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Tannase Production by Solid State Fermentation of Cashew Apple Bagasse

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Abstract

The ability of *Aspergillus oryzae* for the production of tannase by solid state fermentation was investigated using cashew apple bagasse (CAB) as substrate. The effect of initial water content was studied and maximum enzyme production was obtained when 60 mL of water was added to 100.0 g of CAB. The fungal strain was able to grow on CAB without any supplementation but a low enzyme activity was obtained, 0.576 U/g of dry substrate (g_{ds}). Optimization of process parameters such as supplementation with tannic acid, phosphorous, and different organic and inorganic nitrogen sources was studied. The addition of tannic acid affected the enzyme production and maximum tannase activity (2.40 U/g_{ds}) was obtained with 2.5% (w/w) supplementation. Supplementation with ammonium nitrate, peptone, and yeast extract exerted no influence on tannase production. Ammonium sulphate improved the enzyme production in 3.75-fold compared with control. Based on the experimental results, CAB is a promising substrate for solid state fermentation, enabling *A. oryzae* growth and the production of tannase, with a maximum activity of 3.42 U/g_{ds} and enzyme productivity of 128.5×10^{-3} U · g_{ds}^{-1} · h^{-1}.

Index Entries: Cashew apple bagasse; solid state fermentation; tannase; *Aspergillus oryzae*; tannic acid; ammonium sulfate.

Introduction

In the north coast of Brazil, especially in the state of Ceará, the cashew agroindustry has an outstanding role in the local economy. The cashew apple, a pseudofruit or peduncle, is the part of the tree that connects it to the cashew nut, the real fruit and a well-known product around the world. The cashew apple is a hard, pear-shaped, small, and nonclimacteric fruit, and is found in three colors: yellow, orange, and red. The most commonly commercialized ones are the yellow and red fruits. The edible portion,

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Table 1
Physico-chemical Characterization of Natural Bagasses of Cashew (*Anacardium ocidentale* L)

Parameters	Natural cashew bagasse (CAB)
pH	4.01
Acidity (gram of citric acid/100.0 g of sample)	1.34
Soluble solids (Brix)	12.0
Reducing sugars (mg/100.0 g)	6.84
Total sugars (mg/100.0 g)	7.68
Proteins (%)	1.83
Lipids (%)	0.38
Fibers (%)	33.10
Moisture (%)	78.76

From ref. 7.

representing 90% of the fruit, is rich in vitamin C, flavor, and aroma. Internal and external market consumption of cashew nut, in the year of 2004, was about 232,000 t. However, only 12% of the total peduncle is consumed "*in natura*" or processed industrially to produce a wide range of products from concentrated juice to desserts. The industrially processed products are basically consumed by the local market and they do not play an important role in the state or Brazil economy. Furthermore, the majority of the cashew apple spoils in the soil *(1–4)*. When peduncle is industrially processed for the production of juice, 40% (w/w) of bagasse is produced, which is not used for human consumption and is usually discarded by the local industry. These facts, together with its composition (*see* Table 1), turns cashew apple bagasse (CAB) into an interesting and inexpensive (<$0.50/Kg) substrate for several potential applications *(5–7)*, including the production of microbial enzymes by solid state fermentation (SSF).

SSF has been defined as a bioprocess in which microorganisms are grown on solid substances in the absence or near absence of free water *(8)*. Two types of substrates are used in SSF—one in which the solid substrate itself is used by the microorganisms as the carbon and energy source and other in which the substrate acts only as support. SSF mainly deals with the utilization of agroindustrial residues as substrates. Application of such residues as substrates is certainly economical and it also reduces environmental pollution *(9)*. In this work, CAB, a coproduct of cashew apple juice, is investigated for the production of tannase through SSF.

Tannase (tannin-acyl-hydrolase, Enzyme Commission [EC], 3.1.1.20) catalyzes the breakdown of hydrolysable tannis and gallic acid esters *(10)*. Hydrolysable tannins are present in most of the residues obtained from higher plants, which is the case of CAB, and are polyphenolic compounds formed by the association of sugars with ellagic acids through esters linkages *(11)*. Tannase

Table 2
Physico-chemical Characterization of the CAB

Parameters	Natural cashew bagasse (CAB)
Proteins (% [w/w])	16.7
Lipids (% [w/w])	5.22
Glucose (% [w/w])	0.25
Micronutrients (ppm)	
Zn	14.92
Fe	49.72
Mn	14.82
Cu	18.31
Mg	8.13

CAB (*A. ocidentale,* L.) used in this work.

can be obtained from plant sources; the enzyme is present in tannin rich vegetables, mainly in their fruits, leaves, branches, and barks of trees *(10,12,13)* as well as in bovine intestine and other ruminant mucous *(10)*. However, the most important source of industrial tannase is microbial production, as these enzymes are more stable than similar ones obtained from other sources *(10)*.

