academicJournals

Vol. 7(1), pp. 1-6, February, 2016
DOI: 10.5897/JYFR2015.0162
Article Number: C7E8C2657878
ISSN 2141-2413

http://www.academicjournals.org/JYFR

Journal of Yeast and Fungal Research

Full Length Research Paper

An improved method to optimize the culture conditions for biomass and sporulation of mycoparasitic fungus *Trichoderma viride* TV-1

Gao L.

State Key Laboratory for Biology of Plant Disease and Insect Pests, Institute of Plant Protection, Chinese Academy of Agricultural Sciences (CAAS), Beijing, China.

Received 20 October, 2015; Accepted 15 December, 2015

***Trichoderma viride*, the most promising biocontrol agent is able to control a wide range of plant pathogens. It is one of the most widely used species against plant disease and can improve the plant growth and crop yields. Biomass yields and sporulation of this fungus depends on the culture conditions (culture method), nutritional requirements (carbon and nitrogen source, mineral elements, carbon concentration, carbon to nitrogen ratio), together with environmental factors including water potential, pH, dark/light cycle and temperature. The study optimized the best culture conditions for biomass yields of *T. viride* TV-1: 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, $FeSO_4$ 0.01 g and 17.00 g agar (Bactor)) for the first stage culture of 4 days under room condition for fungal growth, then they were transferred to sporulation medium (cellobiose/yeast extract, with the carbon concentration of 2 g/L and carbon to nitrogen ratio of 10:1, $ZnSO_4.7H_2O$ 0.25 g/L, $Na_2MoO_4.2H_2O$ 0.125 g/L, H_3BO_4 0.05 g/L and 17.00 g Bactor) for 4 days, together with the environmental factors combination of water potential -3.9MPa/pH 8/0 h light/23°C for biomass yields, and -3.9 MPa /pH 3/0 h/23 ℃ for sporulation yields. These results provided important information on mass production (including biomass and spore yields) of this potential biocontrol fungus.**

Key words: Nutrition, environment, biomass, sporulation, *Trichoderma viride.*

INTRODUCTION

Increasing concern for health and environmental hazards associated with the use of chemical pesticides lead to serious consideration of sustainability in agriculture. Biocontrol agents (BCA) are promising potential alternatives to reduce the input of chemical pesticides and residues on postharvest fruits (Costa et al., 2001; Pal and Kumar, 2013). *Trichoderma viride* used as BCA has been reported in lots of literature (Bailey and Gilligan,

E-mail: lgao@ippcaas.cn.

1997; Zhao et al., 1998; Ejechi, 2001; Boby and Bagyaraj, 2003; Ji et al., 2005), being the most promising BCA able to control a wide range of plant pathogens (Guo et al., 2002), but also can improve plant growth and crop yields (Kolembet et al., 2001).

Production of the BCA with large numbers of spores or conidia and low cost are the key issues for commercial application of the BCA (Wang et al., 2003). However, production of *Trichoderma* BCA is less prevalent till now. One of the main limitations for commercial application is the high-cost of using raw materials. Many reports mentioned low-cost agro-industrial residues, such as wheat bran (Nidheesh et al., 2015a), oat bran, grape mare, rice straw and animal/seafood byproducts (Nidheesh et al., 2015b), oatmeal (avena sativa) (Motta and Santana, 2012), which were not with defined nutrients. Few studies mentioned synthetic media for production of *Trichoderma* sp., (Lewis and Papavizas, 1983; Gupta et al., 1997). However, no system combination studies of nutrients and environmental were studied. At this crux, a defined nutrient together with combination of environmental factors for growth and sporulation of *Trichoderma* strains is necessary. The primery aim of this study was to optimize the cultural conditions to achieve the highest spores yield with defined nutrients and environmental factors.

