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# Influence of some cultural factors on production of cellulase and β-1, 3-glucanase by the mutant strains of *Trichoderma viride* 1433

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The factors such as incubation period, pH of the medium and incubation temperature were examined to assess their effect in optimizing enzyme production by the wild type and mutant strains of *T. viride* 1433. The influence of carbon and nitrogen sources on enzyme production was also investigated. Among the strains tested, the mutant Tv m6 produced higher concentration of both cellulases and β-1, 3-glucanases. Cellulose and laminarin was found as the best carbon sources for the production of cellulases and β-1, 3-glucanases, respectively. $NaNO_3$ was the best nitrogen source for cellulase production, while $NH_4NO_3$ for β-1, 3-glucanase productions.

**Key words:** Incubation period, pH, incubation temperature, carbon and nitrogen sources, mutant strains, cellulase and β-1, 3-glucanase activity

## Introduction

*Trichoderma* have been reported as most potential biocontrol agents against *Pythium* species (Jayaraj *et al*., 2006; Le *et al*., 2003; Abdelzaher, 2004; Kanjanamaneesathian *et al*., 2003). The antagonistic activity of the *Trichoderma* species might be due to production of cell wall degrading enzymes (Di Pietro *et al*., 1993; Thrane *et al*., 2000). *Trichoderma* attacks the plant pathogen by excreting lytic enzymes such as chitinases, β-1, 3-glucanases and proteases (Elad *et al*., 1982; Haran *et al*., 1996). The cellulases produced by *Trichoderma* species are also known to be involved in antagonistic interactions (De Marco *et al*., 2003). As the cell wall of *Pythium* species are composed of cellulose and 1, 3-β-glucan (Bartinicki-Garcia, 1968), the enzymes, cellulase and β-1, 3-glucanase produced by *Trichoderma* might be involved in hydrolysis of *P. aphanidermatum* cell wall during antagonism (Thrane *et al*., 1997). The production of hydrolytic enzymes from *Trichoderma* is affected by culture

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conditions and by the host (De la Cruz *et al*., 1992; Lorito *et al*., 1994; Schirmbock *et al*., 1994). Kredics *et al.* (2004) considered water activity and pH as the most important environmental parameters affecting the activities of mycoparasitic *Trichoderma* strains. The carbon and nitrogen sources also affect the production of hydrolytic enzymes by the *Trichoderma* species (Gashe, 1992; Shanmugam *et al*., 2008; El-Katatny *et al*., 2000). In view of above, the present study was carried out to investigate the effect of some physical factors, such as, incubation period, pH of the medium and incubation temperature on the production of cellulase and β-1, 3-glucanase activity produced by the wild type and mutant strains of *T. viride* 1433. Attempts were also made to determine the suitable carbon and nitrogen sources for the maximum production of cellulases and β-1, 3-glucanases.

## Materials and methods

### *Microbial strain*

Out of 24 mutants generated by NTG treatment from *Trichoderma viride* 1433, four mutant strains Tv m6, Tv m9 Tv m13 and Tv m21 were found most effective under *in vitro* and *in vivo* condition against *Pythium aphanidermatum* (Khare and Upadhyay, 2009; Khare *et al*., 2010). Therefore, above strains were used in the present study alongwith the wild type strain *T. viride* 1433.

A virulent strain of *Pythium aphanidermatum* was obtained from the Department of Mycology and Plant Pathology, Institute of Agriculture Science, Banaras Hindu University (BHU), Varanasi. The pathogenic and antagonistic strains were maintained on Potato-Dextrose Agar medium (PDA; Merck) at 25±2°C by regular subculturings.

### *Enzymatic activity assay*

For enzyme production, the wild type and the mutant strains of *T. viride* 1433 was grown on minimal synthetic medium (MSM) containing the following components (in grams per liter): $MgSO_4.7H_2O$, 0.2; $K_2HPO_4$, 0.9; KCl, 0.2; $NH_4NO_3$, 1.0; $FeSO_4.7H_2O$, 0.002; $MnSO_4$, 0.002 and $ZnSO_4$, 0.002. The medium was supplemented with the appropriate carbon source (0.2%, w/v) for cellulase (cellulose) and β- 1, 3 glucanase (laminarin), and the pH adjusted to 5.5 unless otherwise noted. The medium was inoculated with a spore suspension to give a final concentration of ~5 $\times 10^6$ conidia per milliliter and placed on a rotary shaker at 150 rpm at 30°C unless otherwise noted for different time intervals. The cultures were harvested after 2, 4, 6, 8 and 10 days

of incubation and were filtered through Whatman No. 44 filter paper and finally centrifuged at 12000 rpm for 10 min at 4°C to get cell-free culture filtrate which were then used as enzyme source.

