Research in Microbiology xxx (xxxx) xxx

Contents lists available at ScienceDirect

Research in Microbiology

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

Brief note

The role of intracellular β-glucosidase in cellulolytic response induction in filamentous fungus *Penicillium verruculosum*

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ARTICLE INFO

Article history:
Received 29 September 2023
Accepted 26 December 2023
Available online xxx

Keywords:
β-glucosidase
Penicillium verruculosum
cbh1 gene
Transcription
CRISPR/Cas9

ABSTRACT

In this study, CRISPR/Cas9 genome editing was used to knockout the *bgl2* gene encoding intracellular β-glucosidase filamentous fungus *Penicillium verruculosum*. This resulted in a dramatic reduction of secretion of cellulolytic enzymes. The study of *P. verruculosum* Δbgl2 found that the transcription of the *cbh1* gene, which encodes cellobiohydrolase 1, was impaired when induced by cellobiose and cellotriose. However, the transcription of the *cbh1* gene remains at level of the host strain when induced by gentiobiose. This implies that gentiobiose is the true inducer of the cellulolytic response in *P. verruculosum*, in contrast to *Neurospora crassa* where cellobiose acts as an inducer.

1. Introduction

Filamentous fungi have the ability to produce large quantities of complex cellulases, xylanases, proteases, laccases and various other enzymes [17]. This capability enables them to be utilized as enzyme producers in bioprocessing, pulp and paper, textile, feed, food industries, among others [4,17].

The fungus *Penicillium verruculosum* (also known as *Talaromyces verruculosus*) is a notable industrial producer of cellulase due to its high productivity (up to 60 g of total secreted protein per litre of culture broth) using a culture medium containing microcrystalline cellulose. The main components of the secreted complex consist of cellobiohydrolase 1 (CBH1) (36–41 %), CBH2 (16–20 %), endoglucanases (EGs) (12–18 %) and β-glucosidase (BGL) (3–5%) [24]. The cellulase complex of *P. verruculosum* is highly efficient due to several reasons. Firstly, its key components exhibit higher specific activity compared to *Trichoderma reesei*, which is the main producer of commercial cellulases. Additionally, the enzymes are less significantly inhibited by lignin. Furthermore, BGL demonstrates a higher level of extracellular activity [25].

A key challenge for biotechnology is to increase the productivity of enzyme producers and enzyme preparations (EPs), in order to reduce the cost of producing the final product [23]. The latest technique to address these challenges is the metabolic engineering approach. For example, BGL knockout of the *bgl2* gene, which encodes intracellular BGL, can increase productivity in the filamentous fungus *Penicillium decumbens*, which is also used for cellulase production [3]. BGL (EC 3.2.1.1.21) is responsible for the final stage of cellulose degradation by releasing glucose from cellooligosaccharides formed after cellulose hydrolysis by the action of CBHs and EGs [22].

Cello-oligosaccharides can be hydrolysed both extracellularly by secreted enzymes and intracellularly by intracellular BGLs in fungal cells. The fungus uses the resulting glucose as an energy source for cell growth and enzyme synthesis.

Nevertheless, the accumulation of glucose within the cell triggers the carbon catabolite repression (CCR) mechanism, which inhibits cellulase expression [1]. While this mechanism enables energy conservation in fungi in nature, it remains one of the factors contributing to reduced productivity in industrial cultivation. In *T. reesei*, *Neurospore crassa*, and *P. decumbens*, intracellular cellooligosaccharides, such as cellobiose, have been identified as essential for the induction of cellulase gene expression [3,30,33]. For example, knocking out the intracellular BGL gene in *P. decumbens*

Abbreviations: BGL, β-glucosidase; EG, endoglucanase; CBH, cellobiohydrolase; CCR, carbon catabolite repression; CMC, carboxymethyl cellulose; FM, fermentation media; MCC, microcrystal cellulose; MM, minimal media; pNPG, p-Nitrophenyl-β-D-glucopyranoside.

