

Full Length Research Paper

# Investigation of stress tolerance of endoglucanases of the cellulosomes of *Clostridium cellulolyticum* to ethanol

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Current energy and environmental challenges are driving the use of cellulosic materials for biofuel production. A major obstacle in this pursuit is poor ethanol tolerance among cellulolytic *Clostridium* species. The objective of this work was to establish a potential upper boundary of ethanol tolerance for the cellulosome itself. The hydrolytic function of crude cellulosome extracts from *Clostridium cellulolyticum* on carboxymethyl cellulose (CMC) with 0, 5, 10, 15, 20 and 25% ethanol was determined. Results indicate that the endoglucanase activity of the cellulosome incubated in 5 and 10% ethanol was significantly different from a control without ethanol addition. Furthermore the endoglucanase activity for the cellulosomes incubated in 5, 10, 15, 20 and 25% ethanol in a standalone experiment was significantly different from the control without ethanol. Endoglucanase activity continued to be observed for up to 25% ethanol, indicating that cellulosome function in ethanol will not be an impediment to future efforts towards engineering increasing production titers to levels at least as high as the current physiological limits of the most tolerant ethanologenic microbes.

**Key words:** Ethanol, *Clostridium cellulolyticum* carboxymethyl cellulose, endoglucanase activity, cellulosome.

## INTRODUCTION

The continual depletion of fossil fuel reserves, increase in the world's population, environmental catastrophes associated with crude oil drilling, price fluctuations, political unrest in major oil producing countries, concern over greenhouse gases (GHG) emissions, and the need to preserve the environment through sustainable practices require an aggressive search for alternative fuels. In spite of the development of numerous alternatives, each with technical merits, bioethanol continues to lead the

pack in adoption and commercial production. Ethanol is readily produced from a variety of agriculture-based renewable materials like sugarcane juice, molasses, potatoes, corn and barley (Panesar et al., 2006) and its potential for use as transportation fuel was conceived as early as 1890 (Esser and Karsch, 1984). The yeast *Saccharomyces cerevisiae* is in use all over the world as the major ethanol-producing microorganism, but despite its extensive use, it has a number of disadvantages

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**Abbreviations:** CMC, Carboxymethyl cellulose; GHG, greenhouse gases; PFO, pyruvate ferridoxin

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including high aeration cost, high biomass production, low temperature requirements, substrate range limitations, and finite ethanol tolerance (Saigal, 1993).

Many efforts to produce biofuel from biomass have focused on members of the genus *Clostridium*, known for their efficient degradation of lignocelluloses. *Clostridium cellulolyticum* is one such member of this group of Gram-positive anaerobic microorganisms. Its ability to utilize cellulose as a carbon and energy source is dependent upon the cell surface expression of multiple enzymes organized into a structure known as the cellulosome. Although, *C. cellulolyticum* is an efficient cellulose degrader, it has a low ethanol production yield, possibly due to pyruvate overflow arising from carbon flux surpassing the level of synthesis of pyruvate ferredoxin (PFO) and lactate dehydrogenase in its sugar utilization pathway (Senthilkumar, 2005). *C. cellulolyticum* ATCC 35319 (formerly identified as strain H<sub>10</sub> was isolated from decayed grass compost at the Université de Nancy, France. It is a Gram-positive bacillus that forms spores in cultures of cellulose media three or more days old (Petitdemange et al., 1984). Growth of *C. cellulolyticum* on cellulose occurs via several cellulases, which are re-grouped into an extracellular enzymatic complex, called the cellulosome. Lignocellulosic fermentation results in mixed products including carbon dioxide, hydrogen, acetate, ethanol, lactate and formate (Desvaux, 2005). The term cellulosome was first introduced with the thermophilic cellulolytic anaerobic bacterium *Clostridium thermocellum* (Lamed et al., 1983). The cellulosome is of particular interest since it permits a highly efficient degradation of crystalline cellulose and offers exceptional biotechnological potential (Bayer et al., 1994). Advantages of the cellulosome include (i) a direct and specific adhesion to the substrate of interest permitting efficient competition with other microorganisms and (ii) ensuring efficient cellular uptake of the soluble cellodextrins by limiting their diffusion in the extracellular milieu (Shoham et al., 1999). The final products of cellulose digestion are oligodextrans, whose subsequent fermentation results in water, CO<sub>2</sub>, and a number of mixed organic acids under anaerobic conditions (Ljungdahl and Eriksson, 1985). Since, the first step in cellulose degradation involves the action of cellulases, many researchers have focused on these enzymes (Bhat, 2000).