***Cellulase assay***

Cellulase activity was assayed following the method of Miller (1959). The assay mixture contained 1 ml of 0.5% pure cellulose (Sigma Co.) suspended in 50 mM phosphate buffer (pH 5.0) and 1 ml of culture filtrates of the wild type and the mutant strain. The reaction mixture was incubated for 30 min at 50°C. The blanks were made in the same way using distilled water in place of culture filtrate. The absorbance was measured at 540 nm and the amount of reducing sugar released was calculated from the standard curve of glucose. One unit of cellulase activity is defined as the amount of enzyme that catalyzed 1.0 µ mol of glucose per minute during the hydrolysis reaction.

***β- 1, 3- glucanase assay***

β-1, 3-glucanase was assayed similarly by incubating 1 ml 0.2% laminarin (w/v) in 50mM sodium acetate buffer (pH = 4.8) with 1ml enzyme solution at 50°C for 1 h and by determining the reducing sugars with DNS (Nelson, 1944). The amount of reducing sugars released was calculated from standard curve for glucose. One unit of β-1, 3- glucanase activity was defined as the amount of enzyme that catalyzed the release of 1 µmol of glucose equivalents per min.

***Effect of culture conditions on enzyme production***

To study the effect of incubation period, enzyme activities were recorded at different time intervals (2, 4, 6, 8 and 10 days). To study the effect of pH, the pH of the MSM medium was adjusted to values from 3.5 to 7.5 using 50 mM of acetate buffer (3.5 to 5.5 pH) or phosphate buffer (6.5–7.5 pH). The tested strain was incubated at 30°C and the enzyme activity was determined at fourth day. To determine the impact of incubation temperature, the tested strain was incubated on MSM (pH 5.5) at 25, 30, 35 and 40°C. The enzyme activity was recorded at fourth day of incubation. Enzyme production was also examined in the presence of five different carbon (cellulose, lactose, glucose, laminarin and sucrose) and five different nitrogen ($NaNO_3$, $NH_4NO_3$, $NH_4Cl$, $(NH_4)_2SO_4$ and urea) sources. The carbon and nitrogen sources were autoclaved separately and then added to MSM medium at a final concentration of 0.2% and

0.1% w/v, respectively. The cellulase and β-1, 3-glucanase activities were recorded by the method as described above at fourth day of incubation.

**Results**

Among the wild type and mutant strains of *T. viride* 1433, the mutant strain Tv m6 exhibited the maximum production of cellulase and β-1, 3-glucanase activities. Therefore, this strain was used for all further experiments.
The influence of incubation period of the tested strain on production of cellulase and β-1, 3-glucanase activities is given in Fig. 1. Cellulase activity was gradually increasing for six days, and then slowly declined. The maximum cellulase activity due to the mutant Tv m6 was 6.4 U/ml at the sixth day. β- 1, 3-glucanase activity was detected after two days of incubation, reaching maximum in four days and then it gradually decreased with increasing the incubation period. The highest β-1, 3-glucanase activity was 42.4 U/ml due to the mutant Tv m6 at the fourth day.

The production of cellulase and β-1, 3-glucanase activities by the tested strain was observed to be affected by the pH conditions of the growth medium (Fig. 2). The maximum production of cellulase and β-1, 3-glucanase activity were recorded when the tested strain was allowed to grow in the medium having pH 5.5. The production of both cellulase and β-1, 3-glucanase activity decreased at below and above this optimum pH. The lowest activity of cellulase and β-1, 3-glucanase was recorded at pH 3.5.
The incubation temperature influenced the production of cellulase and β-1, 3-glucanase activity from the tested strain. The maximum production of cellulase and β-1, 3-glucanase activity were monitored when the strains were incubated at 30°C (Fig. 3). The cellulase and β-1, 3-glucanase activity decreased at below and above this optimum incubation temperature. Next favorable incubation temperature for cellulase production by Tv m6 was 35°C, whereas for β-1, 3-glucanase, it was 25°C. The production of both the enzyme was minimum at 40°C.

The results of effect of carbon sources on production of cellulase and β-1, 3-glucanase activity by Tv m6 is presented in Fig. 4. In the presence of cellulose as sole carbon source, the mutant strain Tv m6 produced maximum cellulase activity (3.1 U/ml). Glucose gave the least degree of cellulase activity (0.4 U/ml), when used as a carbon source. For the production of β-1, 3-glucanase activity, laminarin was found to be the most favorable substrate followed by lactose. The β-1, 3-glucanase activity was 42.2 U/ml with laminarin as carbon source. Cellulose, as carbon source gave the lowest activity of β-1, 3-glucanase (15.2 U/ml).

