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RESEARCH ARTICLE

Determination of Biochemical and Physiological Aspects of a Biocontrol Agent *Trichoderma harzianum Th azad*

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Abstract

Potentiality of *Trichoderma* species as a bioagent has been greatly influenced by the physiological parameters like temperature, moisture, nutrients and pH. These factors greatly influence the growth, sporulation and also in determining the effectiveness of *Trichoderma* against phytopathogens. Among the different solid media, PDA shows excellent growth in average colony diameter (8.72cm) followed by Malt Extract (8.12cm) whereas liquid media such as PDB reveals the excellent average mycelium weight (190.9mg). The most favorable temperature for growth and sporulation of *Trichoderma harzianum Th azad* was found to be 30˚C followed by 25˚C. Similarly, most favorable pH 6.5-7.5 ranges was found in which total dry weight of mycelium also varies between 144.805-142.4 mg. Maximum pH was found at 7.0 (162.5mg). The xylanase activity of the fungus *Trichoderma harzianum Th azad* is profoundly influenced by environmental factors. Effect of different carbon sources such as, glucose, maltose, CMC, sucrose, corncob, wheat bran, oat spelt xylan and birchwood xylan, on xylanase expression was also studied at various time intervals. Highest xylanase activity (5.2 U/mL) was observed at 120 hours with 1 percent birchwood xylan as a carbon source. *T. harzianum* did not show xylanolytic activity without the presence of any carbon source. Expression of xylanase was induced maximally by xylan and repressed by glucose. The pH and temperature optima of the crude xylanase were 5.0 and 28ºC, respectively. Among the lignocellulosic materials tested as carbon source, birchwood xylan was far most effective for xylanase production. By analyzing xylanase activity on various substrates, we can conclude, that among the substrates tested, birchwood was found to be more effective for xylanase production and maximum xylanase activity was achieved on growth medium with the same.

INTRODUCTION

Trichoderma sp. are beneficial free living filamentous fungi. They are cosmopolitan and versatile in nature. They have high biodiversities and have been extensively studied as a model microorganism to analyze and explored its antagonistic action against the phytopathogens. A great number of fascinating characters of *Trichoderma* are responsible for its biocontrol potential. Among them high rhizosphere competency and prolific production of extracellular proteins and also the enzymes that can degrade chitin and cellulose **(Harman et al., 1996)** are also serving as the basis for effective biocontrol applications. Furthermore, they are also known to produce antifungal

antibiotics **(Ghisalberti and Rowland, 1993**).

Xylanase enzyme is the biocatalysts, which are very important for biological reaction and also in several industrial applications, such as paper and pulp processing industries. During the last decade, a large number of studies have been carried out on xylanase activity. A principal hurdle in the commercialization of enzymatic processes is the bulk production of enzymes at a cost effective manner. In order to meet this goal, such strategies should be explored by which cost-efficient bulk production can be achieved. Therefore, coming years will see advancement in production methods to exploit such microbial species that can easily metabolize the available waste material by using the simplest techniques at affordable prices. Research on micro-organisms that utilize xylan, cellulases and on the involvement of enzyme system, is important from ecological and economic point of view. In a time of rising fuel prices, utilization of abundantly available biomass as an alternative resource of energy is one of the thrust area of research. Currently researchers throughout the world focus on the utilization of renewable, economical and abundantly available biomass for the production of useful products.

MATERIALS & METHODS

This part of research work was conducted in the Biocontrol Laboratory, Department of Plant Pathology, C.S.A University of Agriculture & Technology, Kanpur, India.

Isolation and purification of bioagent

Soil sample of potential and effective bioagent *Trichoderma harzianum (Th azad)* collected from rhizospheric soil of infected pigeonpea farm fields of C. S. Azad University of Agriculture & Technology, Kanpur. It was morphologically identified by slide mounting, as well as molecular identified by ITS marker, sequences deposited to NCBI GenBank .(Accession number:KC800922) and re-confirmation by ITCC, IARI, New Delhi (Accession number:ITCC-6796) .Finally, potential and effective bioagent *Trichoderma harzianum* (*Th azad*) was submitted to microbial data bank at NBAIM, Mau (Accession number:F-03109). Isolation of the fungus was done by serial dilution plate technique, (**Johnson and Crul, 1972**). Purification was done by single spore isolation technique.

