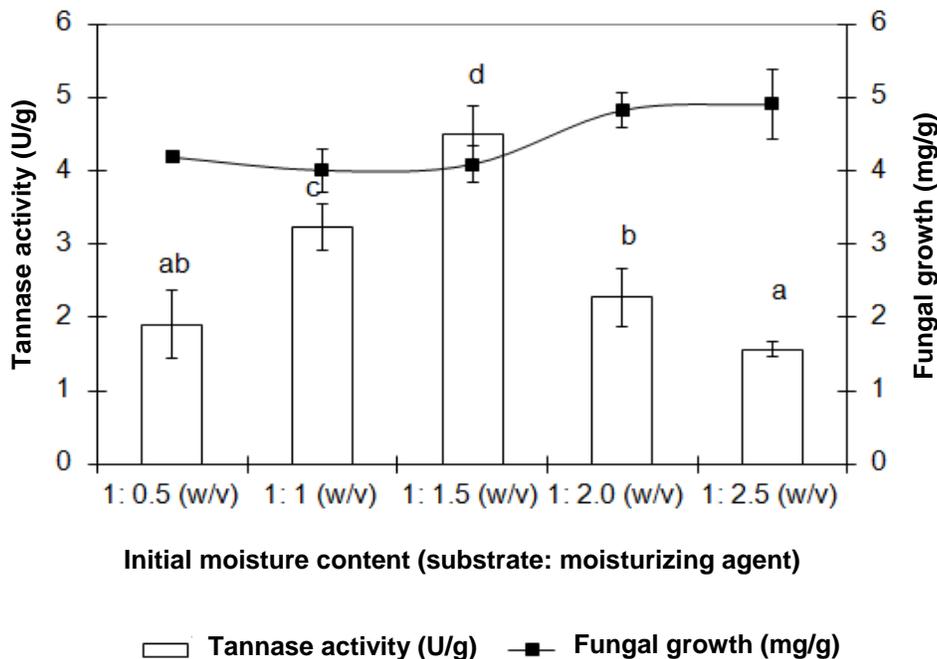


Figure 2. Effects of ratio of initial moisture content on tannase production and fungal growth.

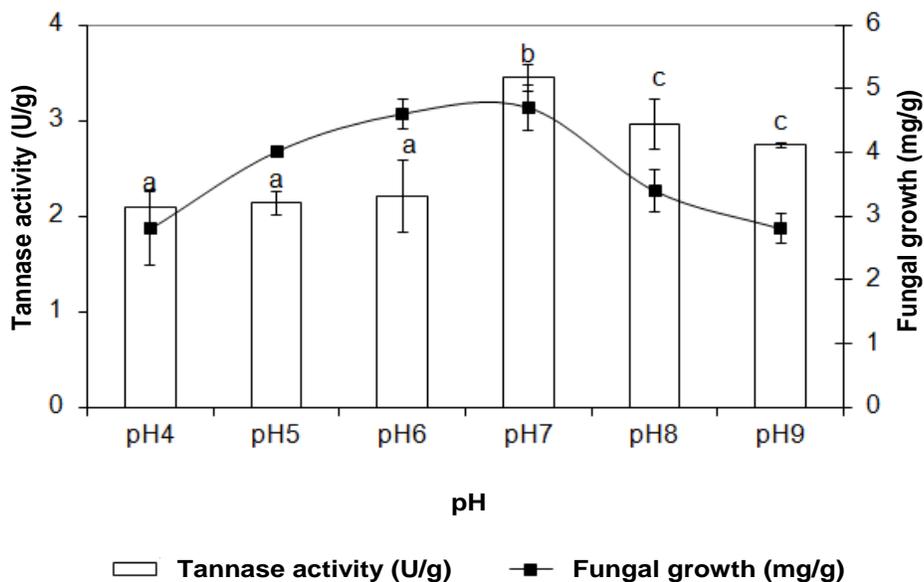


Figure 3. Effects of initial pH value on tannase production and fungal growth.

enzyme compared with acidic Cazpek dox medium but did not favor the optimal growth of fungus as shown in Figure 3.

