Zhang *et al.*
Biotechnology for Biofuels and Bioproducts (2025) 18:11
https://doi.org/10.1186/s13068-025-02610-z

Biotechnology for Biofuels and Bioproducts

RESEARCH

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Involvement of the intracellular β-glucosidase BGL1B from *Aspergillus niger* in the regulation of lignocellulose-degrading enzymes' synthesis

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Abstract

Background *Aspergillus niger* is an important lignocellulose-degrading enzyme-producing strain. Multiple regulatory factors regulate the synthesis of lignocellulose-degrading enzymes in *A. niger*. We previously found that *A. niger* possessed an intracellular β-glucosidase BGL1B, and the intracellular localization of BGL1B and its active transglycosylation action prompted us to explore whether BGL1B was involved in the regulation of the synthesis of lignocellulose-degrading enzymes in *A. niger*.

Results In this study, by investigating the production of lignocellulose-degrading enzymes of *bgl1B* knockout strain (Δ*bgl1B*) and overexpression strain (OE::*bgl1B*), it was found that BGL1B exhibited a repressive role on the expression of lignocellulose-degrading enzyme genes through carbon catabolite repression (CCR) way. On the other hand, BGL1B's transglycosylation products sophorose and laminaribiose were proved to be able to induce the expression of lignocellulose-degrading enzyme genes, which explained why OE::*bgl1B* showed the same enhanced enzyme activity and gene expression as Δ*bgl1B* strain compared to the starting strain (WT).

Conclusions The present study demonstrates that BGL1B plays dual regulatory roles in the regulation of the synthesis of lignocellulose-degrading enzymes in *A. niger*: the repressive role caused by BGL1B's hydrolysis product glucose and the induction role caused by BGL1B's transglycosylation products sophorose and laminaribiose. This study broadens the understanding of the regulatory network of the synthesis of lignocellulose-degrading enzymes in *A. niger*. Also, it provides a strategy to create an engineered strain with high production of lignocellulose-degrading enzymes.

Highlights

- Intracellular β-glucosidase BGL1B in *A. niger* was proven to be involved in the regulation of the synthesis of lignocellulose-degrading enzymes.
- Sophorose and laminaribiose were confirmed to have an induction effect on the synthesis of lignocellulose-degrading enzymes in *A. niger*.

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- BGL1B played dual regulatory roles in synthesizing lignocellulose-degrading enzymes through hydrolysis and transglycosylation products.

Keywords *Aspergillus niger*, Intracellular β-glucosidase, Lignocellulose-degrading enzymes, Sophorose, Laminaribiose

Introduction

Aspergillus niger is a filamentous fungus with important industrial values [1]. It plays a vital role in utilizing crop straw and other lignocellulosic biomass because it can efficiently produce various lignocellulose-degrading enzymes [2, 3]. The synthesis of lignocellulose-degrading enzymes in *A. niger* is strictly regulated. When *A. niger* grows on cellulosic carbon sources such as wheat straw, the cellulosic carbon sources can induce the expression of lignocellulose-degrading enzyme genes [4], and it has been confirmed that low concentration of D-xylose can act as an inducer targeting the transcription factor XlnR to induce the production of *A. niger* cellulases and hemicellulases [5, 6]. When *A. niger* grows on easily available carbon sources such as glucose and sucrose, the lignocellulose-degrading enzyme genes are suppressed due to carbon catabolite repression (CCR) [7, 8].

β-Glucosidase, being a crucial component of lignocellulose-degrading enzymes, has been reported to be involved in the expression regulation of lignocellulose-degrading enzymes in *Trichoderma reesei* and *Penicillium purpurogenum*. In *T. reesei*, β-glucosidase Cel1A was proved to play an induction role in cellulase expression due to the formation of sophorose, an inducer of cellulase, by its transglycosylation activity. In contrast, β-glucosidase BGL3I in *T. reesei* played a repression role in cellulase expression due to degradation of the inducer sophorose [9, 10]. In *P. purpurogenum*, gentiobiose was proposed to be formed by intracellular β-glucosidase, and gentiobiose acted as an inducer to induce the production of cellulose-degrading enzymes [11, 12]. *A. niger* is known for its ability to secrete large amounts of β-glucosidases [13]; however, we recently identified an intracellular β-glucosidase from *A. niger*, BGL1B [14], which exhibited significant transglycosylation activity. When cellobiose was used as the substrate, sophorose, laminaribiose, and cellotriose could be formed through BGL1B's transglycosylation activity, while when cellotriose was used as the substrate, sophorose and laminaribiose could be formed. The intracellular localization of BGL1B, as well as its high transglycosylation activity, prompted us to speculate that BGL1B might also be involved in the regulation of the synthesis of lignocellulose-degrading enzymes in *A. niger*.