Solid media study

Five different media viz. PDA, Czapedox's, Saboraud, Rose Bengal and Malt extract media were selected. All five different media were sterilized at 1.5 kg/cm^2 for 30 min. All media plates inoculated by the pure culture of *Trichoderma harzianum Th azad* (5mm disc) with the help of sterile cork borer. All plates incubated at 25˚C$\pm$2˚C for 2 days. After incubation period the radial hyphal growth is observed in two directions at right angles to each other after every 24 hours and average of the readings in three replicates were measured. The important colony characters like change in the colony colour, rate of growth and sporulation and nature of colony were recorded.

Liquid media study

Studies were carried out to determine the amount of mycelial mat produced by five different liquid media- Potato Dextrose, Rose Bengal, Malt, Sabouraud and Czapek's Broth. 50 ml of each liquid media were poured into 150 ml conical flask in three replications and sterilized in autoclave at 1.5 kg/cm^2 pressure for 20 minutes. The flask were inoculated with 5mm disc of the of *Trichoderma harzianum Th azad* with the help of sterilized cork borer and incubated for 10 days at 23$\pm$2^{0}C. After incubation period, the content of the flask was filtered through Whatman No.4 filter paper. Mycelial mat washed with distilled water and then dried at 60˚C for 72 hours, subsequently cooled in desiccators and weighed on electronic balance. Average dry weight of the mycelial mat was taken as standard value for comparison of growth.

Effect of temperature on growth and sporulation of *Trichoderma harzianum Th azad*

Effect of temperatures on the linear hyphal growth of *Trichoderma harzianum Th azad* was studied in vitro on PDA (10ml) in 9 cm petriplates for each temperature. A bit bit (0.5cm) of pure culture of *Trichoderma harzianum Th azad* was placed at the centre of the petriplates with the help of sterile cork borer and three replications were kept for each treatment. The cultures were incubated at 15, 20, 25, 30, 35 and 40˚C in BOD incubator and daily observation on mycelial growth of *Trichoderma harzianum Th azad* was recorded after every 24 hours up to 7 days.

Effect of pH on growth and sporulation of *Trichoderma harzianum Th azad*

Potato dextrose broths with the set of different pH values (5, 5.5, 6, 6.5, 7, 7.5, 8.0, and 8.5) were prepared and adjusted by adding appropriate amount of citrate phosphate buffer. Three in replicate were kept for each treatment.

The spore count was recorded by using haemocytometer as 1×10^5 spore/ml. 1ml of this spore suspension was then added in PDB set at different pH ranges and incubated in BOD at 25˚C±2 for 7 days. The mycelial mat after 7 days was then harvested from the flask by collecting the culture filtrate through sterilized filter paper (Whatman filter paper No. 4). The harvested mycelium was kept in hot air oven at 35˚C for 48 hours and final weight was measured in mg.

Xylanase enzyme production and biochemical analysis of xylanase

Sporulation medium used for *Trichoderma harzianum Th azad*-The composition of the sporulation medium used for the fungus As described by **Montenecourt and Evelieigh, 1977**.

