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Exploitation of biowaste as natural substrates for biomass production of entomopathogenic fungus *Verticillium lecanii*

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Abstract
Verticillium lecanii is an entomopathogenic fungus (EPF) that occurs naturally in the environment and has the potential as a biological control agent against many insect pests. A laboratory study was conducted to mass production of the entomopathogenic fungus, on different substrates. The maximum biomass was attained on PDB (0.735 g/100ml) followed by SS5, SS3 and SS1 i.e. 0.676, 0.647 and 0.587 g/ 100ml respectively. Maximum viable cell count was found on SS1 i.e. $9x10^8$ per ml on 15th DOI for *V. lecanii*. Medium SS1, SS3 and SS5 were found to be the best substrates among all the tested substrates for mass multiplication of *V. lecanii.*

Keywords: *verticillium lecanii,* mass production, submerged culture

Introduction
Biocontrol agents include a wide variety of life forms, including vertebrates, invertebrates, fungi, and microorganisms. These beneficial species are common in most natural communities and, although their presence is often unnoticed, they help to maintain the "balance of nature" by regulating the density of their host or prey population.
India is bestowed with a rich biodiversity of entomopathogen. Utilization of these natural and renewable resources is important in a successful biocontrol strategy. Among the biocontrol agents, especially entomopathogenic fungi (i.e. fungal pathogens which infect insects) play a predominant role in pest control [4]. Entomopathogenic fungi are the potential microbial alternatives to chemical insecticides and offer several benefits such as growth facility on a variety of substrates, high virulence, transcuticular penetration, broad host range, safety to human beings, animals and environment [7].
V. lecanii has been recognized as a high potential entomopathogen in biological control of aphids. Many isolates of this fungus demonstrate high pathogenicity to several species of aphids [5, 1]. The fungus infects insects by producing hyphae from germinating spores that penetrate the insect's integument. The fungus then destroys the internal contents and the insect dies. The fungus eventually grows out through the cuticle and sporulates on the body surface. Infected insects appear as white to yellowish cottony particles
Various agricultural products and byproducts such as grains, vegetable wastes, seeds, rice husk, sawdust and liquid media such as coconut water, rice and wheat washed water and rice cooked water were evaluated for mass production of three entomopathogenic fungi, *Beauveria bassiana*, *Paecilomyces fumosoroseus* and *Verticillium lecanii*. So the main objective of this investigation was to find out the effects of some agro substrates on the quantity of spore production, biomass production and growth rate.

Materials and methods
Entomopathogenic Fungal culture
The pure culture of *Verticillium lecanii* was procured from ITCC, New Delhi.

Culture and maintenance of culture
A loopful of inoculums from subcultured plates of *V. lecanii* were transferred to autoclaved potato dextrose agar (PDA) slants and maintained as pure cultures. The plates were then incubated at room temperature for ten days. After complete sporulation, conidia from the medium were harvested with the help of a small sterile metal spatula. Spores were stored in 10% glycerol at -20 ^{0}C for further studies.
For liquid culture studies, spore inoculum was produced by growing glycerol stock of *V. lecanii* on PDA plates at room temperature. Spore inoculum was obtained by rinsing the Sporulated PDA plates with deionized water containing Tween-20 (0.2%). One ml (approx. *cfu* $2.8\times10^{5)}$ of the fungal culture was used as standard inoculum for further experimental studies.

Media preparation
Natural substrates *viz.,* wheat bran, rice grain, mustard oilcake, soybean oilcake, sawdust and vegetable waste were tested to assess the suitability of medium to the test fungi by estimating the biomass of *V. lecanii*. For the preparation of substrates wheat bran (S1), rice grain (S2) and vegetable waste (mainly potato peels & carrot peels)(S5) were weighed 20g, washed well and boiled in distilled water (DW) for 1h. It was mashed properly and filtered [8]. Other substrates viz; soybean oilcake (S3), mustard oilcake (S4) and sawdust (S6) were washed and oven-dried at 65° C and then ground to powder form by hammer beater mill and boiled in distilled water (DW) for 1h, then filtered. Twenty gram glucose and 6.5g peptone, as additional inorganic nutrients were supplemented to above mentioned substrates

