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Physiological and biochemical analysis of *Trichoderma* species isolated from different location U. P.

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Seven different species of *Trichoderma* isolated from the different locations of Uttar Pradesh were used in this study. A comparative analysis was done among all the seven species for the determination of glucanase enzyme production and nitrogen content determination the results showed that among all the seven tested species, *Trichoderma harzianum* shows the highest glucanase enzyme production as well as the highest nitrogen content. Optimal physical parameters such as pH, temperature, and effect of agitation were also tested for the biomass production on all the eight species. The results revealed that the optimal pH temp and agitation speed for *Trichoderma* biomass production were 6.0 and 6.5, 25 and 30°C and 150 rpm. In present study we also evaluated the effect of two different carbon sources on the glucanase enzyme induction. Carboxymethyl cellulose (CMC) was found to be the best carbon source for glucanase enzyme induction.

Key words: *Trichoderma,* nitrogen content, biological control.

INTRODUCTION

Soil borne pathogens have a broad host range and persist for longer periods in soil by resistant resting structures. Chemical control of soil borne pathogens provides certain degree of control but at the same time have adverse effects on environment affecting the beneficial soil microorganisms. Therefore, biological control of plant pathogens has been considered as a potential control strategy in recent years and search for these biological agents is increasing. *Trichoderma* is the most commonly used fungal biological control agent and have long been known as effective antagonists against plant pathogenic fungi (Margolles-Clark et al., 1996; Harman et al., 2004; Chet, 1987). In which, *Trichoderma harzianum* has been accepted as one of the most potent biocontrol agents against plant diseases and used as an antagonist against many soil borne phytopathogenic fungi over the past few years (Samuels et al., 1998). Various strategies of biocontrol have been proposed. They include the creation of competition for nutrients or space; the production of antibiotics and lytic enzymes; the inactivation of the enzymes of phytopathogenic fungi; and parasitism (Viterbo et al., 2002). The cell wall-degrading enzymes (CWDEs), mostly chitinases, glucanases, and proteases, are major lytic enzymes that are secreted by biocontrol agents (Bisset, 1991b). CWDEs attack the cell wall of phytopathogenic fungi to cause cell lysis and

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subsequent death. Although the mechanism of mycoparasitism is not completely understood, this process has been assumed to involve the expression of extracellular CWDEs. Recently, homologues of proteins encoded by avirulence genes have been identified in *Trichoderma* strains. These proteins can induce hypersensitive responses and other defense-related reactions in plant cultivars that contain the corresponding resistance genes (Tseng et al., 2008). The knowledge of nutritional requirements is the main need in the cultivation of microorganisms using any cultural technique. The carbohydrates, proteins, lipids, nucleic acids are made up of macro elements like carbon, hydrogen, nitrogen, sulphur, phosphorus and these are involved in mechanisms like host pathogen interaction and self defense mechanisms. Nitrogen is a major element found in many of the simple compounds and nearly all of the complex macromolecules of living cells. Proteins and nucleic acids are especially rich in nitrogen. Thus, it should not be surprising that a substantial cellular investment is made in the metabolic machinery comprising nitrogen catabolic pathways to ensure a constant nitrogen supply for growth. Extensive studies of nitrogen metabolism and its control have been carried out in three fungi, *Neurospora crassa, Aspergillus nidulens* and Saccharomyces cerevisiae. The ability of some filamentous fungal species to produce gram quantities of protein per liter of culture medium has been exploited by enzyme industry e.g. *Trichoderma reesei (*Kendrick and Ratledge, 2006*).*

MATERIALS AND METHODS

Purification and morphological characterization of *Trichoderma* species

Trichoderma isolates collected from soil samples of different lactation of Uttar Pradesh, isolated with the help of serial dilution plate technique, (Johnson and Curl, 1972) were grown on PDA medium for proper identification. These potential isolates of *Trichoderma* species were identified by light microscope for morphological characters such as the branching pattern of conidiophores, the conidiophores apex elongation and shape (coiled, straight or undulate), the phialides shape, structure, size and the conidial shape (Table 1). The cultures were identified using the available literature (Bisset, 1991b; Griffin, 1994) and monographic contribution provided by Bissett (1991a, b) also reconfirmed by ITCC, Division of Plant Pathology IARI, New Delhi.

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 500ml Erlenmeyer flask containing 100 ml broth of PDA media and incubated at 28°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 25, 30, 35 and 40°C in *Trichoderma* specific medium at the determined optimum pH and incubated in BOD incubator 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.

