Bioresource Technology 376 (2023) 128849

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Target integration of an exogenous β-glucosidase enhances cellulose degradation and ethanol production in *Clostridium cellulolyticum*

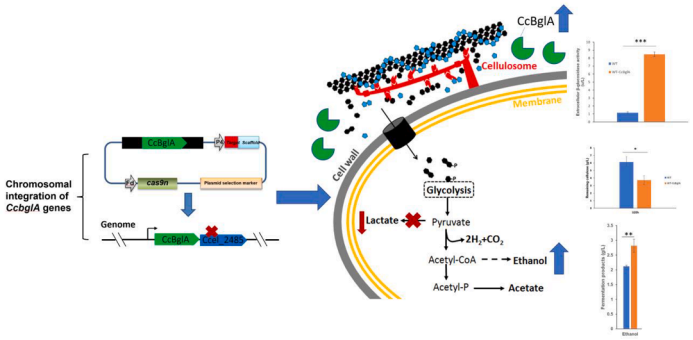
Xuanyu Tao [a,1], Josiah S. Morgan [a,1], Jiantao Liu [a], Megan L. Kempher [a], Tao Xu [a], Jizhong Zhou [a,b,*]

[a] Institute for Environmental Genomics, Department of Microbiology and Plant Biology, and School of Civil Engineering and Environmental Sciences, University of Oklahoma, Norman, OK, USA
[b] Earth and Environmental Sciences, Lawrence Berkeley National Laboratory, Berkeley, CA, USA

HIGHLIGHTS

- First study to integrate an exogenous gene into *Clostridium cellulolyticum*.
- Integration of secretory β-glucosidase increases cellulose degradation efficiency.
- Disruption of *ldh* increases ethanol production.

GRAPHICAL ABSTRACT



ARTICLE INFO

Keywords:
Clostridium cellulolyticum
CRISPR-Cas9n
β-Glucosidase
Cellulose degradation
Ethanol production

ABSTRACT

The bacteria *Clostridium cellulolyticum* is a promising candidate for consolidated bioprocessing (CBP). However, genetic engineering is necessary to improve this organism's cellulose degradation and bioconversion efficiencies to meet standard industrial requirements. In this study, CRISPR-Cas9n was used to integrate an efficient β-glucosidase into the genome of *C. cellulolyticum,* disrupting lactate dehydrogenase (*ldh*) expression and reducing lactate production. The engineered strain showed a 7.4-fold increase in β-glucosidase activity, a 70% decrease in *ldh* expression, a 12% increase in cellulose degradation, and a 32% increase in ethanol production compared to wild type. Additionally, *ldh* was identified as a potential site for heterologous expression. These results demonstrate that simultaneous β-glucosidase integration and lactate dehydrogenase disruption is an effective strategy for increasing cellulose to ethanol bioconversion rates in *C. cellulolyticum*.

* Corresponding author at: Institute for Environmental Genomics, Department of Microbiology and Plant Biology, and School of Civil Engineering and Environmental Sciences, University of Oklahoma, Norman, OK, USA.
E-mail address: jzhou@ou.edu (J. Zhou).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.biortech.2023.128849
Received 6 February 2023; Received in revised form 3 March 2023; Accepted 5 March 2023
Available online 9 March 2023
0960-8524/© 2023 Elsevier Ltd. All rights reserved.

Table 1
List of plasmids and strains used in this study.

Strain or plasmid	Phenotype or genotype	Source or reference
Strains		
Wild type of *C. cellulolyticum* H10	ATCC 35319	Xu et al., AEM, 2015
WT-CcBglA	*CcbglA* gene insertion in upstream of Ccel_2485	This study
Plasmids		
pFdCas9n	Cmp^r in *E.coli* and Tmp^r in *C. cellulolyticum H*10	Xu et al., AEM, 2015
pMS-RNA	$Spec^r$ in *E.coli*	Xu et al., AEM, 2015
pCR/8w p4-4 prom	$Spec^r$ in *E.coli*	Xu et al., AEM, 2015
pCas9n-CcBglAinser-donor	Cmp^r in *E.coli* and Tmp^r in *C. cellulolyticum H*10	This study
pER-*bglA*	Kan^r in *E.coli* and Er^r in *C. cellulolyticum H*10	This study

1. Introduction

Global warming is a well known issue that arose from the excessive release of carbon dioxide (CO_2) and other greenhouse gasses into the atmosphere as a result of the industrial revolution (Zandalinas et al., 2021). The combustion of fossil fuels, which began to be utilized during the industrial revolution, releases a large amount of CO_2 (Naik et al., 2010). Unfortunately, human civilization is still incredibly reliant on this energy source, collecting 84.32% of global energy from fossil fuels in 2020 (Looney, 2020). As a result of fossil fuel usage, 7 Gt of CO_2 is released into the atmosphere yearly. This number is projected to double by 2050, causing a 2 °C global increase relative to 1900, leading to a rise in sea level of up to 6 m (Liao et al., 2016).

In response to the urgent climate dilemma, green alternatives to fossil fuels such as bioethanol and biodiesel are being investigated (Naik et al., 2010; Saravanan, 2022; Srivastava et al., 2023). Bioethanol is a common and easily integrated fuel source that can be synthesized from lignocellulose via consolidated bioprocessing (CBP) by cellulolytic microorganisms (Lynd et al., 1991; Mattila et al., 2017; Morales et al., 2021). While CBP is a promising concept for the production of bioethanol, higher efficiency organisms are needed to meet reasonable industrial production levels (Lynd et al., 2005).

