A NOVEL METHOD TO OPTIMIZE CULTURE CONDITIONS FOR BIOMASS AND SPORULATION OF THE ENTOMOPATHOGENIC FUNGUS *BEAUVERIA BASSIANA* IBC1201

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ABSTRACT

Biomass yields and sporulation of *Beauveria bassiana* was concerned on culture conditions, environmental factors and cultivation method. We optimized the best culture conditions for biomass yields of *B. bassiana* IBC1201 with the novel "two-stage" cultivation method as well as orthogonal matrix method. Firstly, we cultured spore suspension on the basal medium (sucrose 19.00 g, soy peptone 4.06 g, K_2HPO_4 1.00 g, KCl 0.50 g, $MgSO_4$ 0.50 g, $FeSO_4$ 0.10 g and 17.00 g Bactor) for the first stage culture of 4 days under room condition. Then, we transferred them to another defined medium (Cellobiose 9.52 g, urea 1.70 g, $ZnSO_4•7H_2O$ 0.05 g/L, $MnSO_4•H_2O$ 0.005 g/L, $CaCl_2$ 1.00 g/L, $CuSO_4•5H_2O$ 0.05 g/L and 17.00 g Bactor) for more 4 days cultivation with the environmental factors combination of water potential -1.2 MPa /pH 3 /12 h light cycle/23 ℃ for biomass yields, and with the environmental factors combination of water potential -0.8 MPa /pH 3 /24 h light cycle/23 ℃ for spore yields. These results provided important information for mass production (including biomass and spore yields) of this great potential biocontrol fungus.

Key words: Nutrition, Environment, Biomass, Sporulation, *Beauveria bassiana*

INTRODUCTION

Entomopathogenic fungi have attracted substantial attention as biological control agents of insect pests (3). The entomopathogenic fungus *Beauveria bassiana* is widely distributed throughout the world and can be isolated from insects, mites, and soil (25). This fungus can infect a wide range of hosts and shows promise for commercial development as a biological control agent of agricultural insect pests (25). As evidence of its broad host range, *B. bassiana* is an effective pathogen of adult sawtoothed grain beetles (23), and the isolate tested in this paper was obtained from the cuticle of a locust.

The practical use of *B. bassiana* is limited, however, because its mass production is thus far inefficient, and this inefficiency has slowed its commercialization as a mycopesticide (36). The commercialization of potential control agents often depends on the development of a method for efficient, large-scale production.

Development of an efficient method for mass production of a mycopesticide requires detailed knowledge of the nutritional requirements for the growth and sporulation of the fungus. Leite *et al*. (31) examined the effects of carbon and nitrogen sources on the growth of three genera of *Entomophthorales*: *Batkoa, Furia*, and *Neozygites*; the fungi had similar responses

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to various carbon sources but different responses to various nitrogen sources. Liu and Chen (33, 34), who examined the nutritional requirements of three nematophagous fungi (*Hirsutella rhossiliensis*, *Pochonia chlamydosporia*, and Arkansas fungus 18), reported that some carbon and nitrogen sources supported growth in both solid and liquid media, while other carbon and nitrogen sources could not be utilized either with solid or liquid media. Jackson and colleagues found that carbon concentration and C:N ratio of the medium significantly affected the number of conidia produced by the plant-pathogenic fungus *Colletotrichum truncatum* in liquid culture (19,20,21,43). All of these reports, and many others, demonstrate that the nature of the carbon and nitrogen sources, together with carbon concentrations and C:N ratios of culture media, greatly affect fungal growth and sporulation.