Tannases are widely used in the food and pharmaceutical industry, especially in tea clarification and also for the production of pharmaceutically important compounds, such as gallic acid. Gallic acid, a tannin product, is the substrate for chemical synthesis of propyl gallate and trimethoprim, which are important in food and pharmaceutical industries, respectively. Tannase is also used in the treatment of tannery effluents for the stabilization of malt polyphenols, clarification of beer and fruit juices, for the prevention of phenol-induced madeirization in wine and fruit juices, and for the reduction of antinutritional effects of tannins in animal feed *(14–16)*. Therefore, the objective of this work was to study the potential of CAB for the production of tannase, by SSF using *Aspergillus oryzae*.

Materials and Methods

Solid Substrate Preparation

CAB was used as a substrate and was kindly donated by the Kraft Foods unit located in Aracati, State of Ceará, Brazil. Before storage, it was washed three times with water and dried for 24 h at 50°C and characterized, because the CAB composition varies depending on the fruit type and harvest. The average composition of the CAB used in this work is described in Table 2.

Microorganism and Inoculum Preparation

A fungal strain of *A. oryzae* from the Embrapa Agroindústria Tropical, Ceará, Brazil was used in this study. The microorganism was grown and

maintained on Nutrient Agar slants at 32°C. Spore suspensions were extracted with 10 mL of a sterile water solution containing 0.4% Tween 80. Afterwards, 1 mL of spore suspension was placed in flasks, containing 10.0 g of corn brain, 4 mL of a solution 1.7% (w/v) $NaHPO_4$ and 2.0% (w/v) $(NH_4)_2SO_4$, and incubated at 30°C for 3 d. Viable spores were scraped with 40 mL of sterile 0.4% (w/v) Tween 80 solution and determined by plate count technique.

Humidity Determination

Two gram of sample was analytically weighted and dried at 90°C for 24 h in an air circulation oven.

Water Activity Determination

Water activity (a_w) is defined as the relative humidity of the gaseous atmosphere in equilibrium with the substrate. In SSF process, water activity of the substrate quantitatively express water requirements for microbial activity. Pure water has a_w equal to 1.0, which diminishes with increase in amounts of substrate *(17)*. In this work, water activity was determined using an Aqualab CX-2 (Decagon Devices, Pullman, WA) device at 30°C.

Preparation of SSF Medium for Inoculation

SSF was carried out in 500-mL conical flasks containing 40.0 g of medium, CAB enriched or not with tannic acid and moistened with water. Flasks were autoclaved at 121°C for 15 min, cooled to room temperature, and inoculated with fungal spores (10^7 spores/g). The contents were mixed thoroughly and incubated at 30°C for 96 h. Every 24 h, samples were withdrawn by removing a single Erlenmeyer flask from the incubator.

Enzyme Extraction

Tannase was extracted from the fermented substrate by adding 100 mL of acetate buffer (pH 5.0). The contents were incubated at 30°C for 60 min and the crude enzyme was separated by filtration through Whatman No. 1 paper. The filtrate, here denominated as enzymatic extract, was colleted in vials and preserved for further analysis.

Gallic Acid Assay

Gallic acid concentration in the enzymatic extract was determined according to alcoholic rhodanine method that is based on the formation of a chromogen. The developed color was read at 520 nm using a Cary 50 spectrophotometer (Varian, Melbourne, Australia) *(18)*.

Tannase Assay

For tannase determination, the enzymatic extract was incubated with buffered solution (acetate 20 m*M*, pH 5.0) of tannic acid 200 mg/L at 30°C

for 5 min *(19)*. The gallic acid released was quantified by alcoholic rhodanine method *(18)*. One unit of tannase activity was defined as the amount of enzyme that catalyses the production of 1 μmol of gallic acid/min under assay conditions.

Determination of Total Phenolics

Tannic acid concentration in the enzymatic extract was determined with the method proposed by Folin and Denis *(20)*.

Optimization of Process Parameters

SSF was carried out to study the effect of several parameters required for the optimal production of tannase by *A. oryzae*. Initial amount of water added to the substrate (40, 60, 80, and 100 mL for 100.0 g of dry CAB), incubation time (0–96 h), supplementation with tannic acid (2.5, 5.0, 7.5, 10.0, and 12.5% [w/w] of tannic acid for 100.0 g of CAB), phosphorous (0.5, 1.0, 1.5, and 2.0 % [w/v]), and different organic (peptone and yeast extract at 1% [w/v]) and inorganic nitrogen sources (ammonium nitrate at 1% [w/v] and ammonium sulfate from 0.5 to 4.0% [w/v]) were the studied parameters.