Clostridium cellulolyticum is a mesophilic clostridial species that can convert lignocellulose to ethanol, and has potential for use in consolidated bioprocessing (CBP) (Desvaux, 2005). Similar to other cellulolytic bacteria in the *Clostridiales* order, *C. cellulolyticum* creates extracellular enzymatic complexes known as cellulosomes, which efficiently degrade crystalline cellulose and produce cellobiose, the major soluble end product of cellulose hydrolysis (Schwarz, 2001; Gefen et al., 2012). However, earlier studies have found that the accumulation of cellobiose can inhibit both cell growth and cellulase production in cellulolytic clostridia bacteria, such as *Clostridium thermocellum*, further decreasing the rate of cellulose bioconversion (Gefen et al., 2012; Desvaux et al., 2000; Abdou et al., 2008). Cellobiose cleavage to glucose is commonly considered the rate limiting step of cellulose degradation (Parisutham et al., 2017). To address this challenge, previous studies have suggested that the inclusion of β-glucosidase, a cellobiose cleaving enzyme, can improve the efficiency of crystalline cellulose solubilization in *C. thermocellum*. (Gefen et al., 2012; Kadam and Demain, 1989; Lamed et al., 1991).

To increase the conversion efficiency of cellulose in *C. cellulolyticum* for future industrial application, a highly efficient β-glucosidase gene *from Clostridium cellovorans* was inserted (Jeng et al., 2011) into the genome of the *C. cellulolyticum* via a CRISPR-Cas9n editing system (Xu et al., 2015). Meanwhile; a previous study demonstrated that disrupting the lactate dehydrogenase gene in *C. cellulolyticum* can lead to a substantial increase in ethanol production (Li et al., 2012). Drawing from this, an "insertion-disruption" strategy was adopted, seeking to improve cellulose degradation and ethanol production simultaneously by integrating a β-glucosidase gene from *Clostridium cellulovorans* before the lactate dehydrogenase gene (Ccel_2485). The resulting engineered strain was systematically characterized in both physiological and transcriptional aspects. Our findings demonstrate that integrating β-glucosidase into the *C. cellulolyticum* genome at the Ccel_2485 locus can enhance cellulose degradation efficiency and ethanol production. This approach presents an effective strategy that offers new avenues for future genomic engineering of *C. cellulolyticum*, and a potential means to reduce the cost of industrial fermentation.

2. Materials and methods

2.1. Plasmid construction and bacterial strains

Table 1 lists all strains and plasmids used in this study. The plasmid pCas9n-CcBglAinser-donor was constructed via Gibson assembly as previously reported (Xu et al., 2015). In brief, the upstream and downstream homology arms for the vectors were first amplified and purified separately from WT genomic DNA of *C. cellulolyticum*. *CcBglA* was obtained via amplification from the plasmid pER-*bglA* with the primers 2485 BglAF and 2485 BglAR. The P4::gRNA cassette was obtained based on previous study (Xu et al., 2015) using the primers 3FF, 2485GFR, 2RR, and 2485GFF. The linear backbone was digested from pFdCas9n using the KpnI and PvuI restriction enzymes. Nanopore sequencing at the Plasmidsaurus confirmed the plasmid.

2.2. Transformation

The completed pCas9n-CcBglAinser-donor was electroporated into wild-type *C. cellulolyticum* as described previously (Li et al., 2012). Before the transformation, each plasmid was methylated with MspI Methyltransferase (New England Biolabs, Ipswich, MA) and

Table 2
List of primers used for plasmid construction.

Primer Name	Sequences 5′-3′
2485GRF	GTTGTAAATACTAAATGGAAGAGTGTTTTAGAGCTAGAAATAGCAAGT
2485GRR	CTAAAACACTCTTCCATTTAGTATTTACAACTTAATTTTAACTTTAAAAAATAAATTTG
2485LF	GAATTTTATTATGGTACCCGGGTACCCATATATCGGACTGCGGGC
2485LR	TTTCCATTTAGTATTTACTATAAATATACCC
2485BGLAF	TTATAGTAAATACTAAATGGAAAAATTTTAAGGAGGTGTATTTCATATG
2485BGLAR	CATAATCAATATCCTACTGCGTCGGAAAAGTTAAACTGCGA
2485RF	TCCGACGCAGTAGGATATTGATTATGAAAAATAAATCTA
2485RR	CTCATCAATTTGTTGCAACGAGAACCGATGCAGTGGTCGGAGTAG
ID2485R	GGGAATACGTGACTTATTTGGTG
2485SEQR	GTACAATCTCGGAAACTAGTCCGC
3FF	CTCGTTGCAACAAATTGATGAG
2RR	CTGCGCAACTGTTGGGAAGGGCGATCCGCGTCTAGAGCCGATCGA