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https://doi.org/10.1016/j.resmic.2023.104178
0923-2508/

resulted in increased cellulase expression due to both a deactivated CCR mechanism resulting in reduced glucose production from cello-oligosaccharides and an accumulation of inducers in the cell [3]. A comparable outcome was noted in a mutant strain of *N. crassa* lacking activity of two genes encoding extracellular β-glucosidase enzymes and one intracellular β-glucosidase enzyme. However, the strain effectively stimulated cellulase gene expression when cellobiose, cellotriose, and cellotetraose were used as a sole carbon source [33].

Studies have shown that beta-glucosidases play a crucial role in inducing the cellulolytic response in *T. reesei*. In the absence of the genes encoding extracellular beta-glucosidase BGLI and the intracellular beta-glucosidases Cel1a and Cel1b, cellulase expression was simultaneously decreased, but induction with cellobiose leads to the correction of such a defect [30]. However, the overexpression of the *cel1b* gene greatly decreased the synthesis of cellulase, which is likely due to dysfunction in cellular transport and endoplasmic reticulum [18]. On the other hand, when another β-glucosidase gene, *bgl3i*, was deleted in *T. reesei*, it had no effect on growth but significantly increased extracellular protein production and cellulase activity in the Δbgl3i strain cultured on lactose-based media. Experimental evidence suggests that removal of the *bgl3i* gene can result in increased uptake of extracellular lactose from the culture medium into the cell, where it is converted to sophorose through transglycosylation by other β-glucosidases. Sophorose is known to be the most potent inducer of the cellulolytic response in *T. reesei*. Moreover, BGL3i displays significant hydrolytic activity towards sophorose. Thus, deletion of the *bgl3i* gene promotes the accumulation of the inducer within the cell and enhances cellulase expression [32]. The importance of b-glucosidases in the expression of cellulase by filamentous fungi has been illustrated by the example of the extracellular β-glucosidase Cel3j in *T. reesei*. Knocking out the *cel3j* gene inhibited cellulase production regardless of carbon source, lowering the expression of genes involved in cellulase synthesis but increase in genes associated with ribosome biogenesis and RNA polymerase activity [19].

Here we used the CRISPR-Cas9 system to eliminate the *bgl2* gene that encodes intracellular BGL in *P. verruculosum*. Despite anticipating greater productivity for the *P. verruculosum* Δbgl2 strain, we instead observed almost complete absence of cellulolytic enzymes activity on fermentation medium (FM) containing MCC. To clarify the reason for the observed effect, we quantified the relative normalised transcription of the *cbh1* gene that encodes CBHI through quantitative PCR. This was conducted on minimal medium (MM) enhanced with an addition of a variety of mono- and oligosaccharides that trigger CBH1 transcription.

2. Methods

2.1. Knocking out of bgl2 gene

The *bgl2* gene (GenBank ID OR898787) was knocked out using the CRISPR/CAS9 technique adapted in accordance with the procedure detailed in [10]. Briefly, protoplasts of the prototrophic strain B1-221-151 of *P. verruculosum* were transformed with the plasmids p5SniaD and pGCB2 (Supplementary Figure 1). The p5SniaD plasmid carried an sgRNA targeting the *niaD* marker gene for knock-out. The pGCB2 plasmid contained the *cas9* gene regulated by the *PvgpdA* gene promoter and sgRNA for knocking out the *bgl2* gene. To construct the plasmid, a DNA fragment was ligated to the sgRNA after PCR modification of its spacer in the p5SniaD plasmid. The bgl266sgRNAF primers (5'-cgatatcctctcatgtgtacgtacgggttaggttagctagctagaaatagcaag-3') and bgl266sgRNAR primers (5'-ccgtacacacacatgaggatcgatcgctattcgtccttttttcatacaacag-3') were used. PCR was performed using Phire Hot Start II DNA polymerase (Thermo Fischer Scientific, USA). The protospacer sequence (5'-CGATATATCCTCATGTGTGTACGG-3') for the *bgl2* gene knockout was chosen via the ChopChop tool (https://chopchop.cbu.uib.no/).

