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Original article

Production of *Trichoderma harzianum* (127a and 127b) spores by Fermentation (LF and SSF)

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Abstract

In order to produce a bio fungicide, two strains of Trichoderma harzianum (127a and 127b) were used.The results revealed that the mycelium multiplication of Trichoderma harzianum (127a and 127b) strains in liquid fermentation yielded a good mycelial mass (10^6 cells / ml), or the optimal conditions are a pH 5, a temperature of 30° C, and a medium of culture made of Malt extract and yeast extract. However, the more or less unfavorable conditions for the development of mycelium (FMS) favored the appearance of the spore form of our two antagonistic strains, which resulted in a smooth greenish powder at the end of the fermentation, on a solid medium.

Keywords: *Trichoderma harzianum*, Liquid fermentation, Solid-state fermentation, Wheat Bran.

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INTRODUCTION

Agriculture statistic showed that all plant species have been destroyed by plant pathogens. The intensified use of chemical pesticides has resulted in accumulation of toxic compounds in food chain leading to harzardous to humans and environment (Cook and Baker, 1983) and also in the build-up of resistance of the pathogens. In view of this, used of Biological control by an antagonistic organism is apotentiel, non-chemical and ecofriendly approachfoe managing plant diseases (Andrews J.H., 1992).

The power of the antagonist strains of Trichoderma as BCAs has been recognized from 1930 and today there are modern technologies for including them in biological control of various diseases (Gveroska and Ziberoski, 2012) such as *Rhizoctonia, Fusarium, Alternaria, Colletotrichum, Cylindrosporium* and *Helminthosporium*.

Trichoderma harzianum spices are among the most studied fungal biocontrol agents and are successfully used as biopesticides and biofertilizers in greenhouse and field plant production (Harman et al., 2004). These applications are related to their ability to: a) colonizing the soil and/or parts of the plant, occupying a physical space and avoiding the multiplication of the pathogens; b) producing cell wall degrading enzymes against the pathogens; c) producing antibiotics that can kill the pathogens; d) promoting the plant development and e) inducing the defensive mechanisms of the plant (Monte and Llobell, 2003).

Trichoderma harzianum is cosmopolitan, characterized by rapid growth, their ability to use various substrates and resistance to harmful chemical agents (Klein and Eveleigh, 1998). These characteristics have permt of produce it by fermentation.

Our aim, therefore, was to produce spores of two species of *T. harzianum* (*T-127a* and *T-127b*) in a series of two fermentations: liquid and solid state fermentation in laboratory conditions.

Materials and Methods

Microorganisms

Trichoderma harzianum 127a and *T-127b* strains used in this study were obtained from rhizosphere agricultural soil in the region of Constantine (Algeria). This soil is designed for growing *Lens culinaris*. Isolating strains T-127b and 127a was isolated according to suspenssion dillution method (Warcup, 1955). The *Trichoderma* isolates were cultivated on M2 medium (20 g/l malt extract, 2 g/l yeast extract, 15 g/l agar) for 15 days before their used in liquid fermentation.

Liquid fermentation

Spores of T-127a ant T-127b obtained after 15 days from M2 medium were scraping with spatula and suspending in 10 mL distillate water. The concentration was adjusted with Bürker Cell (Niranjan et

al., 2009). 5 mL of this solution was introduced in Erlenmeyer contained 500 ml of M2 liquid medium and incubated at 28±2°C with an agitation of 80 rpm for 24 h (Pedreschi and Aguilera, 1997). The inoculum thus obtained was used as a pre-culture fermenter 20 l.

Production spores of *Trichoderma harzianum*, experiments were conducted at 30°C; medium pH of 5; aeration 1vvm; Vapor 15 psi and at agitation speeds between 350 and 500 rpm according to the fermentation system.

