Madras Agric. J., 2020; doi: 10.29321/MAJ.S.000449

RESEARCH ARTICLE

Characterization of novel cellulosome complex of *Clostridium cellulovorans* TCW 3 from coffee pulp waste

Sugitha TCK*, Ramasamy K, Doraisamy P, Sivakumar U and Maheswari, M
Tamil Nadu Agricultural University, Coimbatore

ABSTRACT

In this present investigation a new bacterial strain *Clostridium cellulovorans* TCW 3 was obtained from coffee pulp waste enriched with goat rumen fluid. The enzyme production profile showed cellulase and xylanase activities when grown on coffee pulp in different incubation periods. Cellulase and xylanase activity were recorded maximum at 60°C and pH 7.5 for cellulase. Endoglucanase, exoglunase and xylanse attained stable at 10- 12 hours where as xyalanse was stable after 6 h of incubation. The apparent k_m and V_{max} values are maximum for soluble substrates like crystalline cellulose and soluble xylan (7.5 and 1.25 mg/mL, respectively). This opens a new paradigm for using coffee pulp waste as a biomass for deriving biofuels and other green chemicals like chlorogenic acid, caffeine, pectin and sugars.

Keywords: *Coffee pulp waste; Xylanase; Cellulase; Kinetic parameters*

INTRODUCTION

Coffee is the second largest traded commodity in the world, after petroleum (Nabais *et al*. 2008). Coffee is being processed either by wet or dry method. Wet processing system consumes huge volume of water (15-20 L Kg^{-1} of coffee beans) and therefore emanates more polluted effluent besides solid wastes (Vincent, 1987. The coffee processing waste water is discharged easily into the nearby streams or water bodies (Damodaran, 1998; Ijanu *et al*., 2019). The coffee processing wastes include pulp (43 %), mucilage (12%) and parchment (6.1%). The effluent is characterized by high organic matter content, suspended solids and highly acidic (Sahana *et al*., 2018). The solid wastes comprises of coffee pulp, spent coffee grounds, cherry husks etc., Presence of proteins, sugars and minerals in coffee pulp favors the rapid growth of microorganisms and if it is not utilized immediately, it causes environmental problems such as ground water pollution and contamination of water streams. The discharge of colored coffee effluent causes opacity, high chemical and biological oxygen demand, consequently leading to eutrophication, and reduced photosynthesis (Takashina *et al*., 2018; Peerez *et al*., 2007).

In terms of chemical composition, crude fibre (cellulose and hemicelluloses) tannins and pectins account for as much 21.4%, 7.5% and 6.8 % of its dry weight respectively. The mucilage contains mainly proteins, sugars and pectins. The pectins make up the gel like constitution of the mucilage by polymerizing galact-uronic acid derived from sugars (Blinova *et al*., 2017). Due to its heterogeneity and complex chemical nature, the biodegradation of coffee processing wastes requires a concerted action of several enzymes including cellulases, xylanases, pectinases, tannases etc. with different specificities to effect extensive hydrolysis to its monomeric components. The majority of glycoside hydrolases (GHs) that attack cellulose and hemicelluloses consist of catalytic modules appended to non-catalytic carbohydrate-binding modules (Davis and Henrissat, 1995). The role of these enzyme subset cellulosomes in the anaerobic conversion of biomass was previously investigated (Chakraborty *et al*., 2015). In this paper, we describe the isolation, partial purification and characterization of an enzyme complex containing both cellulase and xylanase activities from a novel strain *Clostridium cellulovorans* TCW 3.

MATERIALS AND METHODS

Microorganism and enzyme production

Clostridium cellulovorans strain TCW 3 was isolated from the coffee processing wastes *viz.*, coffee pulp waste and coffee processing waste water using enrichment culture technique. The 16S rDNA fragments of the strain were amplified and submitted to sequence in an automatic sequencer (Genei, Bangalore. India). The reference strain *Clostridium cellulolyticum* ATCC 35319 was obtained from the repository maintained at Fermentation laboratory (Tamil Nadu Agricultural University) from previous studies.