Transformants were selected on minimal medium (MM) supplemented with NH_4Cl as nitrogen source and 0.6 M $NaClO_3$ as selective agent. MM contained (in g/L): $(NH_4)_2SO_4$, 5.0; KH_2PO_4, 15; $MgSO_4$, 0.6; $CaCl_2$, 0.6; $FeSO_4 \cdot * 7H_2O$, 0.005; $MnSO_4 \cdot * H_2O$, 0.0016; $ZnSO_4 \cdot * 7H_2O$, 0.0014; $CoCl_2$, 0.002; glucose 10 and supplemented with different nitrogen sources (10 mM $NaNO_3$ for niaD $^+$ or NH_4Cl for niaD$^-$ strains). In addition, 2 % bacto-agar was added for culturing on agar plates. Colonies grown on this medium were reseeded on MM plates without $NaClO_3$ and with NH_4Cl or $NaNO_3$ as nitrogen source. Clones that could grow on $NaNO_3$ were excluded from further analysis [10].

Fragments of the *bgl2* gene were amplified using the colony PCR method with Phire Hot Start II DNA polymerase (Thermo Fischer Scientific, USA) and primers bgl1F81 (5'-gcaacacagccaggtgagatgatgatgctc-3') and bgl1R1098 (5'-gccacgaattcgtcgtccttgttgag-3'). The amplified PCR fragments were sequenced by Syntol LCC (Moscow, Russia).

2.2. Complementation of bgl2 gene

The pBgl2-BleR plasmid (Supplementary Fig. 2) containing the *bgl2* gene from *P. verruculosum* was created through the insertion of the *bgl2* gene, complete with promoter and terminator regions, into the pAN8-1 plasmid [15] using the Gibson cloning method. The pAN8-1 backbone was amplified using pAN8-1F (5'-gttcaccgcatcaggcgcc-3') and pAN8-1R (5'-gaaataaacagcgagacgaaagggc-3') PCR primers. The *bgl2* gene with promoter and terminator was amplified using Pbgl2F (5'-cctgatgcggtgaactcgacgttg-3') and Tbgl2R (5'-cgtctcgctgtttatttccgaccccatg-3'). PCR amplifications were conducted using PhusionTM High-Fidelity DNA Polymerase (Thermo Fischer Scientific, USA). The resulting PCR fragments were assembled by means of the Gibson Assembly® Cloning Kit (NEB, USA). Afterwards, the pBgl2-BleR plasmid was used to transform the *P. verruculosum* Δbgl2 strain, following the modified protocol described in [2]. Briefly, the transformants were selected on MM medium containing 10 mM NH_4Cl and 30 μg/ml bleomycin, and the grown transformants were screened by colony PCR, as detailed in section 2.1.

2.3. Penicillium verruculosum cultivation in Erlenmeyer flasks

P. verruculosum strains were cultured in 750-ml Erlenmeyer flasks for 6 days at a temperature of 32 °C, while being stirred at 220 rpm in 100 ml of cultivation medium (CM). Culture liquids were sampled and fungal biomass was precipitated by centrifugation at 4000 rpm for 10 min. The supernatants were then used for enzyme activity assays and sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). CM for Erlenmeyer flasks comprised of the following (in g/l): cellulose - 40, yeast extract - 10, wheat bran - 10, KH_2PO_4 - 15, $CaCl_2$ - 0.3, and $(NH_4)_2SO_4$ - 5.0 [10].

2.4. Enzymatic activities assay, protein concentration measurement and denaturing polyacrylamide gel electrophoresis

The Nelson-Somogyi method [16,26,27] with modifications [6] was used to determine enzymatic activities towards polymeric substrates by determining the release of reducing sugars. Carboxymethylcellulose (CMC) (Sigma–Aldrich, USA), beechwood xylan (Sigma–Aldrich, USA) and Avicel PH-105 cellulose (MCC) (Serva, Heidelberg, Germany) were used as substrates for the determination of endoglucanase, xylanase and avicelase activities, respectively.

β-glucosidase activity was determined by hydrolysis of p-nitrophenyl-β-D-glucopyranoside (pNPG) (Sigma–Aldrich) as described by [7].

The protein concentration in culture fluids was determined by the Lowry method [14]. The optical density was measured on a Varian Cary 50 UV–Vis spectrophotometer (Agilent Technologies, USA).

