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# Development of a mutant of *Trichoderma citrinoviride* for enhanced production of cellulases

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ABSTRACT

Considering importance of a microbial strain capable of increased cellulases production and insensitive to catabolite repression for industrial use, we have developed a mutant strain of *Trichoderma citrinoviride* by multiple exposures to EMS and ethidium bromide. The mutant produced 0.63, 3.12, 8.22 and 1.94 IU ml-1 FPase, endoglucanase, β-glucosidase and cellobiase, respectively. These levels were, respectively, 2.14, 2.10, 4.09 and 1.73 fold higher than those in parent strain. Glucose (upto 20 mM) did not repress enzyme production by the mutant under submerged fermentation conditions. In vitro activity assay with partially purified cellulase showed lack of inhibition by glucose. Interestingly, the partially purified endoglucanase and β-glucosidase were activated by 2.0 fold and 2.6 fold, respectively, by 20 mM and 30 mM ethanol in the assay mixture. Genetic distinction of the mutant was revealed by the presence of two unique amplicans in comparative DNA fingerprinting performed using 20 random primers.



## 1. Introduction

Cellulases are well known for bioconversion of cellulosic waste to useful products such as ethanol (Lawford and Rousseau, 2003), lactic acid (El-Hawary et al., 2003), single cell protein (Solomon et al., 2000) and other value added products. However, the most widely studied cellulases, namely *Trichoderma* cellulases, have shown several disadvantages. Attempts to use these enzymes in the bioconversion have not been successful for several reasons such as: low enzymatic yields, low specific activities and end product inhibition of the enzyme (Lynd et al., 2002).

One of the reasons which has been reported for low enzyme yields, is catabolite repression of enzyme biosynthesis due to cellobiose; *Trichoderma* are generally deficient in β-glucosidase, therefore cellobiose accumulates and represses enzyme biosynthesis. Most of the cellulolytic strains of *Trichoderma* described in the literature are deficient in the production of β-glucosidase (Zhou et al., 2008).

We have earlier identified a fungus, *Trichoderma citrinoviride,* which produces good levels of β-glucosidase together with endoglucanase and FPase (Chandra et al., 2008). To make this strain more efficient, we attempted further improvement by means of mutagenesis through multiple exposures of two potent chemical mutagens. The study was targeted to develop a strain having features:

(i) Enhanced cellulases production with elevated level of β-glucosidase.
(ii) Reduced sensitivity to catabolite repression of enzyme biosynthesis.
(iii) Reduced sensitivity of cellulase system to glucose and ethanol.

## 2. Methods

### 2.1. Chemicals

Ethyl methyl sulfonate (EMS), ethidium bromide (EtBr), *p*-nitrophenol β-D-glucopyrinoside (*p*NPG), cellobiose and other chemicals were procured from Sigma Chemical Co. USA, whereas Sephadex G-25 from Pharmacia LKB Biotechnology, Piscataway, N.J; *Taq* polymerase, dNTPs, PCR buffer(10x) and custom-made decanucleotide primers were procured from Bangalore Genie (P) Ltd., India.

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### 2.2. Microorganism and culture conditions

*Trichoderma citrinoviride* (MTCC No. 2418) was obtained from Microbial Type Culture Collection Center, Chandigarh, India. The organism was maintained on potato dextrose agar at 28 °C and subcultured after every 15 days.

### 2.3. Mutagenesis and screening of mutants

For EMS mutagenesis, spore suspension of *T. citrinoviride* was treated with EMS (100 μl $ml^{-1}$) and incubated at 28 °C in incubator shaker for 30 min. On the other hand, EtBr mutagenesis was carried out by incorporation of mutagen (10 μg $ml^{-1}$) to the surface agar medium. The spore suspension was serially diluted, spreaded on to the surface agar medium and incubated at 28 °C for 72 h. Screening of mutants was carried by the procedure of Durand et al. (1988). The mutants that displayed better enzyme activity and/or resistance for catabolite repression were selected and sub-cultured at least 20 times on PDA medium to test their stability.

### 2.4. Enzyme assays

Endoglucanase activity (carboxymethyl cellulase activity) in the culture supernatant was determined as described by Mandels et al. (1971). β-Glucosidase was assayed by the procedure of Kubicek (1982). FPase activity (filter paper activity) was determined by the method of Ghose (1987). Cellobiase activity was assayed using a glucose oxidase (GOD) kit (Bayer Diagnostics Vadodara, India) as described by Ghose (1987).

