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Relationship between Agitation Speed and the Morphological Characteristics of *Verticillium lecanii* CS-625 during Spore Production

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Abstract *Verticillium lecanii* is recognized as an entomopathogenic fungus, and has high potential in the biological control of pests. In this study, it was investigated that the relationship between agitation speed in a 2.5 L stirred tank reactor (STR) at 25°C and initial pH 5.5, and the morphological characteristics of *V. lecanii* CS-625, such as hyphal length/width, spore length/width, and the number of tips during spore production. The agitation speed affected the hyphae patterns and the number of tips. The number of spores rapidly increased at 48 to 60 h of cultivation, and the highest spore productivity (2.5×10^{10} spore/L·h at 60 h) occurred with an agitation speed of 350 rpm and an aeration rate of 1.0 vvm. The number of tips increased in proportion to the increase in spore production during the same culture time. The highest number of tips (4.8×10^{8} tip/mL) was obtained at 72 h of cultivation. The shortest mean spore length (2.8 μm) was obtained at 60 h of cultivation. Therefore, it was determined that the increased number of tips and decreased mean spore length were closely related to the production of *V. lecanii* spores. © KSBB

Keywords: entomopathogenic fungi, spore, morphological characteristics, Verticillium lecanii

INTRODUCTION

The entomopathogenic fungus, *Verticillium lecanii*, has the ability to biocontrol a wide range of insect hosts, including Homoptera, Orthoptera, Coleoptera, and Lepidoptera [1,2]. Its spores have received much attention with respect to the biological control of insects and pests [3-5].

It is recognized that agitation speed and aeration are the most critical parameter and plays significant roles in determining the productivity of the sporulation process [6,7]. Thus, a proper agitation speed and adequate aeration may positively influence the morphological characteristics and spore productivity of *V. lecanii* because the primary roles of agitation are to improve the mixing as well as mass and heat transfer in the bioreactor. Although agitation provides increased mixing and mass transfer [8], it has many negative effects on the morphological characteristics of the spores, such as cell rupture, vacuolation, and autolysis, and causes a decrease in productivity [9]. The agitation speed improves the oxygen transfer rate in liquid culture systems [10], and it can aid in maintaining a normal cell growth rate. However, excessive agitation increases shear stress, which damages mycelial growth. An optimized agitation rate is needed to cultivate fungi that are sensitive to shear stress caused by mechanical agitation [11].

The morphological differentiation of *V. lecanii* presents a complex pattern during spore production. Fungal morphology is an important parameter that influences the physical properties of the fermentation broth [12,13]. It was reported that submerged spore grows into aerial growth pattern with many branches on surface cultures. This growth pattern, irrespective of the source of the spores, such as aerial or submerged, indicates that the growth differentiation is controlled by the environmental conditions such as the agitation speed and aeration rate; similarly, the type of spore that will germinate, grow, and sporulate. To our knowledge, there is no study on agitation and morphological characteristics of *V. lecanii* CS-625 in a 2.5 L stirred tank reactor. Thus, this requires further examination in order to understand the control and the differentiation of the spores under different environ-

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mental conditions [14].

The objective of this study was to investigate the relationship between agitation speed and morphological characteristics of *V. lecanii* in a bioreactor for the high spore production.

MATERIALS AND METHODS

Chemicals

Both soy sauce cake [23~30% (w/w) crude protein and 12~14% (w/w) crude fiber] and high fructose syrup [55% (w/v) fructose and 22% (w/v) glucose] were used as the main medium in the cultivation of *V. lecanii* CS-625, and were supplied by CJ Products Inc., Korea.

Strain

V. lecanii CS-625, which was supplied by Mycoplus Ltd., Korea, was used for the spore production. It was maintained by incubating monthly on potato dextrose agar plates (PDA, potato infusion 4 g; dextrose 20 g; agar 15 g; distilled water 1 L; Difco) at 25°C.

Seed Culture

One hundred mL of potato dextrose broth (PDB, potato starch 4 g; dextrose 20 g; distilled water 1 L, Difco) in a 250 mL Erlenmeyer flask was inoculated with 10 agar pieces (5 × 5 mm) from a 7-day cultured plate of *V. lecanii* CS-625, and incubated at 25°C for 2 day in a shaking incubator at 200 rpm.

