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# Comparative evaluation of cellulase activity in *Trichoderma harzianum* and *Trichoderma reesei*

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**Cellulase activity of two promising species of *Trichoderma harzianum* and *Trichoderma reesei* were assessed for agro-industrially important cellulase (E.C.3.2.1.4) production. Both species were used for media optimization studies and effect of pH, temperatures and incubation periods on cellulase activity. The maximum cellulase activity was found to be 1.76 U/ml (EXG) in *T. reesei* at pH 4 in comparison with *T. harzianum* with a maximum of 0.76 U/ml (EXG). The optimum temperature for increased cellulase activity was 35°C in *T. reesei and* incubation period of 112h was found ideal for increased cellulase activity in *T. harzianum* and *T. reesei.* Similarly, with 1% sucrose (w/v), maximum cellulase activity was achieved in *T. reesei* was 0.76 U/ml (EXG,EG). 1% yeast (w/v) was found most suitable nitrogen source for increased cellulase activity in *T. reesei*, that is, 1.96 U/ml (EXG) in comparison with *T. harzianum* where it was found to be maximum (1.29 U/ml (EG)). The potential of these lignocellulytic fungi for industrial cellulase production was tested through cellulase activity assay.**

**Key words:** Cellulase, *Trichoderma harzianum*, *Trichoderma reesei*, enzyme activity.

## INTRODUCTION

Plant cell walls are majorly comprised of cellulose, hemicellulose and lignin, where cellulose is the most abundant component (Han et al., 2003). Plant biomass comprises of an average of 23% lignin, 40% cellulose and 33% hemicellulose by dry weight (Sa-Pereira et al., 2003). Rauscher et al. (2006) showed that about 830 Gt of renewable plant biomass is formed annually consisting mainly of cellulose and hemicelluloses. Cellulases are industrially important enzymes (Schulein, 2000). They are known for their role in exhibiting high substrate specificity and less side chain products formation. These have created an interest in the market because of their wide-spread applications mainly in textile industries and biorefineries because a large amount of consumption of cellulase for biomass saccharification (Zhang et al., 2006; Zhu et al., 2009).

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**Abbrevations: EXG,** Exoglucanase; **EG,** endoglucanase; **PDA,** potato dextrose agar; **CMC,** carboxymethylcellulose; **CBH,** cellobiohydrolase.

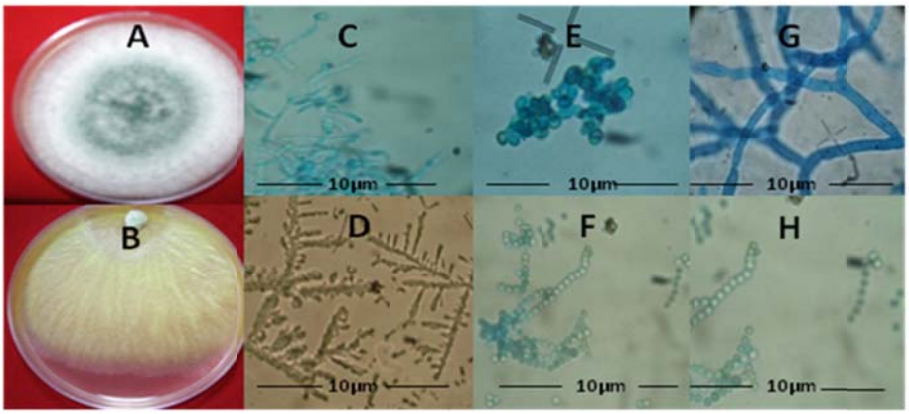


**Figure 1.** Morphological characteristics of *T. harzianum* and *T. reesei* (A and B) on PDA plates. Spore morphology and mycelia bearing phialides after staining with lactophenol blue. (C and D) Branched mycelia ribbons; (E and F) Conidiospres. Scale bar = 10 µm