The cellulosome from an enzymatic viewpoint (i) allows optimum concerted activity and synergism of the cellulases, (ii) avoids non-productive adsorption of the cellulases, (iii) limits competition between cellulases for the sites of adsorption and (iv) allows optimal processivity of the cellulases all along the cellulose fiber (Schwarz, 2001). Given that *C. cellulolyticum* fermentation results in poor ethanol yield, efforts have been made to improve overall yield, including one attempt to improve productivity by introducing heterologous genes from *Zymomonas mobilis*, with mixed results (Senthilkumar, 2005). The

cellulosome of various cellulolytic bacteria have been subjects of intense studies in recent times with the genus *Clostridia* receiving the most attention partly due to the advantages listed above and due to its readily available substantial genomic information. For this reason, various attempts are geared towards the cell surface expression of the cellulosome in more efficient ethanol producers and these attempts have necessitated studies describing bioengineered *Clostridia*, stress tolerance of *Clostridia* under various environmental conditions, cellulosome activity etc. In a study by Brown et al. (2011) on ethanol adapted *C. thermocellum*, it was discovered that a mutant alcohol dehydrogenase (*adhE*) confers increased ethanol tolerance. The results show that strain of *C. thermocellum* DSM 1313 WT carrying the mutant allele showed marked improvement in growth in the presence of 20 and 24 g/L added ethanol and was also the only strain able to grow in the presence of 40 g/L added ethanol.

Xu et al. (2010) in studies of factors influencing cellulosome activity in Consolidated Bioprocessing of cellulosic ethanol concluded that formate, acetate and lactate with concentrations below 100, 200 and 50 mM, respectively, could increase cellulosome activities for cellulosome degradation. They further added that cellulosome exhibited higher ethanol tolerance and thermostability than cellulase and was tolerant up to 5 mM furfural, 50 mM *p*-hydroxybenzoic acid and 1 mM catechol and finally 491 mM ethanol was generated. In another study by Yang et al. (2012), the transcriptomic, metabolic and proteomic profiles of *C. thermocellum* ATCC27405 after ethanol stress, revealed several previously unknown information which include (i) medium supplementation with ethanol negatively influenced *C. thermocellum* growth and cellobiose consumption, with cellobiose consumption rate reduced from 0.46 g/L/h in the absence of ethanol to 0.24 g/L/h after ethanol treatment; (ii) Ethanol treated cells indicated a decline in glutamic acid, a 2.8-fold increase in phenylalanine and a doubling of sugar phosphates that were significant at 60 and 120 min post treatment; (iii) 326 genes showed high levels of expression whereas 361 genes had relatively low expression intensity; (iv) 77 proteins exhibited a  $\geq 1.5$ -fold and significant change ( $p \leq 0.05$ ) in abundance. Of the 77 proteins, 42 were more-abundant within ethanol-treated cells while 35 were down regulated following ethanol treatment; (v) Out of the 158 ethanol-responsive genes, six cellulosomal genes were defined as ethanol-responsive with only one Cthe\_3078 (*olpB*) down-regulated while the rest which were up-regulated at earlier stages, had no or few peptides detected following ethanol treatment. Several researchers have studied the surface assembly and expression of cellulosome primarily on the yeast *S. cerevisiae* and a few bacteria. In the study of the heterologous expression of a *Clostridium* minicellulosome in *S. cerevisiae*, Lilly et al. (2009) reported the establishment of the phenotypic evidence for

cohesion-dockerin interaction with a detection of a twofold increase in tethered endoglucanase activity in *S. cerevisiae* expressing the scaffoldin protein Scaf3 compared with the parent strain. In another study, Fan et al. (2012) reported the self-surface assembly of the minicellulosome of *C. cellulolyticum* on *S. cerevisiae*. The engineered *S. cerevisiae* was applied in the fermentation of carboxymethyl cellulose (CMC), phosphoric acid-swollen cellulose (PASC), or Avicel. It showed a significant hydrolytic activity towards microcrystalline cellulose, with an ethanol titer of 1412 mg/L. They concluded that the simultaneous saccharification and fermentation of crystalline cellulose to ethanol can be accomplished by the yeast, engineered with minicellulosome. Other researchers have similarly studied surface assembly varying degrees of success for example Anderson et al (2011) successfully attached a three-enzyme-containing minicellulosome on the cell surface of *Bacillus subtilis*. Other studies include the works of Tsai et al 2009 and Wen et al 2010.

