RESEARCH ARTICLE

Parthenium sp. as substrate for production of cellulolytic enzymes by *Trichoderma* spp.

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ABSTRACT

High cost of saccharifying enzymes is one of the major constraints for commercialization of bioethanol production from lignocellulosic biomass. Production of cellulolytic enzymes using lignocellulosic biomass such as agro-residues and weeds is an economically viable option in bioenergy industry. In this study, a noxious weed, *Parthenium* sp. was investigated as a substrate for production of cellulolytic enzymes CMCase, FPase, ß-glucosidase and xylanase. Two isolates namely *Trichoderma harzianum* and *T. longibrachiatum* were cultivated on *Parthenium* sp. under submerged (1% w/v) and solid-state fermentation (20% w/v) for production of cellulolytic enzymes. Under SSF, maximum production of CMCase (63.32 IU/g), FPase (9.87 IU/g) and cellobiase (16.36 IU/g) were achieved from *T. harzianum* while xylanase (393.34 IU/g) was achieved from *T. longibrachiatum*. Structural and crystallinity changes of biomass were examined through Scanning Electron Microscope (SEM) and X-Ray Diffraction analysis (XRD), respectively. Decrease in crystallinity index (CrI) of fungal treated biomass revealed the effective degradation of cellulosic components in biomass. The results showed that the menace of *Parthenium* sp. in agriculture can be managed by using it as substrate for production of cellulolytic enzymes which in turn reduces the cost of bioethanol production.

Keywords: Cellulolytic enzymes, *Trichoderma* spp., *Parthenium* sp., fermentation, bioethanol

Introduction

A crucial step for the bioconversion of lignocellulosic biomass into biofuel is a cost-effective meth od for enzymatic saccharification of cellulose and hemicellulose to fermentable sugars (Hu *et al*., 2011). These polysaccharides, which accounts for 50-60% of total biomass, are usually saccharified by hydrolytic enzymes composed of cellulases and hemicellulase that provides better yield with less toxic compounds than acid catalysed hydrolysis (Pan *et al*., 2005). It is an effective and economically feasible method to achieve maximum yield of fermentable sugars from pretreated lignocellulosic biomass under eco-friendly reaction conditions (Wyman *et al*, 2005). But, high cost of enzymes hampers the commercialization of enzymatic saccharification process in bioenergy industry. Thus, reducing the costs of these enzymes is essential to make the bioenergy production economically competitive for industrial applications.

Lignocellulosic biomass obtained from agriculture and forest is an abundant, inexpensive and renewable source of sugars and a desirable substrate for sustainable production of bioenergy and industrially important enzymes (Singhania *et al*. 2013). Among the crop residues, rice straw, wheat straw,
corn straw and sugarcane bagasse are the major agricultural wastes. Despite of being abundant substrate for industrial use these agro-residues also have other uses such as feed or fuel. Weedy biomass would be the alternative source to substitute food/feed-based

lignocellulosic biomass. These weedy biomasses do not require additional economic input as they normally grow on agriculturally degraded land, road sides and water bodies (Huber and Dale, 2009). These are regarded as a nuisance as they pose a serious threat to the biological diversity and ecological environment. The noxious weed, *Parthenium* sp. (congress grass), which have no particular use and their eradication is a major challenge, caused decline in yield up to 40% in agricultural crops (Swati *et al*., 2013). Attempts have been made to use this as substrate for compost preparation but very negligible reports are available for enzyme production. As it contains 31% cellulose and 16.5% hemicelluloses it can be used as substrate for cultivation of fungi for production cellulolytic en- zymes.

Due to high secretion capacity in an industrial context, the genus *Trichoderma* remains the crowned king in production of cellulase and hemicellulase for industrial purposes (Kubicek, 2013). They widely grow on different renewable substrates such as rice straw, wheat straw, bagasse wastepaper, dairy manure (Sun *et al* 2008). In this study, two *Trichoderma* species were evaluated for their production potential using *Parthenium* sp. as substrate under submerged and solid-state fermentation.