Many fungal strains are known for their secretion of higher amounts of cellulases than bacterial strains, where *Trichoderma* is a leading one (Amouri and Gargouri, 2006). The complex structure of lignocellulose and its heterogeneous substrate hampers an efficient conversion to simple sugars and presents a number of technical and economic challenges in bringing cellulosic biofuels to the market. One of the major economical barriers for the production of biofuels is the intrinsic recalcitrance of lignocellulosic plant matter (Himmel et al., 2007). The synergestic decomposition power of mesophilic enzymes and uncomplicated multi-enzyme complex of filamentous fungi like *Trichoderma* spp. particularly *Trichoderma reesei* and *Aspergillus niger* made them effective agents for cellulase production. Cellulases produced by *Trichoderma harzianum*, is the most efficient enzyme system for the complete hydrolysis of cellulosic substrates into its monomeric glucose, which is a fermentable sugar. Mainly filamentous fungus, *T. reesei* and *T. harzianum* are commercially explored now-a-days for the large scale production of different cellulases and hemicellulases in bioreactor cultivations and many scientific groups are working for the improvisation of other strains of *Trichoderma* for the over-production of cellulases and cellulose degrading property of the biocontrol organism (Tiwari et al., 2013). Besides, with well established applications of these enzymes in pulp, paper, food, feed or textile processing industries, these plant cell wall degrading enzymes are now-a-days also employed for the saccharification of cellulosic plant biomass to simple sugars for biofuel production (Bouws et al., 2008; Harman and Kubicek, 1998; Kumar et al., 2008). A cellulosic enzyme system of *Trichoderma* spp. comprises of three major components: endo-β-glucanase (EC 3.2.1.4), exo-β-glucanase (EC 3.2.1.91) and β-glucosidase (EC 3.2.1.21). The exo-β-glucanase causes disruption in cellulose hydrogen bonding, which was later followed by hydrolysis of the accessible cellulose with endo-β-glucanase (Reese et al., 1950). The whole process occurs simultaneously and the rate limiting step is the depolymerisation of the insoluble cellulose by the CBHs and EGs. It is the synergestic action of both types of enzymes exoglucnases and endoglucanases which are involved in degradation of cellulase (Beguin and Aubert, 1994; Tomme et al., 1995).

The main objective of the present study was to comparatively evaluate two potential strains of *Trichoderma*, that is, *T. harzianum* and *T. reesei* by comparing crude cellulase activity, anticipating their possible fruitful role in the production of commercially important cellulases.

## MATERIALS AND METHODS

### Microorganisms for the study

Fungal cultures of laboratory developed strain of *Trichoderma harzianum* strain Th3 obtained from Indian Type Culture Collection (ITCC: 5593) isolated from carnation rhizosphere from IARI field submitted by the author to ITCC in 2005 and *T. reesei* (ITCC:4026) submitted to ITCC by S. Verma were taken for this study from Biocontrol Laboratory, Division of Plant pathology, IARI, New Delhi. The experimental *Trichoderma* spp. were multiplied in potato dextrose agar media, with the combination of peeled potato: 250 g, dextrose: 20 g, agar: 15 g and distilled water: 1000 mL. It was multiplied at 30°C (Barnett and Hunter, 1972) in a BOD incubator.

### Morphological characterization of *T. harzianum* and *T. reesei*

*T. harzianum* was fast growing and produced branched mycelia on PDA plate after 24 h, whereas *T. reesei* was quite sluggish in growth. Mycelial stage of *T. harzianum* was whitish after sporulation, it changed to greenish. Sporulation phase appeared after 4 days of incubation at 30°C.The mycelia in both *T. harzianum* and *T. reesei* were profusely branched with fruiting bodies (phialides). The greenish spores were spherical and biconcave in the case of *T. harzianum* while in *T. reesei*, spores were whitish and form chains as shown in Figure 1.

### Enzyme production

The culture was grown in 250 ml Erlenmeyer flask that contained 50 mL of the medium. Concentrations of the nutrients were 5 g/L trisodium citrate, 5 g/L $KH_2PO_4$, 2 g/L $NH_4NO_3$, 4 g/L $(NH_4)_2SO_4$, 0.2 g/L $MgSO_4$ (Ahmed et al., 2007) supplemeted with different carbon and nitrogen sources. After adjusting pH 5 of the medium prior to sterilization, the flasks were then inoculated with 2 agar discs (2 mm in diameter) of 5 days old culture grown on PDA plates and later incubated under stationary condition at 25, 30 and 35°C for up to 5 days. The crude enzyme was filtered and centrifuged at 12000 xg for 20 min.

### Enzyme assay

Cellulase (exoglucanase activity) was assayed by measuring the release of reducing sugar by DNS (Miller, 1959). The assay mixture contained 1 ml of 0.5% cellulose (Sigma Co.) suspended in 50 mM citrate phosphate buffer (pH 4.8) and 1 ml of culture filtrates of different *T. harzianum* and *T. reesei* strains, respectively. The reaction mixture was incubated for 30 min at 50°C and then centri-fuged at 12000 rpm for 15 min at 4°C. The reaction was arrested by adding 3 ml of 1% DNS (dinitrosalicylate) reagent in 1 M NaOH and followed by 1 mL of 40% Rochelle salt (potassium sodium tartarate) which was added to stabilize the colour. Endoglucanase activity (CMCase) was measured using a reaction mixture containing 1 ml of 1% carboxymethyl cellulose (CMC) made in 0.5 M citrate acetate buffer (pH 5.0) into the culture filtrates. The blanks were made in the same way using distilled water and absorbance was measured at 540 nm. One unit of cellulase activity was defined as the amount of enzyme in 1 ml of the reaction mixture that released 1 µmol of reducing sugar under assay condition.

### Optimization of parameters for cellulase production

#### *Effect of temperature and incubation period on enzyme activity*

In this study, the cellulase activity of fungal isolates grown under different temperature conditions of 25, 30 and 35°C and at time interval of 96, 112, 128, 134, 150, 166, 172h and 188 h was monitored.