Development of an efficient method of mass production of a mycopesticide also requires detailed knowledge of the effects of environmental factors. Hyphal extension and pycnidial production of four isolates of *Coniothyrium minitans*, a fungus that parasitizes plant-pathogenic fungi, occurred over a pH range of 3–8, with optimum values between pH 4.5 and 5.6 (37). A pH range between 3 and 6 allowed active mycelia extension of the plant-pathogenic fungus *Potebniamyces pyri* (47). Sporulation of the entomopathogenic fungus *Metarhizium anisopliae* was directly related to the water content of the medium (6). The water potential that supported mycelial growth and sporulation of the plant-pathogenic fungus *Verticillium dahliae* ranged from -100 to -120 bars (18). Temperature is another important factor affecting fungal growth and sporulation (35). The effect of temperature on *B. bassiana* has been studied (7,12,13,22,44). A temperature of 25°C was reported optimum for *B. bassiana* by Fargues *et al.* (12), while James *et al.* (22) reported that *B. bassiana* grew most rapidly at a continuous temperature of 25–32°C. Light can also significantly influence mycelial growth and conidiation (30). The mycelial growth of the plant-pathogenic fungus *Sphaeropsis pyriputrescens* was enhanced by

fluorescent light (26), and light greatly affected pycnidia formation by some Sphaeropsidales species (1,28), including *Ascochyta pisi* (29) and *Botryodiplodia theobromae* (8). Light significantly increased the pycnidial production but not the growth of four isolates of *C. minitans* (37).

In all of the reports cited thus far in this Introduction, fungal sporulation was studied on the same medium that supported the initial vegetative growth. Because the vegetative growth changes the nutrition of the medium, the nature of the medium that supported sporulation was poorly defined. To deal with this problem and to improve our understanding of the often different requirements for sporulation vs. vegetative growth, Sun *et al.* (46) developed a "two-stage" cultivation method. In this method, the fungus grows vegetatively on one medium before it is transferred to a second medium for sporulation.

The long-range goal of the current study was to develop *B. bassiana* IBC1201 as a mycopesticide. We expect that the response of this isolate to nutrition and environment could differ from that of other isolates because *B. bassiana* is genetically variable; for example, isolates differed in their response to high temperature and low water availability (11,12,13). The specific objectives of this study were to quantify production of biomass and spores by *B. bassiana* IBC1201 as affected by combinations of carbon and nitrogen sources, carbon concentration, C:N ratios, and environment (pH, water potential, temperature, and light). The "two-stage" cultivation method was used to obtain better definition of the requirements for vegetative growth vs. sporulation.

MATERIALS AND METHODS

Fungal strain and inoculum

The tested biocontrol fungi *B. bassiana* IBC1201 was originally isolated from locust by C.S. Deng from Tianjin province in China, and now was deposited in the Center of General Microorganisms Culture Collection (CGMCC) in the

Institute of Microbiology, Chinese Academy of Sciences

Conidial inoculum was prepared according to Gao *et al*. (15). The conidial concentration was determined using a haemocytometer and adjusted to 10^7 spores/mL, and the suspension was used for inoculation.

Nutrition requirements for the sporulation of *B. bassiana* IBC1201 by this novel method

The source of chemicals used: The chemicals used were sucrose, glucose, cellobiose, urea, K_2HPO_4, $MgSO_4$, $FeSO_4$ (Beijing Chemical Reagents Company, Beijing China); yeast extract (Sigma Chemical Co.); soy peptone (Shanghai Chemical Reagents Company, Shanghai China) and KCl (Nanjing Chemical Reagents Company, Nanjing China).

The basal medium: The basal medium was composed of sucrose 19.00 g (equal to 8.00 g carbon), soy peptone 4.06 g (equal to 0.33 g nitrogen), K_2HPO_4 1.00 g, KCl 0.50 g, $MgSO_4$ 0.50 g, $FeSO_4$ 0.10 mg and 17.00 g Bactor (Difco) agar per liter. We used this medium for the first stage vegetative culture of 4 days.

Effects of carbon concentration and carbon to nitrogen ratio: Carbon concentrations were adjusted with sucrose (42 % carbon) to 1, 2, 4, 8 and 16 g/L, and nitrogen concentrations were adjusted with soy peptone (8 % nitrogen) to 0.2, 0.4, 0.8 and 1.6 g/L, which respectively replace the carbon and nitrogen source in basal medium. The combinations of different carbon and nitrogen concentrations resulted in C:N ratios from 0.625:1 to 80:1. These carbon concentrations and C:N ratios were used in the second stage culture for the sporulation of another 4 more days. After this experiment, we had the optimal carbon concentration of 4 g/L with C:N ratio of 5:1.