Induction of β-1-3 Glucanase enzyme taken from *Trichoderma* sp. on different carbon sources

Two different carbon sources were selected for the induction of glucanase enzyme *viz*- CMC and wood dust. These carbon sources were added in Czapek Dox medium at the rate of 1%. Cultures were incubated for 10 days at 28°C on orbital shaker at 150rpm. At the end of the incubation time, wood dust residues were removed and filtrate was centrifuged at 5000 rpm for 10 min. The clear supernatant was considered as a source of crude enzyme. This obtained supernatant was used for measuring enzyme activity. Glucanase enzyme activity was assayed using 1% (w/v) CMC as a substrate. Enzyme activity is expressed as U/mg.

1 ml enzyme solution was added to 1ml of 1% carboxymethyl cellulose dissolved in 50 mM sodium acetate buffer, pH 5.0. After incubation at 50°C for 60 min, the reaction was stopped by the addition of 3 ml DNS reagent. After incubating for 10 min in a boiling water bath enzymatic activity was determined at 540 nm. One unit of CMCase activity was expressed as the amount of protein that liberate reducing sugar equivalent to glucose per minute under assay conditions.

Determination of protein concentration

Protein content of the crude enzyme preparation was assayed by Lowry (1951) method, using BSA, as standard. 0.5 mg/ml of BSA standard was used. We made different dilutions of this standard. To each tube 2 ml of complex forming reagent was added and keep for 10 min at room temperature. After this 0.2 ml of solution Folin-Ciocalteu reagent was added and the sample was incubated for 20 to 30 min. The same steps used for the test sample, absorbance was taken at 660 nm. Calibration curve was constructed by plotting absorbance reading on Y axis against standard protein concentration (mg/ml). Sample concentration was calculated through this standard graph (Figure 2).

Kjeldahl method for nitrogen estimation

Total Nitrogen content of different strains of *Trichoderma* sp. was estimated by Kjelplus nitrogen analyzer. About 1 g dried and well-powdered sample of *Trichoderma* was accurately weighted on a piece of filter paper and transferred along with the filter paper to 30 ml Micro Kjeldahl digestion tube. Then 10 ml of concentrated H_2SO_4 and 500 mg digestion mixture ($CuSO_4$: K_2SO_4, 1: 5) were added to digestion tube. Sample was digested on an electric heater at 400°C for 1 h. After cooling, the digested sample was transferred to micro Kjeldahl distillation apparatus using successive small quantities of

Table 1. Identification of potential strains of *Trichoderma* species.

Strain No.	Name of bioagent	ITCC Acc. No.	GenBank accession no.	Strain code	Source	GPS Location
T1	*T. harzianum*	6796	KC800922	*Th* azad	CSA Kanpur Nagar	Latitude: 25˚ 8′ 34.821″ Longitude: 81˚ 59′ 2.979″
T2	*T. viride*	8315	JX119211	01PP	Hardoi	Latitude: 27˚ 23′ 40.729″ Longitude: 80˚ 7′ 47.751″
T3	*T. asperellum*	8940	KC800921	T_{asp}/CSAU	CSA Kanpur Nagar	Latitude: 25˚ 8′ 34.821″ Longitude: 81˚ 59′ 2.979″
T4	*T. koningii*	5201	KC800923	T_K (CSAU)	CSA Kanpur Nagar	Latitude: 26˚ 29′ 33.384″ Longitude: 80˚ 18′ 6.518″
T5	*T. atroviride*	7445	KC 008065	71 L	Hardoi	Latitude: 26˚ 29′ 28.323″ Longitude: 80˚ 18′ 26.361″
T6	*T. longibrachiatum*	7437	JX978542	21 PP	Kaushambi	Latitude: 26˚ 34′ 27.61″ Longitude: 79˚ 18′ 24.623″
T7	*T. virens*	4177	KC800924	T_{vi} (CSAU)	CSA Kanpur Nagar	Latitude: 25˚ 21′ 39.794″ Longitude: 81˚ 24′ 11.414″

water. 10 ml of 40% NaOH solution was poured in it and NH_3 liberated by steam distillation was collected in 100ml conical flask containing 10ml of 4% boric acid solution in which few drops of mixed indicator was added. Boric acid containing ammonium borate (NH_3) was titrated against N/10 standard HCl until the first appearance of violet colour at the end point. A reagent blank with filter paper (no sample, only digestion mixture and $H_2SO_{4)}$ was also run and titrate value for blank was also recorded. Nitrogen percent in the sample was calculated by using following formula.

$$\text{Nitrogen \% (in 100 g)} = \frac{1.4 \times N \times V \times 100}{W}$$

Protein % = Nitrogen (%) × 6.25

RESULTS AND DISCUSSION

Isolation and identification of bioagent

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. They were morphologically identified by slide mounting and re-confirmation by ITCC, IARI, New Delhi and allotted with unique accession number, as well as molecular identified by ITS marker, sequences deposited to NCBI GenBank. Finally, potential and effective bioagent were submitted to microbial data bank at NBAIM, Mau, India (Table 2).

