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Full Length Research Paper

Synchronous production of conidial powder of several fungal biocontrol agents in series fermentation chamber system

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A series solid-state fermentation (SSF) chamber system was designed to produce superior conidial powder of several fungal biocontrol agents. The synchronous conidia production of six fungal biocontrol candidates in three genera including *Metarhizium flavoviride* ARSEF 5628, *M. anisopliae* ARSEF 456, *Beauveria bassiana* ARSEF 734 and ARSEF 2860, *Paecilomyces fumosoroseus* ARSEF 6206 and ARSEF 3843 were assessed for the feasibility of chamber system. The conidial powder of those strains harvested from 5-kg rice cultures in a chamber weighed 147.1, 137.5, 101.2, 89.2, 55.0 and 40.0 g, and obtained 2.2×10^{12}, 2.7×10^{12}, 5.4×10^{12}, 4.6×10^{12}, 1.8×10^{12}, 1.3×10^{12} conidia kg^{-1} rice, respectively. Experimental data from the new series chamber system were obviously better than those of previous reports. Moreover, conidial viability of those isolates was up to 95% entirely. Results suggest that the chamber system have the capability to meet the quick and simple biocontrol evaluation activities such as biological characteristic of various biocontrol agents, from synchronous production to multiplicate trials.

Key words: Aerial conidia, fungal pathogens, synchronous production, series fermentation chamber.

INTRODUCTION

Most entomopathogenic hyphomycete fungi are of increasing importance in the field application due to more and more negative effects of chemical pesticides on the environment. In microbial control programme, production of sufficient quantities of good quality inoculum becomes essential to its success. Solid-state fermentation (SSF) is currently the best method of obtaining fungal spores by aerial hyphae (Holker, 2004) and can be used for the production of a wide range of biotechnological products (Pandey et al., 1999; Wraight et al., 2001; Bapat et al., 2003; Krishna, 2005; Nava et al., 2006; Ohgren et al., 2007; Nizamuddin et al., 2008).

Aerial conidia produced by SSF are similar to those produced naturally on the surface of insect cadavers and are superior to mycelia and blastospores produced under submerged fermentation conditions (Feng et al., 1994; Deshpande, 1999; Wraight et al., 2001; Roberts and St. Leger, 2004). Many fungal agents, such as *Beauveria bassiana*, *Metarhizium anisopliae* and *Paecilomyces fumosoroseus*, responsible for dispersal and infection under natural conditions are the aerial conidia.

Conditions which there were no quick and effective methods to compare the conidia production crafts among various biocontrol agents restricted extremely the mycopesticide registration development. Previous laboratory scale SSF was processed by several types of equipment such as petri dish, bottles, wide-mouth Erlenmeyer flasks, etc, which were used for screening of substrate or microorganisms in the preliminary stage. Recently, we developed an upright multi-tray conidiation chamber for large-scale conidial production for pest control at effective cost (Ye et al., 2006).

However, this big-size SSF bioreactor does not suit

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Table 1. The ARSEF codes, host insects and geographic origins of strains in this study.

Strain	Host insect	Location
ARSEF 5628	*Schistocerca gregaria* [Orthoptera:Acrididae]	Ethiopia: Shelsela.
ARSEF 456	*Nilaparvata lugens* [Homoptera: Delphacidae]	Philippines: Manila.
ARSEF 734	*Chalcodermus aeneus* [Coleoptera: Curculionidae]	Brazil: Goiânia, Goiás.
ARSEF 2860	*Schizaphis graminum* [Homoptera:Aphididae]	USA: Idaho.
ARSEF 6206	*Lymantria dispar* [Lepidoptera:Lymantriidae]	France: Pujaudrau.
ARSEF 3843	*Bemisia tabaci* [Homoptera:Aleyrodidae]	India: Padappai.