In previous studies mentioned ealier, continuous culture on agar plates and (or) in liquid media has generally been used to study the effects of nutrition and (or) environmental factors on fungal growth and sporulation, which could not be able to define certain nutrients and/or environmental conditions for growth and/or sporulation. This study used an improve two-stage cultivation method to study the combination effects of culture conditions (nutrition and environmental factors) on fungal growth and sporulation. Differences in the optimal conditions needed for growth versus sporulation were detected. This method has also been used to determine optimal nutritional requirements and environmental factors with one-time-at-a-factor method (Gao et al., 2009b). The information is greatly important to understand their physiology and ecological characteristics such as mass production, colonization, survival, and competitive ability under field conditions.

MATERIALS AND METHODS

Fungal strain

The tested fungus *T. viride* TV-1 was originally isolated on *Alternaria alternata* by Dr. G. Wang from Yunnan province in China, and was deposited in the Center of General Microorganisms Culture Collection in Institute of Microbiology, Chinese Academy of Sciences.

Materials used and Nutrition requirements for the sporulation of *T. viride* TV-1

The chemicals used were yeast extract (Sigma Chemical Co.); cellobiose, D-fructose, D-glucose, Tryptone, yeast extract, sucrose, K_2HPO_4, $MgSO_4$, $FeSO_4$ (Beijing Chemical Reagents Company, Beijing China); soy peptone (Shanghai Chemical Reagents Company, Shanghai China) and KCl (Nanjing Chemical Reagents Company, Nanjing China). The basal medium was the same with that of Gao et al. (2010). The optimal carbon concentration was followed by 2 g/L with carbon to nitrogen ratio of 10:1, and the combination of cellobiose/yeast extract from carbon source of cellobiose, D-fructose, D-glucose and nitrogen source of tryptone and yeast extracts were used separately (Sun et al., 2009).

Effect of mineral elements

After testing the components and concentration gradients of six mineral elements for sporulation of the isolate with one-factor-at-a-time method, the study got the optimal components for the sporulation of *T. viride* TV-1, including $ZnSO_4.7H_2O$ - 50mg/Land $Na_2MoO_4.2H_2O$- 125 mg/L.

Effect of environmental factors on sporulation of *T. viride* TV-1

The improved 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 for 4 days on sporulation of the biocontrol fungi. Water potential including -0.3 to -7.3 MPa; pH including 3 to 9, dark/light cycle including 24 h/0 h, 12 h/12 h, 0 h/24 h, temperature of 20 to 32°C were included. The study selected two better levels of each factor for the orthogonal research. The selected levels of environmental factors are shown in Table 2 (Gao and Liu, 2009a).

Orthogonal matrix method

The orthogonal $L_{16}(2^{15})$ was used to obtain the optimal culture conditions on solid for pH, water potential, dark/light cycle and temperature on the certain sporulation medium, successively, after the test of carbon concentration. The study got the best nutrition combination after full experiment was carried out (Gao and Liu, 2010), the study also tried to get the optimal combination of nutrition together with environmental factors for sporulation of *T. viride* TV-1 by orthogonal matrix method under selected two levels for sporulation of four environmental factors.

Statistical analysis

The data were analyzed by one-way ANOVA. Tests of significant differences were determined by Duncan's multiple range tests at $P = 0.05$ using Statistical Analysis System (Version 8.2, SAS Institute, Cary, NC).

RESULTS

To investigate the relationships between variables of environmental factors and certain medium components, and also to 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 1), the experimental conditions for each experimental group were listed in Table 2 with the experimental results concluded in the last two columns. According to the orthogonal method, the effect of environmental factors

Table 1. $L_{16}(2^{15})$ orthogonal desigh of optimization of culture environment of *Trichoderma viride*TV-1.

Factors	Water potential (MPa)	pH	Light (h)	Temperature (℃)
Level 1	-7.3	8	12	23
Level 2	-3.9	3	0	26

Table 2. Orthogonal experiment of $L_{16}(2^{15})$ of biomass yields and sporulation of *Trichoderma viride* TV-1.

