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Verticillium lecanii spore production in solid-state and liquid-state fermentations

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Abstract *Verticillium lecanii* has been recognized as an entomopathogen with high potential in biological control of pests. Two types of cultivation methods, the solid-state fermentation (SSF) and the liquid-state fermentation (LSF), were examined for *V. lecanii*. In SSF, the substrate types including rice, rice bran, rice husk, and the mixtures of these components were tested. The results showed that both cooked rice with appropriate water addition and rice bran gave significantly higher spore production of 1.5×10^9 spores/g substrate and 1.4×10^9 spores/g substrate, respectively. In LSF, SMAY liquid medium was used as a base, and the effects of environmental conditions on the spore production of *V. lecanii* were investigated. From the time course study, on the 9th day the spore yield reached 1.2×10^9 spores/ml of broth at 24 °C, 150 rpm for this strain. A series of medium volumes in the shaker-flask have been tested for the requirement of aeration. The largest surface aeration test, one tenth of the medium volume in the shaker-flask for cultivation, gave the highest spore count. The optimal pH value was tested and the initial pH 5 in the SMAY medium produced a high spore density. Finally, *V. lecanii* spores from SSF and LSF were different in size, shape, and size distribution; while mean spore length from SSF was 6.1 μm, and mean spore length from LSF was 5.0 μm.

1 Introduction

Verticillium lecanii is an entomopathogenic fungus, which has a broad range of hosts including four large orders of insects: Homoptera [1–4], Coleoptera [5], Orthoptera [6, 7], and Lepidoptera [8]. In addition, it parasites soybean cyst nematode [9] and some plant disease pathogens such as cucumber powdery mildew [10] and chrysanthemum rust fungi [11]. Thus *V. lecanii* is a very effective biological control agent. It could be further used together with other insecticide and fungicide to enhance the effectiveness of pest control [12, 13]. Generally, for control of the pests, by spraying of the fungal spores under a high humidity condition such as in a green house, host mortality could arise to the peak shortly.

The common method used to propagate fungal spore is the solid-state fermentation (SSF) or the liquid-state fermentation (LSF). SSF usually needs lower manufacturing costs by utilizing unprocessed or readily accessible raw materials. Other advantages are low wastewater output and essentially complete product recovery. LSF is usually faster, and is easier to control the parameters of the physical and chemical properties of the system [14].

To increase productivity, generally, several factors are involved: the strain improvement, the improvement of the fermentation medium and fermentation conditions, and even fermentation processes. Growth of each isolate is influenced by different substrates, moisture content of substrate, temperature, pH, and supply of air etc. In the past, techniques for mass culturing fungi have been developed towards harvesting fungal metabolites using submerged liquid fermentation. However, for biological control, infective fungal spores are required. Production of well-known entomopathogenic fungi such as *Beauveria bassiana* and *Metarhizium anisopliae* on the surface phase methods have been developed throughout the years. Most works to produce *V. lecanii* for laboratory bioassay tests or small scale field trials, the fungal spores are propagated in the large petri dishes or flasks filled with solid medium. We used an autoclave bag filled with solid substrate, and with moisture content tested, high yield of spore could be accomplished. This method is convenient and space saving, many autoclave bags could be packed into a single incubator.

Most entomopathogenic Hyphomycetes (*Verticillium* belongs to this category) do not sporulate readily in liquid culture [15]. Literature regarding mass production of *V. lecanii* in submerged liquid medium is limited. However, this isolate strain can grow and produce abundant spores by liquid-state fermentation in our laboratory.

In this study, we looked into the optimal cultivation substrate in solid-state fermentation, and the effect of environmental conditions in liquid-state fermentation on the spore production of a native Taiwan isolate of *V. lecanii*. In addition, quantitative image analysis has been primarily used for cell counting and sizing, and for assessment of cell morphology. *V. lecanii* spores from SSF and from LSF were compared in size distribution. Spore size measurement may contribute to a parameter of spore quality.

