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Optimal mass production technology for sporulation of Verticillium lecanii and Trichoderma harzianum

Saba Hasan*

* Amity Institute of Biotechnology, AMITY University Uttar Pradesh, Lucknow Campus, Lucknow – 226 028 INDIA;

ABSTRACT

The present study aims at optimization of a suitable mass production technology for growth and sporulation of the biocontrol agents Verticillium lecanii and Trichoderma harzianum. Combinations of various natural solid substrates (Rice, Maize, Arhar, Defatted Soybean, Gram) and laboratory media (SMYA, Complete media, SDA, Czapek's Dox media) were evaluated for growth and sporulation of V. lecanii and T. harzianum. Data on CFU, concentration of propagules, dry mycelial weight, conidia production and conidia yield were analyzed. It was observed that among the substrate types tested, the fungal strains grew better and produced high quantity of spores in Rice as growth medium after 21 days of incubation followed by Maize and Arhar. The best liquid media which supported maximum conidial production was SMYA and Complete medium. Also, rate of germination of spores as well as their viability at different aeration rates was tested. It was observed that maximum length of germ tube in minimum duration was observed in Rice as substrate. The viability percentage of the biocontrol agents produced at various aeration rates and agitation speeds showed that change in aeration and agitation did not significantly affect spore viability. Results demonstrated that the fungal species could be rapidly produced with a high conidial yield on natural solid substrates as compared to liquid media by fermentation technology.

Indexing terms/Keywords

Biological control; Entomopathogenic fungi; Solid State Fermentation; Verticillium lecani;, Trichoderma harzianum; Liquid fermentation.

Academic Discipline And Sub-Disciplines

Biological Sciences.

SUBJECT CLASSIFICATION

Micro organisms as Mycopesticides.

TYPE (METHOD/APPROACH)

Optimization of suitable mass production technology for spore production of Entomopathogenic fungi and their formulation as effective mycopesticides.

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INTRODUCTION

Chemical pesticides have been predominantly employed for control of pests and diseases in agriculture [1]. New restrictions on application of these chemicals and environmental considerations have led to an increased interest in biocontrol agents. As a result, an economic increment of 20% has been directed to explore biotechnological products of microbial origin, such as biofertilizers, biopesticides and microbial enzymes used for crop bioprocesses [2]. Companies such as Cyanamid, Ciba, Dupont, Monsanto, Sandoz and Zeneca have designed genetic engineering programs in order to develop crops resistant to insects, diseases and chemical herbicides, with use of natural biological organisms [3]. Strains of entomopathogenic fungi are the basis of diverse commercial products such as Mycotal, Biogreen, Mycotrol GH, Laginex [4]. In the development of biocontrol agents and improvements in the effectiveness of mycoinsecticides, it is necessary to find methods for the mass production of inocula [5]. Hence fungi, which have the potential for control of insect pathogens are produced by Solid State Fermentation (SSF) as well as Submerged (liquid) Fermentation [6, 7]. The insecticides based on *Beauveria bassiana (Balsamo) Vuillemin* [8, 9], *Paecilomyces fumosoroseus (Wize) Brown and Smith [*10, 11] *and Verticillium lecanii (Zimm.) Viegas* [12] have been used to control various insect pests. Solid State Fermentation is an alternative cultivation system for the production of value added products from microorganisms, especially enzymes or secondary metabolites [13,14] and has been used for the production of some biological agents, such as *Talaromyces flavus*[15], *Coniothyrium minitans* [16], entomopathogenic fungi such as *Metarhizium anisopliae*, or bioherbicides [17]. Submerged liquid culture is usually preferred for large-scale fermentations [18], and has been used extensively for industrial production of antibiotics, amino acids, ethanol, organic acids, baker's and distiller's yeasts. Submerged fermentation is considered more readily available, economical, and practical than other methods for mass production of biopesticides in developed countries [19, 20, 21]. The present study was undertaken to evaluate substrate types in Solid State Fermentation as well as Liquid (Submerged) Fermentation for the mass production of *Verticillium lecanii* and *Trichoderma harzianum*.

