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Optimal Physical Parameters for Growth of *Trichoderma* Species at Varying pH, Temperature and Agitation

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Abstract

The study was aimed to carry out experiments to determine the optimal parameters for the biomass production of *Trichoderma*. It is quite essential to determine the physical conditions that are favorable for the growth of *Trichoderma* species. The seven species under study have been isolated from the rhizospheric soils of chickpea; pigeon pea and lentil crops of different areas of an Indian State (Uttar Pradesh) and these were later tested in vitro at different pH, temperatures and varying agitation speed. A significant difference in the biomass production was recorded among the species at tested pH levels i.e. 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0. The most favorable pH ranges between 5.5 and 7.5 in which total dry weight of mycelium varies between 1.41 and 1.35 g. Although all the species of *Trichoderma* produced sufficient biomass at different temperatures viz. 20°C, 25°C, 30°C and 35°C but they were found to be best grown at a temperature range of 25°C to 30°C. Aeration by agitation was also checked at different speeds such as 100, 150, 200 and 250 rpm but greatest biomass was recorded at 150 rpm.

Keywords: *Trichoderma*; pH; Biomass production; Optimization

Introduction

Trichoderma species are ubiquitous soil-borne Ascomycetes noted for their biocontrol capabilities against many economically important plant pathogens. In general, commercial preparations of *Trichoderma* sp. for biological control consist of bulk produced conidia, which are the asexual reproductive units of this fungus. Bulk production of conidia typically relies on manipulation of nutrients and substrates to promote conidiation, which has led to much research into the optimal growth conditions for in vitro conidiation in many species of *Trichoderma*. Together, these studies have suggested that the carbon and nitrogen (C:N) ratio, in addition to the ambient pH, are the main environmental factors influencing conidiation in *Trichoderma* [1-5]. *Trichoderma* strains are of great importance as biocontrol strains should have better stress tolerance levels than the plant pathogens against which they are going to be used for biological control [4]. The abiotic factors deteriorated the antagonistic properties of pH that also influence the mycelial growth of phytopathogenic fungi as well as biocontrol agents. As in all microorganisms even in *Trichoderma*, the external factors modify its morphological characteristics as well as physiological functions. Among these factors, pH is probably the most important environmental parameter affecting the mycoparasitic activities of *Trichoderma* strains [4]. A specific value of pH is required to note the maximum growth where these biocontrol agents can be multiplied and pathogen can be controlled. The studies on the variation of pH by different workers revealed that *Trichoderma* isolates showed optimum growth and sporulation rate at different pH values ranging from 2 to 7 [6,7]. In India, there is great diversity in soil characteristics especially with respect to soil pH. *Trichoderma* species are able to grow in a wide range of pH from 2.0 to 6.0 with maximal growth rates at 4.0, the optimum range being 4.6 to 6.8. However, there is a need to have strains specifically for saline soils and acidic soils. Similarly, in major parts of country, high soil temperature is an important factor for the survival of *Trichoderma* species. The residual toxicity due to fungicides used for the control of soil borne pathogens is an important environmental concern. Therefore, the improvement of stress tolerance in *Trichoderma* strains could result in increasing their efficacy against plant pathogenic fungi even under unfavorable environmental conditions. So, for exploiting the optimal antagonistic potential of *Trichoderma* which is to be applied as biocontrol agent (BCA) the effect of pH on their mycelial growth should be tested. Hence, an investigation was undertaken to study, compare, and assess the effect of pH on biomass production of *Trichoderma* sp. at different days of incubation.

Materials and Methods

Isolation of *Trichoderma*

Isolates of *Trichoderma* were isolated from soil samples collected from rhizospheric of chickpea, pigeonpea and lentil crop from different places of Uttar Pradesh, India (Table 1). All the isolates were isolated on PDA medium by following serial dilution plate technique as described by Johnson and Curl [8] and isolates were identified up to species level based on phenotypic characters like colony colour and growth; size and shape of conidiophore, phialides and conidia. The cultures were identified using the available literature [9-12] and confirmed by morphological characters and also confirmed by ITCC, Division of Plant Pathology IARI, New Delhi-12.

