

# Production of naphtho-gamma-pyrones by *Aspergillus niger* under solid-state-fermentation

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**Abstract** *Aspergillus niger* is one of the most important microorganisms used in biotechnology sector. This species is able to produce naphtho-gamma-pyrones (N $\gamma$ Ps), which have excellent antioxidant activity. In the present study, spores and N $\gamma$ Ps production has been investigated through solid state fermentation (SSF) of *A. niger* on different agricultural by-products which, from an economical perspective, constitute cheaper substrates than the synthetic ones, in addition of being more eco-friendly. An optimized N $\gamma$ P yields of 8,12 mg/g dry matter was obtained after 10 days of SSF at 27°C using a mix of sugarcane bagasse, wheat bran and potatoes flour (50/30/20 (w/w/w) respectively. Cultures were carried out in ORSTOM bioreactor using Raimbault glass columns (diameter: 4cm, length: 20cm) packed with 3g solids. Initial moisture content was 75% with an initial aeration rate of 60 ml/min of saturated humid. A significant positive correlation between ergosterol – specific sterol produced by fungi and a biomass marker – and N $\gamma$ P contents was observed.

**Key words:** *Aspergillus niger*, solid state fermentation, naphtho-gamma-pyrone, antioxidant activity, sugarcane bagasse

**Introduction:** Antioxidants used in the food, cosmetic and detergent industries are products of great commercial significance. Existing technologies of antioxidant production by microorganisms have opened up alternative sources of both individual and complex antioxidant mixtures. In this area, filamentous fungi hold much promise. Literature reports indicate that *A. niger* has been used to produce extracellular enzymes (food industry) and citric acid (Geiser *et al.*, 2007; Samson *et al.*, 2009 ; Hawksworth 2011). Today, while more than 60 secondary metabolites produced by *A. niger* have been identified, the

**2.3. Solid state fermentation:** SSF experiments were carried out using a mix of sugarcane bagasse (SCB), wheat bran (WB) and potatoes flour (PF) in the ratio 50/30/20 (w/w/w) respectively. Mixed substrates were sterilized at 120°C for 30 min. After, the medium was inoculated by concentrated spore suspension (2.10<sup>7</sup> spores/g dry matter) and then placed in a Raimbault column. The column was connected to a humidifier which allows a circulation of moist air in the column at a rate of 60 ml/min.

**2.4. Extraction of secondary metabolites:** A sample of 1g fermented substrate was dissolved in 10 ml of ethanol and

N $\gamma$ Ps are the most abundant secondary metabolites produced in this fungus (Choque *et al* 2014). These natural compounds possess very important antioxidant potentialities (Zhang *et al.* 2007). As a result, they could potentially be used in food and cosmetic industries to substitute some chemical antioxidant additives used in the agri-food industry and which represent health problems to the human body (Choque *et al* 2014). Among the microbial fermentation possibilities, many articles suggest that SSF can give higher yields of secondary metabolites than submerged fermentation (Mienda *et al.* 2011). Agro-industrial residues are generally considered the best substrates for solid-state fermentation process due to their low cost (Ghosh *et al.* 2013).

The present study discusses the production of spores and N $\gamma$ Ps by *A. niger* in potato dextrose agar (PDA) plates and under SSF, in order to improve the possibility to produce these metabolites on agricultural waste and the important yield offered by this type of fermentation compared to other types of cultures. On the other hand, this research presents a simplified method for extracting the ergosterol contained in the *A. niger* mycelium, grown on solid substrates.

## 2. Materials and methods

**2.1. Microorganisms and culture conditions:** The *A. niger* strain G131 was cultivated and conserved on PDA medium. The strain was activated in sterilized PDA and inoculated during 5 days at 27°C then conserved at 4°C.