Culture liquid electrophoresis was carried out under denaturing conditions according to [13] with modifications. A Mini Protein II chamber (Bio-Rad Laboratories, USA) was used for electrophoresis. The Prestained Protein Marker II G2058-250UL (Servicebio, China) was used as a protein marker. After separation by SDS-PAGE, proteins were stained with Coomassie G-250 dye (Ferak, Germany).

All experiments and measurements were performed in triplicate. Margin of error was calculated in Microsoft Excel, $p < 0.05$ was considered a significant difference.

2.5. *Determination of* P. verruculosum cbh1 *gene transcription by quantitative PCR*

The induction of *cbh1* gene transcription in *P. verruculosum Δbgl2* and the wild-type strain was measured as previously described [9]. Briefly, spore suspensions of *P. verruculosum* B1-221-151 and Δbgl2 strains were inoculated at the level of 3×10^6 spores in the 750 ml Erlenmeyer flasks with 100 ml liquid MM medium containing 0.8 % glucose and 50 mM citrate-phosphate buffer (pH 5.6) (to prevent osmotic shock in the next step), with addition of 10 mM NH_4Cl as nitrogen source, and incubated on a rotary shaker (220 rpm) at 32 °C for 48 h. To remove glucose, the mycelium was separated from the liquid culture by centrifugation at 4000*g* for 12 min, then washed with MM medium containing citrate-phosphate buffer and 10 mM NH_4Cl; the resulting precipitate was resuspended in the same medium, after which 4 ml of the suspension was transferred into 15 glass tubes. The tubes containing the mycelial suspension were then incubated under the same conditions on an orbital shaker. This allowed the glucose residues to be utilised and the CCR mechanism to be switched off. After 45 min of incubation, 1 ml of a 10 mM induction sugar solution was added to the tubes: cellobiose, cellotriose, gentiobiose and xylose dissolved in MM medium with citrate-phosphate buffer. 1 ml of this medium without sugars was added to the control tube. The tubes were then incubated on an orbital shaker under the same conditions, with 1.2 ml of culture fluid picked off from each tube after 1, 2 and 4 h. RNA was isolated from the selected samples and the number of mRNA copies of the *cbh1* gene was determined by quantitative PCR [9].

Amplification was performed with a CFX96 amplifier (Bio-Rad, USA) in compatible 96-well low profile plates according to the qPCR protocol described by [9]. All samples were analysed in triplicate. To compare Cq values (the number of cycles required for fluorescence to rise above background noise) on different plates, identical samples were loaded in triplicate on each plate and for each gene. Results were analysed using Bio-Rad CFX Manager v.3.1 software. Relative normalised expression and standard errors of the mean were calculated automatically based on gene amplification efficiency values previously reported by [9].

3. Results and discussion

3.1. *Knockout of* bgl2 *in* Penicillium verruculosum

We used the Blast-P search algorithm to identify the homologous intracellular BGL2 in *P. verruculosum*, following the demonstration that knockout of the *bgl2* gene in *P. decumbens* results in increased cellulase production (Chen et al., 2012). The Clustal Omega web tool (https://www.ebi.ac.uk) was then employed to align the identified protein sequence with BGL2 *P. decumbens* (*Penicillium oxalicum*), revealing a 73 % identity (Fig. 1). The presence of a signal peptide was not detected, as confirmed by the SignalP-6.0 web tool (https://services.healthtech.dtu.dk/services/SignalP-6.0/). Next, the *bgl2* gene encoding the homologous protein to BGL2 *P. decumbens* was found in the genome of *P. verruculosum*. Furthermore, the *bgl1* gene was previously discovered, encoding a secreted β-glucosidase [21,31]. To investigate the role of BGL2 in regulating the cellulolytic response in *P. verruculosum*, the CRISPR/Cas9 genome editing method was employed to knock out the *bgl2* gene in the prototrophic strain *P. verruculosum* B1-221-151. The gene mutation, which led to the shift of open reading frame and formation of stop codon, was verified through gene sequencing (Fig. 2). The growth of the mutant strain on a minimal medium (MM) supplied by NH_4Cl as a nitrogen source did not exhibit any visual changes upon the *bgl2* gene knockout (Supplementary Fig. 3).

