Increased expression of β-glucosidase A in *Clostridium thermocellum* 27405 significantly increases cellulase activity

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β-glucosidase A (bglA) in *Clostridium thermocellum* 27405 was increased by expression from shuttle vector pIBglA in attempts to increase cellulase activity and ethanol titers by lowering the end product inhibition of cellulose. Through a modified electrotransformation protocol *C. thermocellum* transformant (+M) harbouroing pIBglA was produced. The β-glucosidase activity of +M was 2.3- and 1.6-fold greater than wild-type (WT) during late log and stationary phases of growth. Similarly, total cellulase activity of +M was shown to be 1.7-, 2.3- and 1.6-fold greater than WT during log, late log and stationary phases of growth. However, there was no significant correlation found between increased cellulase activity and increased ethanol titers for +M compared with the WT. *C. thermocellum* has industrial potential for consolidated bioprocessing (CBP) to make a more cost effective production of biofuel; however, the hydrolysis rate of the strain is still hindered by end product inhibition. We successfully increased total cellulase activity by increased expression of bglA and thereby increased the productivity of *C. thermocellum* during the hydrolysis stage in CBP. Our work also lends insights into the complex metabolism of *C. thermocellum* for future improvement of this strain.

**Introduction**

The production and commercialization of biofuels has gained a great deal of attention and support yet there remain some major bottlenecks with its current status; namely, there is a lack of bio-catalysts that can work efficiently at high temperatures and under extreme pH conditions. Moreover, few microorganisms produce all the required enzymes for efficient hydrolysis of hemicellulose and cellulose. Additionally, hydrolysis and fermentation of lignocellulosic biomass-derived sugars requires separate steps (i.e., fungal enzymes and *Saccharomyces cerevisiae*, respectively), which in turn is less cost-effective. However, *Clostridium thermocellum* is a great potential candidate for biofuel production due to its ability to combine cellulase production and saccharification of biomass with fermentation in a process referred to as consolidated bioprocessing (CBP). In addition, *C. thermocellum* is a Gram positive, anaerobic, thermophilic, ethanologenic and cellulosome-producing bacteria. This means that during the production of biofuels from cellulolic and hemicellulosic biomass, *C. thermocellum* could decompose the biomass using highly versatile cellulosomes in addition to free cellulases and hemicellulases, and ferment 6-carbon sugars to ethanol without the addition of oxygen. Also, the thermophilic nature, (growth optimum 60°C), would allow easier extraction of the ethanol which requires higher temperatures to volatilize and precipitate. Currently, *C. thermocellum* has yet to be widely exploited in the production of biofuels due to several limiting factors. For example, *C. thermocellum* can hydrolyze both cellulose and hemicellulose; however, it can only ferment 6-carbon sugars and thus 5-carbon sugars are not being utilized. Additionally, end products of fermentation such as lactic and acetic acids, as well as ethanol can be toxic to *C. thermocellum*. Also, stresses such as toxicity and oxygen exposure can cause sporulation because it is a spore-forming bacterium. Further, cellulase activity in *Clostridium cellulolyticum* is shown to be inhibited by end products such as cellulose. Inhibition of cellulase activity ultimately represents one of the greatest limitations to using *C. thermocellum* for biofuels. Without maximum hydrolysis of cellulosic biomass we cannot begin to consider optimum ethanol production and we cannot begin to change the economic viability of biofuels. Researchers have suggested and shown that by adding exogenous β-glucosidase isolated from *Aspergillus niger* one can increase the total cellulase activities of the cellulosome from *C. thermocellum* by 10-fold, in vitro. However, the purification and then addition of exogenous β-glucosidase would not be cost-effective for large-scale biofuel production. The genetic modification of the thermophilic anaerobe *C. thermocellum*, has been limited due to the strict restriction endonuclease system, which is described as a Dam+ phenotype. Also, many Gram positive bacteria with their thick cell walls have been reported as difficult to electrotransform. In this study, we have increased the copy number of β-glucosidase A gene (bglA) in *C. thermocellum* 27405 by electrotransforming it with a newly constructed shuttle vector.
Results