Media and culture preparation

Seven species of *Trichoderma* were assessed for biomass production on *Trichoderma* Specific Medium (TSM) for the optimization study on 4^{th}, 7^{th}, 10^{th}, 13^{th} and 16^{th} days at pH level 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0. The pH of the medium was adjusted at 4.0 to 8.0 with HCL or NaOH prior to sterilization. The medium was sterilized at 121°C for 15 min in an autoclave.

Preparation of standardized inoculums

Spore suspensions were prepared by adding 15 ml of sterile distilled water to mature (4-5 days) fungal colonies on PDA plates to dislodge the spores from the mycelium. The spores were counted using a heamocytometer (Neubauer, Germany) to obtain a spore concentration

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of about 105 spores/ml. These suspensions were then used to inoculate 100 ml TSM broth in 500 ml Erlenmeyer flasks [13]. The cultures were incubated at 25°C in an incubator shaker operating at 150 rpm for 48 hours. The resultant active growing cultures were aseptically washed three times with sterilized distilled water to remove remaining media. This resulting culture was then used as standard inoculum for further experiments. A total of 10% (v/v) of standard inoculum was inoculated in each experiment and performed in triplicate. Biomass production was used as an indicator for growth after 4th, 7th, 10th, 13th and 16th days of incubation. The biomass was calculated by obtaining the dry weight of mycelium using oven dry method (Figure 1).

Physical parameters

pH: The influence of initial medium pH on fungal growth was investigated at pH 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0. A 10% (v/v) standard inoculum was inoculated in a 500 ml Erlenmeyer flask containing 100 ml broth of TSM and incubated at 25°C in an orbital shaker at 150 rpm for 7 days. The pH that promoted the highest biomass production was used for subsequent steps of the investigation.

Temperature: The effects of temperature on fungal growth were studied at 20,25,30 and 35°C in *Trichoderma* specific medium at the determined optimum pH and incubated in an orbital shaker at 150 rpm for 7 days. The temperature that promoted the highest biomass production was used for the subsequent steps of the investigation.

Speed of agitation: The effects of agitation during incubation on growth were carried out in *Trichoderma* specific medium at optimum pH using an orbital shaker at 100, 150, 200 and 250 rpm. Incubation was conducted at the determined optimum pH and temperature. The agitation speed that promoted the highest biomass production was used for the subsequent steps of the investigation.

Statistical analysis

The results obtained were analyzed statistically and the means were compared using one-way ANOVA to indicate any significant difference among parameters and the variables. The result was considered significant if $p<0.05$.

Results

Isolation of *Trichoderma*: Seven isolates of *Trichoderma* were isolated from soil samples collected from different places of Uttar Pradesh, India, were identified as *T. harzianum* (*Th Azad*) which is isolated from soil sample of chickpea crop of Kanpur district. *T. viride* (01pp) isolated from soil sample of pigeon pea crop of Hardoi district. *T. asperellum* (Tasp/CSAU) and *T. koningii* (Tk/CSAU) were isolated from rhizospheric soil sample of Nawabganj farm, Kanpur. *T. atroviride* (71L) isolate which is isolated from rhizospheric soil sample of Hardoi district. Whereas, *T. longibrachiatum* (21pp) isolated from soil sample of Neveda block of Kaushambi and *T. virens* (Tvi/CSAU) isolated from soil sample of chickpea field of Student farm, CSAU Campus, Kanpur.

Effect of pH on the biomass of *Trichoderma* sp.

The mycelial growth was observed among all isolates of *Trichoderma* species described in Table 1 at all tested pH values of 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0 each of every 0.5 interval of pH range. Maximum number of isolates showed high biomass production at pH 6.5 followed by 7.5 and 5.5 and minimum at pH 4.0 and 4.5. The biomass production after sixteen days i.e., at the end of the experiment ranged from 0.80-0.97 g in all treatments. With increasing time all isolates showed a significant increase in biomass at all pH levels. The biomass production of *T. harzianum* (*Th Azad*), *T. viride* (01pp) and *T. asperellum* (Tasp/CSAU) were significantly higher than any other species at all pH levels whereas, *T. longibrachiatum* (21pp) and *T. atroviride* (71L) showed moderate biomass production and minimum was observed with *T. koningii* (Tk/CSAU) and *T. virens* (Tvi/CSAU) when incubation period was increased from 4 days to 16 days. All the species like *T. harzianum* (*Th Azad*), *T. viride* (01pp) and *T. asperellum* (Tasp/CSAU) isolated from soil sample initially produced high biomass at pH 7.0 but slowly the preference was shifted to pH 5.5