Xylanase activity

Trichoderma species was maintained at 4ºC after growing for 7 days in a culture medium containing 0.2% malt extract, 0.2% yeast extract, 2% glucose and 2% agar (MYG medium) at 28ºC. Sporulation medium was prepared by using Trisodium citrate (0.5g), KH_2 PO_4(0.5g), NH_4NO_3 (0.2g), $(NH_4)_2$ SO_4 (0.4g), $MgSO_4$ (0.02g), peptone (0.1g), yeast extract (0.2g), glucose (0.2g), and agar – agar (2.5g) and the volume was made upto 100ml with double distilled water at 5.5 pH and 28ºC temperature. Inoculation medium (100ml) was prepared in the same way but without agar –agar. A loopful culture of *Trichoderma* species from sporulation medium was transferred aseptically into the Erlenmeyer flask, containing 100ml Vogel's medium prepared as per standard method. The flasks were incubated on rotary shaker at 150 rpm at 28ºC for 5 days. Liquid state cultures were harvested by centrifugation at 10,000 rpm for 20 min. at 4ºC, and the resulting supernatant was used as crude enzyme preparation. Xylanase activity of this crude enzyme was measured as per method described by Khanna and Gauri using Xylose as standard. The unit (1U) of enzyme activity was defined as the amount of enzyme required to liberate 1 m mole min-1 of xylose from the appropriate substrate under the standard conditions. *Trichoderma* species was grown in Vogel's medium with different carbon sources like glucose, maltose, CMC, sucrose, corn-cobs, wheat bran, oat splet xylan and birch wood xylan to check their effect on the expression of xylanase. The crude xylanase activity was measured in pH range of 4.0 to 8.0 with different buffers. Sodium acetate buffer was used to maintain pH of 4-5.5 and citrate phosphate buffer was used to maintain pH of 6-8. The reactions were carried out at 40, 50, 60 and 70ºC for determination of optimum temperature and xylanase assay was carried out.

Effect of various carbon sources on xylanase activity

Trichoderma harzianum Th azad was grown in Vogel s medium with different carbon sources like glucose, maltose, CMC, wheat bran, corn cob, sucrose, birch wood xylan and oat spelt xylan to check its effect on the expression of xylanase.

Effect of pH on xylanase activity

The activity of the crude xylanase was measured in pH range 3.0 to 6.5 with different buffers. Sodium acetate buffer was used for pH 4-5.5 and citrate phosphate buffer was used for pH 6-8.

Effect of temperature on xylanase activity

The reaction was carried out at 26˚C to 40˚C for determination of optimum temperature and xylanase assay.

Effect of time factor on xylanase production

The time course of xylanase production by the *Trichoderma harzianum Th azad* in Vogel's medium containing 1% xylan as sole carbon source was assayed from cell supernatant at 28°C and pH 5 for every 24 hours.

RESULTS AND DISCUSSION

Effects of different solid media on the growth and sporulation of the identified *Trichoderma harzianum Th azad* was studied to determine the linear hyphal growth rate. Five different media viz. PDA, Czapedox, Sabouraud, Rose Bengal and Malt Extract media were chosen for growing the cultures in three replicates. The radial growth was assessed and nature of colony and sporulation were studied and recorded. The recorded data are shown in Table 1.

From the data presented in **Table 1**, it is concluded that the highest radial hyphal growth of the *Trichoderma harzianum Th azad* was recorded on potato dextrose agar media (8.72cm) followed by malt extract agar media (8.12cm). Growth on Rose Bengal agar media (8.02cm) and Sabouraud's agar media (7.90cm) were good but growth on Czapeks Dox medium (6.53cm) was found poor.

Table 1: Effect of solid media on growth and sporulation of *Trichoderma harzianum Th azad*

Solid media	Average colony diameter (cm)	Fungal growth (category)	Nature of colony and sporulation
Potato dextrose agar	8.72	Excellent	Heavy sporulation, greenish colony colour
Malt extract agar media	8.12	Excellent	Profuse sporulation, whitish, slightly yellowish green in colour, colony puffy in nature
Rose Bengal agar media	8.02	Good	Compact and dark greenish colony, profuse sporulation
Sabouraud's agar media	7.90	Good	Light greenish and compacted and profuse sporulation
Czapeks (Dox) agar	6.53	Poor	Whitish thin and flaky colony and restricted growth.
C.D (0.05)	**0.35**		
S. E. (Diff.)	**0.159**		

Liquid Media

Studies were carried out to determine the average weight of the dry mycelial mat produced maintained from the identified bioagent *T. harzianum* in five different liquid media including Potato Dextrose, Rose Bengal, Malt, Sabouraud and Czapedox, broth in three replications. Average mycelial weight was taken as a standard value for comparison of growth for different treatments. The radial growth, nature of colony and sporulation was observed and recorded. The data are summarized here as under:

From the data presented in Table 2, it is evident that the highest mycelial weight were recorded in potato dextrose broth as 190.9mg, followed by malt broth (186.8mg), rose bengal broth (173.45 mg), Sabouraud's broth (133.21 mg) and Czapek's dox broth (98.29mg). The excellent sporulation was observed in PDB and Malt broth. Good and fair sporulation was observed on Rose Bengal and Sabouraud's, respectively but it was fairly poor on Czapek's Dox broth.