Units (IU) of FPase, endoglucanase and cellobiase were defined as the 1 μ mol of glucose equivalent liberated per minute under assay conditions. One unit of β-glucosidase was defined as the amount of enzyme liberating 1 μ mol of *p*-nitrophenol per minute.

### 2.5. Estimation of reducing sugar and protein

Reducing sugar was estimated as glucose by Somogyi method (Somogyi, 1945). Protein was estimated by the method of Lowry et al. (1951) and protein in the column effluents was monitored by measuring $A_{280}$.

### 2.6. Partial purification

The culture supernatant (100 ml) was filtered by glass wool filter and treated with ammonium sulfate (40–75% saturation) and left overnight. The precipitate was collected by centrifugation at 10,000 rpm for 20 min, dissolved in 50 mM acetate buffer (pH 4.8) and passed through G-25 chromatography (gel filtration chromatography, 2.2 × 20 cm) to remove ammonium sulphate.

### 2.7. Induction, repression studies and saccharification

For catabolite repression studies, sugar (1.0–100 mM) was added to the fermentation medium; for sugar inhibition studies, different concentrations (1–100 mM) of glucose were added to reaction mixture. Effect of ethanol was studied using 1.5 ml reaction mixture with 5–30 mM of ethanol and 30% (v/v) of dimethyl sulfoxide (DMSO) with partially purified β-glucosidase or endoglucanases along with their respective substrates (carboxymethyl cellulose for endoglucanase and *p*-NPG for β-glucosidase). For saccharification, substrate @ 50 g $l^{-1}$ (w/v) in 50 mM sodium acetate buffer (pH 4.8) along with partially purified enzyme was incubated at 50 °C in water bath shaker (@100 rpm). The degree of saccharification was assayed on the basis of released reducing sugar at different time intervals (1 h, 3 h, and 6–81 h) measured at every 3 h. The percentage saccharification was calculated as follows:

$$\text{Saccharification} = \frac{\text{glucose (mg/ml)} \times 100}{\text{substrate (mg/ml)}}$$

### 2.8. DNA fingerprinting

DNA was isolated from parent strain and its mutants as described in Doyle and Doyle (1987). The mutated gene was PCR-amplified using custom synthesized primers (Khanuja et al., 2000).

## 3. Results

### 3.1. Mutagenesis and screening of T. citrinoviride

Random mutagenesis is an easy tool to achieve genetic and functional modifications of an organism. Use of progressive stepwise mutagenesis-selection protocols and various mutagens with differing mode of action has been proved effective in increasing product yield. In our study, mutagenesis was carried out by multiple exposures of the fungus to two potent chemical mutagens viz; EMS and EtBr. After each mutagenic treatment, enzyme production by the mutants that showed larger clearing zones was assessed in shake flask cultures, and the most promising strain was subjected to the next mutagenic treatment. During this mutagenesis processes, we screened >7500 mutants, and of these, nine mutants were found superior, where a mutant designated as *T. citrinoviride* EB-104, was finally selected on the basis of tremendously elevated β-glucosidase production and ability to produce enzyme even in 45 mM of glucose under submerged fermentation condition (Table 1). This mutant showed 2.14, 2.10, 4.09 and 1.73 fold increase in FPase, endoglucanase, β-glucosidase and cellobiase activities, respectively, over the parent one (0.29, 1.49, 2.01 and 1.12 IU $ml^{-1}$ FPase, endoglucanase, β-glucosidase and cellobiase activities respectively in parent strain, and 0.63, 3.12, 8.22 and 1.94 IU $ml^{-1}$ FPase, endoglucanase, β-glucosidase and cellobiase activities, respectively, in mutant strain) with altered enzyme production profile (Fig. 1). Chand et al. (2005) reported 2.0 and 1.3 fold increased FPase and endoglucanase production, respectively, using successive treatments of different mutagens (EtBr and NNMG followed by UV) to spores of *Trichoderma sp.*, while incorporation of two mutagens (EtBr and NNMG) to medium resulted 2.9 and 2.1 fold increase in FPase and endoglucanase activities, respectively. Dillon et al. (2006) also reported 1.5 fold more cellulase productivity in *Penicillium echinulatum* by three repeated mutagenic treatment steps of UV, EMS and $H_2O_2$. It should be noted that the level of β-glucosidase observed for mutant strain (8.22 IU $ml^{-1}$) is unusually high for a species of *Trichoderma.* The enhancement of enzyme activities might be due to increased level of modulator proteins or co-factors promoting the enzyme activities.

