J Ind Microbiol Biotechnol (2009) 36:605–609
DOI 10.1007/s10295-009-0544-9

SHORT COMMUNICATION

# Cellulase production by six *Trichoderma* spp. fermented on medicinal plant processings

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Received: 2 December 2008 / Accepted: 2 February 2009 / Published online: 10 March 2009


**Abstract** Capabilities of cellulase production, using delignified bioprocessings of medicinal and aromatic plants, viz. citronella (*Cymbopogon winterianus*) and *Artemisia annua* (known as marc of *Artemisia*) and garden waste (chiefly containing *Cynodon dactylon*), by the six species of *Trichoderma* were comparatively evaluated. Among the members of *Trichoderma* studied, *T. citrinoviride* was found to be the most efficient producer of cellulases along with a high level of β- glucosidase (produced 102.4 IU $g^{-1}$ on marc of *Artemisia*; 101.33 IU $g^{-1}$ on garden waste; 81.86 IU $g^{-1}$ on distillation waste of citronella and 94.77 IU $g^{-1}$ on pure cellulose). Although *T. virens* was noticed to be the minimal enzyme producer fungus, it interestingly could not produce complete cellulase enzyme complex on any test waste or pure cellulose, except on marc of *Artemisia*, where it produced all three enzymes of the complex. Immediate reduction in pH was also noticed during fermentation in the case of pure polymer (cellulose) by all tested fungi, while it was delayed with delignified agrowastes. The pH profile varied with the substrate used as well as with individual species of *Trichoderma*. On the other hand, no alteration in pH with any species of *Trichoderma* was noticed when grown on marc of *A. annua*, which might be due to the buffering capacity of this marc.

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## Introduction

Lignocarbohydrate degrading microbes produce various and multiple forms of cellulases, hemicellulases, pectinases and ligninases, which are increasingly used in the bioprocessing of plant materials, bio-fuel, feed, chemical feedstocks, silage and feed additives, and they are also used in textile and paper industries [1–3]. Increasing demand of these enzymes has intensified the search for microorganisms producing high levels of cellulases and better and cost-effective substrates [21].

Although *Trichoderma reesei* has been extensively studied and reported to produce a highly active extracellular cellulase system (endoglucanases, exoglucanases and β-glucosidases) [4–8], most of its strains have low activity of β-glucosidase and need its supplementation from other sources to increase the rate and extent of cellulose hydrolysis [9]. So far, no *Trichoderma* sp. that could produce high levels of β-glucosidase has been reported [10]. Therefore, strains producing high levels of β-glucosidase that might be a better candidate for commercial exploitation need to be identified. Moreover, cellulase production has been shown to be influenced, apart from efficient strains, by a number of factors, such as the cultural conditions, type of fermentation and, most importantly, the nature of the substrate. The relationship among these variables has a critical effect on the ultimate production of cellulases [11].

This study primarily aims at identifying a species of *Trichoderma* that, apart from FPase and endoglucanase, could also produce high levels of β-glucosidase specifically utilizing bioprocessings of selected medicinal and aromatic plants and pure fermentation under submerged conditions.

## Materials and methods

### Substrate

Among the processings of medicinal and aromatic plants, distillation waste of citronella (*Cymbopogon winterianus*), an essential oil-bearing crop, and bioprocessings of *Artemisia annua* (an industrial pharmaceutically important plant and source of artemisnin, an antimalarial compound) were selected on the basis of their capability to support higher levels of production of total cellulases (unpublished data), whereas garden waste, primarily consisting of mowed lawn grass (*Cynodon dactylon*), was considered as the control, representing other lignocellulosic waste. All these wastes were obtained from the Central Institute of Medicinal and Aromatic Plants (CIMAP), Lucknow.

### Substrate preparation

All the wastes were sun dried for 2 days and subjected to pretreatment with 1% NaOH. The pretreated materials were repeatedly washed to bring the pH of substrate to near neutral, oven dried for 2 days at 80°C and ground.

### Culture conditions and enzyme production

*Trichoderma reesei* (MTCC no. 164), *T. citrinoviridae* (MTCC no. 2418), *T. koningii* (MTCC no. 796), *T. fasciculatum* (MTCC no. 2771) and *T. virens* (MTCC no. 794) were obtained from the Microbial Type Culture Collection Center, Chandigarh, and *T. harzianum* (ATCC no. PTA 3701) was obtained from the CIMAP culture collection, India. All fungal strains were maintained on potato dextrose agar at 28°C. An inoculum of $5 \times 10^8$ spores/ml was prepared by harvesting 1-week-old culture, and 3 ml of spore suspension was used as inoculum for 50 ml medium in a shake flask containing mineral salt medium [12] together with 1% (w/v) of substrate. The flasks were incubated at 28°C on a shaker at 180 rpm. Samples were drawn at 24, 48, 72, 96, 120 and 144 h after inoculation and centrifuged at 10,000 rpm at 4°C to analyze enzyme activities in the supernatant. All the experiments were carried out in triplicate and subjected to statistical analysis of mean and variations with the help of Sigma plot 8.0.

