



RESEARCH ARTICLE

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

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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, exoglucanase and xylanase attained stable at 10-12 hours whereas xylanase was stable after 6 hours 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, sugars etc.

Key words: Coffee pulp waste, Xylanase, Cellulase, Kinetic parameters

Introduction

Coffee is the second largest traded commodity in the world, after petroleum, and therefore, the coffee industry is responsible for the generation of large amount of residues (Nabais et al. 2008). Coffee pulp waste is considered as a major pollutant of rivers and lakes located near the coffee processing regions (Damodaran, 1998). Wet processing of coffee generates huge quantity of solid and liquid wastes (Vincent, 1987). The solid wastes comprises of coffee pulp, spent coffee grounds, cherry husks etc., and coffee processing waste water is the major liquid waste emanating from coffee industry. 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. 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 galacturonic acid derived from sugars. Due to its heterogeneity and complex chemical nature, the biodegradation of coffee processing wastes requires the coordinated action of several enzymes including cellulases, xylanase, pectinase, tannase etc. with different specificities to effect extensive hydrolysis to its monomeric components. 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 cellulyticum* ATCC 35319 was obtained from the repository maintained at Fermentation laboratory (Tamil Nadu Agricultural University) from previous studies. For cellulase, pectinase, xylanase and tannase production, 2.0 ml of spore suspensions of *C. cellulovorans* strains TCW 3 and reference strain, obtained from 7 day old culture, were grown at $35 \pm 2^\circ\text{C}$ for 7 days (early stationary phase) in a pre-reduced liquid state medium under anaerobic conditions containing 2% (w/v) of powdered coffee pulp waste. After the growth procedure, the cultures were centrifuged at 40C at 10,000 rpm for 15 min and the supernatants were used as crude enzyme extract. The harvested cells were ruptured by ultrasonicator 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 40C 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 calorimetrically (Nelson-Somogyi, 1944).

β 1, 4 – Exoglucanase (C1) (E.C.3.2.1.91)

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) were 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 $35 \pm 2^\circ\text{C}$. 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, 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 (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.

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 IU ml⁻¹) at 7 days of cultivation. For both strains, cellulase activity 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.

An enzyme complex containing xylanase and cellulase activities was 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 ultrafiltrate 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 ultrafiltration 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 (14). The optimum temperature and pH of the isolated cellulosome-type enzyme of *Bacteroides* sp. strain P-1 were 50°C and 6.0, respectively (Ponpium et al., 2000). 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 Damodaran, A. (1998). Pollution abatement strategy for coffee industry: Regulations and location specific technology options. **Indian Coffee**, 2: 22-25.

Nabojs, J.M., P. Nunes, P. Carrott and M.A.D. Diez. (2008). Production of Activated Carbons from Coffee Endocarp by CO₂ and Steam Activation. *Fuel Processing Technology*, 9(3):262-268

in Table 1. The V_{max} and K_m values are maximum for

Nelson-Somogyi, N. (1944). A photometric adoption of the somogyi method for the determination of glucose. crystalline cellulose followed by CMC and filter paper.

The cellobiase sactivity attained maximum at minimum time interval when compared to endoglucanase and exoglucnase. The kinetic parameters viz, *KJ.Biol. Chem.*, 153: 315-380.

Ponpium, P.; Ratanakhanokchai, K.; KYU, K.L. (2000). Isolation and properties of a and cellulosome-type multien-

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^mzyme complex of the thermophilic *Bacteroides* sp.
^{max} values on soluble and insoluble xylans from
oat spelt were measured. The xylanase activity from
the partially purified enzyme complex of TCW 3 was most active on insoluble xylan (Table 2).
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 dif-
ferent subsites on the heterogeneous substrate, the interaction of the subunits within the
cellulose de- grading enzyme system and the probable presence of binding molecules in
addition to the catalytic modules (which have different affinities for soluble and insolu- ble
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 iso- lated from coffee pulp waste can be used for deriving
bioproducts or biofuel from coffee processing wastes. The organization of the cellulosome
complex have to further studied. Further studies are necessary quanti- fy other associated
enzymes like mannose, pectinase and tannase.

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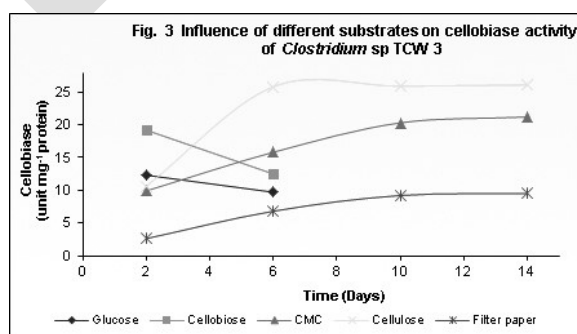
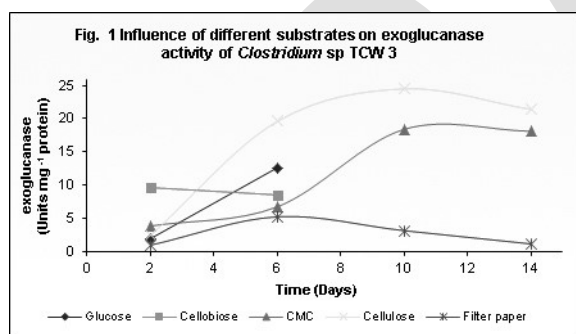
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Substrate	Exoglucanase		Endoglucanase		Cellobias _e	
	K_m	V_{max}	K_m	V_{max}	K_m	V_{max}
Crystalline cellulose	24.8	2.06	23.6	1.96	26.5	4.42
CMC	16.3	1.33	14.3	1.19	18.3	3.05
Filter paper	4.2	0.35	13.8	1.01	4.55	0.76

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



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