Here we constructed *A. niger* intracellular β-glucosidase BGL1B gene knockout strain (noted as Δ*bgl1B*) and overexpression strain (noted as OE::*bgl1B*) to systematically explore the possible regulatory function of BGL1B in the synthesis of lignocellulose-degrading enzymes in *A. niger*. This study may add more knowledge to the mechanisms underlying the regulation of lignocellulose-degrading enzymes' synthesis in *A. niger*, meanwhile may provide a new strategy for engineering an *A. niger* strain with a high production of lignocellulose-degrading enzymes.

Materials and methods

Strains, reagents, and media

Aspergillus niger used in this study was CBS 513.88 Δ*kusA* strain (*kusA* knockout leading to a high efficiency of homologous recombination [15]). Sophorose was purchased from Shanghai yuanye Bio-technology Co., Ltd. (Shanghai, China), and laminaribiose from Megazyme Ltd. (Megazyme, Wicklow, Ireland). Microcrystalline cellulose used was Avicel® PH-101 purchased from Sigma-Aldrich (St. Louis, USA). The substrates sodium carboxymethylcellulose, beech xylan, and *p*-nitrophenyl-β-D-glucopyranoside for enzyme assay were also from Sigma-Aldrich (St. Louis, USA). The substrate *p*-nitrophenyl-β-D-cellobioside was from MacLean Biochemical Technology Co., Ltd. (Shanghai, China). Cellulase and snail enzyme were purchased from Solarbio Co., Ltd. (Beijing, China), and lyticase was purchased from Guangdong Microbial Culture Collection Center (Guangzhou, China).

Potato dextrose agar (PDA) medium was used for the solid culture of *A. niger*, and Mandel's medium [16] supplemented with 1 g/L of tryptone and 10 g/L of sucrose was used as the growth medium. Mandel's medium supplemented with 10 g/L of microcrystalline cellulose Avicel was used for transcriptome and proteome analysis.

Construction of *A. niger bgl1B* knockout strain Δ*bgl1B* and overexpression strain OE::*bgl1B*

This study used homologous recombination to construct *bgl1B* knockout strain Δ*bgl1B* and *bgl1B* overexpression strain OE::*bgl1B*. For the construction of the Δ*bgl1B* strain, the overlap extension PCR method was used to build a knockout cassette containing a 1500 bp homology arm upstream of *bgl1B*, a hygromycin B resistance gene *hph*, and a 1500 bp homology arm downstream of *bgl1B*. To construct the overexpression strain OE::*bgl1B*, a strong promoter derived from the *Aspergillus nidulans gpdA* gene was introduced for *bgl1B*. The constructed

overexpression cassette included the upstream homology arm of *bgl1B*, the hygromycin B resistance gene *hph*, the *gpdA* promoter, and a 1500-bp fragment of the N-terminus of *bgl1B* used as the downstream homology arm (Fig. 1).

Preparation of *A. niger* protoplasts: *A. niger* CBS 513.88 Δ*kusA* strain was cultured in PDA medium, and spores were collected with sterile water to prepare a spore suspension. The spore suspension was added to the malt extract medium containing malt extract 5 g/L, tryptone 30 g/L, and Tween-80 1 g/L, and then cultured at 30 ℃, 250 r/min for 10 h to obtain young mycelium for preparing protoplasts. The mycelium was hydrolyzed with 50 mL of the enzyme solution containing 1% cellulase, 1% snail enzyme, and 2% lyticase at 33 ℃ and 110 r/min for 2–2.5 h. The resulting protoplasts could reach 3×10^7 protoplasts·mL^{-1}.

