Production of a Biological Control Agent: Effect of a Drying Process of Solid-State Fermentation on Viability of *Trichoderma* Spores

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**Abstract:** Biological control is an environmentally effective technology for the reduction of pests through the use of natural enemies called biological control agents (BCA). Among BCA, *Trichoderma* is an important biopesticide fungus against several plant pathogens. The present study, shown the evaluation of a solid-state fermentation process in order to produce spores by *Trichoderma harzianum*. Sugarcane bagasse, wheat bran, and potato flour were evaluated as a mix of substrates to produce fungal spores. CO₂ released during mycelial growth and sporulation was monitoring by respirometry. Effect of dry air was evaluated on the decrease stage of fungal metabolism as an inducer of sporulation, and also the effect of drying on the viability of the spores produced. Under these conditions, the maximum concentration of spores was 8.4x10⁹ spores/g carbon source (CS) at 96 h. Results demonstrated that dry air applied on SSF did not have influence on the increase of spore produced, but it can accelerate this process (at least 24 hours) and also to increase the spore viability (1.3 x10⁹ spore/ g carbon source, with 24.7 % of viability, respectively). SSF without application of dry air showed a production of 8.9x10⁸ spore/g CS. In parallel, the endoglucanase and exoglucanase activities were evaluated obtaining 11.6 and 0.86 U/g CS, respectively.

**Keywords:** Cellulases, Biocontrol agents, Biopesticide, Conservation methods.

1. INTRODUCTION

Biopesticides are synthesized from natural sources. They are formulated to kill or inhibit the growth of fungi on crops and plants. As most of the chemical pesticides cause environmental and health issues, many agrochemical companies are focusing on the development of biopesticides through extensive research and development strategies [1,2].

Several fungi can be considered as biopesticides because they act as natural enemies of fungal phytopathogens. Particularly, filamentous fungi have physiological, enzymatic and biochemical properties which give them the capacity to use as biopesticides [3]. *Trichoderma* is one of the most studied biopesticides around the world. Different species of this genera had been tested against several phytopathogens with good results, such as *Fusarium oxysporum*, *Botrytis cinerea*, *Crinipellis perniciosa*, *Rhizoctonia solani*, *Macrophomina phaseolina* [4-8]. To apply *Trichoderma* as biopesticide on the field crops is necessary to do it in form of spores because they are the most infective units. It is demonstrated that spores produced by solid-state fermentation (SSF) are more resistant to the real conditions found in nature [9].

SSF is a microbial process carried out on solid materials surface on the absence of free water. The materials absorb the water to support the fungal growth and metabolism [10,11]. Generally, the substrates for SSF are agroindustrial wastes, which offer attractive advantages for applications on fermentation processes, including low cost and good availability [12]. SSF is the best way to produce spores of biofungicides, but there are still challenges to be addressed, such as high yields production and viability of spores.

On the present study, it was used the SSF to produce spores by *Trichoderma harzianum*. The aims of the work were: (1) evaluate the application of dry air on the decrease stage of fungal metabolism as an inducer of sporulation, and (2) determinate the effect of drying on the viability of the spores produced.

2. MATERIALS AND METHODS

2.1. Microorganism

The crio-preserved fungal strain of *Trichoderma harzianum* IRDT22C was provided by the Institute Mediterranean of Biodiversity and Marine Ecology and Continental (IMBE), Aix-Marseille University, France. The strain was inoculated and cultured on potato dextrose agar (PDA). The incubation was done for 5 days at 29 °C. After this time, 20 mL of Tween 80 (0.1 g/L) was added to the flask with the inoculum in order to recover the spores. Spore concentration was determined using the cell counter chamber of Malassez.

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2.2. Solid-State Fermentation (SSF)

Solid state fermentation experiments were carried out using a mix of sugarcane bagasse (SCB), wheat bran (WB) and potato flour (PF) as substrates (50, 30 and 20 %, respectively). Mixed substrates were sterilized at 121 °C for 30 min. After cooling the medium was inoculated with a suspension of 2x10⁷ spores/g substrate. Initial pH and moisture contents were adjusted at 5.6 and 66 %, respectively. The material (20 g) was packed into Raimbault columns and incubated at 29 °C [13]. A saturated wet air flow of 25 mL/min was applied in SSF until 72 h. After this time, it was applied dry air at a flow of 40 mL/min until the end of culture. As control was used a SSF keeping wet air flow (25 mL/min) during all fermentation process. Each treatment was done in triplicates and monitored kinetically. The values of air flow, temperature, humidity percentage and CO₂ profile from SSF were monitoring through respirometry analysis using the PNEO equipment.