Sl. No.	ITCC No.	Culture Code	Source	Fungus identified
1	ITCC-6796	*Th Azad*	Kanpur Nagar	*Trichoderma harzianum*
2	ITCC-8315	01PP	Hardoi	*Trichoderma viride*
3	ITCC-8940	T_{asp}/CSAU	Kanpur Nagar	*Trichoderma asperellum*
4	ITCC-7437	21PP	Kaushambi	*Trichoderma longibrachiatum*
5	ITCC-7445	71 L	Hardoi	*Trichoderma atroviride*
6	ITCC-5201	T_k/ CSAU	Kanpur Nagar	*Trichoderma koningii*
7	ITCC-4177	T_{vi}/CSAU	Kanpur Nagar	*Trichoderma virens*

Table 1: *Trichoderma* sp. isolated from different places of Uttar Pradesh, India.

Figure 1: Effect of different pH on biomass of *Trichoderma* sp.

when incubation time was increased. *T. asperellum* (Tasp/CSAU) and *T. longibrachiatum* (21pp) always produced more biomass at pH 5.5 and this state has not changed across the days of incubation (Figures 2-10). The statistical analysis showed no significant difference between pH 6.0, 6.5 and 7.0 with p value at 0.0304.

Effect of temperature on the biomass of *Trichoderma* sp.

All the species of *Trichoderma* produces good biomass at different temperatures. Maximum biomass produced by *T. harzianum* (1.42 g) when incubated at 25°C compared to incubation at 20°C and 35°C which resulted in the production of 0.97 g and 0.82 g biomass respectively. The next highest biomass produce (1.35 g) by *T. viride* (01pp), 1.27 g by *T. asperellum* (Tasp/CSAU) and 1.24 g biomass produce by *T. longibrachiatum* (21pp). Whereas, 1.23 g biomass produced by *T. atroviride* (71L) and 1.21 g by *T. koningii*. Minimum biomass (1.18 g) produced by *T. virens* (Tvi/CSAU). There was no significant difference between 25°C and 30°C with p-value at 0.041 (Figure 11).

As for the effects of aeration, *T. harzianum* showed an increase biomass as the rate of agitation increased up to 150 rpm, then reduced when the speed of agitation increased up to 250 rpm (Figure 12). Statistical analysis showed no significant difference between speed of agitation of 150 and 200 rpm with p-value at 0.059, although species of *Trichoderma* produced higher biomass at 150 rpm than at 250 rpm.