#### *Effect of pH on enzyme production*

The most suitable pH optimum for the growth of *Trichoderma* sp. was obtained by adjusting the pH of the growth medium from 2-5 using 50 mM sodium phosphate and 50 Mm citrate buffers and the cellulase activity was measured at 540 nm at each pH to know the ideal condition.

#### *Effect of carbon sources on enzyme production*

Effect of various carbon compounds viz., cellulase, CMC, glucose, sucrose and maltose were used for the study. The broth was distributed into different flasks and 1% of each carbon sources were then added before inoculation of the strain at 28°C.

#### *Effect of nitrogen sources on enzyme production*

In the present study, the whole idea was to detect the appropriate nitrogen source for getting maximum cellulase enzyme activity by *T. harzianum* and *T. reesei*. The influence of peptone, beef extract, ammonium nitrate and yeast extract procured from HIMEDIA, India, by supplementing the growth medium with the organic and inorganic compounds was studied.

### Statistical analysis

Average value of cellulase activity was determined for multiple mean comparisons obtained through three separated experiments and to use the values for analyzing data for calculating standard deviation (±SD) from there independent experiments ranging between ±0.01 to ±0.05, respectively.

## RESULTS

### Effect of incubation period on enzyme production

Time of incubation has great bearing with the production of enzyme and operation of other metabolic systems. To a certain extent, *Trichoderma* sp. showed most active cellulolytic species along different incubation period (96, 112, 128, 134, 150, 166, 172 and 188 h, respectively). Cellulase activity values ranges from 0.1 to 0.47 U/ml (EXG) and 0.13 to 0.46 U/ml (EG) in *T. harzianum* with peak cellulolytic (EXG) activity of 0.7U/ml achieved at 112 h whereas activity value ranges from 1.30 to 1.76 U/ml (EXG) and 1.30 to 1.76 U/ml (EG) at different hours of incubation with the peak value of 1.96 U/ml at 112h in *T. reesei* shown in Figure 2.

### Effect of pH on enzyme production

Cellulase yield by *Trichoderma* sp. depends on the pH value. Results illustrated in Figure 3 indicate that cellulase activity increased gradually as pH increase from 2 to 4 with the increase of 0.02 to 0.76 U/ml (EXG) and 0.03 to 0.07 U/ml (EG) in *T. harzianum* and remain maximum (0.76U/ml) for *T. harzianum* at pH 5. Almost, similar trend was observed in *T. reesei* from pH 2 to 5 with the maximum value of cellulose activity, 1.76 U/ml (EXG) at pH 4. Effect of pH on cellulase production by *Trichoderma* supports the findings of Lee et al. (2002) who reported that CMCase exhibit the pH optimum of 4 and β-glucosidase ranges between pH 4-5.

### Effect of temperature on enzyme activity

Like pH, temperature is also an important factor that influences the cellulase activity. It was found to be 0.45 U/ml at 25°C and 0.57 U/ml at 30°C and maximum (EXG) activity of *T. harzianum* was found to be 0.95 U/ml. In the case of *T. reesei*, it was 1.22 U/ml at 25°C and 1.44 U/ml at 30°C. If we see the EG activity of *T. harzianum,* it was found to be 0.33 U/ml at 25°C and 0.47U/ml at 30°C which was low in comparison with *T. reesei* with EG activity of 1.22 and 1.33 U/ml activities at 25 and 30°C as shown in Figure 4. Maximum activity in *T. harzianum* was found at 35°C that is, 0.94 U/ml (EXG), 0.77 U/ml