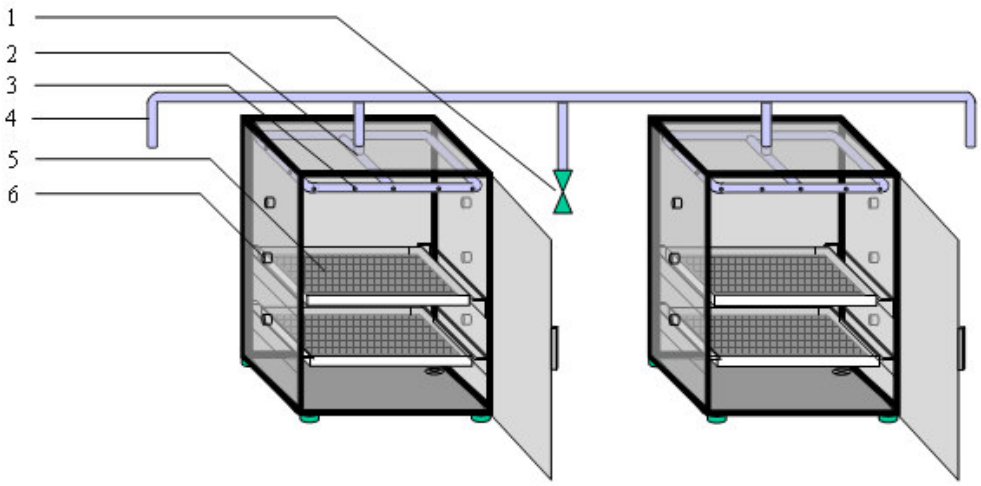

Figure 1. Diagram for the structure of series SSF chamber. (1) Inlet of distilled water supported by moisture generator. (2) An "日" type PVC circle through which sterile mist was slowly piped into the series chamber system. (3) The distilled steam pores. (4) Connecting PVC-pipe among the SSF chambers. (5) Rust-proof metal tray with meshed bottom. (6) Multifunction hole.

appropriately to synchronous production of kinds of hyphomycete fungus and is not ready for multiplicate trials of laboratory-scale. The present study sought to develop a series chamber system for synchronous conidia production. We also assessed the possibility of a research project aiming to build up a quick and simple evaluation system on the biological characteristic of various biocontrol agents candidate based on the series SSF chamber system.

MATERIALS AND METHODS

Fungal isolates and cultures

Six fungal biocontrol candiadates including *M. flavoviride* (Mf5628), *M. anisopliae* (Ma456), *B. bassiana* (Bb734, Bb2860) and *P. fumosoroseus* (Pf6206, Pf3843) were maintained on plates of Sabouraud dextrose agar (w/v: 4% glucose, 1% peptone, 1% yeast extract and 2% agar) at 4℃ prior to revival. All isolates were requested from ARSEF (Collection of Entomopathogenic Fungal Cultures, USDA-ARS Plant Protection Research Unit, U.S. Plant, Soil and Nutrition Laboratory, Ithaca, New York, USA). The ARSEF codes, host insects and geographic locations of six strains used in this study listed in Table 1. Some desirable potential such as high virulence or resistance to high temperature have been assessed through peach aphid *Myzus persicae* in previous research (Shan and Feng, 2006; Yu and Feng, 2006).

Series fermentation chamber

The series solid-state fermentation chamber (Figure 1) has dimensions of 40 × 50 × 60 cm and equipped with 10 trays parallel to each other equally spaced. Each tray, 3 cm depth and 38 cm length, accommodates 0.5 kg solid substrate. The bottom of each tray is an open mesh (with 0.5-mm pores) to maintain temperature and relative humidity (RH) as uniformly as possible in the chamber during SSF. Thus, a chamber has an overall SSF area of 1.2 (0.32 × 0.38 × 10) m^2 and may hold ≥5 kg solid substrate fitted on laboratorial scale. The chambers are chained tightly by 2 cm-diameter PVC pipes for synchronous production. Sterile mist from a moisture generator containing 3.5 liter distilled water was slowly piped into the series chamber system to maintain nearly saturated RH through an "日" type circle. Subsequently, moisture supply was ceased after 12 h but high RH was retained in the following 4-5 days for growth. The transparency of its door and walls permits