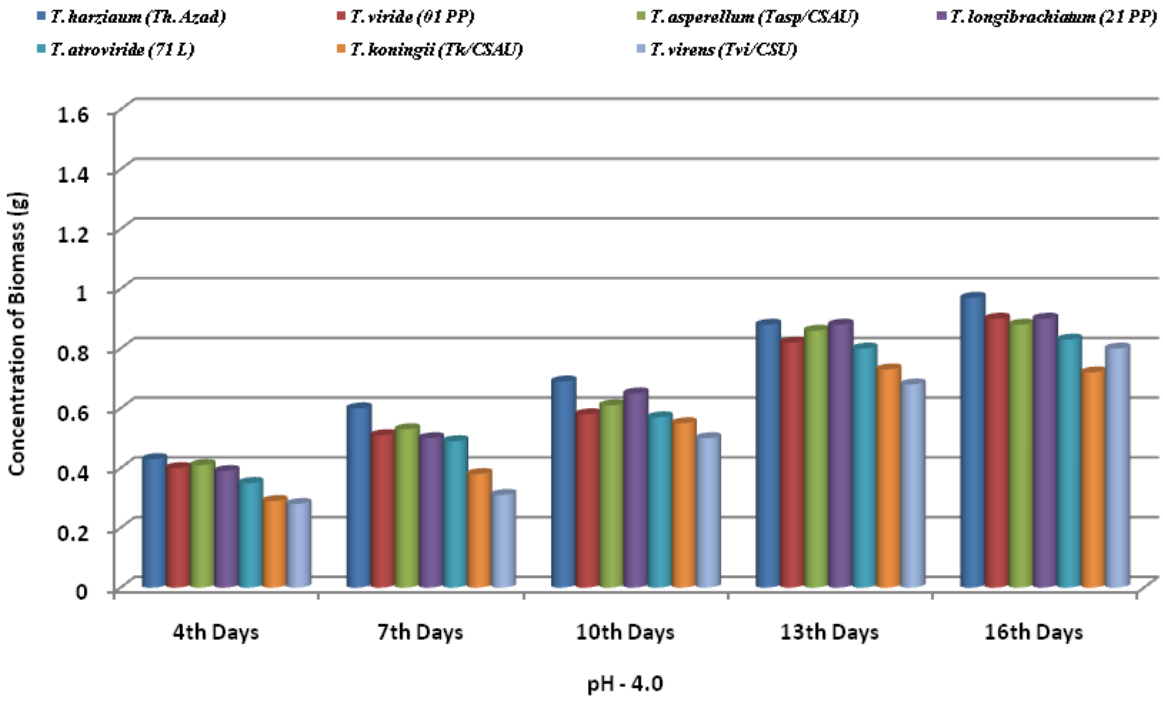

Figure 2: Biomass production of *Trichoderma* sp. at pH - 4.0.

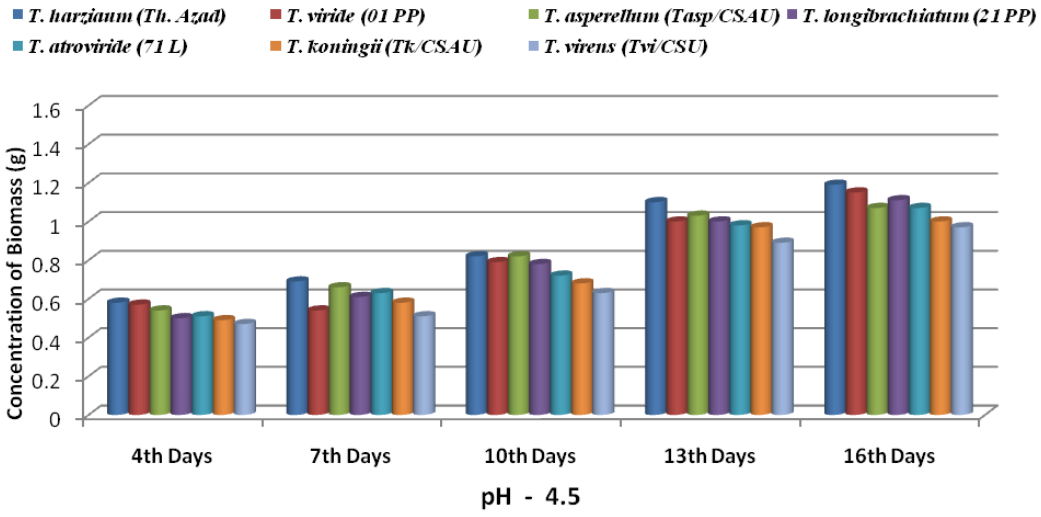

Figure 3: Biomass production of *Trichoderma* sp. at pH - 4.5.