Effects of carbon and nitrogen source: The combinations of carbon sources including sucrose, glucose, cellobiose and nitrogen sources including of soy peptone, urea and yeast extract were tested. Based on the carbon concentration 4 g/L (pure carbon per liter calculated by percentage of carbon element in the molecule) and C:N ratio of

5:1 (1 g/L nitrogen concentration, pure liter calculated by percentage of nitrogen element in the molecular), we had the combinations of different carbon and nitrogen sources for sporulation with this novel method. For each combination, we added them to the basal medium to replace the sucrose and soy peptone as sporulation medium for the second stage culture of more 4 days. The basal medium for sporulation of another 4 days was used as control.

Effects of mineral elements: After tested the components and concentration gradients of six mineral elements for sporulation of the isolate with one-factor-at-a-time method, we had the optimal components for the sporulation of *B. bassiana* IBC1201, including of $ZnSO_4 \cdot 7H_2O$ 0.05 g/L, $CaCl_2$ 1.00 g/L, $MnSO_4 \cdot H_2O$ 0.05 g/L, and $CuSO_4 \cdot 5H_2O$ 0.05 g/L.

Effects of environmental factors on sporulation of *B. bassiana* IBC1201 by this novel method

The novel method of "two-stage" cultivation in plates was applied to evaluate the effects of pH, water potential, dark/light cycle and temperature on the second stage culture of 4 more days on sporulation of the biocontrol fungi. Water potential including -0.3, -0.8, -1.2, -2.1,-3.9 and -7.3 MPa; pH including 3, 4, 5, 6, 7, 8, 9, dark/light cycle including 24 h/0 h, 12 h/12 h, 0 h/24 h, temperature including 20 ℃, 23 ℃, 26 ℃, 29 ℃, 32 ℃ (14).

Orthogonal matrix method

The orthogonal $L_{16}(2^{15})$ was used to obtain the optimal culture conditions in solid for pH, water potential, dark/light cycle and temperature on the certain sporulation medium, successively, after the testing of carbon concentration, carbon to nitrogen ratio, the combination of carbon and nitrogen source, and environmental factors by one-factor-at-a-time with this novel method. We have been got the best nutrition combination by full experiment, now we try to get the optimal combination of nutrition together with environmental factors for sporulation of *B. bassiana* IBC1201 by orthogonal matrix

method under the selected two levels of four environmental factors by orthogonal matrix method under the selected two levels of four environmental factors by 16 experiments, including water potential, pH, light, temperature, water potential and pH, water potential and light, pH and light, water potential and temperature, pH and temperature, light and temperature.

Assays of fungal growth and sporulation

The method for optimize the combination of nutrition and environmental factors was the same according to Gao *et al*. (14), colonies on each plate were collected with a sterile scalpel, weighed and transferred to a 50 ml centrifuge tube containing 10 ml sterile 0.05 % Tween 80 solution. Spores were dislodged, and the resulting suspension was vigorously mixed in a tube with a vortex shaker for 3–5 min, the number of spores per colony was determined using a haemocytometer (15).

Statistical analysis

The data (spore number and dry mycelial mass) were analyzed by one-way analyses of variance (ANOVA) with Statistical Analysis System software (Version 8.2, SAS Institute, Cary, NC), and means were separated using Fisher's protected least significant difference (LSD). A logarithmic transformation was applied to the sporulation data before statistical analysis.

RESULTS

Effects of nutrition on sporulation of *B. bassiana* IBC1201

Results (Table 1) indicated that the optimal carbon concentration was 4 g/l and C:N ratio was 5:1 for sporulation of *B. bassiana* IBC1201 by "two-stage" cultivation method.

There were significant effects of the combination of carbon and nitrogen sources on sporulation of the isolates (Table 2). The combination of cellobiose and urea obtained the

most spore yields. In the following experiments, we used this combination as carbon and nitrogen source together with other components in basal medium for optimization of culture conditions.