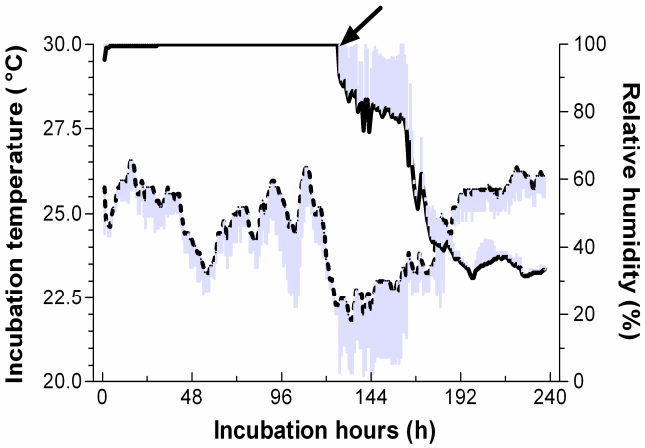

Figure 2. Trends in the hourly mean records of temperature (Dotted lines) and relative humidity (solid lines) within the fermention chamber. The arrow in graph indicates the time at which the conidiation was terminated by opening the moisture outlets and multifunction holes or slightly opening the door.

entry of natural light, favoring fungal conidiation and solid culture observation during SSF. There are also three multifunction holes for spare use on the side of wall.

Preparation of solid substrate

Low-quality rice (e.g., indica rice grown in early season in Southern China or long stored) is chosen as solid substrate due to larger surface area in smaller particles of an equal volume. Prior to SSF, preparation steps of rice cultures are carried out following previous reports (Ye et al., 2006). The rice ready for inoculation is usually in moderate firmness with a water content of ~38%.

Synchronous production trials in series system

To test the performance of the series SSF chamber system, six chambers were involved simultaneously for production of fungal conidia in Hangzhou, Zhejiang Province. Liquid culture of six strains with mycelial biomass of ~18 mg ml^{-1} was mixed with the steamed rice at the ratio of ~100 ml kg^{-1}. Uniform inoculation was achieved by agitating the mixture in a stirrer (VFM-20, Hengfeng Co.). Well mixed rice with no excessive water dripping was then spread *ca* 1 cm thick in trays (0.5 kg per tray in dry weight).

Synchronous production included 5-kg rice per chamber standing in a 10-m^2 room, in which temperature was controlled around 25°C by a 1.18-kw air conditioner. Before use, room was treated overnight under ultraviolet light. A modified moisture generator maintained the nearly saturated RH through pipe transmission for 12 h as the switch was open and ventilated the filtrated air as the switch was closed. When rice grains were generally covered with a heavy layer of yellowish powder, conidiation was terminated by rapid RH reduction, which was achieved by opening moisture outlets and multifunction holes or slightly opening the door. The resultant rice cultures were dried overnight in a ventilation oven at 32°C, followed by harvest of fine conidial powder using a cyclone spore separator 'MK- V' (CABI Bioscience, Silwood Park, Ascot, Berks, UK). Three samples of the conidial powder from each tray were then taken for the estimates of percent water content (based on weight loss of 1 g powder dried at 120°C for 2 h), of conidia g^{-1} powder (based on microscopic counts in hemocytometer) and of viability percent (based on counts of germinated conidia in 24 h liquid culture shaken at 25°C). Rice consumption rate in each trial was estimated equally based on the weight of the rice residues after harvest. These estimates and calculations were used as indices for the production of aerial conidia.

To monitor temperature and RH inside the six chambers during series SSF, both variables were hourly recorded by three external input of HOBO® data logger (Onset Computer Corporation, USA) insert in the multifunction hole which centre in consolidated glass side of the chamber and analyzed using BoxCar® Pro 4 software.

RESULTS AND DISCUSSION

Simultaneous controllability of temperature and RH

Hourly records from the three data loggers during SSF provide an overview to the controllability of both temperature and RH within the series chamber standing in the workshop-like room (Figure 2). During fungal growth and conidiation (days 1-5), daily means (± SD) of the two variables were 24.9 (± 0.8) and 99.8% RH (± 0.2%) in trial. Generally, both variables were well controlled in different periods despite slight variation within the chamber. Recorded time lasted for 10 days until RH was decreased to ~30%. Temperature in day 5 was down to almost 22°C because of the volatilization of water bead in chamber when door and multifunction holes were opened. Then it rose to 25°C smoothly with the air between chamber and room exchanged adequately. The RH was controlled more readily through chained pipes by the mist generator than was the temperature by air conditioner.

