Jurnal Rekayasa Kimia dan Lingkungan
Vol. 6, No. 1, hal. 35-40, 2007
ISSN 1412-5064

Spore Production of Biocontrol Agent *Trichoderma Harzianum*: Effect of C/N ratio and Glucose Concentration

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Abstract

Effects of medium condition on spore production and spore viability of *Trichoderma harzianum* UPM 29 isolated from oil palm rhizosphere were studied. The carbon to nitrogen (CN) ratio and glucose concentration have significant effect on spore production and spore viability of the fungus. Highest spore production ($1.6x10^8$spores/ml) was obtained in a medium containing 30 g/l glucose with a CN ratio of 24. The highest spore viability (52.5%) was produced in the culture grown on media with glucose concentration of 30 g/l and with a C/N ratio of 44.

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

1. Introduction

The effort for isolating and developing the indigenous biocontol agents against various soil-borne plant pathogenic fungi continually conducted in several countries for alternative in reducing application of chemical pesticide which cause decrease of environment quality standard. There are some advantageous to apply indigenous biocontrol agents for solving local plant disease problems, such as naturally, they are available in the local plant rhizospheres, there is no obstacle for climate change and it gives opportunity for domestic income. In order to commercialize these biocontrol agents for biofungicide, suitable cultural methods and fermentation production systems should be developed, and also the optimal conditions for spore production should be determined.

Several authors (Lewis and Papavizas, 1983; Harman *et al*., 1991; Agosin *et al*., 1997) have studied the effects of environmental factors such as carbon and nitrogen sources, carbon to nitrogen ratio (C/N ratio), pH, and temperature on spore production and spore viability of some strains of *Trichoderma harzianum*. But for new or local strains such effects should be studied again, because each strain has its own characteristic responses for these factors, besides that, insufficient information is available on the influence of culture conditions on the quantity of spore produced and spore viability for industrial purposes. Our present work was focused on the effects of C/N ratio and glucose concentration on spore production and spore viability of biocontrol agent *Trichoderma harzianum* UPM 29 in submerged fermentation.

2. Methodology

2.1 Microorganisms

The organism used for biofungicide production was biocontrol agent *Trichoderma harzianum* (UPM 29). The organism was periodically (once in a month) subcultured on potato dextrose agar plates.

2.2 Media

Media for inoculum 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^{o} C. The resulting biomass (which concentration 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. 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. 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.

2.5 Glucose Concentration and CN Ratio Studies

To evaluate the effect of initial glucose concentration on spore production and spore viability, a series of experiments were carried out at different initial glucose levels of 10, 30 and 50 g/l, where initial CN ratio was maintained 44. The effect of initial CN ratio was studied at 24, 44 and 64 where glucose concentration was maintained at a constant level of 30 g/l. Carbon concentration and CN ratios in medium were calculated using the values, carbon in glucose is 40 % and carbon and nitrogen in yeast extract is 70% and 11% respectively. The cultivation was conducted at temperature 30^{o}C, agitation speed 400 rpm, aeration 1.0 vvm and medium pH of 7.

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 suspensions were 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 conidia 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 Waring blender at full speed for 3 min. Samples for viability tests were taken at maximum spore production time.

3. Results and Discussion

3.1 Effect of Initial CN Ratio on Spore Production

Initiation of sporulation began during the deceleration phase i.e. after 18 h of incubation (Figure 1). This sporulation was probably induced by the physiological

nutrient limitation as a result of the reduction in the yeast-extract transport from the medium to the pellet. Although the residual yeast-extract in all cultures grown was still significant after 18 h of incubation (2.0, 0.9, and 0.7 g/l respectively, its consumption by cultures slowed considerably or nearly stopped around this time. This nature of sporulation was also observed by Morton (1961). According to him, the most general condition for induction of sporulation is the reduction or exhaustion of assimilable nitrogen while carbohydrate is still available. Andrew and Harris (1997), explained that sporulation initiated response to nutrient limitation involves reorganization of endogenous resource as well as the use of exogenous substrate.

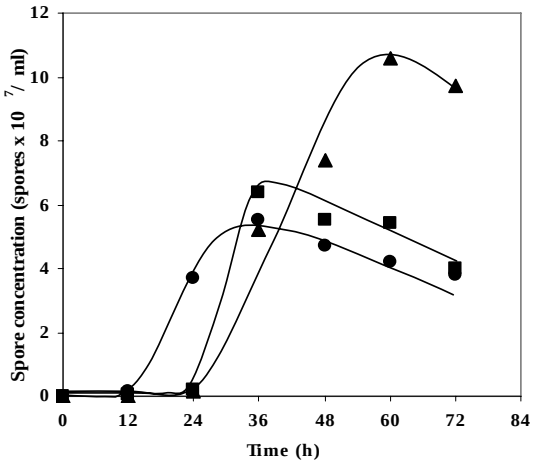

Figure 1. Spore production of biocontrol agent *trichoderma harzianum* in media with different CN ratio (▲-24, ■ -44, ● -64)

Figure 1 shows that the spore concentration increased over time until it reached a maximum value. In cultures with initial CN ratio of 24 and 44 the increase in spore concentration began after 24 h of incubation but in the culture with initial CN ratio of 64, it began after 18 h of incubation itself. Lower the CN ratio in culture media, longer the incubation time required to reach maximum spore concentration. In cultures with an initial CN ratio of 44 and 64 the spore concentration increased until it reached a maximum value at 36 h of incubation and then it began to decrease. In the culture with an initial CN ratio of 24, spore concentration continued to increase until 60 h of incubation time and then decrease with time.