Exp. group	A	B	A×B[a]	C	A×C	B×C		D	A×D	B×D		C×D				Biomass yields (mg per colony)	Sporulation (10^5 per colony)
1[b]	1[c]	1	1	1	1	1	1	1	1	1	1	1	1	1	1	765.67 ± 45.54[§]	16.83 ± 3.69
2	1	1	1	1	1	1	1	2	2	2	2	2	2	2	2	738.67 ± 63.31	24.83 ± 9.75
3	1	1	1	2	2	2	2	1	1	1	1	2	2	2	2	926.00 ± 38.51	46.00 ± 37.27
4	1	1	1	2	2	2	2	2	2	2	2	1	1	1	1	805.33 ± 148.29	39.83 ± 15.80
5	1	2	2	1	1	2	2	1	1	2	2	1	1	2	2	808.33 ± 50.85	60.67 ± 13.99
6	1	2	2	1	1	2	2	2	2	1	1	2	2	1	1	761.00 ± 116.05	20.50 ± 7.81
7	1	2	2	2	2	1	1	1	1	2	2	2	2	1	1	786.00 ± 73.78	59.83 ± 20.04
8	1	2	2	2	2	1	1	2	2	1	1	1	1	2	2	735.00 ± 34.70	16.33 ± 11.93
9	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	758.67 ± 90.07	7.17 ± 2.02
10	2	1	2	1	2	1	2	2	1	2	1	2	1	2	1	671.33 ± 79.50	17.33 ± 6.21
11	2	1	2	2	1	2	1	1	2	1	2	2	1	2	1	789.00 ± 40.27	15.17 ± 1.89
12	2	1	2	2	1	2	1	2	1	2	1	1	2	1	2	763.00 ± 72.13	16.33 ± 4.37
13	2	2	1	1	2	2	1	1	2	2	1	1	2	2	1	1010.33 ± 247.97	39.50 ± 13.94
14	2	2	1	1	2	2	1	2	1	1	2	2	1	1	2	813.00 ± 67.85	16.17 ± 10.49
15	2	2	1	2	1	1	2	1	2	2	1	2	1	1	2	756.00 ± 34.22	36.33 ±12.69
16	2	2	1	2	1	1	2	2	1	1	2	1	2	2	1	849.00 ± 94.02	42.00 ± 22.12

[a] A×B, A×C, B×C, A×D, B×D, C×D 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, successively; [b] Every row of the experimental group number represents one experimental replicate, and every experimental group was replicated thrice; [c] Values are mean ± SD of triple determinations.

(pH, water potential, dark/light cycle, temperature) on the growth and sporulation of *T. viride* TV-1 was evaluated and shown in the bottom five rows of Table 2. According to the magnitude order of R (maximum difference) in Table 3, the order of effect of all factors on mycelia growth could be determined as 57.60 (temperature) > 21.00 (pH) > 10.54 (water potential) > 10.29 (light), the results indicated that the effect of 57.6 (temperature) was more important than that of the other three environmental factors; the order of effect of all factors on sporulation could be determined as 13.48 (pH) > 11.85 (water potential) > 11.02 (temperature) > 8.60 (light), the results indicated that the effect of 13.48 (pH) was more important than that of the other three environmental factors.

To test the effects of the four factors, ANOVA was used. As shown in Table 4, the pH factor had

Table 3. Analysis of environmental factors on biomass production and sporulation of *Trichoderma viride* TV-1 with this novel method.