Tannase Productivity

In this work tannase productivity (P_{EN}) is defined as the amount of the desired product formed (tannase activity—A_{EN}) per reaction time (t_{max}) to achieve the highest enzyme activity *(21)*, *see* Eq. 1.

$$P_{EN} = \frac{A_{EN}}{t_{max}} \tag{1}$$

Results and Discussion

Effect of Initial Water Content

Several studies have showed that initial water content is a critical factor for growth and enzyme production and it is intimately related to the definition of SSF because it is necessary for new cell synthesis *(9,17,22–24)*. Water is needed for cooling and also for incorporation into new microbial cells. Moreover, some authors *(25)* observed that fungal growth could be hampered by limited water availability. Therefore, in this work, the effect of initial water content on tannase production was investigated and results are pictured in Fig. 1.

It can be observed that maximum enzyme production was obtained when 60 mL of water was added to 100.0 g of dry CAB and 0.576 U/g of dry substrate (g_{ds}). In this medium the humidity and water activity were 40.4% and 0.978, respectively (Table 3). The increase in water content beyond 60 mL inhibited enzyme production. With increasing water content, keeping

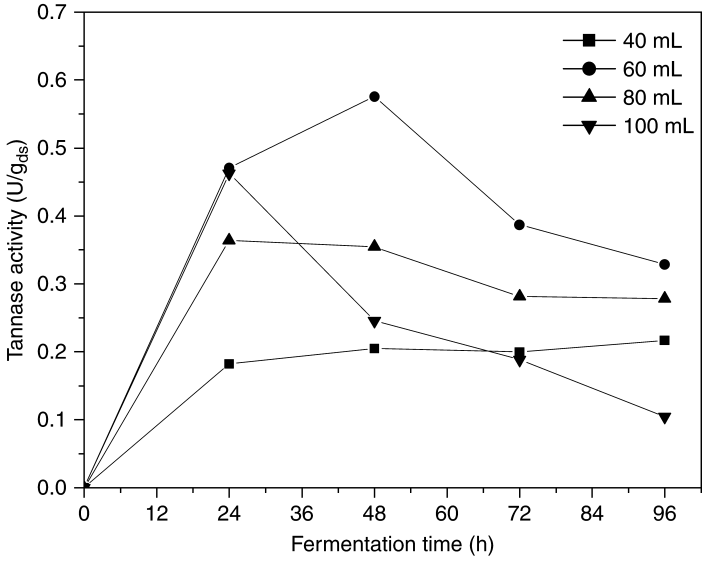

Fig. 1. Effect of water content on tannase yield production by *A. oryzae* in SSF of CAB (100.0 g) at 30°C.

Table 3
Humidity and Water Activity of the CAB Used in This Work for Different Amounts of Added Water

Added volume (mL)	Humidity (%)	a_w
40	31.6	0.965
60	40.4	0.978
80	47.2	0.983
100	51.8	0.993

substrate volume constant, the air content of the substrate occupied within the interparticle space, decreases. Moreover, the scarcity or excess of water affects the decomposition rate of the organic matter, which was found to decrease, affecting enzyme production *(9,24)*. Low substrate water content may result in poor microbial growth and product formation owing to the poor access to nutrients arising from reduced mass transfer of gas and solute to the cells *(26)*. Some experiments have showed the influence of water content on the metabolism of microorganisms. The influence of water content on a solid substrate on growth rate and sporogenesis of filamentous fungi was reported *(27)*. Others authors *(28)* studied the influence of water activity on enzyme biosynthesis and enzyme activities produced by fungi. The water content of the medium is a fundamental parameter for mass transfer of water and solutes across the cell membrane. The control of this parameter could be used to modify the metabolic production or excretion of a microorganism *(17)*. Optimal water content allows the entry of nutrients