3.2. *Knockout of* bgl2 *reduces extracellular enzyme production*

The lack of BGL2 resulted in a significant increase in extracellular cellulase and xylanase activities when submerged

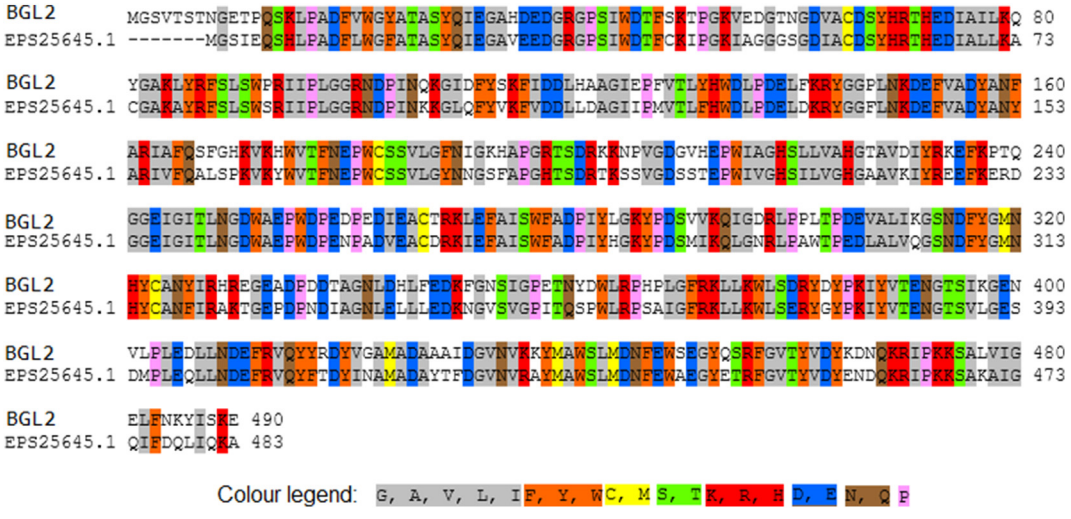

Fig. 1. Alignment of BGL2 *P. verucullosum* and BGL2 *P. decumbense*.

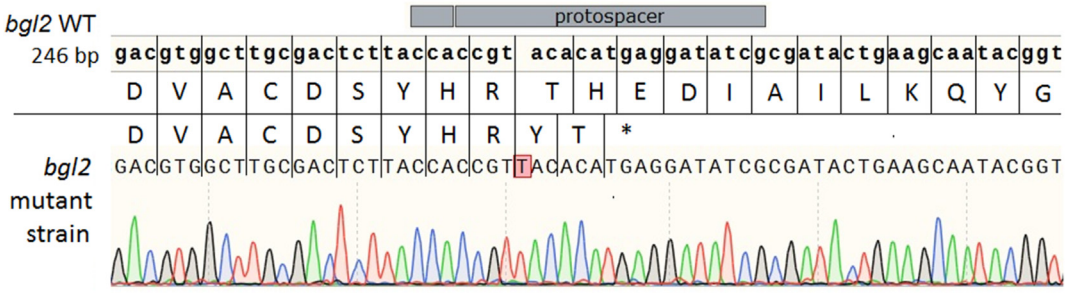

Fig. 2. Alignment of amplified fragment of *bgl2* gene in mutant strain with wild type *bgl2* gene. An insertion of one nucleotide is observed at the site of double-strand DNA break by Cas9 nuclease, which led to a frameshift and gene knockout.