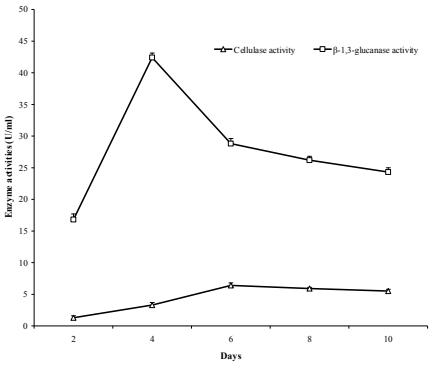


**Fig. 1.** Effect of incubation period on the cellulase and β-1, 3-glucanase production by the mutant Tv m6.

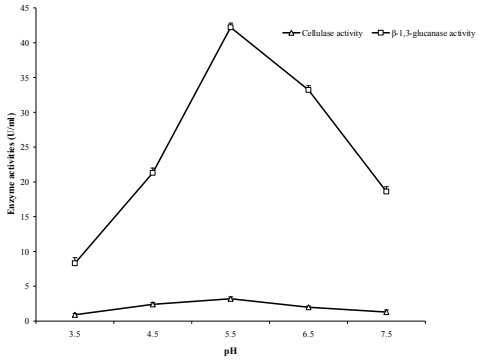


**Fig. 2.** Effect of pH of the medium on the cellulase and β-1, 3-glucanase production by the mutant Tv m6.

Fig. 5 presents the effect of nitrogen sources on production of cellulase and β-1, 3-glucanase activities by the mutant strain Tv m6. $NaNO_3$ was observed to be the most stimulative for production of cellulase activity (3.5 U/ml), followed by $NH_4NO_3$ (3.0 U/ml). In contrast, $NH_4NO_3$ was the best nitrogen source for production of β-1, 3-glucanase activity (42.3 U/ml), followed by $NaNO_3$ (38.6 U/ml). The least activity of cellulase (0.7 U/ml) and β-1, 3-glucanase (30.1 U/ml) was recorded with $NH_4Cl$ and $(NH_4)_2SO_4$, respectively, as the nitrogen source.