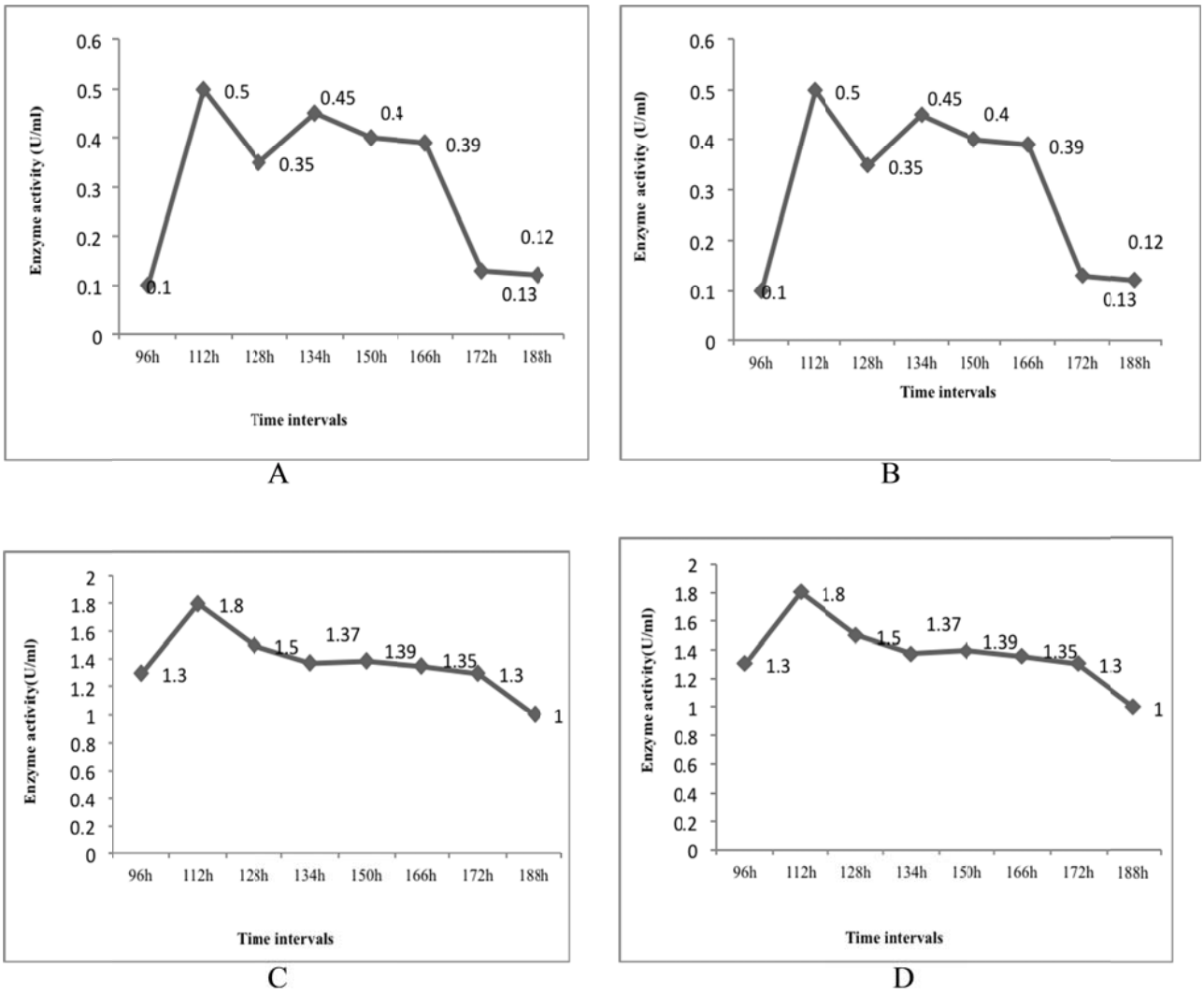


**Figure 2.** (A) Effect of incubation period on cellulase (EXG) activity (U/ml) in *T. harzianum* (B) cellulase (EG) activity (U/ml) in *T. harzianum*; (C) Effect of incubation on cellulase (EXG) activity (U/ml) in *T. reesei* (D) cellulase (EG) activity (U/ml) in *T. reesei.*

(EG) and in *T. reesei* it was 1.83 U/ml (EXG) and 1.78 U/ml (EG) at 35°C.

## Effect of carbon source on enzyme activity

Data presented in Figure 5 showed that cellulase activity by *Trichoderma* sp. under test was significantly influenced by the type of carbon source in the medium. Sucrose was found to be most effective as a sole carbon source for cellulase enzyme production, which results in increased enzyme activity, being 1.16 U/ml (EXG), 1.24U/ml (EG) in *T.harzianum* while 1.76U/ml (EXG) and 1.76U/ml (EG) were obtained in *T. reesei* when grown in Vogel growth medium containing 1.0% sucrose followed by cellulose, glucose and maltose as shown in Figure 5. It was also reported that maximum yields of cellulase were obtained on 1% different carbon substrate using *T. viride.* Cellulase production reached nitrogen limiting conditions and the yield of cellulase decreased when excess peptone was presented, various inorganic nitrogen sources have been optimized by different workers for cellulase production.

## Effect of nitrogen sources on enzyme activity

Various inorganic nitrogen sources have been optimized by different workers for hemicellulase production to evaluate the effect of nitrogen source on cellulase formation. In this study, different nitrogen sources were supplemented in the growth medium to optimize cellulase activity. Data revealed in Figure 6 showed that the supplementation of organic and inorganic nitrogen sources of 1% (w/v) of peptone, beef extract, yeast extract, ammonium nitrate, stimulated the cellulase yield and activity.

