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Optimization of Solid-State Fermentation for Improved Conidia Production of *Beauveria bassiana* as a Mycoinsecticide

Tuan Anh Pham[1], Jeong Jun Kim[2] and Keun Kim[1]*

[1]*Department of Bioscience and Biotechnology, The University of Suwon, Hwaseong 445-743, Korea*
[2]*Applied Entomology Division, National Academy of Agricultural Science, Suwon 441-707, Korea*

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The production of conidia of entomopathogenic *Beauveria bassiana* by solid-state fermentation was studied for the development of a biocontrol agent against aphid *Myzus persicae*. The optimal conditions for conidia production on polished white rice were 40% moisture content, 25°C culture temperature, 2-day-old seeding culture grown in 3% corn meal, 2% rice bran, 2% corn steep powder medium, initial conidia concentration of 10^7 conidia/g in the wet rice, 10% inoculum size, and use of a polyethylene bag as a container. The polyethylene bag containing inoculated rice was hand-shaken every 12 hr during fermentation. Using optimal conditions, the maximum conidia production obtained was 4.05 g conidia/100 g dry rice after 14 days of cultivation, a rate 2.83 times higher than conidia yield of pre-optimization.

KEYWORDS : *Beauveria bassiana*, Conidia production, Optimal culture conditions, Solid-state fermentation

Entomopathogenic fungi are recognized as important natural enemies of insect pests. Such species include *Verticillium lecanii* (Zimmerman) Viegas, *Metarhizium anisopliae* (Metsch.), *M. flavoviride* (Metsch.), *Nomuraea rileyi* (Farlow) Samson, *Beauveria bassiana* (Balsamo) Vuillemin (Hypocreales: Clavicipitaceae), and *Paecilomyces farinosus* (Holm ex S. F. Gray) Brown & Smith [1]. Mycoinsecticides are garnering increased attention as environmentally-friendly insect control agents. Although thousands of fungal species infect insects, few have received serious consideration as potential commercial candidates. *B. bassiana* appears to have the broadest potential as a viable insect control agent. Adhesion of fungal spores to the host cuticle along with germination are prerequisites for the efficacy of fungal pathogens [2-4].

Aphids are distributed worldwide but are most common in temperate climates. Aphids transmit various pathogenic plant viruses and are very difficult to control using only organic pesticides due to increased insecticide resistance and rapid increases in population size. Among the various biocontrol agents using entomopathogenic fungi to control aphids, *B. bassiana* (Balsamo) is the most promising [5-7].

Production of *B. bassiana* spores can be achievied using different methodologies, which can be classified as either low input or industrial technologies [2]. Both solid and liquid fermentation systems are used for the mass production of biocontrol agents [8, 9]. Solid state fermentation (SSF) allows several types of fungi to produce hardly and healthy conidia [10]. Though blastospores are produced by submerged liquid fermentation, they are hydrophilic and lose viability relatively quickly during storage [11, 12]. Therefore, SSF is considered the proper system for the mass production of conidia to be used in oil formulation [13]. SSF has additional advantages compared to submerged fermentation; SSF is more simple and productive, requires lower capital and energy, uses simpler fermentation media, does not require rigorous control of fermentation parameters, uses less water, produces less wastewater, easily controls bacterial contamination, and has cheaper downstream processing [14, 15].

Previously, we isolated the entomopathogenic fungus *B. bassiana* KK5, which has high infectivity and virulence to aphids (*Myzus persicae*). Despite its high infectivity, the large scale application of this fungus for aphid control is only possible if high titers of spores are grown and produced on widely available substrates.

Therefore, the aim of this study was the optimization of *B. bassiana* KK5 conidia production on polished rice using SSF.

Materials and Methods

Microorganism. The original strain of *B. bassiana* KK5 was preserved at −80°C in sterile cryovials containing 10% glycerol (in sterilie 0.02% of Tween 80 solution). The fungal strain was cultured on potato dextrose agar (PDA) at 25°C for 10 days and stored at 4°C until use.

Preparation of the seeding inoculums. Aerial conidia taken from a stock culture growing on a PDA agar plate were suspended in sterile water containing 0.02% Tween

*Corresponding author <E-mail : kkim@suwon.ac.kr>

80. The number of conidia in the suspension was counted using a haemocytometer (Superior Marienfeeld, Lauda-Königshofen, Germany), followed by dilution to 1.0×10^8 conidia/mL with 0.02% Tween 80. One milliliter of the conidium suspension was inoculated into a 250 mL Erlenmeyer flask containing 100 mL of liquid media, followed by culture at $25 \pm 0.1^{\circ}C$ using a rotary shaking incubator operated at 200 rpm. The resulting suspension was used as the seeding inoculum for SSF.

