GLOBAL JOURNAL OF BIO-SCIENCE AND BIOTECHNOLOGY

www.scienceandnature.org

ASPERGILLUS NIGER GROWTH ON POLYPHENOLIC CARBON SOURCES AND OPTIMIZATION OF THE TANNASE PRODUCTION

Souad Akroum
Department of Applied Microbiology and Food Sciences, faculty of Natural Sciences,
University of Jijel, Ouled Aissa City, Jijel 18000, Algeria.

ABSTRACT
Aspergillus niger is a mould known to be the best tannase producer. In this study we tried to test its ability to growth in some media containing flavonoids and tannins as principal or only carbon source. The inoculation was done on a medium containing the acid tannic as only carbon source; medium containing the ethanolic extract of verbena which was rich in flavonoids and medium containing the acetonic extract of tea which was rich in tannins. These extract were used as only carbon source. The evaluation of growth was done with the conidia counting. Then the tannase production was measured before and after optimization. The results showed that *A. niger* had an average growth on the tannic acid and the acetonic extract of tea. But the growth on the ethanolic extract of verbena was very low. This was confirmed by the conidia count. So, both tannins and flavonoids were considered as a real difficult carbon source for the species. The tannase optimization increased the enzyme activity from 20.6 U /ml to 23.1 U / ml.

KEYS WORDS: *A. niger*, Polyphenols, Mycelium aspect, Conidia count, Tannin degradation.

INTRODUCTION

Aspergillus niger is a mould known for its ability to produce different kinds of enzymes and thus to degrade a big diversity of organic compounds. This characteristic allows the growth of the species in different media and environments (Schuster *et al.,* 2002). On the other hand, polyphenols are secondary plant metabolites which have big antibacterial and antifungal activities. In fact, these molecules can inhibit several types of microorganisms, principally bacteria, yeasts and moulds (Beil *et al.,* 1995; Patel *et al.,* 2011; Xiang-bo *et al.,* 2013). In this study we tried to test the capacity of *A. niger* to growth in some media containing polyphenols as only or principal carbon source. Knowing that if the mould grew this is signified that it was able to resist to the present polyphenols and also able to degrade them. Then we compared the conidia production and growth in the tested media in order to compare the growth quantity in each medium. Finally, we tried to measure and optimize the tannase production of *A. niger*.

MATERIALS & METHODS

a) *Origin of A. niger: Aspergillus niger* provided from the microbiological department of the Constantine 1 university (Constantine, Algeria).
b) *Origin of plants*: Tea was obtained from herbalists and verbena was picked just before the flowering. The plants were identified at the botanical and ecological department of the Constantine 1 university (Constantine, Algeria).

Growth on the Medium Containing Tannic Acid

The acid tannic is commercial hydrolyzable tannin. The inoculation was effectuated on a specific growth medium which contained the acid tannic as only carbon source (TA medium). The constitution of the AT medium was: Tannic acid :10 g, $NaNO_3$: 3 g, KH_2PO_4: 1 g, $MgSO_4.7H_2O$: 00.5 g, KCl: 0.5 g, $FeSO_4$: 0.07 g, Agar: 30 g, pH: 5.5, the incubation was occurred at 25 °C for 72 h (Pinto *et al.,* 2001).

Growth on the Medium Containing Ethanolic Extract of Verbena

a) Preparation of the ethanolic extract

The ethanolic extract was prepared by three successive macerations of verbena on the aqueous ethanol (80%). Then, the solvent was evaporated and the extracted molecules were recuperated with 10 ml of distilled water (Marston and Hostettmann, 2006).

b) Quantification of flavonoids

The quantification of flavonoids on the ethanolic extract was made using the UV–visible spectrophotometry method (Chen *et al.*, 2010).