**2.2. Inoculum preparation:** Erlenmeyer flasks (250 ml) containing the mycelia fungal strain was cultivated on 25 ml PDA during three days. The mycelia were suspended into 100 ml of distilled water with tween 0.01%, and 1ml of this suspension was used to inoculate the cultures.

homogenized for 5 min with an *Ultra-Turrax*®. The assembly was passed through an ultrasonic bath BioBlock Scientific® at frequency of 20 kHz for 60 min. The ethanolic extract was then filtered on Whatman® filter paper to remove solid residues before analysis.

**2.5. Quantitative analysis of secondary metabolites:** N $\gamma$ Ps analysis was performed by High Performance Liquid Chromatography (HPLC, Agilent®) on reversed phase (Zorbax C18 column, 15cm x 4.6mm). The eluents used were water milliQ and acetonitrile at 1 ml/min flow rate, starting with water/acetonitrile (70:30, v/v) to 100%

acetonitrile (45 min). The UV detection (diode array detector) was at 280 nm. The N $\gamma$ P contents were determined using rubrofusarine (commercial standard from BioVitica®) as external standard and are given in mg.l<sup>-1</sup> expressed in equivalent rubrofusarine.

**2.6. Biomass:** Fungal growth was determined by ergosterol analyses. 50 mg of lyophilized fermented substrate in 1 ml of methanol was stirred and kept overnight at 8°C. After incubation, the mixture was centrifuged for 10 min at 14000 rpm. The supernatant containing the ergosterol is recovered. The analysis of ergosterol was carried out by HPLC (WATERS 1525 system) on reversed phase C18 column. The system was completed with an oven and a WATERS 2487 UV detector. For each sample, the supernatant was injected in triplicate with 1 min interval. The oven was set at a temperature of 40 °C and the detection was done at 280 nm. The eluent was pure methanol at a flow rate of 1 ml.min<sup>-1</sup>. The peak corresponding to ergosterol was detected at a retention time of 16 min.

### 3. Results

The strain culture on PDA reveals that *A. niger* has a powdery appearance and a black coloration (due to the appearance of the reproductive structures in the mycelium after 24 hours of culture). The apical growth of *A. niger* on PDA at 27°C was 4.02 mm/day. This allows to deduce that this fungus has an apical growth rate more slowly compared to *Trichoderma* (17.99mm /day) which is considered the fastest in terms of apical growth rate (a petri dish can be entirely colonized in two days (Roussos, 1985)).

**Spores production by *A. niger* cultivated in SSF:** A sporulation kinetics based on direct counting of the spores using the cell of Malassez was carried out for the *A. niger* (Table 1) strain in order to evaluate their sporulation

The sporulation kinetics of *A. niger* increases in the range of 16.6.10<sup>9</sup>sp/g.DM<sup>-1</sup> and 10.9.10<sup>10</sup>sp /g.DM<sup>-1</sup> between 48h and 120h. In terms of sporulation *A. niger* is therefore a very sporulating strain compared to other filamentous fungi, such as *Microdochium dimerum*, which exhibits very weak indices of sporulation which varied generally between 0.21 sp / g.DM<sup>-1</sup> and 7.5 sp/g.DM<sup>-1</sup> (Roussos *et al* 2008).

**Analysis of N $\gamma$ Ps in the PDA and SSF of *A. niger*:** The follow-up of the NGP concentration as a function of time according to the two cultivation methods investigated (Figure 1), showed a considerable increase of the latter as a function of incubation time.

The production of these metabolites increases with time on both PDA and SSF media and for both media, the occurrence of N $\gamma$ Ps is correlated with the establishment of secondary metabolism by the fungus (Calvo *et al.* 2002). However, there is a marked difference in the concentration of N $\gamma$ Ps in *A. niger* depending on the nature of the culture medium. These mean values are 1.6 mg NGPs/g of dry matter obtained on PDA and 8 mg of NGPs/g of dry matter obtained on solid SSF medium, after 10 days of incubation. This important production of N $\gamma$ Ps in SSF with respect to the synthetic medium can be explained by this mode of culture (SSF culture). However, SSF makes it possible to get as close as possible to the natural conditions of development of the fungus. It depends on the composition of the environment, especially which relates to the source of carbon and nitrogen, or the control of thermodynamic parameters such as temperature or humidity. Similar results have been described by Hölker *et al.* (2004) for the production of secondary metabolites using SSF. Moreover, the concentration of all the N $\gamma$ Ps reached a threshold value after 4 to 5 days of culture for the two cultivation methods and then remains constant throughout the duration. This may be explained by the fact that the N $\gamma$ Ps