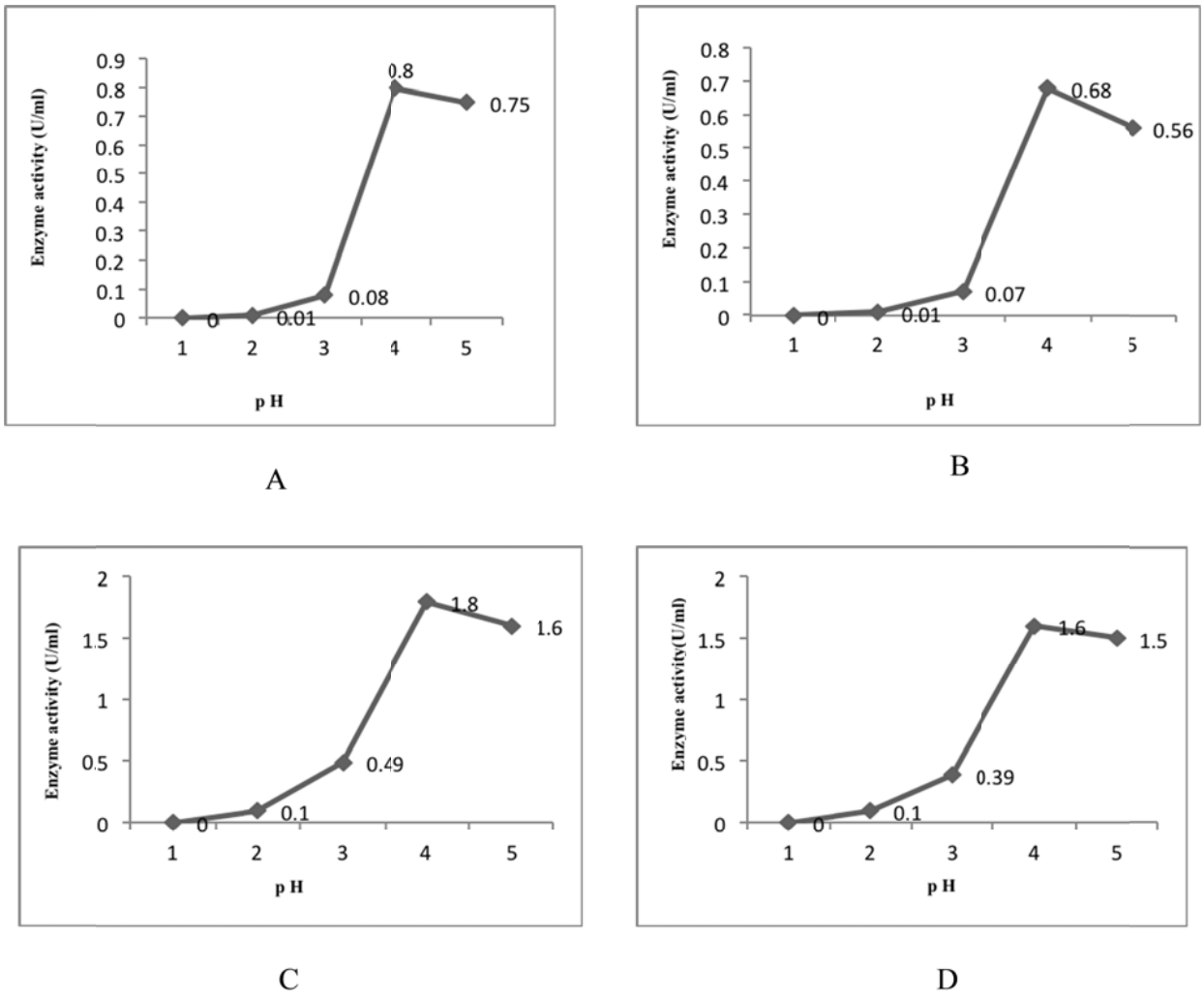


**Figure 3.** (A) Effect of pH on cellulase (EXG) activity (U/ml) in *T. harzianum;* (B) cellulase (EG) activity (U/ml) in *T. harzianum* (C). Effect of pH on cellulase (EXG) activity (U/ml) in *T. reesei* (D) changing cellulase (EG) activity (U/ml) in *T. reesei.*

The maximum enzyme activities were obtained with yeast extract (1.0%) which brought about an improvement in all the two cellulase components, including EXG and EG was found to be 1.96 and 1.76 U/ml in *T. reesei* and 0.88 and 1.29 U/ml in *T. harzianum* shown in Figure 6, respectively. Peptone was second important nitrogen source used by *Trichoderma* sp. in cellulase production. It was reported that good cellulase yield can be obtained with ammonium compound as the nitrogen source.

## DISCUSSION

Cellulases are commercially famous enzymes known for their vast roles in biomass consumption and others. Keeping this view, a comparative study was done on two important species of *T. harzianum* and *T. reesei* and the effect of different parameters which have direct or indirect bearing on the crude cellulase activity were assessed. It was observed that the cellulase activity was less at 20, more at 30°C and maximum enzyme production was found at 35°C at 112 h in both species under study. This observation was similar to the finding of Mekala et al. (2008) which showed that cellulase production was maximum at 33°C incubation and decreased with high temperature in case of *T. harzianum*. Rajshekharan et al. (2011) also stated that optimum temperature for the fermentation of media components in *T. reesei* was found to be ideal at 32°C. Many workers have also reported different temperatures for maximum cellulase production either in flask or in fermentor studies using *Trichoderma* sp. suggesting that the optimal temperature for cellulase production also depends on the strain variation of the microorganism (Murao et al., 1988; Lu et al.; 2003).