During the production of the biomass by liquid fermentation, three parameters were followed: Glucose concentration (YSI SELECTglucometer MODEL 2700), Optical Density (VWR® Spertrophotometer V-1200) and the spore concentration ((Bürker cell).

Solid state fermentation (SSF)

At the end of the liquid fermentation, the contents of the bioreactor was filtered under aseptic conditions. The biomass obtained was measured and mix with Wheat Bran (W/W). The biomass / Wheat Bran was mixed well, covered and incubated at 28± 2°C until the maturation of spores and the middle of drying.

In the SSF, three parameters were measured daily, namely: weight, moisture and the spores concentration.

At the end of the SSF, the spores were separated from their culture medium.

Results and Discussion

Research of strains of *Trichoderma hazrianum* in agricultural soil has done tow strains: *Trichoderma harzianum 127a* and *Trichoderma 127b* (Figure 1). Strains showed a green coloring at degree variants depending on the isolate. Microscopic observation showed globular unicellular conidia; phialides shaped keel, in whorls on branched conidiophores with right angle or on their side branches.

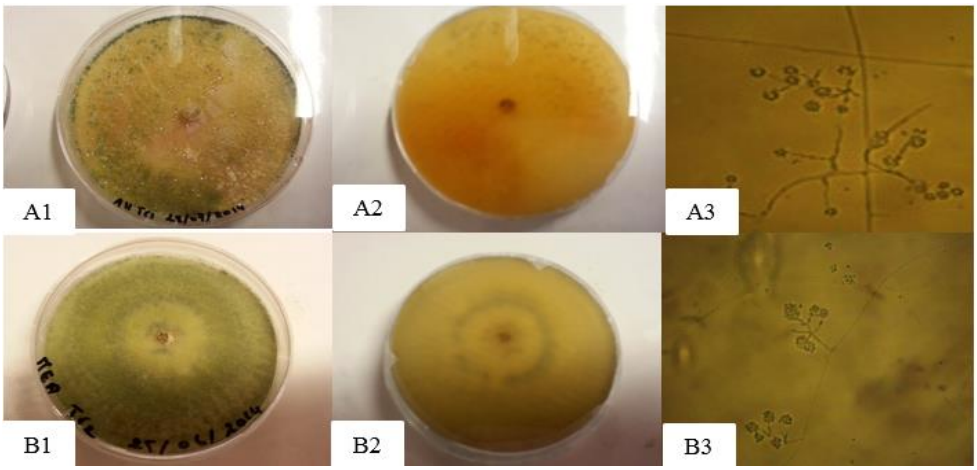


Figure 1. Macroscopic observation (A1, A2) and microscopic (A3) of *Trichoderma harzianum 127a*; and *Trichoderma harzianum 127b* (B1, B2, B3).

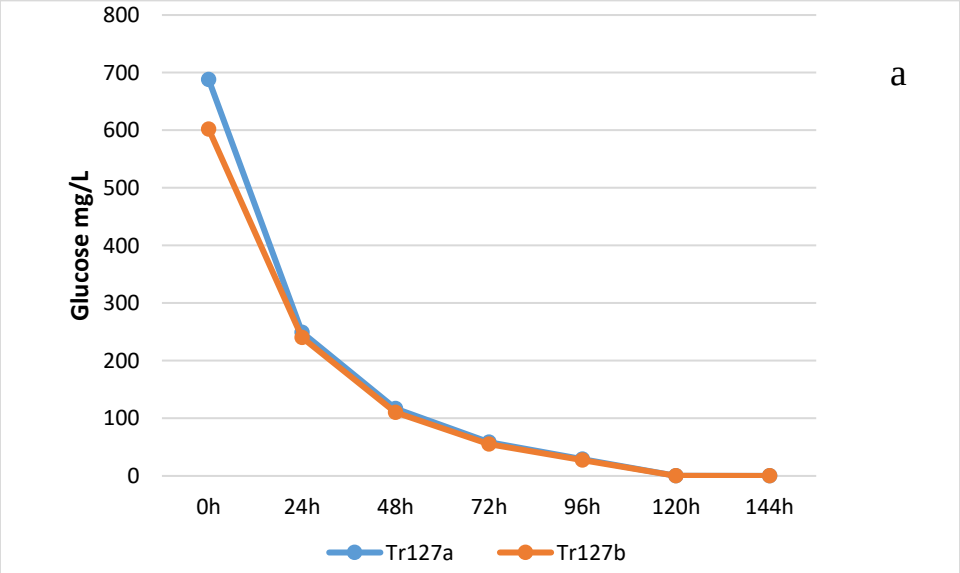
Analysis of the results obtained at the end of the liquid fermentation, showed a steady decrease in the concentration of Glucose in the bioreactor during the all times (144h) of the fermentation for both strains of *Trichoderma harzianum*. By cons, followed by the optical density showed that it has undergone a rapid increase during the first 48 hours and less rapid in the last hours of fermentation (Figure 2a).

