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RESEARCH ARTICLE

Isolation and Optimized Production of Xylanase under Solid State Fermentation Condition from *Trichoderma* sp.

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Abstract

Seven strains of *Trichoderma* were isolated and selected for the xylanase enzyme production under optimized conditions. Various process variables were optimized using conventional 'one-variable-at-a-time' approach which involves varying a single independent variable and maintaining others at a constant level. All culture conditional variables had profound influence on enzyme production and a significant increase in xylanase enzyme production was occurred when 2 ml of inoculum is added in the growth media and incubated for 7 days at pH 6.0 and temperature 55˚C under static conditions in solid state fermentation.

Introduction

Trichoderma sp. is a well known biocontrol agent against many plant pathogents such as *Rhizoctonia, Botrytis, Pythium, and Fusarium* which cause various plant diseases (Chet *et al.*, 1987). There are about 41 species in the genus *Trichoderma. Trichderma* species are potent agents for the biocontrol of plant pathogens. 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. The cell wall-degrading enzymes mostly chitinases, xylanases glucanases, and proteases, are major lytic enzymes that are secreted by biocontrol agents. CWDEs attack the cell wall of phytopathogenic fungi to cause cell lysis and subsequent death. Although the mechanism of mycoparasitism is not completely understood, this process has been assumed to involve the expression of extracellular CWDEs. Xylanases, chitinolytic beta glucanases or cellulases are the enzymes most frequently considered in biocontrol mechanism (Chet *et al.*, 1987). Xylanases (β-1,4-xylan xylanohydrolase, E.C.3.2.1.8) is the major component of xylanolytic enxymes and randomly cleaves the β-1,4-glycosidic bond of xylan backbone. Xylan is the second most abundant natural polysaccharide. Xylan is a heteropolysaccharide consisting of β -1,4-linked D-xylose monomers in connection with side branches of arabinosyl, glucuronosyl, acetyl, uronyl, and mannosyl residues (Tsujibo *et al.*, 1997). Complete degradation of xylan structures requires the concerted and synergistic function of several enzymes including endo-β-1,4-xylanases (EC 3.2.1.8) (Jun *et al.*, 2009). Due to the broad applications in biopulping and biobleaching in paper industry, xylanase has been one of the major research focuses for researchers (Tsujibo *et al.*, 1997).

Materials and Methods

Microorganism and inoculums

Isolation was carried out from soil samples collected from different locations of an Indian state Uttar Pradesh. Isolates of *Trichoderma* species weres isolated and identified in potato dextrose agar medium thereafter submitted to the Indian Type Culture Collection at IARI (Pusa, New Delhi, India) and allotted with specific ITCC numbers. The inoculums was prepared in potato dextrose medium (PDM) containing birch wood xylan as the carbon source, by harvesting spores from 120 h old cultures grown at 30˚C (Table 1).

Fermentation parameters

Solid state fermentation (SSF) was carried out with 50 ml of Vogel's medium with some modifications and 1% of birchwood xylan in 250 ml Erlenmeyer flasks and incubated at 30°C for 7 days in a BOD incubator. After 8 days, the contents of the flask were filtered through filter paper and the obtained culture broths were centrifuged at 3000 × g for 10 min and supernatant was assayed for extracellular xylanase activity. For maximum production of xylanase, various culture conditions viz., carbon and nitrogen source, inoculum size, pH, temperature and incubation period were optimized by conventional 'one variable at a time' approach which involves varying a single independent variable at a time while maintaining the others at a constant level. Fungal isolates were grown in different media containing 1% birchwood xylan, at pH 6.0 for 192 h. Medium giving maximum growth was further used to standardize the inoculum size by inoculating different concentrations (1-5 ml) of inoculums. Similarly, the temperature and time of incubation were optimized by growing the fungal isolate at different temperatures (25˚-65˚C) for different time periods (96-192 hrs). Different organic viz., yeast extract (YE), beef extract (BE), peptone (PPT), soybean residue (SR) and corn powder (CP) were supplemented separately to a final concentration of 0.3 % (w/v) to study the microbial growth and xylanase activity. Glucose, maltose and sucrose at a final concentration of 0.1% while wheat bran (WB), corn cob (CC) birchwood xylan (BW) and carboxy methyl cellulose (CMC) were used as the carbon source at a final concentration of 1.0%.