Solid-state fermentation for aerial conidia production. The moistened solid medium was transferred into a container (see "Effect of solid medium container on aerial conidia production" in Materials and Methods) and autoclaved at 121°C for 25 min. The container with moistened solid media was then cooled, after which the seeding culture was added at 10% inoculum size (10^7 conidia/g wet medium) to each container, followed by thorough mixing using a sterilized spoon. The specimens were then incubated at various temperatures and relative humidities (RH) under still culture conditions for a desired time period, depending on experimental protocols. The contents of the container were gently hand-shaken every 12 hr. Unless otherwise mentioned, these conditions were maintained throughout all experiments. Conidia were counted using a haemocytometer after growth for 10 days. For all experiments, three replicates of each treatment were used.

Counting conidia. Two grams of conidiated rice was mixed with 18 mL of 0.02% Tween 80. The 100 mL flask containing the mixture of conidiated rice was agitated in a rotary shaking incubator operated at 200 rpm. After 60 min of agitation, the mixture of conidiated rice was filtered through three layers of cheese cloth. The number of conidia was determined using a haemocytometer (Neubauer improved; Superior Marienfeld, Germany).

Harvesting conidia. Conidiated rice cultivated for various desired times were dried at 35°C for 24 hr and then harvested through three sieves with mesh sizes of 25 (710 μm), 80 (180 μm), and 200 (75 μm) using a sieving vibrator (Model 3PRO; Fritsch Co. Ltd., Idar-Oberstein, Germany). The conidia were then collected and dried using a vacuum dryer at room temperature in order to lower the moisture content to less than 5%.

Effect of different substrates on aerial conidia production. Several kinds of solid substrates were tested, including polished rice, brown rice, and rice husk. The substrates were mixed with water to achieve a moisture level of 35%, then put into a plastic bottle ($62 \times 92 \times 160$ mm^3). The solid medium in the plastic bottle was inoculated with 2-day-old liquid culture grown in medium composed of 3% corn meal, 2% rice bran, and 2% corn steep powder, followed by incubation at 25°C, 75% relative humidities (RH). After 14 days of cultivation, the fermented medium was dried at 35°C for 24 hr, after which the conidia yield was determined.

Effect of temperature on aerial conidia production. Inoculated, steamed polished rice at a moisture level of 35% was incubated in a plastic bottle at 25, 27, and 30°C at 75% RH. The number of conidia per g wet conidiated rice was determined after 12 days of cultivation.

Effect of liquid seeding culture on aerial conidia production. Sterilized steamed rice at a moisture level of 35% in plastic bottles ($62 \times 92 \times 160$ mm^3) were inoculated with two-day-old cultures grown in different liquid media, such as potato dextrose broth (PDB), wheat bran (WB; 3% corn meal, 2% wheat bran, 3.5% corn steep liquor), corn step liquor (CSL; 3% corn meal, 2% rice bran, 3.5% corn steep liquor), and rice bran (RB; 3% corn meal, 2% rice bran, 2% corn steep powder), at an inoculum size of 10% (v/w). Conidia production on rice was conducted at 25°C and 75% RH for 14 days, after which the conidia were dried at 35°C for 24 hr and then harvested using a sieve vibrator.

Effect of seeding culture time on aerial conidia production. Seeding cultures grown in RB medium for different time periods (2, 3, or 5 days) were inoculated onto rice. The initial inoculum sizes were 10% (v/w). Conidia production on rice was conducted at 25°C and 75% RH for 14 days, after which the conidia were dried at 35°C for 24 hr and then harvested using a sieve vibrator.

Effect of inoculum size on aerial conidia production. Sterilized polished rice in plastic bottles were inoculated with different inoculum sizes (10, 15, and 20% v/w). The inoculated rice were incubated at 25°C and 75% RH for 14 days, after which the conidia were dried at 35°C for 24 hr and then harvested using a sieve vibrator.

Effect of inoculum concentration on aerial conidia production. The steamed rice in plastic bottles at a 35% moisture level were inoculated with different concentrations of 10% inoculum in order to achieve an initial concentration of 10^6, 10^7, and 10^8 conidia/g wet rice. And then conidia were produced on the rice as described above.

