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A SIMPLE METHOD FOR HARVESTING *Trichoderma asperellum* SPORES

Domenico Pavone-Maniscalco[*1], *Massiel Pinto*[1], *Renny Pacheco-Calderón*[2], *Douglas Mora*[1], *Carmen Andara*[2], *Anakarina Bello*[2], *Maira Hernández*[1], *María Alejandra Correa*[1] *& Oscar Valbuena*[1]

Address(es):
[1]Centro de Biotecnología Aplicada (CBA), Departamento de Biología, Facultad de Ciencias y Tecnología, Universidad de Carabobo, Campus de Bárbula, Municipio Naguanagua, Estado Carabobo, Venezuela. Phone: +58 424 4737054.
[2]Centro de Zoología Aplicada (CEZA), Departamento de Biología, Facultad de Ciencias y Tecnología, Universidad de Carabobo, Campus de Bárbula, Municipio Naguanagua, Estado Carabobo, Venezuela.

*Corresponding author: dfpavone@gmail.com

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ABSTRACT

The fungus *Trichoderma asperellum* has a great potential to be used as biocontrol agent against plant pathogens in field. For this purpose it is necessary to optimize mass production on an industrial scale. This work proposes a method for the recovery and concentration of *T. asperellum* spores. The addition of the fertilizer Urfos 44®, decreased pH value of spore suspension from 6.31 to 1.76. This condition allowed spores to flocculate in few hours, rendering a more concentrated suspension by a factor of 6 - 7. Spore viability did not significantly decreased in 72 hours under these conditions. These results will contribute to obtain more concentrated products based on *T. asperellum* using a fast and low cost method.

Keywords: Solid state fermentation, flocculation, aggregation, Urfos 44®, urea, phosphoric acid

INTRODUCTION

The fungus *Trichoderma asperellum* (Ascomycota) is a well-known biocontrol agent in agriculture (**Wu *et al*., 2017; Rosmana *et al*., 2016; Li *et al*., 2016; El Komy *et al*., 2015; Elsharkawy *et al*., 2013; Mbarga *et al*., 2013; Segarra *et al*., 2010**). The industrial production of *T. asperellum* is generally based on solid state fermentations (SSF), in which substrates as rice or maize are usually used. In our laboratory, spore recovery after fermentation is carried out by washing fermented material with water, followed by a concentration step. There are several methods to concentrate spores, such as centrifugation, filtration and decantation, among others; however such methods could be expensive or time consuming.
Flocculation is a useful method for harvesting biomass in yeast and microalgae industry (**Kim *et al*., 2017; Vallejo *et al*., 2013; Vandamme *et al*., 2013; Schlesinger *et al*., 2012; Soares, 2010**), but there is no report in literature using this method for harvesting *Trichoderma* spores.
Urfos 44® is a fertilizer containing urea and phosphorous, when dissolved in water forms urea and phosphoric acid as described in equation 1, resulting in the decreasing of pH values.

$$[H_3PO_4{:}(NH_2)_2CO]_{crystal} \longrightarrow H_3PO_4 + (NH_2)_2CO \quad \text{(Eq. 1)}$$

In this work, the use of Urfos 44® as a flocculant agent for harvesting *T. asperellum* spores will be described, as well as, its effect on some physicochemical parameters and viability of spore suspension.

MATERIALS AND METHODS

Biological material

The strain of *Trichoderma asperellum* used (TV190), was isolated from corn field soil in Monagas State, Venezuela, and identified by molecular analysis (**Pavone & Dorta, 2015**). It was maintained by alternated culture on potato dextrose agar (PDA) and sterilized soil.

Flocculant agent

Urfos 44® produced by Tripoliven C.A., Carabobo, Venezuela (www.tripoliven.com/documentos/URFOS-44.ppt), is a fertilizer used in several agricultural crops. The composition and some physicochemical characteristics of Urfos 44® are described in Table I.

Table I Composition and some physicochemical characteristics of Urfos 44®

Composition/ Physicochemical Characteristic	Value
Phosphorous, P_2O_5	44 %
Nitrogen, N	17 %
pH (solution at 1 % w/v)	1.7 – 1.9
Conductivity, CE (1 g/L)	0.84 dS/m
Solubility 20 ºC	960 g/L H_2O
Chemical purity (dry base)	>99%

Production of *T. asperellum*

The production of *T. asperellum* was based on a solid state fermentation using maize or rice. The substrate (150 g), hydrated (50% dry basis) in cellophane bags (20x30 cm), was cooked by wet heat for two hours. A 10^7 spores/mL suspension of the fungus (10 mL) was inoculated in each bag, mixed and incubated at 27±2 ºC for 5-6 days. After sporulation, fermented material was washed twice using potable water. The resulting spore suspension was passed through a sieve with 1.5 mm pores and reserved for subsequent experiments.