Most studies on environmental stress have concentrated on cultures of *Clostridia* species with little or none addressing stress response of the cellulosome itself *in vitro*. There is lack of sufficient literature detailing ethanol stress on the cellulosome of the genus *Clostridia* and other genera that possesses the cellulosome, particularly at the time researchers have found novel ways to express the genes coding for the cellulosome proteins in microorganisms that are better suited to produce industrially useful titers of ethanol. The aim of this study was to determine the upper boundary of *C. cellulolyticum* cellulosome ethanol tolerance. The data will serve as a guide to a better understanding of the cellulosome of this and other cellulose degraders, as well as identify potential limitations to increasing ethanol yield in biofuel systems.

## MATERIALS AND METHODS

### Bacterial strain and media

*C. cellulolyticum* (ATCC 35319) was purchased from the American Type Culture Collection (Manassas, Virginia USA). The culture was first grown in *C. cellulolyticum* medium (ATCC, 1368) using glucose as the carbon source. Medium preparation involved the addition of resazurin to indicate for the presence of oxygen. The medium was boiled under pressure, with the removal of oxygen using a vacuum pump. At the point at which the resazurin changed color from blue to pink, the boiling was stopped and the medium allowed to cool down to room temperature. The medium was transferred into the Coy Anaerobic chamber (Great Lakes, Michigan USA) where 10 ml each were dispensed into sterile serum bottles. The bottles were sealed to prevent any introduction of oxygen and the medium sterilized under standard autoclave conditions. Thereafter, the *C. cellulolyticum* culture was aseptically transferred into the 10 ml medium, gassed for 1 minute using 90% hydrogen and 10% carbon dioxide gas mix. The culture was incubated for 24 h in 37°C shaking incubator at 225 revolutions per minute. After 24 h, they were routinely transferred into *C. cellulolyticum* medium (ATCC 1368) containing microcrystalline cellulose as carbon source and

incubated for 72 h under the same conditions.

### Cellulosome purification

Cultures were harvested according to the methods as described by Gal et al. (1997). Cells were cultivated anaerobically in two 500-ml flasks at 37°C and 160 revolutions per minute (rpm) in *C. cellulolyticum* medium. After 120 h of growth, cells and residual cellulose were harvested by centrifugation at 13000 g for 20 min. The pellets were washed five times in 50 ml of 25 mM Tris-HCl (pH 8.0), re-suspended in the same medium and filtered through a 3- $\mu$ m pore size glass filter (glass microfiber filter GF/D; WHATMAN; GE Healthcare Pittsburgh Pennsylvania USA) to remove cellulose fibers, washed on the filter, first with 100 ml of 25 mM Tris-HCl (pH 8.0) and second with 100 ml of 12.5 mM Tris-HCl (pH 8.0) and the cellulosome eluted with 150 ml of water on the filter. The eluted fraction was then filtered on a 0.2- $\mu$ m pore size nylon membrane filter and concentrated using Amicon Ultra centrifugal filters (30 KDa molecular weight cutoff filter, Billerica Massachusetts USA). The final 1-ml sample of filtrate (eluate) extract containing the crude cellulosome preparation was subjected to vacuum drying for 20 min to concentrate the dilute cellulosome extracts to approximately 0.5 ml.

### Protein quantification of cellulosome preparations

Total protein concentrations of crude cellulosome extracts were determined by the Bradford assay using Bovine serum albumin (BSA) as a standard. Bovine serum albumin standards at concentrations of 0, 20, 40, 80 and 100  $\mu$ g/ml were prepared in 0.1 M sodium acetate buffer pH 5.5, the standard and cellulosome extracts incubated at 37°C for 30 min, and absorbance readings were taken at 595 nm on a Synergy 2 Multi-Detection Microplate Reader (BioTek Instruments Inc., Winooski VT USA).