Enzyme isolation and assay

After 7 days of growth, the fungal broth was filtered through filter paper and centrifuged at 3000 × g for 15 min at 4˚C in a refrigerated centrifuge. The supernatant taken as enzyme extract containing extracellular xylanase was used to assay the enzyme activity. Concentration of protein in crude enzyme was determined by Lowry's method (Lowry 1951) of protein estimation in which enzyme was reacted with the Lowry's reagents and the absorbance obtained was compared with a standard graph plotted by reacting a standard protein with known concentrations with the Lowry's reagents and plotting a graph between concentration of standard protein (BSA) on X axis and absorbance at 660 nm on Y axis.

Enzyme characteristics

Effect of EDTA and SDS: Effect of EDTA and SDS at the conc of 20 µg/ml of reaction mixture was studied.
Effect of alcohols: Enzyme activity was measured in the presence of methanol, ethanol and n-propanol.

Result and discussion

Isolation of Fungi

The identified *Trichoderma* isolates were confirmed by morphological descriptors and deposited in Indian Type Culture Collection (ITCC), IARI, Pusa, New Delhi, India.

Table 1: Identification of potential strains of *Trichoderma* sp.

Strain No.	Name of Bioagent	ITCC Acc. No	GenBank Accession No.	Strain code	Source	GPS Location
T1	*T. viride*	8315	JX119211	01PP	Hardoi	Latitude: 27˚ 23′ 40.729″ Longitude: 80˚ 7′ 47.751″
T2	*T. harzianum*	6796	KC800922	*Th* Azad	CSA, Kanpur Nagar	Latitude: 25˚ 8′ 34.821″ Longitude: 81˚ 59′ 2.979″
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″

Table 2: Morphological descriptors used for characterization of native isolates of *Trichoderma* sp.

Name of Strains	Colony Growth rate (cm/day)	Colony colour	Reverse colour	Colony edge	Mycelial form	Mycelial colour	Conidiation	Conidiophore branching	Conidia wall	Conidial colour	Chlamydospores
T. viride	8-9 in 3 days	Dirty green	Dark greenish	Smooth	Floccose to Arachnoid	Watery white	Ring like zones	Ball like structure	Rough	Green	Not observed
T. harzianum	8-9 in 3 days	Dark green	Colourless	Wavy	Floccose to Arachnoid	Watery white	Ring like zones	Highly branched, regular	Smooth	Dark Green	Not observed
T. asperellum	5-6 in 3 days	Snow white green	Orange	Smooth	Floccose	Watery White	Ring like zones	Branched, regular	Smooth	Green	Not observed
T. koningii	7-8 in 3 days	Dirty green	Yellowish	Smooth	Floccose to Arachnoid	Watery white	Ring like zones	Highly branched, regular	Rough	Grayish Green	Not observed
T. atroviride	5-6.5 in 3 days	Light dark effuse	Colourless	Effuse	Floccose to Arachnoid	Watery white	Irregular	Irregular	Rough	Yellowish Green	Not observed
T. longibrachiatum	8-9 in 4 days	White to green	Colourless	Effuse	Floccose to Arachnoid	Watery white	Circular zones	Rarely re-branched	Smooth	Green	Not observed
T. virens	8-9 in 3 days	Snow white	Colourless	Smooth	Floccose to Arachnoid	Watery White	Flat	Highly branched, regular	Smooth	Dirty Green	Not observed