**Table1.** Evolution of the number of spores during the culture of *A. niger* on PDA and SSF

Time (hours)	Number of spores (10 <sup>9</sup> sp/g.DM)	
	PDA	SSF
48	16.03	42.01
72	20.01	72.05
96	55.04	96.03
120	109.01	142.03

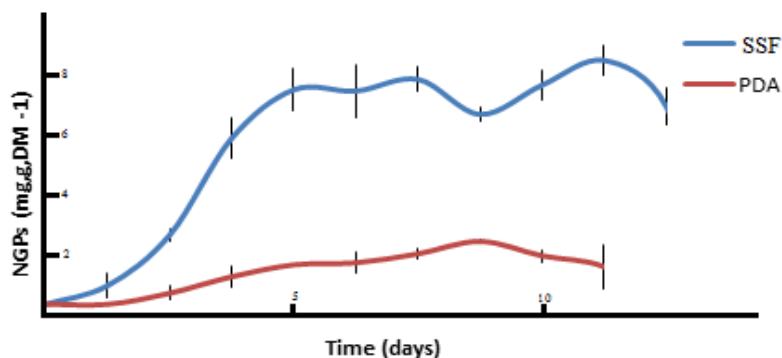


Figure. 1: Influence of the medium nature on the production of NGPs by *A. niger*

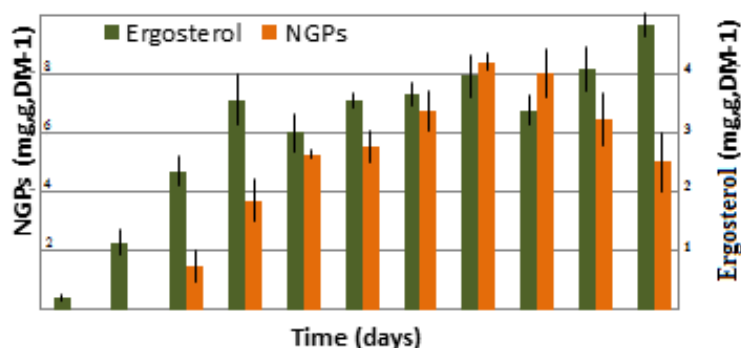


Fig. 2. Evolution of ergosterol and N $\gamma$ Ps concentration during the SSF culture of *A. niger*

production begins after the phase of vegetative multiplication, concomitantly with the conidiogenesis, which is a common trait of secondary metabolites (Calvo *et al.*, 2002).

**Evolution of ergosterol and N $\gamma$ P concentrations during the SSF of *A. niger*:** A method for analyzing ergosterol by HPLC has been developed to estimate the biomass produced by *A. niger* grown in SSF. The extraction of ergosterol was carried out by maceration in pure methanol using lyophilized-dried samples obtained from 1 to 11 days of culture. By comparing the concentration variation of N $\gamma$ Ps and ergosterol, these metabolites evolve continuously. Then it decreases with respect to the concentration of ergosterol which remains high. In SSF analysis of ergosterol extracted from biomass lyophilisation shows that the concentration of ergosterol in *A. niger* cultures has fairly similar values and does not vary much as a function of incubation time. On the other hand, there is a clear difference with respect to the N $\gamma$ P concentrations. These mean values are 4.96 mg ergosterol /g of dry matter and 8.2 mg of N $\gamma$ Ps dry matter. From

ergosterol quantification, it was possible to deduce that the biomass growth of the strain *A. niger* G131 on standard SSF medium (SCB, WB and PF) was faster than on PDA. Indeed, indirect estimation of fungal biomass using ergosterol estimation was already used by different research teams (Durand *et al.*, 1997; Trigos et Franco, 1993; Trigos *et al.*, 1994). However, it is important to keep in mind that a true estimation of the biomass in SSF and on PDA remains a delicate point. Because of the solid nature of the substrate, fast measurements such as turbidimetry or nephelometry, commonly used in liquid fermentations, are impossible to apply in SSF. It is necessary to carry out a sampling and chemical assays made difficult by the entanglement existing between the substrate and the biomass.

