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Effect of pH on Growth, Spore Production and Spore Viability of Biocontrol Agent *Trichoderma Harzianum* in Submerged Fermentation

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The effects of pH medium on growth, spore production and spore viability of biocontrol agent *Trichoderma harzianum* were investigated in a batch fermenter in the range of 4 to 7. The biomass concentration of this fungus at pH 4, 6 and uncontrolled pH were higher than at pH 5 and 7 after 18 h incubation. The spore produced by the fungus at 6, 7 and uncontrolled pH was higher than others. The highest level of spore production (6.67×10^7 spores/ml) occurred at uncontrolled pH, but the spore produced in the culture grown at 7 has a higher viability (52.5%) compared to those grown at other pH.

Keywords: *Trichoderma harzianum*, Biocontrol agent, Biofungicide, Spore production, Spore viability, Effect of pH

INTRODUCTION

Diseases caused by fungi are a major problem in agriculture and can substantially reduce crop yield. The common approach used by farmers for crop treatment to control fungal infections is by spraying fields with fungicides. Alternatives for these often toxic chemical, potentially harmful to farmers and the environment, are biofungicides.

Biofungicides are based on living microorganisms that attack and kill pathogenic fungus. These biofungicides attack fungi through a variety of biological pathways and considered by regulatory agencies to be intrinsically-safe to the environment. Some advantages of using biofungicides are: (1) they help reduce the use of chemical-based fungicides and the risk of developing pathogen resistant to traditional chemicals. (2) in most cases they are safer to use. (3) they tend to be more stable than chemical pesticides if stored properly. (4) in most cases they have lower re-entry interval (R.E.I.) times, and (5) they are less phytotoxic (Nameth, 2001).

Most reports in the literature (Papavizas and Lewis, 1987; Sivan and Chet, 1987; Elad *et al.*, 1980) indicate that *Trichoderma harzianum* is an effective biological control agent in reducing disease caused by soil-borne fungal pathogens. To mass produce this fungus commercially for use as a biofungicide, the studies related to nutritional and operating conditions are needed. As a living organism, the bioprotectant fungus must be well adapted to commercial production to yield a large quantity of biomass. It also must survive well in each processing step, such as harvesting, desiccation, storage, and delivery. One of the criteria of a high quality biofungicide is its viability. It is imperative that most of the propagules in the biomass of a bioprotectant to be used for biocontrol be viable and able to germinate rapidly.

High viability is also related to economics because if only a low percentage of the propagules are viable, then most of the bioprotectan is wasted (Harman *et al.*, 1991).

Some studies have been done by several authors (Jackson *et al.*, 1991 (a); Jackson *et al.*, 1991 (b); Lewis and Papavizas, 1983) on the types of media and nutritional environment such as C/N ratio and substrate level on the growth, spore production and spore viability, mostly in shake flasks. However, studies on operating conditions is little known. One of the important operating parameters in liquid fermentation is pH and its influence on spore production by *Trichoderma spp* have been studied by very few authors. Hence, studies on the effect of pH on spore production and spore viability of biocontrol agent *Trichoderma harzianum* is still very limited.

This research is directed towards optimizing the operating conditions for maximizing the spore production with high viability in submerged fermentation. The effect of various medium pH on the growth, spore production and spore viability of *Trichoderma harzianum* UPM 29 have been studied using a laboratory-scale fermenter.

MATERIALS AND METHODS

Organism

The organism used for the biofungicide production was a locally isolated *Trichoderma harzianum* (UPM 29). The organism was periodically subcultured on potato dextrose agar (PDA) plates.

Media

A semi-defined glucose-yeast extract basal medium based on Czapek mineral salts medium was used for preparing inoculum and media. It contained: KH_2PO_4, 1.0 g/l; $MgSO_4.7H_2O$, 0.5 g/l; KCl, 0.5 g/l; $FeSO_4.7H_2O$, 0.01 g/l; $ZnSO_4.7H_2O$, 0.01 g/l; $CuSO_4.5H_2O$, 0.005g/l; glucose, 30 g/l and yeast extract, 2.75 g/l.

Inoculum

Aerial spores of *T. harzianum* were removed from 20 days old PDA agar plates by scraping with a spatula and suspended in a sterile 1% (w/v) NaCl solution. This suspension of spores was used to inoculate 200 ml medium in a 500 ml Erlenmeyer flask to give a concentration of 10^6 spores/ml. The flask was incubated for 60 hours in an orbital shaker at 200 rpm and at a temperatures of 30°C. The resulting biomass was used as an inoculum for the fermenter.

Experiment

The experiments were performed in a laboratory-scale fermenter (Biostat B, B. Braun) with a working volume of 4 liters. The cultivation was conducted for 3 days at various pH, 4, 5, 6, 7 and uncontrolled pH, temperature at 30°C, aeration 1 vvm and at an agitation rate of 400 rpm. The pH of the medium was controlled using 4M NaOH or 2M H_2SO_4 during fermentation. For evaluating the effect of uncontrolled medium pH, the initial pH was adjusted to 6. An inoculum of 400 ml volume was used.