Effect of incubation temperature

Among the different temperatures (Figure 4), 30°C was

found to be the optimum temperature for the production of tannase by *A. niger* (4.21 U/g of fermented substrate) with the growth at 3.83 mg/g of fermented substrate. The fungus achieved maximum growth at 40°C (4.10 mg/g of fermented substrate). Low yield of enzyme was observed at room temperature, which fluctuated tremendously from 24 to 30°C throughout the incubation periods. Enzyme 30°C. activity started to decrease at temperatures

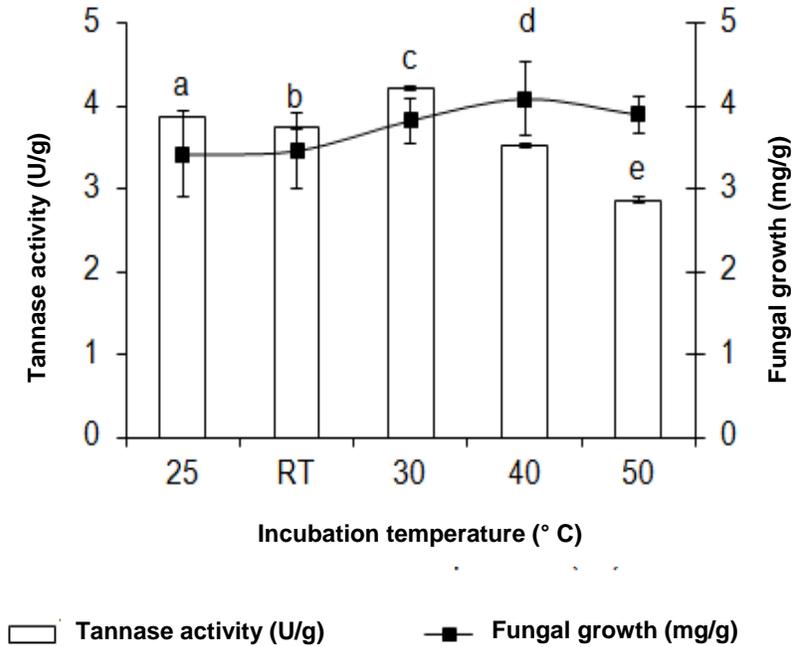


Figure 4. Effects of incubation temperature on tannase production and fungal growth.

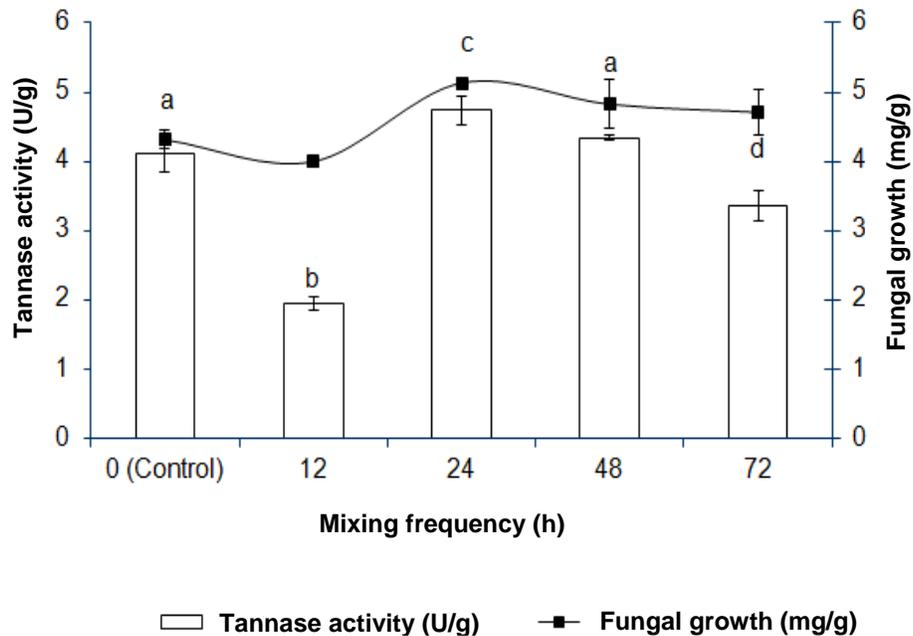


Figure 5. Effects of mixing frequency on tannase production and fungal growth.

higher than

Effect of mixing frequency

The results of mixing frequency on enzyme production

and fungal growth are shown in Figure 5. Among the frequencies tried, mixing done at every 24 h, was found to be the best frequency for the maximum production of tannase enzyme (4.73 U/g of fermented substrate) as well as fungal growth (5.13 mg/g of fermented substrate). Mixing at every 12 h gave the lowest yield of