Construction of plasmid pIBglA. Plasmid pIBglA was constructed and transformed into Dam+ E. coli JM109. Due to the enzymatic limitations observed during experiments using SacI, which according to the distributor notes has several inhibitors causing low cutting efficiency, transformation of ligation products resulted in 1 positive bglA-containing transformant from 68 ampicillin positive transformants. Plasmid extraction of pIBglA followed by 1% agarose gel electrophoresis confirmed an approximate size of 7 kb. Sequencing revealed the complete cloned sequence of bglA of approximately 1,800 bp.

Verification of C. thermocellum electrotransformation with pIBglA. The transformation of C. thermocellum with pIBglA was completed and selection was based on a combined resistance to appropriate concentrations of ampicillin and lincomycin in semi-solid agar. Electrocompetent C. thermocellum cells were transformed at a rate of 5.17 ± 3 transformants ml⁻¹ of C. thermocellum media agar supplemented with appropriate concentrations of ampicillin and lincomycin. Thus, strain C. thermocellum-MC-glA (+MC-glA) was created. No spontaneous ampicillin-lincomycin resistant C. thermocellum cells were detected after 6 d incubation. PCR was performed with primers designed for the ampicillin gene using total DNA from the ampicillin-lincomycin resistant clones as a template. As shown in Figure 1, the presence of the ampicillin gene resulted in a ~530 bp product (lane 2), confirmed by pIBglA plasmid DNA as a positive control (lane 3) and negative control (WT total DNA) (lane 4).

β-glucosidase activity of wild-type C. thermocellum and C. thermocellum-MC-glA. The β-glucosidase activity, was evaluated for WT- and +MC-glA- C. thermocellum and was found to be an average 1.9-fold greater in +MC-glA directly correlating with an increase in expression of β-glucosidase from plasmid pIBglA. As seen in Figure 2, the β-glucosidase activity increased during late log and stationary phases of growth for +MC-glA and were found to be 2.3- and 1.6-fold greater than WT with a statistical significance of p < 0.05 (Student’s t-test). However, biological and technical replicates revealed there was no significant difference in β-glucosidase activity during early log phases of growth for WT and +MC-glA.

Total cellulase activity of wild-type C. thermocellum and C. thermocellum-MC-glA. The total cellulase activity, the amount of glucose equivalents released (μM) from 1% Avicel, of WT- and +MC-glA- C. thermocellum was evaluated to determine if an increase in expression of bglA could increase the overall cellulase activity during batch fermentation trials. The results in Figure 3 show that total cellulase activity of the +MC-glA was observed to be 1.7-, 2.3- and 1.6-fold significantly greater than the activity of WT during log, late log and stationary phases of growth, respectively, p < 0.05 (Student’s t-test). Thus, total cellulase activity was an average 1.9-fold greater for +MC-glA compared with the WT.

Analytical ethanol analysis for wild-type and +MC-glA C. thermocellum WT- and +MC-glA- C. thermocellum were grown in 1.5% cellobiose and 1% Avicel medium. Samples were
taken when the cultures reached log and stationary phases of growth and analyzed for ethanol concentrations (Fig. 4). When +MCbgA reached stationary phase, the average ethanol concentration in the media was 2.5 g/l. This is slightly higher than what was observed for the WT C. thermocellum (1.9 g/l), but this difference was deemed not to be statistically significant. Likewise, +MCbgA produced more ethanol during log phase growth, but it was not significantly different from the WT. Ethanol production by +MCbgA was also investigated in Avicel medium, and it yielded similar results to the cellobiose trials. No significant differences were observed between the bglA copy number mutant and the WT in both the log and stationary phases. Little difference was observed between ethanol concentrations at log and stationary phases.