MATERIALS AND METHODS

Isolation of the pathogen

Microconidial inoculum of *Verticillium lecanii* and *Trichoderma harzianum* parasitizing hyphae of plant pathogens viz. Sclerotium rolfsii, Rhizoctonia solani, Alternaria alternata were isolated, purified and microscopically identified from these plant pathogenic fungal cultures in SMY (Sabouraud's Maltose Yeast extract) broth.

Spore production in Liquid fermentation

V. lecanii and *T. harzianum* was produced in liquid fermentation similar to the method described by Pascual et al.(2000) [22] for Penicillium oxalicum. Both plant pathogenic fungi were grown in four liquid mediums respectively, SMYA, SDA, Czapek's medium, Complete medium, adjusted to pH 7. Flasks were inoculated with 1 ml of a spore suspension (1×10^7 conidia ml^{-1}) of *V lecanii* and *T. harzianum* and incubated at 28±1°C and 150 rpm. Harvesting of conidia was done at the end of 7th, 14th and 21st day respectively, and the conidial count/ml was determined microscopically.

Spore production in Solid State fermentation

Grains were cleaned, washed and dried prior to use. Ten grams of each grain was added to 150 ml capacity Erlenmeyer flask. They were moistened by addition of tap water (Table 1) and were autoclaved at 121°C at 15 lb/in2 for 30 minutes. Equal concentration of spores were added to the flasks as initial inoculums and were incubated at 28±1°C. Harvesting of conidia was done at the end of 7th, 14th and 21st day respectively, and the conidial count/ml was determined microscopically.

Table 1. Ratio of substrate to moistening agent

Substrate	Ratio to moistening agent
Arhar (*Cajanas cajan*)	10 g + 8 ml
Gram (*Cicer aretinum*)	10 g + 8 ml
Defatted Soybean (*Glycine max*)	10 g + 8 ml
Rice (*Oryza sativa*)	10 g + 8 ml
Maize (*Zea mays)*	10 g + 8 ml

Germination Test

Germination time and germ tube lengths were chosen as parameters for Solid State Fermentation of *Verticillium lecanii* and *Trichoderma harzianum* because of importance of spore germination in enhancing infectivity of the fungal strain. A loopful of fungal spore suspension was placed asceptically on the moist chamber slide. It was covered with a coverslip and was incubated at 28±1^{o}C. Length of germ tube was measured after every four hours by using a micrometer.

Viability determination

The substrate/media that gave best result for conidial production during Solid state and Liquid (Submerged fermentation) was selected for assessment of viability of spores. Viability was determined by comparing CFU (Colony forming units) with total number of spores in Rice and SMYA. For viability determination, harvested medium was filtered through a compacted glass wool. The filtrate was centrifuged at 10,000rpm for 10 minutes and the supernatant was discarded. The resulting spore pellet in each centrifuge tube was resuspended in 5ml of distilled water and centrifuged again at 12,000 rpm for 10 mins, discarding the supernatant [23]. The pellet was removed from each centrifuge tube, spread in a petridish, dried in a dessicator with silica gel for three days [24]. Dry preparations were used to examine the total number of conidia and colony forming units (CFU). The number of spores were counted directly in a haemocytometer. The CFU was determined by plating the serial dilutions of various conidial preparations onto potato dextrose agar to limit colony diameter. The germination percentage, as a parameter of dessication tolerance of conidia in dry preparations was determined by comparing Colony Forming Units (CFU) with total spores. Prior to enumeration or plating, dry conidial preparations were soaked in sterile distilled water for two hours and then ground in a blender at full speed for 3 minutes. To study the effect of agitation and aeration on viability of spores, experiments were conducted at 25^{o} C, pH 7, aeration 1 vvm and at various agitation speeds: 300, 400, 500, 600, 700 rpm. Effect of aeration was studied at three different rates : 1.0, 1.5 and 2.0 vvm.