Synchronous conidia production in series SSF

Overall, the chamber worked very well in trials. The first 3 days witnessed rapid growth of the fungal agents on rice, followed by desirable conidiation in the following 2 days. Under controlled conditions, the whole production process was completed within 10 days (Figure 2). Good quality powder of aerial conidia was readily separated from the dried cultures by the cyclone spore separator MK-V and automatically collected into its front product cylinder. Larger particles such as substrate and mycelial powder collected in its rear cyclone enable to be obtained synchronously.

Indices for conidial production in the chamber before or after Nomal Vaccum Dehydration (BVD) were listed in Table 2. Harvested spores may require further drying (using a desiccator) to maximize storage period before packaging. The conidial powder of six strains (Mf5628, Ma456, Bb734, Bb2860, Pfr6206, Pfr3843) harvested from 5 kg rice cultures weighed 137.5, 147.1, 101.2, 89.2, 55.0 and 40.0 g, respectively and had a variable range of water content.

On average, conidial yield of two strains (Mf5628, Ma456) reached 34.4 and 36.8 g powder kg^{-1} rice, including 0.6×10^{11} and 0.8×10^{11} conidia g^{-1} powder. Those

Table 2. Indices for the synchronous production of *M.* spp., *B. bassiana* and *P. fumosoroseus* conidia in the series solid-state fermentation chamber system.

Production indices	Strains (Mean ± SD) [a]					
	M. spp.		*B. bassiana*		*P. fumosoroseus*	
	Mf5628	Ma456	Bb734	Bb2860	Pfr6206	Pfr3843
Before NVD [b]						
Conidial powder (g /kg rice)	34.4±5.7a	36.8±9.3a	25.3±1.6b	22.3±9.4bc	13.8±4.0cd	10.0±3.1d
Water content (%)	6.6 ±2.0a	14.1±6.0a	13.0±8.0a	6.8±1.6a	6.7±0.7a	8.1±0.6a
Conidia/g powder ($\times10^{11}$)	0.6±0.1 c	0.8±0.1 c	2.1±0.2 a	2.1±0.1 a	1.3±0.1 b	1.3±0.1 b
Conidia/kg rice ($\times10^{12}$)	2.2±0.0 d	2.7±0.0 c	5.4±0.0 a	4.6±0.0 b	1.8±0.0 e	1.3±0.0 f
Conidial viability (%)	96.3±0.6a	95.7±0.9a	91.9±0.4b	95.1±1.3a	95.3±0.3a	96.4±0.4a
Mycelial powder (g/kg rice)	5.7±1.0a	9.3±6.5a	4.3±0.3a	9.4±2.5a	4.0±1.0a	3.1±0.4a
Overall biomass (g/kg rice) [c]	40.1±9.1ab	46.1±7.9a	29.6±1.8bc	31.7±5.1b	17.7±2.7cd	13.1±0.7d
Rice Water content (%)	8.2±2.0bc	15.5±3.5a	10.1±1.1b	15.2±3.4a	4.9±1.1c	9.3±0.4bc
Rice consumption rate (%)	13.9±8.8a	9.5±4.2a	6.4±3.4a	4.3±2.0a	14.0±5.8a	12.2±3.3a
After NVD						
Conidial Water content (%)	4.0±0.1b	6.9±0.4a	1.1±0.3d	1.8±0.3d	3.0±0.6c	1.5±0.1d
Conidial viability (%)	95.0±1.0a	96.0±1.0a	93.7±1.2a	95.3±0.6a	95.0±1.0a	95.7±1.5a

[a] Each table entry was based on measurements or observations of three rice culture samples from each of the 10 trays (0.5 kg rice per tray) in each trial. Means with different lowercase letters in each line differed significantly (Tukey's HSDs, $P<0.05$; df =5, 18 for all F tests).
[b] NVD means Nomal Vaccum Dehydration.
[c] The sum of fine conidial powder and coarse mycelial powder.