Spore Production

The composition of the spore production medium was as follows: 1.6% soy sauce cake and 4.7% high fructose syrup. It was adjusted to pH 5.5 with 1 M HCl. The main culture in a 1.8 L working volume was carried out in a 2.5 L stirred tank reactor (STR) at various agitation speeds (250, 300, 350, and 400 rpm) and aeration rates [0.5 and 1.0 vvm (air volume/medium volume/minute)] at 25°C. The main cultures were inoculated with 10% (v/v) of the seed culture. The spores produced by *V. lecanii* were collected out of hyphae using sterilized gauze. The collected spores have been used throughout the studies [15]. The number of spores was counted by a hemocytometer under a microscope.

Analysis of Morphological Characteristics

Through image analysis using a digital camera (TK-1070U, JVC, Japan) and an imaging program (ImagePro 3.0 software, Mediacybernetics. INC., USA), the morphological factors were measured quantitatively according to the various agitation speeds in the 2.5 L stirred tank reactor at 25°C, 1.0 vvm, and initial pH 5.5. The means and standard deviations of the number of tips and the spore length were automatically calculated by the ImagePro software. The morphological characteristics were analyzed at 600 × magnification under a microscope connected to the image analysis system (ImagePro 3.0 software). Once an image was captured in the digital form, it was processed to improve its quality or to select its random features, and then analyzed to obtain the morphological characteristics information (hyphal length/width, spore length/width, and the number of tips). The increasing rate in the number of tips during culture was determined using V_{tips}, which was calculated from formula (1):

$$V_{tips} = \frac{\Delta N_i / \Delta t_i}{N_i} \quad (1)$$

Here, V_{tips} is the increasing rate of the number of tips, Δt_i is a 12-h interval of cultivation, ΔN_i is the increased number of tips at Δt_i, and N_i is the number of tips at t_i. The unit of measure was micrometer (μm) and the means and standard deviations were calculated from four replicates.

RESULTS AND DISCUSSION

Spore Production at Various Agitation Speeds in a 2.5 L Stirred Tank Reactor

The *V. lecanii* CS-625 spores were produced at various agitation speeds (250, 300, 350, and 400 rpm) in a 2.5 L stirred tank reactor (1.0 vvm, 25°C, and initial pH 5.5) containing soy sauce cake and high fructose syrup. A liquid-state fermentation with raw materials was carried out for spore production because liquid-state fermentations are usually faster; also it is easier to control the physical and chemical properties as well as raw materials, including the soy sauce cake and high fructose syrup, of such systems [16] decreasing the manufacturing costs. It was found that the spore concentration rapidly increased between 48 to 60 h of culture time at the agitation speed of 350 rpm while spore production was not as high at the other agitation speeds (Fig. 1).

The culture at the 350 rpm agitation speed gave the highest number of spores (2.0×10^9 spore/mL) at 120 h, as compared to those of the other agitation speeds, which was similar to the results (5.0×10^9 spore/mL broth) of a liquid culture performed by Ekbom [17]. For spore production, in general, several important factors are needed: an improved strain, proper fermentation medium/condition, and even a fermentation process [14]. The amount of dissolved oxygen in the bioreactor is supported by the agitation speed, aeration, viscosity, medium, *etc*. The results indicate that 350 rpm was an appropriate speed, so that a high level of dissolved oxygen was gained within a range of low shear stress in the bioreactor. The effect of the agitation speed on spore productivity, meaning an increasing rate of spores [spore production (spore/mL) per 1 h], was investigated in a 2.5 L stirred tank reactor under the same conditions as in Fig. 1 (Fig. 2).

The spore productivity rapidly increased from 48 to 60 h

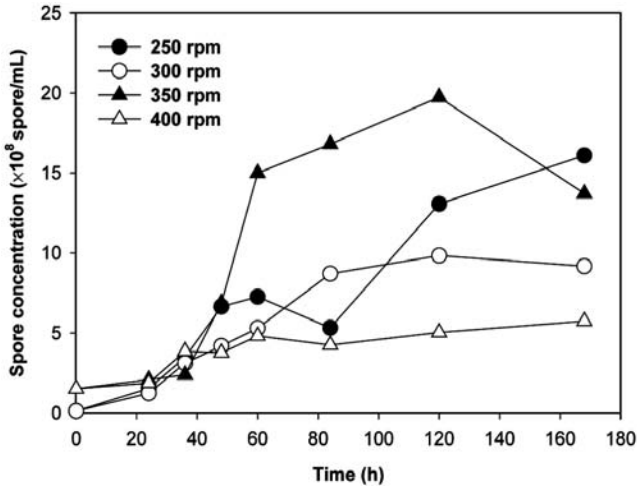

Fig. 1. Time course of spore production by *V. lecanii* CS-625 in a 2.5 L stirred tank reactor at various agitation speeds. The experiment was performed in a 2.5 L stirred tank reactor (25°C, initial pH 5.5) containing soy sauce cake and high fructose syrup. ○, 250 rpm; ●, 300 rpm; △, 350 rpm; ▲, 400 rpm.