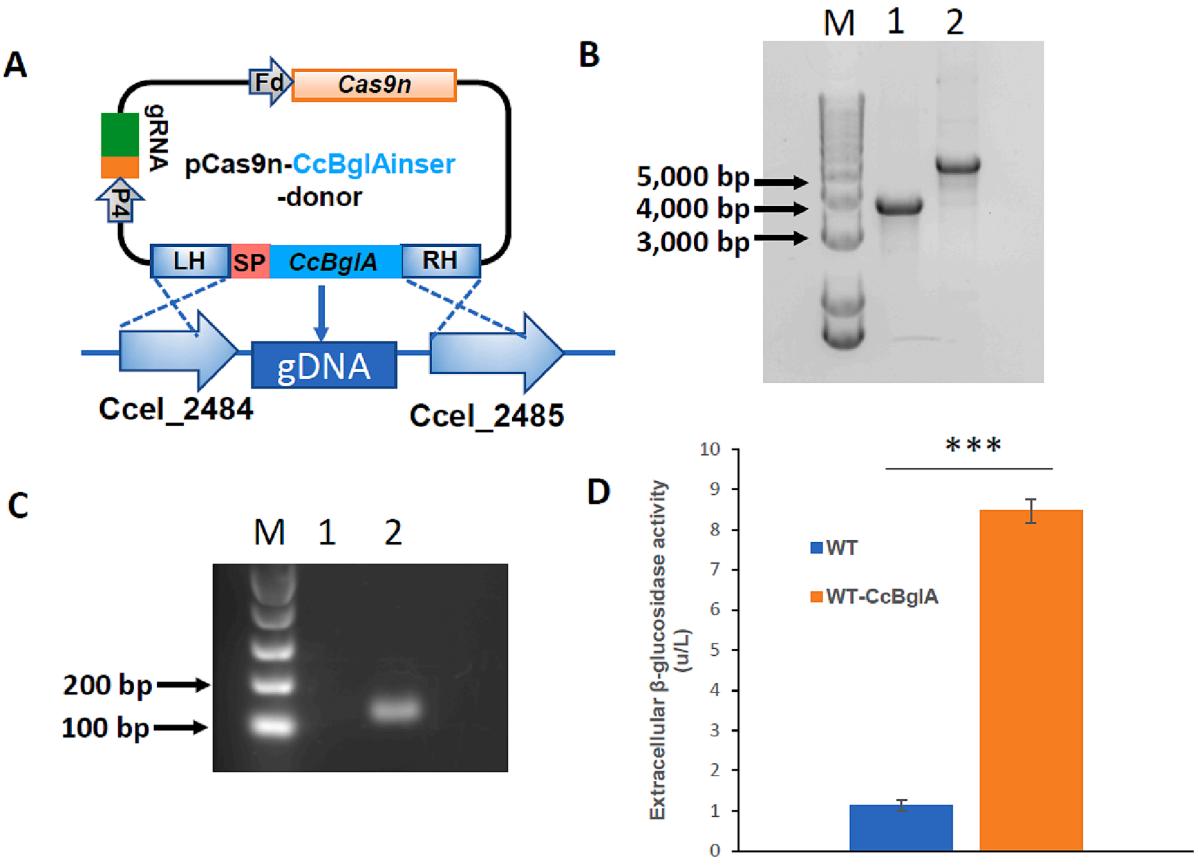


Fig. 1. Exogenous *bglA* gene integration increased the extracellular β-glucosidase activity. (**A**) An overview of the integration of the exogenous *bglA* gene in *C. cellulolyticum*. Plasmid pCas9n-CcBglAinser-donor was used for the integration of β-glucosidase gene from *C. cellulovorans* to the genome of *C. cellulolyticum*. SP, signal peptide; CcBglA, *C.cellulovorans* β-glucosidase. (**B**) PCR identification of WT-CcBglA. Lane 1, WT; lane 2, WT-CcBglA. M: 1 kb plus DNA ladder (Invitrogen). (**C**) RT-PCR identification of expression of *C. cellulovorans* β-glucosidase gene in *C. cellulolyticum*, which was incubated in the defined VM medium (20 g/L cellulose). Lane 1, WT; lane 2, WT-CcBglA. M: 1 kb plus DNA ladder (Invitrogen). (**D**) *In vitro* measuring the extracellular β-glucosidase activity in WT and WT-CcBglA. Data are presented as the mean ± SD (n = 3). Significance is indicated by ***, $p < 0.001$, determined by unpaired two-tailed *t* test.

subsequently purified. The plasmid for the transformant was cured as reported previously (Li et al., 2012).

2.3. Mutant verification and measurement of β-glucosidase activity

The genomic DNA of WT-CcBglA was obtained using the GenElute™ Bacterial Genomic DNA Kit (Sigma-Aldrich, St. Louis, MO). The primers 2485LF and ID2485R were used for the PCR verification of WT-CcBglA (Table 2). The β-glucosidase Activity Assay Kit (Sigma-Aldrich, St. Louis, MO) was used to measure β-glucosidase activity. In short, WT and WT-CcBglA were grown in defined VM media containing crystalline cellulose (20 g/L) at 34 °C. When grown to the mid-exponential phase, cultures were pelleted, and extracellular β-glucosidase activity was measured in the supernatant. P-nitrophenyl-β-D-glucopyranoside (β-NPG) was used as a substrate and variation of optical density was measured at 405 nm after 20 min incubation at 34 °C. Units/L were used to scale the assay results. One unit (U) corresponds to the quantity of enzyme needed to catalyze the hydrolysis of 1.0 μmole of substrate at pH 7 per minute.

2.4. Quantification of cell growth, remaining cellulose, and fermentation products

The *C. cellulolyticum* wild type and engineered strain were grown on complex VM media with 5 g/L cellobiose and then transferred to defined VM media with 5 g/L cellobiose. When grown to mid-exponential phase (Optical Density (600 nm) of 0.5–0.6), each strain grown was used to inoculate 50 mL of defined VM media with crystalline cellulose (20 g/L). 1 mL of cell culture was sampled at each time point during growth, and then frozen at −80 °C for later measurement of metabolites, cell biomass, and remaining cellulose. Total protein estimates were used to generate growth curves (Li et al., 2012). The major products of fermentation (ethanol, acetate, and lactate) and dissolved sugars (glucose and cellobiose) were measured using the supernatant of exhausted medium using high-performance liquid chromatography (HPLC) (Li et al., 2012). The phenol sulfuric acid method was used to measure remaining cellulose in the medium (Tao et al., 2020).