(S1, S2, S3, S4, S5 and S6) to make supplemented medium (SS1, SS2, SS3, SS4, SS5 and SS6)[10]. The volume was made 1L with DW for each substrate. Now from each substrate, 100ml aliquots were packed separately in 250 ml conical flasks. They were plugged with cotton wool and autoclaved at 15 psi for 1h at 121˚C. After cooling, 1ml (~2.8x10^5 spores) of the fungal culture was inoculated into each flask, separately. All these procedures were done under a laminar airflow chamber. All flasks were incubated for 15 days, at 23 °C for two days and at ambient temperature (~25-30 °C) for further days of incubation with agitation on a rotary shaker. Biomass production was used as an indicator for growth in 3, 6, 9, 12 and 15 days of incubation.

Growth determination of fungus

The mycelial growth produced in the culture medium was determined by dry weight measurement after 3, 6, 9 and 12 till 15 days of incubation, which was recovered by filtration using Whatman filter paper (No. 1) of each substrate. The content of the flask was filtered through the filter paper to separate the mycelial mat and the culture filtrate. Filter paper was dried in an oven at 65°C till constant weight was achieved and cooled in a desiccator for 10-20 min prior to weighing. The dry weight of mycelia was calculated according to the formula as given below [9]

Growth (g/100mL) = Weight of culture + filter paper- initial weight of filter paper

Comparative analysis of the dry mycelial weight of fungus *V. lecanii* was done in each substrate to find out the cheapest and best natural substrate for the mass multiplication of fungus.

Preparation and count of Spore Concentrate

Spores were harvested from liquid culture media following the procedure with some modifications [5]. The liquid media was filtered through sterilized Whatman No.1 filter paper to remove mycelia. The filtrate was centrifuged at 12000 rpm for 25 min in the centrifuge (Remi, India) to obtain spore pellet. The pellet was repeatedly washed with sterile distilled water and resuspended in 10 ml sterile distilled water and this concentrated spore suspension was utilized for viable cell count.

The total count of suspension was obtained by multiplying the number of cells per plate by the dilution factor which is the reciprocal of the dilution.

CFU/plate = No. of colonies obtained× Dilution factor

Statistical analysis

Statistical analysis of all the data for fungal growth was subjected to one way ANOVA by MATLAB R2018a software. Growth curve data (slopes) were analyzed by random coefficient regression analysis using Excel 10.

Results and Discussion

For selection of a suitable, cheaper and easily available substrate for mass multiplication of the *V. lecanii*, different categories of substrates were evaluated. Substrates selected for biomass and spore production were wheat bran, rice grains, oil cakes, saw dust and vegetable waste. Potato Dextrose Broth (PDB) was taken as control.

Biomass production and exponential growth analysis of *V. lecanii* in different substrate media