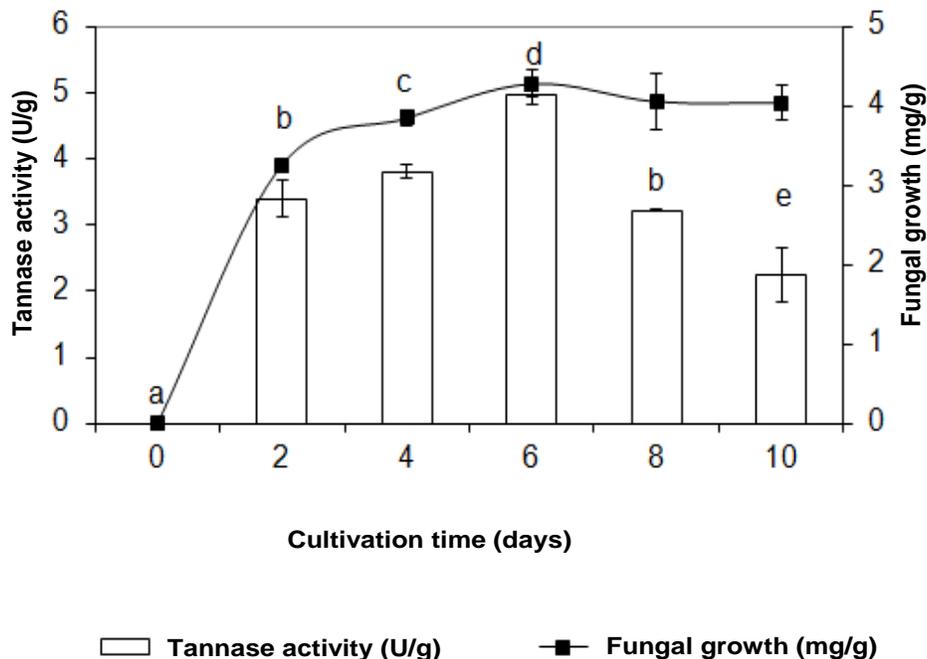


Figure 6. Time course study on the production of tannase and fungal growth.

tannase as well as fungal growth.

Effect of inoculum size

Maximum tannase production (4.40 U/g of fermented substrate) was observed with an inoculum of 1×10^6 spores/ml (Results not shown). The fungal growth at this inoculum was 5.65 mg/g of fermented substrate. The yield of enzyme was found to decline with the further increment of inoculum size.

Effect of cultivation time

Finally, all the optimized parameters were incorporated and fermentation was carried out for different periods of incubation. The results after optimization of culture conditions show that, the maximum yield of enzyme was observed on the 6th day of cultivation (4.97U/g of fermented substrate) with fungal growth achieved at 4.28 mg/g of fermented substrate (Figure 6).

DISCUSSION

A. niger produced significant amounts of tannase enzyme (2.85 U/g of fermented substrate) using *R. apiculata* barks on day 8 of initial profile. Optimization of substrate particle size revealed that, combination of 0.75 and 2 mm produced the highest yield of tannase and fungal growth. Small sized substrate particles increased the surface

area for microbial action, facilitating bioconversion (Kar et al., 1999), advantageous for heat transfer and exchange of oxygen and carbon dioxide between air and solid surface (Sato and Sudo, 1999), but might result in substrate agglomeration if the particles are too small (Krishna, 2005). Larger particles give a better respiration and aeration efficiency but limited surface for microbial action and also very low in water holding capacity (John et al., 2006). Hence, the combination of suitable size of fine and coarse particles enables them to compliment among each other to promote a suitable culture condition for the optimal growth of culture and production of enzyme.

Tannase is an adaptive and induced enzyme (Lokeswari and Jaya Raju, 2007) and the presence of tannic acid in the modified Czapek dox medium enhances the yield of tannase. Besides, the presence of basal salts in modified Czapek dox medium also favors the optimal growth of the fungus. Even when distilled water and tap water were used, the production of tannase was not insignificant, which means that *R. apiculata* bark is a nutrient-rich substrate which is suitable for the production of tannase with minimum external nutrients. Additionally, initial moisture content of solid substrate is an important factor which dictates the growth of the organism, new cell synthesis and enzyme production (Sabu et al., 2006). Highest yield of tannase was obtained when initial moisture ratio was adjusted to 1: 1.5 (w/v). Tannase yield reduced with the further increment in initial moisture ratio. Krishna (2005) reported that, fungi are well known to be favoured by a moist environment, for their growth while filamentous fungi are known to grow

at water deficient substrates (Hölker et al., 2004).