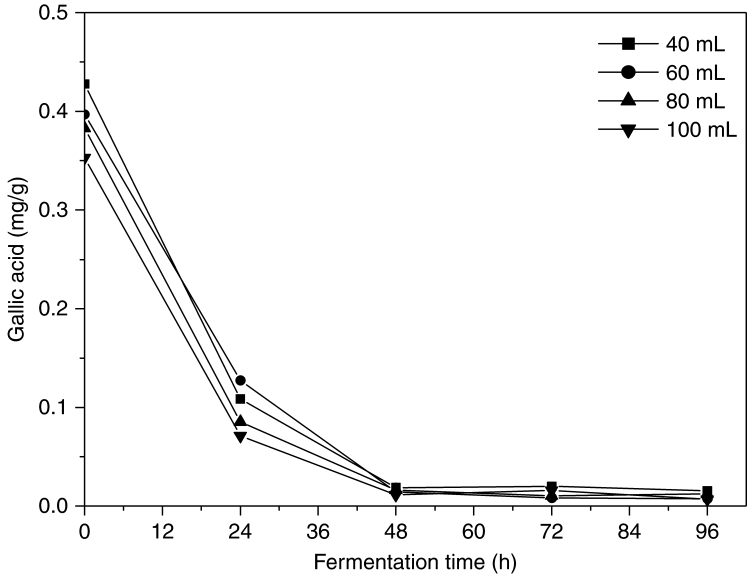

Fig. 2. Gallic acid concentration during SSF of CAB supplemented with different amounts of water.

through the cell walls, which enhances enzyme production. Any deviation from optimal may decrease enzyme production owing to osmotic imbalance inside the cells *(29)*.

Tannase production is a response of an induction process *(10)*. However, in this stage no inducer was added to the culture media, but tannase activity was detected. The analysis of fermentation extracts showed that phenolic compounds were presented in all fermentation media, from 0.50 to 0.55 mg/g (data not shown). Specifically, gallic acid, a pointed tannase inducer, was observed as a natural component of culture media from 0.35 to 0.43 mg/g (Fig. 2). In fermentation process, the gallic acid content decreased, probably as a result of microorganism consumption (Fig. 2). Based on the obtained results, all the subsequent fermentations were performed using 60 mL of initial water content.

Effect of Addition With Tannic Acid

The supplementation of CAB with different amounts of tannic acid was investigated and the results are presented in Fig. 3. It can be observed that addition of tannic acid affected the enzyme production and maximum tannase activity (2.40 U/g_{ds}) was obtained with 2.5% (w/w) supplementation. Moreover, the addition of 2.5% of tannic acid stimulated tannase production about 4.2-fold, when compared with the results presented in Section *Effect of Initial Water Content*. Other authors observed an improvement in tannase production when tannic acid was added to the culture media *(9,24)*. An increase in tannic acid concentration over 2.5%, did not correspond to more tannase production. These results are similar to those observed by

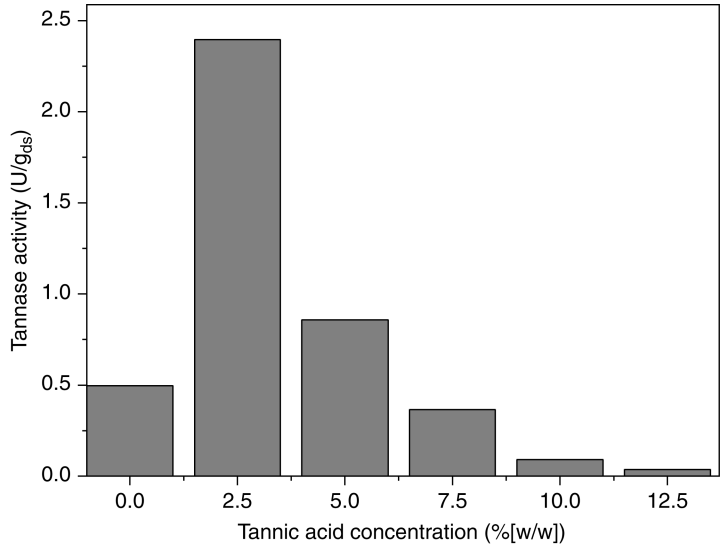

Fig. 3. Effect of tannic acid supplementation (% [w/w]) on tannase production by SSF at 30°C, 40.4% humidity, 40.0 g of CAB, and 48 h of incubation period.

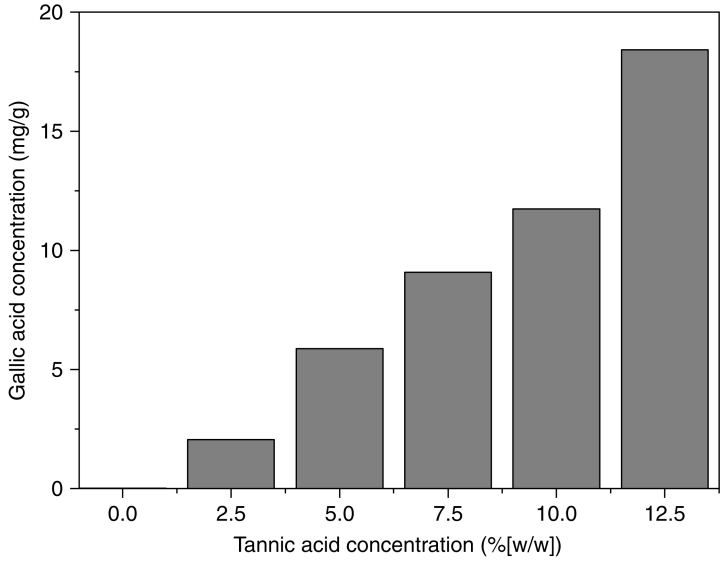

Fig. 4. Gallic acid concentration during SSF of 40.0 g of CAB supplemented with 2.5% (w/w) tannic acid at 30°C, 40.4% humidity, and 48 h of incubation period.