*Corresponding author's e-mail: usiva@tnau.ac.in

For cellulase, pectinase, xylanase and tannase production, 2.0 ml of spore suspensions of *C. cellulovorans* strains TCW 3 and reference strain ATCC 35319, of 7 day old culture, grown at 35 ± 2 °C (early stationary phase) in a pre-reduced liquid – state medium under anaerobic conditions containing 2% (w/v) of powdered coffee pulp waste was employed. Well grown cultures were centrifuged at 4°C at 10,000 rpm for 15 min and the supernatants served as crude enzyme extract. The harvested cells were ruptured by ultrasonicater to release the intracellular enzyme complex. The resulting cell suspensions were centrifuged at 16,000x g at 40 °C for 10 min. Both the extracellular and intracellular fragments were pooled and stored at 4°C for further enzymatic assays. For enzyme induction, aliquots were harvested every 24 h for 15 days and used to estimate the enzyme activity.

ASSAY OF ENZYMES

β 1, 4 – Endoglucanase (Cx) (E.C.3.2.1.4)

The extracellular fractions (100 µl) were incubated with one ml of 0.1% CMC and one ml of McIlvaine's buffer (pH 7.0) for 1 hour (Wood, 1968) and the reducing sugars released were estimated calorimetically (Nelson-Somogyi, 1944).

β 1, 4 – Exoglucanase (C1) (E.C.3.2.191)

One hundred µl of the samples were added to one ml of McIlvaine buffer (pH 7.0) and 2.5 ml of one per cent crystalline cellulose. The reaction mixture was incubated for an hour (Wood, 1968) and the reducing sugars released were estimated (Nelson -Somogyi, 1944).

Cellobiase (aryl β-glucosidase, 1,4 β-glucosidase) (E.C.3.2.1.2.1)

One hundred µl of the samples were incubated with one ml of 0.2% cellobiose and one ml of McIlvaine buffer (pH 7.0) for one hour (Raabo and Terkildsen, 1960) and the reducing sugars were estimated (Nelson -Somogyi, 1944).

Assay of xylanase enzymes

The extracellular fractions (100 µl) incubated with one ml of 0.8% xylan and one ml of McIlvaine's buffer (pH 7.0) for one hour (Wood, 1968) and the reducing sugars released were estimated (Nelson-Somogyi, 1944). The isolates performed best were screened and used for further studies. Protein concentration was measured by the method of Bradford (Bradford, 1976), using bovine serum albumin as standard.

Factors influencing enzyme activity

To optimize the conditions for maximum xylanase and cellulase activity, the efficient strains were grown on xylan and cellulose at 1% concentration at 35 ± 2°C .The enzyme activities were determined by incubating the culture under different pHs (5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0 and 9.0) and temperature (28°C, 37°C, 40°C, 50°C and 60°C). The effect of different sub-strates *viz*., glucose, cellobiose, carboxymethyl cellulose, crystalline cellulose, Whatman No.1 filter paper, insoluble and soluble oat spelt xylan on various enzyme activities of the strains was also studied.

For kinetic experiments, the enzyme activities at different concentrations of the substrates *viz*., 0%, 0.1% 0.5%, 1.0%, 1.5% and 2.0% was also determined under the same assay conditions as described above. Km and Vmax Values were estimated from Michaelis-Menten equation with a non-linear regression data analysis program using GraphPad Prism version 5.0 (Silva *et al.,* 1999). Appropriate controls were maintained in all cases.

RESULTS AND DISCUSSION

The partial 16S rDNA sequence of TCW 3 was compared with the 16S rDNA sequences of organisms available in NCBI. Phylogenetic analysis of the partial 16S rDNA sequence of the present strain showed that the highest similarity (93%) was obtained with the 16S rDNA of *C. cellulovorans*. Zhilina *et al*. (2005) described a new strain of *Clostridium* (Z-7026) showing 94.8, 94.9 and 95.5% of similarity with 16S rDNA sequences of *Acetivibrio cellulolyticus*, *C.aldrichii* and *C. thermocellum*, respectively. The strain was able to grow well on coffee pulp waste (NCBI Accession No. for *C. cellulovorans* TCW3: KF672708).

The growth profile of strain TCW 3 on coffee pulp waste was accompanied by a highest peak of xylanase activity 15.50 IU ml^{-1}) at cultivation interval of 5 days, while the production of xylanase activity by the reference strain reached its maximum (8.70 $IUml^{-1}$) at 7 days of cultivation.