indices were significantly greater than that of Bb734 and Bb2860 (25.3 and 22.3 g powder kg^{-1} rice, including 2.1 $\times10^{11}$ and 2.1 $\times10^{11}$ conidia g^{-1} powder), and also greater than that of Pfr6206 and Pfr3843 (13.8 and 10.0 g powder kg^{-1} rice, including 1.3 $\times10^{11}$ and 1.3 $\times10^{11}$ conidia g^{-1} powder). In a series chamber, Mf5628 and Ma456 produced 2.2 $\times10^{12}$ and 2.2 $\times10^{12}$ conidia kg^{-1} rice, respectively. While conidial powder yield of *Beauveria* per kg rice were less than *Metarhizium*, the conidia counts (5.4 $\times10^{12}$ conidia kg^{-1} rice and 4.6 $\times10^{12}$ conidia kg^{-1} rice) were greater.

The conidia counts of *Peacilomyces* were the least. It was 1.8 $\times10^{12}$ and 1.3 $\times10^{12}$ conidia kg^{-1} rice. Before NVD, water content of six strains ranged from 6.6% to 14.1%, however, conidial viability of them was not significant different, obviously over 91% (91.9%~96.4%). After NVD, this conidial powder could be easily dried to 3.05%(1.1%~6.9%) water content in a vacuum drier for long-term storage under cool conditions and then used to prepare oil formulation for ultra-low volume spray or emulsifiable formulation for field trials onto crops (Pu et al., 2005). Those results suggested that the series chambers could effectively product high-quality and sufficient-quantity conidia powder for crop protection.

The average counts per gram conidia produced in series SSF, such as Bb2860 strain, reached 2.1 $\times$ 10^{11}, which was greater than 1.7 $\times$ 10^{11} in upright multitray conidiation chamber reported by Ye et al. (2006). Furthermore, the Bb2860's absolute conidia yield approached to 4.6 $\times$ 10^{12} conidia kg^{-1} rice in series SSF chamber, a greater than threefold increasing over the previous operational chamber.

In addition to cyclone extraction of conidial powder, total average concentration (density) of conidia in the end product (pure conidial powder) is 2.1 $\times$ 10^{11} g^{-1} produced in a series SSF chamber, which was approximately 21 times higher than 1.1 – 1.2 $\times$ 10^{10} g^{-1} produced in a packed-bed bioreactor reported by Kang et al. (2005). It seems that the advanced dual-cyclone technology (separating the conidia from solid substrate) should be effectively explored deeply for further formulation and application. This technology plays a great role in the isolating process of high quality conidia powder.

Moreover, a small amount of mycelial debris in the form of coarse powder was also obtained at the yield of 6.0 (3.1-9.4) g kg^{-1} rice (Table 2). This coarse powder was potentially useful for preparations of certain fungi, or discarded.

Concluding remarks

In summary, series fermentation chamber especially designed for synchronous conidia production of fungal biocontrol agents is featured with high production, stable work-function, low manufacture cost, and easy operation and monitoring. To testify the amplification ability of series chamber system, the spore production indices of strain Ma456 had been detected in the big-size chamber. Data taken from the series fermentation chamber and big-size chamber were compared with the same way. Conidial

powder (g /kg rice) reached 60.84% of the smaller chamber (22.4 ± 9.3 VS 36.8 ± 9.3), and the other indices such as water content (14.5% VS 14.1%), counts of conidia powder per gram (0.7×10^{11} VS 0.8×10^{11}) and conidial viability (96.7% VS 95.7%) were close (unpublished data). Those results seemed satisfactorily to embody the amplification efficiency of bulk series fermentation and its competence. And data measured from sorts of stains would be detected considerately for its efficiency in the following studies.