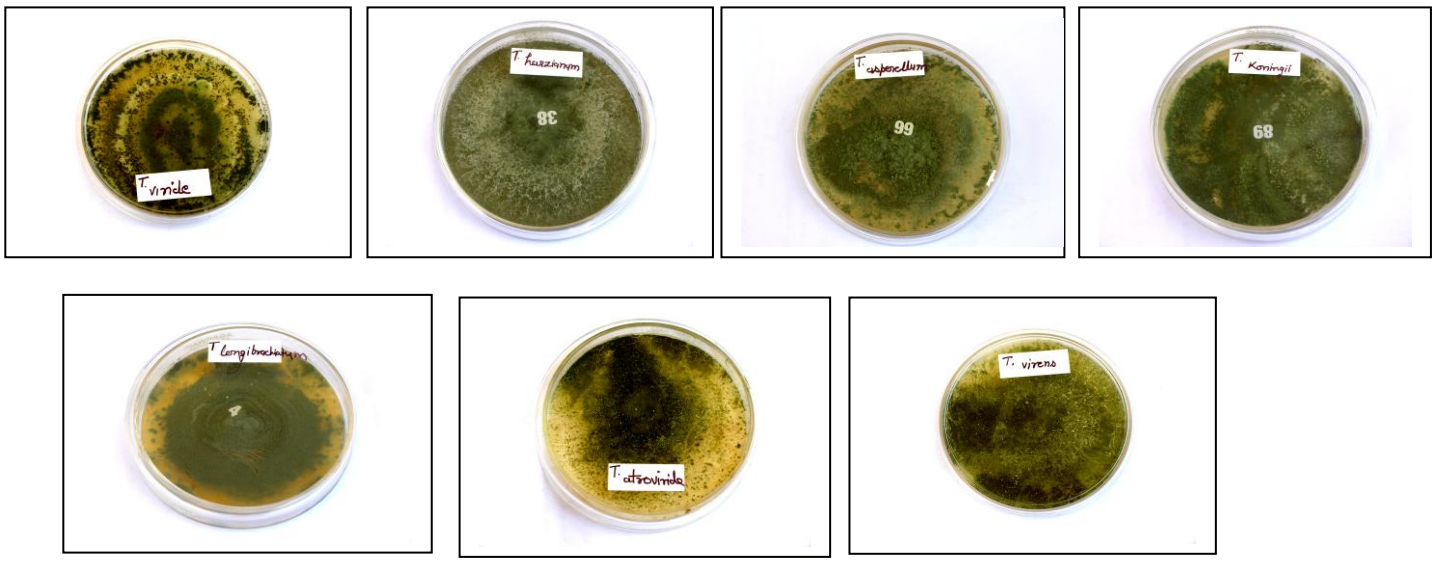

Figure 1: Seven different isolated strains of *Trichoderma*

Optimization of culture conditions

Medium: Vogel's medium with some modifications containing 1% Birchwood Xylan was used for for growth and enzyme production. Medium was sterilized in autoclave at 121˚C, 1.5 atm for 15 min and trace element solution at 0.1 % concentration was added. The trace element solution contained (in g/l): $FeSO_4$ 0.05, $ZnSO_4.7H_2O$ 0.014, $CoCl_2$ 0.02, $MnSO_4$ 0.016.

Inoculum concentration/size: Each 250 ml Erlenmeyer flask containing 50 ml modified growth media (pH 6.0) was inoculated with different amount of working inoculum ranging from 1-5 ml taken from spore stock prepared in DW in a BOD incubator at 30˚C for 7 days to determine the optimum inoculum size for xylanase production. 2 ml inoculums gave the maximum activity of xylanase, this size was used for further optimization process (Figure 2).During the cultivation of *A. foresides* maximum xylanase activity occurred when inoculums had a concentration of $1.5x10^8$ spores/ml (Shah *et al.*, 2005).

