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Spore Production by Biocontrol Agent *Trichoderma Harzianum* in Submerged Fermentation: Effect of Agitation and Aeration

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Abstract

Effects of agitation and aeration on spore production and spore viability by biocontrol agent UPM 29 were investigated. The optimum fermentation at different agitation speeds, ranging from 300 rpm to 700 rpm show that there is an optimum agitation speed gives the highest spore production. The spore production increased with increasing agitation speed from 300 rpm to 500 rpm. At the higher agitation speed the spore production dropped. The highest spore concentration ($9.2x10^7$ spores/ml) was produced in culture grown on media with agitation speed of 500 rpm. The rate of aeration effected spore production and spore productivity in the range 1.5 to 2.0 vvm, and increased the spore production from $4.7x10^7$ to $6.0x10^7$ spores/ml (28% increase) and $4.7x10^8$ to $8.1\ x10^8$ spores /l/h respectively. The spore viability was not affected significantly by aeration rate or agitation speed.

Keywords: agitation, biocontrol agent, biofungicide, spore viability, *Trichoderma harzianum*

1. Introduction

Biofungicide is an alternative to substitute or to replace the use of chemical fungicide which has potential for decreasing the environmental quality. Most reports indicate that propagules biomass of biocontrol agent *Trichoderma harzianum* is an effective biofungicide in reducing diseases caused by soil-borne fungal pathogens (Elad *et al*., 1980b; Lewis and Papavizas, 1984; Papavizas and Lewis, 1987; Sivan and Chet, 1987; Jackson *et al*., 1991). They have been applied to either soil or plants in the form of mycelial or conidial biomass (Lewis and Papavizas, 1985; Papavizas and Lewis, 1989; Nelson *et al*., 1988; Inbar *et al*., 1996). Conidium (spore) is more preferred for it can be produced abundantly in liquid fermentation (Harman *et al.* 1991) and its desiccation tolerant (Jin, *et al*., 1996).

As in nature, most fungi form spore in an aerial environment (Jackson, 1997), so that to mass produce spores in submerge fermentation the agitation and aeration are important to supply oxygen. In an aerobic submerged fermentations the oxygen distribution and concentration in culture is critical thing. A few numbers of works in the literature state effect of agitation or aeration in spore production. Jackson *et al*., (1991a) described that biocontrol agent *Trichoderma* sp. produced more spore in the shake flask compare with static culture. Felse and Panda (2000), reported that extensive sporulation of *Trichoderma harzianum* observed in the culture with higher speed agitation.

Although increased agitation may provide increased mixing and mass transfer, it may also have many negative effects such a rupture of cells, change in morphological state, decreasing in productivity, vacuolation and autolysis (Smith and Lilly, 1990, Cui *et al*., 1997). Hence, each of fermentation has its own optimum agitation speed. This agitation speed will depend upon the resistance of the organism to shear, its morphological state, the nutrient composition, pH and many other conditions (Smith and Lilly, 1990). As there is insufficient information in literature, studying on the effect of agitation speed and dissolved oxygen concentration on spore production and spore viability becomes very important.

In the present work, the effect of agitation speed and aeration rate on the growth, spore production, and spore viability of local biocontol agent *Trichoderma harzianum* UPM 29 in submerged fermentation were studied.

2. Methodology

2.1 Microorganisms

The organism used for biofungicide production was local biocontrol agent *Trichoderma harzianum* (UPM 29). This organism was isolated from rhizosphere of oil

palm in Malaysia and it is a biocontrol agent against *Ganoderma boninense*. The organism was periodically (once in a month) subcultured on potato dextrose agar plates.

2.2 Media

Media for inoculums and fermentation in bioreactor consisted of defined basal salts medium, glucose, 30 g/l and yeast extract, 2.8 g/l. The defined basal salts medium (based on Czapek mineral) was composed of 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. Yeast was chosen over inorganic nitrogen source as the former is known to serve as an excellent N-source for spore production of *Trichoderma* (Papavizas *et al*., 1984; Jackson, *et al*., 1991).

2.3 Inoculum

Aerial spores of *T. harzianum* were removed from 20 days old potato dextrose agar plates (Pedreschi *et al*., 1997) by scraping with a spatula and suspending in 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 (Pedreschi *et al*., 1997). The flask was incubated for 60 hours (Pedreschi *et al*., 1997) in an orbital shaker at 200 rpm and 30°C. The resulting biomass (which concentration of 5 g/l) was used as an inoculum for the bioreactor.