Effect of solid medium container on aerial conidia production. The rice were submerged and drained in order to achieve a moisture level of 40%. The moistened rice were then placed into either plastic bottles ($62 \times 92 \times 160$ mm^3 and $70 \times 92 \times 160$ mm^3) or polyethylene bags [325×435 mm^2 with two small aeration filters (diameter, 36 mm)

or two large aeration filters (diameter, 79 mm)]. The plastic bottle was plugged with a ventilated cap in order to minimize contamination and allow passive aeration during growth and conidiogenesis. The moistened rice were next sterilized, cooled, and inoculated with 2-day-old RB culture, followed by additional incubation as shown above. After 14 days of cultivation, the conidiated rice were dried at 35°C for 24 hr, after which conidia were harvested using a sieve vibrator and dried using a vacuum dryer at room temperature to a moisture level lower than 5%. Lastly, the conidia yield was determined.

Effect of moisture content of rice on aerial conidia production. The polished rice were submerged in tap water for 2~3 hr, followed by draining for different time periods to achieve moisture levels of 30, 35, 40, and 50%. One hundred grams of moistened rice was dried at 105°C for 24 hr, followed by weighing to determine the water content. Polyethylene bags with small aeration filters containing the inoculated rice were then incubated at 25°C for 12~17 days, after which the number of conidia/g wet conidiated rice was determined.

Effect of different ambient RH on aerial conidia production. Sterilized moistened rice in the polyethylene bag with two small aeration filters were inoculated with 2-day-old liquid culture as shown above, followed by incubation at 25°C at different ambient RHs of 45, 60, and 75%. The conidia yield was determined after 15 days after inoculation.

Effect of incubation period on aerial conidia production. Conidia production on sterilized rice at a moisture level of 40% was conducted at 25°C and 75% RH in a polyethylene bag with two small aeration filters. After 10 days of cultivation and a 1 day interval, the number of conidia per g rice was determined.

Results and Discussion

Effect of different substrates on aerial conidia production. After 14 days of cultivation, the highest amount of aerial conidia was produced using steamed polished white rice (2.69 g conidia/100 g substrate), followed by brown rice (1.43 g conidia/100 g substrate) (Fig. 1). Conversely, the results show that rice husk was not suitable for producing aerial conidia of *B. bassiana* KK5. According to Feng *et al.* [16], the conidia yields of *V. lecanii* on cooked rice and rice bran are 1.5×10^9 and 1.4×10^9 conidia/g solid culture, respectively, which are significantly higher than those of rice husk and a mixture of rice and rice bran under identical culture conditions. The maximum conidia yield for *B. bassiana* is 4.38×10^9 conidia/g wet rice [17]. The conidia yield of *B. brongniartii* cultured on media consisting of 70% cotton seed-shell powder, 25% wheat bran, and 5% corn flour (plus water) is 2.9×10^9 conidia/g dry powder [18]. When *B. brongniartii* was cultured for 42 days on a mixture of shelled barley, sunflower oil, and water, the conidia yield was found to be 2×10^9 [19]. Therefore, the optimal substrate for the high yield production of conidia varies depending on the fungal species.

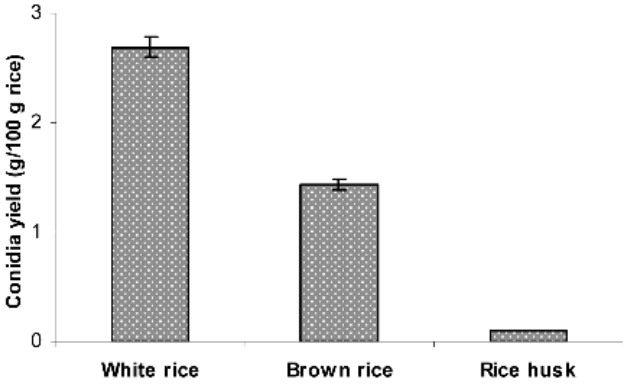

Fig. 1. Aerial conidia production on different solid culture media. Polished rice, brown rice, and rice husk at a 35% moisture level in plastic bottles were inoculated with 2-day-old liquid culture and then incubated at 25°C and 75% relative humidities for 14 days.

The most commonly selected substrate for the production of fungal conidia is by far white rice [2, 3, 20-23], which was also selected as the best solid substrate in this study. This is probably due to a combination of factors including nutritional balance, cost, worldwide availability, physical characteristics such as grain size and shape, hydration properties, and structural integrity even after colonization by fungi [13].