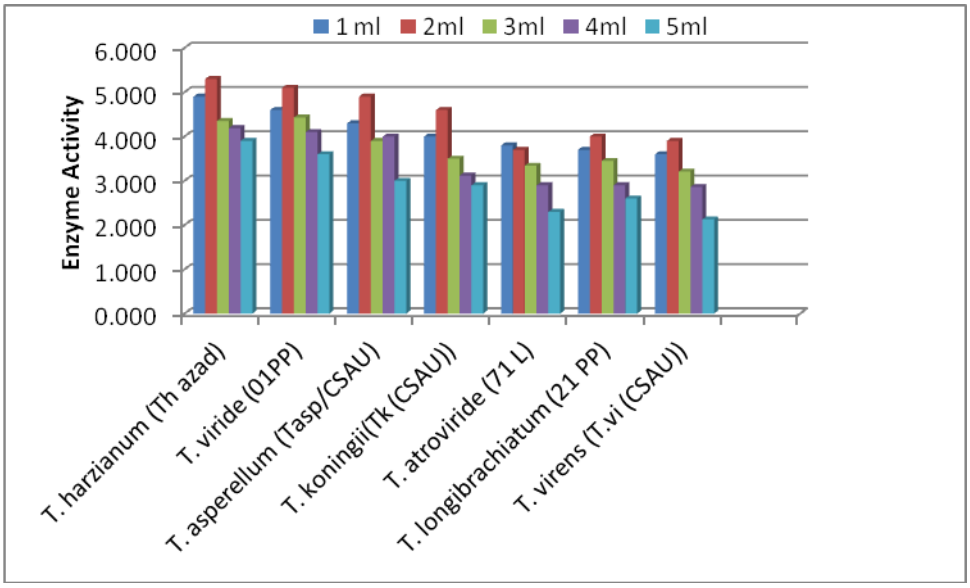

Figure 2: Effect of inoculums size (spore concentration) on xylanase production by *Trichoderma* sp.

pH: To obtain maximum xylanase production by *Trichoderma* sp., each Erlenmeyer flask containing 50 ml growth media with pH ranging from 4.0-7.0 was incubated at 30°C with 2 ml inoculums for 7 days. After 7 days of incubation, xylanase activity was determined.

Growth medium having pH 6.0 gave the maximum activity and was used for further studies (Figure 3). On either side of the optimum pH (pH 6.0) of the medium, the enzyme production decreased. Similar results have been reported in *A. terreus* where maximum xylanase activities were observed at pH 6.0 (Chidi *et al.*, 2008). The effect of initial culture pH on xylanase production in *P. thermophila* has been reported and found maximum activity when the initial pH was adjusted to 7.0 (Yang *et al.*, 2006). High level of xylanase production by this strain was observed in the range of pH 5.0-8.0. *Aspergillus* sp. RSP- 6 was active in xylanase production over a broad range of pH from 2.0- 6.0 with maximum production at pH 3.0 (Sathish, 2008). A pH of around 5.0, in general, has been observed to be optimum for xylanase production (Subramaniyan (2002), Shah *et al.* (2005), Gupta, (2009) and Sridevi, (2011)).

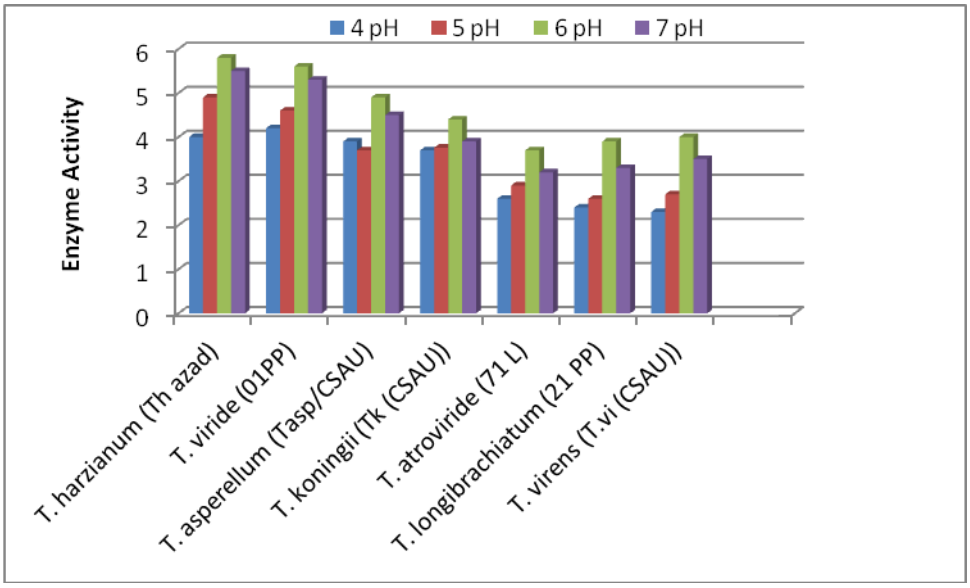

Figure 3: Effect of broth pH on xylanase production by *Trichoderma* sp.

Incubation period: To study the effect of incubation period, each 250 ml flask containing 50 ml growth media (pH 6.0) was incubated at 30°C. After 96 hrs of incubation, the samples were harvested at regular interval of 24 hrs up to 8 days to determine xylanase activity in the supernatant of each sample. Maximum xylanase production was observed when incubated for 7 days (Figure 4). After 7 days, a decline in the activity was observed. Maximum xylanolytic activity in *A. terreus* was observed after 4 days at 35°C and remained at that level until 6.5 days when the activity started to decrease (Sathish, 2008). In *F. solani*, enzyme production started after 24h of inoculation but showed maximum production on 6th day of incubation period at 30°C (Gupta, 2009). Maximum xylanase production by *Aspergillus sp*. RSP-6 occurred on 5th day of incubation and further increase in fermentation time resulted in reduction of activity (Sathish, 2008).