Table 1. Kinetic parameters on the cellulase activity of TCW 3

Substrate	Exoglucanase		Endoglucanase		Cellobiase	
	K_m	V_{max}	K_m	V_{max}	K_m	V_{max}
Crystalline cellulose	24.8	2.06	23.6	1.96	26.5	4.42
CMC	16.3	1.33	14.3	1.19	18.3	3.05
Filter paper	4.2	0.35	13.8	1.01	4.55	0.76

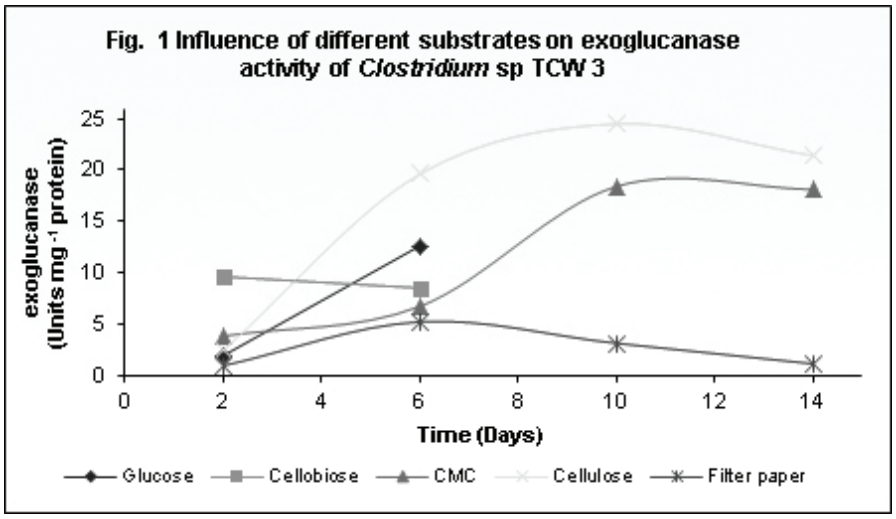
For both strains, cellulase activ- ity was expressed at 72 h of cultivation in coffee pulp waste containing medium. The strain TCW 3 recorded endoglucanase activity of 28.31 IU ml^{-1}, exoglucanase (26.51 IU ml^{-1}) and cellobiase (30.12 IU ml^{-1}). Cellulase activity remained constant after 72 h cultivation.

Table 2. Kinetic parameters on the xylanase activity of TCW 3

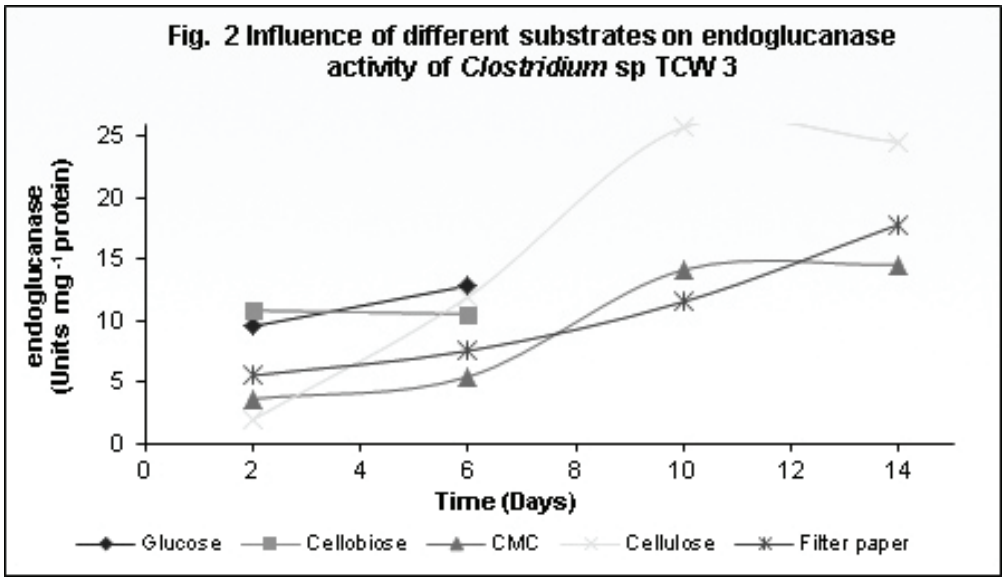
Substrate	K_m	V_{max}
Soluble xylan	7.5	1.25
Insoluble xylan	3.8	0.32

An enzyme complex containing xylanase and cellulase activities were isolated from *C. Cellulovorans,* strain TCW 3 when grown in coffee pulp waste and purified by ultra filtration techniques. The culture supernatant was concentrated by ultrafiltration with a 300 kDa cut-off point membrane (PM 300). The ultra- filtrate and concentrate were assayed for activity as a matter of course. Cellulase and xylanase activities were found in the concentrate, while pectinase and small molecular mass xylanase permeated the ultra- filtration membrane.