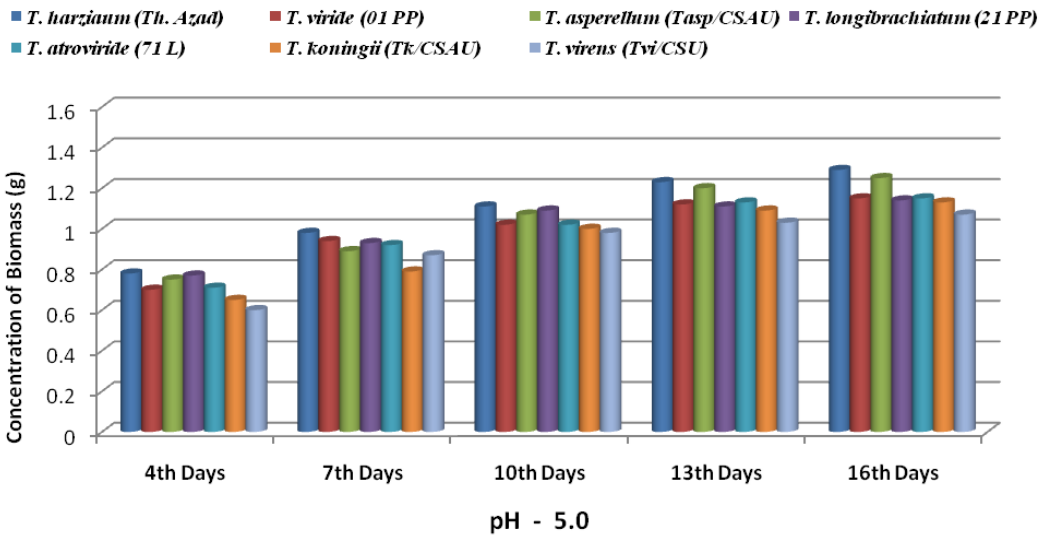

Figure 4: Biomass production of *Trichoderma* sp. at pH – 5.0.

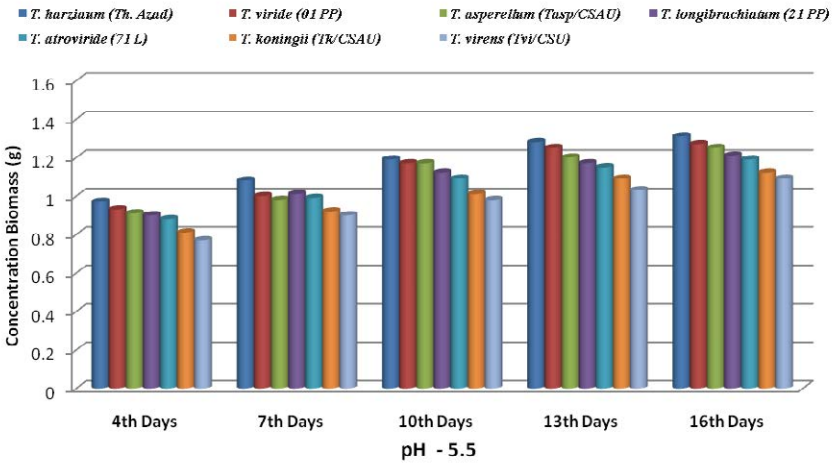

Figure 5: Biomass production of *Trichoderma* sp. at pH – 5.5.

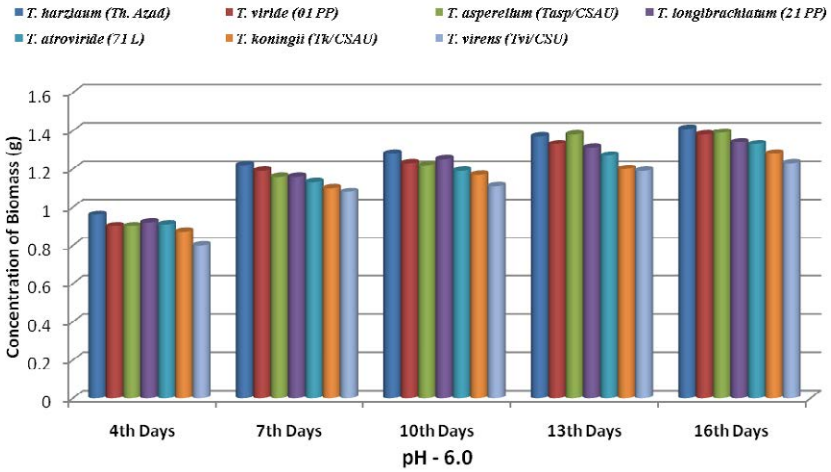

Figure 6: Biomass production of *Trichoderma* sp. at pH – 6.0.