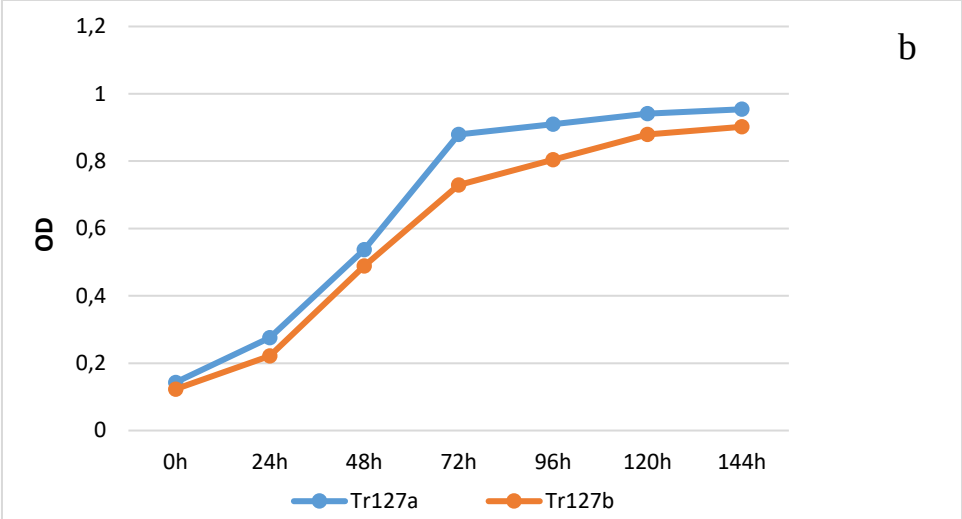
In addition, the follow-up of the optical density (OD) measurement shows a rapid increase during the first (48h) hours, but it becomes less rapid in the last hours of fermentation (Figure 2b).

Furthermore, the concentration of spores was linear during the first 48 hours and has grown rapidly since the 48th hour to the end of fermentation. The maximum concentration reached at the end of the fermentation is of the order of 10^6 cells / ml for the two antagonistic strains (Figure 2c).

The results obtained in LF, show that the spore concentration, the concentration of sugars and the OD, are in close relationship. According to Tarus et al. (2003), the development of mycelium is increasing during fermentation as long as the concentration of sugars and nutrients is sufficient. However, as of the fourth day (96 h) of fermentation, this concentration becomes more and more stable with the depletion of the carbon and nitrogen substrate and probably the influence of various physicochemical factors that become unfavorable to growth. This also explains the beginning of the appearance of some spores at this stage. These results are consistent with those of Papavizas and Lumsader (1982).

At the end of the liquid fermentation, a creamy biomass of mini pellets is harvested and weighed under aseptic conditions. It should be noted that the biomass obtained from the strain “T-127a” is greater than that obtained by the strain “T-127b”. This can be explained by the possibility of adaptation of the first strain to the fermentation conditions in comparison with the second strain.