### Substrates

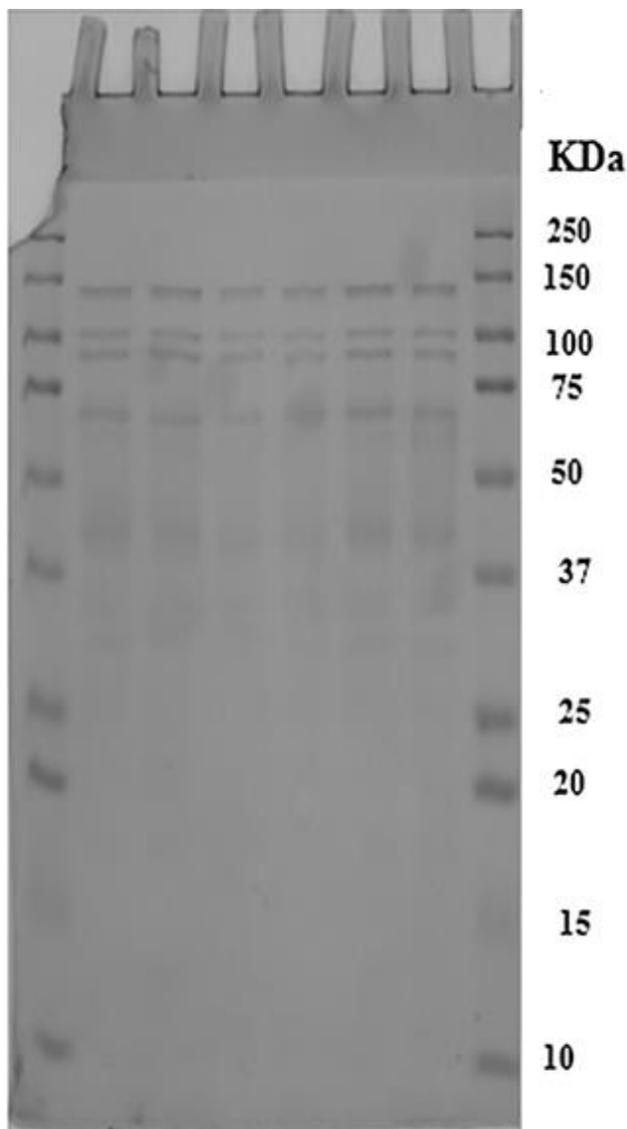
Medium-viscosity carboxymethyl cellulose (CMC) originally purchased from Nutritional Biochemicals Corp. (Cleveland, Ohio USA) was used.

### Hydrolysis with crude cellulosome preparation

Reactions were carried out according to the method of King et al., (2009) for the quantification of free reducing ends of released mono and oligosaccharides as described below. Each tube contained 90  $\mu$ L of 0.25% CMC in 0.1 M sodium acetate buffer pH 5.5. Five tubes were set up as follows: (a) 90  $\mu$ L crude cellulosome with 90  $\mu$ L of water (negative control); (b) 90  $\mu$ L CMC substrate with 90  $\mu$ L of water (negative control); (c) 90  $\mu$ L CMC substrate in 0% ethanol with 90  $\mu$ L of crude cellulosome (d) 90  $\mu$ L CMC substrate in 10% ethanol with 90  $\mu$ L of crude cellulosome (5% final ethanol concentration), and (e) 90  $\mu$ L CMC substrate in 20% ethanol with 90  $\mu$ L of crude cellulosome (10% final ethanol concentration), these were incubated at 37°C first for 24 h and then for another 24 h. These time points were chosen to allow for adequate hydrolysis of the substrate and release of the reducing sugars. This exact procedure was replicated two additional times under the same conditions. A standalone fourth sample was analyzed and it included 15, 20 and 25% final ethanol concentrations in the set to gauge the upper ethanol concentration limit of activity.

### Reducing sugar quantification

The DNS reagent was prepared according to method of Ghose



**Figure 1.** Cellulosomes Extract on SDS-PAGE. Wells 1 and 8 contain 250 KDa protein ladder. Wells 2 to 7 are replicates of the cellulosome proteins.

(1987). The DNS reagent is non-specific and reacts with both five and six carbon reducing sugars (King et al., 2009). Glucose standards curve of 0, 0.25, 0.50, 0.75, and 1.0 mg ml<sup>-1</sup> were prepared in 0.1 M sodium acetate buffer pH 5.5 with additional sets of the same standards containing 10% and 20% ethanol, respectively. The glucose standards prepared under different conditions were used to determine if the presence of ethanol would interfere with quantification of reducing sugars. 60 µL of each standard or reaction hydrolysis product was added to 120 µL DNS reagent in a 2.0 ml PCR microtube for a total volume of 180 µL. The DNS reactions were carried out in thermocyclers (Bio-Rad, Hercules California USA) by heating at 95°C for 5 min followed by cooling to 4°C for 1 minute and holding at 20°C. A 36 µL aliquot of each completed DNS reaction was added to 160 µL of deionized water in a flat bottom, 96-well microplate and mixed thoroughly using the micropipette. Absorbance was immediately determined at 540 nm.

### Statistical analyses

The data obtained from the glucose standard assay and the endoglucanase activities were analyzed using ANOVA and the t-test of means from the statistical software package SigmaPlot (San Jose California USA, [www.sigmaplot.com](http://www.sigmaplot.com)).