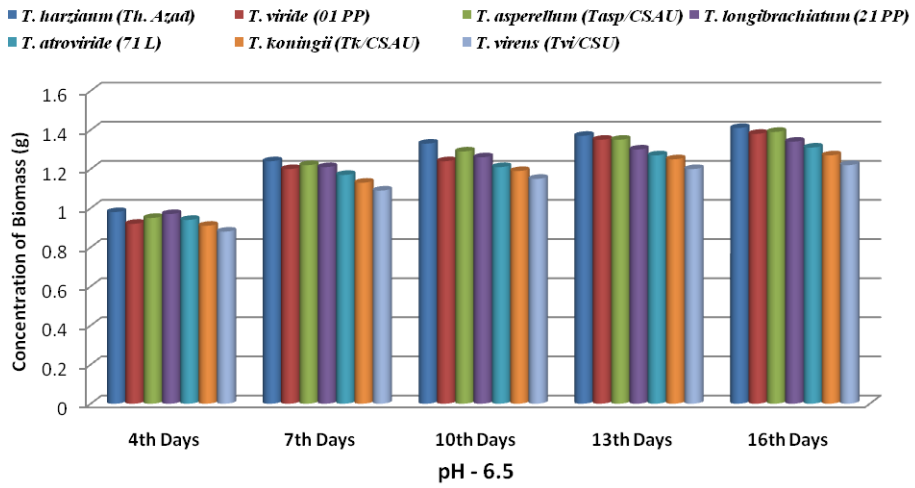

Figure 7: Biomass production of *Trichoderma* sp. at pH – 6.5.

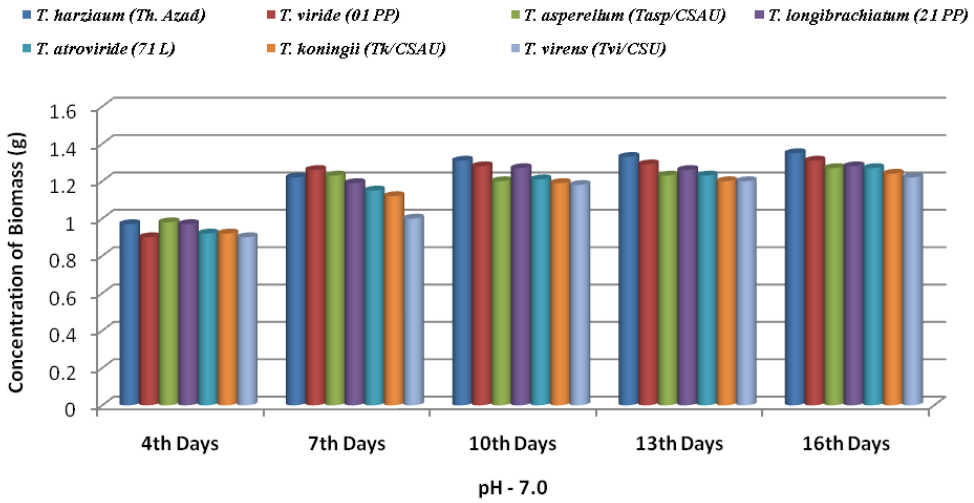

Figure 8: Biomass production of *Trichoderma* sp. at pH – 7.0.