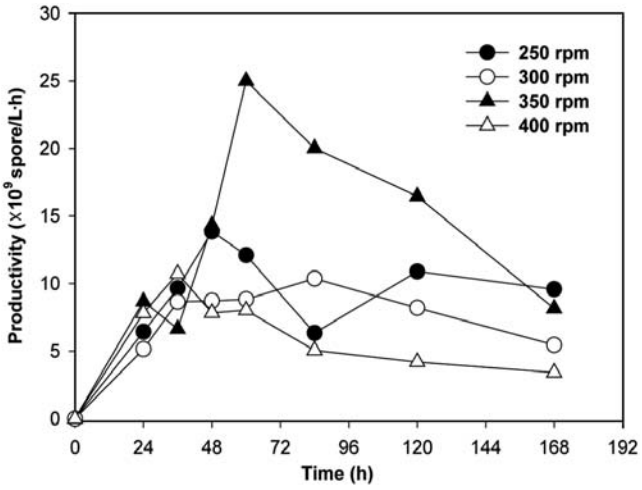

Fig. 2. Time course of spore productivity by *V. lecanii* CS-625 in a 2.5 L stirred tank reactor at various agitation speeds. The experiment was performed in a 2.5 L stirred tank reactor (25°C, initial pH 5.5) containing soy sauce cake and high fructose syrup. ○, 250 rpm; ●, 300 rpm; △, 350 rpm; ▲, 400 rpm.

of cultivation at the 350 rpm agitation speed and reached its highest level (2.5×10^{10} spore/L·h) at 60 h of cultivation. Then, for all the agitation speeds, the spore productions of *V. lecanii* decreased after 60 h. These results for the spore production and productivity indicate that *V. lecanii* can produce abundant spores in this sort of culture, where the agitation speed of 350 rpm greatly affected the sporulation of *V. lecanii* between 48 and 60 h of culture time.

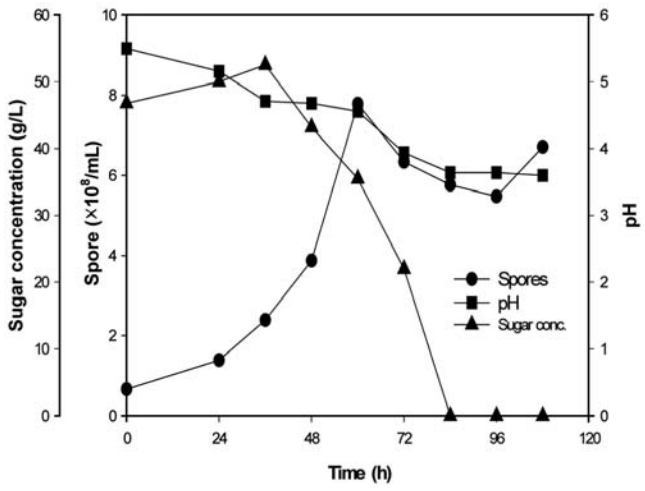

Fig. 3. Time course of spore production by *V. lecanii* CS-625 at 0.5 vvm and 350 rpm. The experiment was performed in a 2.5 L stirred tank reactor (25°C and initial pH 5.5) containing soy sauce cake and high fructose syrup. ●, spore production; ■, pH; ▲, sugar concentration.

Effects of Aeration Rates (0.5 and 1.0 vvm) on Spore Production

In order to compare the results of spore production at 1.0 vvm and 350 rpm, spore another experiment was carried out at 0.5 vvm and 350 rpm in a 2.5 L stirred tank reactor (25°C and initial pH 5.5) containing soy sauce cake and high fructose syrup. The results are shown in Fig. 3.

The spore concentration decreased in the low aerated culture condition. The sugar concentration was rapidly reduced and almost exhausted at 84 h, and the initial pH of 5.5 was gradually reduced to pH 3.6 at 108 h. The spore production (9.26×10^8 spores/mL) at 0.5 vvm and 350 rpm was lower than the (2.0×10^9 spore/mL) at 1.0 vvm and 350 rpm shown in Fig. 1. And the number of spores rapidly increased between 48 to 60 h of culture time. Although lower spore production was obtained at 0.5 vvm, it showed a similar pattern to the spore production at 1.0 vvm. The aeration rate leads to varied growth in *V. lecani*. When the air supply is inadequate during culture, oxygen transfer is a problem, resulting in pellet formation. Therefore, the aeration rate seems to be an important factor for the growth of *V. lecanii* and its sporulation.