Effect of temperature on aerial conidia production. The result depicted in Fig. 2 indicates that incubation tem-

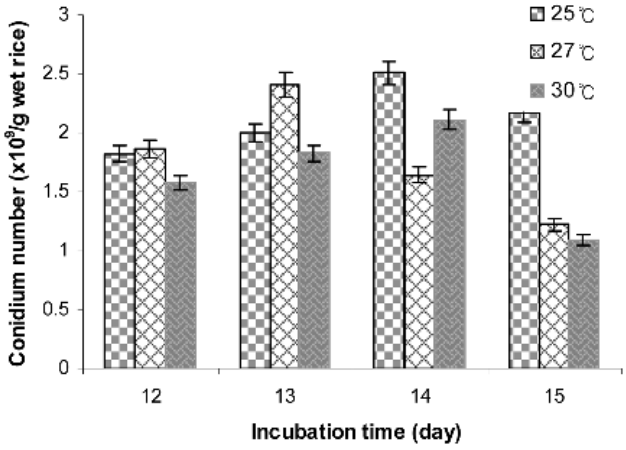

Fig. 2. Effect of temperature on aerial conidia production. The inoculated steamed polished rice at a 35% moisture level in a plastic bottle was incubated at 25, 27, and 30°C and 75% relative humidities for 12~15 days.

perature markedly affected conidia yield. The optimal temperature for conidia production was 27°C for 12 and 13 day incubations, whereas 25°C was optimal for 14 and 15 day incubations. The optimal temperature for conidia production was 25°C for the 14 day incubation. In this case, the number of conidia produced was 2.51×10^9/g wet conidiated rice. According to Vu *et al.* [9], the optimal temperature for conidia production of *V. lecanii* 41185 is 25°C, with the number of conidia produced 6.13×10^9 conidia/g. In this study, the optimal temperature of 25°C matched that for liquid culture of *B. bassiana* KK5 [8]. Furthermore, Thomas and Jenkins [24] showed that the incubation temperatures of the liquid and solid production stages matched those of the germination and mycelial growth of *M. flavoviride*.

Effect of liquid seeding culture medium on aerial conidia production. Different seeding culture media including PDB, WB, CSL, and RB were tested. The results in Fig. 3 show that conidia production significantly depended on liquid culture medium. Rice inoculated with the culture grown in RB medium produced a significantly higher number of conidia (2.69 g conidia/100 g rice) compared to those grown on other seeding culture media. Rice inoculated with the PDB culture produced the lowest number of aerial conidia.

Effect of seeding culture time on aerial conidia production. The RB seeding culture was inoculated onto rice for 2, 3, or 5 days at 10% inoculum size, after which conidia yield after 14 days of cultivation was determined. The results in Fig. 4 show that conidial production on rice

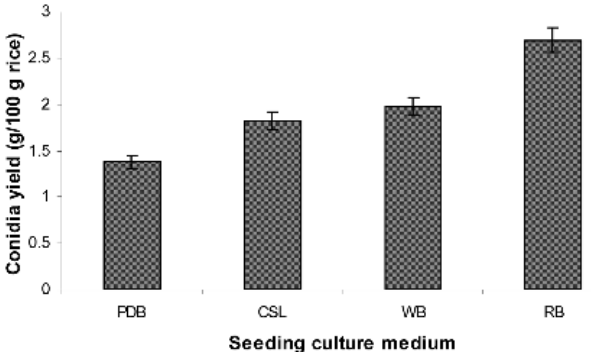

Fig. 3. Effect of seeding culture medium on aerial conidia production. PDB, potato dextrose broth; wheat bran (WB), 3% corn meal, 2% wheat bran, 3.5% corn steep liquor; corn step liquor (CSL), 3% corn meal, 2% rice bran, 3.5% corn steep liquor; and RB, 3% corn meal, 2% rice bran, 2% corn steep powder. Various seeding cultures grown in different liquid media were inoculated onto sterilized steamed rice at a 35% moisture level in plastic bottles at an inoculum size of 10% (v/w). Determination of conidia production was conducted at 25°C and 75% relative humidities for 14 days.