Lekha and Lonsane *(10,30)*. For high concentrations of tannic acid, ranging from 4 to 20%, tannase production by *A. niger* PKL104 and *A. oryzae* were negatively affected. The authors suggested that a growth inhibition, and consequently less enzyme synthesis, was related to high tannin concentration.

Figure 4 presents gallic acid profile in culture media. In all media supplemented with tannic acid, a gallic acid accumulation until 48 h was

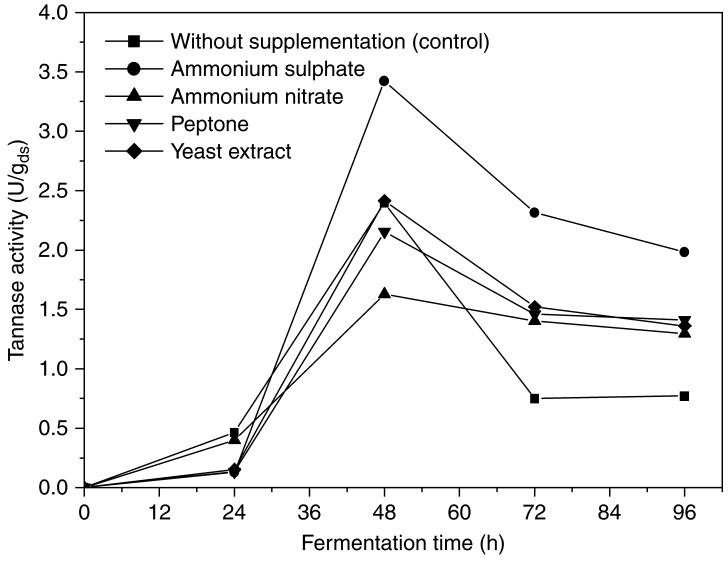

Fig. 5. Effect of supplementation of different nitrogen sources (1% [w/v]) on tannase production by *A. oryzae* in SSF of 40.0 g of CAB supplemented with 2.5% (w/w) tannic acid at 30°C and 40.4% humidity.

observed. The relation between the presence of high amounts of this compound and enzyme activity is not clear. Some authors *(10, 31–33)*, showed that gallic acid acts as inductor of synthesis. In opposite way, other authors *(34,35)* point gallic acid out as a great feedback repressor. Based on the obtained results, all the subsequent fermentation media were supplemented with 2.5% (w/w) of tannic acid.

Effect of Supplementation of Nitrogen Sources

The effect of supplementation of different organic (peptone and yeast extract) and inorganic (ammonium nitrate and ammonium sulfate) nitrogen sources on tannase production was evaluated and results are shown in Fig. 5. It can be observed that ammonium nitrate, peptone, and yeast extract exerted no influence on tannase production. These results were similar to those obtained by other authors studying the production of tannase by *A. niger* 3T5B8 in wheat straw *(33)*. These authors point the distinct assimilation of inorganic ions and the possibility of complex formation between tannins and proteic structures of yeast extract and peptone. Ammonium sulphate, at a concentration of 1%, on the other hand, improved the enzyme production in 1.43-fold compared with control (fermentation in the absence of nitrogen sources). Nitrogen can be an important limiting factor in the microbial production of enzymes. The presence of an additional nitrogen source in the substrate may have promoted cell growth and enzyme production *(9)*.