The total flavonoids content (TFC) was calculated with the following equation:

$$\text{TFC (\% in dry matter)} = \frac{A\ 500\ nm\ \times Dilution\ factor}{A1cm1\%\ \times (w\ -\ Id)}$$

Where: A= absorbance of the sample at 500 nm; w = mass of plant material (g), ld = loss on drying of plant material (8%, w / w); A1cm 1% = specific absorption for rutin-$AlCl_3$ complex (259.4).

c) *Growth of the species*

A medium containing the ethanolic extract of verbena as only carbon source (EE medium) was prepared by modification of the precedent medium (Akroum *et al.*, 2009): Ethanolic extract: 10 g, $NaNO_3$: 3g, KH_2PO_4: 1g, $MgSO_4.7H_2O$: 00.5 g, KCl: 0,5 g, $FeSO_4$: 0.07 g, Agar: 30 g, pH: 5.5. The medium was inoculated with the suspension of *A. niger* (10^6 CFU) then incubated at 25°C for 72 h.

Growth on the medium containing acetonic extract of tea

a) Preparation of the acetonic extract

The acetonic extract was prepared by three successive macerations of tea on the aqueous acetone (70%). After evaporation of the solvent, the extracted molecules were recuperated with 10 ml of distilled water (Noweer and Dawood, 2009).

b) Quantification of condensed tannins

The condensed tannins on the acetonic extract of tea were measured by the butanol/ HCl method (Phale and Madibela, 2006): 0.5 ml of the extract was added to 4,5 ml of butanol / HCl reagent (butanol / HCl 95:5 v / v). After boiling at 90 °C for 2 h, the absorbance was measured at 550 nm.

$$\text{Condensed tannins (\% in dry matter)} = \frac{A550\,nm \times 78.26 \times Dilution\,factor}{\%\ \text{dry matter}}$$

Where: A550 nm = absorbance of the sample at 550 nm. The formula assumes that the effective E5501 % of leucocyanidin is 460.

c) Growth of the species

A medium containing the acetonic extract as only carbon source (AE medium) was prepared with the following constitution : Acetonic extract : 10g, $NaNO_3$: 3g, KH_2PO_4 : 1 g, $MgSO_4.7H_2O$: 00.5 g, KCl : 0.5 g, $FeSO_4$: 0.07 g, Agar : 30 g, pH : 5.5. The incubation was occurred at 25 °C for 72 h. The suspension of *A. niger* (10^6 CFU) was inoculated on the medium, then the incubation was occurred at 25 °C for 72 h (Akroum *et al.*, 2009).

Growth on the Malt Agar Extract Medium

The malt agar extract medium (MAE medium) and incubated by a suspension of 10^6 CFU then inoculated at 25 °C for 72 h (Palacios-Cabrera *et al.*, 2005). The growth on this medium is considered as a reference for comparison with the other media.

Conidia Count

Suspensions of conidia were prepared for each medium (MAE, TA, EE, AE) by submerging the Aspergillus cultures with 3 ml of distilled water and agitation. The suspensions were prepared at 21 days of culture and the counting of conidia was done with the Malassez cell (Sautour *et al.*, 2001).

Optimization of the Tannase Production

The tannase production was optimized for *A. niger* by growing the species on a medium containing 100 % of glucose as only carbon source, then by transplanting the species on some other media where the carbon source was progressively replaced by the tannic acid. The used medium had the following composition:

Medium 1 (per liter): Glucose 10 g, $NaNO_3$ 3 g, KH_2PO_4 1 g, $MgSO_4.7H_2O$ 00,5 g, KCl 0.5 g, $FeSO_4$ 0.07 g, Agar 30 g, pH 5.5

Medium 2 (per liter): Glucose 7.5 g, tannic acid 2.5 g, $NaNO_3$ 3 g, KH_2PO_4 1 g, $MgSO_4.7H_2O$ 00,5 g, KCl 0.5 g, $FeSO_4$ 0.07 g, Agar 30 g, pH 5.5

Medium 3 (per liter): Glucose 5 g, tannic acid 5 g, $NaNO_3$ 3 g, KH_2PO_4 1 g, $MgSO_4.7H_2O$ 00.5 g, KCl 0.5 g, $FeSO_4$ 0.07 g, Agar 30 g, pH 5,5

Medium 4 (per liter): Glucose 2.5 g, tannic acid 7.5 g, $NaNO_3$ 3 g, KH_2PO_4 1 g, $MgSO_4.7H_2O$ 00,5 g, KCl 0,5 g, $FeSO_4$ 0,07 g, Agar 30 g, pH 5,5