The partially purified enzyme complex exhibited maximal cellulase and xylanase activities at 65°C. Xylanase displayed a higher activity at pH 6.0 and maintained more than 60% of its activity at pH range of 3.5-8.5. It retained 85% of xylanase activity, after incubation at 65°C for 12 h. Xylanase from *C. acetobutylicum* was stable at 60°C for 1 h at pH 5.0-6.5 (Yang *et al*., 2013). The optimum temperature

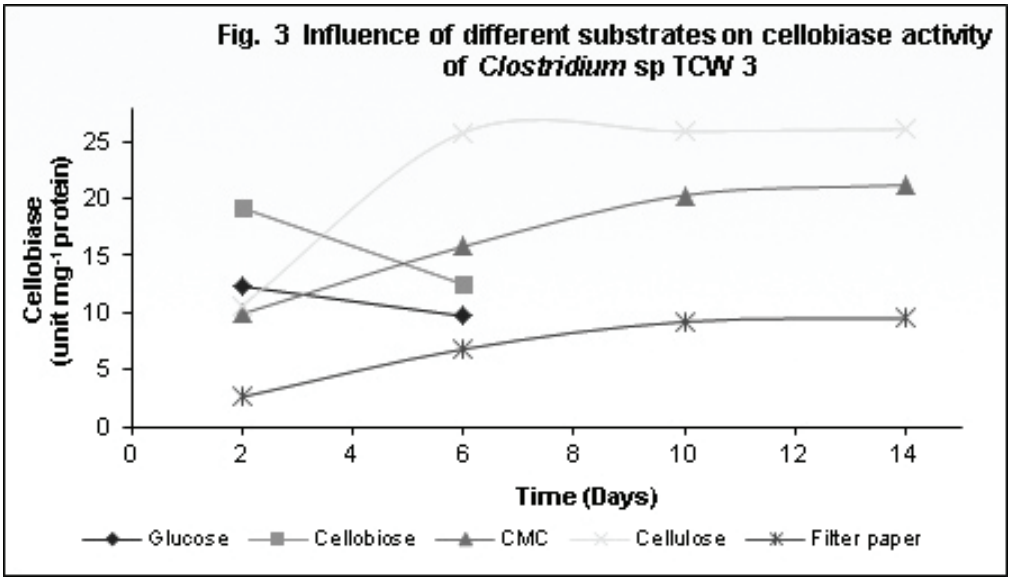


and pH of the isolated cellulosome-type enzyme of *Bacteroides* sp. strainP-1 were 50°C and 6.0, respectively (Ponpium *et al*., 2000; Zhillina et al., 2005). Maximum cellulase activity is achieved at pH 7.5 and temperature 60°C. Influence of different substrates on various cellulase enzyme showed that crystalline cellulose is the preferred source. It is clearly depicted in the Fig (1,2 and 3). The k_m and



V_{max} values for cellulase are depicted in Table 1. The V_{max} and K_m values are maximum for crystalline cellulose followed by CMC and filter paper. The cellobiase activity attained maximum at minimum time interval when compared to endoglucanase and exoglucanase

The xylanase activity from the partially purified enzyme complex of TCW 3 was most active on insoluble xylan (Table 2). Further the Vmax values on soluble and insoluble xylans from oat spelt were measured. The Km value for crystalline cellulose and soluble xylan was much higher than the insoluble



one. The reference strain showed less activity (data not shown). The cellulose and xylan breakdown is dependent on several factors, including enzyme synergism, the interaction with different subsites on the heterogeneous substrate, the interaction of the subunits within the cellulose degrading enzyme system and the probable presence of binding molecules in addition to the catalytic modules (which have different affinities for soluble and insoluble substrates).

This might suggest a steric hindrance due to the presence of side-chains groups in insoluble substrates (Silva *et al.,* 1999).