Material and Methods

Isolation and Screening of fungal isolates for cellulo- lytic enzymes

Various samples including leaf litter, barks and soils were collected from hilly forests of Pachmarhi hills (22.47°N 78.43°E) in Bhopal, Isolation of cellulolytic fungi was done by inoculating the samples on Reese's mineral medium (/L) KH_2PO_4 2g; $(NH_4)_2SO_4$ 1.4g; KNO_3 1.4g; $MgSO_4.7H_2O$ 0.3g; $CaCl_2.2H_2O$ 0.3g; $FeSO_4.7H_2O$ 5mg; $MnSO_4.2H_2O$ 1.6mg; $ZnSO_4.7H_2O$ 1.4mg; $CoCl_2.6H_2O$ 2mg; pH 4.8 supplemented with carboxymethyl cellulose (CMC) (1%) as a sole source of carbon. The cultures were further purified and sub-
cultured on Potato Dextrose Agar (PDA) and stored at 4°C. All the isolates were screened for cellulolytic activity in petri dishes on Reese's mineral medium (RMM) supplemented with 1% CMC and incubated for 3 days at 30˚C. The plates were flooded with Con- go red (1mg/ml) solution for 15 min and destained by 1M NaCl solution for 15 min. Development of hydro-lysis zone around growth indicates positive for cellulolytic activity (Teather and Wood, 1982).

Molecular characterization of fungal isolates

The promising isolates were selected and their total DNA extraction was done by using ZR Fungal/ Bacterial DNA Kit (ZymoResearch). For sequence analysis ITS1-5.8-ITS2 region of the fungus was am- plified by using universal fungal primers homologous to conserved sequences with the small subunit (SSU) rDNA gene, pITS-1 (5'-TCCGTAGGTGAACCTGCGG-3') and pITS-4 (5'-TCCTCCGCTTATTGATATGC- 3') (White *et al*, 1990). The PCR program was as follows: a) initial incubation at 94°C for 5 min b) followed by 35 cycles (94°C for 50s, 55°C for 1 min and 72°C for 90s) and c) final extension at 72°C for 10 min. DNA sequencing of amplified fragments (approx. 550 bp) was done at Eurofins Genomics India Pvt. Ltd, Bangalore. The com- parison of partial sequence of the isolate was done with sequences available by the blast search in the National Centre for Biotechnology Information (NCBI– http://www.ncbi.nlm.nih.gov) database to identify the nearest taxa.

Submerged fermentation (SmF)

Two isolates namely *Trichoderma harzianum* and *T. longibrachiatum* were selected for further study as they are well known to produce high cellulolytic enzymes. They were grown under submerged fermentation in RMM with 1% *Parthenium* sp. as a lignocellulosic substrate by inoculating an agar plug (6 mm diameter) of one week old fungal isolates for the production of cellulolytic enzymes. Inoculated flasks were incubated statically at 30 ˚C for 7 days. The supernatant was filtered through whatman filter paper and the filtrate was used for the assay of various extracellular cellulolytic enzymes.

Solid state fermentation (SSF)

The fungal isolates were inoculated with *Parthenium* sp. under solid state fermentation for production of cellulolytic enzymes. Five grams of air dried and chopped *Parthenium* sp. biomass was suspended in 25 ml of Reese's mineral medium (RMM) with solid to liquid ratio of 1:5 and sterilized at 121˚C for 20 min. *Parthenium* sp. was inoculated with 5 plugs (6 mm diameter) of one week old fungal mycelium and incubated at 30 ˚C for 7 days. The flasks were suspended in 50 ml of 0.05 M Citrate buffer (pH 4.8) and kept in shaking for 1 h. It was centrifuged at 8000 rpm for 20 min and the supernatant was stored at -20 °C for estimation of cellulolytic activity.