Medium 5 (per liter): tannic acid 10 g, $NaNO_3$ 3 g, KH_2PO_4 1 g, $MgSO_4.7H_2O$ 00,5 g, KCl 0,5 g, $FeSO_4$ 0,07 g, Agar 30 g, pH 5,5

The tannase production was measured for the mould taken from the medium 5 (the optimized mould) and for the one taken from the medium 1 (original mould). The spore suspensions were prepared and inoculated on a specific medium constituted by modification of the original medium described by Costa and his collaborators (2008). The specific medium contained KH_2PO_4 1.0g; Mg $SO_4.7H_2O$, 2.0, $CaCl_2$ 1 g; NH_4Cl 3g; yeast extract 1g and Quebracho 3g.

The tannase activity was measured by the rhodanin method and the activity was expressed in International units per milliliter (Costa *et al.*, 2008).

RESULTS & DISCUSSION

Growth on the Medium Containing Tannic Acid

Aspergillus niger showed on the TA medium a green mycelium, powdery with conidial production. Sporocysts were small and distant; they displayed a low density during the 14 first days then an average density. The mycelium was initially low then it became high after 14 days. Its diameters were 1.7 cm, 4.2 cm, 7 cm and 7 cm at 3, 6, 14 and 21 days of culture. The dissemination of the species was present and it allowed the apparition of some little mycelia. Reverse could not be observed because the medium was dark (Fig. 1).

FIGURE 1: *A. niger* culture on the TA medium after 21 days.

Growth on the Medium Containing Ethanolic Extract of Verbena

a) Quantification of flavonoids

The TFC of verbena was quantified according the quoted equation and the results showed that the value was 85 mg per g of material plant, that to say 0.085 %. So, the ethanolic extract of verbena was rich in flavonoids in step with the results of another investigation (Xiang-bo *et al.*,

2013). Some other researchers demonstrated that verbena contained mainly flavones and flavanones, including quercetin and luteolin (Beil *et al.*, 1995).

b) Growth of the species

Aspergillus niger had in the 3 first days a white and woolly mycelium, and after it became olive-green to gray-green and powdery. Sporocysts were also white becoming green; they were very small and with an average density. The growth of the mycelium on the Petri dish was characterized with a big spreading and a poor development (low height and density). The diameters were respectively 1.4 cm, 4.3 cm, 7.5 cm and 9 cm at 3, 6, 14 and 21 days of culture. The dissemination of the central mycelium was low in this medium (Fig. 2) and the reverse was whitish. The growth of *A. niger* on the this medium testified that the species was able to degrade the flavonoids present in the ethanolic extract. But it is important to signal that the development of the mycelium was very low, so the flavonoids were a very difficult carbon source for *A. niger*.

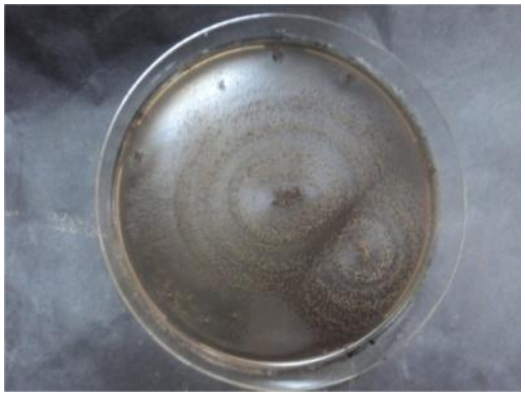

FIGURE 2: Low growth of *A. niger* on the EE medium

Growth on the Medium Containing Acetonic Extract of Tea

a) Quantification of condensed tannins

The amount of condensed tannins in the acetonic extract of tea was about 0,076 % (or 76 mg per g). So, these compounds were found in a big quantity in accordance with the results of a previous work (Calani *et al.*, 2012). For more precision, the last work affirmed that tea contained mainly monomeric flavan-3-ols and proanthocyanidins.

b) Growth of the species

The mycelium was white fluffy at the center becoming yellow then brown and powdery. Sporocysts were absent until 6 days of the culture. They were at first very small and in a low quantity becoming after 14 days in a high quantity and density. The diameters were respectively 1,4 cm, 3 cm, 4 cm and 4 cm at 3, 6, 14 and 21 days of culture. We could easily observe that the species growth was more important in this medium then in the TA and EE media. The dissemination of the species was observed it yielded several small mycelia which covered the quasi-totality of the Petri dish after 21 days of growth (Fig. 3). The reverse could not be observed because the medium was dark.