Table 2: Effect of liquid media on growth and sporulation *Trichoderma harzianum Th azad*

Liquid media	Average mycelium wt. in (mg)	Sporulation
Potato dextrose broth	190.9	Excellent
Malt broth	186.8	Excellent
Rose Bengal broth	173.45	Good
Sabouraud's	133.21	Fair
Czapeks dox	98.29	Poor
C.D (0.05)	**5.38**	
S.E.Difference	**2.41**	

The growth of the bioagent (*Trichoderma harzianum Th azad*) was tested on five different solid as well as liquid media in which Potato dextrose agar in solid and potato dextrose broth in liquid media supported excellent radial growth and sporulation. These results were conformed to the findings of **Samuels et al., 1998** and **Gupta et al., 2003**, **Singh et al., 2011. Kumar and Singh, 2008**. The study on the liquid media showed that the potato dextrose broth was the best liquid media for growth and sporulation of Alternaria helianthi **(Gupta et al., 1969**) and Alternaria pori **(Raju and Mehta, 1982).** Similar results were observed by **Shahid et al., 2011** in studying the growth and sporulation of T. longibrachiatum. **Kumar et al., 2007** also gave the similar results while studying the growth and sporulation of Colletotrichum dematium var. truncata. In another study, **Kumar et al. (2008)** also found that Potato dextrose broth medium to be the best medium for the growth of Sclerotium rolfsii.

Effect of temperature on growth of *Trichoderma harzianum Th azad*

Study was undertaken to find out the optimum as well as the best temperature for the growth of *Trichoderma harzianum Th azad* by growing the bioagent at different temperatures on potato dextrose agar medium. After 7 days of incubation the average mycelial dry weight was recorded and presented below:

From the data presented in **Table 3**, it can be concluded that the growth of the fungi was excellent at temperature range of 25-30˚C, there after the growth decreases. Maximum average dry weight was observed at

30˚C. The statistical analysis shows that the growth of the bioagent significantly affected with either increase or decrease of the temperature.

Table 3: Effect of temperature on growth of *Trichoderma harzianum Th azad*

Temperature (^{0}C)	Dry weight of mycelium (mg)	Sporulation
15	110.165	Fair
20	146.5	Good
25	190.5	Excellent
30	212.6	Excellent
35	176.00	Good
40	56.5	Poor
C. D. (0.05)	**8.17**	
S. E. Difference	**3.75**	

In assessing the optimum as well as suitable temperature for the growth of *Trichoderma* sp., the microorganism was grown at six different temperatures ranges from 15˚C to 40˚C on potato dextrose agar medium. The growth of the microorganism was increased upto 30˚C thereafter the growth started decreasing. The maximum colony growth of the mycelium was observed at 30˚C temperature**. Bandopadhyay et al., 2003** also found that the growth and sporulation of *Trichoderma* species were optimum at temperature 25-30˚C**. Singh and Sudhir, 2009** also reported the same findings and observed that temperature had a significant in influence on the growth and sporulation on *Trichoderma* species in which the most favourable temperature for the eight *Trichoderma* species was found in between 25-30˚C (53-90 mm dia). **Kunming, 2004** examined the potential application value of *Trichoderma harzianum Th azad* strains (Th-B) in culture media on different temperatures and observed that the strain grew best at 15-35˚C and optimum growth is seen at 25-30˚C. **Sharma et al., 2005** reported that none fungi could grow at temperature 40˚C. **Singh et al., 2011** reported that the most favourable temperature for growth and sporulation of *Trichoderma atroviride* was between 25- 30˚ C (240-260 mg dry wt.), followed by 40˚C (60 mg dry wt.).

