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CLADOSPORIUM TENUISSIMUM - A NEW PRODUCER OF XYLOGLUCAN-SPECIFIC ENDOGLUCANASE

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ABSTRACT

Lignocellulosic material generated as agro-industrial wastes can be used as cheap and easily available substrates for the production of many industrial enzymes. A strain of *Cladosporium tenuissimum,* capable of producing xyloglucanase using tamarind kernel powder as the sole carbon source, was isolated from a soil sample collected near Bangalore. Viscometric analysis showed that it is an endoglucanase with very low activity towards carboxymethylcellulose, suggesting that it might be a xyloglucan-specific endoglucanase. The enzyme was extracellular and produced preferentially at alkaline pH, thus enhancing its viability for commercial applications. Apart from xyloglucanase activity, the culture filtrate showed FPase, β-glucosidase and β-galactosidase activities. As per our knowledge, this is the first report on *C. tenuissimum* as a producer of xyloglucanase.

KEY WORDS: **Xyloglucanase; *Cladosporium tenuissimum*; Tamarind xyloglucan; β-glucosidase**

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1. INTRODUCTION

Enzyme based technologies for bioconversion are considered to be most efficient and environment friendly[1]. Agro-industrial residues, containing lignocellulosic material, are attracting wide attention as raw material for the production of many industrial enzymes, as they have the potential to be used as carbon sources for microbial growth and enzyme production[2]. This approach reduces the cost of enzyme production, as the major limiting factor presently is the cost of growth substrates. Many seed endosperms contain only hemicelluloses including xyloglucan which is also the predominant hemicellulose found in the primary cell wall of dicots and several monocots. Its structure is similar to cellulose as it has a backbone of β-1,4-linked d-Glc*p* residues (β-D-glucopyranose). Glucose residues are covalently linked to xylose at the O-6 position, to which other saccharides such as galactose and fucose, may be attached. While the extent of xylose substitution varies considerably, the glucose backbone structure is typically substituted by 50 or 75% xylose[3]. Microbial degradation of lignocelluloses requires the participation of many carbohydrases, which act synergistically and co-dependently. Major β-1,4-glucan degrading enzymes include cellobiohydrolase, endo-1,4-β-endoglucanase and β-glucosidase. Based on their sequence and 3-dimensional structure, endoglucanases are grouped, along with other enzymes, into 11 glycoside hydrolase families[4]. Xyloglucan-degrading enzymes, which belong to this class, have attracted wide attention recently. Only few, but not all, cellulases (endo-1,4-β-glucanases) are able to hydrolyze the xyloglucan backbone at unsubstituted d-Glc*p*. Xyloglucan specific β-(1-4)-glucanases (XG), (endo as well as exo) have been identified in families GH5, 12, 16, 44, and 74[3]. Tamarind and tamarind kernel powder production in India is about 2,50,000 and 20,000 tons per annum respectively[5]. Tamarind seeds are rich (65-72%) in the polysaccharide galactoxyloglucan. These xyloglucans can serve as cheap substrates for production of lignocellulolytic enzymes. Of the many organisms that are employed for production of enzymes, fungi are presently the most exploited class for industrial applications as they offer advantages of a secreted enzyme complex, relative easiness and economy of producing the enzyme[1]. The focus of the present study was to use tamarind kernel powder (TKP) as the sole carbon source for screening of XG producing fungal strains and partial characterization of the enzyme so produced.

2. MATERIALS AND METHODS

2.1 Chemicals and Substrate

All chemicals and media components were of analytical grade. TKP, which was used as the carbon source for isolation and production of XG, was prepared as per the procedure described by Rao and Srivastava[6]. This was then pretreated by suspending 1.0 g powder in 100 ml of distilled water, boiled for 10 min and centrifuged at 10,000×g for 10 min. The supernatant was used as carbon source. Total carbohydrate content in the supernatant was determined by phenol-sulphuric acid method[7] and found to be between 50-60%. Tamarind xyloglucan for assays was procured from Megazyme, Ireland.