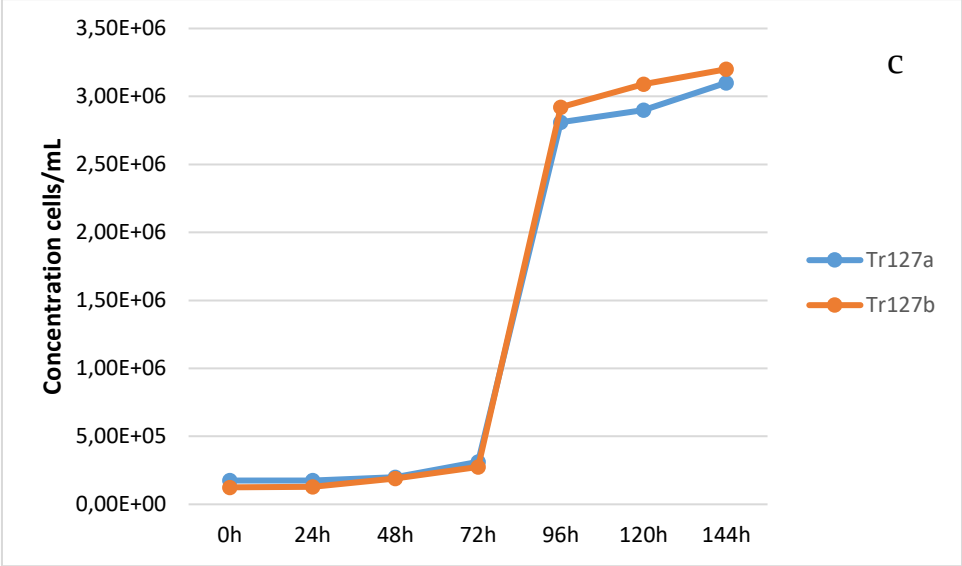
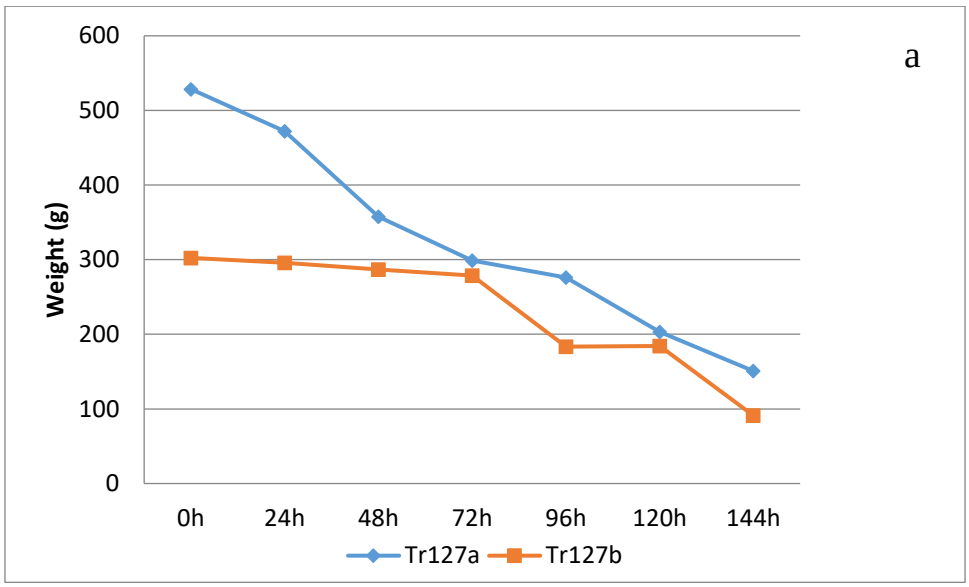