Effect of Urfos 44® on pH suspension, spore viability and concentration

Spore suspension was transferred to 1 L bottles adding Urfos 44® at different concentrations (0; 0.1; 0.5 and 1 % w/v). The bottles were shaken by handle and five samples were taken to measure pH and initial spore concentration. After 24 h, spore concentration and volume occupied by spores were determined. Spore concentration was determined using a Neubauer chamber and pH was measured using a pH meter (Sper Scientific). Finally, spore viability was determined at different times (0, 24, 48 and 72 h) and storage conditions (5 ºC or 25 ºC), by cultivating dilutions (10^{-7} and 10^{-8}) of spore suspensions on PDA plates (three replicates). Results were expressed in CFU/mL and analyzed through ANOVA to determine statistical differences using the Program Statistix v.8. All experiments were repeated twice.

RESULTS & DISCUSION

Concentration and suspension pH

One of the most important effects of Urfos 44® on spore suspension is the decreasing of pH. Table II resumes pH values for spore suspension at different Urfos 44® concentrations. Suspensions without Urfos 44® presented pH values of 6.31, whereas increasing Urfos 44® concentration to 1 % w/v, decreased pH to 1.76. In suspensions without Urfos 44® or 0.1 % w/v, volume occupied by spores did not vary (975 mL). However, when adding Urfos 44® at 0.5 or 1 % w/v, volume occupied by spore after 24 h, decreased to 150 mL. Under these conditions, aggregation and flocculation of spores occurred in few hours. Initially, spore concentration was $2.81x10^8$ spore/mL (value not shown in Table II), whereas when adding Urfos 44® at 0.5 and 1 % w/v, spore concentration reached $1.83x10^9$ and $2.12x10^9$ spore/mL, respectively (Table II). The spore concentration was 6-7 times higher than the untreated spore suspension. Finally, a brown color only in treatments without Urfos 44® at 25 ºC was evident after 24-48 hours, suggesting an oxidation process and a protective effect of Urfos 44®.

Table II Effect of Urfos 44® on pH, volume of spores and spore concentration of *T. asperellum*

Urfos 44® Concentration (% w/v)	pH	Volume (mL) occupied by spores after 24h	Concentration (Spores/mL) (24h)
0	6.31±0.013	975	$1.01x10^8$±$1.87x10^7$a
0.1	4.53±0.008	975	$1.32x10^8$±$1.09x10^7$a
0.5	2.20±0.009	150	$1.83x10^9$±$2.53x10^8$b
1	1.76±0.011	150	$2.12x10^9$±$2.08x10^8$b

It was demonstrated that the addition of Urfos 44® to *T. asperellum* spore suspension accelerates flocculation. Under these conditions, spores maintained its viability at least for 72 hours. This is the first report of flocculation of *T. asperellum* spores using Urfos44®. However, the molecular mechanism involved in this process is unknown; it will be necessary more studies to understand the role of pH decreasing, urea, phosphoric acid or other microelements present in suspension.

Viability of *T. asperellum* spores in Urfos 44® suspensions

High spore viability in a biocontrol agent is crucial to obtain adequate control in field. For this reason, all methods used to produce and recovery spores must be suitable to maintain viability. In Figure 1, viability of *T. asperellum* spores at different Urfos 44® concentrations and storage conditions is shown. Statistical analysis demonstrated that viability did not change significantly between 0 and 72 hours in all treatments, with p-values of U25=0.86, W25=0.73, U5=0.47 and W5=0.86.

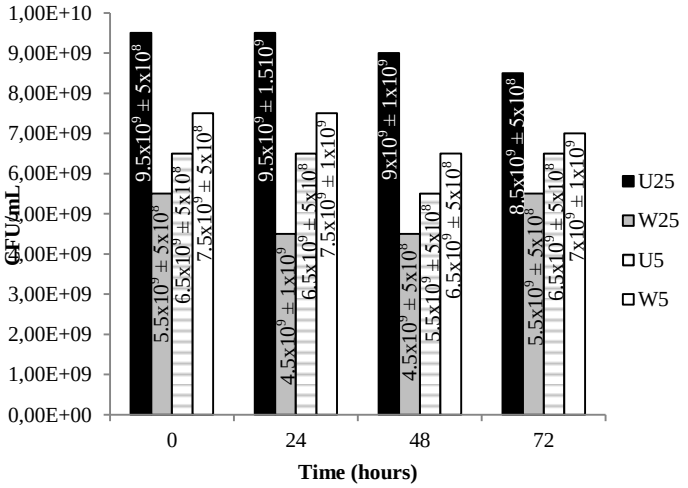

Figure 1 Viability (CFU/mL) of *T. asperellum* spores in Urfos44® suspension (0.5% w/v).
U25: Urfos 44® suspension at 25ºC; W25: Water suspension at 25ºC; U5: Urfos 44® suspension at 5ºC; W5: Water suspension at 5ºC.