Effect of pH on growth and sporulation of *Trichoderma harzianum Th azad*

Like other physiological parameters, pH also plays an important role in the growth and sporulation. For assessing the optimum pH for the growth and sporulation of *Trichoderma harzianum Th azad*, a set of different pH values (5.0, 5.5, 6.5, 7.0, 7.5, 8.0, and 8.5) were maintained on potato dextrose broth media in three replicates. pH were adjusted by adding appropriate amount of citrate phosphate buffer. The spore count of 1×10^5 spore/ml was maintained by using haemocytometer in PD broth media for every pH value and after 7 days the mycelial mats were harvested and their average dry weight were recorded as presented below:

From the data presented in Table 4, that the maximum weight was recorded as 162.5 mg at pH 7, followed by 144.805 mg at pH 6.5. The minimum dry weight was recorded at pH 8.5 (100mg) and excellent sporulation was observed at pH 7.

Table 4: Effect of pH on growth and sporulation of the bioagent *Trichoderma harzianum Th azad*

pH range	Average weight (mg)	Sporulation
5.0	111.56	Poor
5.5	130.00	Fair
6.5	144.805	Good
7.0	162.5	Excellent
7.5	142.4	Good
8.0	103.0	Poor
8.5	100.0	Poor
C.D. (0.05)	**6.32**	
S.E. difference	**2.95**	

pH also plays an important role for growth and spore germination. The maximum weight was recorded as 160mg at pH 7 followed by 143.805mg at pH 6.5 whereas, the minimum dry weight was recorded at pH 8.5

(100mg). Excellent sporulation was observed at pH 7. The results were in conformity with the findings of **Bandopadhyay et al., 2003** also observed the same findings on growth and sporulation of *Trichoderma* strains. **Singh et al., 2011** also confirms the above results where pH range 7-7.5 has a significant influence on growth and sporulation on *T. atroviride*. However, **Kunming (2004**), examined the potential application value of *Trichoderma harzianum Th azad* strains in culture media on different pH and favourable growth was seen at pH 5-8 and optimally at pH 5-6.

Biochemical analysis of xylanase enzyme produced by *Trichoderma harzianum Th azad*

Optimum pH and temperature for xylanase production from *Trichoderma harzianum Th azad*

The optimum pH and temperature for the growth of *Trichoderma harzianum Th azad* and xylanase production was found to be 5 and 28˚C.

Optimization of xylanase activity

Three different buffers (sodium citrate, sodium acetate, and citrate phosphate) were used to evaluate for assessing xylanase production activity by DNS method (**Miller, 1959**) with xylose as a standard from *Trichoderma harzianum Th azad*. Maximum xylanase activity was achieved with citrate phosphate buffer. Therefore, citrate phosphate buffer was used in all subsequent experiments of xylanase activity determination.

Effect of different carbon sources on xylanase induction

Effect of different carbon sources on xylanase expression was also studied. It is well established fact that the culture conditions effect significantly the production of xlyan degrading enzymes. The choice of appropriate substrate is of great importance in producing the xylanase in large scale level. These substrates not only serve as the carbon sources but also they are necessary inducing compounds for the growth of the compounds.

Since the cost of the substrate plays a crucial role in the economics of an enzyme production, therefore different lignocellulosic substrates utilized by *Trichoderma harzianum Th azad* for xylanase production were compared. . All the carbon sources were testing 1% (w/v). *Trichoderma harzianum Th azad* was grown in Vogel's medium with various carbon sources including Glucose, Maltose, Carboxy Methyl Cellulose, Sucrose, Corn cobs, Wheat bran, Xylan and expression of xylanase were studied **(Figure 1 (a-i).** After 5 days the culture mixture was taken out of orbital shaker. Supernatant obtained after centrifugation was tested for xylanase activity.