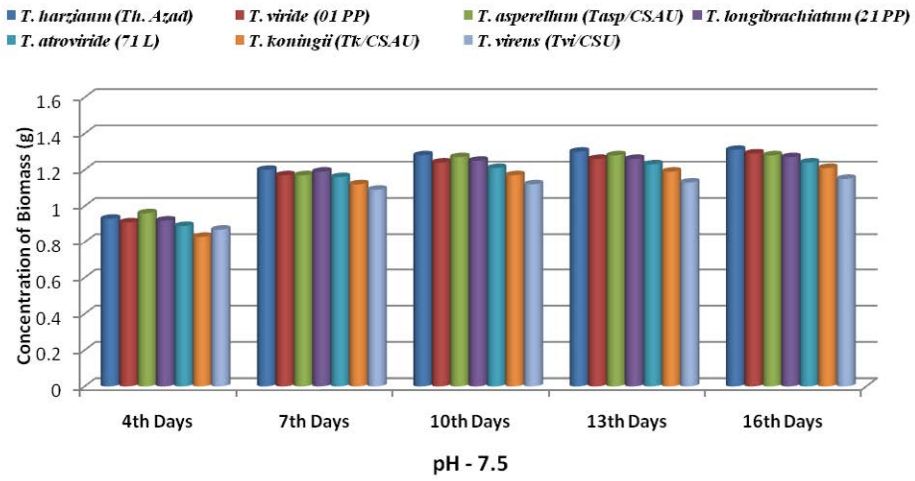

Figure 9: Biomass production of *Trichoderma* sp. at pH – 7.5.

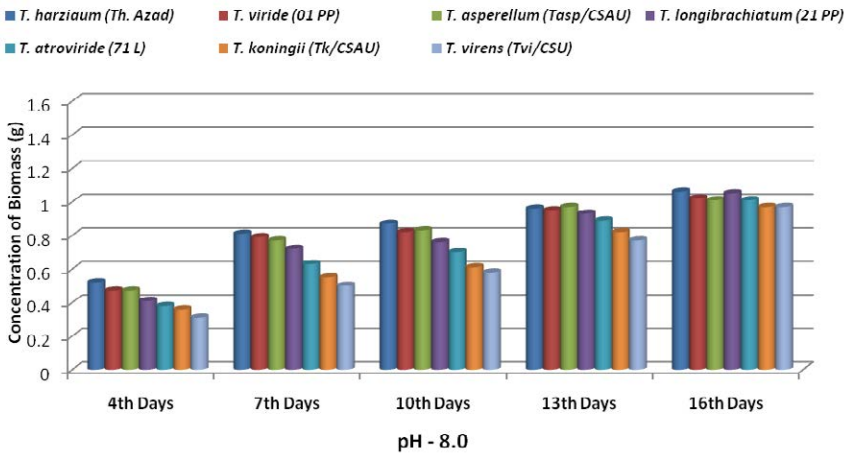

Figure 10: Biomass production of *Trichoderma* sp. at pH – 8.0.

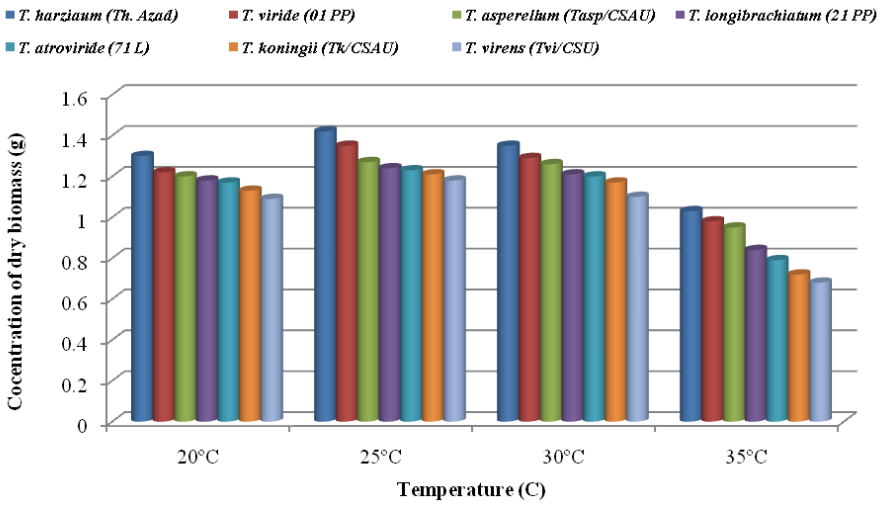

Figure 11: Effect of Temperature.