#### **Production of spores and N $\gamma$ Ps in SSC:**

Conidiogenesis and the production of N $\gamma$ Ps follow a similar trend at the beginning of cultivation; the amount of conidia thereafter tends to decrease while the amount of N $\gamma$ Ps is maintained stable over time.

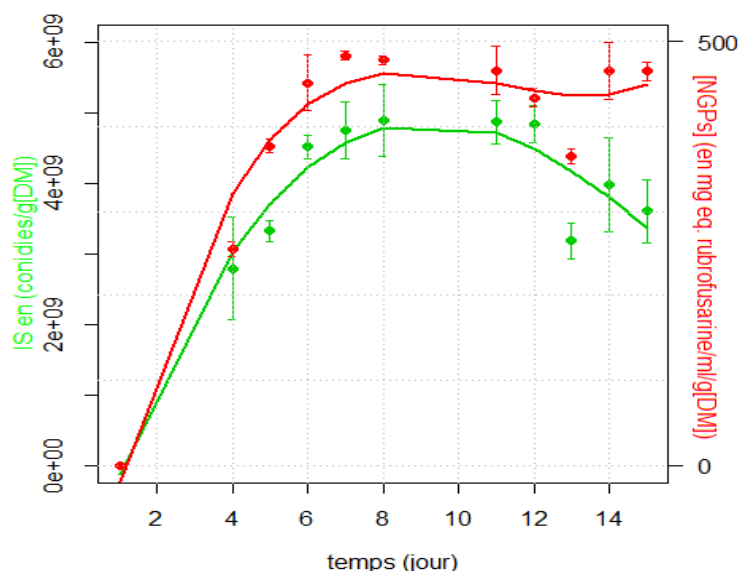


Figure3: Spores and NγPs productions using SSF

Conidia are abundantly produced on the environment, production shows a maximum of spores around day 6, this value is maintained for about 6 days, and thereafter tends to decline. This pattern of production is similar to that found in the literature for similar growing conditions (Hassouni 2007). The production kinetics curves shows that production of NγPs begins after the vegetative growth phase, more or less at the time of conidiogenesis, which is a common feature of secondary metabolites (Calvo *et al.*, 2002)

**Conclusion:** Under the present culture conditions, the production of spores and NγPs by *A. niger* in solid medium is possible and offers very interesting quantitative and qualitative advantages. Indeed, the molecules are produced in greater quantity than on the synthetic medium (PDA) The substrate mixture was chosen because it is demonstrated that bagasse provides effective culture support and that wheat bran is a rich source of *A. niger* food and allows it to produce a variety of molecules. Because of the use of agricultural by-products, the SSF is more economical than liquid media with many advantages. These include the simplicity of this technology and the cost of equipment. Moreover, the absence of free water makes it possible to reduce considerably the volume of fermentation plants and the contamination bacteria. Applied to lyophilized-dried samples and reduced to fine powder, the method of extracting ergosterol by macerating the samples in pure methanol is very effective. The method of analysis of ergosterol is simple, reliable and reproducible. It is easy to start and does not require heavy equipment. Moreover, it is not expensive. Finally, these secondary antioxidant metabolites can be used in the food industry as the preservation of aliments through the antioxidant action and in the cosmetic industry as the creation of anti-aging skin products.