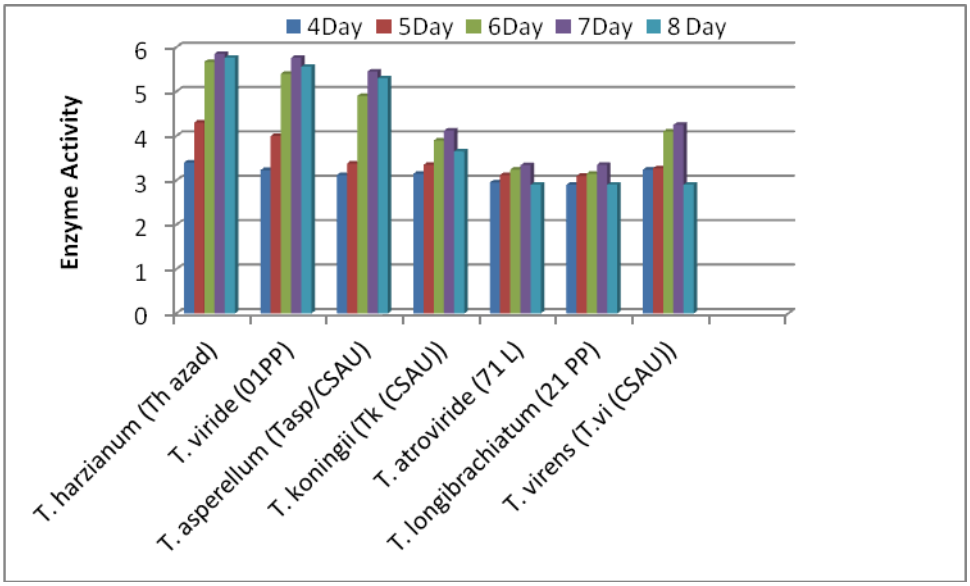

Figure 4: Effect of culture incubation time on xylanase production by *Trichoderma* sp.

Effect of temperature on xylanase: Xylanases purified from the culture filtrate of *Trichoderma* sp. was assayed at different temperatures ranging from 25, 35, 45, 55 and 65°C and the optimal temperature was 55°C. Enzymes show highest activity at 55°C (Figure 5) and their activity decreases beyond 55°C. Gupta *et al.*, 2009 stated that the optimal temperature for xylanases was found to be 50°C. The best temperatures for xylanase production by *A. japonicum* have been reported to be 25°C (Simoes, 2005). With cultivation temperature lower and higher than the optimum, decline in xylanase activity has been reported (Kheng (2005) and Gupta (2009)). A slightly higher temperature of 45°C and 50°C has been reported to be optimum for xylanase production by *P. oxalicum* and *T. aurantiacus*, respectively (Muthezhilan, (2007) and Dhillon, (2006)).