### SDS-PAGE

SDS-PAGE was performed by the procedure of Laemmli (1970) by preparing 10% polyacrylamide gels. The samples used for the SDS-PAGE analyses were boiled in sample buffer before use. The SDS-PAGE was run under a constant flow of cold water using the Hoefer slab apparatus (Holliston, Massachusetts USA) for approximately 3 h.

## RESULTS

### SDS-PAGE

The cellulosome extracts were run on SDS-PAGE containing 0.25% CMC to determine the likely fractions of the cellulosome retained in the extract. The largest fraction at approximately 150 KDa suggests the presence of the scaffoldin protein CipC. The individual enzymes of the cellulosome range between 40 and 90 KDa in size. Figure 1 shows the SDS-PAGE with the inclusion of 0.25% CMC.

### Glucose Standard Curve

Glucose standard curve without ethanol, with 10% ethanol, and with 20% ethanol was determined and plotted. The regression values under the three conditions were determined using the R programme to be 0.992, 0.993 and 0.992 respectively. This experiment was repeated two more times and designated as batch 1, 2 and 3 with each batch consisting of glucose standard 0%, 10% and 20% ethanol. To further determine whether the addition of ethanol had any significant effect on the measurement of Absorbance in the reducing sugar reaction for the standard curves, ANOVA was applied to each batch ( $\alpha = 0.05$ ) and the values obtained were 0.9994, 0.9962 and 0.9916, respectively. From the values obtained, it was concluded that ethanol up to 20% did not interfere with the reducing sugar quantification reaction and would therefore not affect the determination of the endoglucanase activity of the crude cellulosomes.

### Measurement of endoglucanase activity

To determine endoglucanase activity of the cellulosome from the Absorbance values obtained at 540 nm, the standard endoglucanase determination formula for carboxymethyl cellulose was calculated using the method of (Xiao et al., 2005).

**Table 1.** Endoglucanase activities measured from CMC per batch determined at 24 and 48 hours of incubation. The numbers in parentheses represent the standard deviations of triplicate values

Parameter	Endoglucanase activity (10 <sup>-6</sup> , IU/ml) after 24 h	Endoglucanase activity (10 <sup>-6</sup> , IU/ml) after 48 h
<b>Samples (Batch 1)</b>		
Cellulosome without substrate	3.000	3.000
CMC without cellulosome	3.000	3.000
CMC in 0% ethanol with cellulosome	11.22 (1.025)	10.80 (0.177)
CMC in 5% ethanol with cellulosome	8.72 (0.537)	10.76 (0.042)
CMC in 10% ethanol with cellulosome	8.28 (0.170)	8.90 (0.113)
<b>Samples (Batch 2)</b>		
CMC in 0% ethanol with cellulosome	8.19 (0.244)	9.24 (0.100)
CMC in 5% ethanol with cellulosome	8.66 (0.330)	9.83 (0.127)
CMC in 10% ethanol with cellulosome	8.17 (0.140)	9.25 (0.159)
<b>Samples (Batch 3)</b>		
CMC in 0% ethanol with cellulosome	7.55 (0.138)	8.85 (0.052)
CMC in 5% ethanol with cellulosome	7.10 (0.216)	8.46 (0.132)
CMC in 10% ethanol with cellulosome.	6.97 (0.261)	8.15 (0.360)
<b>Samples (Batch 4)</b>		
CMC in 5% ethanol with cellulosome	4.0775 (0.03)	4.210 (0.04)
CMC in 10% ethanol with cellulosome	3.960 (0.08)	4.09 (0.111)
CMC in 15% ethanol with cellulosome	3.820 (0.09)	3.905 (0.03)
CMC in 20% ethanol with cellulosome	3.7175 (0.02)	3.845 (0.02)
CMC in 25% ethanol with cellulosome	3.68 (0.04)	3.63 (0.05)