2.2 Isolation & Screening of Fungi for XG Production

Czapek Dox (CD) agar[8] (pH 4.5±0.2), where dextrose was replaced by 0.2% TKP as the sole carbon source and amended with yeast extract (0.02%) & streptomycin (200 µg/ml), was used for isolation. Thirteen soil samples were collected from different locations in Bangalore. All samples were serially diluted, plated using 10^{-3} dilution by spread plate technique and incubated at 28±2 °C for 72 hrs. The isolated colonies were individually sub-cultured onto the same medium and screened for XG production. After three days of growth, they were flooded with 0.03% (w/v) Congo red solution (in distilled water) for 30-45 mins, destained with 1 M NaCl and observed for the zone of clearance against red color of agar[9]. Positive producers were compared by calculating the enzymatic index, EI (ratio of the radius of the zone of clearance formed on the

solid medium to that of the radius of colony). All measurements were taken in triplicates. Positive XG producers were identified upto genus level based on macroscopic and microscopic appearance when stained with lactophenol cotton blue and by using standard reference manuals[10,11]. The isolate showing maximum EI was used for all further studies and sent for species identification to Agharkar Research Institute, Pune.

2.3 Fermentation Studies

Submerged fermentation was carried out in 250 ml Erlenmeyer flasks using 50 ml medium containing 1.0% TKP as substrate. The medium was inoculated with $1x10^5$ spores/ml and incubated at 28±0.2°C, 150 rpm. After required fermentation time, the medium was centrifuged at 10,000 rpm for 10 min. and the resultant culture filtrate (CF) was used as the source of enzyme for all assays. Two different media were tested: CD broth and Mandels and Weber[8] (M and W) broth, pH 4.5±0.2, amended with 1% TKP as the sole carbon source. Fermentation was carried out as described earlier. The medium that showed better XG activity was chosen for further studies. Effect of medium pH, on the production of XG, was determined by adjusting the pH of the chosen medium to 4.5±0.2 and 8.5±0.2. To identify optimum fermentation time, aliquots of the CF, from both acidic and alkaline medium, were removed at intervals of 24 hrs for seven days and assayed.

2.4 Enzyme Profile of Culture Filtrate

Tamarind xyloglucan (TXg) (p-Xyloglucan, Amyloid) was used as the substrate for all XG assays. XG activity was assayed by quantifying the reducing sugars using dinitrosalicylic acid method[12]. The assay mixture consisted of 0.4 ml CF, 0.5 ml of 1% TXg (in distilled water) and 0.1 ml acetate buffer (0.2M, pH 4.5). FPase, CMCase, xylanase, chitinase, laminarinase, amylase and pectinase activities were assayed by quantifying the reducing sugars liberated from filter paper, 1% (w/v) CMC, oat spelt xylan, colloidal chitin, laminarin, starch and apple pectin respectively using dinitrosalicylic acid method as per the standard protocol described by the Commission on Biotechnology, IUPAC. One unit of enzyme activity was expressed as micromoles of reducing sugars liberated per milliliter of CF per minute under assay conditions. Glucose was used as the standard. β-glucosidase and β-galactosidase activities were assayed using respective p-nitrophenyl derivatives, prepared in 0.2M acetate buffer, pH 4.5 (0.75 ml of 1.2 mM substrate + 0.25 ml of CF at RT)[13]. One unit of enzyme activity was expressed as micromoles of p-nitrophenol liberated per milliliter of CF per minute under assay conditions. Protein was estimated by the method of Bradford[14].

2.5 Mode of action of enzyme

Viscometric assays were carried out by using a reaction mixture containing 4.0 ml of 0.3% TXg (in 0.2M acetate buffer; pH 4.5) and 0.8 ml of CF. Flow time of reaction mixture was determined using a Schott-Gerate AVS 310 automated viscometer thermostatted at 37°C. After various incubation times, the flow time was determined and specific viscosity was calculated from the observed flow time of the reaction mixture with the enzyme and corrected to that of the buffer alone using the formula $(T_0-T)/T_0$ where T_0 & T are the flow time measured for buffer and reaction mixture respectively. Aliquots of the same reaction mixture were assayed for reducing sugars.

2.6 Enzyme Localization

Enzyme localization studies were performed in order to identify whether the enzyme was membrane bound or intracellular. The entire mycelia obtained after filtration of the fermentation broth, was homogenized with chilled acetate buffer (0.05M, pH 4.5) and centrifuged at 8000 rpm for 10 min. The pellet was resuspended in buffer and the activity was determined with the supernatant and the resuspended pellet fractions.