		A	B	A×B	C	A×C	B×C		D	A×D	B×D		C×D			
B[a]	K_1	6326.0	6217.7	6664.0	6327.0	6230.7	6060.3	6400.7	6600.0	6382.3	6397.3	6388.3	6495.3	6143.7	6208.7	6437.7
	K_2	6410.3	6518.7	6072.3	6409.3	6505.7	6675.6	6335.7	6136.3	6354.0	6339.0	6348.0	6241.0	6592.7	6527.7	6298.7
	k_1	790.75	777.21	833.00	790.88	778.83	757.54	800.08	825.00	797.79	799.67	798.54	811.92	767.96	776.08	804.71
	k_2	801.29	814.83	759.04	801.17	813.21	834.45	791.96	767.04	794.25	792.37	793.50	780.13	824.08	815.96	787.33
	R	10.54	21.00	73.96	10.29	34.37	76.96	8.13	57.60	3.54	7.29	5.04	31.79	56.13	39.87	17.37
	O	2	2	1	2	2	2	1	1	1	1	1	1	2	2	1
S[b]	K_1'	284.80	183.52	261.52	203.04	232.66	220.65	204.99	281.52	275.16	180.17	209.15	238.66	218.64	212.99	250.99
	K_2'	190.00	291.36	213.36	271.84	242.16	254.16	269.84	193.36	199.68	294.64	265.68	236.16	256.16	261.84	223.84
	k_1'	35.60	22.94	32.69	25.38	29.08	27.58	25.62	35.19	34.40	22.52	26.14	29.83	27.33	26.62	31.37
	k_2'	23.75	36.42	26.67	33.98	30.27	31.77	33.73	24.17	24.96	36.83	33.21	29.52	32.02	32.73	27.98
	R'	11.85	13.48	6.02	8.60	1.19	4.19	8.11	11.02	9.44	14.31	7.07	0.31	4.69	6.11	3.40
	O'	1	2	1	2	2	2	2	1	1	2	2	1	2	2	1

[a]Biomass yields (mg per colony);[b] Sporulation (10^5 conidia per colony);K_1 and K_2 are the total content of biomass yields from the level 1 and level 2 separately; k_1 and k_2 are the mean value of levels 1 and 2 separately; K_1' and K_2' are the total spore yields from the level 1 and level 2 separately; k_1' and k_2' are the mean value of levels 1 and 2 separately; R is the maximum of k_1, k_2 minus the minimum of k_1, k_2 and R' is the maximum of k_1, k_2 minus the minimum of k_1, k_2 respectively; O is the optimal level of biomass yields and O' is the optimal value of spore yields.

significant effects on sporulation. Table 5 shows the effect of combinations of four factors on biomass yields and sporulation. It is demonstrated that the combinations of B1/A2, A2/C2, B1/C2, A2/D2, D1/B2, D1/C2 have the best effect on biomass yields, producing 857.08, 836.08, 888.67, 815.58, 838.42, 799.08 (mg per colony) biomass yields respectively. To obtain a high mycelia yields, the optimum factors should be water potential -3.9MPa (A2) /pH 8 (B1)/0 h light (C2) /23 ℃ (D1), which was not consistent with intuitive analysis (Table 4). It is demonstrated that the combinations of B2/A2, A2/C1, B1/C2, A2/D1, B2/D1, D1/C2 have the best effect on sporulation, producing 39.33, 39.88, 35.38, 44.67, 38.54, 34.83 spore yields respectively. To obtain a high spore yields, the optimum factors should be water potential -3.9 MPa (A2) /pH 3 (B2) /0 h (C2) light /23°C (D1), which was not very much consistent with intuitive analysis water potential -7.3 MPa (A1) / pH 3 (B2) / 0 h light (C2) /23°C (D1) in Table 3.

DISCUSSION

The first level the study choose was the best for sporulation of *T. viride* TV-1 by one-factor-at-one-time method, while after the orthogonal matrix method, the study found that certain nutrition was not combined in the first level of environment, which also means that the orthogonal method was necessary for optimizing the sporulation culture conditions including nutritional and environment factors.

Though other studies concern is mainly on the orthogonal matrix method, which focuses on nutritional components or just environmental factors, while this study combined them together. After doing the full experiment of nutrition and environmental conditions, the study used the former two levels of environmental factors with certain nutrition to optimize the culture conditions with orthogonal matrix method.