2.4 Cultivation Conditions in the Bioreactor

Biofungicide cultivation experiments were performed in a 5L working volume bioreactor (Biostat B, B. Braun) with diameter of 160 mm. This bioreactor was completely equipped with the following: four baffles; two Rushton turbines impellers with diameter of 64 mm; gas supply pipe with ring sparger; harvest pipe; exhaust cooler; temperature probe; pH electrode; pO_2 electrode and antifoam probe. The bioreactor was also equipped with a digital measurement and control system. Standard measurement and control functions were for temperature, pH, antifoam and level. Both pO_2 and pH electrodes were calibrated before proceeding with the fermentation. The pO_2 electrode was calibrated after mounting into the culture vessel and after sterilization of the equipment. For the calibration of the electrode for zero pO_2, the culture solution was gassed with nitrogen to remove any oxygen dissolved in the solution. Air was required for saturation of the culture solution and to set pO_2 reading of the electrode to 100%. The pH electrode was calibrated before mounting into the culture vessel, that is prior to the sterilization. The pH of the medium was adjusted by using 2M NaOH or 1M H_2SO_4 during fermentation. The uncontrolled dissolved oxygen concentration was measured during fermentation. An inoculum of 400 ml volume was added into 3600 ml volume of sterilized culture medium. This addition made total working volume of the culture in bioreactor to 4 L. The cultivation was carried out for 3 days. The experimental run was then repeated to verify the data.

To study the effect of agitation speed, experiments were conducted at 30°C; medium pH of 7; aeration 1vvm; and at various agitation speeds: 300, 400, 500, 600, and 700 rpm. Effect of aeration was studied at 30°C; medium pH of 7; agitation speed of 300 rpm and at three different aeration rates: 1, 1.5, and 2 vvm.

2.5 Measurement of Biomass Dry Weight

In preliminary experiments, it was found that the concentration of biomass (in pellet formation) in the bioreactor was homogenous and hence 20 ml of the culture sample for determination of biomass weight was considered sufficient. The cultures were filtered through a preweighed 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. The culture samples were taken every 6 h from 0 h to 36 h of incubation, but from 36 h to 72 h of incubation, the samples were taken every 12 h of the fermentation period.

2.6 Spore Quantification

Spore production was determined after macerating the fungal biomass in a Waring blender for 2 min at high speed in the growth medium (10 ml) and the mixture filtered through glass wool (Pitt and Poole, 1981). Flask containing spore suspension was shaken on a vortex mixer for 1 minute. Three 1-ml aliquots were diluted to 5 ml separately, and samples of these dilutions were shaken again on a vortex mixer for 1 minute. They were then placed on a hemacytometer and the number of spores was counted in 25 squares for each diluted sample (Aube and Gagnon, 1969). The spore concentration was

determined as millions of spores per ml of sample (Pitt and Poole, 1981). Culture samples were taken after 12, 24, 36, 48, 60, and 72 h of incubation for spore quantification.

2.7 Viability Determination

Viability was determined by comparing the colony forming units (c.f.u.) with total spores. For viability determination, harvested medium was filtered through a compacted glass wool. The filtrate was centrifuged at 10,000 rpm for 10 min and the supernatant was discarded. The resulting spore pellet in each centrifuge tube was resuspended in 5 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 preparations were used to examine the total number of conidia and colony forming units (cfu). The number of spores was counted directly in an hemacytometer. The cfu numbers were determined by plating serial dilutions of various conidial preparations onto potato dextrose agar amended with 0.1% (v/v) Igepal (CA-630 SIGMA) to limit colony diameter (Norton and Harman, 1985). The germination percentage, as a parameter of desiccation tolerance of conidia in dry preparation, was determined by comparing colony forming units (cfu) with total spores (Agosin *et al*., 1997). Prior to enumeration or plating, dry conidial preparations were soaked in sterile distilled water for 2 h and then ground in a blender at full speed for 3 min.

3. Results and Discussion

3.1 Effect of Aeration Rate

The growth profiles of biocontrol agent *Trichoderma harzianum* in cultures grown in media with various aeration rates during fermentation are shown in Figure 1. There is no significant difference on biomass production among the cultures grown in this range of aeration rate. The biomass concentration of all cultures increased continuously over time from 3 h until the end of incubation at 72 h. The increasing of aeration rate did not affect significantly the maximum growth rate. This is probably due to dissolved oxygen concentration in culture medium was above the critical oxygen concentration.