cultivation occurred on cellulose-inducing medium that contained 1 % MCC and 1 % wheat bran as demonstrated for *P. decumbens* [3]. Therefore, when *P. verruculosum* Δbgl2 was cultivated on the FM medium containing MCC, we expected similar results. Surprisingly, instead of an increase in cellulolytic production during the fermentation of *P. verruculosum* Δbgl2 as it was with *Penicillium decumbence* [3], we observed an almost complete absence of cellulolytic production (Fig. 3 and Table 1). Therefore, the *P. verruculosum* Δbgl2 strain demonstrated a reduction in protein productivity by 4-fold, a decline in endoglucanase activity when assayed with carboxymethylcellulose (CMC) by over 10-fold, β-glucosidase activity when assayed with p-nitrophenyl-β-D-glucopyranoside (pNPG) by nearly 10-fold, avicelase activity by 11-fold, and xylanase activity by more than 18-fold) (see Table 1). To confirm the causality between the *bgl2* gene knockout and the observed effect, we transformed the Δbgl2 strain with the pBgl2-BleR plasmid that contains *bgl2* gene. We selected positive clones on bleomycin-containing media and verified replacement of the defective gene with the original one by gene sequencing (Supplementary Figure 4). Upon

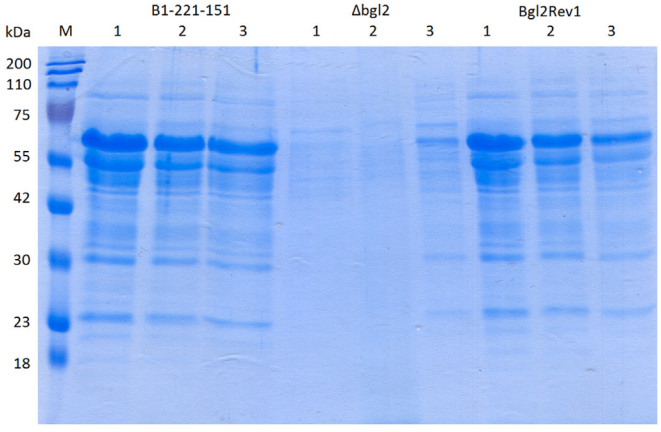

Fig. 3. SDS-PAGE gel electrophoresis of culture fluids after flask cultivation of *P. verruculosum* B1-221–151, Δbgl2, Bgl2Rev1 strains on the FM medium with MCC at 6 day in triple series. Track numbers 1–3 correspond to the numbers of flasks in the series.

cultivation of this strain, named Bgl2Rev1, on CM medium (see Table 1), restoration of cellulolytic response, typical for wild-type strain, was observed. It is noteworthy that elimination of the genes responsible for intracellular BGL in *N. crassa* and *T. reesei* leads to a notable reduction in cellulase expression when grown on MCC-containing media. However, the cellulolytic activity of both mutant strains was restored to the level of the initial strain when grown on media containing cellobiose as a carbon source [30,33].

3.3. *The lack of cellobiose and cellotriose induction on* cbh1 *gene expression in* P. verruculosum *Δbgl2 strain*

The relative transcript level of the *cbh1* gene in response to different oligosaccharides was analysed to investigate the termination of the cellulolytic response in *P. verruculosum* Δbgl2 strain. Prior studies have demonstrated that *cbh1* gene transcription is induced by cellotriose, cellobiose, gentiobiose, and to a lesser extent, xylose, xylobiose, and xylotriose [9]. In contrast, lactose, which induces cellulase expression in *T. reesei* [29] and *Acremonium cellulolyticus* [5], does not stimulate *cbh1* gene transcription in *P. verruculosum* [9].

The transcription induction of the *cbh1* gene in *P. verruculosum* Δbgl2 was impaired when it was exposed with cellobiose and cellotriose. This was observed throughout all time intervals when sampling was carried out, over a period of 4 h. However, transcription was not impaired when it was induced by gentiobiose and xylose (Fig. 4). Moreover, the transcription of the *cbh1* gene under the influence of gentiobiose even increased and turned out to be longer in the strain with a knockout of the *bgl2* gene. It is apparently could be explained by the absence of an enzyme capable of hydrolyzing it. These unexpected results deviated from the known inducers of cellulases in *P. decumbens* such as cellobiose and cellotriose [3] or only cellobiose in *T. reesei* and *N. crassa* [8,30,33]. However, this explains why the *P. verruculosum* Δbgl2 strain did not exhibit cellulolytic activity during cultivation on FM medium.