Effect of pH, temperature and agitation on the biomass of *Trichoderma* sp

The mycelial growth was observed among all isolates of *Trichoderma* species described in Table 3 at all tested pH values of 4.5, 5.0, 5.5, 6.0, 6.5, 7.0 and 7.5 each of every 0.5 interval of pH range. Maximum number of isolates showed high biomass production at pH 6.0 and 6.5 followed by 5.5 and 7.0 and minimum at pH 4.5 and 7.5. 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)

Table 2. Morphological and cultural observation of *Trichoderma* species.

Isolates/ characters	*T. harzianum* (*Th* azad)	*T. viride* (01PP)	*T. asperellum* (T_{asp}/CSAU)	*T. longibrachiatum* (71 L)	*T. atroviride* (21 PP)	*T. koningii* T_K (CSAU)	*T. virens* T_{vi} (CSAU)
Colony growth rate (cm/day)	7-8 in 5 days	7-8 in 5 days	6-7 in 5 days	7-8 in 5 days	7-8 in 5 days	5-6 in 5 days	6-7 in 5 days
Colony colour	Light green to dark green	Bright green to dark green	dark green	Yellowish green	Dark Green	Dull green	Yellowish green
Reverse colony colour	Whitish ring like zones	Colourless	Colourless	Colourless	Colourless	Light yellow	Light yellow
Colony edge	wavy	Smooth	Smooth	Smooth	Smooth	crysty	Smooth
Culture smell	Coconut like	Coconut like	Coconut like	Coconut like	Coconut like	Malt	Malt
Mycelial form	Floccose to arachnoids	Floccose to arachnoids	Floccose to arachnoids	Floccose to arachnoids	Floccose to arachnoids	Crysty	Crysty
Myceilial colour	Cottony white	Whitish white	Watery white	Watery white	Watery white	Watery white	Watery white
Conidiation	Circular	Ring like zones	Irregular	Circular	Circular	Irregular	Circular
Conidiophores branching	Flexuous regularly highly branched	Irregularly branched often paired	Repeated, highly branched	Sparingly, rarely branched	Irregularly, crowed smooth, branched	Simple, pyramidal, smooth,	Simple, rebranched, few lateral branches
Phialide shape	Flask shape, swollen in middle, narrow at the tip	Flask shape swollen in middle, narrow at the base	Long, swollen in middle, horn shaped	Lageniform, slightly swollen near the middle	Cylindrical, slightly flask shape, swollen in middle	Nine-pin bowling shape	Lageniform to ampuliform swollen at the middle
Conidial shape	Subglobose to ovoid	Globose to ovoid	Globose to subglobose	Ellipsoidal to obvoid	Subglobose	Oblong, obvoid, ellipsoidal	Ellipsoidal to obvoid
Conidial color	Light green	Light green	Yellowish green	Light green	Light green	Light green	Yellowish green
Clamydospore	Observed	Not observed	Not observed	Not observed	Not observed	Not observed	Not observed

Table 3. Effect of pH on the biomass of *Trichoderma* species.

Stains name	pH 4.5	pH 5.0	pH 5.5	pH 6.0	pH 6.5	pH 7.0	pH 7.5
T.harzianum (T. Azad)	0.107	0.152	0.313	0.607	0.709	0.301	0.223
T. viride (01PP)	0.098	0.119	0.210	0.564	0.702	0.321	0.122
T. asperellum (T-asp)/CSAU	0.105	0.117	0.398	0.667	0.732	0.438	0.235
T. longibrachiatum (21PP)	0.098	0.138	0.295	0.655	0.803	0.465	0.463
T. atroviride (71 L)	0.089	0.122	0.284	0.583	0.789	0.378	0.148
T.koningii (T.k/CSAU)	0.086	0.109	0.376	0.540	0.653	0.366	0.073
T.virens (T.vi /CSAU	0.078	0.102	0.270	0.398	0.537	0.302	0.980

Table 4. Effect of Temperature on the biomass of *Trichoderma* species.