Polyethylene glycol (PEG)-mediated transformation and transformant screening: 20 μL of the knockout cassette (800 ng/μL) for constructing Δ*bgl1B* strain and 20 μL of the overexpression cassette (800 ng/μL) for constructing OE::*bgl1B* strain obtained above were added to the protoplast suspension and mixed thoroughly. After adding 1 mL of 25% PEG buffer and mixing, the mixture was allowed to stand on ice for 20 min. The PEG and protoplast mixture was transferred into a 15-mL enzyme-free sterile centrifuge tube, and 10 mL 1.33 M STC buffer (10 mM pH 7.5 Tris–HCl buffer containing 1.33 M sorbitol, 35 mM NaCl, and 50 mM $CaCl_2$) was added and resuscitated at 33 ℃ and 90 r/min for 2 h. After resuscitation, the centrifuged and concentrated *A. niger* suspension was transferred into a regeneration medium containing 0.95 M sucrose, 2% (v/v) 50×ASPA + N solution ($NaNO_3$ 297.5 g/L, KCl 26.1 g/L, KH_2PO_4 74.8 g/L, pH 5.5), 0.2% (v/v) 1 M $MgSO_4$ buffer, 0.1% (v/v) 1000×Trace element buffer (EDTA 10 g/L, $ZnSO_4{\cdot}7H_2O$ 4.4 g/L, $MnCl_2{\cdot}4H_2O$ 1.01 g/L, $CoCl_2{\cdot}6H_2O$ 0.32 g/L, $CuSO_4{\cdot}5H_2O$ 0.315 g/L, $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$ 0.22 g/L, $CaCl_2$ 1.11 g/L, $FeSO_4{\cdot}7H_2O$ 1.0 g/L, pH 4.0) and 0.7% agarose for regeneration. The regenerated culture was transferred into a PDA plate containing 200 mg/L hygromycin B for transformant screening. For those colonies capable of growing on that plate, the genome of each colony was extracted for PCR verification and sequencing verification of the *bgl1B* site. Furthermore, the expression level of *bgl1B* in each mutant strain was detected by fluorescence quantitative PCR using 18S rDNA as the internal reference gene: the total RNA of each colony was extracted using RNAiso plus reagent (Takara Bio Inc., Shiga, Japan) followed by cDNA synthesis using ReScript™ II kit (Beijing Nolai Enzyme Biotechnology Co., Ltd.), and the fluorescence quantitative PCR was performed using 2×SYBR Premix WizTaq II kit (Beijing Nolai Enzyme Biotechnology Co., Ltd.).

Extraction and detection of intracellular proteins of *bgl1B* overexpression strain

The spore suspensions of the starting strain (noted as WT) and *bgl1B* overexpression strain OE::*bgl1B* were inoculated into the growth medium, respectively. After incubating at 28 ℃ for 24 h, the mycelium was collected and an equal amount of mycelium was transferred into Mandel's medium supplemented with 10 g/L of wheat straw for another 24 h. The mycelium was harvested and washed with distilled water for three times. Mycelium weighing 0.6 g for each strain was ground into fine powder under liquid nitrogen protection and then suspended in 1 mL pH 7.2 PBS buffer. After shaking in an incubator at room temperature for 10 min, the suspension was centrifuged at 14,000×*g* for 10 min to remove the cell debris. The resulting supernatant was taken as the intracellular proteins and was detected by SDS–PAGE with a 12% resolving gel [17].

Biomass determination

The spore suspension of WT, Δ*bgl1B*, and OE::*bgl1B* were prepared from PDA medium, respectively, and an equal amount of spores were transferred into the growth medium which was taken as sucrose carbon source. For wheat straw or microcrystalline cellulose Avicel carbon

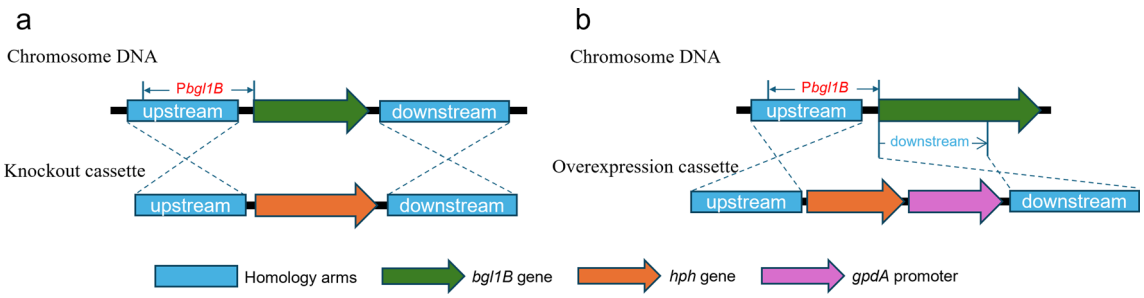

Fig. 1 Schematic diagram of the construction of *bgl1B* knockout (**a**) and overexpression (**b**) strains by homologous recombination method