2.3. Enzyme Assay

Fermented material (5 g) were mixed with 50 mL of distilled water, the suspension was homogenized using an Ultra-turax for 1 min and then pH was measured. The endoglucanase and exoglucanase activities were determined by the methodology of Mandels et al., [14]. An international unit of cellulase activity is the amount of enzyme to release a micromole of glucose per minute of the reaction. Reducing sugars were determined by spectrophotometry [15]. The sample solution (2 mL) and DNS reagent (3 mL) were placed in a tube test. This mix was stirring on a vortex and after it was placed in a bath for 5 min (100 °C). The reaction was stopped in a frozen bath. A calibration curve was done from 0 to 1 g/L of glucose and the measure was done at 575 nm.

2.4. Spore Production of T. harzianum on SSF

Fermented material (1 g) was placed in an Erlenmeyer flask with 100 mL of Tween 80 at 0.01 % and stirring for 10 min. The spore counting was done every 12 hours, using a cell counter chamber (Malassez). The results were expressed as the spore number per gram of carbon source initially present in the culture media (spore/g CS). A culture of T. harzianum on PDA medium was used as a control.

2.5. Spores Viability of T. harzianum Under Different Stock Conditions

The viability of the spores produced by T. harzianum was determined. Preservation by PDA cooled, lyophilization and frozen material were the methods compared against drying process. One gram of samples (lyophilized, frozen and dried) was placed in a conic tube with 40 mL of Tween 80 at 0.1 % and stirring. PDA cooled sample was mixed with 10 mL (Tweem 80 at 0.1 %) in the Erlenmeyer flask. Serial dilutions of each suspension were spread (0.2 mL) onto Petri plates (PDA/ Rose Bengal). After incubation at 28 °C for 4 days, colonies of T. harzianum were counted.

2.6. Experimental Design and Data Analysis

For each fermentation process, an experimental design with a monofactorial fix was used. The ANOVA was done by the software UANL version 2.5 and the mean comparison tests by Tukey.

3. RESULTS AND DISCUSSION

3.1. Enzyme Assays

Endoglucanase and exoglucanase activities were kinetically evaluated on SSF3 by T. harzianum during 180 h. Exoglucanase did not show any activity in the first 24 h, but it does at 41 h. The activity increased up to 89 h reaching the maximum enzyme activity (0.86 U/g). This activity was maintained up to 96 h, but after this time the activity disappeared. Endoglucanase was not detected in the first 48 h of culture, however at 89 h the activity achieve its maximum value of 11.6 U/g. The enzyme activity was stable through the time until the end of culture (Figure 1).

3.2. CO₂ Concentration and Effect of Dry Air Application on Spore Production on SSF by T. harzianum

The behavior of spore production and CO₂ released during the solid-state fermentation by T. harzianum were evaluated. The CO₂ is produced by fungal metabolism during the growth development. The Figure 2 shows that the release of CO₂ begun from the 5 hours of culture, reaching the maximal concentration at 72 h of growth (1.02 % CO₂). CO₂ decreased until negligible percentage at 80 h and that level was maintained constant until the end of culture. Drying process was applied at 72 h because of the diminution of fungal metabolism indicated by CO₂ decrease. Dry process on SSF showed a maximal sporulation of 8.4x10⁹ spores/g CS at 96 h, after that the values were maintained without significant change. Wet SSF (control condition) showed a value of 8.9x10⁹ spores/g CS at 120 h. Both fermentations maintained these concentrations of spores during the process until the end of culture (144 h). The time of maximal spore production was variable, so it was
possible to compare in terms of productivity (spores/g * h) (Table 1).

3.3. Effect of Conservation Method on Spore Production on SSF by *T. harzianum*

Four treatments were evaluated to conserve spores of *Trichoderma harzianum* produced by SSF. The spores conserved on PDA media at 4 °C were the control, showing 26.75 % of viability. The spores obtained from the dry samples of DA-SSF reached 24.7 % of viability (Table 2). 15.64 and 3.17 % of viable spores were values showed by frozen and lyophilized samples, respectively.