Biomass and Spore Quantification

For the estimation of biomass, the harvested medium was filtered through a weighed Whatman filter paper no. 1, and then washed with sodium chloride (1%) solution. The washed biomass was oven dried at 80°C for 48 hours and weighed. Spore production was determined after macerating the fungal biomass in a Waring blender for 2 min at high speed in the growth medium (15 ml) and the mixture filtered through glass wool. Spores were counted using an hemacytometer and spore concentration was determined as described by Pitt and Poole (1981). For the determination of spore production, culture samples were taken after 12, 24, 36, 48, 60, and 72 h of incubation. Residual glucose concentration was determined by the DNS method (Miller, 1959).

Viability Determination

Viability is determined by comparing colony forming units (c.f.u.) with total spores. For viability determination, harvested medium at 48 h incubation are filtered through compacted glass wool. The filtrate is centrifuged at 10,000 rpm for 10 min and supernatant discarded. The resulting spore pellet in each centrifuge tube is resuspended in 1 ml of distilled water and centrifuged again at 12,000 rpm for 10 min, discarding the

supernatant (Agosin *et al.*, 1997). The pellet was removed from each centrifuge tube, spread in a petri dish, dried in a desiccator with silica gel for 3 days (Pedreschi and Aguilera, 1997). Dry preparation were used to examine the total numbers of conidia and colony forming units (cfu). The number of conidia were counted directly in a hemacytometer. The cfu numbers were determined by plating serial dilutions of various conidial preparations onto potato dextrose agar amended with 1% (v/v) Igepal to limit colony diameter. Prior to enumeration or plating, dry conidial preparations were soaked in sterile distilled water for 2 h and then ground in a Waring blender at full speed for 3 min (Jin *et al.*, 1991). Samples were taken at 48 h incubation.

RESULTS AND DISCUSSION

The growth

The growth profile of *Trichoderma harzianum* at different pH is shown in Fig. 1. The biomass concentration of all cultures increased within 12 h incubation. The biomass concentration of cultures grown in medium at pH 5 or 7 decreased after 18 h incubation while the cultures grown at pH 4, 6 or uncontrolled pH continuously increased over time until 48 h incubation and then reached stationary phase. In general, in the range of 24 to 48 h incubation, the biomass produced by the fungus increased as pH was decreased from 7 to 4, excepted at pH 5. The change of pH values in medium with uncontrolled pH during fermentation was in the range of 5.85 to 6.14.

The residual glucose concentration in the broth during incubation is shown in Fig. 2. The uptake profile of glucose for all cultures are similar. However, glucose in all media was not completely utilized. Final residual glucose content in media is around 9 g/l.

Spore Production and viability

The spore production profiles are shown in Fig. 3. The increases in biomass concentration, did not support higher spore production yields (see Fig. 1 and 3). This suggests that some inhibitory compounds could be produced together with sporulation (Bodo *et al.*, 1985).

High levels of spore were produced by *Trichoderma harzianum* in cultures at pH 6, 7 and