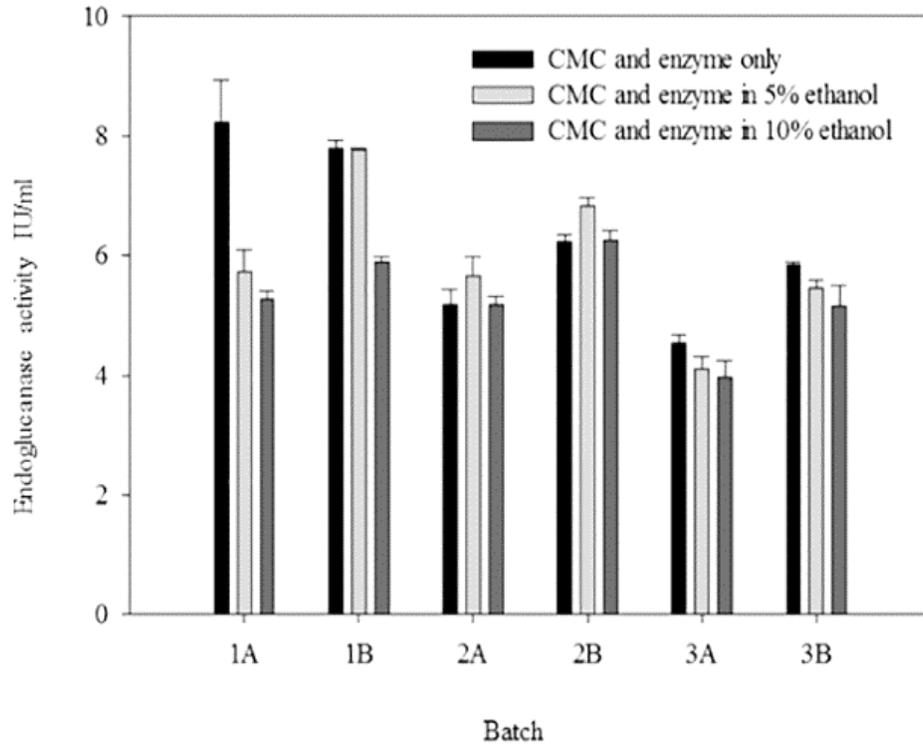
IU/ml =  $(A_{540} \text{ sample}/A_{540}/\mu\text{g standard}) (1/180 \mu\text{g}/\mu\text{mol glucose}) (1/30 \text{ min}) (1/x \text{ ml})$  Where one international unit (IU) is defined as an average of 1  $\mu\text{mol}$  of glucose equivalents released per min in the assay reaction.  $A_{540}$  sample is the absorbance obtained from the reducing sugar assay for CMC at  $\lambda = 540 \text{ nm}$ ;  $A_{540}/\mu\text{g standard}$  is the absorbance for 1  $\mu\text{g}$  of glucose as derived from the glucose standard curve. 180  $\mu\text{g}/\mu\text{mol}$  glucose is the amount of glucose in 1  $\mu\text{mol}$ ; 30 min is the assay incubation time; and x ml is the volume of the enzyme used in the assay (25), (in this case 0.09 ml). Table 1 shows the calculated values for the endoglucanase activities under the different ethanol concentrations after 24 and 48 h, the standard deviations of triplicate samples are shown in parenthesis.

Figure 2 shows the bar plot of endoglucanase activity for batch 1, 2, and 3 determined after 24 and 48 h. The time points were arbitrarily chosen. The bar plots clearly indicate an improvement in reducing sugars released after 48 h (5 and 10%, respectively) and the extracts incubated in substrates containing 10% ethanol showed slightly lower values of reducing sugars released overall when compared with those without ethanol and those with 5% ethanol concentration. The values obtained from the three independent experiments for 0, 5 and 10% conditions were normalized before plotting and subjected to statistical analyses using ONE-WAY ANOVA. There is

