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

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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 ethanol 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 ethanol 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, NaNO₃: 3 g, KH₂PO₄: 1 g, MgSO₄·7H₂O: 0.5 g, KCl: 0.5 g, FeSO₄: 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 using the following equation:

\[
TFC \text{ (% in dry matter)} = \frac{A_{500 \text{ nm}} \times \text{Dilution factor}}{A_{1cm 1\%} \times (w – ld)}
\]

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-AI CI₃ 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, NaNO₃: 3g, KH₂PO₄: 1g, MgSO₄·7H₂O: 0.05 g, KCl: 0.5 g, FeSO₄: 0.07 g, Agar: 30 g, pH: 5.5. The medium was inoculated with the suspension of A. niger (10⁶ 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).

\[
\text{Condensed tannins (\% in dry matter)} = \frac{A_{550\text{nm}} \times 78.26 \times \text{Dilution factor}}{\% \text{ dry matter}}
\]

Where: \( A_{550\text{nm}} \) = absorbance of the sample at 550 nm. The formula assumes that the effective E5501 % of leucocyanidin is 460.

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.

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, NaNO₃: 3g, KH₂PO₄: 1 g, MgSO₄7H₂O : 0.5 g, KCl : 0.5 g, FeSO₄ : 0.07 g, Agar : 30 g, pH : 5.5. The incubation was occurred at 25 °C for 72 h. The suspension of A. niger (10⁶ 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 10⁶ 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 5 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, NaNO₃ 3 g, KH₂PO₄ 1 g, MgSO₄7H₂O 0.05 g, KCl 0.5 g, FeSO₄ 0.07 g, Agar 30 g, pH 5.5

Medium 2 (per liter): Glucose 7.5 g, tannic acid 2.5 g, NaNO₃ 3 g, KH₂PO₄ 1 g, MgSO₄7H₂O 0.05 g, KCl 0.5 g, FeSO₄ 0.07 g, Agar 30 g, pH 5.5

Medium 3 (per liter): Glucose 5 g, tannic acid 5 g, NaNO₃ 3 g, KH₂PO₄ 1 g, MgSO₄7H₂O 0.05 g, KCl 0.5 g, FeSO₄ 0.07 g, Agar 30 g, pH 5.5

Medium 4 (per liter): Glucose 2.5 g, tannic acid 7.5 g, NaNO₃ 3 g, KH₂PO₄ 1 g, MgSO₄7H₂O 0.05 g, KCl 0.5 g, FeSO₄ 0.07 g, Agar 30 g, pH 5.5

Medium 5 (per liter): tannic acid 10 g, NaNO₃ 3 g, KH₂PO₄ 1 g, MgSO₄7H₂O 0.05 g, KCl 0.5 g, FeSO₄ 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 KH₂PO₄ 1.0g; MgSO₄7H₂O 2.0, CuCl₂ 1 g; NH₄Cl 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.](image)

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](image1)

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, $10^5$ conidia / ml. The TA medium gave a near result with $3.3,10^5$ 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 3: Average growth of *A. niger* on the AE medium](image2)
Aspergillus niger growth and tannase 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