2.5. RT-PCR and microarray analysis

Four biological replicates of the C. *cellulolyticum* wild type strain and the engineered strain were grown in defined VM medium with cellulose (20 g/L). Each strain was collected during mid-log phase and the collected pellets were subsequently frozen with the liquid nitrogen. The total RNA extraction, microarray hybridization, microarray data analysis and PCA analysis were performed as described previously (Tao et al., 2020). Primers BglA-RTF and BglA-RTR were used for verification of the expression of the CcBglA gene in the engineered strain by RT-PCR.

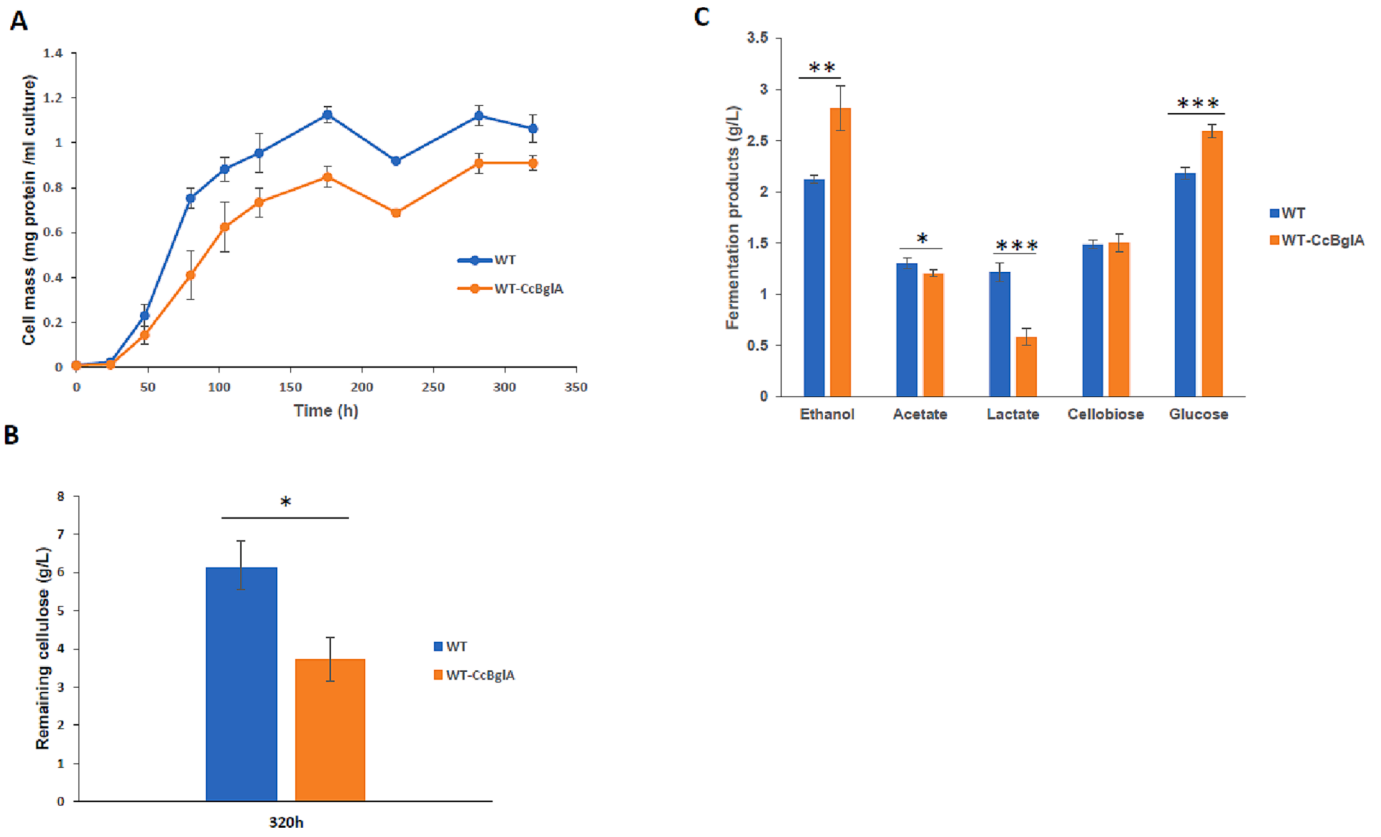


Fig. 2. Integration of the *CcBglA* gene improved the cellulose degradation and ethanol production. **(A)** Growth profiles of WT and WT-CcBglA grown at defined VM medium (20 g/L cellulose). **(B)** Remaining cellulose for WT and WT-CcBglA at the end fermentation time. **(C)** Titers of primary products and soluble sugars for WT and WT-CcBglA at the end fermentation time. Data are presented as the mean ± SD (n = 3). Significance is indicated by *, $0.010 < p \leq 0.050$, **, $0.001 < p \leq 0.010$ or ***, $p < 0.001$ determined by unpaired two-tailed *t* test.