Cellulase activity was found to be increased at acidic pH 4 and decrease at pH 5 due to the fact that cellulases are acidic proteins and affected by neutral pH values

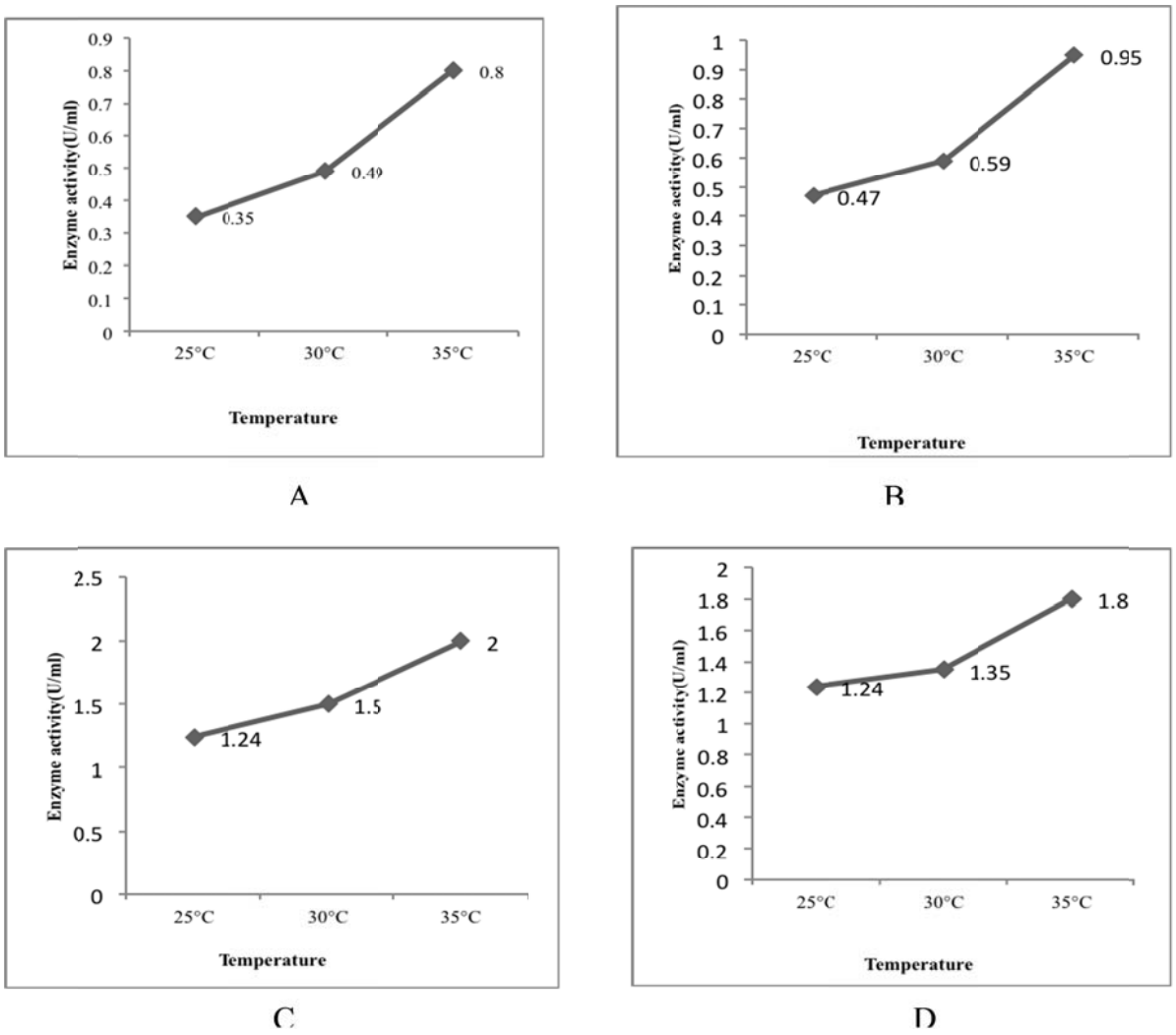


**Figure 4.** (A) Effect of temperature on cellulase (EXG) activity (U/ml) in *T. harzianum;* (B) cellulase (EG) activity (U/ml) in *T. harzianum;* (C) Effect of temperature on cellulase (EXG) activity (U/ml) in *T.reesei;* (D) changing cellulase (EG) activity (U/ml) in *T.reesei.*

(Juhasz et al., 2004). The optimal pH for fungal cellulases varies from species to species, though in most cases, the optimum pH ranges from 3.0 to 9.0 (Ishfaq et al., 2011). *T. harzianum* strain Ce 17A which showed maximum exoglucanase production at a pH range of 3 - 5 and then decreased above pH 5 (Mekala et al., 2008).

The optimal pH for fungal cellulases varies from species to species though in most cases the optimum pH ranges from 3.0 to 6.0 (Niranjane et al., 2007).