Fig. 4. Effect of seeding culture time on aerial conidia production. Ten percent (v/w) seeding cultures grown in 3% corn meal, 2% rice bran, 2% corn steep powder medium for different times were inoculated onto steamed rice, after which the inoculated rice were incubated at 25°C and 75% relative humidities for 14 days.

inoculated with 2-day-old liquid culture was the highest, whereas rice inoculated with 5-day-old liquid culture produced the lowest yield of aerial conidia.

Effect of inoculum size on aerial conidia production. Rice were inoculated with different sizes of inoculum and then incubated for 14 days, after which the conidiated rice was dried at 35°C for 24 hr. The dried conidia powder was then harvested to determine conidia yield. The results shown in Fig. 5 indicate that the conidia yield of rice inoculated with increasing sizes of inoculum was decreased, due possibly to increased moisture content and, in turn, decreased aeration in rice. The highest conidial yield was achieved after 14 days of cultivation using 10% inoculum

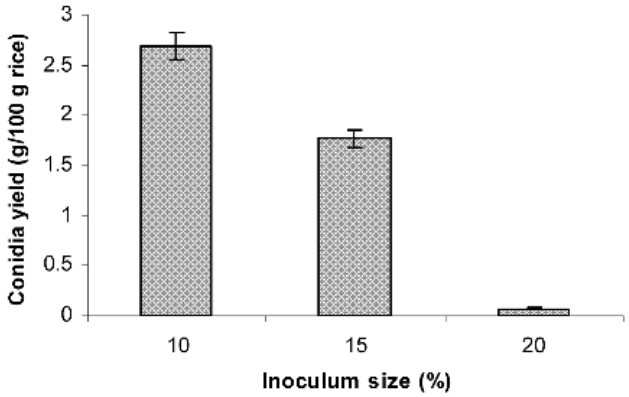

Fig. 5. Effect of inoculum size on aerial conidia production. The sterilized polished rice in plastic bottles were inoculated with different inoculum sizes, such as 10, 15, and 20%, of seeding culture. The inoculated rice were incubated at 25°C and 75% relative humidities for 14 days.

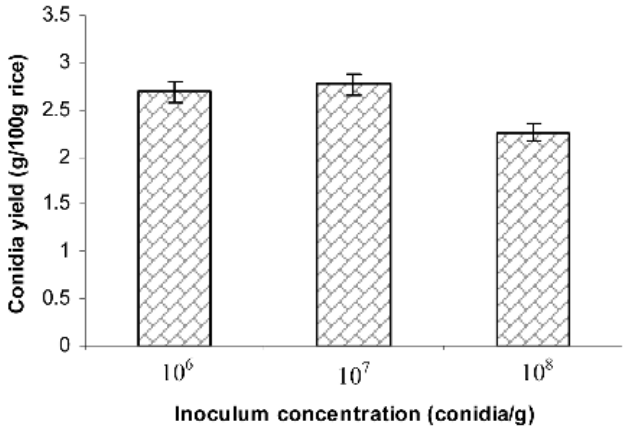

Fig. 6. Effect of initial conidia concentration on aerial conidia production. The steamed rice at a 35% moisture level in plastic bottles were inoculated with different conidium concentrations of 10% inoculum in order to achieve initial substrate concentrations of 10^6, 10^7 and 10^8 conidia/g wet rice, followed by incubation of inoculated rice at 25°C and 75% relative humidities for 14 days.

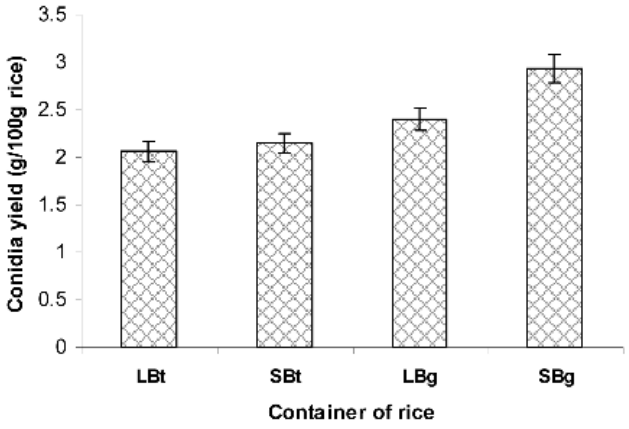

Fig. 7. Effect of rice container on aerial conidia production. LBt, large plastic bottle; SBt, small plastic bottle; SBg, small aerated area polyethylene bag; and LBg, large aerated area polyethylene bag. The polished rice at a 40% moisture level were placed into different containers such as plastic bottles ($62 \times 92 \times 160$ mm^3 and $70 \times 92 \times 160$ mm^3) with a ventilated cap and polyethylene bags (325×435 mm^2) with small aeration filters (diameter, 36 mm) or large aeration filters (diameter, 79 mm). The moistened rice in the container were sterilized, cooled, and inoculated with 2-day-old 3% corn meal, 2% rice bran, 2% corn steep powder culture, followed by incubation at 25°C and 75% relative humidities for 14 days.

size of 2-day-old seeding culture.