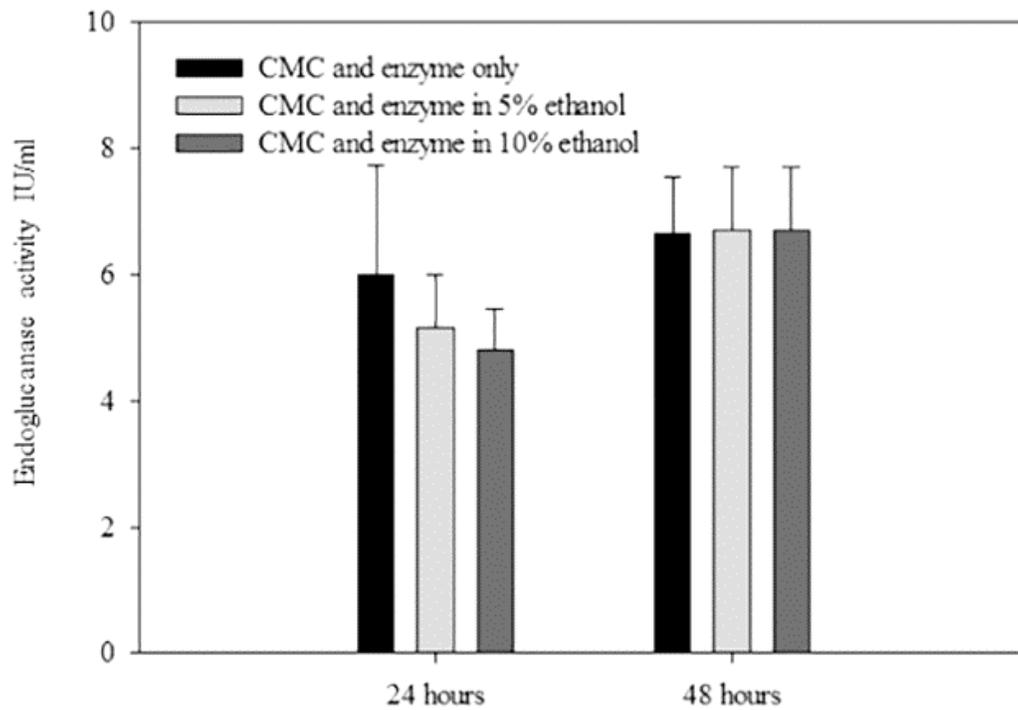
a statistical significant difference in the amount of reducing sugar released under the three conditions,  $p = 0.033$  ( $\alpha = 0.05$ ). Additionally, the t-test of means (using the Mann-Whitney Ranked Sum Test) was applied to the values to determine whether any difference existed between the 24 h samples and the 48 h sample. The difference in the median values between the two groups is greater than would be expected by chance; there is a statistically significant difference ( $P = <0.001$ ) (Figure 3). The data indicate that the reaction was highly affected by the addition of ethanol up to 10% and that endoglucanase activity could have continued beyond 48 h. Figure 4 shows the bar plot of a standalone batch 4 with 15, 20 and 25% ethanol concentrations included for 24 and 48 h incubation time. Replicates of the sample were statistically analyzed separately from other batches using the Kruskal-Wallis One Way Analysis of Variance on Ranks. The differences in the median values among the ethanol concentrations are greater than would be expected by chance; there is a statistically significant difference ( $P = <0.001$ ).

## DISCUSSION

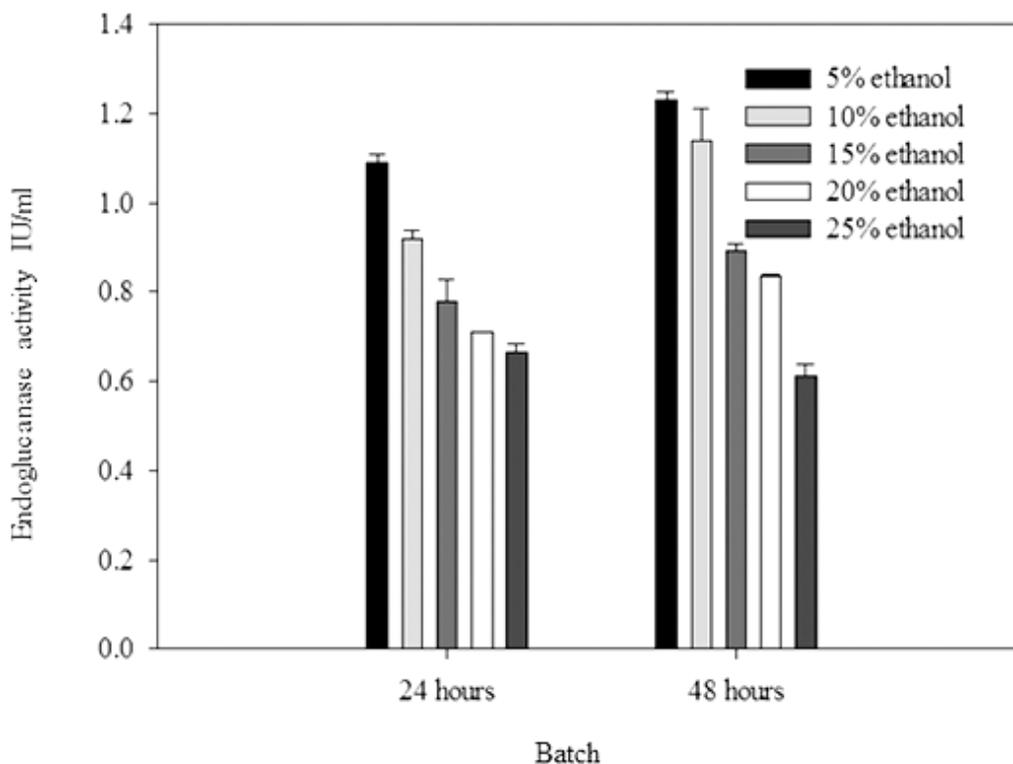
The result of the statistical analysis obtained for the batch 4 supports the result earlier seen in batch 1, 2 and 3 even



**Figure 2.** Effects of 0%, 5% and 10% ethanol on endoglucanase activities. A, 24-hour incubation; and B, 48-hour incubation. Error bars represent standard deviations among three replicates (One-way ANOVA).