In yeasts, there are many reports of flocculation as a common process used in industry. Several mechanism of flocculation have been described in *Saccharomyces cerevisiae*, including: (a) cell wall charge and hydrophobicity: at physiological pH values, *S. cerevisiae* cell wall bears a net negative charge due to the ionization of carboxyl and phosphodiester groups, which drives repulsion of charges reducing cell aggregation. Therefore, the reduction of cell charge should facilitate yeast flocculation. Additionally, cell-surface is partially responsible by the triggering of flocculation of brewing strains (**Smit *et al*., 1992; Straver *et al*., 1993; Speers *et al*., 2006; cited by Soares, 2010**); (b) Lectin theory, in which a specific lectin-like protein interacts with carbohydrate residues of □-mannans and (c) Flo1: cell surface proteins present in yeast related to the flocculation process (**Soares, 2010**). The cell wall composition of *Trichoderma pseudokoningii* mycelium was determined by **Jennah *et al*., (1982)**, whom treating it sequentially with enzymes detected a layered structure comprising glucan, protein/glycoprotein, and chitin. For the genus *Trichoderma* there is no report of Flo1 gen on GenBank, whereas it has been reported in yeasts (**Van Mulders *et al*., 2010**) and some species of the filamentous fungus *Metarhizium rileyi* (**Shang *et al*., 2016**).
Culture filtrates and mycelial extracts of two mycoparasitic *Trichoderma* species were tested for the presence of lectins, by haemagglutination with human and marsupial erythrocytes (**Neethling & Nevalainen, 1996**). In *Trichoderma viride*, haemagglutinating activity was present in both mycelial extracts and culture filtrate. While secreted lectins were only detected after 6 days of growth, the presence of mycelium-associated lectins was first noted in 3 day old cultures. Agglutinating activity was also demonstrated in the mycelium of 6 to 13 days old cultures of *Trichoderma harzianum*. This is the first report on the occurrence of lectins in *Trichoderma* spp. Spores of *Trichoderma harzianum* have been harvested for seed application by scrapping from PDA plates, obtaining 11–12 log10 CFU/g (**Singh & Nautiyal, 2012**).
A pH increase for harvesting microalgae have induced flocculation with efficiency up to 90% for freshwater microalgae (*Chlorella vulgaris*, *Scenedesmus* sp. and *Chlorococcum* sp.) and marine microalgae (*Nannochloropsis oculata* and *Phaeodactylum tricornutum*) (**Wu *et al*., 2012**). In this case, magnesium in the hydrolyzed growth medium drives the formation of magnesium hydroxide precipitate, which coagulated microalgal cells by sweeping flocculation and charge neutralization. Additionally, the microalgal biomass concentration and released polysaccharide from microalgae could influence the flocculation efficiencies. It has been proposed that alkaline flocculants neutralize the repelling surface charges of algal cells, allowing coalescence into a floc. Flocculation can also be due to multi-valent cross-linking or co-precipitation with phosphate, magnesium and/or calcium (**Schlesinger *et al*., 2012**).
In microalgal cultures, flocculation often occurs spontaneously when pH increases above 9 (autoflocculation) as a result of photosynthetic CO_2 depletion. As stated below, this process is associated with the formation of calcium or magnesium precipitates, which can induce flocculation through charge neutralization and/or sweeping flocculation. High phosphate concentrations are required for this type of flocculation to occur (**Vandamme *et al*., 2013**). For harvesting *Chlorella* sp biomass, a new flocculation approach utilizing metal coagulant ($Fe_2(SO_4)_3$) and sulfuric acid (H_2SO_4) is actually used. The method induces a reduction of pH which releases precipitates attached to the microalgae (**Kim *et al*., 2017**).
In fungal biocontrol formulation for industry, the methodology to harvest and concentrate spores is a key step. Current methods have an important impact on sale prices due to reactive, high cost equipment or time consuming processes. The method proposed in this work utilizes a very inexpensive agricultural commodity in a fast and simple way to concentrate spores of *T. asperellum*, reaching a gel consistence from which it is possible to formulate directly or initiate a complete dehidratation process.
Concentrated spore suspension of *T. asperellum* obtained by the above described method, was air dried and mixed with starch, and finally processed to obtain a granular formulation with $2x10^9$ spore/g and commercialized in bags with 50 g/ha. Albeit, this product acts efficiently in field, a more detailed study related to the effects of Urfos 44® on spore physiology and presence of contaminants is necessary.

CONCLUSIONS

Our results suggest that the addition of Urfos 44® to *T. asperellum* spore suspension is a suitable, fast and low-cost method to concentrate spores which does not affect its viability. However, the effect of the flocculation agent on other physiological characteristics of *T. asperellum*, as well as, the mechanism by which flocculation is induced (pH, presence of urea, phosphoric acid, etc.), remains to be determined.

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