Assay for cellulolytic enzymes

Endoglucanase (CMCase; E.C: 3.2.1.4), Filter paper lyase (FPase), β-D- glucosidase (cellobiase; E.C: 3.2.1.21) and xylanase (E.C: 3.2.1.8) were assayed in culture filtrate using substrates carboxymethyl cellulose (CMC), Filter paper, p-nitrophenyl- β-D- glucopyranoside and xylan, respectively as described by Ghose (1987) and Wood and Bhat (1988). The enzymatic reaction mixture (1ml) contained 500µl of filtrate and 500µl of respective substrates along with control were incubated at 50 °C for 30 min or 1 hour for FPase activity. After incubation 3 ml of Dinitro salicylic acid (DNSA) reagent was added to each tube to stop the reaction and kept in boiling water bath for 10 min and cooled. The amount of glucose released was measured at 575 nm using spectrophotometer (Miller, 1959). The β-D- glucosidase activity was estimated by adding glycine buffer (pH 10.8) after incubation to stop the reaction and amount of p- nitrophenol was measured at 430 nm. The activity was represented as IU ml^{-1} (one IU was defined as the amount of enzyme capable of liberating 1µmol of reducing sugar per min under assay conditions).

Analysis of structural changes in *Parthenium* sp.

The structural and crystallinity changes of *Parthenium* sp. were characterized by scanning electron microscopy (SEM) and X-ray diffraction (XRD).

Scanning Electron Microscopy (SEM)

For SEM, the untreated and fungal pretreated samples were gradually dehydrated with acetone gradually increasing the concentration up to 100%. Finally, samples were coated with gold/palladium (Au/Pd) and imaging was done at different magnifications between 500 X to 5000 X with ZEISS (EVOMA Scanning Electron Microscope at 20 kV/ EHT and 10 Pa.

X-ray diffraction (XRD)

For XRD, samples of particle size less than 1mm were scanned at a speed of 1°/min in the range of 2θ between 10 ° and 35° by using Cu-Kα radiation at 40 kV and 25 mA (PW 1729 Philips X-ray generator with PW 1710 diffractometer). Biomass crystallinity as ex- pressed by crystallinity index (CrI) was determined ac- cording to a method by Segal *et al*. (1959) as follows:

$$CrI = [(I_{002} - I_{amorphous})/I_{002}] \times 100$$

in which, I_{002} is the intensity for the crystalline portion of biomass (i.e., cellulose) at about 2θ = 22.5 and $I_{amorphous}$ is the peak for the amorphous portion (i.e., cellulose, hemicelluloses and lignin) at about 2θ = 16.2.

Results and Discussion

Isolation and Screening of fungal isolates for cellulolytic enzymes

Twenty-eight fungal strains were isolated and screened for cellulolytic enzyme production. All the twenty-eight fungal strains had shown clear hydrolyt- ic zones when stained with congo red and destained by 1M NaCl on RMM agar plates which indicates that they are capable of producing cellulolytic enzymes. Four promising isolates were selected and molecularly identified to the closest genera as follows; *Trichoderma harzianum*, *T. longibrachiatum*, *Aspergillus niger* and *A. flavus* and their partial sequence was submitted to NCBI GenBank.

Submerged (SmF) and Solid-state fermentation (SSF)

Cellulolytic enzyme production was analysed after 7 days of incubation with *Parthenium* sp. under SmF and SSF at 30 °C. The main aim of this experiment was to select a potent cellulolytic *Trichoderma* species that shows maximum production of cellulolytic enzymes using *Parthenium* sp. as a sole carbon source. On the basis of screening two isolates *Trichoderma*

harzianum and *T. longibrachiatum* were selected as promising isolates for further study. Under SmF & SSF, *T. harzianum* showed high activity of Endoglucanase (CMCase) (0.22 & 63.32 IU ml^{-1}), FPase (0.09 & 9.87 IU ml^{-1}) and β-glucosidase activity (0.58 & 16.63 IU ml^{-1}) and isolate PK-23 produced high xylanase activity (14.75 & 393.34 IU ml^{-1}). The results of four promising isolates are presented in Table 1.