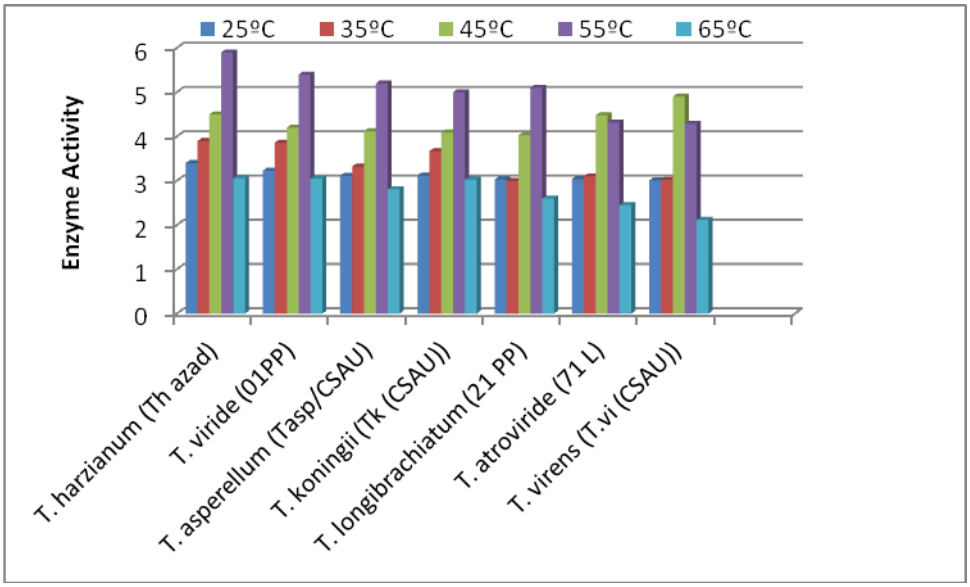

Figure 5: Effect of culture incubation temperature on xylanase production by *Trichoderma* sp.

Carbon source: Different carbon sources were used to determine their effect on xylanase production. Each 250 ml Erlenmeyer flask containing 50 ml growth media (pH 6.0) contained either of these carbon sources viz. various commercially available sugars, wheat bran, corn cob, birchwood xylan and carboxy methyl cellulose at 1% concentration each. The medium was inoculated with 2 ml inoculums and incubated at 30˚C for 7 days and xylanase production was determined. Birchwood xylan at a concentration of 1% showed highest xylanase production (Figure 6). None of the monosaccharides and disaccharides tested viz. glucose, maltose and sucrose were found suitable for xylanase production. However, xylanase production in *Aspergillus sp*. RSP-6 to be constitutive in nature and none of the monosaccharide or disaccharide improved the xylanase production compared to palm fiber as carbon source (Sathish, 2008). In contrast, xylanase activity in *A. pullulans* Y-2311-1 was induced by xylose and xylan (Li, 1994). Suppression of xylanase synthesis by readily metabolizable sugars such as glucose and/or xylose has been reported in *Streptomyces* sp. (Beg, 2000). The use of wheat straw and wheat bran as a carbon source for xylanase production has been reported (Bakri (2008) and Okafor (2007)). Other agro-residues such as rice straw (Dhillon *et al.*, 2000), sugarcane bagasse (Sandrim, 2005), corn cob (Pal (2010), da Silva (2005) and Gomes (1993)), oatspelt xylan (Muthezhilan, 2007) and Brewer's spent grain (Terrasan, 2010) have also been reported as suitable substrates for xylanase production.