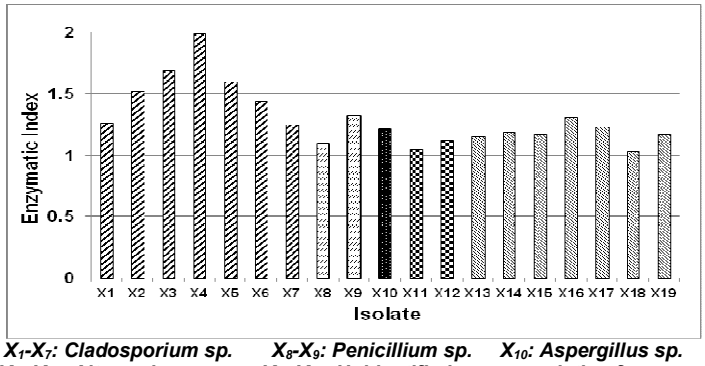
3. RESULTS AND DISCUSSION

3.1 Isolation & Screening of Fungi for XG Production

From the 13 soil samples, a total of 105 fungal colonies were isolated. Only 19 isolates were able to produce zone of clearance and were considered positive producers. They were identified to belong to the genus *Cladosporium,*

Penicillium, Aspergillus and *Alternaria*. Some non-sporulating sterile forms were also isolated. The EI of these isolates ranged from 1.03 to 1.99 (Fig. 1), with isolate X4 showing the maximum (1.99).

Figure 1
Comparison of enzymatic index of positive isolates

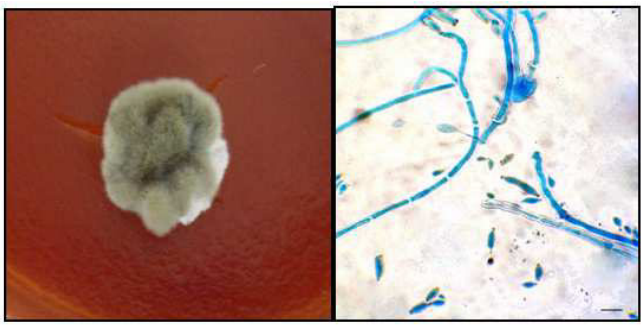


X_1-X_7: Cladosporium sp. X_8-X_9: Penicillium sp. X_{10}: Aspergillus sp.
X_{11}-X_{12}: Alternaria sp. X_{12}-X_{19}: Unidentified non-sporulating forms

3.2 Identification of Isolate X4

Isolate X4 was identified at Agharkar Research Institute, as a strain of *Cladosporium tenuissimum* Cooke *NFCCI 1841.* Colonies of X4 on CD agar were deep olive in colour and velvety, growing convexly on CZ agar (Fig. 2a). Reverse side was black with irregular boundary. Conidiophores were tall, branched, straight, carrying catenate conidia occurring in branched chains (Fig. 2b). Conidia were ellipsoidal, smooth and verruculose with prominent scars at each end. *C. tenuissimum* is a dematiaceous hyphomycete and a well known hyperparasite of several rusting fungi occurring in soil, on plant surfaces and on dead plant material[11], the latter suggesting that it could be a probable producer of cell wall degrading enzymes.

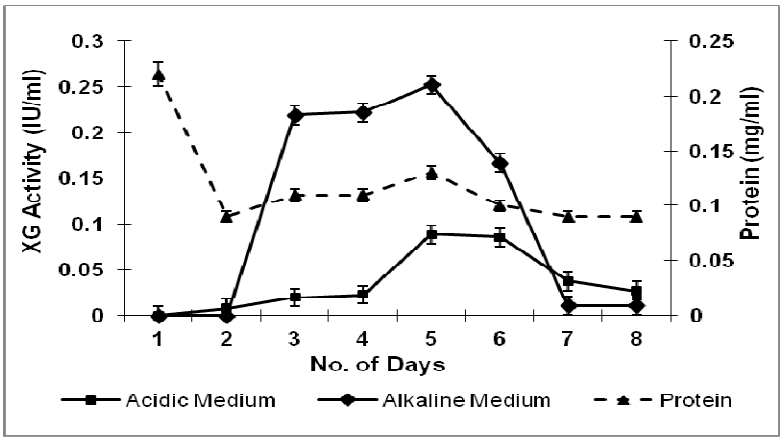
Figure 2
(a) Colony characteristics on Czapek-Dox agar; (b) micrograph showing spores under 40X of Cladosporium tenuissimum (— = 10 µM)



3.3 Fermentation Studies

Studies were carried out to determine the effects of nutritional sources, incubation time and pH on XG production. All assays were carried out in triplicates and results represented as mean±sd. Among the two media tested, *C. tenuissimum* showed higher activity in CD (0.14±0.02) than in M and W medium (0.03±0.002). Hence, CD broth was chosen for all further experiments. Comparison of the activity in acidic and alkaline media indicated that *C. tenuissimum* had higher activity in alkaline medium (0.25±0.04) than in acidic medium (0.09±0.003) indicating that it is an alkalophilic strain. Activity was found to be maximum on the 4th day, both in acidic as well as in alkaline medium (Fig. 3).

