XII Seminário Brasileiro de Tecnologia Enzimática
ENZITEC 2016

Comparison of commercial cellulase blends to that produced by *Clostridium thermocellum* B8

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Abstract

*In the present work two different sets of enzymes produced by Clostridium thermocellum B8 were used as source of holocellulases, supernatant and residual substrate bound proteins (cellulosome enriched sample, **CES**). During growth time in the presence of Avicel Clostridium thermocellum presented highest growth rate after 48h at 60ºC and produced xylanases, pectinases and endoglucanases. Comparatively to commercial enzymatic blend, enzymatic mixtures produced by Clostridium thermocellum (supernantant and CES) showed highest activity values in basic pH whereas commercial enzymes were only active in acid pH range. Temperature effect showed that both Clostridium thermocellum's enzymatic mixtures and Viscozyme L (Novozymes) had maximal pectinase, endoglucanase and xylanases activities at 50, 60 and 70°C respectively.*
Key words: *Clostridium thermocellum*, *holocellulases*, *bioenergy*.

INTRODUCTION

One of the main barriers to obtain an economic and efficient process to second generation ethanol production is the development of enzymatic blends capable of deconstructs completely plant cell wall polysaccharides to its fermentable monomeric units. Up to now few microorganisms were successfully studied as candidates to consolidated bioprocessing, among them *Clostridium thermocellum*, an anaerobic, thermophilic bacterium has emerged as one of the most promising candidates to simultaneously hydrolyze cellulosic materials and metabolize reducing sugars into value added chemicals as ethanol, malate, acetate, and gaseous hydrogen (Soto, 2014).

Notwithstanding *C.thermocellum* natural ability to ferment cellulosic materials into ethanol, yield obtained through consolidated bioprocessing using this bacterium is far from that nowadays obtained in industrial levels. However, *C.thermocellum* is capable of organizing a enzymatic complex named cellulosome, this organization is one of the most efficient system to plant cell wall deconstruction.

Enzymes synthesized and secreted by *C. thermocellum* are less studied as candidates to industrial application and their potential is not explored yet in comparison to filamentous fungus enzymes. But, there is a crescent demand for holocellulases presenting higher thermal-stability as those secreted by *C.thermocellum*.

Aiming to assess the potential of *C.thermocellum* holocellulases, in the present study the main goal is characterize two different set of proteins secreted by *C.thermocellum* B8 in comparison to the commercial blend (Viscozyme L, Novozymes) taking under investigation their catalytic properties and thus development of new enzymatic blends.

MATERIAL AND METHODS

C. thermocellum B8 was isolated, maintained and grown under anaerobic conditions using as carbon source Avicel® 1% (*w/v*) as previously published in Hamann et al. (2015).

Supernatant (free-enzymes) was obtained after vacuum filtering the culture and centrifuging at 10000 *g* 4°C for twenty minutes, ten milliliters from the clarified blend were subjected to overnight dialyses against five liters of distilled water using a 12 kDa cut-off membrane. Cellulosomes were obtained by vacuum washing residual Avicel with 300 mL of Tris-HCl 100 mM buffer to removed unspecific bounded proteins followed by a second washing step with 50 mL of ultra-pure deionized water to elute proteins strongly bounded to cellulosic fibers. Commercial holocellulases were obtained from Novozymes (Viscozyme L), dilute in distillated water in the proportion of 1:1000 and then subject to dialyses as already described for supernatant preparation.

Hydrolytic activities against mannan, xilan and carboxymethilcellulose polysaccharides were evaluated as described in Hamann et al. (2015). Enzymatic characterization was conducted with ten times fold concentrate free enzymes and cellulosomes by ultrafiltration using 1 kDa cut-off membrane, effect of pH on enzymatic activities was assessed by conducting enzymatic assays changing the buffer system, sodium acetate 100 mM pH 3 to 5, Sodium phosphate 100 mM pH 6 and 7, and Tris-HCl 100 mM pH 7 to 9. Temperature effect on hydrolytic activities was evaluated by conducting standard enzymatic assay in sodium acetate buffer pH 5.0 50 mM in a temperature range from 30 to 80°C.

RESULTS AND DISCUSSION

C.thermocellum B8 was capable of growing on liquid media containing Avicel as sole carbon source with highest growth rate after 48h (figure 1). Up to now, few microorganism were capable of degrading cellulosic materials, agro industrial residues and Avicel in such efficiency as *C.thermocellum* , reinforcing the importance of this specie to the development of new strategies on plant polysaccharides deconstruction.