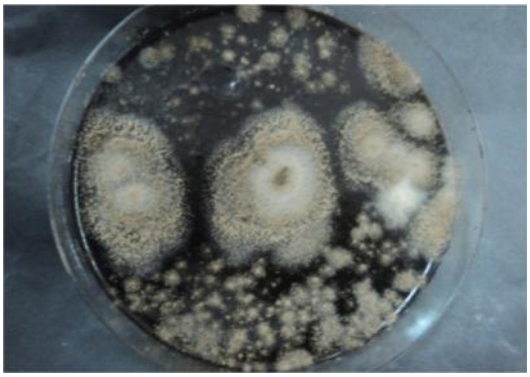

FIGURE 3: Average growth of *A. niger* on the AE medium

The growth of *A. niger* in the AE media. This is indicated that the species was able to degrade condensed tannins of tea. No work was found on this information, however some researchers have previously reported the capacity of the species to produce tannase and thus to degrade condensed tannins (Pinto *et al.*, 2001 & Rodríguez-Durán *et al.*, 2013). This explained the mould growth on the TA medium. In fact by comparing with the other prepared media, we could observe that *A. niger* had also an non negligible growth on this medium.

Growth on the Malt Agar Extract Medium

The mycelium was first white velvety then becoming black powdered and very dense. Sporocysts were more numerous, big and dense in comparison with the previous media. The mycelium showed radial septa. The diameters of the mycelium were respectively 2.3 cm, 3 cm, 4 cm and 4 cm at 3, 6, 14 and 21 days of culture. The dissemination of the species was very important, after 21 days, it allowed the recovery of the entire Petri dish surface. Reverse was whitish with radial septa.

Conidia Count

The conidia count showed that the EA medium had the most important conidia production with a value of 3,6. 10^6 conidia / ml. The TA medium gave a near result with $3.3.10^6$ conidia / ml. But the counting on the EE medium revealed a very poor conidia production. On the MAE medium, which was used as a reference, the conidia count was higher than all the other values with 28, 2.10^6 conidia /ml. This signified that the growth of the mould was difficult on media rich or contained only tannins and more difficult on media rich in flavonoids. In other words, both tannins and flavonoids were difficult to degrade by *A. niger*, but the flavonoids were the most difficult. Also, the obtained results meant that *A. niger* was not sensitive to the polyphenols presents in the prepared media. In fact the species growth indicated that the tannic acid and the extracted tannins and flavonoids had not an antifungal activity on it.

Optimization of the Tannase Production

The results showed that the optimum of the tannase production was obtained after two days of the culture. The tannase activity was 23,1 U / ml of the optimized mould, and 20,6 U /ml for the original mould (Fig. 4).

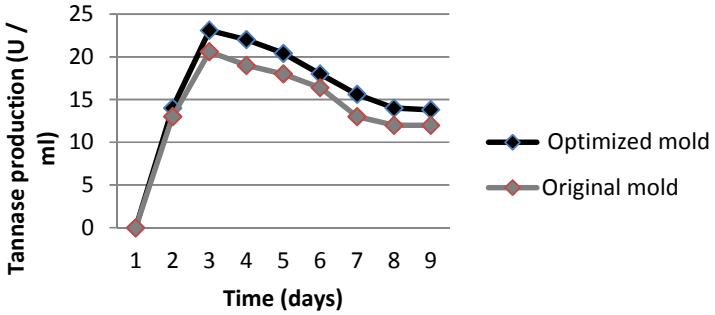

FIGURE 4: Tannase activity before end after optimization

The gradual replacing of the glucose by the tannic acid had really optimized the tannase production of *Aspergillus niger*. The value obtained for the tannase production by the original mould was already reported. In fact, *A. niger* was considered as the producer of tannase, this species was able to degrade both hydrolyzables and condensed tannins (Akroum *et al.*, 2009). But no work was found on the optimization of the species by the used method.