According to Shuler and Kargi (1992), when dissolved oxygen concentration in the medium is above the critical level, the growth rate becomes independent of dissolved oxygen concentration. The critical oxygen concentration is about 10% to 50% of the saturated dissolved oxygen concentration for mold cultures, depending on the pellet size of the molds (Shuler and Kargi, 1992). The minimum dissolved oxygen concentration in each culture grown in media at 1.0, 1.5 and 2.0 vvm were around 20%, 50% and 60% respectively.

3.2 Effect of Aeration on Spore Production

The effect of aeration rate on spore production is shown in Figure 1. Increasing aeration rate from 1.0 to 1.5 vvm did not affect spore production, but further increase of aeration rate to 2.0 vvm increased spore production from $4.7x10^7$ to $6.0x10^7$ spores/ml (28% increase). Increasing aeration rate above 1 vvm resulted in decreased time necessary to reach the maximum spore production from 48 h to 36 h. Increasing aeration rate from 1.0 to 2.0 vvm caused minimum dissolved oxygen concentration in medium to rise by three-fold. Dissolved oxygen concentration affecting the spore production was also reported by Pitt and Poole, (1981). They found that between dissolved oxygen concentration of 2% and 20%, 鞴here was approximately a five-fold increase in the sporulation index of *Penicillium notatum*.

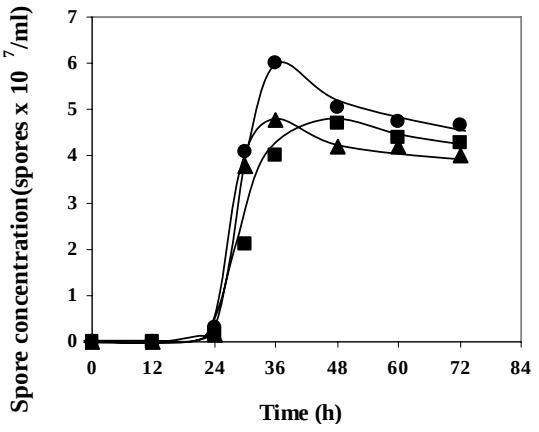

Figure 1. Spore production of biocontrol agent *trichoderma harzianum* in cultures with different aeration rates (■ - 1 vvm, ▲- 1,5 vvm, ● – 2 vvm)

3.3 Effect of Agitation Speed on Spore Production

The effect of agitation speed on spore production is shown in Figure 2. The spore production increased with increasing agitation speed from 300 to 500 rpm. Further increase in spore production was not observed at higher speeds. The effect of agitation speed on spore production was also observed by Felse and Panda (2000).

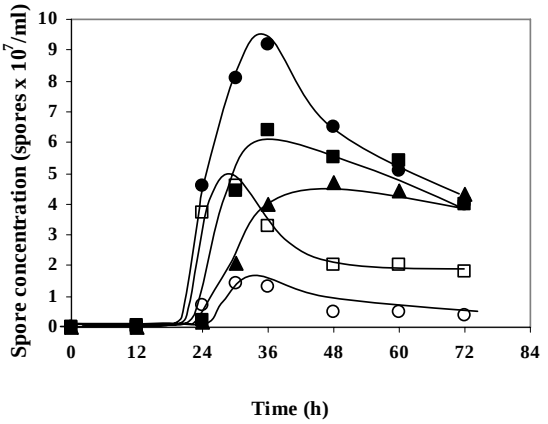

Figure 2. Spore production of biocontrol agent *Trichoderma harzianum* in cultures with different agitation speeds (▲-300, ■ -400, ● -500, □ -600, o -700)

They reported extensive sporulation of *T. harzianum* in culture grown at 300 rpm, at 72 h of fermentation, while in culture grown at 224 rpm a very few spores were produced after 96 h and no spores were observed in culture grown at 150 rpm even after 120 h of fermentation.

Figure 3 shows the profile of maximum spores produced in cultures grown at various agitation speeds. At lower agitation speed (300 rpm), spore concentration attained was only 4.7×10^7 spores/ml due to oxygen transfer limitation. Nearly the same concentration (4.6×10^7 spores/ml) was attained at 600 rpm, which was probably due to excessive shear rate. The highest production of spores (9.2×10^7 spores/ml) was in the culture grown at an agitation speed of 500 rpm.