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2 Materials and methods

2.1 Microorganism and inoculum

Verticillium lecanii strain Hualien F091 was used. This strain was isolated from aphid by Dr. S.S. Kao of Taiwan Agricultural Chemicals and Toxic Substances Research Institute (TACTRI). The culture was maintained on SMAY (Sabouraud maltose agar with yeast extract) or PDA (potato dextrose agar) slant. The fungus was grown on SMAY agar at 24 °C for 9 days. The spores produced were suspended in 10% glycerol of sterile distilled water, and then divided into 1 ml centrifuge tubes, frozen and stored at −70 °C for long term storage. For immediate inoculation, by pouring 0.1% Tween 80 in sterile distilled water to wash off the spores, and the spore suspension ($\sim 5 \times 10^6$ spore/ml) could be used as seed culture for the experiments.

2.2 Culture media and preparation

2.2.1 Solid-state fermentation (SSF)

Rice (100 g in 90 g water), rice bran (100 g in 100 g water) and rice husk (100 g in 100 g water) were boiled and cooked in a rice-cooker until done (about 20 min.). Then 100 g cooked rice, rice bran, or rice husk was each packed into an autoclave bag. The bag was plugged with a ring and a cheese' cloth covered cotton stopper. To test the effect of the water content in the rice substrate, water of different volume had been added into the cooked rice bag. In the experiments of cultivation using the different substrates and the combinations of those, rice, rice bran, rice husk, and mixtures in several different ratios, e.g. 1:1, 1:1:1 etc were tested. The autoclave bags with the prepared substrate were then autoclaved at 121 °C for 20 min. Each bag was inoculated with the seed culture, and incubated in an incubator at 24 °C for 2 weeks. The spores were harvested by pouring 100 ml of 0.1% Tween 80 in distilled water into the bag and rinsing off the substrate thoroughly, twice. Spore counts were determined by using an improved Neubauer hemocytometer.

2.2.2 Liquid-state fermentation (LSF)

SMAY liquid medium (maltose 40 g, yeast extract 20 g, neopeptone 10 g in 1 liter of water) was chosen as the basic medium to test the effects of the environmental conditions. The same seed culture was inoculated into 50 ml SMAY in 250 ml. Erlenmeyer flask, and fermented at 24 °C, 150 rpm for 7 days. For time course test, every day except the 7th day, 1 ml of broth was taken from the flask to count the spore yield with an improved Neubauer hemocytometer. To examine the effect of surface aeration, different initial volume of medium: 50, 100, 150, 200, and 250 ml in a 500 ml Erlenmeyer flask with a baffle was used. Cell density of inoculation was tested by adding $1\times = 1$ ml (2.5×10^6 spore/ml), 3×, 5×, 7× and 9× of inoculum to make the same volume 50 ml in 250 ml flask. The PH was adjusted with 4N NaOH or 4N HCl to test the effect of initial pH to the sporulation. Autoclave condition was set at 121 °C, 20 min. In this study, 24 °C was used all through the experiments in SSF and LSF. In both SSF and LSF experiments, the data were polled in from three replicates of each experiment, and the spore yield was the mean of six samplings. The vertical bars in the graphs represent the standard deviation (SD) from each triplicate run.

Optimas 6 Image analysis system (Optimas Co., Bothell, Washington, USA) was used for quantitative studies of spore numbers and sizes. With an Olympus bright field light microscope at 400×, each sample of 300 SSF spores and 300 LSF spores were captured and analyzed in the measurement of spore length.

3 Results and discussion

3.1 Effect of moisture of rice substrate in SSF

Rice has been commonly used as the basic substrate for cultivation of fungi in solid-state fermentation. Cooked rice was our first choice. The culture media were prepared on the basis of cooked rice moistened with various amount of distilled water. In detail, distilled water from zero to 110 ml was added to every 100 g of cooked rice by each increment of 20 ml. The results showed that the one with 50 ml water addition gave the highest spore yield of 1.5×10^9/g of substrate (Fig. 1). Although steamed rice has been prepared in growing many other microorganisms, it was proved successful to grow *V. lecanii* strain Hualien F091 on solid cooked rice in a convenient autoclave bag.