Table 1. Effects of carbons concentrations (1, 2, 4, 8 and 16) and carbon-to-notrogen ratios (from 0.625 to 80) on the sporulation of *B. bassiana* IBC1201 (10^5/ml) by "two-stage" cultivation method.

Carbon concentration (g/L)	Carbon-to-nitrogen ratio	Spore yields	
1	5.000	38.800	h
1	2.500	143.800	d
1	1.250	104.000	e
1	0.625	101.000	e
2	10.000	18.700	ijk
2	5.000	11.000	kl
2	2.500	37.400	h
2	1.250	71.000	f
4	20.000	70.400	f
4	10.000	23.400	ij
4	5.000	293.700	a
4	2.500	242.400	b
8	40.000	14.000	kl
8	20.000	15.300	jlk
8	10.000	26.000	i
8	5.000	8.200	lm
16	80.000	76.000	f
16	40.000	54.000	g
16	20.000	209.800	c
16	10.000	2.300	m
8(CK)	24.000	38.200	h

*Values are means of three replicates. Values in the same column followed by a same letter are not significantly different (LSD; P ≤ 0.05)

Table 2. Effects of carbon and nitrogen sources on the sporulation of *B. bassiana* IBC1201 (10^5/ml) by "two-stage" cultivation method.

Carbon source	Nitrogen source			CK
	Yeast extract	Soy peptone	Urea	
Cellobiose	1914.3 d	642.7 g	3032.7 a	
Sucrose	2823 b	580 h	327.3 i	35 j
Glucose	1060 f	2078.7 c	1560 e	

*Values are means of three replicates. Values in the same column followed by a same letter are not significantly different (LSD; $P \leq 0.05$)

Effects of environmental factors on sporulation of *B. bassiana* IBC1201

We selected two better levels for sporulation of each environmental factor for the orthogonal research, respectively was water potential -1.2 and -0.8 MPa, pH 3 and 6, 0 h/ 24 h and 12 h/12 h dark/light cycle, temperature 23ºC and 29ºC. The levels of environmental factors are shown in Table 3.

Table 3. Effects of environmental factors (pH, water potential, dark/light cycle and temperature) on growth and sporulation of *B. bassiana* IBC1201 by "two-stage" cultivation method.

Fungal growth (g per cm²)	pH	3	4	5	6	7	8	9
		0.034 ab	0.031 b	0.039 a	0.032 ab	0.022 c	0.034 ab	0.031 b
	Water potential (Mpa)	-0.3	-0.8	-1.2	-2.1	-3.9	-7.3	
		0.031 b	0.043 ab	0.038 ab	0.042 ab	0.034 b	0.052 a	
	Temperature (ºC)	20	23	26	29	32		
		0.046 a	0.047 a	0.038 a	0.039 a	0.038 a		
	Dark/Light cycle	0 h /24 h	12 h/12 h	24 h/0 h				
		0.043 a	0.035 a	0.032 a				
Sporulation (10^5 conidia per cm²)	pH	3	4	5	6	7	8	9
		2.34 a	0.41 b	0.84 b	1.39 ab	1.20 ab	0.85 b	0.86 b
	Water potential (Mpa)	-0.3	-0.8	-1.2	-2.1	-3.9	-7.3	
		0.69 c	6.97 b	13.53 a	2.21 bc	2.56 bc	0.08 c	
	Temperature (ºC)	20	23	26	29	32		
		128.95 ab	158.41 a	119.24 ab	135.10 ab	82.15 b		
	Dark/Light cycle	0 h /24 h	12 h/12 h	24 h/0 h				
		30.12 a	22.50 b	0.83 c				

*Values are means of three replicates. Values in the same column followed by a same letter are not significantly different (LSD; $P \leq 0.05$)

Optimization by orthogonal matrix method

To investigate the relationships between variables of environmental factors and certain medium components and optimize the culture conditions for sporulation, the orthogonal layout of $L_{16}(2^{15})$ was employed based on the design of four factors and two levels (Table 3). According to the orthogonal method, the effect of environmental factors, including pH, water potential, dark/light cycle, and temperature on growth and sporulation was evaluated and shown in the bottom five rows of Table 4. According to the magnitude order of R (maximum difference) in Table 5, the order of the effect of all factors on mycelia growth could be determined as 43.13 (pH) > 38.63 (water potential) > 9.13 (dark/light cycle) > 3.13 (temperature), the results indicated that the effect of 43.13 (pH)

was more important than that of the others three environmental factors; the order of effect of all environmental factors on sporulation could be determined as 0.16 (dark/light cycle) > 0.12 (pH) > 0.09 (temperature) > 0.06 (water potential), the results indicated that the effect of 0.16 (light) was more important than that of the others three environmental factors.