Statistical analysis

Statistical analysis of different parameters of the liquid and solid-state fermentation studies followed a completely randomized design in a 2 × 7 and 2 × 8 factorial schemes, respectively. Data of number of CFU, concentration of propagules, dry mycelial weight, conidia production and conidia yield were analyzed through two-way analysis of variance (two-way ANOVA) using a generalized linear model (PROC GLM). Means were compared by the TukeyKramer HSD test and considered to be statistically different at the 5% significance level. Data on CFU number, propagules concentration and conidia production were subjected to logarithmic transformation [log10 (x)] to improve homogeneity of variances and to be normalized before the data analysis. All data analyses were performed using the SAS 8.02 software [25].

RESULTS

Liquid Fermentation

The alternative liquid culture media were composed of inexpensive and largely available laboratory media. The main ingredients used, were SMYA, SDA, Czapek's Dox and Complete medium. SMYA and Complete medium (CM) had the maximum influence on the CFU number for *V. lecanii* and *T. harzianum* ($p<0.0001$). The mean propagules concentration and the dry mycelial weight of the fungal strains were better on CM ($p=0.0001$). The fungal strains showed the highest CFU number in SMYA, highest concentration of propagules in SMYA and CM and highest dry mycelial weight in SMYA and CM ($p=0.0001$). The SMYA medium promoted better development for the isolates. These results suggested that the presence of Maltose in the media generated higher concentration of propagules and dry biomass than the other alternative media for these fungal isolates (Table 2).

Solid State Fermentation

The solid substrates which resulted in the highest conidia productions were rice, arhar and maize ($p<0.0001$). The mean values were 1.4 × 10^{9}, 1.1 × 10^{9} and 1.0 × 10^{9} conidia.g^{-1}, respectively. Gram and defatted Soybean presented low conidia concentration and yield for *V. lecanii* and *T. harzianum*, probably because C/N ratio and nutritional requirements were not appropriated for these fungi (Table 3). Other factors such as particle size (aeration) and grain moisture may have affected the fungal growth and sporulation. A study using agro-industrial residues revealed that the proportion of 60% potato refuse and 40% sugarcane bagasse promoted high production of aerial conidia of *B. bassiana* (3.4 × 10^{9}conidia.g^{-1}dry mass) in Erlenmeyer flasks [26], demonstrating the feasibility of these byproducts as alternative nutritional sources to produce entomopathogenic fungi.

Germination time and germ tube length of spores

Germ tube lengths of spores were selected as criterion for characterization of the *V. lecanii* and *T. harzianum* isolates. Germination of spores of the fungal isolates was tested on four substrates showing prolific growth and sporulation – Rice, Maize, Arhar and Defatted Soybean. Germ tube lengths were measured in every four hours of incubation at 28±1^{o}C. It was concluded that at the end of four hours of incubation, maximum length of germ tube was observed with Arhar and Rice as substrates (7.812 μm & 7.409 μm). Least increase in germ tube length was reported in Defatted soybean (4.34 μm & 4.08 μm). In the 8th hour, Rice showed good results in terms of germ tube lengths (18.72 μm) whereas poor results were obtained with defatted soybean (13.214 μm). Similarly, in the 12th hour, maximum germ tube length was reported in Rice (30.724 μm & 31.450 μm).

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DISCUSSION

Liquid Fermentation

The SMYA medium favored the tested fungi for all evaluated parameters. The medium seems to be more practical and less costly than the others, because their ingredients can be obtained from the sugar factories. Humphreys et al. (1990) [27] showed that the sporulation of *I. farinosa* in submerged culture decreased when poor carbon sources were used or under limited amount of nitrogen, as observed here for the SDA medium. During liquid fermentation, the media achieved viscous consistency due to fungal growth, and the color of the media also differed ranging from light cream to the yellow color when they were grown with *V. lecanii*, and from being white to greenish when growing with *T. harzianum*. Color changes and medium consistency could be related to the intense growth of fungal mycelia, nutrient metabolism and production of several metabolites. After 48 h of fermentation, mycelial pellet formation begins in the medium, increasing it's size until the last 120 h. Mycelial pellets in liquid fermentation have already been observed for *I. farinosa* and *I. fumosorosea* previously [27,28]. The maximum production of dry biomass for I. fumosorosea obtained by Torre and CardenasCota, (1996) [28] was 7.5 mg.mL^{-1} in submerged culture after four days at 37°C with 12:12 (L:D) h photoperiod. Conversely, the highest biomass production obtained for *V. lecanii* and *T. harzinaum* were in SMYA after four days of fermentation at 26°C (Table II). After 48 h of submerged fermentation, high number of viable blastospores were obtained that showed a faster germination rate than aerial conidia.