Stains name	15°C	20°C	25°C	30°C	35°C	40°C
T.harzianum (T. Azad)	2.1	3.2	5.9	6.7	6.5	4.9
T. viride (01PP)	1.9	3.1	5.7	6.6	6.8	4.6
T. asperellum (T-asp)/CSAU	1.9	2.7	5.5	6.7	6.7	4.9
T. longibrachiatum (21PP)	2.0	2.8	5.7	6.5	6.4	4.0
T. atroviride (71 L)	1.8	2.2	5.4	6.3	6.2	3.8
T.koningii (T.k/CSAU)	1.6	2.0	5.5	6.4	6.0	3.6
T.virens (T.vi /CSAU	1.4	1.9	4.2	5.5	5.9	3.2

Table 5. Glucanase enzyme production by *Trichoderma* spp. grown on different carbon sources.

Carbon source (1%)	*T. viride*	*T. harzianum*	*T. asperellum*	*T. koningii*	*T. atroviride*	*T. longibrachiatum*	*T. virens*
CMC	1.66	2.01	1.42	1.39	1.35	1.20	0.82
Wood dust	0.5	0.41	0.4	0.39	0.35	0.33	0.3

and *T. virens* (Tvi/CSAU) after 7 days (Table 4). Along with pH all the species of *Trichoderma* produces good biomass at different temperatures. In which maximum biomass produced by *T. harzianum*, *T. viride*, *T. asperellum* followed by *T. longibrachiatum T. atroviride, T. koningii and* T. *virens* when incubated at 25, 30 and 35°C compared to incubation at 15, 20 and 40°C. There was no significant difference between 25 and 30°C with p-value at 0.041 (Table 4). As for the effects of aeration, *Trichoderma* species showed an increase biomass as the rate of agitation increased up to 150 rpm and reduced when the speed of agitation increased up to 250 rpm. 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.

Calculation of results

The general equation for the protein content is:

$$\text{Percent protein} = \frac{\text{Adolescent } [(V_b - V_s)\ (N)\ (1.4007)}{(W)] \times f}$$

V_b = ml titrant for the blank; V_s = ml titrant for the individual samples; N = Normality of the acid titrant (norminally 0.1); 1.4007 = A single factor that takes into account the molecular weight of nitrogen; The conversion of the mille – equivalent result of V × N, and the conversion to 1; W = the weight of the sample in grams. The error is sufficiently small that, for Sample weighted to 1.000 g + / - 0.0005 g. This can be assumed to be 1; F = the factor for converting the percent nitrogen in sample to percent protein.

N content of the sample was calculated on the basis of the following formula.

$$N\% = \frac{(\text{ml } H_2SO_4 \text{ in sample}) - (\text{ml } H_2SO_4 \text{ in blank}) \times \text{normality of acid}}{\text{Weight of the sample (mg)}}$$

Total protein content was determined by, multiplying a factor with the observed nitrogen values.

To achieve maximum production two different carbon sources were added in the culture media for maximum glucanase enzyme production (Figure 1). CMC was found to be the best glucanase inducer as compare to wood dust (Table 5). *T. harzianum* and *T. viride* produced the highest amount of glucanase enzyme. So these strains can be commercialized on large scale for the control of phytopathogens

The chemical factors which in turn influenced the occurrence of *Trichoderma* sp. *Trichoderma* species that were high in nitrogen favored the occurrence of this fungus. From the data presented in Table 6, it was revealed that the maximum Nitrogen was recorded in *T. asperellum* (10.2%) followed by *T. longibrachiatum* (7.1%), *T. harzianum* and *T. koningii* (6.8%) but in *T. viride* (4.1%) Nitrogen content was found to be too low. Mineral nutrition is essential for growth, sporulation and stimulation of fungal secondary metabolism (Griffin, 1994). High total N availability increased sporulation, production of antifungal anthroquinone pigments, hyphal growth rate (Fargasova, 1992), and the antagonistic activity of *Trichoderma* sp. against wood rot fungus *Serpula lacrymans* (Score and Palfreyman, 1994). Soil nitrate levels were positively correlated with cellulose production (Widden and Breil, 1988) and may favor competitiveness of the biocontrol agent with the pathogen. Nitrogen is a major element found in most simple compounds and nearly all of the complex microorganisms of living cells. Proteins and nucleic acids are especially rich in nitrogen.