The capacity to produce 10^{13} spores at a cost competitive with the per hectare costs of chemical insecticides has historically represented an important goal in commercial development (Feng et al., 1994; Wraight et al., 2001; Faria and Wraight, 2007). One series chamber can produce a quantity of fungal pathogens conidia for a spray of 2.3 ha at the cost of 5 kg rice within ~10 days, such as Bb2860 strain. Furthermore, this cost is likely to be decreased if the residue rice is recycled in production. Thus, the series chamber system would be of high potential production of aerial conidia, of effective and quick evaluation system, for fungal biocontrol agents as well as other filamentous fungus.

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REFERENCES

Bapat PM, Kundu S, Wangikar PP (2003). An optimized method for *Aspergillus niger* spore production on natural carrier substrates. Biotechol. Prog. 19(6): 1683-1688.

Deshpande MV (1999). Mycopesticide production by fermentation: Potential and challenges. Crit. Rev. Microbiol. 25(3): 229-243.

Faria M, Wraight SP (2007). Mycoinsecticides and Mycoacaricides: A comprehensive list with worldwide coverage and international classification of formulation types. Biol. Control, 43: 237-256.

Feng MG, Poprawski TJ, Khachatourians GG (1994). Production, formulation and application of the entomopathogenic fungus *Beauveria bassiana* for insect control: current status. Biocontrol. Sci. Technol. 4: 3-34.

Holker U, Hofer M, Lenz J (2004). Biotechnological advantages of laboratory-scale solid-state fermentation with fungi. Appl. Microbiol. Biotech. 64(2): 175-186.

Krishna C (2005). Solid-state fermentation systems -An overview. Crit. Rev. Biotechnol. 25(1-2): 1-30.

Nava I, Gaime-Perraud I, Huerta-Ochoa S, Favela-Torres E, Saucedo-Castaneda G (2006). *Penicillium commune* spore production in solid-state fermentation of coffee pulp at laboratory scale and in a helical ribbons rotating reactor. J. Chem. Technol. Biotechnol. 81(11): 1760-1766. Nizamuddin S, Sridevi A, Narasimha G (2008). Production of -galactosidase by *Aspergillus oryzae* in solid-state fermentation. Afr. J. Biotechnol. 7(8): 1096-1100.

Ohgren K, Vehmaanpera J, Siika-Aho M, Galbe M, Viikari L, Zacchi G (2007). High temperature enzymatic prehydrolysis prior to simultaneous saccharification and fermentation of steam pretreated corn stover for ethanol production. Enzyme Microb. Technol. 40(4): 607-613.

Pandey A, Selvakumar P, Soccol CR, Nigam P (1999). Solid state fermentation for the production of industrial enzymes. Curr. Sci. 77 (1): 149-162.

Pu XY, Feng MG, Shi CH (2005). Impact of three application methods on the field efficacy of a *Beauveria bassiana*-based mycoinsecticide against the false-eye leafhopper, *Empoasca vitis* (Homoptera: Cicadellidae) in tea canopy. Crop Prot. 24: 167-175.

Roberts DW, Leger RJS (2004). *Metarhizium spp.*, cosmopolitan insect-pathogenic fungi: Mycological aspects. Adv. Appl. Microbiol. 54: 1-70.

Shan LT, Feng MG (2006). Comparative susceptibility of *Myzus persicae* to 16 strains of *Metarhizium spp.* from different host insects and geographic regions. Acta Microbiologica Sinica, 46(4): 602-607.

Wraight SP, Jackson MA, de Kock SL (2001). Production, stabilization and formulation of fungal biocontrol agents. In: Butt T, Jackson C, Magan N (eds), Fungal biocontrol agents-progress, problems and potential, CAB International, Wallingford, UK, pp. 253-287.

Ye SD, Ying SH, Chen C, Feng MG (2006). New solid-state fermentation chamber for bulk production of aerial conidia of fungal biocontrol agents on rice. Biotechnol. Lett. 28: 799-804.

Yu J, Feng MG (2006). Screening of thermotolerant *Beauveria bassiana* strains based on conidial response to thermal stress. Acta Mycosystem, 25(2): 278-283.

Kang SW, Lee SH, Yoon CS, Kim SW (2005). Conidia production by *Beauveria bassiana* (for the biocontrol of a diamondback moth) during solid-state fermentation in a packed-bed bioreactor. Biotechnol. Lett. 27: 135-139.