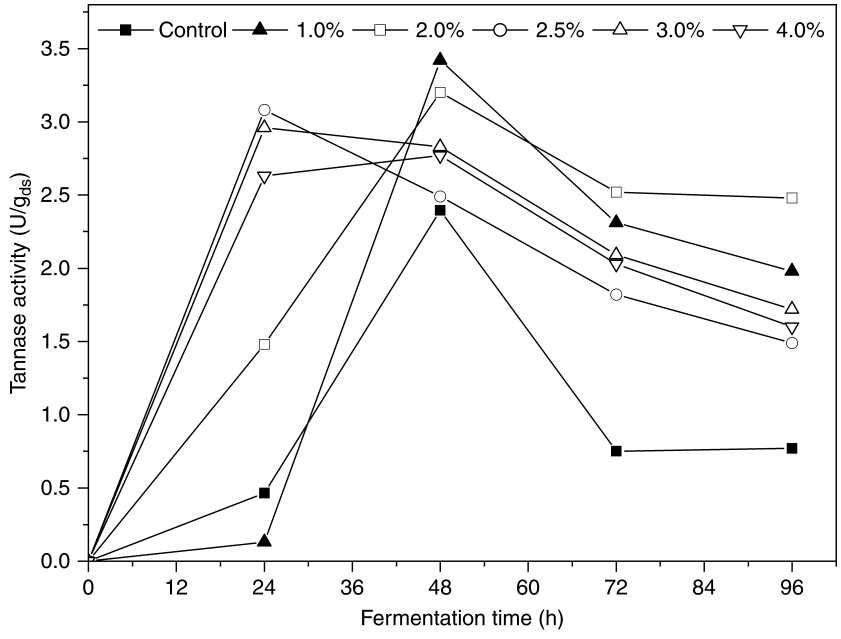

Fig. 6. Effect of ammonium sulphate concentration on tannase production by *A. oryzae* in SSF of 40.0 g of CAB supplemented with 2.5% (w/w) tannic acid at 30°C and 40.4% humidity.

Media supplementation with different amounts of ammonium sulfate was investigated and the results are presented in Fig. 6. It can be observed that increasing ammonium sulfate concentration exerted no influence on the amount of tannase produced; however, it has influenced the kinetics of enzyme production. Table 4 shows enzyme activity and productivity of tannase obtained in this work (SSF using *A. oryzae* and CAB supplemented with 2.5% (w/w) of tannic acid and ammonium sulphate) and by other authors *(9,24)* using different substrates and microorganisms.

Results pictured in Fig. 7 show an acceleration of the microbial metabolism when more than 2.0% (w/v) of ammonium sulphate was added to CAB, and consequently, better tannase productivities (128.5×10^{-3} $U \cdot g_{ds}{}^{-1} \cdot h^{-1}$) were achieved, *see* also Table 4. These productivity results, compared with other authors' results, indicate that CAB is a promising substrate for tannase production.

Effect Phosphorous Supplementation

The effect of supplementation of CAB, enriched with 1% (w/v) of ammonium sulphate, with $NaH_2PO_4 \cdot H_2O$ on tannase production was evaluated and results are shown in Fig. 8. It can be observed that $NaH_2PO_4 \cdot H_2O$ exerted no influence on tannase production. Pinto *(33)* observed that the addition of phosphorus to the fermentation media (wheat

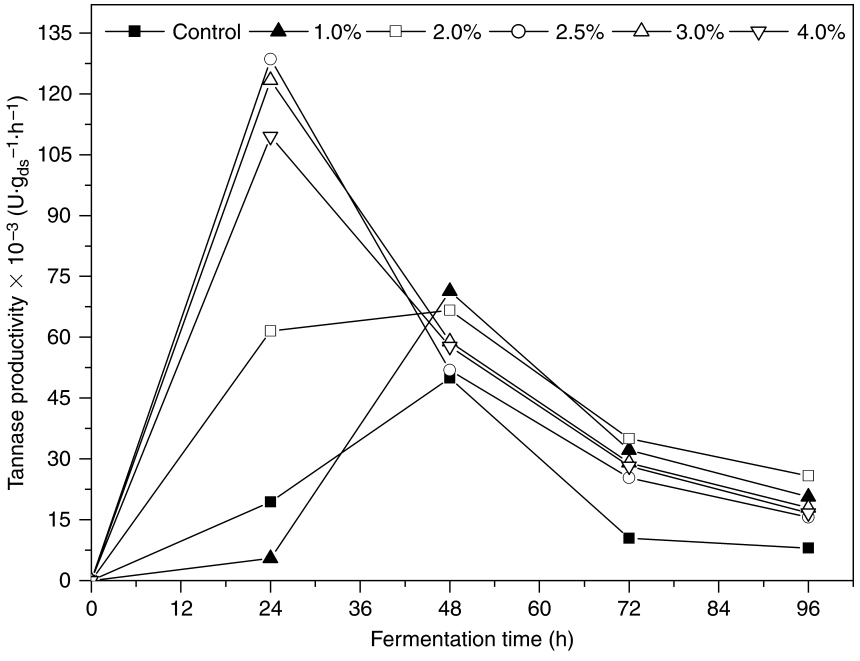

Fig. 7. Effect of ammonium sulphate concentration on tannase productivity by *A. oryzae* in SSF of 40.0 g of CAB supplemented with 2.5% (w/w) tannic acid at 30°C and 40.4% humidity.