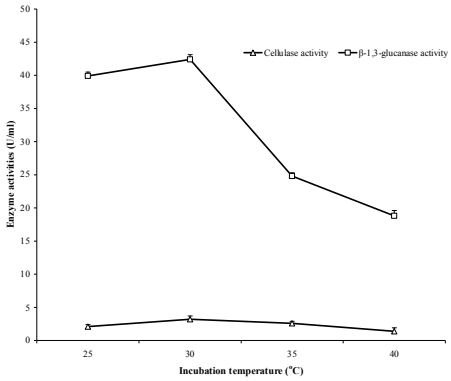


**Fig. 3.** Effect of incubation temperature on the cellulase and β-1, 3-glucanase production by the mutant Tv m6.

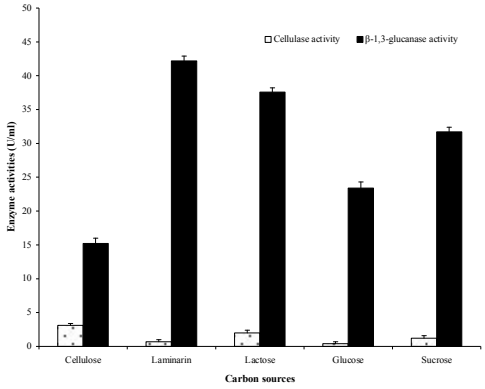


**Fig. 4.** Effect of different carbon sources on the cellulase and β-1, 3-glucanase production by the mutant Tv m6.

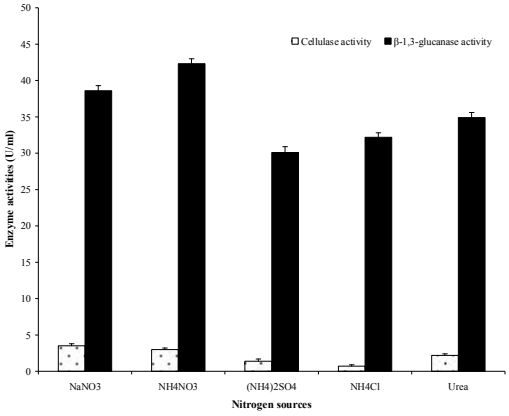


**Fig. 5.** Effect of different nitrogen sources on the cellulase and β-1, 3-glucanase production by the mutant Tv m6.

## Discussion

In the present study, investigation was carried out on the effect of incubation period on the production of cellulase and β-1, 3-glucanase activities by the tested strain (Fig. 1). The maximum production of cellulases by the mutant strain Tv m6 was observed after 6 days of incubation, and thereafter the enzyme activity decreased. The result was in consistent with De Marco *et al*. (2003) who reported cellulase activity of the *Trichoderma* isolate to be maximum after 5 days of incubation. The production of β-1, 3-glucanase by mutant strain Tv m6 was optimum after 4 days. The results were consistent with El-Katatny *et al*. (2000), who found highest activity of β-1, 3-glucanase produced by *Trichoderma harzianum* at fourth day of incubation. However, in another study El-Katatny *et al*. (2006) found β-1, 3-glucanase activity to be maximum after 3 days of incubation. The study suggests that the production of cellulase and β-1, 3-glucanase was optimum after a definite period of incubation, however further increase in the incubation time, reduced the enzyme production. The reason for this may be because of autolysis of mycelium in prolonged incubation period leading to enzyme instability (Shanmugam *et al*., 2008) or due to the depletion of macro and micronutrients in the medium with the lapse in time (Ikram-ul-Haq *et al*., 2006).

The pH has a direct effect on the uptake of mineral nutrients, which are present in the medium, therefore, the production of enzymes are affected. The optimal pH for the production of cellulase and β-1, 3-glucanase by the mutant strain Tv m6 was 5.5 (Figure 2). El-Katatny *et al*. (2000) too reported that the production of β-1, 3-glucanase by *Trichoderma harzianum* was favored by acidic pH of 5.5. Both high acidic and high basic pH shows negative effects, but a medium with low acidic pH i.e. 5.5 was ideal for enzyme production. This might be due to the fact that fungal cultures require slightly acidic pH for their growth and enzyme biosynthesis (Haltrich *et al*., 1996). Acidic pH was reported to be an important growth parameter in the production of chitinases and β-1, 3-glucanases in mycoparasite *T. harzianum* (Elad *et al*., 1982). There are many reports on the requirement of pH of culture medium for extracellular enzyme production by fungi and bacteria, and in most cases the maximum lies between pH 4.5 and 5.5 (Coughlan, 1985).

Temperature plays an important role in the metabolic activities of microorganisms. The optimum incubation temperature of the tested strain for the production of cellulase and β-1, 3-glucanase activities was 30°C (Figure 3). This might be due to better growth of the strains at this temperature. Further increase in incubation temperature resulted in the gradual decrease in enzyme production. The

reason might be because a higher temperature (above 30°C) alters the cell membrane composition and stimulates protein catabolism (Ikram-ul-Haq *et al*., 2006).

Although different carbon sources induced production of cellulase, the level produced was variable (Figure 4). The reason for this may be because of influence of carbon sources on the growth of cellulolytic organisms (Mandels *et al*., 1974; Lakshmikant, 1990; Lakshmikant and Mathur, 1990). In this study, cellulose was a good inducer for cellulase biosynthesis by the tested strain. The role of a compound to act as inducer of cellulase biosynthesis varies from organism to organism (Shanmugam *et al*., 2008). The production of β-1, 3-glucanase by the mutant strain Tv m6 was highest in the presence of laminarin as carbon source. This is because β-1, 3-glucanase induction depends on the type of linkage (Vasquez-Garciduenas *et al*., 1998). El-Katatny *et al*. (2000) reported that induction of the enzyme β-1, 3-glucanase may vary in response to the glucan structure and found highest production of β-1, 3-glucanase in laminarin (1, 3-glucan) followed by pustulan (1, 6-glucan) and pullulan (1, 6-glucan).

The nitrogen source used in the production medium is one of the major factors affecting enzyme production. The production of cellulase and β-1, 3-glucanase by the tested strain was maximum in the medium having $NaNO_3$ and $NH_4NO_3$, respectively, as the nitrogen source (Figure 5). Abdel-Satar and El-Said (2001) reported $NaNO_3$ and peptone as the best nitrogen sources for *Trichoderma harzianum*. Rajoka (2004) reported that nitrates were the best nitrogen sources for the production of cellulases in *Cellulomonas flavigena* while $NH_4Cl$, $(NH_4)_2SO_4$, $NH_4H_2PO_4$, corn steep liquor and urea were the poor nitrogen sources. Spiridonov and Wilson (1998) observed that $NH_4$ compounds were the most favorable nitrogen sources for protein and cellulase synthesis. El-Katatny *et al*. (2000) reported peptone-casein to be the best nitrogen source for β-1, 3-glucanase productions by *Trichoderma harzianum*, followed by corn steep solid and then $NH_4NO_3$.

In conclusion, the mutant strain Tv m6 was better adapted to the changed cultivation conditions and produced significant amount of enzymatic activities. Therefore, the mutant strain Tv m6 may be promising to apply as biocontrol agent against *P. aphanidermatum* in different conditions.

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