### 3.2. Glucose tolerance

The inhibition of cellulases by glucose is a common characteristic in *Trichoderma* (Lynd et al., 2002). However, in our study, no significant inhibition of partially purified FPase, endoglucanase β-glucosidase and cellobiase from mutant EB-104 occurred by glucose upto 45 mM. On the other hand, activities of all four enzymes from parent strain were profoundly inhibited by 15 mM glucose (Fig. 2).

### 3.3. Stimulation of enzyme activity by ethanol

Although, ethanol has been reported to be inhibitory in the process of saccharification and fermentation (Wu and Lee, 1998),

**Table 1**
Insensitivity to catabolite repression of cellulases biosynthesis in mutant strain, *T. citrinoviride* EB-104, under submerged fermentation condition

| Strain | 10 mM Glucose | | | | 15 mM Glucose | | | | 20 mM Glucose | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | FPase | EG | BG | CB | FPase | EG | BG | CB | FPase | EG | BG | CB |
| Parent | 0.19 | 0.87 | 1.80 | 1.02 | nd | nd | nd | nd | nd | nd | nd | nd |
| Mutant, EB-104 | 0.63 | 3.03 | 8.23 | 1.76 | 0.61 | 2.97 | 6.46 | 1.55 | 0.60 | 2.90 | 6.09 | 1.49 |

EG-endoglucanase, BG-β-glucosidase, CB-cellobiase; enzyme activities are given in terms of IU $ml^{-1}$.

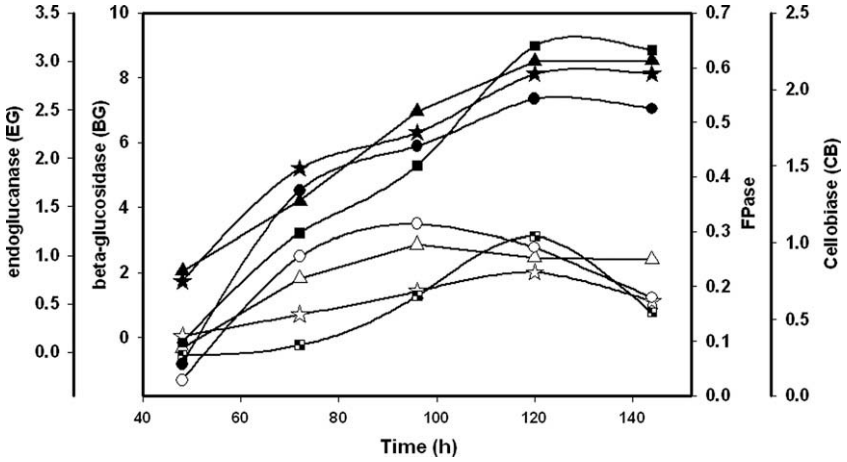


**Fig. 1.** Cellulases production profile of *T. citrinoviride* and its mutant *T. citrinoviride* EB-104 under submerged fermentation condition. Data points represent means of three replicates. [endoglucanase (mutant); endoglucanase (parent); β-glucosidase (mutant); β-glucosidase (parent); FPase (mutant); FPase (parent); cellobiase (mutant); Cellobiase (parent)].