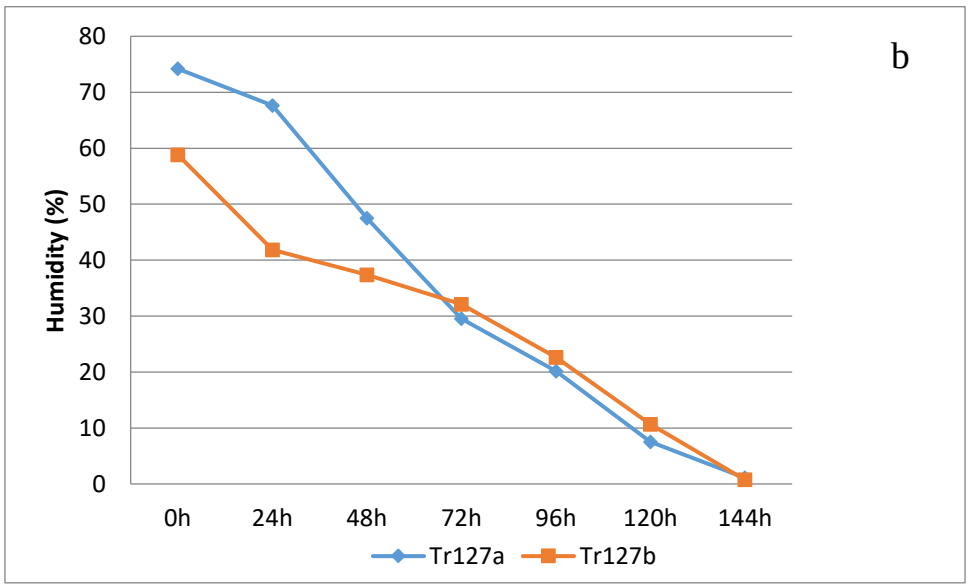
Figure 2. The different parameters measured during LF**:** the concentration of sugars (a); the optical density (b) and the concentration of spores (c)

Analysis of the results obtained at the end of the SSF showed that the weight of the trays gradually decrease until a stable weight (weight measurements after the 6th day gives values very close). Unlike the initial weight between the two *Trichoderma* isolates is due to the difference of biomass after liquid fermentation (Figure 3a).

In addition, moisture-monitoring results in the interior trays shows that the percentage of moisture fell during the FMS until a solid medium (about 1% moisture) (Figure 3b). The trays obtained at the end of the FMS was dry texture, cracked, solid, breakable and wearing a white greenish staining.

The measurement results of the spore concentration shows that the latter increases regularly exponentially until 96 h where it waits around 10^9 UFC/g for two fungal antagonists (Figure 3c).