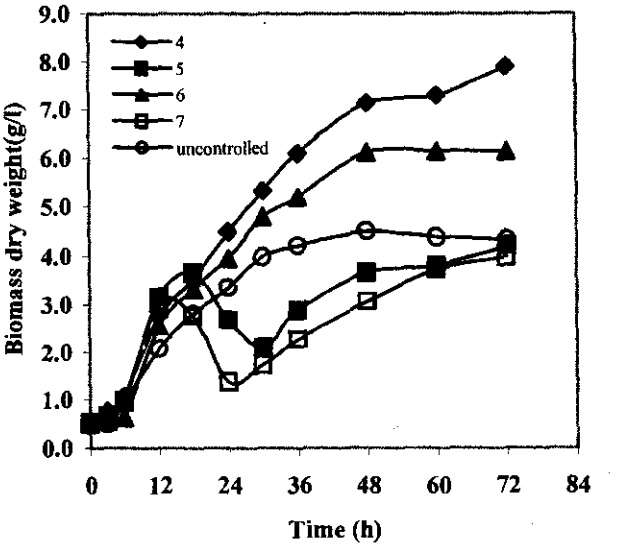

Figure 1. Growth profile of Trichoderma harzianum at various pH

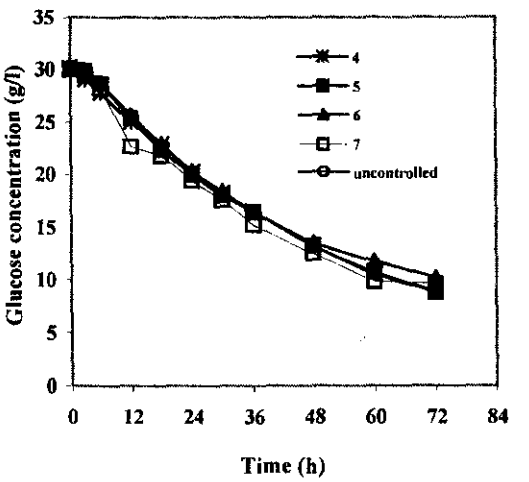

Figure 2. The residual glucose concentration during fermentation

uncontrolled pH within 24 h of fermentation and reached their maximum level in 36 h of fermentation. At pH 6 and uncontrolled pH the spore concentration in the broth decreased after 36 h, but for pH 7 it is nearly constant. At pH 5 the production of spore is low and at pH 4 it is nearly zero. The highest maximum yield of spore (6.67×10^7 spores/ml) occurred at uncontrolled pH. Agosin *et al.* (1997) found that the conidia produced by *Trichoderma harzianum* was higher at pH 7 than at pH 4.

The decrease in spore level after 36 h fermentation in cultures grown at pH 6 or uncontrolled pH, and at pH 7 the level remaining nearly constant, could probably be explained as

mentioned by Agosin *et al.* (1997). They stated that pH appeared to be a key parameter to manipulate for both growth and sporulation. The most striking structural feature of spores produced at low pH, was the absence of both an outer wall layer and pigmentation. Spore walls in *T. harzianum* are formed by two layers, the outer being notably more electron-dense than the inner. The outer layer is the spore's first barrier and therefore the first possible defense against adverse conditions. Electron-dense material, present in electron dense bodies (EDB), disappeared over time, concomitantly with a decrease in spore viability and shelf-life. While EDB degraded, the number of mithocondria increased. These facts indicate that, with time, spores would assume a germination-like state. Activated spores of *T. harzianum* would be more susceptible to changes in environmental conditions (Agosin *et al.*, 1997).

The pH of the medium affected dessication tolerance of spores of *Trichoderma harzianum* The germinating percentage (viability) at 6, 7 and uncontrolled pH was found to be 40.7, 52.5 and 39.92% respectively. The germination percentage of spore was higher in biomass produced at pH 7 than in biomass produced at pH 6 or uncontrolled pH. In comparison, Agosin *et al.* (1997) found that the percentage of viable spore after drying for seven days at 25°C and 75% relative humidity was higher in biomass produced at pH 7 than in biomass produced at pH 4.

Linoleic acid was found to be a major membranes component of *T. harzianum* spores. The decrease in its content with cultivation time, and especially in spores produced under acidic conditions, would imply that deterioration or senescent processes will readily occur (Agosin *et al.*, 1997). During dehydration there is an inherent loss of viability which has been ascribed to alteration in DNA or RNA and intracellular proteins, but it is now generally agreed that damage occurs first in the cell membrane (Pedreschi and Aguilera, 1997). Accumulation of trehalose in spores of *T. harzianum* was correlated with desiccation tolerance (Harman *et al.*, 1991; Jin *et al.*, 1991). Trehalose is responsible for stabilizing membranes of cells during desiccation (Jin *et al.*, 1996). High viability is correlated with trehalose content (Pedreschi *et al.*, 1997).

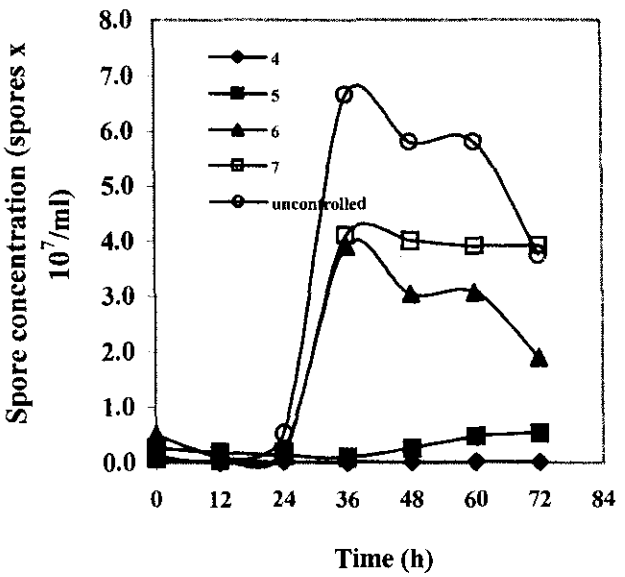

Figure 3. Effect of time and pH on spore production of Trichoderma harzianum

CONCLUSIONS

One of the commercial condition in order the harvested fungal biomass could be used for biological control of plant pathogens is to have high spore viability. This study shows that viability of *T. harzianum* spores can be manipulated by culture pH. Results obtained in this study demonstrate that the fungus grown in medium at pH 7 produced high spore level and with higher viability than at other pH.

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