### Enzyme assay

FPase activity (filter paper activity), using a 1 × 6-cm strip of Whatman no. 1 filter paper, was assayed by the method of Ghosh [13]. Endoglucanase activity (carboxymethyl cellulase activity), using carboxy methyl sodium salt, was determined as described by Mandels and Werber [12]. β-Glucosidase was assayed using para-nitrophenyl-β-D-glucopyranoside by the procedure of Kubicek [14].

Units (IU) of endoglucanase and FPase were defined as the micromole of glucose equivalent liberated per minute of culture filtrate under assay conditions. One unit of β-glucosidase is defined as the amount of enzyme liberating 1 μmol of *p*-nitrophenol per minute.

Reducing sugar was estimated as glucose by the Somogyi method [15], and soluble protein was measured by the Lowry method [16].

## Results

The different *Trichoderma* species tested showed activities of all three enzymes of the cellulase system in shake flask cultures containing 1% pure cellulose in the fermentation medium, except for *T. virens*, where only β-glucosidase activity was observed (Fig. 1a). The enzyme production varied with the species tested. *T. citrinoviride* exhibited the highest production of all three enzymes of cellulases system, whereas *T. reesei* (13.01 IU $g^{-1}$) and *T. citrinoviride* (14.61 IU $g^{-1}$) showed relatively higher FPase activities. No FPase activity could be detected in the case of *T. virens*. *T. citrinoviride* also showed the highest endoglucanase activity (97.60 IU $g^{-1}$), followed by *T. reesei* (94.13 IU $g^{-1}$), *T. fasciculatum* (72.80 IU $g^{-1}$), *T. koningii* (63.09 IU $g^{-1}$) and *T. harzianum* (28.66 IU $g^{-1}$). Again, no endoglucanase activity could be detected in *T. virens*. Interestingly, higher levels of β-glucosidase (94.77 IU $g^{-1}$) were produced by *T. citrinoviride*, i.e., more than five times higher than that produced by *T. reesei* (16.85 IU $g^{-1}$) and *T. fasciculatum* (16.34 IU $g^{-1}$).

All the *Trichoderma* species were also compared for their enzyme production capabilities using processings of selected medicinal and aromatic plants and garden waste as substrate in submerged fermentation conditions. Induction of a particular enzyme of the cellulase system was found to be species and substrate specific.

Distillation waste of citronella, however, induced higher production of β-glucosidase with *T. fasciculatum*; a considerable reduction in the production of this enzyme was noticed in *T. harzianum*, when compared with cellulose as substrate. Similarly, a marked reduction in activity of endoglucanase was noticed in *T. reesei* with all the lignocellulosics. *T. citrinoviride, T. fasciculatum* and *T. virens* responded favorably when marc of *A. annua* was used, but there was a sharp decline in the production of all three cellulases in the other three species (*T. reesei, T. harzianum* and *T. koningii*). One of the interesting observations with marc of *Artemisia* was that *T. virens* produced all three enzymes of the cellulase system on this substrate, whereas it was not able to produce them on any other test substrate, including pure polymer cellulose.