Table 4. Orthogonal experiment of $L_{16}(2^{15})$ of biomass yields and sporulation of *B. bassiana* IBC1201 by "two-stage" cultivation method

Exp. group	A	B	A×B*	C	A×C	B×C		D	A×D	B×D		C×D				Biomass yields (mg per colony)	Sporulation (10^5 per colony)
1‡	1†	1	1	1	1	1	1	1	1	1	1	1	1	1	1	139.00 ± 17.52	2.97 ± 0.10
2	1	1	1	1	1	1	1	2	2	2	2	2	2	2	2	58.33 ± 24.54	3.14 ± 0.07
3	1	1	1	2	2	2	2	1	1	1	1	2	2	2	2	169.33 ± 9.29	2.77 ± 0.02
4	1	1	1	2	2	2	2	2	2	2	2	1	1	1	1	126.00 ± 15.10	2.77 ± 0.04
5	1	2	2	1	1	2	2	1	1	2	2	1	1	2	2	82.33 ± 14.64	3.05 ± 0.03
6	1	2	2	1	1	2	2	2	2	1	1	2	2	1	1	39.33 ± 11.37	2.83 ± 0.06
7	1	2	2	2	2	1	1	1	1	2	2	2	2	1	1	74.00 ± 18.19	2.82 ± 0.02
8	1	2	2	2	2	1	1	2	2	1	1	1	1	2	2	80.00 ± 10.15	2.89 ± 0.01
9	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	132.67 ± 10.97	2.81 ± 0.01
10	2	1	2	1	2	1	2	2	1	2	1	2	1	2	1	193.33 ± 93.22	3.00 ± 0.12
11	2	1	2	2	1	2	1	1	2	1	2	2	1	2	1	119.67 ± 38.11	2.65 ± 0.23
12	2	1	2	2	1	2	1	2	1	2	1	1	2	1	2	164.33 ± 61.58	2.90 ± 0.06
13	2	2	1	1	2	2	1	1	2	2	1	1	2	2	1	120.67 ± 7.57	3.15 ± 0.02
14	2	2	1	1	2	2	1	2	1	1	2	2	1	1	2	108.33 ± 0.58	3.19 ± 0.01
15	2	2	1	2	1	1	2	1	2	2	1	2	1	1	2	89.67 ± 7.64	2.92 ± 0.02
16	2	2	1	2	1	1	2	2	1	1	2	1	2	2	1	143.67 ± 25.15	3.10 ± 0.11

*AxB, AxC, BxC, AxD, BxD, CxD represent the interactions between the factors water potential and pH, water potential and light, pH and light, water potential and temperature, pH and temperature, light and temperature, sucessively.
†The arragement of colunms were decided by orthogonal design for $L_{16}(2^{15})$.
‡Every row of the experimental group number represents one experimental replicate, and every experimental group was replicated thrice.
§Values are mean ± SD of triple determinations.

Table 5. Analysis of environmental factors on biomass production and sporulation of *B. bassiana* IBC1201 with this novel method.