The time needed to reach maximum spore production was also affected by agitation speed. The maximum spore production was reached at 48 h in culture with agitation speed of 300 rpm, and 36 hours of cultivation was needed in cultures with agitation speeds of 400 and 500 rpm, while it was 30 h in cultures with an agitation speed of 600 and 700 rpm.

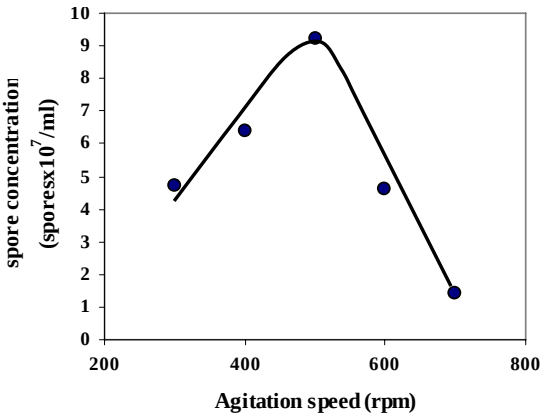

Figure 3. Effect of agitation speed on maximum spore production by biocontrol agent *Trichoderma harzianum*

The drop in spore production at an agitation speed higher than 500 rpm could be explained in terms of increasing mycelium damage. The dissolved oxygen (DO) concentration in culture medium affected the spore production. Increasing agitation speed resulted in increased oxygen transfer rate which in turn resulted in increasing dissolved oxygen level in the medium. However increasing agitation also increased the disruption or damage of cell in culture medium and this caused spore production to decrease. So the optimal speed (500 rpm) created a balance between the constructive effects viz. increased mixing and mass transfer and the destructive effects, viz. fragmentation and shear damage brought about by increased agitation speeds. Effect of agitation speed on productivity has been discussed by some researchers. Musilcova, *et al*. (1981) observed that citric acid production by *Aspergillus niger* depended on the mixer speed. The low productivity in the range of low mixer speeds was attributed to the shortage of dissolved oxygen. The maximum production was reached at 600 rpm. They suggested the drop in the production at higher speeds is due to damage of the mycelium. Makagiansar *et al*. (1993) observed decrease in the specific penicillin production rate with increase in impeller speed. They concluded that the low production rate at higher agitation intensities was due to severe fragmentation.

3.4 Effect of Aeration Rate and Agitation Speed on Spore Viability

The viability percentage of biocontrol agent *T. harzianum* spores produced at various aeration rates and agitation speeds is shown in Table 1 and Table 2. The data shows that

changing aeration rate or agitation speed did not significantly affect the spore viability.

Table 1. Spore viability at various aeration rate

	Aeration rate (vvm) (300 rpm)		
	1.0	1.5	2.0
% viability	48.0	49.0	48.5

Table 2. Spore viability at various aeration rate

	Agitation speed (rpm) (1.0 vvm)				
	300	400	500	600	700
% viability	48.0	53.0	51.2	50.0	49.5

4. Conclusion

Aeration rate did not affect significantly the maximum biomass dry weight reached and spore viability. But it affected spore production and spore productivity in the range 1.5 to 2.0 vvm, and increased the spore production from $4.7x10^7$ to $6.0x10^7$ spores/ml (28% increase) and $4.7x10^8$ to $8.1\ x10^8$ spores /l/ h, respectively.

The growth of *Trichoderma harzianum* was affected by the agitation speed. The biomass dry weight decreased with increasing the agitation speed from 300 to 700 rpm. It is attributed to the increasing the number of wounds when the speed of agitation was increased. The maximum spore production reached increased with increasing agitation speed from 300 to 500 rpm, but further increase in rpm resulted in a decrease. The drop in spore production at an agitation speed higher than 500 rpm could be explained in terms of increasing mycelium damage. The increasing of the agitation speed resulted in increased dissolved oxygen level in the medium. However, increasing the agitation speed also increased the damage to the cells in the culture medium and this caused spore production to decrease. The highest production of spores ($9.2x10^7$ spores /ml) and spore productivity ($13.1\ x\ 10^8$ spores/l/h) was obtained in the culture grown at an agitation speed of 500 rpm. The spore viability was not affected significantly by aeration rate or agitation speed.

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