The results of maximum xylanases production from fungi on xylan as a carbon source are in accordance with earlier reports. In T. hamatum (**Da Silva and Carmona, 2008**), Clostridium absonum (**Rani and Nand, 2000**), Thermomyces lanuginosus (**Damaso et al. 2000**) and A. giganteus (**Coelho and Carmona, 2003**), xylan induced highest level of xylanase activity. In studies carried out with xylanase production by *Trichoderma* spp, Sugarcane bagasse was used as substrate for xylanase production by means of a strain of *Trichoderma harzianum Th azad* Rifai (**Maria et al., 2002).**

Results of similar experiments by **Meenakshi et al., (2008)** studies about cultural and nutritional conditions for enhanced production of xylanase by a local soil isolate of *Trichoderma viride,* using various lignocellulosic substrates in submerged culture fermentation have been optimized. Of the lignocellulosics used, maize straw was the best inducer followed by jowar straw for xylanase production.

Effect of different carbon sources on Xylanase activity of *Trichoderma harzianum Th azad*

Effect of different carbon sources on xylanase expression was also studied as shown in **Table 5**. All carbon sources tested were 1% taken at a concentration of (w/v).

Table 5: Effect of different carbon sources on Xylanase activity of *Trichoderma harzianum Th azad*

S. No.	Carbon source (1%)	*Xylanase activity (1U/ml) Mean ± SD
1.	Glucose	0.64 ± 0.001
2.	Maltose	0.44 ± 0.008
3.	CMC	2.58 ± 0.04
4.	Sucrose	0.172 ± 0.006
5.	Corncobs	1.12 ± 0.04
6.	Wheat bran	3.06 ± 0.06
7.	Oat spelt xylan	3.05 ± 0.03
8.	Birch wood xylan	3.36 ± 0.01