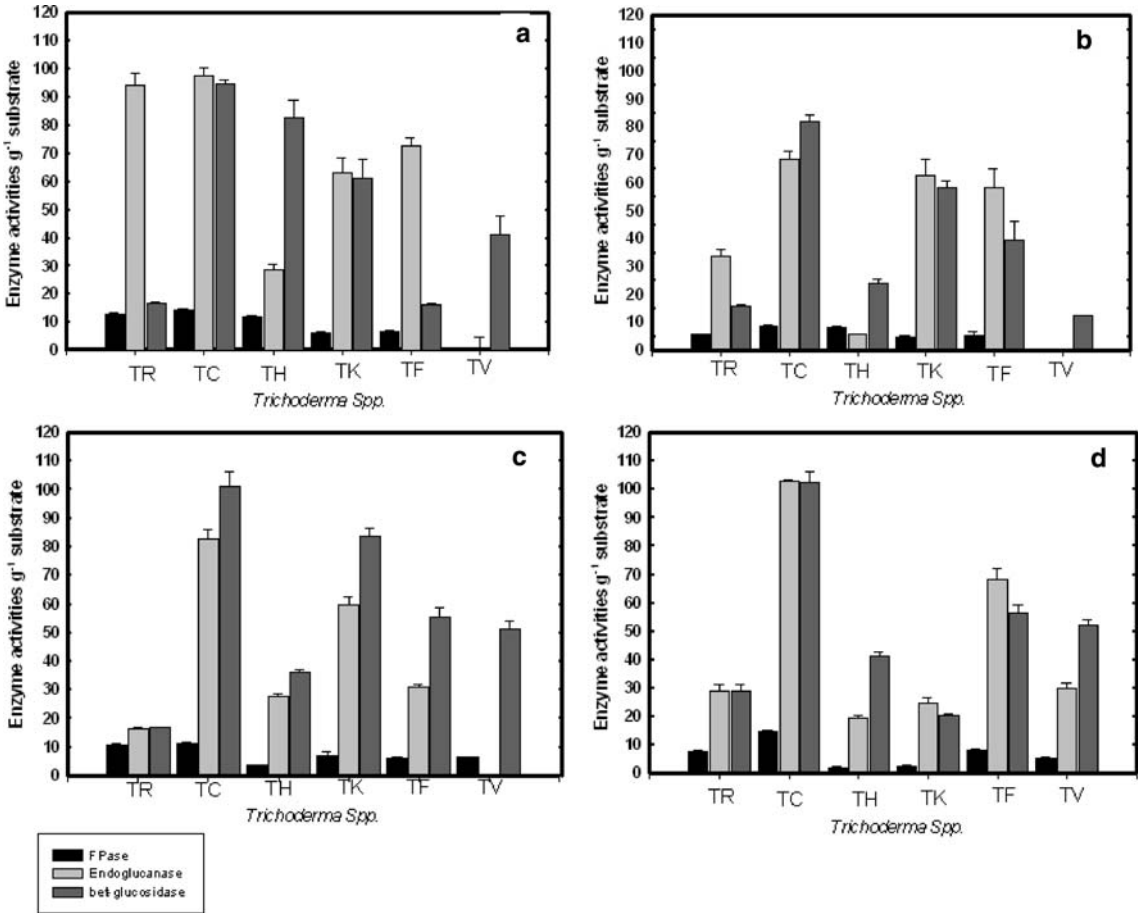


**Fig. 1** Specific activities of FPase, endoglucanase and β-glucosidase by different species of *Trichoderma* fermented on **a** cellulose powder, **b** distillation waste of citronella (*C. winterianus*), **c** mowed lawn grass (*C. dactylon*) and **d** marc of *A. annua*. TR = *T. reesei*, TC = *T. citrinoviride*, TH = *T. harzianum*, TF = *T. fasciculatum*, TV = *T. virens*. Values are mean of three replicates with SE

The alteration in pH (with an initial pH of 5.6) with time course in pure cellulose-containing media was also studied during the fermentation period. *T. reesei, T. citrinoviride* and *T. koningii* showed an immediate reduction in pH (5.6–3.5) within 24 h, which, however, subsequently increased. On the other hand, there was a gradual decline in pH for *T. harzianum, T. fasciculatum* and *T. virens*, which ranged from 3. 5 to 5.0 in a span of 144 h (Fig. 2). In media containing lignocellulosic substrates, the changes in pH were minimal in comparison to media containing pure cellulose, where pH was observed to decline at a faster pace. Surprisingly, no change in pH was noticed in medium supplemented with marc of *Artemisia* irrespective of the *Trichoderma* species involved (Fig. 3).

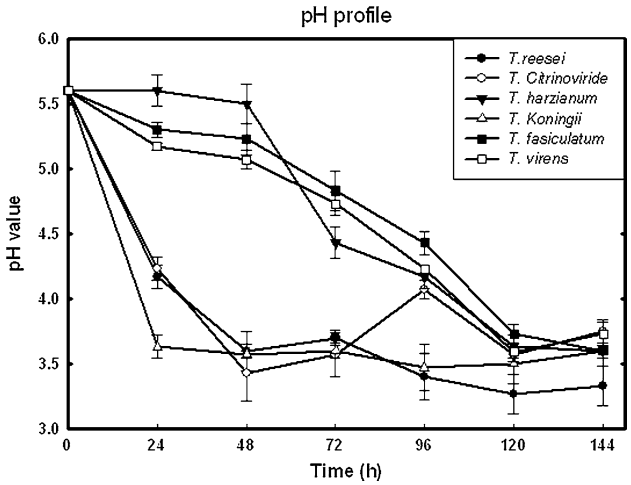


**Fig. 2** The pH alteration during the fermentation period (different spNSecies of *Trichoderma* were grown on cellulose powder-containing media in shake flask cultures, and pH was monitored after every 24 h). Values are mean of three replicates with SE