The decrease in spore concentration with time after reaching its maximum value was also observed by Frey and Magan (2001) in biocontrol agent *Ulocladium atrum* cultures grown in liquid oatmeal extract broth medium. The decrease in spore level could probably be explained as hypothesized by Agosin *et al*. (1997). They noted, the linoleic acid is one of the most important phospholipids components in *T. harzianum* spores and this component is affected by the culture conditions. The decrease in its content with cultivation time would imply that deterioration or senescent processes will readily occur.

The decrease in spore production by biocontrol agent *Trichoderma harzianum* UPM 29 is related to the decrease in biomass dry weight concentration in culture with increasing initial CN ratio. Figure 2 shows the correlation between maximum spore and biomass concentration, where maximum spore concentration reached increased with increase in maximum biomass dry weight in the stationary phase.

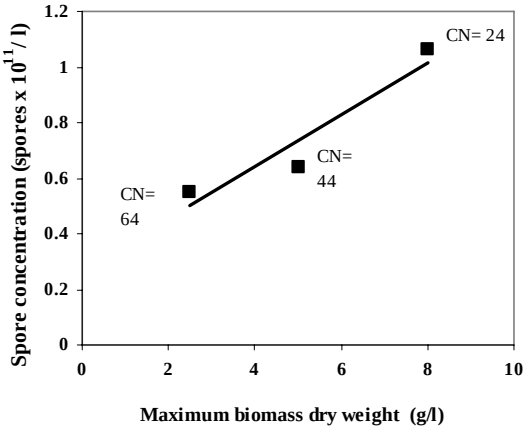

Figure 2. Correlation between maximum spore consentration and maximum biomass dry weight produced in cultures grown in media with CN ratio of 24, 44 and 64

3.2 Effect of Initial Glucose Concentration on Spore Production

Spore production was significantly affected by glucose level (Figure 3). In cultures with initial glucose concentration of 30 and 50 g/l, spore production increased after 24 hours of incubation but in culture with an initial glucose concentration of 10 g/l, it started just after 12 hours. In all cultures, spore concentration increased with time. It reached the maximum value at 36 h and then decreased for culture with an initial glucose concentration of 10 g/l and 30 g/l. For the culture with an initial glucose concentration of 50 g/l, it reached the maximum value at 48 h of incubation and then decreased. The reason for the decrease in spore concen-

tration with time after reaching the maximum value has already been discussed previously.

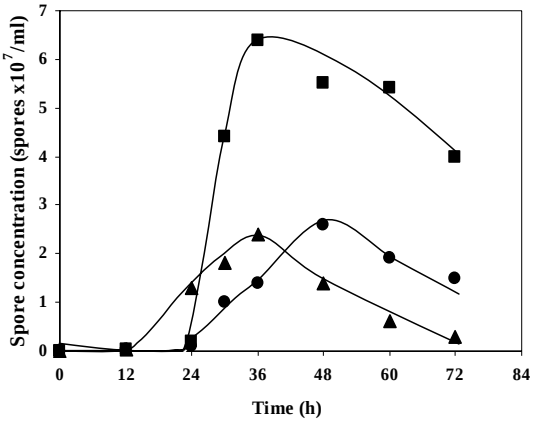

Figure 3. Spore production of biocontrol agent trichoderma harzianum in cultures with different initial glucose level (▲-10 g/l, ■ -30 g/l, • -50 g/l)

In these experiments, the yeast-extract content in each culture media depended on the glucose level because the media were maintained at a constant CN ratio. As expected higher the yeast-extract content, longer the incubation time required to reach maximum spore yield. The latter is related to time required to fully consume yeast-extract in the culture media.

In general, there was an increasing tendency for spore production with increase in glucose level in the range of 10 to 30 g/l. The highest spore concentration ($6.4x10^7$/ml) was obtained in culture grown in the medium with a glucose level of 30 g/l. Yu et al., (1998) also showed similar nature of glucose effect on spore production. They noted that spore concentration of biocontrol agent *Colletotrichum cocodes* increased with the increase in carbon concentration from 5 to 20 g/l at a constant CN ratio. Jackson and Bothast (1990) also studied the influence of glucose concentration at a constant CN ratio of 5 on biocontrol agent *Colletotrichum trucantum* spore production. Their results showed that spore production in 5-day old cultures increased with increasing glucose concentration in the range of 10 to 20 g/l. They also observed that spore production decreased with further increase in glucose level to 30 and 40 g/l. At a glucose level of 50 g/l or higher, they observed, spores were not production. Based on our and other researchers observations, we can say that the lower spore concentration reached in medium with a glucose concentration of 50 g/l is perhaps due to inhibition of spore formation at higher glucose levels.