Morphological Characteristics at Various Agitation Speeds

The morphological characteristics of *V. lecanii* CS-625 in a 2.5 L stirred tank reactor were investigated via image analysis (ImagePro 3.0) at various agitation speeds. The experiment was performed in a 2.5 L stirred tank reactor (25°C, initial pH 5.5, and 1.0 vvm) containing soy sauce cake and high fructose syrup. Fig. 4 shows the morphological changes at 48 and 60 h of cultivation according to the various agita-

Table 1. Increasing rate (V_{tips}, h^{-1}) of the number of tips at various agitation speeds

Agitation speed (rpm)	Culture time (h)					
	24~36	36~48	48~60	60~72	72~84	84~96
250	0.14 ± 0.02	0.08 ± 0.01	–0.06 ± 0.02	–0.01 ± 0.03	0.08 ± 0.13	0.17 ± 0.07
300	0.24 ± 0.19	0.22 ± 0.41	0.10 ± 0.23	–0.26 ± 0.10	0.24 ± 0.18	–0.51 ± 0.24
350	0.53 ± 0.17	0.35 ± 0.37	0.99 ± 0.40	0.78 ± 0.42	–1.39 ± 0.24	–0.57 ± 0.08
400	0.08 ± 0.01	0.01 ± 0.05	0.08 ± 0.01	0.33 ± 0.06	0.07 ± 0.06	0.01 ± 0.10

$V_{tips} = (\Delta N_i/\Delta t_i)/N_i$; V_{tips}, the increasing rate for the number of tips; Δt_i, 12-h interval of cultivation; ΔN_i, the increased number of tips at Δt_i; N_i, the number of tips at t_i. The unit of measure is micrometers (μm), and means and standard deviations were calculated with four replicates. The experiment was performed in a 2.5 L stirred tank reactor (25°C, initial pH 5.5, and 1.0 vvm) containing soy sauce cake and high fructose syrup.