Fungal tannases are acidic proteins in general (Lokeswari and Jaya Raju, 2007) and many had reported that the optimum initial pH for tannase production is around 5.5 (Lekha et al., 1997). However, in the present study, the maximum tannase yield as well as fungal growth was observed at modified Czapek dox medium with pH 7.0 (3.45 U/g). This can be explained by considering the chemical property of substrate used in this fermentation system. *R. apiculata* bark is rich in tannin and highly acidic in nature (Rahim et al., 2008). Due to its acidic and complex chemical composition property, the incorporation of neutral moistening agent in this study (Figure 3) has been found to be essential to create an optimal condition for the growth and production of tannase. Kheng et al. (2006) reported that, the mechanism of pH effect on the growth and metabolite production by microorganisms on solid substrates remains unclear. This is because the natural habitat of microorganisms on solid substrates can be highly influenced by rapid changes in several governing physical parameters in its environment, including changes in environmental pH.

John et al. (2006) reported that, the incubation temperature affects the viability of culture and the yield of product formation in solid substrate fermentation system. Metabolic heat generation will also be added up during the course of fermentation and it may or may not be good for the culture. The present study found that, 30°C was the optimum temperature for the production of tannase and the growth of *A. niger* was maximal at 40°C. Furthermore, this study revealed that a constant incubating temperature is important to ensure the growth and production of enzyme as low yield of enzyme was observed at room temperature, which fluctuated between 24 and 30°C throughout the period of incubation. Kumar et al. (2007) and Sabu et al. (2005) also reported an optimal incubation temperature of 30°C for the production of tannase when *Aspergillus ruber* and *A. niger* respectively, were used. The temperature range of 28 to 35°C is the optimum for the growth of many fungi (Kheng et al., 2006).

Mixing at every 24 h maximized the yield of tannase and fungal growth. However, frequent mixing (at every 12 h) hampered the growth of fungi and low yield of enzyme was observed. Chatterjee et al. (1996) reported that, the absence of mixing in solid substrate fermentation contributes to a compact substrate and thus, lessening the mass transfer processes. Besides, Krishna (2005) also pointed that, intermittent agitation is more appropriate than continuous agitation to prevent damage to the mycelia and disruption of mycelial attachment to solids in certain cases. Therefore, mixing at suitable frequency lends a hand to the system to maximize the utilization of substrate for the growth as well as tannase production.

Pandey et al. (2000) reported that, a balance between the biomass proliferation and available substrate material

is important as it ensures the yield of maximum enzyme. In this study, inoculum at the concentration of 10^6 spores/ml was found to be at an optimum concentration for the maximal yield of tannase. Lower tannase was obtained at inoculum concentration lower than 10^6 spores/ml. Kheng et al. (2006) reported that, higher enzyme production at higher inoculum is related to the rapid growth of the fungus which resulted in higher degradation of the substrates and increased availability of the nutrients. Furthermore, Kashyap et al. (2002) also evaluated that, lower level of inoculum may not be sufficient to initiate the growth and enzyme synthesis on different substrates. The enzyme production will start to decline after a certain limit of time because of depletion of nutrients due to enhanced biomass, which would result in a reduction in metabolic activities.

Maximum enzyme activity (4.97 U/g of fermented substrate) obtained after optimization experiments increased by 74.39% when compared to the maximum enzyme activity obtained from the initial experiment, which was 2.85 U/g. Fungal growth after optimization (Figure 6) was found to be higher than fungal growth achieved in initial experiment. Moreover, the time course study also shows that, the optimization of physical parameters was not only able to enhance the high yield of tannase, but also reduced the incubation time, which is 6 days instead of 8 days as observed from initial profile.

Conclusion

This is the first report on the use of *R. apiculata* bark as solid substrate for the production of tannase under solid substrate fermentation. The study clearly reveals the potentials of this tannin-rich charcoal industry waste as one of the cost effective substrate to be explored and used in large-scale production. This research was to convert "waste to wealth".

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