*Values are the means of 3 replicates

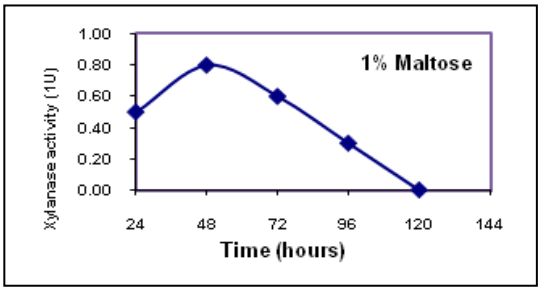

Fig. 1(a). Effect of glucose on xylanase activity

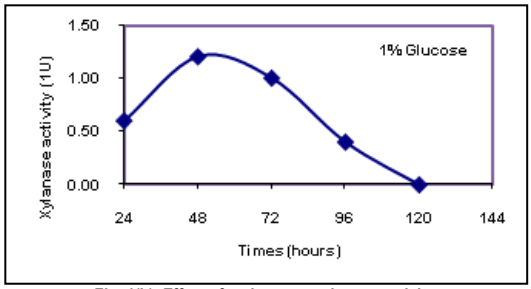

Fig. 1(b). Effect of maltose on xylanase activity

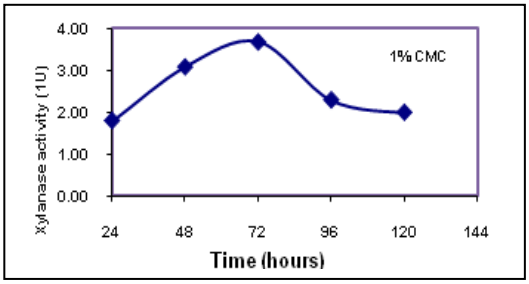

Fig. 1(c). Effect of CMC on xylanase activity

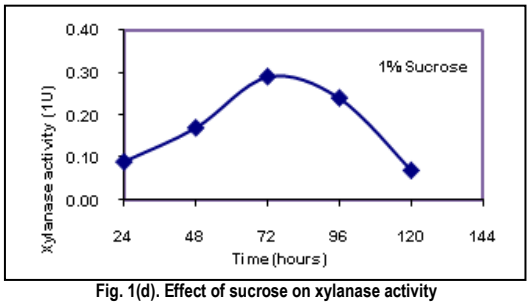

Fig. 1(d). Effect of sucrose on xylanase activity

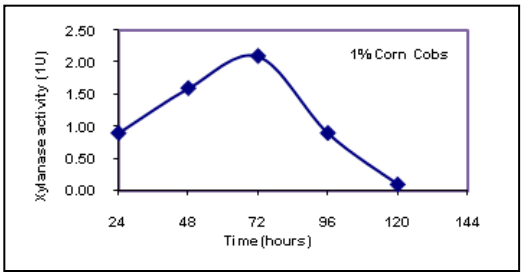

Fig. 1(e). Effect of corn cobs on xylanase activity

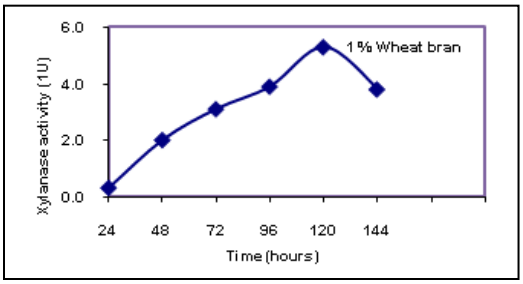

Fig. 1(f). Effect of wheat bran on xylanase activity

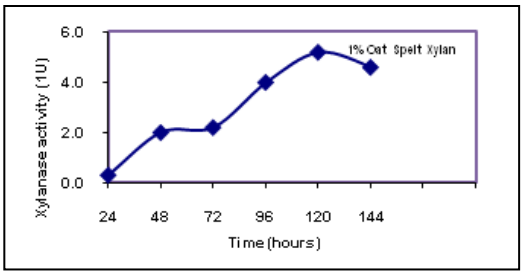

Fig. 1(g). Effect of Oat spelt xylan on xylanase activity

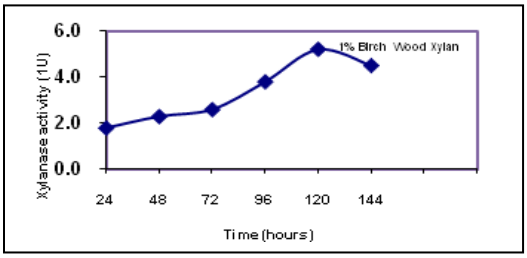

Fig. 1(h). Effect of birch wood xylan on xylanase activity

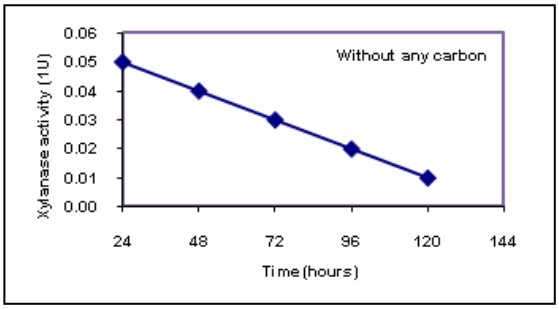

Fig. 1(i). Xylanase enzyme activity without any carbon source

Fig. 1 (a-i): Effect of different carbon sources on the Xylanase activity of *Trichoderma harzianum Th azad*

Shahid et al., 2012 concluded that the various substances used for the maximum xylanase production, birch wood xylan at a concentration of 1 per cent proved to be the best carbon source (substrate) for *Trichoderma viride 01PP* as it observed highest xylanase activity at a concentration of 1 percent.

Conclusion

Trichoderma species did not show xylanolytic activity without the presence of any carbon source.
The fungi as enzyme sources have many advantages that, the enzymes produced are normally extracellular, making easier for downstream process. To identify the xylanase enzymes and to study the activity of the xylanase complexes of *Trichoderma* species a series of experiments were conducted. In this study comparative analysis and substrate optimization was conducted among various cultures of *Trichoderma* species isolated from decomposing soil sample. The results clearly show that culture of T. harzianum Th-azad exhibited better activity. The optimum pH of xylanase and xylanase enzyme was found to be pH 5.0 and the optimum reaction temperature and incubation period to be at 28^0 C and 120 h. During our investigation we also found that the fungal isolates shows better xylanase and xylanase activity with wheat bran as sole carbon source. Finally, it should be noted that wheat bran is a cheaper raw material, since it is the by-product of agricultural wheat production and readily available in large amounts. Therefore, it is concluded that wheat bran can be utilized as a cheap substrate in various industrial sectors for production of various useful products such as biofuels

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