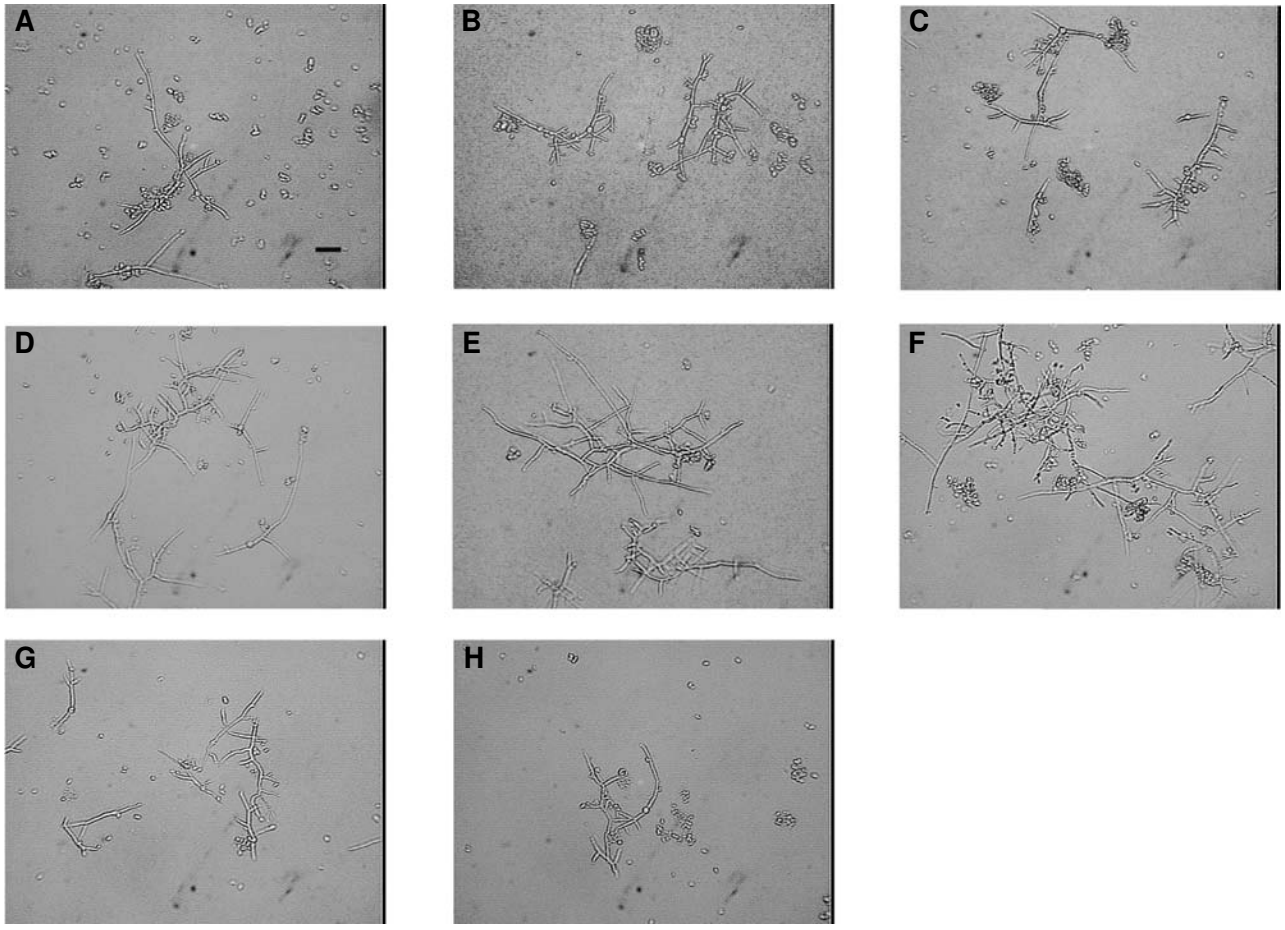

Fig. 4. Image analysis of morphological characteristics of *V. lecanii* CS-625 in a 2.5 L stirred tank reactor from 48 to 60 h of cultivation. The experiment was performed in a 2.5 L stirred tank reactor (25°C, initial pH 5.5, and 1.0 vvm) containing soy sauce cake and high fructose syrup. (A) 250 rpm and 48 h, (B) 250 rpm and 60 h, (C) 300 rpm and 48 h, (D) 300 rpm and 60 h, (E) 350 rpm and 48 h, (F) 350 rpm and 60 h, (G) 400 rpm and 48 h, (H) 400 rpm and 60 h. Magnification was 600 × and bar interval was 11.2 μm.

tion speeds (250, 300, 350, and 400 rpm).

The agitation speeds changed the morphological characteristics greatly. An abundant cell mass was observed at the 350 rpm agitation speed, the morphological characteristics of *V. lecanii* at the agitation speed were considerably changed in proportion to the culture time. In particular, numerous tips and spores were produced and long hyphae started to entangled at 350 rpm from 40 to 60 h of cultivation. In the growth patterns of *V. lecanii* linear-formed filamentous hyphae and small tips are contained in the initial culture medium. The amount of tips and spores increased in proportion to decreased in hyphal length. While the hyphal length decreases and separates into fragments, new small hyphae are found in the broth. The individual hyphae of the mycelium branches

Table 2. Length (μm) of *V. lecanii* CS-625 spore at various agitation speeds

Agitation speed (rpm)	Culture time (h)							
	0	24	36	48	60	72	84	96
250	4.4 ± 1.4	4.3 ± 0.5	4.7 ± 0.4	3.8 ± 0.2	3.7 ± 0.4	4.7 ± 0.6	3.8 ± 0.5	4.6 ± 0.9
300	4.5 ± 0.9	5.1 ± 0.2	4.2 ± 0.4	4.1 ± 0.9	3.1 ± 0.2	3.6 ± 0.4	3.9 ± 0.2	3.8 ± 0.9
350	3.1 ± 0.5	3.7 ± 0.5	3.8 ± 0.2	3.9 ± 0.2	2.8 ± 0.5	3.7 ± 0.3	4.2 ± 0.4	4.4 ± 0.6
400	3.7 ± 0.5	4.1 ± 0.2	4.6 ± 0.3	4.4 ± 0.1	4.0 ± 0.1	4.2 ± 0.1	4.2 ± 0.2	4.4 ± 0.2

The unit of measure is micrometers (μm), and mean and standard deviations were calculated with four replicates. The experiment was performed in a 2.5 L stirred tank reactor (25°C, initial pH 5.5, and 1.0 vvm) containing soy sauce cake and high fructose syrup.