Effect of inoculum concentration on aerial conidia production. Rice were incubated with 10^6, 10^7, or 10^8 conidia/g wet rice for 14 days after which conidial yield were examined. The results shown in Fig. 6 indicate that the highest conidial yield (2.77 g conidia/100 g rice) was achieved using an initial concentration of 10^7 conidia/g wet rice.

Effect of rice container on aerial conidia production. Four different types of rice containers were compared regarding their conidia production (Fig. 7). The two polyethylene bags produced more conidia than the two platic bottles. The conidia yield in the container with two small aeration filters was higher than that containing a larger aeration filter. In general, aeration during cultivation was important for conidia production. However, the production of conidia can be affected by the fact that aeration through a large aeration filter alters the water content of rice via absorption of outside moisture from the humid culture environment. Rice in the polyethylene bag with two small aeration filters produced the most number of conidia (2.91 g conidia/100 g rice) compared to other containers.

Effect of moisture content of rice on aerial conidia production. Conidia production on rice with different moisture contents of 30, 35, 40, and 50% were examined and the results are shown in Fig. 8. The results show that rice with a moisture level of 40% had the highest conidia yield (3.17×10^9 conidia/g wet conidiated rice) after 15 days of incubation. Moisture content of the substrate plays a significant role in the final yield of conidia [13]. Most

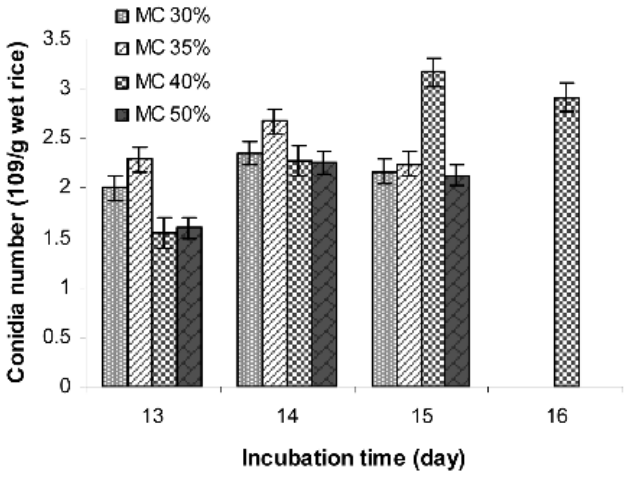

Fig. 8. Effect of moisture content of rice on aerial conidia production. The polished rice with desired moisture contents (MC) of 30, 35, 40, and 50% in polyethylene bags with small aeration filters were inoculated with 10% inoculum in order to achieve an initial concentration of 10^7 conidia/g wet rice. The inoculated rice were then incubated at 25°C for 12~17 days in order to determine the conidia number/g wet conidiated rice.

mitosporic fungi prefer humid environments. However, different substrates vary in their moisture sorption curves, reaching maximum adsorption at different moisture levels.

The conidia yield in this study was increased significantly with increasing incubation time. The results demonstrate that conidia yield strongly depended on the moisture content of rice. In another report [25], initial moisture levels from 35 to 80% were utilized in solid fermentation, depending on the fungal strain and substrate.

The moisture content of the medium changed during fermentation as a result of evaporation and metabolic activities. Therefore, the optimum moisture level of the substrate is very important [26]. There was a close relationship between moisture content and oxygen availability; increases in the moisture content of the substrate tends to decrease oxygen availability as the inter-particular spaces become filled with water and air is forced out [13, 27].

Effect of different ambient RH on aerial conidia production. The conidia were produced on rice at a moisture level of 40% inoculated with 10% inoculum size of 10^7 conidia/g wet rice, followed by incubation at 25°C at different ambient RHs of 45, 60, and 75%. The conidial yield was determined after 15 days of inoculation. The results in Fig. 9 indicate that the amount of aerial conidia significantly depended on ambient RH. For example, the amount of conidia produced at 75% RH (3.43 g conidia/100 g rice) was significantly higher than that at 45 or 60% RH.