The influence of moisture of substrate on the performance in SSF is important; however, to control the moisture during process is difficult. The water availability could change during the course of fermentation as a result of variations in the moisture content. The sources of the variations are evaporation caused by metabolic heat evolution, water consumption for polysaccharide hydrolysis, and water produced by the carbohydrate metabolism. According to our results, substrate with limited moisture

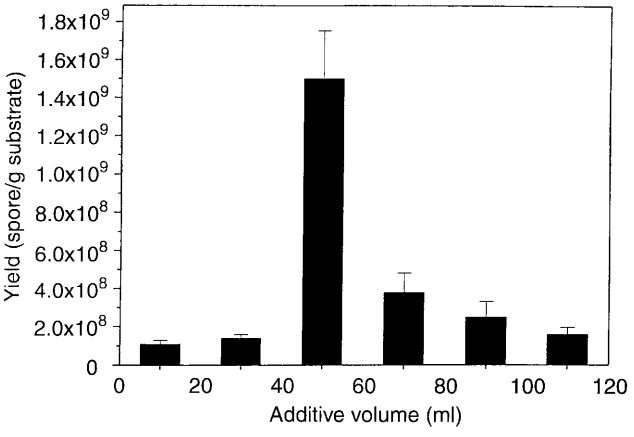

Fig. 1. Effect of substrate moisture on spore production of *V. lecanii* in SSF by using various water additive volumes in rice culture fermented at 24 °C

content (100 g cooked rice with 10 ml water added), and over-supplied moisture in the substrate (100 g cooked rice with added 110 ml water) both showed inferior spore yield, 1.1×10^8 spores, 1.6×10^8 spores per g of substrate, respectively. While the optimal amount of water of 50 ml to 100 g cooked rice had produced 10 fold more spores. Thus, to determine the optimal water portion in a SSF substrate preparation should have the priority among the tests in SSF.

3.2
Effect of mixed substrates in SSF

The use of mixed substrates in SSF was to combine a culture medium rich in nutrient substance and characterized of a suitable physical structure that would have improved gas exchanged in the substrate layer. Preliminary tests were with rice, rice bran, and rice husk mixed in different ratio. The results are demonstrated in Fig. 2. Rice bran alone had the highest spore yield (1.4×10^9 spores/g substrate), and rice/rice bran/rice husk with 1:1:1 ratio also provided a good yield (8.0×10^8 spores/g substrate). Grajek [16] used wheat bran and sugar-beet pulp mixture to cultivate *V. lecanii* in Poland, and found 9 parts of wheat bran and 1 part of sugar-beet pulp produced good spore yield (3.2×10^9 spore per 1 g of the medium dry matter). The spore yield was better than wheat bran alone (3.0×10^9 spore/g medium). Dorta et al. [17] used the rice bran, and rice bran/rice husk mixture (1:1) for producing *Metarhizium anisopliae* spore to evaluate the amount of water released from the fermentors in a SSF process. Their results showed that the mixed media and the more initial dry weight of solids had less water losses. They concluded that the right selection of the initial weight of the solid substrate and the initial water activity could prevent losses of water from the vessels, thus improving the performance of the process. Our results showed rice bran alone had the highest yield, while rice bran/rice husk mixture (1:1) gave 20% less production. Rice bran may have an unique nutrient and texture property suitable for *V. lecanii* F091 strain growth and sporulation.

3.3
Time course of spore production in LSF

Data of *V. lecanii* growth in liquid culture are limited. The results of growing *V. lecanii* in SMAY liquid medium in a shaker-flask at 24 °C, 150 rmp for up to 12 days are shown in Fig. 3. On the 9th day, the spore yield reached 1.2×10^{10} spores/ml of broth. Then the spore production dropped back to 3.5×10^9 spores/ml of broth on the 12th day. Kao et al. [18] propagated the original isolate of this strain at 24 °C on SMAY agar plate. The fungal colony had the highest growth diameter on the 9th day, but no spore yield was shown in their experiments. The growth rate shown on Fig. 3 indicated that this strain sporulated very fast in the LSF, and the exponential phase lasted quite long for 7 days.