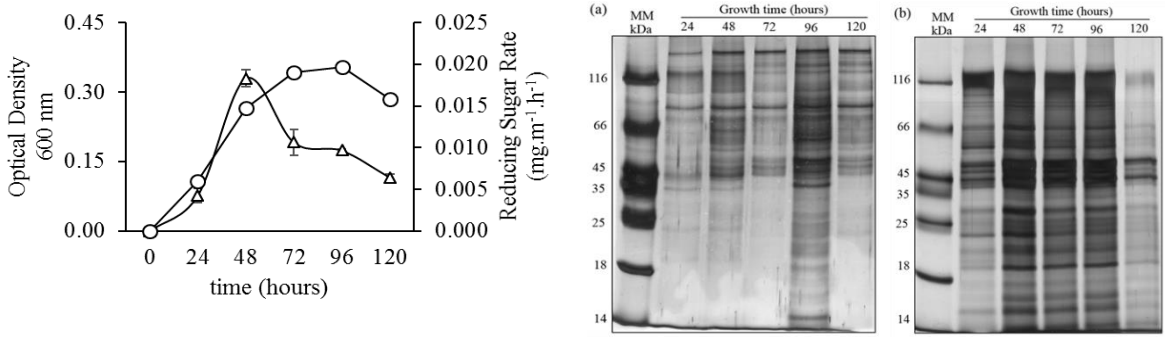

Figure (1): Evaluation of C. thermocellum B8 growth and reducing sugar production during growth in the presence of Avicel 1% (w/v) as carbon source. Vertical bars represent standard deviation from biological duplicate. Electrophoresis analyses on SDS-PAGE (10%) of (a) Proteins bounded to residual substrate (cellulosome) and (b) secreted proteins on supernatant (free enzymes) during *C.thermocellum* B8 growth time.

To accomplish lignocellulosic material degradation, *C.thermocellum* must secrete a set of enzymes to hydrolyze plant cell wall, in this study isolate B8 secreted enzymes with catalytic activity against xylan, pectin, carboxymethyl cellulose and mannan (figure 2). But contrary to expected, secretion of enzymes not related to cellulose degradation was more present than endoglucanases.

In addition to the contrasting enzyme production from cellulosome fraction and free enzymes secreted on supernatant, SDS-PAGE profile showed predominance of proteins with

masses above 45 kDa for cellulosomic fraction, whereas for supernatant fraction a more diverse population of proteins was observed (figure 1). A similar profile was obtained on xylanase activity gel, both cellulosomic and supernatant fraction showed activity bands above 45 kDa, however, for cellulosomic fraction a more varied group of catalytic bands was found (figure 3).

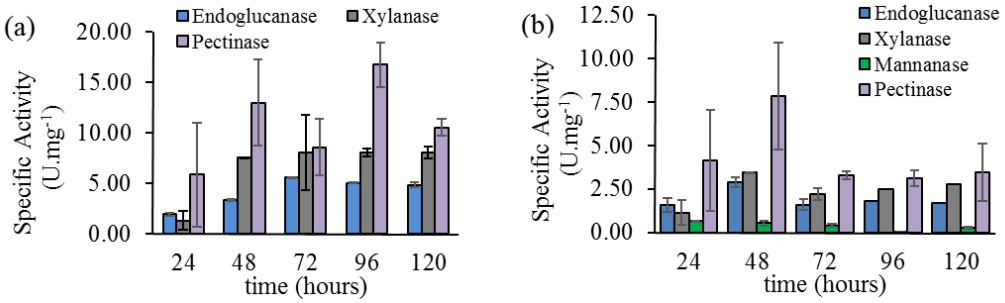

Figure (2): Time course production of pectinases, xylanases, endoglucanases and mannanases synthetized by C.thermocellum B8 during growth time. (a) enzymatic activities bound to residual substrate (cellulosomes) and (b) supernatant (free enzymes). Vertical bars represent standard deviation from biological duplicate.

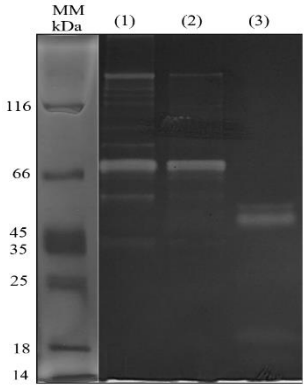

Figure (3): Xylanase activity on gel under denaturing conditions. 1) enzymes bounded to residual substrate (cellulosomes); (2) supernatant (non-celulossomal free enzymes); (3) commercial blend (Viscozyme L).