Table 2. Number of colony forming units (CFU), concentration of propagules and dry mycelial weight of *Verticillium lecanii* and *Trichoderma harzianum* for different liquid substrates

Liquid Media	Number of colonies		Concentration		Dry mycelial weight	
	(10^4 CFU.mL-1)[1]		(10^4 propagules.mL-1)[1]		(g.flask-1)[2,3]	
	V. lecanii	*T.harzianum*	*V.lecanii*	*T.harzianum*	*V.lecanii*	*T.harzianum*
SMY	370.00±157	359.00±142	290.63±32.92	264.39±30.72	0.78±0.06	0.72±0.02
CM	12.5±4.10	11.2±3.86	3.75±0.44	2.99±0.31	0.44±0.01	0.32±0.01
SDA	2.35±0.36	2.03±0.28	6.09±2.95	6.00±2.66	0.56±0.03	0.49±0.01
Czapek's	12.00±2.71	11.63±2.21	164.22±22.90	148.12±21.14	0.65±0.03	0.58±0.01
			Dox			
CV (%)[4]	6.8		8.91		11.90	

[1]Letters obtained from log10 (x) transformed data.

[2]Letters obtained from untransformed data. Means (± standard error, SE) followed by the same upper case letters (within the columns) and lower case letters (within the rows) do not differ significantly by Tukey-Kramer HSD test (α=0.05).

[3]Medium in flask : 50ml

[4] Coefficient of variation. Untransformed data

Solid State Fermentation

In the present study, rice and maize as a substrate promoted the highest conidia yield for *V. lecanii* and *T. harzianum* ($p<0.0001$) (Table 3). The final moisture, after the drying, varied according to the solid substrates and, subsequently, affected the spore extraction. There was an abundant mycelial growth for Gram as substrate but low sporulation, and for defatted Soybean, poor mycelial growth was observed. For the other substrates the fungal sporulation occurred all over the grains. The conidial viability of the isolate was good for all the solid media tested. Alves and Pereira (1989) [29] obtained between 6 and 11.4% of the product yield with 1010 conidia.g^{-1} for *M. anisopliae*. It was observed that the conidia yield, as well as its concentration, might vary according to the method used in the solid fermentation, incubation time, grain size (aeration), substrate moisture and nutritional composition, the fungal species and also their isolates tested.

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Table 3. Conidia production and yield of *Verticillium lecanii* for different solid substrates.

Solid medium	Conidia production (10^7 conidia.g-1)[1]		Conidia yield (%)[2]	
	V. lecanii	*T.harzianum*	*V. lecanii*	*T.harzianum*
Rice	108.06±18.12	105.06±16.25	2.04±0.10	1.99±0.06
Arhar	49.84±3.98	47.88±3.66	0.89± 0.01	0.97± 0.02
Maize	41.69±1.98	40.22±1.25	0.48±0.02	0.36±0.01
Gram	16.75±2.46	14.57±2.19	0.51±0.03	0.49±0.01
Defatted Soybean	6.09±0.42	5.63±0.25	0.11±0.03	0.12±0.01
CV (%)[3]	8.12		17.28	

[1]Letters obtained from log10 (x) transformed data.

[2]Letters obtained from untransformed data. Means (± SE) followed by the same upper case letters (within the columns) and lower case letters (within the rows) do not differ significantly by Tukey-Kramer HSD test (α=0.05).