3. Results and discussion

3.1. Targeted integration of β-glucosidase into the genome of C. cellulolyticum

Based on annotative analysis, the gene Ccel_0374 is theorized to encode for β-glucosidase in *C. cellulolyticum.* However, no previous studies have proved this, and so the efficiency of this supposed enzyme is elusive. As such, integration of a known highly efficient exogenous β-glucosidase gene into *C. cellulolyticum* is a suitable strategy for enhancing enzymatic conversion from cellobiose to glucose. We opted to insert the β-glucosidase gene, *bglA,* from *C. cellulovorans (CcbglA).* This gene is shown to have the strongest activity in comparison to other β-glucosidase genes in a previous study, and has an optimal temperature of 30–50 °C, which is compatible with the growth of *C. cellulolyticum* (Jeng et al., 2011). Since cellobiose is normally accumulated extracellularly (Desvaux, 2005), the integrated β-glucosidase will be most efficient if secreted. To increase the extracellular presence of BglA, we slightly modified the gene by adding the secretionary signal peptide from the *ce19E* gene from *C. cellulolyticum.*

In a previous study (Xu, 2015), twelve operons in *C. cellulolyticum* were shown to be significantly upregulated in response to cellulose via RNA sequencing. Additionally, these operons contain only one promoter and one gene (monocistronic), indicating other genes will likely not be affected by the alteration of these sites. As a result, we chose to insert *BglA* at three genomic sites, Ccel_1380, Ccel_1432, and Ccel_2485. The first two genes were selected because they each encode a respective hypothetical protein, and as such, disruption of the genes has a small chance of disrupting cellulose metabolism or ethanol production. The third gene chosen, Ccel_2485, encodes a L-lactate dehydrogenase (*ldh*). Inactivation of *ldh* has been shown to enhance ethanol production by cutting off the lactate pathway (Li et al., 2012). This gene is predicted to have a downstream non rho-independent terminator (Li et al., 2012). For each insertion, a comprehensive Cas9 nickase-based genome editing vector was constructed for the specific site (Fig. 1A). However, successful integration only occurred upstream of Ccel_2485, which was confirmed via PCR and Sanger sequencing (Fig. 1B). The corresponding engineered strain was named WT-CcBglA.

The reverse-transcription PCR (RT-PCR) showed that the *BglA* was successfully expressed (Fig. 1C) and the β-glucosidase activity assay found that WT-CcBglA had a 740% increase in supernatant activity compared to WT (Fig. 1D). This result suggests the expression of *CcbglA* was normal and that the secreted CcBglA can function properly outside the cell.

Inserting a foreign gene into the genome of *C. cellulolyticum* is particularly challenging due to the very low transformation efficiency of this organism (~100 transformants per μg plasmid DNA), even when using the CRISPR-Cas9n-based editing method (Xu et al., 2015; Jennert et al., 2000). Furthermore, successful expression of a foreign gene over 1.6 kb in size while utilizing a native promoter has not been previously achieved in *C. cellulolyticum*. Additionally, secretion of a foreign gene using a signal peptide has not been demonstrated in this organism. Therefore, the successful expression and secretion of the exogenous β-glucosidase gene in *C. cellulolyticum* is a significant achievement towards engineering this microorganism to meet the requirements for consolidated bioprocessing (CBP).