In comparison to commercial enzymes, plant cell wall degrading enzymes synthetized by *C.thermocellum* B8 regardless of their source as free enzymes or bounded to residual substrate displayed a contrasting effect of pH, and temperature.

In general for all the enzymatic activities evaluated, maximal values were reached in different pH zone varying according to the protein source. Maximal activities for commercial blend were achieved in acid pH. *C.thermocellum's* endoglucanases and xylanases were maximum in pH 6 and pectinases in pH 9, these results are in conformity to previous results (Hamann et al., 2015). Indeed, enzymes secreted by *C.thermocellum* B8 had a broader pH range, and in this context is important to emphasize that pectinase, xylanase and endoglucanases were detect in pH 8 and 9, under this condition just residual activity was observed for commercial blend.

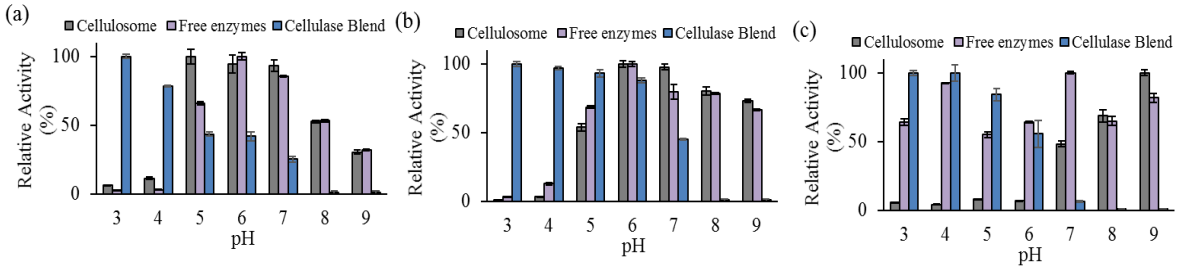

Figure (4): pH effect on different plant cell wall degrading enzymes activities. (a) Endoglucanase (CMCase); (b) Xylanase and (c) Pectinase. Vertical bars represent standard deviation from technical repetition (n=3).

Despite the opposition on pH effect observed for enzymatic activities according to the protein source, the temperature effect on hydrolytic activities is quite similar for both samples enzymes secreted by *C.thermocellum* and commercial enzymes (Viscozyme L) (figure 4).

Maximal xylanase activity was observed between 60 and 70°C for all samples. Enzymes presenting maximal activity in high temperatures are extremely sought after for industrial purposes. Regarding endoglucanase activities, maximum activity in a similar way as xylanases were reached at 60°C, in addition, at 70°C was noted a substantial decreasing on this activity for all enzymatic samples, in this temperature commercial enzymes had higher activity than cellulosomal and free-enzymes secreted by *C.thermocellum* B8. Differing from endoglucanase and xylanase activities, pectinases displayed peak activity at 50°C independently of their source (figure 5).

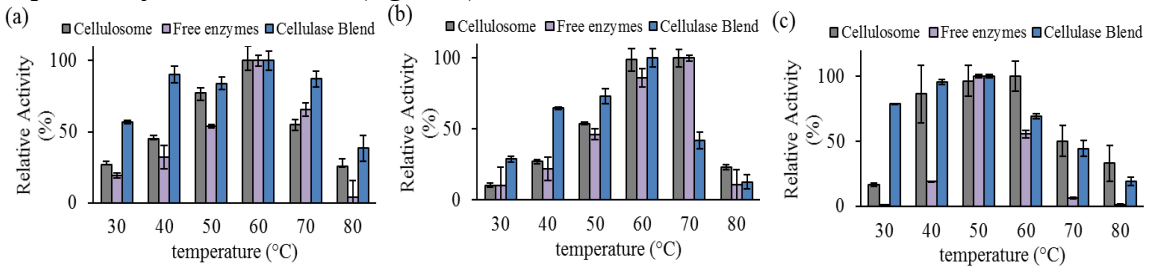

Figure (5): Temperature effect on different plant cell wall degrading enzymes activities. (a) Endoglucanase (CMCase); (b) Xylanase and (c) Pectinase. Vertical bars represent standard deviation from technical repetition (n=3).

CONCLUSIONS

In this study, enzymes secreted by *C.thermocellum* B8 during avicel degradation showed attractive physical-chemical properties for industrial applications. In comparison to a well established commercial enzymatic blend, *C.thermocellum*'s holocellulases exhibited higher activity in basic pH, however, temperature effect on enzymatic activities showed a similar profile among all tested blends.

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