## References

- Boukcim H and Mousain D (2001). Effets de la fertilisation phosphatée sur la mycorhization, la croissance et la nutrition en phosphore et en azote de semis de Cèdre (*Cedrus atlantica* Manetti) inoculés en pépinière par *Tricholoma tridentinum* Sing. Var. *cedretorum* Bon. *Annals of Forest Science* 58: 289-300.
- Calvo AM, Wilson RA, Bok JW and Keller NP (2002). Relationship between Secondary Metabolism and Fungal Development. *Microbiology and Molecular Biology Reviews*, 66, 3: 447-459.
- Choque E., El Rayess Y., Raynal J and Mathieu F. (2014). Fungal naphtho-alpha-pyrones secondary metabolites of industrial interest, 99:1081-1096.
- Durand A., Vergoignan C. and Desgranges C. (1997). Biomass estimation in solid state fermentation. In *Advances in Solid State Fermentation*, (Eds.), Roussos S., Lonsane B. K., Raimbault M. & Viniegra-Gonzalez G., Kluwer Acad. Publ. Dordrecht p. 23-38.
- Geiser D.M., Klich M.A., Frisvad J.C., Peterson S.W., Varga J., Samson R.A. (2007). The current status of species recognition and identification in *Aspergillus*. *Studies in Mycology* 59: 1-10.
- Ghosh S., Murthy S., Govindasamy S. and Chandrasekaran M. (2013). Optimization of Lasparaginase production by *Serratia marcescens* (NCIM 2919) under solid state fermentation using coconut oil cake. *Sustainable Chemical Processes*, 1: 1-9.
- Hawksworth D.L. (2011). Naming *Aspergillus* species: progress towards one name for each species. *Medical Mycology* 49: S70-S76.
- Hölker U., Höfer M. and Lenz J. (2004). Biotechnological advantages of laboratory-scale solid-state fermentation with fungi. *Applied Microbiology and Biotechnology*, 64, 2: 175-186.
- Mienda B.S., Idi A. and Umar A. (2011). Microbiological features of solid state fermentation and its applications - An overview. *Research in Biotechnology*, 2, 6: 21-26.
- Montgomery H.J., Monreal C.M., Young J.C. and Seifert K.A. (2000). Determination of soil fungal biomass from soil ergosterol analyses. *Soil Biology and Biochemistry* 32: 1207-1217.
- Newell S., Arsuffi T.L. and Fallon R.D. (1988). Fundamental procedures for determining ergosterol content of decaying

plant material by liquid chromatography. *Applied and Environmental Microbiology* 54(7): 1876-1879.

**Ooijkaas L.P., Tramper J. and Buitelaar RM.** (1998). Biomass estimation of *Coniothyrium minitans* in solid-state fermentation. *Enzyme and Microbial Technology* 22: 480-486.

**Pandey A., Soccol C.R., Nigam P.** (2001). *Solid-State Fermentation in Biotechnology: Fundamentals and Applications*, API, New Delhi, 221 pp.

**Roussos S., Hassouni H., Ismaïli-Alaoui M.** (2008). Procédé de production de spores et de métabolites provenant de microorganismes d'origine fongique et leurs utilisations. Brevet N° FR 07/05519; PCT FR 2008/001064.

**Roussos, S.** (1985). Croissance de *Trichoderma harzianum* par fermentation en milieu solide: physiologie, sporulation et production de cellulases. Thèse Doctorale d'Université, Université de Provence, Marseille, 78.

**Samson R.A., Varga J.** (2009). What is a species in *Aspergillus*? *Medical Mycology*, 47: S13-S20.

**Trigos A. and Franco G.** (1993). Determinación de ergosterol en hongos silvestres mexicanos. *Micología Neotropical Aplicada* 6: 105-108.

**Trigos A., Zayas T., Ortuño L., M. Sobal and Morales P.** (1994). Contenido de ergosterol en algunas especies cultivadas de *Pleurotus*. *Micología Neotropical Aplicada*. 7: 43-46.

**Zhang Y., Li X.M. and Wang B.G.** (2007). Nigerasperones AC, new monomeric and dimeric naphtho-gamma-pyrones from a marine alga-derived endophytic fungus *Aspergillus niger* EN-13. *The Journal of antibiotics*, 60, 3: 204-210.