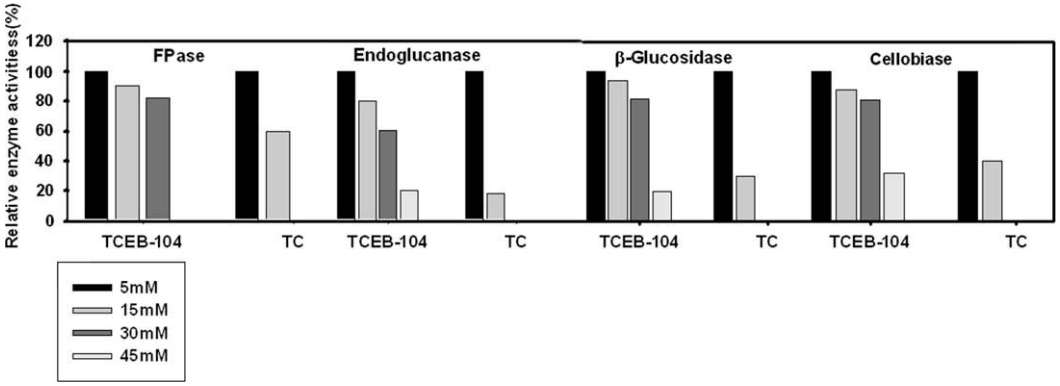


**Fig. 2.** Effect of various glucose concentrations (5–45 mM) on activities of partially purified cellulases, produced by *T. citrinoviride* and its mutant EB-104. Values are mean of three replicates. (TCEB = *T. citrinoviride* EB-104; TC = *T. citrinoviride*).

however, in our study, ethanol at 20 mM and 30 mM concentrations increased the activity of β-glucosidase of *T. citrinoviride* EB-104 by 1.5 fold and 2.6 fold respectively. On the other hand, ethanol at 20 mM concentration increased the endoglucanase activity by 2.0 fold. The enzyme system showing the insensitivity to glucose and ethanol, offers a better candidate for higher bioconversion of cellulosics.

### 3.4. Saccharification

Cellulases produced by both parent and mutant strain were evaluated for their efficiency of bioconversion or saccharification. The results demonstrated that enzyme system produced by parent, *T. citrinoviride* and mutant EB 104 resulted in 41% and 57% saccharification (data not shown). The enzyme system produced by *T. citrinoviride* EB 104, therefore, has potential to hydrolyze the cellulosic substrate more efficiently, which may be due to the elevated level of β-glucosidase in its cellulase system, as scarcity of this enzyme limits the bioconversion of cellulosic substrates (Lynd et al., 2002).

### 3.5. DNA finger printing

Several factors have been associated for the alteration in secretion of enzyme from the mutated organism. We expected that there could be detectable genetic changes in mutated gene of the *T. citrinoviride* EB-104. It was found that the DNA banding pattern was similar in case of parent and mutants strain except with primer MAP-2 (sequence GTCCTACTCG), where two unique bands could be detected in mutant *T. citrinoviride* EB-104 (Fig. 3). Similarly, a unique band was also observed in mutant *T. citrinoviride* EM-563 and *T. citrinoviride* EB-782 with primer MAP-2. The

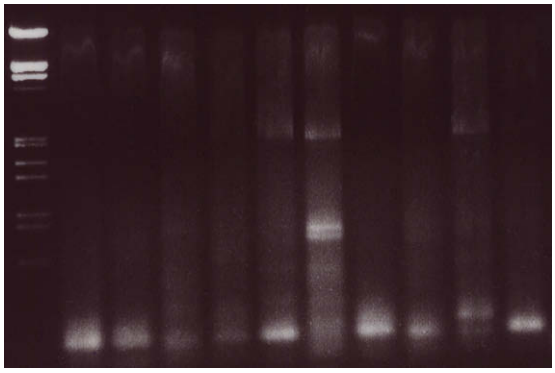

**Fig. 3.** DNA finger printing of different mutants of *T. citrinoviride* with MAP-2 primer. (Lane 1 = M-marker; Lane 2 = Parent strain; Lane 3 = mutant, Em-1026; Lane 4 = mutant, Eb-36; Lane 5 = mutant, Eb-81; Lane 6 = mutant, EM-563; Lane 7 = mutant, EB-104; Lane 8 = mutant, EB-109; Lane 9 = mutant, EB- 279; Lane 10 = mutant, EB- 782; Lane 11 = mutant, EB-1421).

observed specific bands reveal that sequence of mutated gene has been changed.

## 4. Conclusion

The present work shows that the improvement of cellulase production capability in *T. citrinoviride,* through chemical mutagenesis, resulting 2.14, 2.10, 4.09 and 1.73 fold increase in FPase, endoglucanase, β-glucosidase and cellobiase production, respectively, with higher insensitivity for catabolite repression. Furthermore, partially purified enzyme system of mutant strain was also found to be insensitive to glucose and ethanol. Interestingly, ethanol at lower concentrations increased activities of β-glucosidase and endoglucanase, produced by mutant strain. Thus, this mutant strain could be a potential candidate for the bioconversion process as it produces good levels of endoglucanase, cellobiase and FPase together with the exceptionally high levels of β-glucosidase.

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