Analysis of structural and chemical changes in *Parthenium*

Scanning Electron Microscopy

SEM images of fungal pretreated and untreated samples are shown in Fig. 1. The images of pretreat- ed sample reveals the formation of some holes on the biomass surface and disruption of the biomass network especially degradation of cellulose from lignocelluloses. This indicates the potential of *Trichoderma* isolates to utilize *Parthenium* sp. as a substrate through release of extracellular cellulolytic enzymes.

X-ray Diffraction

The crystallinity index of samples from solid state fermentation was calculated from the XRD data and the results are summarized in Fig 2. The crystallinity index for control is CrI 9.65%, which showed a significant decrease (CrI 33.1%) in biomass incubated with *Trichoderma harzianum* for 7 days. The decrease in crystallinity index (CrI) of fungal treated sample may be attributed to the breakdown of inter/intra hy- drogen bonding in the crystalline cellulose resulting in modified crystal structure as reported by Zhang *et al*. 2012.

Conclusion

Parthenium sp., a noxious weed, has been demonstrated as a suitable substrate for production of cellulolytic enzymes from *Trichoderma* sp. The structural changes in biomass revealed an effective degradation of noxious weedy biomass which oth erwise creates nuisance to health and in agriculture. These findings paved a way for utilization of weedy and harmful lignocellulosic biomass as cheap sub- strate to reduce the cost of production for cellulolytic enzymes.

Table 1. Cellulolytic activity of promising isolates under submerged fermentation (SmF) and solid-state fermentation (SSF) condition with *Parthenium* sp. as substrate.

Isolates	Cellulolytic activity (IU ml^{-1})			
	CMCase (Endo β-D glucanase)	FPase (Exo β-D glucanase)	Cellobiase (β-D glucosidase)	Xylanase
Trichoderma harzianum (SmF)	0.22	0.09	0.58	10.48
T. harzianum (SSF)	63.32	9.87	16.63	122.35
T. longibrachiatum (SmF)	0.20	0.08	0.40	14.75
T. longibrachiatum (SSF)	36.86	8.06	13.91	393.34

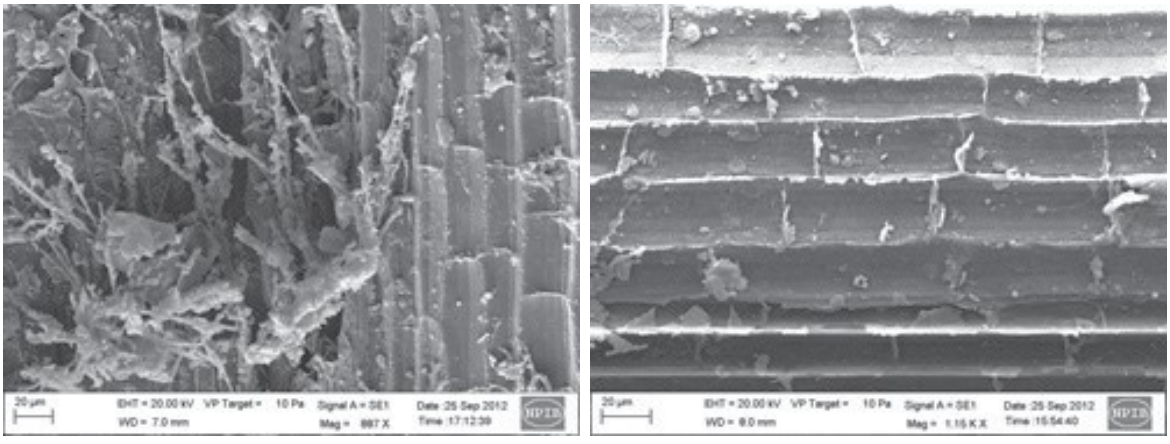

Fig.1 SEM images at various magnifications for fungal (T. *harzianum*) treated and raw substrates. A – T. *harzianum* treated, B – Raw *Parthenium* sp.