## Discussion

Although *T. reesei* is a well-known and commercially exploitable fungus for the production of cellulases, its cellulase system is deficient in the production of a satisfactory level of β-glucosidase resulting in the accumulation of disaccharide cellobiose/cellooligomers, which repress enzyme synthesis and activity [10, 17]. In an effort to identify a *Trichoderma* species producing a higher level of

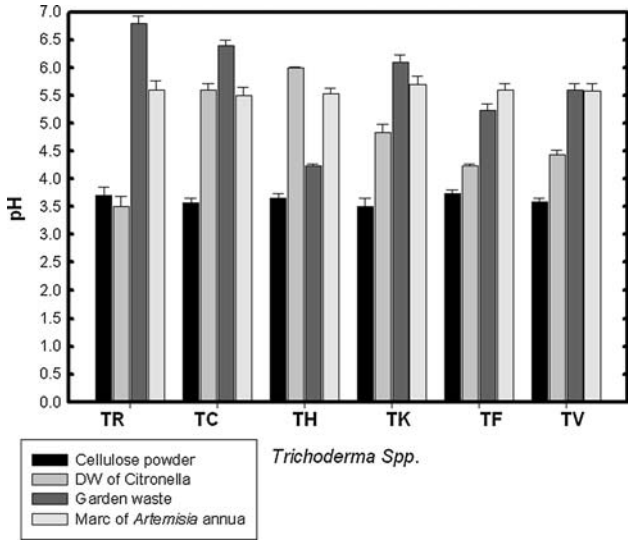


**Fig. 3** The pH of different polymer-containing media (different species of *Trichoderma* were grown on cellulose powder, distillation waste of citronella, mowed lawn grass and marc of *A. annua*-containing media in shake flask cultures, and pH was measured after 120 h of the fermentation period). TR = *T. reesei*, TC = *T. citrinoviride*, TH = *T. harzianum*, TF = *T. fasciculatum*, TV = *T. virens*. Values are mean of three replicates with SE

β-glucosidase along with a sufficient amount of endoglucanase and FPase, various species of *Trichoderma* were screened by growing them on cellulose and different lignocellulosic materials. The study has revealed a clear difference within the *Trichoderma* species with respect to their ability to produce cellulases on different substrates. Among them, *T. citrinoviride* was noticed as the most potent and efficient cellulase producer, whereas *T. virens* was the poorest enzyme producer. *T. citrinoviride* produced a high level of β-glucosidase concomitant with the appreciable levels of production of FPase and endoglucanase. Furthermore, the fungus was found to grow well on a range of lignocellulosic substrates.

The time course of pH alterations during the fermentation period was also monitored over a period of 144 h, deviating from its initial pH adjustment of 5.6. The substrate in the culture medium caused lowering in pH of the medium as generally observed with cellulase enzyme-producing organisms. In the medium supplemented with cellulose, *T. reesei, T. citrinoviride* and *T. koningii* showed a sudden decline in pH from 5.6 to 3.5 with slight fluctuations later, whereas in the case of *T. harzianum, T. fasiculatum* and *T. virens* the decline was slow. The fall in pH might have arisen as a result of the formation of oxidation products from cellobiose, such as cellobionolactone [18], which could be subsequently hydrolyzed to carboxylic acids [19, 20]. Surprisingly, marc of *A. annua* restricted the decline in pH in all *Trichoderma* species studied probably because of the substrate composition, which might have a buffering effect/mechanism as the buffering capacity of several other natural products, such as agar, is well known.

In summation, our study has shown that *T. citrinoviride* is a potent and efficient species for the production of all the constituent enzymes of the cellulase system with a higher level of β-glucosidase. Marc of *A. annua*, a waste produced in huge amounts after the processing of the *A. annua* herb, was found to be the suitable substrate for this fungus for maximizing the production of all three constituents of cellulase. Apart from being cheap with easy and sufficient availability from Artemisnin-producing industries, the marc of *A. annua* has the additional advantage of maintaining the pH of fermentation medium, which requires frequent pH correction during fermentation.

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