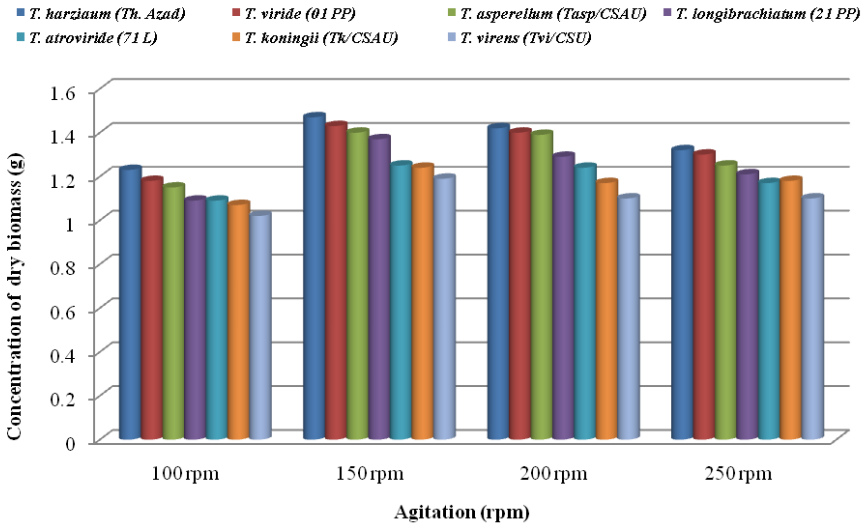

Figure 12: Effect of Agitation.

Discussion

The influence of pH on mycelial growth which clearly demonstrates that the acidic ambient pH has a major regulatory factor for biomass production in all these species that optimize the growth of the microorganism [14]. As expected, pH, temperature and aeration are important physical parameters and play significant roles in enhancing biomass production. This study confirmed that *Trichoderma* species grew better in acidic conditions. The studies of Limón et al. [15] showed that acidic pH favoured fungal growth than alkaline pH. Bitton and boylan [16] reported that growth of *Trichoderma* is more efficient in acidic than alkaline soils and they modify the rhizosphere soil by acidifying the soil. This explains the reason for isolates which prefer acidic pH. Our study showed none of the isolates showed higher biomass production at pH 4.0. Verdin et al. [16] reported that most fungi do not grow at very low pH values. Jackson et al. [2] reported that *T. harzianum* isolate showed optimum mycelial growth between pH 4.8 to 6.8. Even though at the end of our experiment, we checked the pH of the medium at all test pH values and it was found that the pH of the medium was constant. Previous studies also reported that several fungal isolates such as *Fusarium solani*, *F. oxysporum*, *Trichoderma viride* [17] and *Aspergillus niger* [18] cultured in MSM medium at pH 5.5 also gave a good growth. Among the parameters that could affect biomass production is temperature, generally considered the most important factor. The common incubation temperature for the growth of fungi such as *A. niger* [19], *Trichoderma* sp., *Fusarium* sp., *Penicillium* sp. and *Graphium* sp. [20] is taken to be 30°C. Sharma et al. [21] reported that media, temperature and pH had profound effect on growth of fungi. They also reported that none of the *Trichoderma* species grew at or above 40°C. Singh et al. [22] also got similar results. Agitation influenced the microbe to absorb more nutrients and the amount of dissolved oxygen in the cultivation medium [23]. Agitation speed has also been proven to be a critical factor influencing mycelial biomass. Similarly, this study found that production of biomass increased with the speed of agitation. Aeration could be beneficial to the growth and performance of microbial cells by improving the mass transfer characteristics with respect to substrate, product or by-product and oxygen.

Conclusion

Isolation, characterization and morphological description of *Trichoderma* species are important before further dissemination is done leading to the biomass production at different environmental and cultural conditions. An attempt has been made to grow different species of *Trichoderma* at varying pH, temperature and agitation speeds in order to reveal all the relevant and favorable parameters. The isolates from the soils of legume fields are more adaptive to the tested pH ranges than the isolates from virgin soils where there is no intervention of agricultural practices. As *Trichoderma* is an ecofriendly biological control agent against other soil borne plant pathogens, it is necessary to grow it at suitable conditions before it is used for commercial purposes. Different pH, temperatures and agitation speeds have been tested in this study for a better growth of different isolates of *Trichoderma* species.

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