Figure 3
Kinetics of the xyloglucanase production in acidic and alkaline CD medium



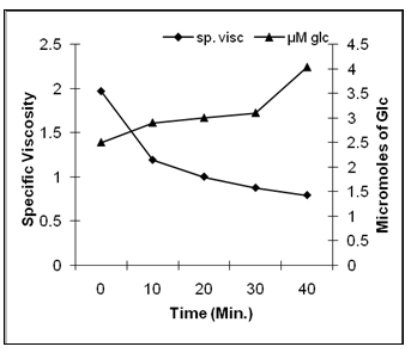
3.4 Enzyme Profile of Culture Filtrate

Carbohydrase activities in the CF were determined using the 4th day's aliquot of the alkaline media. Apart from showing consistent and appreciable XG activity, the filtrate also had Fpase-0.37±0.08, CMCase-0.07±0.01, amylase-0.04±0.003, β-glucosidase-0.04±0.001 and β-galactosidase-0.02±0.005 IU/ml activity**.** The CF possessed no activity towards other substrates and contradicts the report of Assante *et al.*[15] who found that *C. tenuissimum* was able to grow on a medium containing laminarin (a linear polysaccharide of glucose with a β(1→3):β(1→6) linkage), but not when chitin was used as the sole carbon source. The specific activity of XG produced during this study from *C. tenuissimum* (1.9 U/mg) is much higher than that reported by Yaoi & Mitsuishi[16] from *Geotrichum* (0.8 U/mg), using a similar medium with tamarind seed polysaccharide as substrate. Sipos *et al*.[17] have reported similar activities from *Trichoderma reesei* (approx. 2.0 U/mg) using different substrates like Solka Floc, steam pre-treated corn stover and lactose. Hakamada *et al.*[18] have reported comparable activity from *Aspergillus oryzae* when grown on wheat bran.

3.5 Mode of enzyme action

Polysaccharide-degrading enzymes are classified based on their mode of action on the polymeric substrate as endo and exo. To confirm the mode of action of this enzyme, viscosity reduction studies were carried out. CF, when added to the substrate, caused nearly 40% reduction in viscosity within 10 min. and approx. 67% reduction in 1 hr, with a small and gradual increase in reducing sugars when the same reaction mixture was assayed by DNS method (Fig. 4). This strongly indicates the endo – mode of action of *C. tenuissimum* XG.

Figure 4
Viscosity reduction and generation of reducing groups during tamarind xyloglucan hydrolysis by C. tenuissimum xyloglucanase



3.6 Enzyme Localization

Enzyme localization studies were carried out using the reconstituted pellet and supernatant fractions from homogenized mycelia. If there is greater activity in the pellet, the enzyme may be membrane bound and diffusible while greater activity in the supernatant indicates intracellular localization of the enzyme. *C. tenuissimum* showed total XG activity (1.85±0.08 IU/min) in the reconstituted pellet fraction and no activity in the supernatant fraction. Hence the major enzyme could be membrane bound.

CONCLUSION

In this study, we report, for the first time, the production of xyloglucanase from the fungus *Cladosporium tenuissimum*. The isolate is alkalophilic and produces XG utilizing a cheap and locally available substrate. Observed high alkalophilic production of *C. tenuissimum* XG may be preferable in industrial processes that operate at higher pH. The XG so produced has greater substrate specificity to tamarind xyloglucan than to CMC. Similar substrate specificity has been reported for XG isolated from *Penicillium canescens* and *P. verruculosum* by Sinitsyna *et al.*[19], *Aspergillus japonicus* by Grishutin *et al.*[20] and *Aspergillus aculeatus* by Pauly *et al.*[21]. Enzymes whose activity toward xyloglucan exceeds the activity toward CMC at least by 10 times or more could be classified as xyloglucan – specific endoglucanases (Grishutin *et al.*, 2004, Vlasenko *et al.*, 2010) with EC 3.1.2.151 and subgrouped under family 5 and 12 of glycoside hydrolases[22]. Though detailed studies, especially with the purified enzyme, are necessary to confirm the same, based on substrate specificity and mode of action alone, it can be assumed that the enzyme from *C. tenuissimum* may belong to the above mentioned class.

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CONFLICT OF INTEREST

Conflict of Interest: Conflict of interest declared none.