The method was developed by Johan Kjeldahl, and is used extensively in the determination of protein, since

Table 6. Glucanase enzyme protein content of seven fungal strains.

Fungal strain	Glucanase enzyme protein concentration (mg/ml)
T. harzianum	0.23
T. viride	0.21
T. koningii	0.19
T. asperellum	0.17
T. atroviride	0.15
T. longibrachiatum	0.12
T. virens	0.09

Table 7. Evaluation of N% of *Trichoderma* species.

Trichoderma species	Titration value	Nitrogen percentage	Protein percentage
T. harzianum	0.79	1.10	6.8
T. viride	0.48	0.67	4.1
T. koningii	0.80	1.1	6.8
T. asperellum	1.17	1.63	10.2
T. atroviride	0.54	0.75	4.6
T. longibrachiatum	0.82	1.14	7.1
T. virens	0.49	0.68	4.2

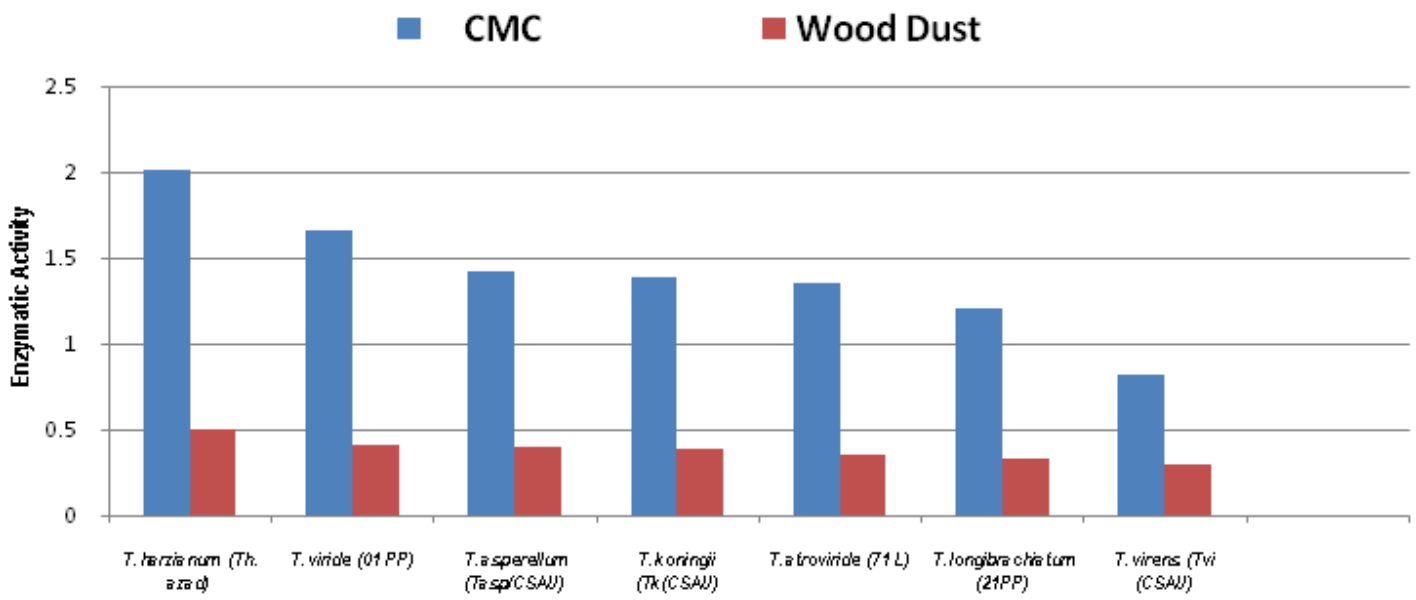

Figure 1. Effect of carbon sources on glucanase enzyme induction.

protein is a macromolecule and made up of nitrogen containing amino linkage together. For the given sample of *Trichoderma* sp., the percent nitrogen measured is converted to the equivalent protein content by the use of an appropriate numerical factor. For this sample, the factor is 6.25 since fungal protein is approximately 16% of nitrogen. Data revealed (Table 7) that highest protein is found in *T. asperellum* followed by *T. longibrachiatum.*

Conflict of Interests

The authors have not declared any conflict of interests.