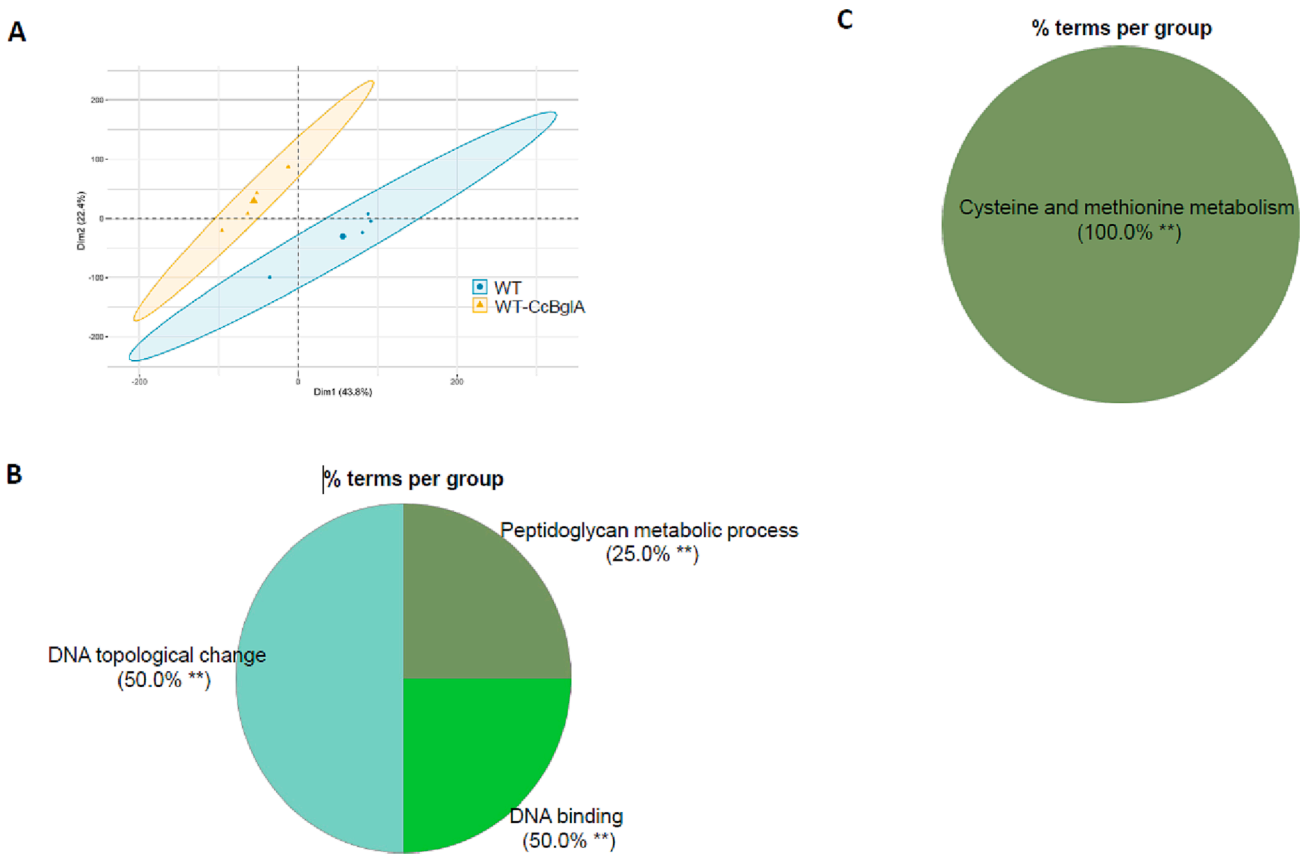


Fig. 3. Engineered strain presented different gene expression profiles compared to wild type strain. **(A)** PCA analysis of differentially expressed genes (DEGs) between wild type and engineered strain grown on cellulose. **(B)** Enrichment map of gene ontology (GO) terms in upregulated DEGs between wild type and engineered strain grown on cellulose. Only GO terms with Bonferroni-Hochberg corrected $p < 0.05$ are displayed. **(C)** Enrichment map of GO terms in down-regulated DEGs between wild type and engineered strain grown on cellulose. The statistical significance is indicated by **, P < 0.001, adjusted by Benjamini-Hochberg method.

3.2. Conversion of cellulose to end products was improved in the engineered strain WT-CcBglA

To compare the cellulose degrading activity of WT and WT-CcBglA, we inoculated both strains separately into the defined VM medium containing cellulose (20 g/L). Generally, WT-CcBglA performed better than WT in the degradation of cellulose and production of ethanol. However, the engineered strain had a longer doubling time (WT-CcBglA: 21 h, WT: 18 h), and less maximum cell biomass (Fig. 2A). Ultimately, WT-CcBglA could degrade 12% more cellulose than WT at the final time point (Fig. 2B), indicating a higher cellulose degradation efficiency. Regarding the end products of cellulose fermentation, WT-CcBglA produced 32% more ethanol than WT, and generated less lactate and acetate (Fig. 2C). Additionally, the engineered strain had an 18% increase in glucose accumulation compared to WT (Fig. 2C). Despite there being no significant change regarding the accumulation of cellobiose, WT-CcBglA accumulated more glucose, indicating an increase in cellulose degradation, in congruence with the phenotypic result (Fig. 2 B and C). The excess glucose likely arose as a result of increased cellobiose cleavage by the newly introduced CcBglA. Meanwhile, the microarray analysis showed no change in any genes belonging to the *cip-cel* operon, a gene cluster coding for the major elements of the cellulosome in *C. cellulolyticum* (Abdou et al., 2008), suggesting that the enhanced degradation efficiency was a result of synergistic degradation catalyzed by the cellulosomes and the introduction of CcBglA, rather than an increase in the abundance of cellulases. Additionally, this also indicates that the accumulation of cellobiose causes the repression of cellulase genes that may not exist in *C. cellulolyticum*.

At the end of the fermentation (320 h), approximately 20% of the cellulose remained in the broth, indicating that the cellulase dose may not have been sufficient to fully degrade the substrate. To enhance cellulose degradation, we could increase the cellulase dose (Tao et al., 2020), the activity of cellulases was increased by inserting a native promoter from *C. cellulolyticum* into the genome to enhance the expression of the "*cip-cel*" gene operon. Thus, the cellulose degradation efficiency in *C. cellulolyticum* may be further enhanced by combining this strategy with the integration of exogenous β-glucosidase. Additionally, fermentation conditions including pH, temperature, (Desvaux et al., 2000) and metabolic electron flow (Guedon et al., 1999) can affect cellulose degradation efficiency. Future studies could investigate the optimization of these parameters in combination with genetic engineering to further improve cellulose degradation and bioconversion efficiency in *C. cellulolyticum*.

As a result of the *ldh* disruption, microarray analysis showed a 70% decrease in *ldh* expression in the engineered strain, ultimately resulting in a 50% decrease in lactate production compared to WT. Due to the diminished presence of this enzyme, carbon flow was diverted to ethanol and acetate production pathways. As a result, ethanol produced was increased by 32% and acetate production decreased by 9% (Fig. 2C). In *C. cellulolyticum,* the acetate production pathway produces more ATP than either the ethanol or lactate pathways (Desvaux et al., 2000). Therefore, less acetate production indicates decreased formation of ATP, potentially resulting in the longer doubling time and smaller maximum biomass of WT-CcBglA. It is unknown why more carbon was not directed to the acetate production pathway; this will be investigated by further study.

Furthermore, it seems that the WT-CcBglA experienced a mild diauxic growth pattern. Previous studies (Giallo et al., 1983; Petitdemange

et al., 1984) have shown that the growth rate of *C. cellulolyticum* on cellobiose is nearly twice as fast as on glucose, indicating a preference for cellobiose. During cellulose degradation, *C. cellulolyticum* preferentially consumes cellobiose over glucose. The increased availability of glucose due to enhanced β-glucosidase activity in the engineered strain may have caused a delay in the utilization of cellobiose and led to the mild diauxic growth pattern and longer doubling time in the WT-CcBglA strain. Further studies are needed to confirm this hypothesis.