Environmental factors which have different effects on biomass and sporulation, this study cultured them under different environment conditions to get the certain yields needed, for example, biomass or spores. It is demonstrated that the combinations of different environmental factors have different effect on biomass and spore yields. On biomass production, the study found the result got by combination factors was consistent with intuitive analysis. While on sporulation, the result was not consistent with intuitive analysis. Maybe some system error took place which could not be controlled.

It has been shown that alternative nutritional components can significantly influence growth and sporulation of many fungi (Rao et al., 1997; Tigano

Table 4. The variance analysis of $L_{16}(2^{15})$ orthogonal test on optimization of environmental factors for biomass yields and sporulation of *Trichoderma viride* TV-1.

Variables	Variance source	Sum of square deviation (SS)	Degree of freedom (v)	Mean square (MS)	*F*-ratio	Significance level[a]
Biomass yields (mg per colony)	A	444.50	1	8555.78	0.11	
	B	5662.30	1	1764.21	1.38	
	C	423.61	1	1.00	0.10	
	D	13437.04	1	1024.16	3.27	
	A×B	21859.10	1	21859.10	1.06	
	A×C	4730.53	1	4730.53	0.23	
	A×D	41.66	1	41.66	0.00	
	B×C	23038.92	1	23038.92	1.12	
	B×D	217.06	1	217.06	0.01	
	C×D	4047.17	1	4047.17	0.20	
	Error	20533.86	5			
Sporulation (10^5 conidia per colony)	A	561.93	1	561.93	3.77	
	B	726.84	1	726.84	4.88	*
	C	296.01	1	296.01	1.99	
	D	485.98	1	485.98	3.26	
	A×B	147.73	1	147.73	0.20	
	A×C	4.62	1	4.62	0.01	
	A×D	356.47	1	356.47	0.48	
	B×C	68.69	1	68.69	0.09	
	B×D	817.49	1	817.49	1.10	
	C×D	-0.64	1	-0.64	0.00	
	Error	745.50	5			

[a]$F_{0.1}(1, 5) = 4.06$, $F_{0.05}(1, 5) = 6.610$, $F_{0.01}(1, 5) = 16.3$; * *F*-ratio >$F_{0.1}$;**F 0.1 < *F*-ratio < $F_{0.05}$; *** *F*-ratio < $F_{0.01}$.

Table 5. Effects of combinations of environmental factors on biomass yields and sporulation of *Trichoderma viride* TV-1.

B, C or D	A				B				C			
	A_1		A_2		B_1		B_2		C_1		C_2	
	B[a]	S[b]	B	S	B	S	B	S	B	S	B	S
B_1	808.92	31.87	857.08	33.50								
B_2	745.50	14.00	772.58	39.33								
C_1	764.09	18.29	793.58	39.88	777.34	30.00	780.33	28.17				
C_2	790.33	27.58	836.08	32.96	888.67	35.38	737.75	26.17				
D_1	781.50	24.12	814.08	44.67	838.42	30.25	757.17	38.54	796.50	33.96	799.08	34.83
D_2	772.92	21.75	815.58	28.17	827.58	35.12	760.92	14.79	761.17	24.21	827.33	25.71

A1, A2, B1, B2, C1, C2, D1, D2 represent the 1 and 2 levels of water potential, pH, light and temperature; [a] Represent the biomass yields (mg per colony); [b] Represent spore yields (10^5 conidia per colony).

et al., 1995; Culbreath et al., 1986). This 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. This study also found that fungal biomass was not necessarily correlated with fungal sporulation under the

orthogonal matrix method. This result showed the essential of two-stage method. All of this information are all important to mass production with lower cost, also reflecting some physiology on nutrients and environmental characteristics of this fungus, and also gives the essential information for its industrialization.

The soil fungistasis as an obstacle for the efficiency of biocontrol, the culture conditions can be improved (nutrition and environmental) to help overcome the soil fungistasis in order to colonize and to have better mycelia and sporulation in soil, together with combined environmental conditions, which will be a great help for biocontrol efficiency (Sun et al.,1997).

Conflict of Interests

The authors have not declared any conflict of interests.

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