The amount of spore in cultures depended on the biomass dry weight concentration. Figure 4 shows the correlation between maximum spore concentration and maximum biomass dry weight concentration for each culture grown in media with glucose level of 10, 30 and 50 g/l. The maximum spore concentration reached increased with increase in maximum biomass dry weight reached for initial glucose concentration of 10 and 30 g/l but decreased for the glucose level of 50 g/l. The decrease in spore production at this level is related to inhibition of spore production at high glucose levels.

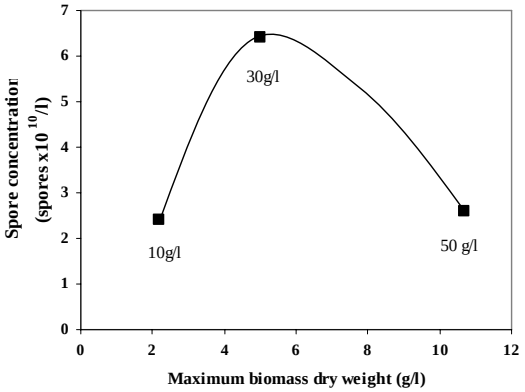

Figure 4. Correlation between maximum spore consentration and maximum biomass dry weight produced in cultures grown in media with CN ratio of 24, 44 and 64

3.3 Effect of Initial CN Ratio on Spore Viability

It can be seen from Table 1 that initial CN ratio did not significantly affect the desiccation tolerance of spores of *Trichoderma harzianum*. The germinating percentage of spores was slightly higher but not greatly significant in the biomass produced at an initial CN ratio of 44 and 64 than in biomass produced at an initial CN ratio of 24.

Table 1. Spore viability at various initial C/N ratios.

Initial CN ratio	% viability
24	46.0
44	53.0
64	53.0

The effect of initial CN ratio on spore viability was also observed by Agosin *et al*, (1997). After 7 days of storage at 25°C, they found that the spore produced by biocontrol agent *Trichoderma harzianum* in medium with a CN

ratio of 14 had higher spore viability (64.5%) than the spores produced in a medium with a CN ratio of 3 (0.5%). Spores of other fungi produced in nitrogen-limited cultures had improved survival. It has been observed that the tolerance to heat and desiccation is improved by using media containing excess glucose (McBride and Ensign, 1987). According to Jin *et al*. (1991), accumulation of trehalose in the spores of *Trichoderma harzianum* could be correlated with desiccation tolerance. In view of the above observations, probably the excess glucose contained in the medium with an initial CN ratio of 44 and 64 resulted in increased trehalose content of spores in these cultures, resulting in higher spore viability.

3.4 Effect of Initial Glucose Concentration on Spore Viability

Glucose level also affected the desiccation tolerance of spores of *Trichoderma harzianum*. The percentage germination of spores increased with increasing glucose level Table 2. The effect of initial glucose concentration on viability of biocontrol agent *Trichoderma harzianum* was also observed by Agosin *et al*, (1997). They studied the effect of glucose concentration on viability of the spores in the range of 3 to 48 g/l at a constant CN ratio. They found that there was an increase in viable spores with increasing glucose concentration. The increase in desiccation tolerance or spore viability of *Trichoderma harzianum* has been correlated with accumulation of trehalose in spores (Jin et al. 1991). Probably the increase in glucose concentration in culture medium produces spore with increased trehalose content. McBride and Ensign (1987) also noted that the trehalose content of *Streptomyces griseus* spore was dependent on the glucose concentration in the culture medium and it increased with increasing glucose concentration in the culture media.

Table 2. Spore viability at various glucose concentrations

Glucose concentration (g/l)	% viability
10	10.0
30	53.0
50	56.0

4. Conclusions

Spores produced of biocontrol agent *Trichoderma harzianum* UPM 29 was significantly affected by glucose concentration and CN ratio of the culture media. Biomass increased with increase in initial glucose concentration in the range of 10 to 50 g/l. The same correlation was also observed for the maximum spore concentration reached in the range of 10 to 30 g/l. Biomass and spore concentration decreased with increase in initial CN ratio from 24 to 64. Decreasing of biomass and spores concentration with increasing initial CN ratio is due to different amounts of nitrogen supplied to the culture. At higher CN ratio, less nitrogen is contained in the medium for a given amount of carbon. The CN ratio however did not significantly affect the spore viability, but increasing glucose concentration from 10 to 50 g/l increased spore viability.

Acknowledgement

We thank Prof. Dr. Sariah Meon (Center for Biological Control of Tropical Pest, Universiti Putra Malaysia) for kindly providing the isolates.

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