Table 4
Tannase Activity (U/g_{ds}) and Productivity ($U \cdot g_{ds}^{-1} \cdot h^{-1}$) in SSF Using Different Microorganisms and Culture Media

Substrate	Microorganism	Tannase activity (U/g_{ds})	Tannase productivity $\times 10^3$ ($U \cdot g_{ds}^{-1} \cdot h^{-1}$)
CAB supplemented with 2.5% (w/w) tannic acid and 1% (w/v) ammonium sulphate	*A. oryzae*	3.42	71.3
CAB supplemented with 2.5% (w/w) tannic acid and 2.5% (w/v) ammonium sulphate	*A. oryzae*	3.08	128.5
Coffee husk *(24)*	*Lactobacillus* sp.	0.70	14.5
Tamarind seed power *(24)*	*Lactobacillus* sp.	0.65	13.5
Palm kernel cake *(9)*	*A. niger* ATCC16620	13.03	135.7

In this work, *A. oryzae* was able to grow in 40.0 g of CAB supplemented with 2.5% (w/w) tannic acid and ammonium sulphate at 30°C and 40.4% humidity.

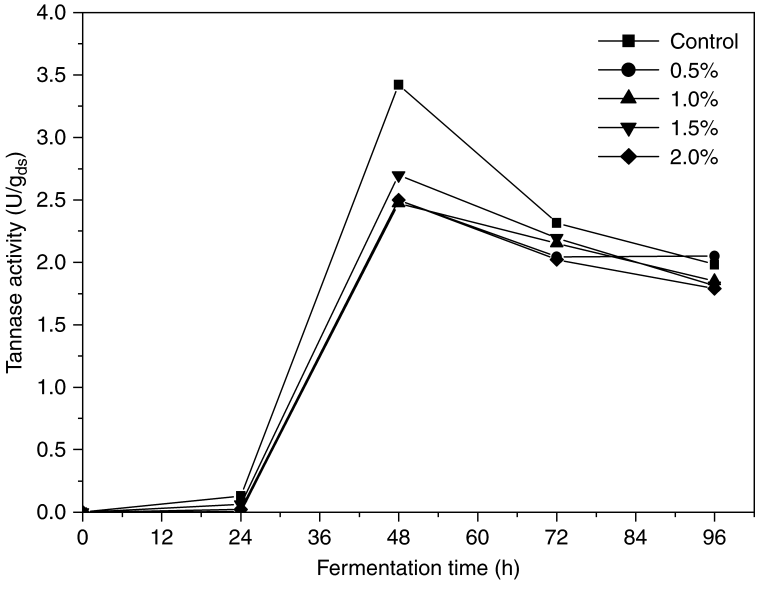

Fig. 8. Effect of sodium phosphate supplementation on tannase production by *A. oryzae* in SSF of 40.0 g of CAB supplemented with 2.5% (w/w) tannic acid and 1% (w/v) ammonium sulphate at 30°C and 40.4% humidity.

straw) promoted an increase in enzyme yield and productivity, which was not the observed in this work.

Effect of Incubation Period

Maximum amount of tannase activity was obtained after 48 h or 24 h of fermentation (Figs. 1, 5, 6, and 8) for all conditions studied in this work. A decrease in enzyme yield with further increase of incubation time was also observed. This behavior could be owing to the reduced nutrient level of the media, affecting the enzyme synthesis. Some authors claim that tannase is produced during the primary phase of growth, and therefore, the activity decreases either because of decrease in production or because of enzyme degradation *(9)*. The consumption of gallic acid (Fig. 2) may contribute to a decrease in the level of induction. Nevertheless, the increase of ammonium sulphate concentration in culture media improved enzyme productivity (Figs. 6 and 7), allowing to produce almost the same amount of enzyme in 24 h of fermentation.

Conclusions

Results obtained in this work showed that CAB was a promising substrate, enabling *A. oryzae* growth and the production of tannase. The physico-chemical parameters that influenced the enzyme production were: water content (60 mL), supplementation with tannic acid (2.5% [w/w]), and supplementation with nitrogen source (2.5% [w/v] ammonium sulphate).

The increase in ammonium sulphate concentration in the media improved enzyme productivity to 128.5×10^{-3} U $\cdot$ $g_{ds}{}^{-1} \cdot h^{-1}$, when 2.5% was added. Supplementation of the medium with phosphorous ($NaH_2PO_4 \cdot H_2O$), ammonium nitrate, peptone and yeast extract exerted no influence on tannase production.