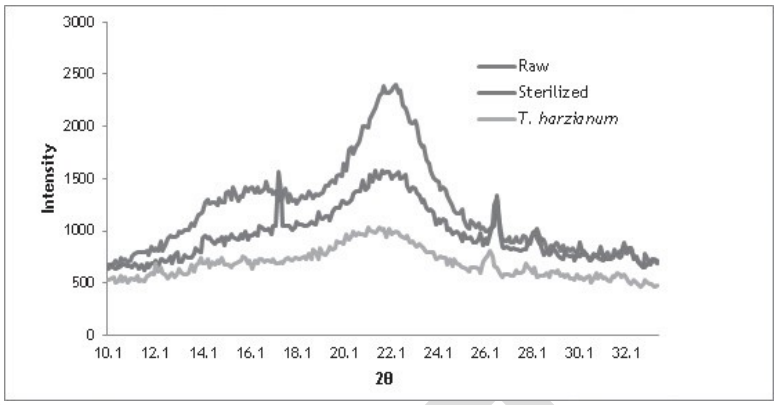

Fig.2 XRD pattern of untreated and *Trichoderma harzianum* treated *Parthenium* sp.

References:

Ghose, T.K. (1987) Measurement of cellulose activities.
Pure Appl. Chem., **59** (2): 257-268

Hu, J., Arantes, V. and Saddler, J.N. (2011) The enhance- ment of enzymatic hydrolysis of lignocellulosic sub- strates by the addition of accessory enzymes such as xylanase: is it an additive or synergistic effect? Bio- technol. Biofuels, 4: 36

Huber, G.W., Dale, B.E. (2009). Grassoline at the pump. Sci- entific American Ind. 4: 40-45.

Kubicek, C.P. (2013) Systems biological approaches towards understanding cellulase production by *Trichoderma reesei*. J. Biotechnol., 163: 133–142.

Miller, G.L. (1959) Use of dinitrosalycilic acid reagent for determination of reducing sugar. Anal. Chem., **31:** 426-428

Pan, X.J., Arato, C., Gilkes, N., Gregg, D., Mabee, W., Pye, K., Xiao, Z., Zhang, X., Saddler, J. (2005) Biorefining of softwoods using ethanol organosolv pulping: pre- liminary evaluation of process streams for manufac- ture of fuel-grade ethanol and co-products. Biotech- nol. and Bioengg., 90: 473-481

Segal, L., Creely, J.J., Martin, A.E.Jr., and Conrad, C.M. (1959) An empirical method for estimating the de- gree of crystallinity of native cellulose using X-ray diffractometer. Tex. Res. J., 29: 786-794

Singhania, R.R., Patel, A.K., Sukumaran, R.K., Larroche, C., Pandey, A. (2013) Role and significance of beta-glu- cosidases in the hydrolysis of cellulose for bioethanol production. Bioresour. Technol., 127: 500–507

Sun, W.C., Cheng, C.H., Lee, W.C. (2008) Protein expres- sion and enzymatic activity of cellulases produced by Trichoderma reesei Rut C-30 on rice straw. Process Biochemistry, 43: 1083-1087

Swati, G., Halder, S. Ganguly, A., and Chatterjee, P.K. (2013) Review on *Parthenium hysterophorus* as a potential energy source. Renew. Sust. Energ. Rev., **20:** 420-429

White, T. J., Bruns, T. D., Lee, S. B. and Taylor, J. W. (1990). Amplification and direct sequencing of fungal ribo- somal RNA genes for phylogenetics, p. 315–322. *In*
M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White (ed.), PCR protocols. A guide to methods and applica- tions. Academic Press, San Diego, Calif.

Wood, T.M. and Bhat, K.M. (1988) Methods for measuring cellulose activities. Methods Enzymol., **160**, 87-112

Wyman, C.E., Dale, B.E., Elander, R.T., Holtzapple, M., La- disch, M.R., Lee, Y.Y. (2005) Comparative sugar re- covery and fermentation data from laboratory scale application of leading pretreatment technologies. Bioresour. Technol., 96: 2026-2032

Zhang, S., Xu, Y., and Hanna, M.A. (2012) Pretreatment of corn stover with twin-screw extrusion followed by enzymatic saccharification. Appl. Biochem. Biotech- nol., 166 (2): 458-469