There are several studies where it is reported the production of cellulolytic enzymes under SSF [16,17]. Currently, the SSF is one of the most systems used

![Endoglucanase and exoglucanase activities on SSF by *T. harzianum*.](image1)

![Spore production and CO2 released during the treatment SSF3 by *T. harzianum*.](image2)

![Spore production by *Trichoderma harzianum* on SSF](image3)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Spore /g CS</th>
<th>Time (h)</th>
<th>Productivity (spores/g * h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDA control</td>
<td>8.6x10⁹ a</td>
<td>147</td>
<td>5.9x10⁷ c</td>
</tr>
<tr>
<td>SSF control</td>
<td>8.9x10⁹ a</td>
<td>120</td>
<td>7.4x10⁷ b</td>
</tr>
<tr>
<td>Drying process</td>
<td>8.4x10⁹ a</td>
<td>96</td>
<td>8.8x10⁷ a</td>
</tr>
</tbody>
</table>

*Different letters in the same column are statistically different.*
because the raw materials are cheaper, such as sugarcane bagasse, wheat bran, corn cob, among others [18]. Cellulases: The lytic enzymes such as cellulases are very important to filamentous fungi because they need this kind of enzymes to obtain nutrients and energy from fibrous substrates without free simple sugars. Mainly, cellulases break the biopolymer cellulose resulting in a liberation of glucose monomers needed to the development of microorganisms [19].

In the present study, exoglucanase activity started between 36-48 h, increased over the time and achieved a maximum value at 89 h, however after 96 h the activity was not detected. The exoglucanase has the ability to break cellulose into two-sugar segments called cellobiose, however this compound could cause a catabolic repression of the enzyme activity [20]. This effect could support the low detection of activity after 96 h. On the other hand, endoglucanase activity breaks internal links β-1,4 of cellulose, releasing monomers and/or polymers of glucose [19]. Endoglucanase started to act after 48 h, rapidly achieve the maximum value at 89 h and that it is possible to see a very slowly decrease but maintaining high enzyme values at 180 h. This effect suggests that endoglucanase produced under the present SSF condition by *Trichoderma harzianum* is not as susceptible to repression and it maintains its activity for a long time.

Spore production (CO2 Monitoring). It is well known, when culture conditions (Environmental and nutritional) become critical the sporogenesis begins on fungi [1,21]. CO2 concentration shows the metabolic activity by fungal respiration (*T. harzianum*). The exponential phase of CO2 concentration is related to the development of mycelial growth (active metabolism) when CO2 concentration decrease indicates the end of mycelium formation, but the start of sporulation. Spore production (dry air applied).

According to the experiment, the decrease of CO2 started at 72 h, it was tried to accelerate the desiccation process applying dry air after 72 h to induce critical culture conditions, using an air flow of 40 mL/min. This experiment was done to evaluate the effect of dry air on the sporulation index by *T. harzianum*. The number of spores produced did not increase, in fact, it was possible to produce the same quantity in both experiments. However, the production was done 24 hours soon than the normal SSF with forced humid air. On the solid-state fermentation, the dry air applied cause over *T. harzianum* an effect of hydric stress. It could favor the sporulation and minimize the time of production, because the excess of water is evaporated, causing free space to be occupied by spores, which contribute to achieving the maximum sporulation in less time. The levels of spores obtained are better than other researchers, such as Kancelista et al., [22] who reported 3.13x10^9 spores/g using corn cob under SSF by *T. asperellum*, and Motta and Santana [23], who reported 4.4x10^9 spores/ g using empty fruit bunch under SSF by *Trichoderma spp*.

Conservation methods. The viability of dry spores obtained in the previous experiment was evaluated. In short term is common to store fungal spores on PDA (Erlenmeyer or Petri plates) into the fridge at 4 °C. Therefore, this conservation method was used as a control test. Dried spores showed good viability and very similar with PDA at 4 °C (means comparison did not show significant differences). Lyophilization and frozen methods showed low viability. Lyophilization is not a process for all fungi, some kind of spores tend to coll apse and the structural damage caused is not reversible. Freezing process has several disadvantages, such as physical damage and death of the spores by the formation of ice crystals [24].

The spore production of *T. harzianum* increased and achieved its maximal spore concentration at the same time CO2 was decreasing until zero (After 80 h of culture). The results of this study demonstrated that the application of dry air after 72 h of culture, can accelerate the spore production at least 24 h, than the SSF without application of dry air.
4. CONCLUSION

Under the present experimental conditions, the system of solid-state fermentation using the Raimbault columns by *Trichoderma harzianum* demonstrated great functionality of sugar cane bagasse and wheat bran as substrates to the development of *Trichoderma harzianum* growth. High yield of spores and high enzyme activities were reached under the present culture conditions. Close relationship between CO$_2$ released and sporulation was determined, because the level of CO$_2$ decreased resulting proportional to the spores produced by *T. harzianum*. Dry air applied on the decrease stage of CO$_2$ did not affect positively on the increase of spores produced by *T. harzianum*, but the sporulation process was accelerated 24 hours. The process of spore drying through dry air allow to keep viable 25 % of spores, this is 1.6 and 7.9 folds better than frozen and lyophilization, respectively. The dry process represents a potential method to store fungal spores, reducing the cost of energy used in the other process mentioned, however, operational conditions of the dry process should be further optimized.

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