**Discussion**

The opportunity to use C. thermocellum for CBP in the biofining industry has exceptional potential if we can overcome some of the challenges facing its development. The majority of difficulty working with thermophilic anaerobic bacterial systems arises from the slow progress in genetic manipulation of these systems. Plasmid pIKM1 bearing a kanamycin cassette and with Gram negative and positive origins of replication was constructed by Mai et al. in 1997 and transformed into Thermocellum sp strain JW/SL-YS485 a close thermophilic anaerobic strain to C. thermocellum. Then, it was not until 2004, that Tyurin et al. developed an efficient protocol to transform C. thermocellum 27405 among two other C. thermocellum strains DSM 1313 and 4150 with plasmid pIKM1 using a uniquely designed electroporation system. More recently in 2010, Lin et al. developed a minimally invasive ultrasound-based sonoporation method for simple and rapid transformation of thermophilic Gram positive anaerobes. In doing so, they transformed Thermocellum strain X514 with pIKM2 harboring a C. thermocellum β-1,4-glucanase gene, endoglucanase activity was observed in both electroporated and sonoporated X514 samples. Now for one of the first times reported, we expressed a functional gene (β-glucosidase A) from a Dam methylated plasmid pIBglA (constructed from pIKM1) in C. thermocellum 27405 using a modified electroporation protocol. In this research article, we strived to develop a C. thermocellum strain which can have greater total cellulase activity by reducing substrate specific end product inhibition. Thus, we proposed increasing the copy number of β-glucosidase. The BglA gene was chosen because it is fully sequenced and also because it’s native to C. thermocellum. Thus, this gene is suitable for expression in its thermophilic host. Due to the current lack of confident recombinant systems for C. thermocellum and for a concern in disruption of vital genes we chose to use plasmid pIKM1 shuttle vector with low copy number to increase expression of BglA without overburdening the cell. Growth in ampicillin remained constant; however, the loss of ampicillin resistance when continuously subculture without antibiotics was observed at approximately the 5th generation. One of the greatest limitations to using any whole microorganism for hydrolysis of cellulose and hemicellulose is the end product inhibition. Cellobiose is an inhibitor of cellulase activity in C. thermocellum and a previous report showed that the exogenous addition of β-glucosidase purified from Aspergillus niger could increase cellulase activity by 10-fold in C. thermocellum. Moreover, in Trichoderma reesi transformants the heterologous expression of a β-glucosidase gene from Penicillium decumbens resulted in an average 30% increase in filter paper activity (representing total cellulase activity). Therefore one may hypothesize that if there is an increase in copy number of β-glucosidase in C. thermocellum, there would also be an increase in β-glucosidase activity.
and this would ultimately increase total cellulase activity. In this study, the increase in copy number of \( bglA \) significantly increased both \( \beta \)-glucosidase activity as well as total cellulase activity during late log and stationary phases of growth by an average of 2.0-fold and 1.9-fold, respectively, for +MC. \( C.\) thermocellum over WT. Thus it appears the increase in \( \beta \)-glucosidase activity of \( C.\) thermocellum is also proportional to the observed increase in total cellulase activity. However, it was hypothesized here that the increase in total cellulase activity would also result in a likewise increase in ethanol production during batch fermentation trials, due to an increase in glucose catabolism. Nonetheless, there was no significant difference in ethanol production between WT- and +MC. \( C.\) thermocellum suggesting that total cellulase activity is not directly proportional to fermentative metabolism of glucose under batch fermentation conditions. We suggest that this could be due to the metabolic overburden in +MC from the presence of the shuttle vector pIBglA. This could potentially decrease the resistance of +MC to toxic end products such as ethanol, lactic, and acetic acids. The toxicity of end products such as ethanol in the fermentation of glucose for ethanogenic microorganisms such as \( C.\) thermocellum has gained a lot of attention in research for biotechnological implications. Ethanol is known to inhibit glycolytic enzymes and cause damage to cell membranes, thus inhibiting cell growth. It is also proportional to the observed increase in cellulase activity. In this study, the increase in copy number of \( bglA \) from +MC was done inside a C. Anaerobic Chamber (Coy Laboratories) under 5% hydrogen, 95% nitrogen mixed atmosphere, except applying the electric potential to the cells, centrifugation of cells and genomic DNA as enzyme extraction. The other strain used in this study \( E.\) coli JM109, was grown at 37°C in Luria Bertani broth or on Lura Burri agar containing 100 \( \mu \)g/ml of ampicillin for selection of transformants when appropriate.