3.4
Effect of surface aeration in LSF

Various initial volumes of medium in the shaker-flask provided different surface aeration for the growth and sporulation of the fungus. By varying the volume of liquid culture in the flasks so that the ratio of the surface area of the liquid exposed to air to the liquid volume was varied. Using a set of 500 ml Erlenmeyer flasks with a baffle, the flasks were filled with different amount of medium: 50, 100, 150, 200, 250 ml of SMAY liquid medium. The flasks were shaken in a standard manner at 150 rpm. The results are shown in Fig. 4. Fifty milliliters of medium in 500 ml flask had the highest spore production of 2.1×10^{10}/ml. The results indicated that this strain of *V. lecanii* grew very well in a highly aerated culture condition. The effect of aeration on the growth of fungi is complex. The fast growth of the microorganism, and the condensation of the characteristics of the mycelia network structure caused the viscosity of the medium to increase. The oxygen supply to the interior of the mycelium mass is decreased. In the fermentation suspension, the oxygen transfer had to be enhanced. Diffusion of oxygen from the medium into the mycelium mass is assumed to be the limiting factor for growth of fungal mycelium and sporogenesis.

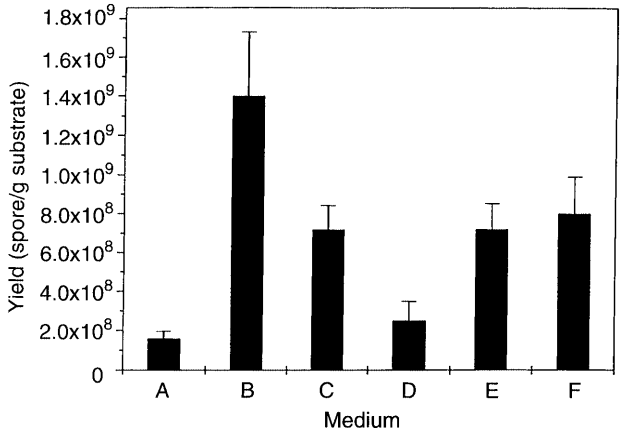

Fig. 2. Effect of substrate components on spore production of *V. lecanii* in SSF: A. rice; B. rice bran; C. rice/rice bran (1:1); D. rice/rice-husk (1:1); E. rice bran/rice husk (1:1); F. rice/rice bran/rice husk (1:1:1) fermented at 24 °C

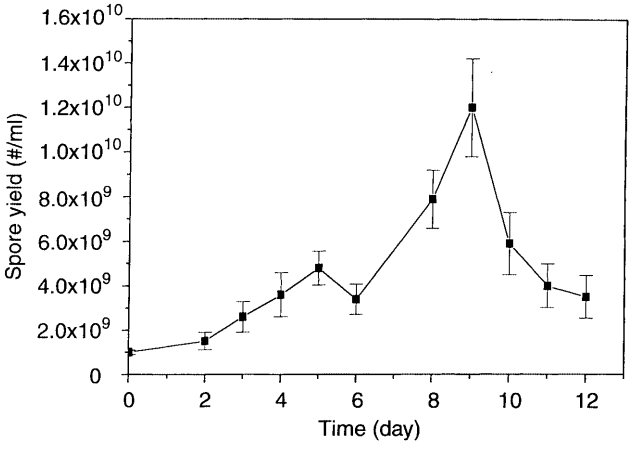

Fig. 3. Time course of spore production of *V. lecanii* in LSF by using liquid SMAY medium in shaker-flask incubated at 24 °C, 150 rpm