CONCLUSION

Aspergillus niger was able to degrade tannic acid, condensed tannins extracted from tea and flavonoids extracted from verbena. The conidia production in media containing acid tannic and condensed tannins was almost similar; also it was more important than the conidia production on the medium containing flavonoids. This meant that tannins were easier to degrade than the flavonoids. But these two types of polyphenols remained considered as carbon source difficult to degrade for the mould. The tannase production was really optimized when the carbon source was gradually changed from a conventional substrate to the tannic acid.

REFERENCES

Akroum, S., Haddi, M. L. and Lalaoui, K. (2009) Fungal tannase degrading condensed tannins of *Camellia sinensis* and measure of the enzyme activity on quebracho. Middle-East J. Sci. Res. 4, 237-241.

Beil, W., Birkholz, C. and Sewing, K. F. (1995) Effects of flavonoids on parietal cell acid secretion, gastric mucosal prostaglandin production and *Helicobacter pylori* growth. Arzneimittelforscung 45, 697-700.

Calani, L., Del Rio, D., Callegari, M. L., Morelli, L. and Brighenti, F. (2012) Updated bioavailability and 48 h excretion profile of flavan-3-ols from green tea in humans. Int. J. Food. Sci. Nutr. 63, 513-521.

Chen, Y., Wang, J. and Wan, D. (2010) Determination of total flavonoids in three sedum crude drugs by UV–Vis spectrophotometry. Pharmacogn. Mag. 6, 259-63.

Costa, A. M., Ribeiro, W. X., Kato, E., Monteiro, A. R. G. and Peralta, R. M. (2008) Production of tannase by *Aspergillus tamarii* in submerged cultures. Braz. Arch. Biol. Technol. 51, 399-404.

Marston, A. and Hostettmann, K. (2006) Separation and quantification of flavonoids. CRC Press Taylor and Francis group, Berlin, New York.

Noweer, E. M. and Dawood, M. G. (2009) Efficiency of propolis extract on faba bean plants and its role against nematode infection. Commun. Agric. Appl. Biol. Sci. 74, 593-603.

Palacios-Cabrera, H., Taniwaki, M. H., Hashimoto, J. M. and Castle de Menezes, H. (2005) Growth of *Aspergillus ochraceus*, *A. carbonarius* and *A. niger* on culture media at different water activities and temperatures. Braz. J. Microbiol. 36, 24-28.

Patel, K. D., Scarano, F. J., Kondo, M., Hurta, R. A. and Neto, C. C. (2011) Proanthocyanidin-rich extracts from cranberry fruit (*Vaccinium macrocarpon Ait.*) selectively inhibit the growth of human pathogenic fungi *Candida spp*. and *Cryptococcus neoformans*. J. Agri. Food Chem. 59, 12864-12873.

Phale, O. and Madibela, O. R. (2006) Concentration of soluble condensed tannins and neutral detergent fibre-bound tannins in fodder trees and forage crops in Botswana. J. Biol. Sci. 6, 320-323.

Pinto, G. A. S., Leite, S. G. F., Terz, S. C. and Couri, S. (2001) Selection of tannase producing *Aspergillus niger* strains. Braz. J. Microbiol. 32, 75-79.

Rodríguez-Durán, L. V., Spelzini, D., Boeris, V., Aguilar, C. N. and Picó, G. A. (2013) Partition in aqueous two-phase system: its application in downstream processing of tannase from *Aspergillus niger*. Colloids. Surf. B. 101, 392-397.

Sautour, M., Rouget, A., Dantigny, P., Divies, C. and Bensoussan, M. (2001) Prediction of conidial germination of *Penicillium chrysogenum* as influenced by temperature, water activity and pH. Lett. Appl. Microbiol. 32, 131-134.

Schuster, E., Dunn-Coleman, N., Frisvad, J. and van Dijck, P. (2002) On the safety of *Aspergillus niger* – a review. Appl. Microbiol. Biotechnol. 59, 426-435.

Xiang-bo, M. A., Wang, B., Rong, R. and Dang, Z. (2013) Optimization of ultrasonic extraction technology of flavonoids from herba Verbenae and study on the antimicrobial activity. Hubei. Agric. Sci. 3, 645-648.