Cloning \( \beta \)-glucosidase A from \( C.\) thermocellum. The genomic DNA of \( C.\) thermocellum was extracted from 3 ml of 48 h broth cultures using the Fungi/Yeast Genomic DNA Isolation Kit (Norgen Biotek Corporation) according to the instructions for Gram positive bacteria provided by the supplier. Two primers, BglPFW and BglSRV (shown in Table 1), were used to amplify the complete sequence of \( bglA \) from \( C.\) thermocellum with promoter and terminator. The primers were designed to contain the restriction cut sites for PstI and SacI for BglPFW and BglSRV, respectively, in addition to nucleotides complementary to \( bglA \). The PCR reaction mixtures contained 10 ng of \( C.\) thermocellum genomic DNA, 10 pmol of both forward and reverse primers, 10x Taq buffer with 500 mmol KCL, 25 mmol/l MgCl\(_2\), 0.2 mmol deoxyribonucleotide triphosphate, and 5 U Pfu DNA polymerase per 50 \( \mu \)l reaction. The PCR program was as follows: primary denaturation 3 min at 95°C, followed by 35 amplification cycles consisting of denaturing at 95°C for 1 min, annealing for 1 min at 54°C, and extension at 72°C for 1 min, upon completion of 35 amplification cycles a final extension step was done at 72°C for 10 min. The resulting amplicon of approximately 1.5 kb was confirmed by sequencing on ABI 3730xl automatic sequencer (Eurofins MWG Operon). The complete \( bglA \) was confirmed by complementation and alignment of sequencing results using DNAMAN software. The \( bglA \) product was then digested with restriction enzymes PstI and SacI (Fermentas, Canada) by combining 5 \( \mu \)l of \( bglA\) PCR product with 0.6 \( \mu \)l of PstI and 2 \( \mu \)l of 10x Tango buffer, incubated for 3 h at 37°C. Source and construction of plasmid pHglA. Plasmid pHglA was a gift from Lee Lynd (Dartmouth College, USA). The pHglA DNA was isolated from \( E.\) coli using Ultra Clean 6 Minute Mini Plasmid Kit (Mo Bio Laboratories) following the directions provided by the supplier. No sequence information was available for plasmid pHglA therefore we designed the primers KmPFW and KmRV (Table 1). The primers were designed within the kanamycin cassette gene to sequence the flanking multiple cloning sites. Restriction maps were produced from resulting sequences using DNAMAN software and cross referenced to the sequence of \( bglA\). Two restriction endonucleases were chosen and used for digestion of pHglA: PstI and SacI (Fermentas). Restriction digest was performed by combining 0.8 \( \mu \)g/ml of pHglA DNA with 6.3 U of SacI and 2 \( \mu \)l of 10x Tango Buffer (Fermentas), this mixture was then incubated at 37°C in a water bath for 1 h.
After 1 h time, 6.3 U of PstI was added and then the mixture was incubated for an additional 2 h at 37°C. Digestion resulted in two bands, one approximately 1.5 kb and the other approximately 5 kb. The 5 kb representing the remainder of the vector minus most of the kanamycin cassette was gel extracted using the Nucleospin Extraction II kit (Clontech Laboratories), following instructions provided by the distributor. Previously cloned, digested and cleaned pBlgA were ligated to the approximate 5 kb vector using T4 DNA ligase (Fermentas). The ligation reaction mixture contained 2 μl of ligation buffer, 5 U T4 DNA ligase, 15 μl of 80 ng μl-1 pBlgA DNA and 2 μl of 100 ng μl-1 of digested pKm13 DNA, and allowed to incubate at ambient temperature for 3 h. After ligation, the resulting DNA was transformed to E. coli JM109 using 40 μl of prepared electrocompetent cells premixed with 1 μl of ligation reaction in a 0.4 cm cuvette at 55°C for 30 min incubation.15 The reaction was stopped by adding 100 μl of chilled 1M Na2CO3, followed with 10 min incubation at 4°C. The absorbance was measured on a Bio-Rad Laboratories xMark spectrophotometer at 545 nm. 