Cellulase production is also dependent on the concentration of carbon source in the production media. Addition of different carbon sources had both positive and negative effects on cellulase production (Rai et al., 2012). The maximum enzyme production was obtained using the carbon source 1% (w/v) sucrose and with 1% (w/v) of cellulose, this can be due to immediate need of energy. Mandels and Reese (1957) also reported that maximum yields of cellulase were obtained on 1% different carbon substrate using *T. viride.* For other sources of carbon (glucose and maltose) cellulase activity was found to be less as compared to the sucrose and cellulose. Malik et al. (1986) have also reported that negligible cellulases were produced with glucose as carbon source from *T. harzianum*.

Various inorganic nitrogen sources have been optimized by different workers for cellulase production (Sherief et al., 2010; Solomon et al., 1997; Lee et al., 2010). In the present study, yeast followed by peptone was found to have more cellulase activity in both species which was in accordance with the results obtained by Gautam et al. (2010). Though the addition of organic nitrogen sources such as beef extract and peptone resulted in increased growth and enzyme production, as was reported before, they were not an effective replacement for inorganic

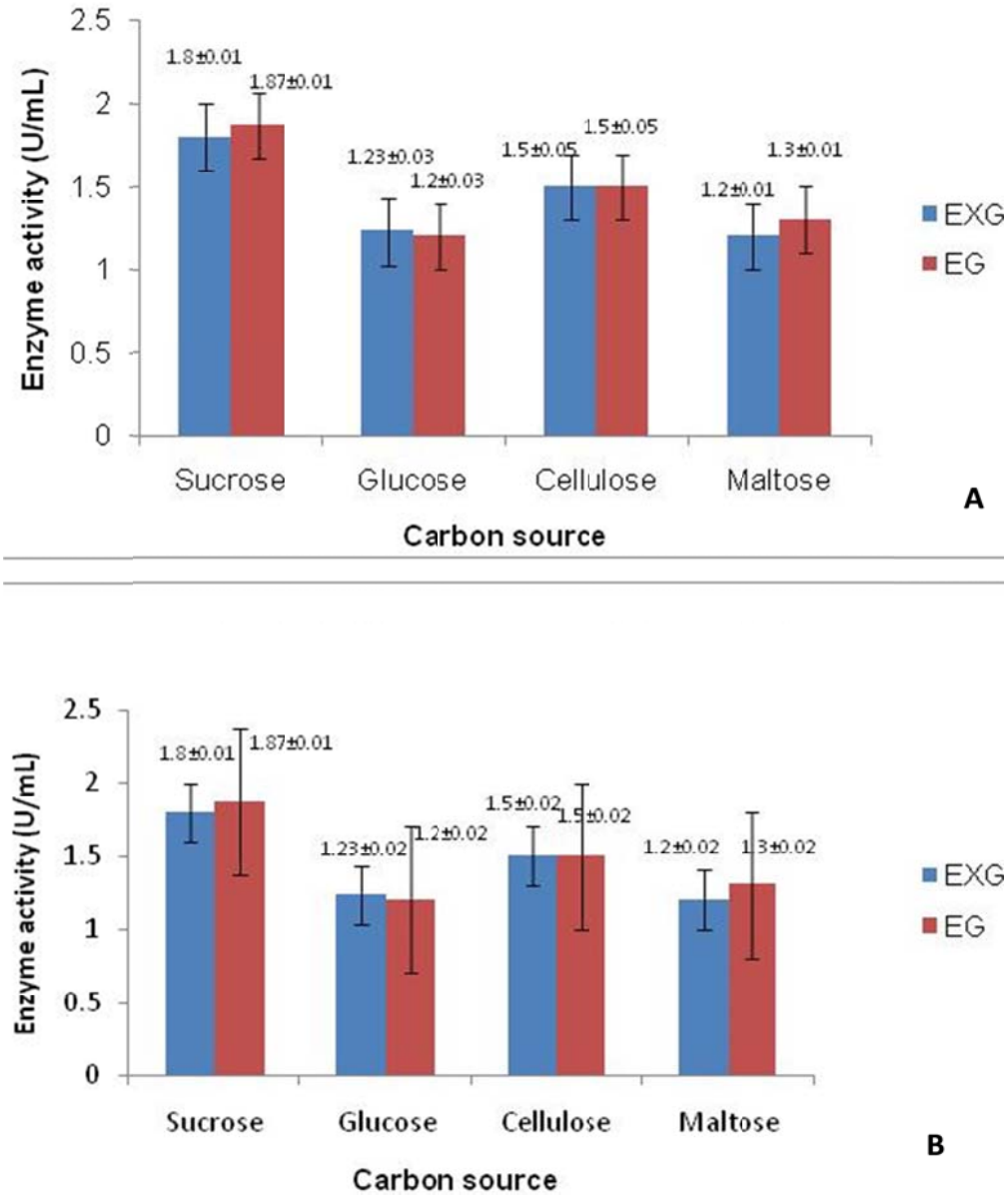


**Figure 5.** (A) Effect of different carbon sources on cellulase (EXG and, EG) activities in *T. harzianum.* (B) Effect of carbon sources on cellulase (EXG and EG) activities in *T. reesei.* The lines in the bar graph represent the standard error (SE).

nitrogen sources because of their higher cost.