Maximum biomass of fungus was observed on day fifteenth in the case of medium S1 i.e. 0.325 g whereas 1.079g in SS1 medium (Figure 1). Exponential growth was observed at 3rd to 9th days both in S1 and SS1 medium with slope value 0.27 and 0.53 respectively (Table 1). The slope values represent the rate of colony growth, in which high values signify profuse growth while low values indicate delayed or inhibited growth. Moreover, these substrates are rich in nutrients and the availability of these nutrients in the liquid media contributed to increase the conidia production [6]. In S2 and SS2 medium, maximum biomass was measured at 12th DOI i.e. 0.236g and 0.573g respectively. Biomass was decreased at 15th day due to the non-availability of sufficient nutrients to fungus. The least slope value was observed with treatment S2 (0.09). S3 and SS3 medium seemed to be very fair with a bit stationary and with less slope value of 0.13 and 0.19. Biomass production was increased from 3rd DOI to 15th DOI i.e., 0.257 to 0.841g respectively. The combination of the liquid media made with soy flour and Dextrose was evaluated for the production of seven *Hirsutella thompsonii* Fischer (1950) isolates, and one *H. nodulosa* Petch (1926) isolate [11]. The dry mycelial weight of fungus on the S4 medium was very less with a very good pattern of exponential growth with a slope value 0.12. Maximum biomass was 0.061 g only, which was very less as compared to SS4 medium (0.821g), which might be due to devoid of glucose and peptone. Vegetable waste consisted of potato and carrot peels (S5) in which the growth of *V. lecanii* has consistent growth from 9th to 12th days of incubation and i.e. 0.357 g to 0.361 g/100ml of substrate media. In contrast to S5, SS5 gave growth in increasing rate and maximum growth was observed on the fifteenth day i.e. 0.898 g/100ml with 0.21 slope in the log growth curve. This might be due to carrot peels, as previous findings reported that carrot was found to be the cheap and best suitable media for the large-scale production of deuteromycota fungi [3, 2]. Very less mycelial growth was observed on S6 as comparison to SS6. An increment in biomass was observed on the 12th day i.e. 0.038 g/100ml. Although initial days of incubation gave very less biomass in case of treatment SS6 but later on it was good (0.279g) might be due to the adaptability of fungus in the new medium (Figure 1). Growth of *V. lecanii* on PDB as control gave very good dry mycelial weight as compared to other media and maximum weight was on the 15th day (0.1 g/100ml).

Comparative analysis of average biomass of *V. lecanii*

Figure 2 depicts the average biomass of observed fungus. In which maximum biomass was attained on Potato dextrose broth (0.735 g/100ml) followed by SS5, SS3 and SS1 i.e. 0.676, 0.647 and 0.587 g/ 100ml respectively. The least average biomass weight was observed on S6 (0.029g) which is in an agreement with the findings where the least biomass *of V. lecanii* was observed on sawdust [8]. Although average biomass of fungus SS5 and SS3 were more than SS1 but the rate of growth was observed slow i.e. 0.21 and 0.19 per day in above mentioned media. All treatments were found significantly different through ANOVA ($P<0.001$) at alpha 0.05, which means all treatments significantly different from each other at a 95% level of confidence. All treatments were found significantly different from control also through multiple comparison tests (Figure 3)

Viable cell count on different substrates

Comparative analysis of viable cell count depicted that

maximum viable cell count was found on SS1 i.e. 9x10^8 per ml on 15th DOI for *V. lecanii*. This observation is in agreement with the report, that wheat supported maximum spore production for *B. bassiana* while sorghum recorded maximum spore production in *Paecilomyces fumosoroseus* and *Verticillium lecanii* [12]. Control, SS3 and SS5 were shown approximately the same CFU i.e. 4x10^8 to 4.9x10^8 per ml on the 15th day. Minimum spore count was found on S6 substrate i.e. 1x10^8 per ml as shown in Table 1.

Medium SS1, SS5 and SS3 were found to be the best substrates among all the tested media for mass multiplication of *V. lecanii*.