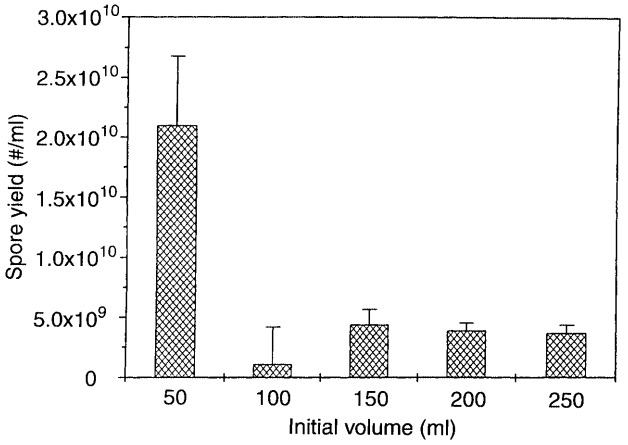

Fig. 4. Effect of initial volume of medium (liquid SMAY) on spore production of *V. lecanii* in LSF by using various volumes of medium in 250 ml shaker flask without baffle incubated at 24 °C, 150 rpm

3.5
Effect of inoculation cell density in LSF

In order to understand cell density in relation to the spore production, various sizes of inoculum were tested. Five levels of inoculum, 1× spores/ml was increased up to 9×, the results are presented in Fig. 5. The spore production was from 1.3×10^9 to 1.8×10^9 spores/ml of broth, the differences were not noticeable. In general, inoculum size on microbial fermentation process is important. An increase in inoculum concentration increases the yield of mycelium and the number of spore produced. On the study of protease production of *Rhizopus oryzae*, Tunga et al. [14] found that the protease production steadily increased with the increasing size of inoculum. However, the inoculum density did not have unlimited effect on fermentation process. If it reached the magnitude the enzyme productivity became maximum, no appreciable change in enzyme activity with high inoculum size could be observed. Later study with 500 dilution of inoculum in our laboratory (results not shown), the spore yield was still in the same range. This strain may not be sensitive to the density of growing population.

3.6
Effect of initial pH in LSF

To most organisms, the standard pH range for growing is from 5 to 7. The optimal initial pH for this native strain was determined in SMAY liquid medium. By adjusting the culture medium pH to 4, 5, 6, 7, and 8 with 4 N HCl or 4 N NaOH, the spore production is shown in Fig. 6. The initial pH 5 of the medium gave the highest spore yield (5.1×10^9 spores/ml of broth). Ekbom [19] used glucose as the carbon source, $(NH_4)_2SO_4$ as the nitrogen source, and phosphate buffered medium at pH 6.3 as the optimal growth medium. The spore production, 5×10^9 spores/ml of broth, was comparable to this study. When ammonium salts were used as the nitrogen source, the pH value decreased further during the mycelium growth because of the process of assimilation of the ammonion ions and the release of acid anions. Thus, the results showed that the sporulation of *V. lecanii* is very much depending on the pH change, it is affected if pH level is higher or lower compared to the optimal value.

3.7
Comparison of SSF and LSF spore sizes

Spore size can be related to some physiological features of the cell. Yeast cells are known to elongate under stress, due to nutrient limitation or inhibition by accumulated by-product. SSF spore shape was most in ellipsoid form, while spores from LSF were varied in shapes from ovoid, oblong to long ellipsoid. Figure 7 panel A, and panel B reveal the size range of *V. lecanii* SSF and LSF spores. Mean spore length of SSF was 6.1 μm, SD = ±0.915, total spore count = 300; mean spore length of LSF was 5.0 μm, SD = ±0.986, total spore count = 300. The size range of SSF spores was from 3.5 μm to about 9.0 μm, while the spores from LSF had much wider range, from 2.5 μm to about 11 μm. According to Jackson et al. [2], small size spores (<3 μm) may be avirulent in some strains of

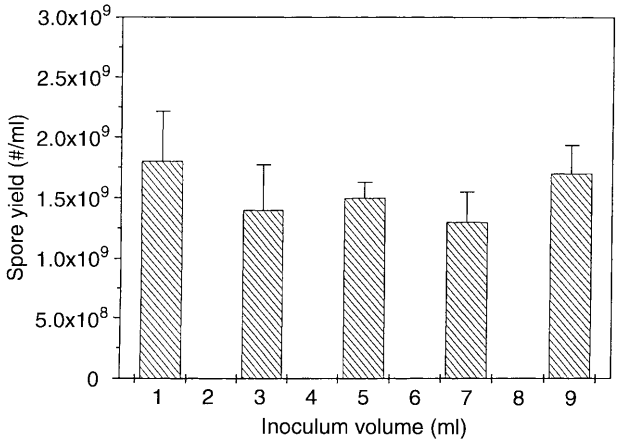

Fig. 5. Effect of inoculation density on spore production of *V. lecanii* in LSF by using cell density from 1× to 9× spore/ml incubated at 24 °C, 150 rpm