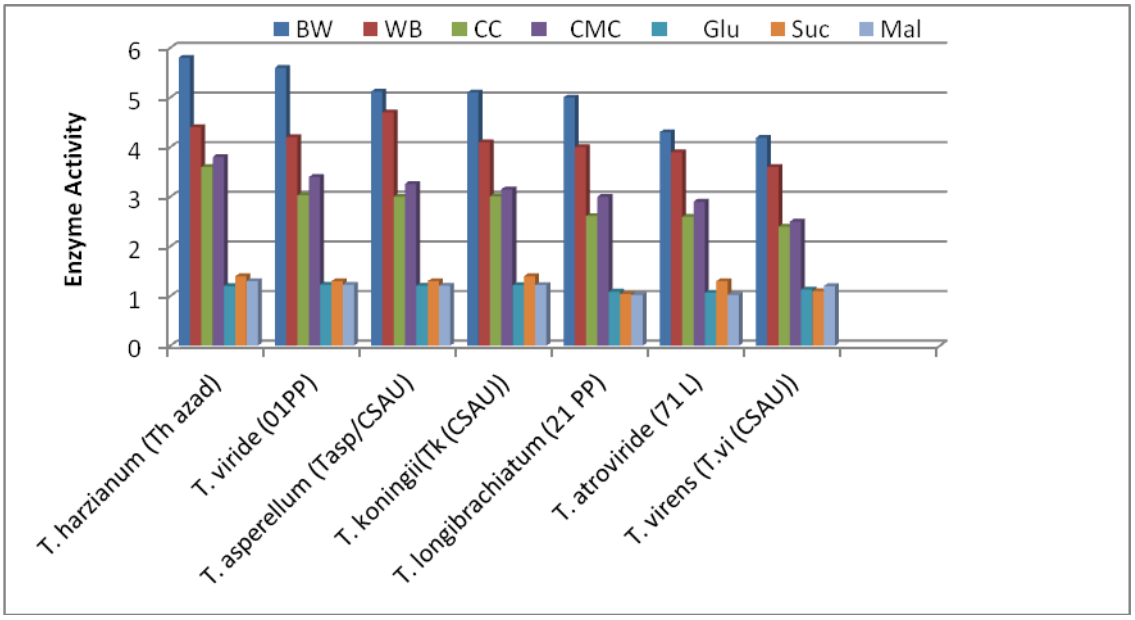

Figure 6: Effect of carbon source on xylanase production by *Trichoderma* sp.

Nitrogen source: Effect of different nitrogen sources viz., yeast extract (YE), beef extract (BE), peptone (PPT), soybean residue (SR) and corn powder (CP) on xylanase production was studied by replacing yeast extract by various nitrogen sources in growth media (pH 6.0). Each 250 ml Erlenmeyer flask containing 50 ml medium (pH 6.0) was incubated with 2 ml inoculums, determined. Corn powder was found to be the best nitrogen source as it gave the maximum xylanase activity. The results obtained during the present investigations are in agreement with those reported (Sathish, 2008) where yeast extract has been reported to be the best nitrogen source for xylanase production by *Aspergillus* sp. RSP-6 and on contrary, nitrogen sources such as peptone, beef extract, soybean meal, peanut meal and corn steep liquor were found to be poor nitrogen sources. Among the other organic nitrogen sources, defatted rapeseed meal induced maximum enzyme production by *Streptomyces* sp. Corn steep liquor and soya bean meal produced maximum xylanase level in *B. licheniformis* A 99 (Archana, 1997). In contrast to our results, peptone as the best source of organic nitrogen for the production of xylanase from *A. niger, F. solani and T. harzianum* (Bakri *et al.* (2008), Gupta *et al.* (2008), Seyis *et al.* (2003)).

Protein Estimation in Crude Enzyme

Amount of protein in the crude enzymes extracted from fermented flasks was determined by Lowry's method and the results of the same can be seen in Table 3 below.

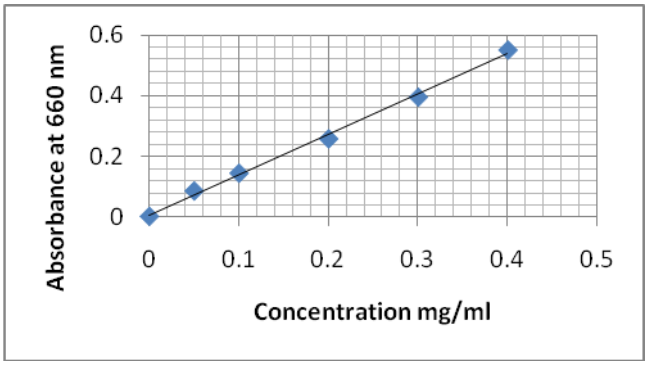

Figure 7: BSA Standard Curve

Table 3: Protein content of seven fungal strains

Sl.No.	Fungal Strain	Optical Density	Protein concentration (mg/ml)
1	*T. harzianum*	0.280	0.21
2	*T. viride*	0.220	0.16
3	*T. koningii*	0.168	0.15
4	*T. asperellum*	0.142	0.10
5	*T. atroviride*	0.113	0.084
6	*T. longibrachiatum*	0.105	0.07
7	*T. virens*	0.063	0.042

Based on the above study it can be concluded that among the seven different isolated strains of *Trichoderma* evaluated for the xylanase enzyme production activity, *T. harzianum* was the most promosing strain followed by *T. viride, T. koningii, T. asperellum, T. atroviride, T. longibrachiatum* and *T. virens*.

Effect of SDS and EDTA

SDS and EDTA have showed inhibitory effect on the xylanase activity. EDTA is a chelating agent (Ali and Sayed, 1992) and its inhibition ability indicates that specific ions might be actively involved in catalytic reaction of the enzyme (Kotchoni *et al.*, 2006).