[3]Coefficient of variation. Untransformed data are presented.

Germination time and germ tube length of spores

Conidium development and fungal development greatly influence potentiality of mycoinsecticides for biocontrol. Dormancy could be caused by adverse environmental factors [30], and is due at least in part to the presence of metabolites present in ungerminated spores [31]. Once germination has been achieved, fungal pathogens penetrate their hosts and usually secrete extracellular enzymes [32]. Hence, acceleration of spore germination could enhance the infectivity and efficacy of spore preparations [33]. Concluding the results at the end of 24 hours, Rice was reported to give the best result (56.24 μm). According to Jackson et al. (1985) [34], the most virulent strains of *V. lecanii* produced in artificial media more conidia than less virulent strains. Furthermore, fast germination rates were also related with virulence. Kassa et al. (2004) [35] studied production of submerged conidia of *M. anisopliae* and concluded that the viability of germination were significantly affected by the freeze drying techniques. Feng et al. (2000) [36] produced spores of *Verticillium lecanii* by SSF and Liquid fermentation. They concluded that V. lecanii spores failed to germinate until 8 hours of incubation, but over 90% spore germination ratio was reached at 18 hours of incubation. germination and growth patterns of *V. lecanii* and *T. harzianum* differed greatly under different culture conditions.

Effect of agitation rate and aeration on spore viability

The viability percentage of the biocontrol agent produced in rice and SMYA at various aeration rates and agitation speeds shows that change in aeration and agitation did not significantly affect spore viability (Table 4).

Table 4. Viability Determination at different Aeration and Agitation rates

	Aeration rate (vvm) (1.0vvm)					
	V. lecanii	*T.harzianum*	*V. lecanii*	*T. harzianum*	*V. lecanii*	*T .harzianum*
	1.0		1.5		2.0	
	% viability					
Rice	51	52	52.6	51.9	49.5	50.6
SMYA	46	48	47	49	48	47.2
	Agitation rate (rpm) (300rpm)					
% viability	300		400		500	
Rice	46.5	47.2	51.2	50.3	47	46.8
SMYA	46	45.9	45	47	43.5	45.2

CONCLUSION

The production of mycelial biomass is a suitable measure to be used when high quantity of dry mycelium is needed for further formulation, with emphasis on its field application. Reliable mass production systems ensure production and commercial application of these biocontrol agents. *V. lecanii* and *T. harzianum* are promising biological control agents. The fungal strains also shows biocontrol against other crop diseases. Hence, in the present investigation, attempts have been made to grow the strain of *Verticillium lecanii* and *Trichoderma harzianum* using several important parameters associated with increase in spore count and spore germination, so that they could be formulated in an effective formulation for pest control. Highest number of viable conidia of *V. lecanii* and *T. harzianum* were produced with Rice as substrate in Solid State Fermentation and SMYA as liquid media during Liquid fermentation. Although *V. lecanii* and *T. harzianum* produced conidia on liquid fermentation, volumetric yield of their conidia is 25-fold higher in solid fermentation than in liquid fermentation at 30 days after inoculation. Also, Solid State Fermentation proved to be an economic and cost effective technology for spore production and formulation. Conidia of *V. lecanii* and *T. harzianum* in solid fermentation had a long shelf life, if stored at –20°C. Research is in progress to improve the shelf life of conidia of these biocontrol agents to stabilize the performance of formulated product.

Conflicts of Interest

The authors declare no conflict of interest.

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Author' biography with Photo

Dr Saba Hasan has completed Ph D (Microbiology) in the year 2008 from R D University. She is presently employed as Assistant Professor in Amity Univeristy Uttar Pradesh, Lucknow Campus. She holds a teaching experience of 11.5 years and research experience of 10 years to her credit. She has published 18 research papers in Journals of National and International repute. Also, she has been appointed as Reviewer of about 9 International and National journals. She is the member of Editorial Board in journals of repute. In addition, she holds recognitions like External Examiner, Paper setter of recognized Universities, Chairperson of prestigious Conference to her credit.