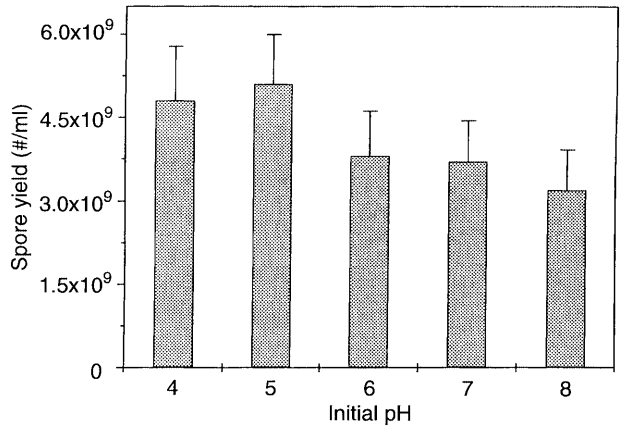

Fig. 6. Effect of initial pH on spore production of *V. lecanii* in LSF by using culture medium adjusted to pH 4–pH 8 incubated at 24 °C, 150 rpm

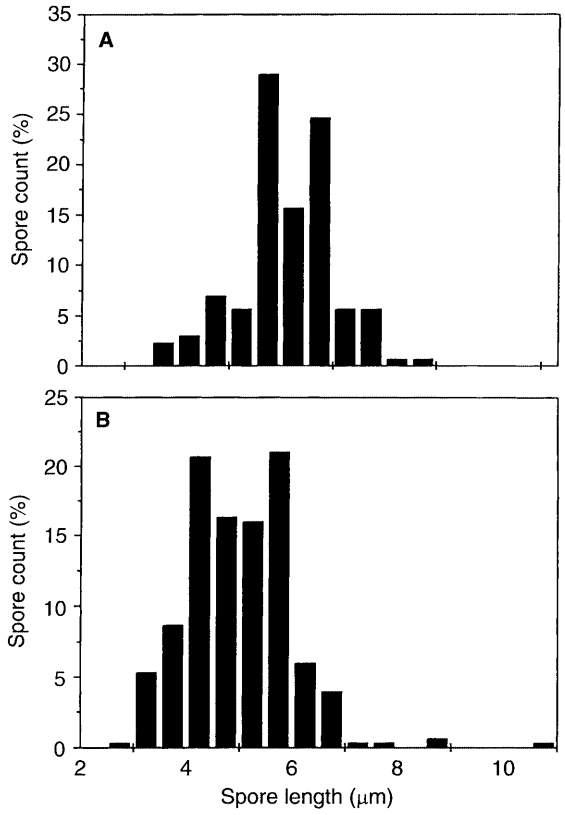

Fig. 7. Size distributions of *V. lecanii* spore length (μm) from SSF (panel A) and LSF (panel B)

V. lecanii. The virulence of the two type spores has to be determined by bioassay tests.

4 Conclusion

As all the microbes, yield and productivity of the fungus *Verticillium lecanii* vary much depending on the strains, substrates, cultivation methods, and conditions. Solid-state fermentation with different substrate composition and different water content in the substrate can be adjusted to produce high yield of spore. By using an autoclave bag filled with cooked rice or rice bran as solid substrate, we have successfully cultivated and produced high spore yield of *V. lecanii* strain Hualien F091. In the liquid-state fermentation, the cultivating conditions such as higher surface aeration (low medium to flask volume, 1:10) and lower initial pH value (pH 5) were demonstrated to produce good yield of spores in this native fungal strain. The spore shape and size distributions indicate the spores produced from SSF and LSF are different morphologically, whether the different types of spores will express the virulence differently remains to be investigated.

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