The study demonstrates that gentiobiose is the primary inducer for cellulases synthesis in *P. verruculosum*. It is interesting to note that a similar result occurs in *Penicillium purpurogenum* (Kurasawa et el. 1992). This fungus is not recognised as a

Table 1
Average enzyme activities in culture fluids after triple flask cultivation of *P. verruculosum* B1-221-151, Δbgl2 strains and complimented Δbgl2 strain with *bgl2* gene on the FM medium with MCC at 6 day.

Strain	Protein, mg/ml	CMC, U/ml	pNPG, U/ml	Avicel, U/ml	Beech xylan, U/ml
B1-221-151	11.5 ± 0.4	70 ± 0.6	0.8 ± 0.1	1.5 ± 0.1	259 ± 2.4
Δbgl2	2.6 ± 0.5	5.9 ± 0.72	0.07 ± 0.03	0.13 ± 0.04	14 ± 0.4
Bgl2Rev1	10.9 ± 0.5	68 ± 1.5	1.1 ± 0.1	1.8 ± 0.2	264 ± 5.1

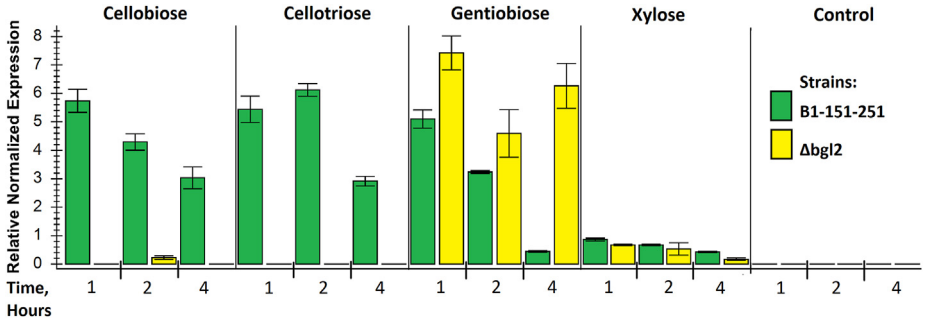

Fig. 4. Transcription levels of *cbh1* gene in *P. verruculosum* B1-221-151 (WT) strain and *P. verruculosum* Δbgl2 strains after induction with oligosaccharides and xylose.

lignocellulose-degrading organism. Gentiobiose (β-D-glucopyranosyl-(1 → 6)-β-D-glucopyranose) is a dimeric pustulan residue [20] and is scarcely formed by direct MCC hydrolysis. The primary route for producing gentiobiose from MCC is through a transglycosylation reaction.

Fungal β-glucosidases (BGLs) from *Penicillium* spp. have been shown to catalyze the formation of gentiobiose from cellobiose *in vitro* [11,12]. Furthermore, BGLs have been found to synthesise gentiobiose at high concentrations of glucose [28]. Apparently, BGL2 of *P. verruculosum* is the main producer of gentiobiose in the cell, possessing transglycosylase activity. After knocking out the *bgl2* gene, the formation of genentiobiose in the cell was hindered along with the induction of the cellulolytic response. This outcome further illustrates the significant diversity of cellulase expression inducers found across various filamentous fungi species.

4. Conclusion

The Δbgl2 strain displayed reduced cellulase expression when grown on MCC-containing medium. We also used this Δbgl2 strain to study the effect of inducers on the transcription of the *cbh1* gene, which encodes cellobiohydrolase 1 (CBH1), the major enzyme in *P. verruculosum* cellulolytic complex. It was found that cellobiose and cellotriose do not directly induce transcription, but that the induction of cellulase expression occurs through the action of gentiobiose in *P. verruculosum*.

The results demonstrate the diverse assortment of cellulase activators in filamentous fungi species, which has of great importance for the understanding of the regulation of cellulase transcription in *P. verruculosum* and related fungi, which is important for the improvement of their productivity.

Author contributions

VK: Investigation, Writing - Original Draft. AC: Methodology. AD: Investigation. IS: Investigation. AS: Supervision. AR- Conceptualization, Writing - Review and 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.

Acknowledgement

This work was supported by the Ministry of Science and Higher Education of the Russian Federation (Grant # 075-15-2021-1071, 28.09.2021).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.resmic.2023.104178.

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