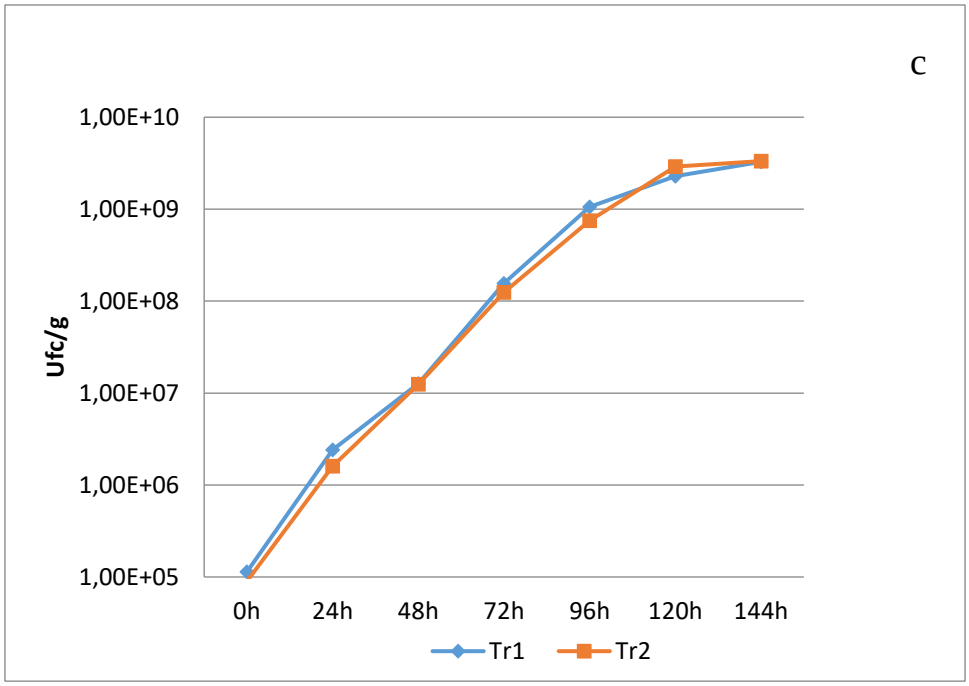


Figure 3. The different parameters measured during LF**:** the weight (a); the humidity (b) and the concentration of spores (c)

At the end of the fermentation process, the trays take on a dry, cracked, solid, breakable texture with a white to greenish appearance (Figure 4a).

The separation of spores from their fermentative support (wheat bran) yielded a dark green powder with a smooth texture, corresponding to the spores of *Trichoderma harzianum* (Figure 4b).

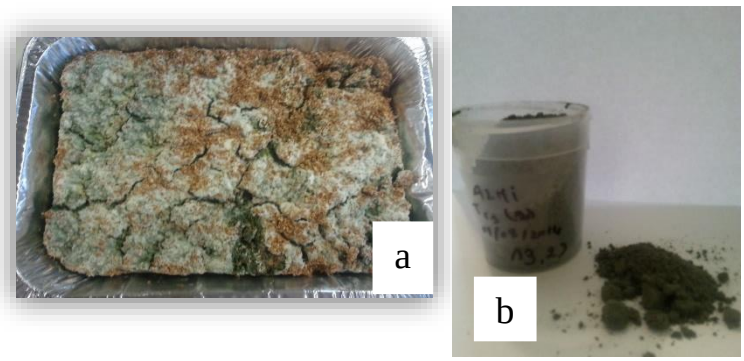


Figure 4. The texture of the tray at the end of the SSF (a); final appearance of fungicide (b).

Several authors for the production of *Trichoderma* spores (Tewari L. and Bhanu C. 2004, Rosane and *al.*, 2008) have advocated the use of wheat bran as a fermentation medium. The analysis of the results we obtained showed an important relationship between the different parameters studied: weight, humidity and spore concentration. Indeed, during the SSF we found that the spore concentration is increasing rapidly at the expense of moisture and weight. Moisture reduction is a factor influencing dry

weight and water activity, leading to unfavorable conditions for mycelia growth, which directs T-harzianum strains to sporulation. Also, it should be noted that the maximum spore concentration reached the order of 10^9 CFU / g, which is in agreement with the work of Tewari and Bhanu (2004) and Zuriash Mamo and Tesfaye Alemu (2012).

Conclusion

The production of a biological fungicide from two *Trichoderma harzianum* strain (127a and 127b) was carried out in three stages: the first two stages are represented by two kinds of fermentation. First, a LF aiming at the multiplication of the mycelium and thus production of a large quantity of biomass of the order of 10^6 cells / ml) and an SSF with unfavorable conditions for mycelial multiplication thus leading to the production of the spores or the final concentration of its last summer of the order of 109 CFU / g. The results obtained allowed us to observe a good relationship between my different control parameters during both fermentation, namely: the decrease in the glucose concentration as the OD and the mycelial concentration increase and this in the case the LF; on the other hand, an increase in the SSF spore concentration as humidity and weight decrease. Lastly, the last production steps made it possible to separate the product (spores) from their fermentation medium (Wheat Bran).

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