3.3. Engineered strain presented different gene expression profiles

Microarray-based transcriptomic analysis revealed there were 147 differentially expressed genes (DEGs) between WT and WT-CcBglA grown on cellulose. PCA analysis revealed distinct gene expression profiles for WT and WT-CcBglA strains (Fig. 3A). These indicate that some genes were affected by the heterologous expression of *CcbglA* and disruption of *ldh* during growth on cellulose. For up-regulated DEGs, the enrichment analysis indicated that the biological processes associated with the metabolic process of peptidoglycan, DNA binding, and DNA topological change were significantly enriched (Fig. 3B). As glucose is a precursor to the two primary components of peptidoglycan (N-acetylmuramic acid and N-acetylglucosamine), it is reasonable that the excess glucose observed in the engineered strain may have resulted in up-regulation of peptidoglycan synthesis genes and a subsequent increase in peptidoglycan (Pazos and Peters, 2019; Raimi et al., 2020; Calvert et al., 2017). Furthermore, as the lactate production pathway is used for NADH consumption, the disruption of the pathway could cause an imbalance between NAD^+ and NADH (Li et al., 2012). This may further influence expressions of genes associated with DNA topological change and binding (Mikolčević et al., 2021; Sorci et al., 2014; Wilkinson et al., 2001). An NAD^+/NADH imbalance can also affect carbohydrate metabolism, potentially affecting gene expression profiles further (Hopp et al., 2019). For down-regulated DEGs, it was found that the biological process associated with methionine and threonine metabolism was significantly enriched (Fig. 3C). In humans, histones are often modified by adding N-acetylglucosamine to methionine and threonine residues. As we see an increase in DNA topology and folding genes, it could follow that histone-like-proteins in the engineered strain are being modified in a similar fashion as a result of an increase in N-acetylglucosamine from the presence of excess glucose, relating to the downregulation of methionine and threonine metabolic genes (Pratt and Cornely, 2021; Wang and Maier, 2015). However, histone-like-proteins have not been thoroughly explored in *C. cellulolyticum.* These speculations will be investigated by future study.

4. Conclusion

Integration of CcBglA into the genome of *C. Cellulolyticum* proves to be an excellent strategy for improving the conversion efficiency of cellulose to ethanol. Furthermore, the site Ccel_2485 (*ldh*) is a viable insertion point, as its disruption cuts off an unwanted pathway of cellulose metabolism, resulting in increased ethanol production. This site proves to be a valuable target for heterologous expression and will be useful for future genomic editing. Together, these results demonstrate the potential of this strategy to improve cellulose degradation and bioconversion in cellulolytic bacteria, providing new insights for genetic engineering.

CRediT authorship contribution statement

Xuanyu Tao: Conceptualization, Investigation, Visualization, Writing – original draft. **Josiah S. Morgan:** Investigation, Visualization, Writing – original draft. **Jiantao Liu:** Investigation, Visualization. **Megan L. Kempher:** Writing – review & editing. **Tao Xu:** Conceptualization, Investigation, Visualization. **Jizhong Zhou:** Conceptualization, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Microarray data are deposited in the ArrayExpress under accession number E-MTAB-12766.

Acknowledgement

This work was supported by the Office of the Vice President for Research at the University of Oklahoma.

References

Abdou, L., Boileau, Céline, de Philip, P., Pagès, S., Fiérobe, H.-P., Tardif, C., 2008. Transcriptional regulation of the Clostridium cellulolyticum cip-cel operon: a complex mechanism involving a catabolite-responsive element. J. Bacteriol. 190 (5), 1499–1506.

Calvert, M.B., Mayer, C., Titz, A., 2017. An efficient synthesis of 1,6-anhydro-N-acetylmuramic acid from N-acetylglucosamine. Beilstein J. Org. Chem. 13, 2631–2636.

Desvaux, M., 2005. Clostridium cellulolyticum: model organism of mesophilic cellulolytic clostridia. FEMS Microbiol. Rev. 29 (4), 741–764.

Desvaux, M., Guedon, E., Petitdemange, H., 2000. Cellulose catabolism by Clostridium cellulolyticum growing in batch culture on defined medium. Appl. Environ. Microbiol. 66 (6), 2461–2470.

Gefen, G., Anbar, M., Morag, E., Lamed, R., Bayer, E.A., 2012. Enhanced cellulose degradation by targeted integration of a cohesin-fused β-glucosidase into the Clostridium thermocellum cellulosome. PNAS 109 (26), 10298–10303.

Giallo, J., Gaudin, C., Belaich, J.P., Petitdemange, E., Caillet-Mangin, F., 1983. Metabolism of glucose and cellobiose by cellulolytic mesophilic Clostridium sp. strain H10. Appl. Environ. Microbiol. 45 (3), 843–849.

Guedon, E., Payot, S., Desvaux, M., Petitdemange, H., 1999. Carbon and electron flow in Clostridium cellulolyticum grown in chemostat culture on synthetic medium. J. Bacteriol. 181 (10), 3262–3269.

Hopp, A.K., Grüter, P., Hottiger, M.O., 2019. Regulation of Glucose Metabolism by NAD(+) and ADP-Ribosylation. Cells 8 (8).

Jeng, W.-Y., Wang, N.-C., Lin, M.-H., Lin, C.-T., Liaw, Y.-C., Chang, W.-J., Liu, C.-I., Liang, P.-H., Wang, A.-J., 2011. Structural and functional analysis of three β-glucosidases from bacterium Clostridium cellulovorans, fungus Trichoderma reesei and termite Neotermes koshunensis. J. Struct. Biol. 173 (1), 46–56.

Jennert, K.C.B., Tardif, C., Young, D.I., Young, M., 2000. Gene transfer to Clostridium cellulolyticum ATCC 35319. Microbiology 146 (12), 3071–3080.

Kadam, S.K., Demain, A.L., 1989. Addition of cloned beta-glucosidase enhances the degradation of crystalline cellulose by the Clostridium thermocellum cellulose complex. Biochem. Biophys. Res. Commun. 161 (2), 706–711.

