Aeration influenced on the production of 6-pentyl-alpha-pyrones, spores and enzymes in solid state fermentation by *Trichoderma* strains

R. Hamrouni[1, 2], J. Molinet[1], M. Claeys-Bruno[1], N. Dupuy[1], A. Masmoudi[2], S. Roussos[1]

[1]Aix Marseille Univ, Avignon Université, CNRS, IRD, IMBE, Marseille, France.

[2]Univ. Manouba, ISBST, BVBGR-LR11ES31, Biotechpole Sidi Thabet, 2020, Ariana, Tunisia.

Presenting author email: sevastianos.roussos@imbe.fr

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Introduction

Commonly the pests causing problems on food crops are treated with chemical pesticides, but the wrong and excessive application are causing severe damages to human health, to the environment by residual generation, to ecology by non-target organisms eliminated and the creation of pests resistant to pesticides (Miranda-Hernández et al., 2016). In recent years biopesticides have placed as a viable alternative to control pests and as a possible substitute for the traditional chemical used (Tranier et al., 2014). Already, exists a big number of studies focused on the selection of microorganisms to inhibit pests even kill them, evaluation of effectiveness against pests, mechanisms of action, production systems, viability, among others (Innocenti et al., 2015; Keswani et al., 2016). *Trichoderma* is a filamentous fungus considered a biocontrol agent (BCA), it has been a very important model for study because of its mechanisms of action; nutrients and space competence, antibiotics production, lytic enzymes production and mycoparasitism, therefore is placed as one of the most effective BCA against several pests (Zachow et al., 2016). The production of microorganisms BCA's is a core point to gain ground to the chemical pesticides. In the particular case of filamentous fungi, the best way to apply on fields is through spores because they are the cell structures very resistant to critical conditions of the environment (De la Cruz Quiroz et al., 2015). The production systems using solid substrates have shown excellent yields for the production of filamentous fungal spores (Mascarin and Jaronski, 2016). Solid-state fermentation allows the valorization of agroindustrial wastes having an impact on the worldwide ecology. This production system has the potential to produce value-added products such as antibiotics, pigments, aromas like 6-pentyl-alpha-pyrones and enzymes of industrial interest like cellulases, chitinases, amylases, etc., (Sarhy-Bagnon et al., 2000; Gutiérrez-Sánchez et al., 2013). On the present study, it was evaluated the application of an aeration through a solid-state fermentation using *Trichoderma* strains to produce 6-pentyl-alpha-pyrone, spores and important enzymes. It was used a mix of vine shoots, jatropha, potatoes flour, olive pomace and olive oil as substrate.

Materials and methods

Microorganisms and culture conditions: The strains of *T. harzianum G7, Trichoderma harzianum G3 and T. harzianum G18,* from the IRD/IMBE fungi collection were used in present study. The fungal strains were activated in sterilized PDA and inoculated during 5 days at 30ºC to conserve at 4º C.

Solid State Fermentation (SSF): Solid state fermentation experiments were carried out using a mix of vine shoots, potatoes flour, jatropha, olive pomace and olive oil as substrate. Mixed substrates were sterilized at 121 °C for 30 min. After cooling the medium was inoculated with a concentrated spore suspension of $2x10^7$ spores/g dry matter. Initial pH and moisture contents were 6 and 55 %, respectively. Cultures were carried out at 29 °C using glass columns and flasks packed with 60 g solids. The fermentation process control was done with the application of forced humid air during all the process. The initial aeration rate was 60 ml of saturated humid air/min, only for columns.

Enzyme Assays: Amylase activity was measured using soluble starch (1%) in phosphate buffer (0.1M, pH 7.0) at 50 °C for 10 min (Singh et al., 2014). Cellulase activity was determined using carboxymethyl cellulose (1%) in sodium citrate buffer (50 mM, pH 4.8) at 50 °C for 30 min, in according with De la Cruz Quiroz et al., 2017. An enzyme activity (U) was defined as the amount of enzyme that catalyzes the release of 1 µmol of glucose per minute. Lipase activity was determined using 0.5 mL of p-nitrophenyl (25 mM) in phosphate buffer (25 mM, pH 7.0) at 30 °C for 30 min (Lopes et al., 2011). An enzyme activity (U) was defined as the amount of enzyme required to release 1 µmole of p-nitrophenol per minute.

Extraction of 6-PP: Volatiles compounds were recovered by soxhlet extraction system from solid fermented materia using heptane. Samples (10 g of the fermented materia) were co-distilled at 60°C with 100 ml heptane during 45 min. γ -undecalactone (0.08 mg) was added as the internal standard before extraction.

Results and discussion

T.harzianum G7 also showed the best results for amylase activity during the aerated SSF (46.33 $U.g^{-1}$). A great difference was noticed between SSC without aeration and the aerated SSF with regards to the cellulase activity (Table.1).

Table 1. Aeration influenced on the production of metabolites and spores by *Trichoderma* strains at 46 h of culture for enzymes activity and 96 h of culture for 6-PP and spores production.

Determination of the Enzyme Activities, 6-PP and spores during the Solid-State culture (without aeration)					
Strains	Cellulase ($U.g^{-1}$)	Amylase ($U.g^{-1}$)	Lipase($U.g^{-1}$)	6-PP (mg.gDM)	Spores ($x10^7$ ·DM)
T. harzianum G7	6.69	4.62	17.55	1.13	12.17
T. harzianum G3	7.34	5.31	9.63	0.034	14.53
T. harzianum G18	9.51	7.63	23.16	0.42	106.44
Determination of the Enzyme Activities, 6-PP and spores During the aerated Solid-State Fermentation					
Strains	Cellulase ($U.g^{-1}$)	Amylase ($U.g^{-1}$)	Lipase($U.g^{-1}$)	6-PP (mg.gDM)	Spores ($x10^7$·DM)
T. harzianum G7	34.25	46.33	20.00	1.45	16.53
T. harzianum G3	18.39	9.72	11.41	0.58	123.1
T. harzianum G18	20.96	14.96	29.30	0.98	145.21

High values of cellulase were also shown by *T. harzianum G7* (34.25 U g_{-1} at 72 h) and *T. harzianum G18* (20.96 U g_{-1} at 46 h).The strains *T. harzianum G7*and *T. harzianum G18* were benefited by the application of aeration on the SSF system for 6-PP production (1.45 and 0.98 mg.gDM at 96 h, respectively). The maximum spore production for all three *Trichoderma* strains was achieved at 96 h of fermentation. *T. harzianum G18* resulted in a production of 145.21× 10^7 spores g_{-1} DM (aerated) and 106× 10^7 spores g_{-1} (not aerated). Generally since the 120 h of culture, the sporulation was maintained without important changes (De la Cruz Quiroz et al., 2017).

Conclusion: On these present study, it was proposed an evaluation of the effect of an aeration for the production of lytic enzymes, volatile molecules and spores by SSF. Conventionally SSF is done using tray bioreactors, however, this kind of bioreactors implies the difficulties of aeration and heat removal, which can be attended by the use of Raimbault columns as bioreactors (Raimbault and Alazard, 1980). A mix of substrates was used in the present study, which was used by the fungi as a source of nutrients and also as a matrix to anchor on it. It is well known that vine shoots plays an important role because its high porosity allowing good water absorption, indispensable to carried out the microbial metabolism.

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