β-glucosidase activity of wild-type C. thermocellum and C. thermocellum + MChIgA. The β-glucosidase activity of wild-type (WT): C. thermocellum and the transformant containing multiple copies of pBlgA (MCChIgA-C. thermocellum) was assayed by measuring the increase of absorbance at 400 nm via the release of p-nitrophenol from p-nitrophenyl-β-D-glucopyranoside (PNPG). Briefly, WT- and MCChIgA-C. thermocellum were pre-cultured for 48 h in 6 ml of Clostridium broth with 1% (v/v) cellulose as the sole carbon source (n = 3). Then 200 μl of each culture were subsequently inoculated into triplicate to a fresh new 40 ml of the same media for batch fermentation. Samples were collected in 1.5 ml aliquots from each vial to determine total cellulase activity during exponential phase (0.25 O.D.600 nm), late exponential phase (0.5 O.D.600 nm) and stationary phase (0.7 O.D.600 nm) in triplicate for each strain during each growth phase. In triplicate, 5 μl aliquots from each strain during each growth phase were pre-mixed under anaerobic conditions with the chilled 40 μl aliquots from each strain during each growth phase and expressed in glucose equivalents (μM). Wild-type C. thermocellum (WT) and C. thermocellum + MChIgA (+MCChIgA) were cultured for 48 h in 6 ml of C. thermocellum media supplemented with 1% (w/v) cellulose as the sole carbon source (n = 3), then 200 μl of each strain was subsequently transferred in triplicate to a fresh new 40 ml of the same media for batch fermentation. 

β-glucosidase activity of wild-type C. thermocellum and C. thermocellum + MChIgA. The β-glucosidase activity of wild-type (WT): C. thermocellum and the transformant containing multiple copies of pBlgA (MCChIgA-C. thermocellum) was assayed by measuring the increase of absorbance at 400 nm via the release of p-nitrophenol from p-nitrophenyl-β-D-glucopyranoside (PNPG). Briefly, WT- and MCChIgA-C. thermocellum were pre-cultured for 48 h in 6 ml of Clostridium broth with 1% (v/v) cellulose as the sole carbon source (n = 3). Then 200 μl of each culture were subsequently inoculated into triplicate to 40 ml of the same broth. Growth was monitored over 48 h during batch fermentation and 3 ml of samples were collected for each strain 

Table 1. Primers used in this study

| DNA target           | Forward 5'-3' | Reverse 5'-3' | Product (bp) |
|----------------------|--------------|---------------|--------------|
| BglA                 | BglFPW-ACA   | KmPFW-CTG AGA AGA AGA CAC TCC A | 1,867 |
| pKm13                | KmPFW-CTG AGA AGA AGA CAC TCC A | KmPFW-TGG AGT GTC TTC TCT CCA G | N/A |
| AmpIClin             | AmpIClin+5'  | -             | 534 |

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The injection port temperature was set at 250°C, the column inlet was run splitless. Nitrogen was used as a carrier gas with a flow rate of 1.5 ml/min and the run time was 5 min/sample. The injection port temperature was set at 250°C, the column temperature was isothermally set at 75°C, and the detector temperature was 300°C. Standards were prepared the day of analysis by using anhydrous ethanol and they were also spiked with 100 μl n-butanol per 1 ml standard.

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