		A	B	A×B	C	A×C	B×C		D	A×D	B×D		C×D			
B*	K_1	765.00	1092.00	936.00	883.00	835.00	920.00	862.00	932.00	1072.00	929.00	999.00	980.00	938.00	881.00	953.00
	K_2	1074.00	747.00	903.00	956.00	1004.00	919.00	977.00	907.00	767.00	910.00	840.00	859.00	901.00	958.00	886.00
	k_1	95.63	136.50	117.00	110.38	104.38	115.00	107.75	116.50	134.00	116.13	124.88	122.50	117.25	110.13	119.13
	k_2	134.25	93.38	112.88	119.50	125.50	114.88	122.13	113.38	95.88	113.75	105.00	107.38	112.63	119.75	110.75
	R	38.63	43.13	4.13	9.13	21.13	0.13	14.38	3.13	38.13	2.38	19.88	15.13	4.63	9.63	8.38
	O	2	1	1	2	2	1	2	1	1	1	1	1	1	2	1
S†	K_1'	23.23	22.96	24.00	24.11	23.55	23.62	23.68	23.12	23.76	23.19	23.44	23.62	23.42	23.19	23.27
	K_2'	23.70	23.92	22.93	22.82	23.36	23.31	23.20	23.84	23.12	23.74	23.52	23.31	23.51	23.74	23.66
	k_1'	2.90	2.87	3.00	3.01	2.94	2.95	2.96	2.89	2.97	2.90	2.93	2.95	2.93	2.90	2.91
	k_2'	2.96	2.99	2.87	2.85	2.92	2.91	2.90	2.98	2.89	2.97	2.94	2.91	2.94	2.97	2.96
	R'	0.06	0.12	0.13	0.16	0.02	0.04	0.06	0.09	0.08	0.07	0.01	0.04	0.01	0.07	0.05
	O'	2	2	1	1	1	1	1	2	1	2	2	1	2	2	2

*Biomass yields (mg per colony)
† Sporulation (10^5 conidia per colony)
K_1 and K_2 are totel content of biomass yields from the level 1 and level 2 seperately; k_1 and k_2 are the mean value of levels 1 and 2 seperately;
K_1' and K_2' are totel spore yields from the level 1 and level 2 seperately; k_1' and k_2' are the mean value of levels 1 and 2 seperately.
R is the maximum of $k_1 \cdot k_2$ minus the minimum of $k_1 \cdot k_2$ and R' is the maximum of $k_1 \cdot k_2$ minus the minimum of $k_1 \cdot k_2$ respectively.
O is the optimal level of biomass yields and O' is the optimal value of spore yields.

To test the effects of four factors, ANOVA was used and shown in Table 6, the factor of water potential and pH had significant effects on biomass yields and the factor of pH, dark/light cycle had significant effects on sporulation. Table 7 shows the effect of combinations of four factors on biomass yields and sporulation. It is demonstrated that the combinations of B2/A1, A1/C2, A1/D1, B1/C2, D1/B1, D1/C2 have the best effect on biomass yields, producing 153.75, 154.25, 165.50, 127.50, 137.25, 137.00 mg per colony biomass yields respectively. To obtain a high mycelia yields, the optimum factors should be water potential -1.2MPa (A1) /pH 3 (B1) /12 h light (C2) /23 °C (D1). It is demonstrated that the combinations of B1/A2, A2/C2, A2/D1, B1/C1, B1/D1, D1/C1 have the best effect on sporulation, producing 3.09, 3.01, 3.04, 3.03, 3.01, 3.00×10^5 per colony spore yields respectively. To obtain a high spore yields, the optimum factors should be water potential -0.8 MPa (A2) /pH 3 (B1) /24 h (C1) light /23 °C (D1).

Table 6. The variance analysis of L$_{16}$(2^{15}) orthogonal test on optimization of environmental factors for biomass yields and sporulation of *B. bassiana* IBC1201.