Lamed, R., Kenig, R., Morgenstern, E., Calzada, J.F., De Micheo, F., Bayer, E.A., 1991. Efficient cellulose solubilization by a combined cellulosome-β-glucosidase system. Appl. Biochem. Biotechnol. 27 (2), 173–183.

Li, Y., Tschaplinski, T.J., Engle, N.L., Hamilton, C.Y., Rodriguez, M., Liao, J.C., Schadt, C. W., Guss, A.M., Yang, Y., Graham, D.E., 2012. Combined inactivation of the Clostridium cellulolyticum lactate and malate dehydrogenase genes substantially increases ethanol yield from cellulose and switchgrass fermentations. Biotechnol. Biofuels 5 (1).

Liao, J.C., Mi, L., Pontrelli, S., Luo, S., 2016. Fuelling the future: microbial engineering for the production of sustainable biofuels. Nat. Rev. Microbiol. 14 (5), 288–304.

Looney, B., 2020. Statistical review of world energy, 2020. Bp 69, 66.

Lynd, L.R., Cushman, J.H., Nichols, R.J., Wyman, C.E., 1991. Fuel ethanol from cellulosic biomass. Science 251 (4999), 1318–1323.

Lynd, L., Zyl, W., Mcbride, J., Laser, M., 2005. Consolidated bioprocessing of cellulosic biomass: an update. Curr. Opin. Biotechnol. 16 (5), 577–583.

Mattila, H., Kuuskeri, J., Lundell, T., 2017. Single-step, single-organism bioethanol production and bioconversion of lignocellulose waste materials by phlebioid fungal species. Bioresour. Technol. 225, 254–261.

Mikolčević, P., Hloušek-Kasun, A., Ahel, I., Mikoč, A., 2021. ADP-ribosylation systems in bacteria and viruses. Comput. Struct. Biotechnol. J. 19, 2366–2383.

Morales, M., Arvesen, A., Cherubini, F., 2021. Integrated process simulation for bioethanol production: Effects of varying lignocellulosic feedstocks on technical performance. Bioresour. Technol. 328, 124833.

Naik, S.N., Goud, V.V., Rout, P.K., Dalai, A.K., 2010. Production of first and second generation biofuels: a comprehensive review. Renew. Sustain. Energy Rev. 14 (2), 578–597.

Parisutham, V., Chandran, S.-P., Mukhopadhyay, A., Lee, S.K., Keasling, J.D., 2017. Intracellular cellobiose metabolism and its applications in lignocellulose-based biorefineries. Bioresour. Technol. 239, 496–506.

Pazos, M. and K. Peters, *Peptidoglycan.* Bacterial cell walls and membranes, 2019: p. 127-168.

Petitdemange, E., Caillet, F., Giallo, J., Gaudin, C., 1984. Clostridium cellulolyticum sp. nov., a cellulolytic, mesophilic: species from decayed grass. Int. J. Syst. Bacteriol. 34 (2), 155–159.
Pratt, C.W., Cornely, K., 2021. Essential biochemistry. John Wiley & Sons.
Raimi, O.G., Hurtado-Guerrero, R., Borodkin, V., Ferenbach, A., Urbaniak, M.D., Ferguson, M.A.J., van Aalten, D.M.F., 2020. A mechanism-inspired UDP-N-acetylglucosamine pyrophosphorylase inhibitor. RSC Chem. Biol. 1 (1), 13–25.
Saravanan, A., et al., 2022. Recent advances and sustainable development of biofuels production from lignocellulosic biomass. Bioresour. Technol. 344, 126203.
Schwarz, W.H., 2001. The cellulosome and cellulose degradation by anaerobic bacteria. Appl. Microbiol. Biotechnol. 56 (5–6), 634–649.
Sorci, L., Ruggieri, S., Raffaelli, N., 2014. NAD homeostasis in the bacterial response to DNA/RNA damage. DNA Repair (Amst.) 23, 17–26.
Srivastava, N., Singh, R., Srivastava, M., Mohammad, A., Harakeh, S., Pratap Singh, R., Pal, D.B., Haque, S., Tayeb, H.H., Moulay, M., Kumar Gupta, V., 2023. Impact of nanomaterials on sustainable pretreatment of lignocellulosic biomass for biofuels production: An advanced approach. Bioresour. Technol. 369, 128471.
Tao, X., Xu, T., Kempher, M.L., Liu, J., Zhou, J., 2020. Precise promoter integration improves cellulose bioconversion and thermotolerance in Clostridium cellulolyticum. Metab. Eng. 60, 110–118.
Wang, G.e., Maier, R.J., 2015. Bacterial histone-like proteins: roles in stress resistance. Curr. Genet. 61 (4), 489–492.
Wilkinson, A., Day, J., Bowater, R., 2001. Bacterial DNA ligases. Mol. Microbiol. 40 (6), 1241–1248.
Xu, C., et al., 2015. Cellulosome stoichiometry in Clostridium cellulolyticum is regulated by selective RNA processing and stabilization. Nat. Commun. 6, 6900.
Xu, T., Li, Y., Shi, Z., Hemme, C.L., Li, Y., Zhu, Y., Van Nostrand, J.D., He, Z., Zhou, J., Spormann, A.M., 2015. Efficient Genome Editing in Clostridium cellulolyticum via CRISPR-Cas9 Nickase. Appl. Environ. Microbiol. 81 (13), 4423–4431.
Zandalinas, S.I., Fritschi, F.B., Mittler, R., 2021. Global warming, climate change, and environmental pollution: recipe for a multifactorial stress combination disaster. Trends Plant Sci. 26 (6), 588–599.