REFERENCES

1. Mathew GM, Sukumaran RK, Singhania RR and Pandey A, Progress in research on fungal cellulases for lignocellulose degradation. J Sci Ind Res, 67(11): 898-907, (2008).
2. Sukumaran RK, Singhania RR and Pandey A, Microbial cellulases-production, applications and challenges. J Sci Ind Res, 64:832-844, (2005).
3. Powlowski J, Mahajan S, Schapira M and Master ER, Substrate recognition and hydrolysis by a fungal xyloglucan-specific family 12 hydrolase. Carbohydr Res, 344:1175–1179, (2009).
4. Cantarel BL, Coutinho PM, Rancurel C, Bernard T, Lombard V and Henrissat B, The Carbohydrate-Active EnZymes database (CAZy): an expert resource for Glycogenomics. Nucleic Acids Res, 37:D233-238, (2009).
5. Menon V, Prakash G and Rao M, Enzymatic hydrolysis and ethanol production using xyloglucanase and *Debaromyces hansenii* from tamarind kernel powder: Galactoxyloglucan predominant hemicelluloses. J Biotechnol, 148:233–239, (2010).
6. Rao PS and Srivastava HC, Tamarind. In: Whistler RL (ed.) *Industrial Gums*, Academic Press: New York, (1973).
7. Sadashivam S and Manickam A, Ed. Biochemical Methods, 2nd Edn, New Age

International (P) Limited Publishers: 25-27, (1991).
8. Atlas RM, Ed. Handbook of Microbiological Media, CRC press, Boca Raton, Fla, USA: (2004).
9. Teather RM and Wood P J, Use of Congo red–polysaccharide interactions in enumeration and characterization of cellulolytic bacteria from the bovine rumen. Appl Environ Microbiol, 43(4): 777–780, (1982).
10. Raper KB and Fennel DJ, The genes of *Aspergillus.* Williams & Wilkins, Baltimore, (1965).
11. Ellis MB, More dematiaceous hypomycetes. CABI, Knew, Survey, England, (1976).
12. Miller GL, Use of dinitrosalicylic reagent for determination of reducing sugars. Anal Chem, 31:426-428, (1959).
13. Kirchner OG, Granados MS and Pascual PR, Effect of media composition and growth conditions on production of β-glucosidase b*y Aspergillus niger* C-6*.* Appl Biochem Biotechnol, 121:347-359, (2005).
14. Bradford MM, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem, 72:248–254, (1976).
15. Assante G, Maffi D, Saracchi M, Farina G, Moricca S and Ragazzi A, Histological studies on the mycoparasitism of *Cladosporium tenuissimum* on urediniospores of *Uromyces appendiculatus*. Mycol Res, 108(2):170-182, (2004).
16. Yaoi K and Mitsuishi Y, Purification, characterization, cDNA cloning, and expression of a xyloglucan endoglucanase from *Geotrichum* sp. M1281. FEBS Let, 560: 45-50, (2004).
17. Sipos B, Benko Z, Dienes D, Reczey K, Viikari L and Siiko-Aho M, Characterisation of specific activities and hydrolytic properties of cell-wall-degrading enzymes produced by *Trichoderma reesei* Rut C30 on different carbon sources. Appl Biochem Biotechnol, 161(1-8): 347-364, (2010).
18. Hakamada Y, Arata S and Ohashi S, Purification and characterization of a xyloglucan-specific glycosyl hydrolase from *Aspergillus oryzae* RIB40. J Appl Glycosci, Advanced publication, (2011).
19. Sinitsyna OA, Fedorova EA, Pravilnikov AG, Rozhkova AM, Skomarovsky AA, Matys VYu, Bubnova TM, Okunev ON, Vinetsky YuP and Sinitsyn AP, Isolation and properties of xyloglucanases of *Penicillium* sp. Biochemistry (Moscow), 75(1): 41-49, (2010).
20. Grishutin SG, Gusakov AV, Markov AV, Ustinov BB, Semenova MV and Sinitsyn AP, Specific xyloglucanases as a new class of polysaccharide-degrading enzymes. Biochim Biophys Acta, 1674:268– 281, (2004).
21. Pauly M, Andersen LN, Kauppinen S, Kofod LV, York WS, Albersheim P and Darvill A, A xyloglucan-specifi*c endo*-b-1,4-glucanase fro*m Aspergillus aculeatus:* expression cloning in yeast, purification and characterization of the recombinant enzyme. Glycobiology, 9(1):93–100, (1999).
22. Vlasenko E, Schulein M, Cherry J and Xu F, Substrate specificity of family 5, 6, 7, 9, 12, and 45 endoglucanases. Bioresour Technol, 101:2405–2411, (2010).