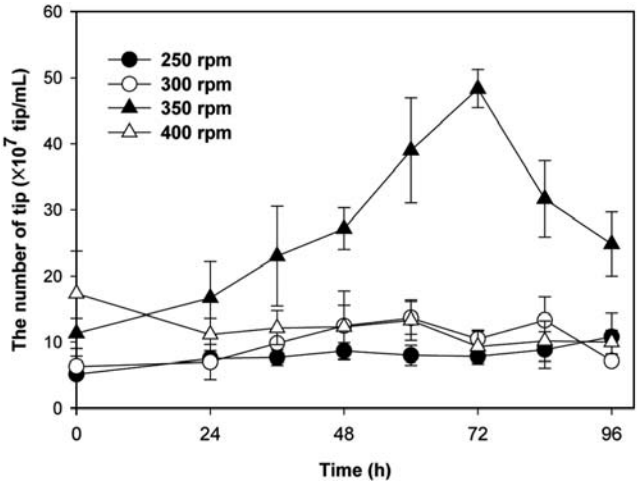

Fig. 5. The number of tips of *V. lecanii* CS-625 branches on various agitation speeds. The experiment was performed in a 2.5 L stirred tank reactor (25°C, initial pH 5.5, and 1.0 vvm) containing soy sauce cake and high fructose syrup. ○, 250 rpm; ●, 300 rpm; △, 350 rpm; ▲, 400 rpm.

grow and stretch in all directions, and then spores are formed on the tips of the hyphae. In the results, the various growth patterns of *V. lecanii* were well presented under the 350 rpm agitation speed in the bioreactor. However, different morphological patterns were shown at the other agitation speeds due to deficient aeration with lower agitation speeds or shear stress with higher speeds.

Fig. 5 shows the number of tips for the *V. lecanii* CS-625 branches, and Table 1 presents the increasing rate (V_{tips}) of tips for each 12 h, in the same culture as in Fig. 4.

In the results, the highest number of hyphal tips (4.8×10^8 tip/mL) at 350 rpm was obtained at 72 h of cultivation and was significantly higher than those of the other agitation speeds. The highest V_{tips} (0.99) was obtained between 48 and 60 h of cultivation at 350 rpm. Wendland and Philippsen [18] reported that hyphae extend continuously from their hyphal tips, and spores are formed at the hyphal tips so that fungal mycelia can grow from the spores. Thus, it was assumed that the increased hyphal tips at 48~60 h of cultivation, due to high mycelia growth, allowed for high spore production to be at 350 rpm, which was the proper agitation speed to supply aeration with low shear stress. Spore size also seemed to be associated with spore production. Table 2 presents the spore length for the same culture of Fig. 4.

The spore length decreased in proportion to increasing numbers of *V. lecanii* spores at 350 rpm from 48 to 60 h of cultivation. The lowest spore length (2.8 μm) was obtained at 60 h of cultivation, when V_{tips} was highest at 350 rpm and 1.0 vvm. Spore size is changed according to certain physiological features of this fungus. The spores from liquid culture have various shapes such as ovoid, oblong, and long ellipsoid [3]. Most of the initial spores from the hyphal tips have variable shapes and then grow into hyphae, while the amount of spores is decreased. In the results, when the number of tips was increased by hyphae growth from 48 and 60 h, many small-sized spores seemed to be produced. The changes of the spore forms were well presented at 350 rpm in the bioreactor.

Hall [19] reported that it is considered that biocontrol by *V. lecanii* with a high epizootic potential for aphids, is generated by large conidiospores. And small size spores may be avirulent in some strains of *V. lecanii* [20], biocontrol abilities of produced spores can be measured by a bioassay.

In this study, it was found that agitation speed was related to the morphological characteristics of *V. lecanii* during spore production. The most appropriate agitation condition (350 rpm), which provided sufficient aeration with low shear stress, offered a good growth pattern of *V. lecanii* with high spore production. When the nutrient supply in a bioreactor is reduced or starved, nitrogen, primary nutrient, is exhausted, and sporulation is usually stimulated [21]. However, sporulation can be generated without mycelium starvation during the growth pattern of *V. lecanii*, and *V. lecanii* spore production increases in proportion to mycelia growth [22]. In this study, results, it was well demonstrated that at 350 rpm, parallel to an increase in hyphal tips (4.8×10^8 tip/mL), high spore production (2.0×10^9 spore/mL) was obtained between 48 and 60 h of cultivation. The spore length was reduced and no significant changes in the hyphal length/width or spore width were identified during that time.

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