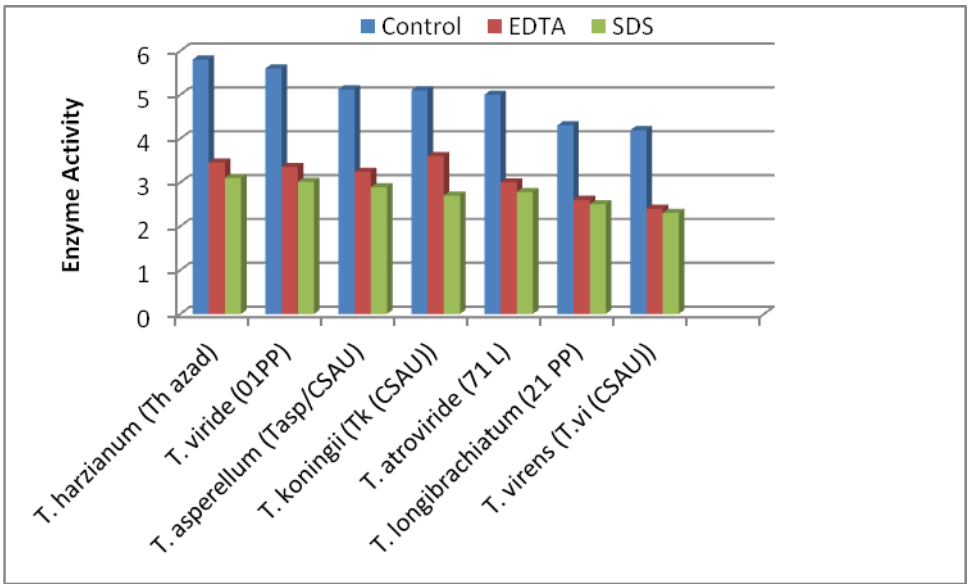

Figure 8: Effect of SDS and EDTA on xylanase enzyme activity

Effect of Alcohols

Alchohols (Ethanol, Propanol and Methonol) have inhibitory effect on xylanase enzyme activity. These findings were in close resemblance with the findings of Khan *et al.*, (2003).

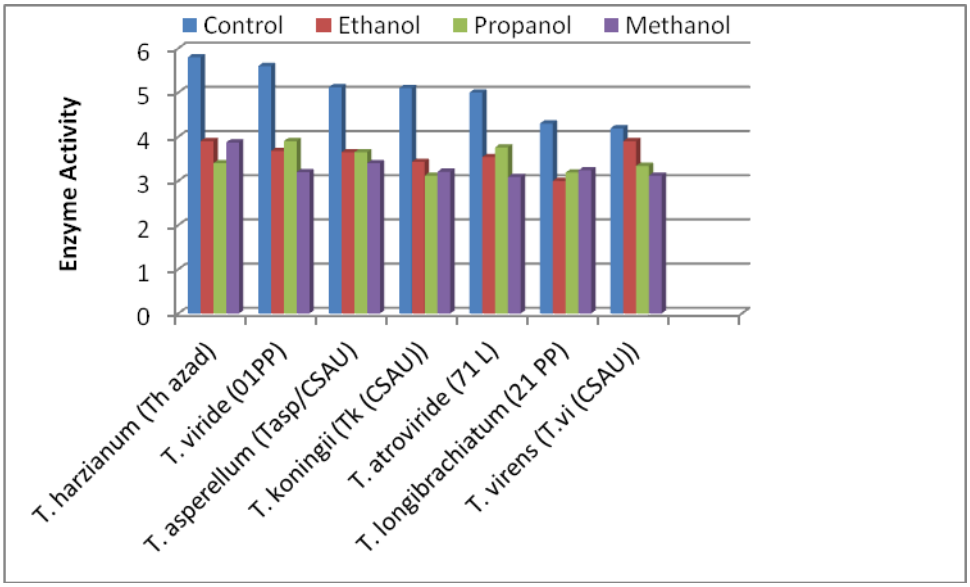

Figure 9: Effect of alcohols on xylanase enzyme activity

Conclusion

Results obtained on optimization of process variables under solid state fermentation revealed that birchwood xylan could be effectively used for xylanase enzyme production. A considerable increase in xylanase production was achieved when 2 ml of the inoculums is used for the inoculation and incubated in growth media at pH 6.0 and 55˚C for 168 hrs. The study also revealed that highest amount of xylanase enzyme is produced by *T. harzianum (Th* Azad) followed by *T. viride* 01PP.

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