Effect of incubation period on aerial conidia production. The conidia production of *B. bassiana* KK5 was conducted at 25°C and 75% ambient RH using rice at a moisture level of 40% in a polyethylene bag with two small aeration filters and 2-day-old seeding culture grown in RB medium. Under these optimal conditions, the effect

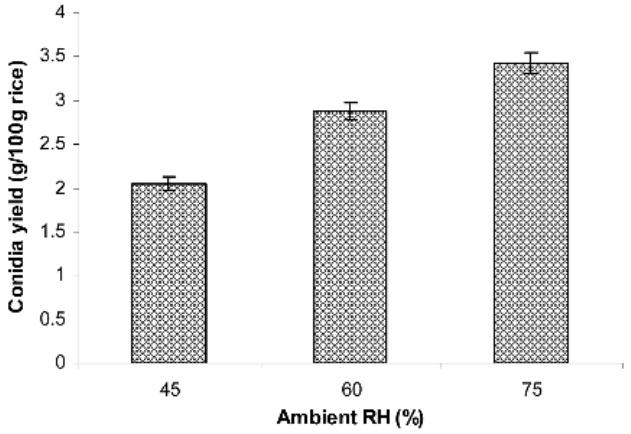

Fig. 9. Effect of ambient relative humidities (RH) on aerial conidia production. The sterilized moistened rice in a polyethylene bag with small aeration filter were inoculated with 2-day-old 3% corn meal, 2% rice bran, 2% corn steep powder liquid culture at 10% inoculum size, followed by incubation at 25°C and different ambient RHs (45, 60, and 75%) for 15 days.

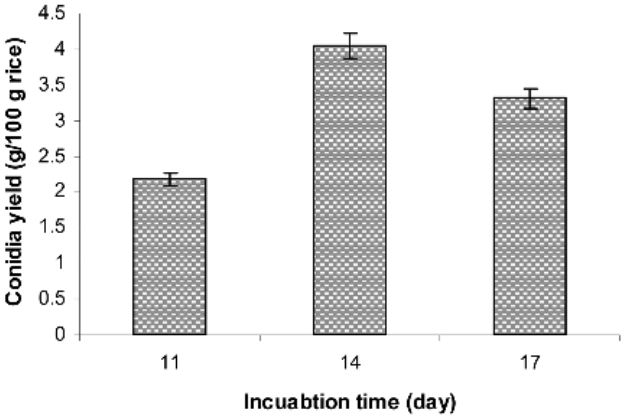

Fig. 10. Effect of incubation period on aerial conidia production. Conidia production was conducted at 25°C and 75% relative humidities using polished rice at a 40% moisture level in a polyethylene bag with small aeration filter for 10~17 days.

of incubation period (11, 14, or 17 days) on conidia production was examined, the results of which are shown in Fig. 10. After 14 days of incubation, the conidia yield was 4.05 g conidia/100 g rice (1.82×10^9 conidia/g rice), which is more than 2.83 times higher than that before optimization (1.43 g conidia/100 g rice). This value was also higher than the conidia yields of 3 g conidia/100 g rice and 2.28 g conidia/100 g as reported by Alves and Pereira [20] and Posada-Flórez [28], respectively, for *B. bassiana*. Using a mixture of 70% cotton seed-shell powder, 25% wheat bran, and 5% corn flour (plus water), the conidia yield of *B. brongniartii* was 2.9×10^9 conidia/g dry powder [17]. However, when *B. brongniartii* was cultured for 42 days on a mixture of shelled barley, sunflower oil, and water, the conidia yield was 2×10^9 [18].

In conclusion, we evaluated the suitability of various substrates, moisture content, temperature, seeding culture time, etc. on the aerial conidia production of *B. bassiana* KK5. The results show that the optimal conditions of conidia production on rice were 40% moisture content of whole polished rice, 25°C culture temperature, 2-day-old seeding culture in 3% corn meal, 2% rice bran, and 2% corn steep powder, inoculum concentration of 10^7 conidia/g wet rice, 10% inoculum size, a fermentation container comprising a polyethylene bag with two small aeration filters. Under optimal conditions, maximum conidia production (4.05 g conidia/100 g dry white rice) was obtained after 14 days of cultivation and was 2.83 times higher than that pre-optimization.

Acknowledgements

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