**Figure 3.** Effects of 0%, 5% and 10% ethanol on endoglucanase activities after 24 and 48 hours respectively. Error bars represent standard deviations among three replicates. (Mann-Whitney T-test).



**Figure 4.** Effects of 0%, 5% 10%, 15%, 20% and 25% ethanol on endoglucanase activities on the standalone batch 4. Error bars represent standard deviations among three replicates. (Kruskal-Wallis One Way Analysis of Variance on Ranks).

though the standalone batch 4 did not produce the same level of endoglucanase activity as previously observed with the first 3 batches that were analyzed. Cellulosome activity could be highly dependent upon other extraneous factors, such as pH, temperature, presence of chemical elements in the aqueous medium etc. For batch 4, samples from 5 to 25% followed a similar pattern with the previously analyzed batch 1, 2 and 3 and a decline was observed in endoglucanase activity with increasing ethanol concentration. An attempt was made to analyze higher ethanol concentration above 30% and the results (not shown) were highly inconsistent with varying figures and therefore were deemed not reproducible and dropped. The Mann-Whitney Ranked Sum Test was performed for the 24 and 48 h reading of batch 3. The difference in the median values between the two groups is not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference ( $P = 0.228$ ). This is not consistent with the previously observed result for the first three batches and could be as a result that of low endoglucanase activities obtained. The actual values for the endoglucanase activities determined could however be a function of the concentration of the cellulosomes, the composition of the cellulosomes, the physiological state of the cells in the culture medium, the culturing

conditions used, the purification, handling and processing conditions etc. The cellulosome of *C. cellulolyticum* retained endoglucanase activity in the presence of ethanol in some cases up to 25%. Cellulosomes activity seemed improved after 48 h of incubation with CMC for 5, 10, 15, 20 and 25% samples, even though activity decreased as the ethanol concentration increased. However, at no point was activity totally eliminated. In a similar study by Skovgaard and Jorgensen (2013), a mixture of mesophilic and thermostable lignocellulolytic enzymes were exposed to a temperature of 55 to 65°C and up to 5% ethanol (w/v), the thermostable and mesophilic mixture remained active at up to 65°C. When the enzyme mixtures reached their maximum temperature limit, ethanol had a remarkable influence on enzyme activity, e.g., the more ethanol, the faster the inactivation. During hydrolysis, it has been found that ethanol is a non-competitive inhibitor binding to the allosteric site of the enzyme, which results in reversible denaturation because of the solvent properties of ethanol (Holtzapfle et al., 1990).

Furthermore, ethanol destroys the tertiary hydrophobic interactions in the enzyme, breaking or loosening the compact structure of the enzyme complex (Shao et al., 2012; Yoshikawa et al., 2012). From these preliminary results, the various bioengineering attempts to increase

ethanol production in microbes will not likely be limited by cellulosome activity but further investigation will need to be carried out to determine whether a “ceiling” exists for ethanol stress that could be tolerated by the cellulosome. Additionally, the actual activity of cellulosome *ex vivo* could be different from that observed under the *in vitro* conditions tested here. This could occur if, for example, other yet-to-be annotated genes and their protein products may contribute to the resilience of the cellulosome *ex vivo*. Cellulosome, in spite of their size and complexity, are remarkably robust complexes. It is likely that these structures can withstand various degrees of other environmental stresses in addition to those discussed here. The use of carboxymethyl cellulose as a substrate for this experiment is a test bed for further investigation. In reality, the cellulosome would degrade other forms of cellulose in its various forms, these would be utilized in further testing of cellulosome behavior under ethanol stress. Additionally, this work provides the grounds for further research into other stressors that could impact on the activities of the cellulosome, taking into consideration that they could be used in different unrelated microorganisms that could be making other products besides ethanol. Future efforts would explore tolerances to alternative biofuel products, such as biodiesel, biobutanol, etc.

### Conflict of interest

The author has not declared any conflict of interest.

### ACKNOWLEDGEMENT

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