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References

1. Morton, J. F. (1997), *Fruits of Warm Climates*, Florida Flair Books, Miami: pp. 239–240.
2. Campos, D. C. P., Santos, A. S., Wolkoff, D. B., Matta, V. M., Cabral, L. M. C., and Couri, S. (2002), *Desalination* **148,** 61–65.
3. Assunção, R. B. and Mercadante, A. Z. (2003), *J. Food Composition Anal.* **16,** 647–657.
4. Azevedo, D. C. S. and Rodrigues, A. (2000), *J. Sep. Technol.* **35,** 2561–2581.
5. Rocha, M. V. P., Oliveira, A. H. S., Souza, M. C. M., and Gonçalves, L. R. B. (2006), *World J. Microb. Biot.* **22,** 1295–1299.
6. Ferreira, A. C. H., Neiva, J. N. M., Rodríguez, N. M., Lobo, R. N. B., and Vasconcenlos, V. R. (2004), *R. Bras. Zootec.* **33,** 1380–1385.
7. Matias, M. F. O., Oliveira, E. L., Gertrudes, E., and Magalhães, M. M. A. (2005), *Braz. Archives Biol. Technol.* **48,** 143–150.
8. Cannel, E. and Moo-Young, M. (1980), *Proc. Biochem.* **15,** 2–7.
9. Sabu, A., Pandey, A., Jaafar Daud, M., and Szakacs G. (2005), *Bioresour. Technol.* **96,** 1223–1228.
10. Lekha, P. K. and Lonsane, B. K. (1997), *Adv. Appl. Microbiol.* **44,** 215–260.
11. Kurmar, R. and Singh, M. (1984), *J. Agric. Food Chem.* **32,** 447–453.
12. Madhavakrishna, W., Bose. S., and Nayudamma, Y. (1960), *Bull. Cent. Leather Res. Inst.* **7,** 1–11.
13. Pourrat, H., Regerat, F., Pourrat, A., and Jean, D. (1985), *J. Fermentation Technol.* **63,** 401–403.
14. Adachi, O., Watanabe, M., and Yamada, H. (1968), *Agric. Biol. Chem.* **32,** 1079–1085.
15. Aguillar, C. N. and Sanchez, G. (2001), *Food Sci. Tech. Int.* **7,** 373–382.
16. Pinto, G. A. S., Leite, S. G. F., Terzi, S. C., and Couri, S. (2001), *Braz. J. Microb.* **32,** 24–26.
17. Pandey, A. (1992), *Process Biochem.* **27,** 109–117.
18. Inoue, K. H. and Hagerman, A. E. (1988), *Anal. Biochem.* **169,** 363–369.
19. Sharma, S., Bhat, T. K., and Dawra, R. K. (2000), *Anal. Biochem.* **279,** 85–89.
20. Folin, O. and Denis, J. (1912), *J. Biol. Chem.* **12,** 239–243.
21. Gonçalves, L. R. B., Giordano, R. L. C., and Giordano, R. C. (2005), *Process Biochem.* **40,** 247–256.
22. Robinson, T. and Nigam, P. (2003), *Biochem. Eng.* **13,** 197–203.
23. Francis, F., Sabu, A., Nampoothiri, K. M., et al. (2003), *Bioch. Eng. J.* **15,** 107–115.
24. Sabu, A., Augur, C., Swati, C., and Pandey, A. (2006), *Process Biochem.* **41,** 575–580.
25. Oriol, E., Schettino, B., Viniegra-Gonalez, G., and Raimbault, M. (1988), *J. Ferment. Technol.* **66,** 57–62.
26. Bradoo, S., Gupta, R., and Saxena, R. K. (1996), *J. Gen. Appl. Microbiol.* **42,** 325–329.
27. Gervais, P., Grajek, W., Bensoussan, M., and Molin, P. (1988), *Biotechnol. Bioeng.* **31,** 457–463.
28. Grajek, W. and Gervais, P. (1987), *Enzyme Microbial. Technol.* **9,** 658–662.
29. Banerjee, R., Mukherjee, G., and Patra, K. C. (2005), *Bioresource Technology* **96,** 949–953.
30. Lekha, P. K. and Lonsane, B. K. (1994), *Process Biochem.* **29,** 497–503.

31. Knudson, L. J. (1913), *Biol. Chem*. **14,** 159–184.
32. Nishira, H. and Mugibayashi, N. (1953), *Hyogo Noka Diagaku Kenkyu hokoku Nogeikagaku Hen* **4,** 113–116.
33. Pinto, G. A. S. (2003), *PhD Thesis,* Universidade do Rio de Janeiro, Rio de Janeiro, Brasil.
34. Bradoo, S. and Gupta, R. (1997), *J. Gen. Appl. Microbiol*. **42,** 325–329.
35. Aguilar, C. N., Augur, C., Favela-Torres, E., and Viniegra-González, G. (2001), *Process Biochem*. **36,** 565–570.