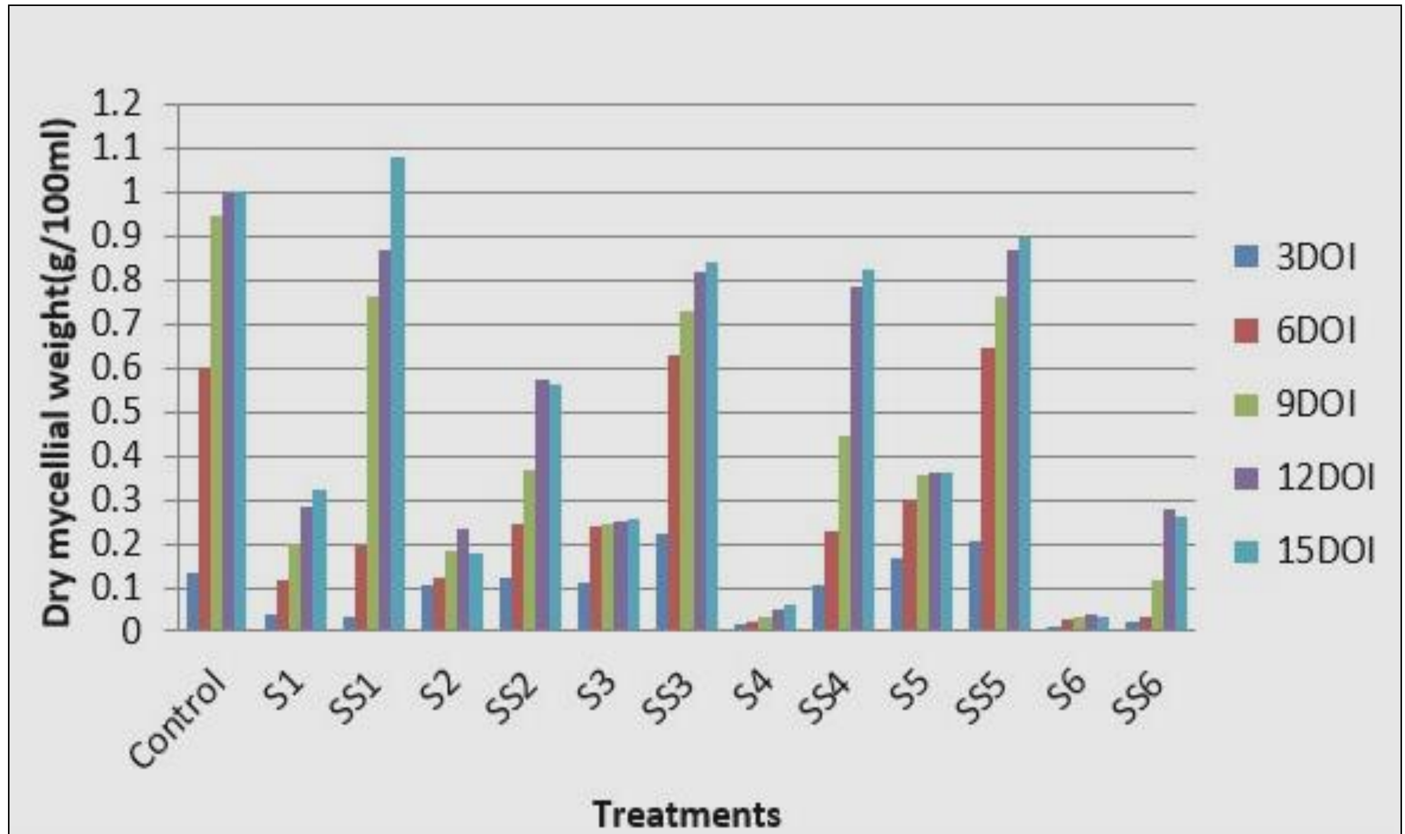

Fig 1: Growth of entomopathogenic fungus on different medium

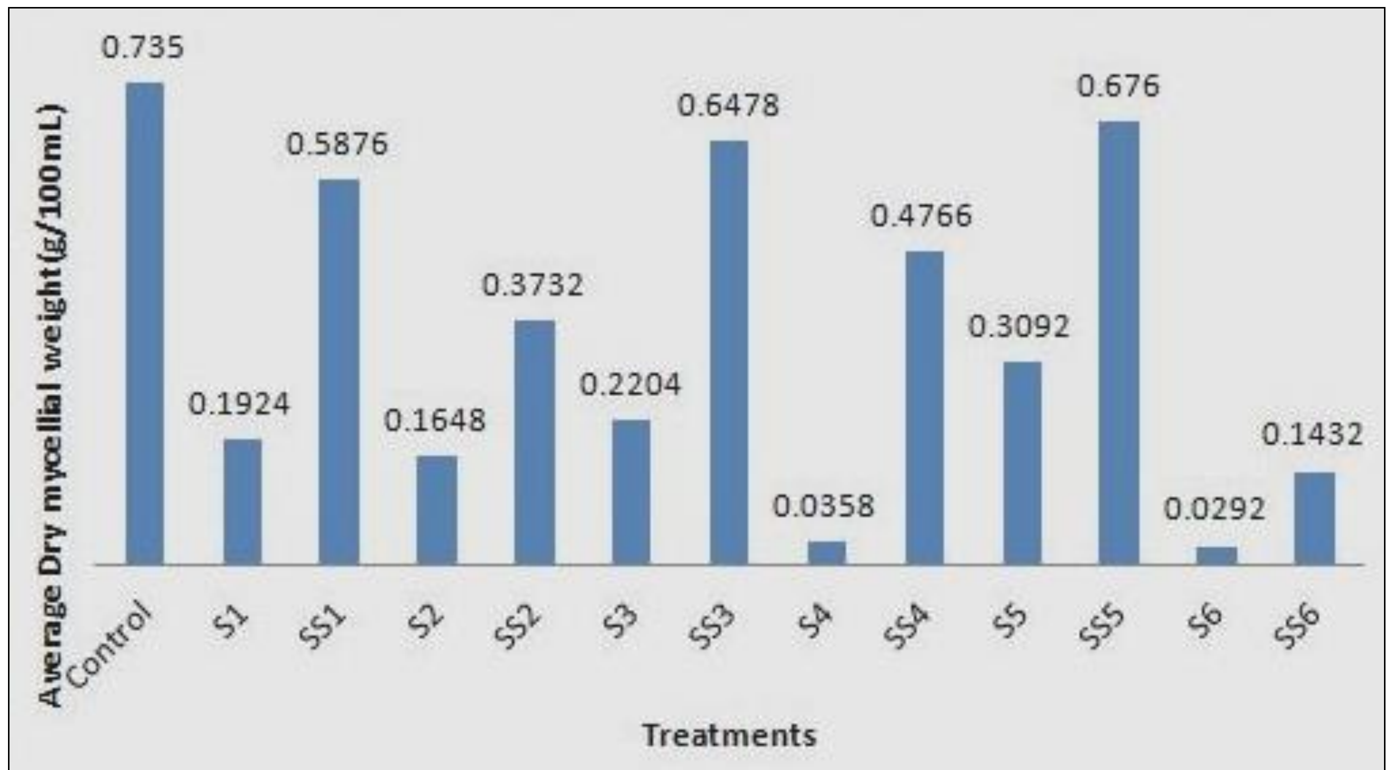

Fig 2: Average Biomass production of V.lecanii on different medium

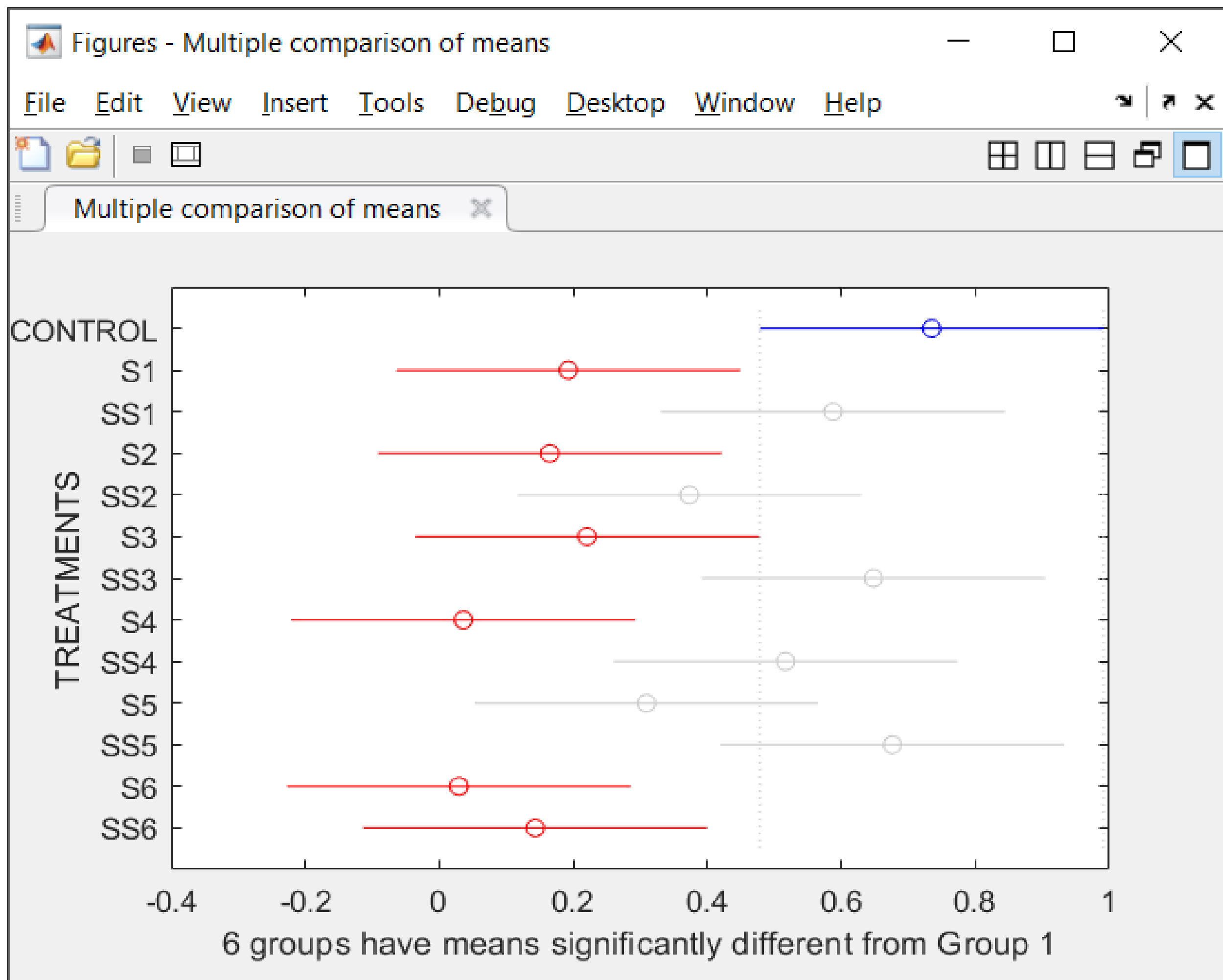

Fig 3: Multiple Comparisons of different treatments.

Table 1: Spore production and slope value of *V. lecanii* on different medium

Treatments (Media)	Spore counts (1×10^8cfu*)		Slope values #
	12th DOI	15th DOI	
Control	3.9	4	0.32
S1	3	3.4	0.27
SS1	6	9	0.53
S2	1.3	1.5	0.09
SS2	2.4	2.7	0.18
S3	3.2	3.9	0.13
SS3	4	4.9	0.19
S4	1.2	1.5	0.12
SS4	3.6	3.9	0.24
S5	3.0	3.2	0.12
SS5	4.2	4.7	0.21
S6	1	1	0.09
SS6	1.2	1.4	0.15

* mean value of three replicates
# The slope values represent the rate of fungus growth. High value Signifies profuse growth
While low value indicated inhibited growth

Conclusion

The successful commercial application of this promising biocontrol agent depends on the production of large quantities of biomass and conidia with minimum manufacturing cost. Keeping this in mind, easily available and inexpensive biodegradable substrates were used in the present study to screen for their potential for producing biomass in submerged culture. Maximum biomass of *V. lecanii* was found on Vegetable waste, Soybean oilcake and Wheat bran, supplemented with glucose and peptone. Highest number of viable conidia of *V. lecanii* was produced with supplemented wheat bran as substrate in liquid media during Liquid fermentation.

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