CONCLUSION

In the present investigation the *C. Cellulovorans* strain TCW 3 produced a enzyme complex with both cellulase and xylanase activity. The enzymes showed stable at temperature above 60°C. This new strain isolated from coffee pulp waste can be used for deriving bio-products or biofuel from coffee processing wastes. Further studies are necessary to understand the structural organization and other associated enzymes like mannose, pectinase and tannases.

ACKNOWLEDGEMENT

The authors acknowledge Coffee Board, Ministry of Commerce, Government of India for sponsoring the project.

REFERENCES

Blinová L., M. Sirotiak, A.Barto ová, M. Soldán.(2017). Review: Utilization of waste from coffee production. Research papers, Faculty of materials Science and Technology: In Trnava Slovak University Of Technology In Bratislava. **25:** 40.

Bradford, M.M. (1976). A rapid sensitive method for the quantitation of microgram quantities of protein uti- lizing the principle of protein dye binding. *Anal. Bio- chem.*, **72**: 248-254.

Chakraborty, S., Fernandes, V. O., Dias, F. M., Prates, J. A., Ferreira, L. M., Fontes, C. M., Goyal, A., & Centeno, M. S. (2015). Role of pectinolytic enzymes identified in *Clostridium thermocellum* cellulosome. *PloS one*, **10*(2)*,** e0116787. https://doi.org/10.1371/journal.pone.0116787

Damodaran, A. (1998). Pollution abatement strategy for coffee industry: Regulations and location specific technology options. *Indian Coffee,* **2:** 22-25.

Davis G, Henrissat B (1995) Structures and mechanisms of glycosyl hydrolases. Struct **3:** 853–859.

Ijanu, E.M., Kamaruddin, M.A. & Norashiddin, F.A. (2020). Coffee processing wastewater treatment: a critical review on current treatment technologies with a proposed alternative. *Appl Water Sci* **10:** 11 https://doi.org/10.1007/s13201-019-1091-9

Nelson N. (1944). A photometric adoption of the somogyi method for the determination of glucose. J.*Biol. Chem.,* **153***:* 375-380.

Péerez TZ, Geissler G, Hernandez F (2007) Chemical oxygen demand reduction in coffee wastewater through chemical flocculation and advanced oxidation processes. *J. Environ. Sci,* **19:** 300–305

Ponpium, P.; Ratanakhanokchai, K.; KYU, K.L. (2000). Isola- tion and properties of a cellulosome-type multi-engineered strain P-1. *Enzyme. Microb. Technol.*, **26**: 459-465.

Sahana M, Srikantha H, Mahesh S, Swamy MM (2018). Coffee processing industrial wastewater treatment using batch electrochemical coagulation with SS and Fe electrodes and its combinations, recovery and reuse of sludge. *Water Sci.Technol.,* **78:** 279–289.

Silva, C.H.C.; Puls, J.; Sousa, M.V.; FILHO, E.X.F. (1999). Purification and characterization of a low molecular weight xylanase from solidstate cultures of *Aspergillus fumigatus* Fresenius. *Braz. J. Microbiol.*, **30:** 114-119.

Takashina TA, Leifeld V, Zelinski DW, Mafra MR, Igarashi-Mafra L (2018). Application of response surface methodology for coffee effluent treatment by ozone and combined ozone/UV. Ozone Sci Eng **40(4):**293–304. https://doi.org/10.1080/01919512.2017.1417112

Vincent, J.C. (1987). Green Coffee processing. **In:** Coffee Technology. (Eds.) R.J. Clarke and R. Macrae, Elsevier Science publishers, New York: pp. 19-32.

Wood, T.M. (1968). Cellulolytic enzyme system of *Tricho- derma koningii*, separation of components attacking native cotton. *Biochem. J.,* **109***:* 217-265

Yang, X., Tu, M., Xie, R. *et al.* (2013). A comparison of three pH control methods for revealing effects of undissociated butyric acid on specific butanol production rate in batch fermentation of *Clostridium acetobutylicum* .*AMB Expr* **3:** 3 https://doi.org/10.1186/2191-0855-3-3

Zhillina, T.N.; Keybrin, V.V.; Tourova, T.P.; Lysenko, A.M.; Kostrikina, N.A.; Zayarzin, G.A. (2005). *Clostridium alkalicellum* sp. nov., an obligately alkaliphilic cellulolytic bacterium from a soda lake in the Baikal region. *Microbiol.*, **74:** 557-566.