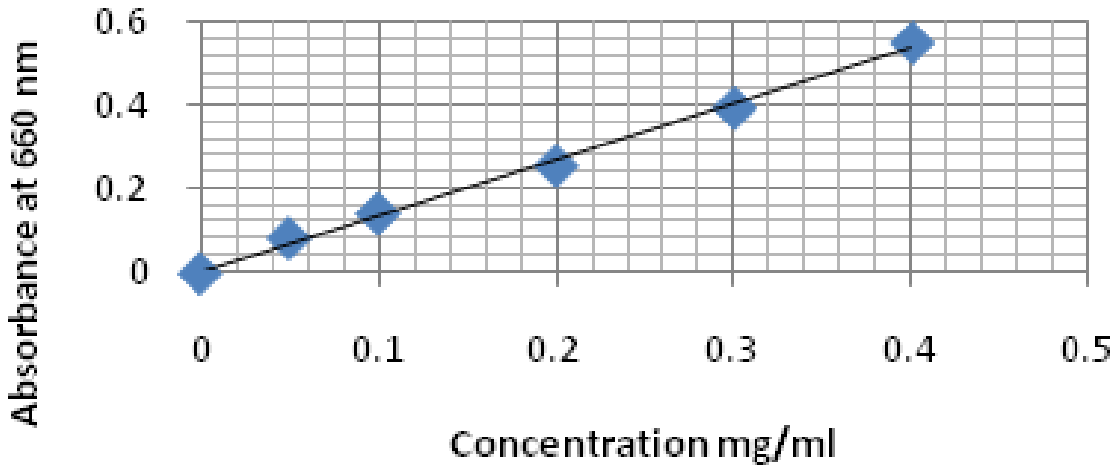

Figure 2. BSA standard curve.

REFERENCES

Bisset J (1991a). A revision of the genus *Trichoderma* II. Infragenric classification Canadian. J. Bot. 69:2373-2417.

Bisset J (1991b). A revision of the genus *Trichoderma* III. Sect. *Pachybasium*. Canadian J. Bot. 69:2373-2417.

Chet I (1987). *Trichoderma*: application, mode of action and potential as a biocontrol agent of soilborne plant pathogenic fungi. In Innovative Approaches to Plant Disease Control. Chet, I., Ed.; Wiley: New York. pp. 137-160.

Fargasova A (1992). The influence of various nitrogen sources on the growth, conidiation and pigmentation production of the brown mutant of *Trichoderma viridae* M-108. Biologia 47:453-194.

Griffin DH (1994). Fungal Physiology, 2nd ed. John Wiley & Sons, New York.

Harman GE, Howell CR, Viterbo A, Chet I, Lorito M (2004). *Trichoderma* species-opportunistic, avirulent plant symbionts. Nat. Rev. 2:43-56.

Johnson LF, Curl EA (1972). Methods for Research on the Ecology of Soil bore Plant Pathogens. Burgess Publishing company. Minneapolis.

Kendrick A, Ratledge C (1996). Cessation of polyunsaturated fatty acid formation in four selected filamentous fungi when grown on plant oil. J. Am. Oil Chem. Soc. 73:431-435.

Lowry OH, Rosebrough AL, Farr RJR (1951). Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193(1):265-275.

Margolles CE, Hayes CK, Harman, GE, Penttila M (1996). Improved production of *Trichoderma harzianum* endochitinase by expression in *Trichoderma reesei*. Appl. Environ. Microbiol. 62:2145-2151.

Samuels GJ, Petrini KO, Lieckfeldt KE, Kubicek CP (1998). The *Hypocrea schweinitzii* complex and *Trichoderma sect. Longibrachiatum*. Stud. Mycol. 41:1-54.

Score AJ, Palfreyman JW (1994). Biological control of the dry rot fungus *Serpula lacrymans* by *Trichoderma* species: the effects of complex and synthetic media on interaction and hyphal extension rates. Int. Biodeterioration Biodegradation. 33:115-128.

Tseng SC, Liu SY, Yang HH, Tsuen LC, Peng KC (2008). Proteomic Study of Biocontrol Mechanisms of *Trichoderma harzianum* ETS 323 in Response to *Rhizoctonia solani* .J. Agric. Food Chem. 56:6914-6922.

Viterbo A, Ramot O, Chernin L, Chet I (2002). Significance of lytic enzymes from *Trichoderma* spp. in the biocontrol of fungal plant pathogens. Antonie Van Leeuwenhoek 81:549-556.

Widden P, Cunningham J, Breil B (1988). Decomposition of cotton by *Trichoderma* species: Influence of temperature, soil type and nitrogen levels. Can. J. Microbiol. 35:469-473.