## Conclusion

In this investigation, cellulolytic potential of the two potential strains of *Trichoderma* based on crude cellulase activity was tested. *T. reesei* was already being commercially exploited due to its higher cellulase activity. *T. harzianum* though depicting lower cellulytic potential than *T. reesei,* also have possibility for its usage in commercial production of enzyme and a have wider scope to be further exploited for the commercial production of cellulases by increasing the cellulase yield by further characterizing it based on stability and applicability of the enzymes from these two species.

## Conflict of Interests

The author(s) have not declared any conflict of interests.

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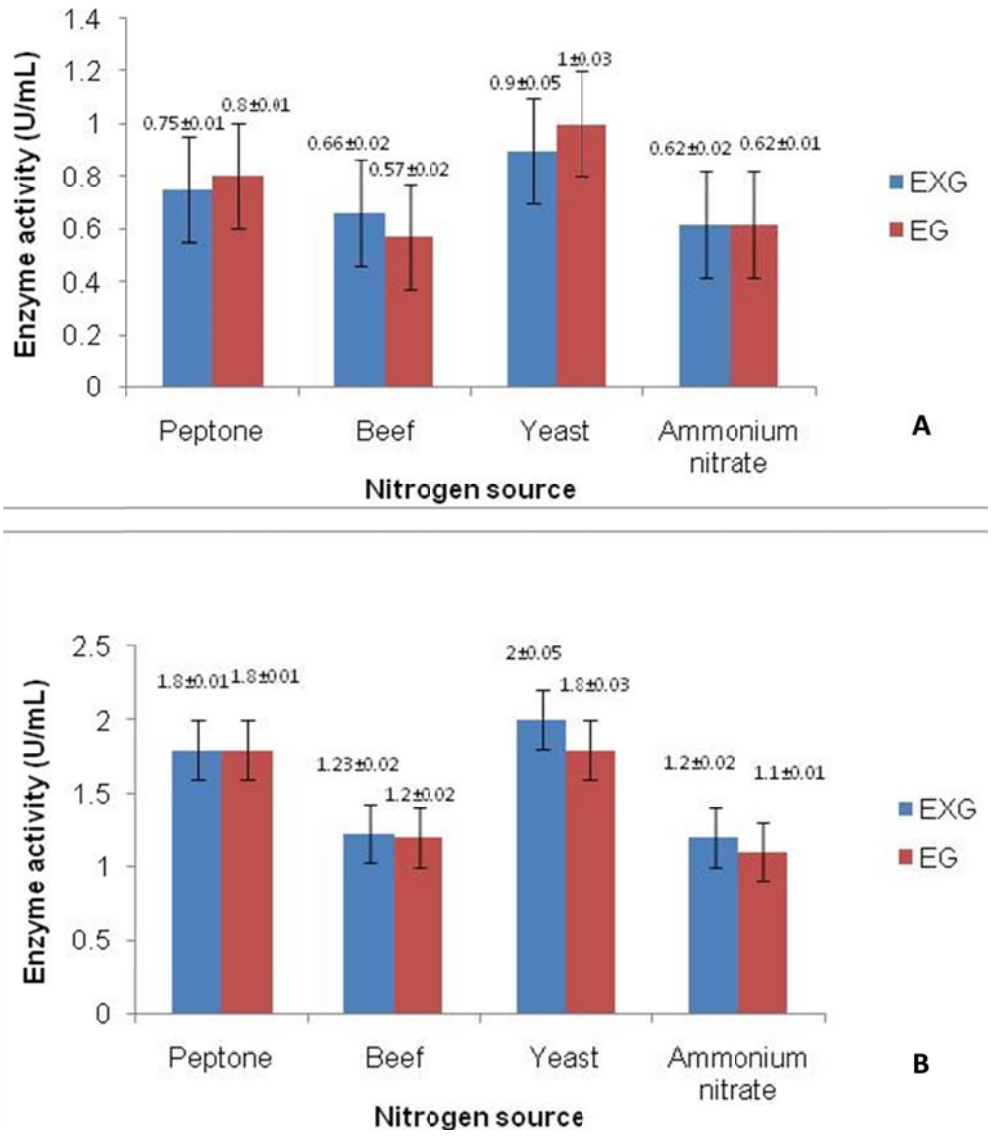


**Figure 6.** (A) Effect of different nitrogen sources on Cellulase (EXG and EG) activities in *T. harzianum.* (B) Effect of nitrogen sources on Cellulase (EXG and, EG) activities in *T. reesei.* The lines in the bar graph represent the standard error.

as antagonist soil borne pathogens'' and we thank the Head, Division of Plant Pathology, Indian Agricultural Research Institute (IARI) where the facilities for the experimentation was provided.

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