	Variance source	Sum of square deviation (SS)	Degree of freedom (v)	Mean square (MS)	*F*-ratio	Significance level†
	A	5967.56	1	5967.56	9.49	**
	B	7439.06	1	7439.06	11.83	**
	C	333.06	1	333.06	0.53	
	D	39.06	1	39.06	0.06	
Biomass yields (mg per colony)	A×B	-313.70	1	-313.70	-0.08	
	A×C	1403.30	1	1403.30	0.37	
	A×D	5432.30	1	5432.30	1.45	
	B×C	-381.70	1	-381.70	-0.10	
	B×D	-359.20	1	-359.20	-0.10	
	C×D	533.30	1	533.30	0.14	
	Error	3743.32	5			
	A	0.01	1	0.22	1.56	
	B	0.06	1	0.01	6.56	**
	C	0.104	1	0.002	11.56	**
	D	0.03	1	0.01	3.33	
Sporulation (10^5 conidia per colony)	A×B	-0.12	1	-0.12	-2.32	
	A×C	-0.28	1	-0.28	-5.59	
	A×D	-0.44	1	-0.44	-8.87	
	B×C	-0.13	1	-0.13	-2.70	
	B×D	-0.12	1	-0.12	-2.44	
	C×D	-0.13	1	-0.13	-2.70	
	Error	0.050	5			

†F$_{0.1}$(1,5) = 4.06, F$_{0.05}$(1,5) = 6.610, F$_{0.01}$(1,5) = 16.3.
*F-ratio >F$_{0.1}$
** F$_{0.1}$ < F-ratio < F$_{0.05}$
*** F-ratio > F$_{0.01}$

Table 7. Effects of combinations of environmental factors on biomass yields and sporulation of *B. bassiana* IBC1201

B, C or D	A				B				C			
	A_1		A_2		B_1		B_2		C_1		C_2	
	B†	S‡	B	S	B	S	B	S	B	S	B	S
B_1	119.25	2.91	114.75	3.09								
B_2	153.75	2.84	72.00	2.90								
C_1	118.75	2.91	90.00	2.98	106.50	3.03	102.25	2.86				
C_2	154.25	2.83	96.75	3.01	127.50	2.97	123.50	2.88				
D_1	165.50	2.91	102.50	3.04	137.25	3.01	130.75	2.94	131.00	3.00	137.00	2.94
D_2	107.50	2.84	84.25	2.95	96.75	3.00	95.00	2.79	77.75	2.89	114.00	2.90

A_1, A_2, B_1, B_2, C_1, C_2, D_1, D_2 represent the 1 and 2 levels of water potential, pH, light and temperature.
†Represent the biomass yields (mg per colony).
‡Represent spore yields (10^5 conidia per colony).

DISCUSSION

"Two-stage" cultivation method

Biological control using entomopathogenic fungi will only become feasible if economic methods of mass production are available (24,27). "Two-stage" cultivation method on solid for spores can be produced in an easier step, and industrial scale-up could be enhanced and have a high spore yields compared with liquid fermentation, from liquid to solid or just solid-state production of aerial conidia.

The novel "two-stage" cultivation method was used for culturing the biomass and sporulation of entomophagous fungi. Basal medium for first stage of 4 days fungal growth culture and the second medium for another stage of 4 days sporulation culture were developed with the aid of supporting membranes of cellophane. After incubating for 4 days on basal medium firstly, fresh mycelium and its underlying cellophane were taken off from the agar plate to second stage medium for sporulation of another 4 days, then to determine its biomass before spore production was quantified. This method we have used in our previous study, we got the nutrition for sporulation of *B. bassiana* IBC1201 on second stage medium, and also got the two better levels of 4 environmental factors on sporulation of *B. bassiana* IBC1201, then we combined them together by orthogonal matrix method to got a better combinations including nutrition and environmental under "two-stage" cultivation method based on biomass and spore yields, which indicating that fungal biomass could be well estimated by mycelia fresh weight (45), which lead to a high production than traditional method (unpublished data).

Effects of nutrition on sporulation of *B. bassiana* IBC1201

Barnes *et al*. (2) have been reported that *B. bassiana* grew best on melizitose but sporulated best on sucrose, trehalose, and glucose, grew least on rhamnose, sporulated least on sorbose. The result also indicated that the difference between the nutrition for mycelial growth and sporulation, while they did not used the "two-stage" cultivation method to define the differences, and also did not combine carbon and nitrogen sources one by one to optimize the combinations together with carbon concentrations and C:N ratios for mycelia and sporulation respectively.

The nutrition combination with this method greatly accelerated the spore production with low cost. Our results proved that different combinations of carbon and nitrogen sources lead to different spore yields. The sporulation of this carbon concentration and C:N ratio resulted in a spore production of 293.7×10^5 spores/mL, which was about 7.7 times

spore yields than control plates by traditional culture method on carbon concentration of 8 g/L with a C:N of 24:1.

Effects of environmental factors on sporulation of *B. bassiana* IBC1201

Environmental factors which have different effects on biomass and sporulation, in our research, pH is important to biomass, while dark/light cycle was the key to sporulation for *B. bassiana* IBC1201. So, we could culture them under different environmental conditions to get the certain yields we want to have, biomass or spores. It is demonstrated that the combinations of different environmental factors have different effects on biomass and spore yields.

Although environmental constraints must ultimately be tested under wild conditions, Fargues and Remaudiere (13) recommend assessment of intra-specific variations in response to abiotic stress in laboratory assays before the commencement of wild tests, to better predict efficacy under wild conditions. A correlation between optimum temperature for fungal growth and infection and subsequent mycosis has been reported by several workers (12). Most in vitro assays to assess fungal response to temperature have been done at constant temperatures. Few entomogenous fungi are active above 32 °C. This temperature is frequently exceeded in tropical habitats. Fortunately such temperatures often only retard fungi, but do not kill them (38). Both temperature and humidity affect survival and germination of *B. bassiana* (5). Although, focus is usually placed on the effect of a particular variable, environmental parameters interact with each other in their impact on entomopathogens. While possible, these factors should be addressed interactively (17). Therefore, the combined effect of high temperature and low water availability on vegetative growth of *B. bassiana* was studied. Variations in isolate response to high temperature and low water availability conditions were observed, as anticipated from previous reports (11,12,13). Hallsworth and Magan (16), and Sivasankaran *et al*. (44) reported that *B. bassiana* isolates require high levels of

water activity in the surrounding medium for germination.

Optimization by orthogonal matrix method

Many reports concern the orthogonal matrix method just on nutritional components, or just environmental factors, while our research combined them together. The first level we choose was the best for sporulation of *B. bassiana* IBC1201 by one-factor-at-one-time method, while after the orthogonal matrix method, we found that certain nutrition not all combined the first level of environment, mostly was the mixture of these two levels, which also means that the orthogonal method was necessary to optimize the sporulation culture conditions including nutritional and environment factors. In this study, we used the former two levels of environmental factors with certain nutrition to optimize the culture conditions with orthogonal matrix method after doing the full experiment of nutrition and environmental conditions.

Combinations of the three fields

For a fungal pesticide, hyphae and conidia are the main biocontrol entities and generally a large mass of inocula of a biocontrol fungus is necessary for efficient application in the fields. However, their limited production outputs restrict the development of fungal agents to a great extent. It has been shown that alternative nutritional components can significantly influence growth and sporulation of many fungi (4,10,31). Higher carbon concentrations or C:N ratios reduced conidiation of the plant-pathogenic fungus *Helminthosporium solani* (9). In contrast, higher C:N ratios increased sporulation for *Talaromyces flavus*, which is a fungal biological control agent of plant-pathogenic fungi (10). Fungal growth was determined on dry weight basis for mycelia cultured in liquid by other researchers (32,39,40,41,42), however, the measurements of hyphal growth and conidiation had to be conducted in two separate experiments (on agar and in liquid cultures). To simplify testing procedures, biomass was determined by fresh mycelia weight and conidiation was determined by spore

numbers per colony. In this paper, we also found that fungal biomass was not necessarily correlated with fungal sporulation under the orthogonal matrix method, which have been proved in our previous study, such as separately the environmental factors and nutrition factors on mycelia growth and sporulation. This phenomenon proved that our method is the right way to separate the mycelia growth and sporulation, and the result could help us have better spore yields under lower cost.

In conclusion, this report provides opportunities to find the most effective and commercially available nutritional and environmental factors, through screening the combination of carbon and nitrogen source, carbon concentration, C:N ratio, together with environmental factors including water potential, pH, dark/light cycle and temperature to facilitate the mass production of a potential high-virulence biocontrol isolate *B. bassiana* IBC1201.

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