source, 10 g/L of wheat straw or Avicel was used instead of 10 g/L of sucrose in the growth medium. After incubating at 28 ℃ for 72 h, the mycelium was collected by filter, rinsed with sterile water, and briefly dried before placing in the oven of 105 ℃ for 6 h. The dry weight of the mycelium was determined with triplicates. At the same time, the culture supernatant obtained was used to estimate enzyme activities.

Assay of lignocellulose-degrading enzyme activities

The activities of endoglucanase (EG) and xylanase (Xyn) were determined using the dinitrosalicylic acid (DNS) method [18]. For EG activity determination, sodium carboxymethylcellulose was used as the substrate. The total reaction mixture of 400 μL contained 200 μL of 1% substrate, 100 μL 0.01 M of pH 5.0 citric acid-phosphate buffer, and 100 μL of the corresponding enzyme solution. The reaction mixture was incubated at 50 ℃ for 3 h. After incubation, 600 μL of DNS solution was immediately added, followed by boiling for 10 min. The absorbance at 540 nm was measured to determine the amount of the released reducing sugar. Beech xylan was used as the substrate for Xyn activity determination. The total reaction mixture of 400 μL contained 100 μL of 2% substrate, 200 μL 0.01 M pH 5.0 citric acid-phosphate buffer, and 100 μL corresponding enzyme solution. The reaction mixture was incubated at 50 ℃ for 1 h. The following procedures were the same as above.

The activities of cellobiohydrolase (CBH) and β-glucosidase (BGL) were determined using *p*-nitrophenyl-β-D-cellobioside and *p*-nitrophenyl-β-D-glucopyranoside as the substrate, respectively. The total reaction mixture of 200 μL contained 50 μL of 20 mM substrate, 100 μL 0.01 M pH 5.0 citric acid-phosphate buffer, and 50 μL corresponding enzyme solution. The reaction mixture was incubated at 50 ℃ for 3 h. After incubation, 1 mL of 0.2 M Na_2CO_3 solution was added to terminate the reaction. The absorbance at 408 nm was measured to determine the amount of the released *p*-nitrophenol.

All assays were performed in triplicates. One unit of the enzyme activity (U) was defined as the amount of enzyme required to produce 1 μmol of product per min.

Transcriptome analysis

An equal amount of mycelium of WT, Δ*bgl1B*, and OE::*bgl1B* collected from the growth medium was transferred into Mandel's medium supplemented with 10 g/L of microcrystalline cellulose Avicel, respectively. After incubating at 28 ℃ for 6 h, the mycelium was collected and milled under liquid nitrogen's protection. The total RNA of *A. niger* was extracted using RNAiso plus reagent, reverse transcribed using ReScript™ II kit, and fluorescent quantitative detection was performed using 2 × SYBR Premix WizTaq II kit aforementioned.

The construction and sequencing of RNA-seq libraries were performed by BGI Genomics (Shenzhen, China). Each sample was sequenced in triplicates, and the sequencing raw data were quality-controlled using SOAPnuke [19] to obtain clean data.

The clean reads were aligned to the reference genome of *A. niger* CBS 513.88 (NCBI reference genome version GCF_000002855.3_ASM285v2) using HISAT2 [20], and then the alignment results were mapped to the reference gene sequence using Bowtie2 [21]. The gene expression level of each sample was calculated using RSEM [22] software. DESeq2 [23] was used to detect differentially expressed genes with a threshold of $Q \leq 0.05$ or FDR ≤ 0.001.

Secreted proteome analysis

An equal amount of mycelium of WT, Δ*bgl1B*, and OE::*bgl1B* collected from the growth medium was transferred into Mandel's medium supplemented with 10 g/L of microcrystalline cellulose Avicel, respectively. After incubation at 28 ℃ for 24 h, the supernatant was collected as the secreted proteins' solution and sent to BGI for mass spectrometry analysis. The proteins were digested with trypsin and separated using the Thermo UltiMate 3000 UHPLC. The separated peptides were then detected using the tandem mass spectrometer Q-Exactive HF X in data-dependent acquisition (DDA) mode. The mass spectrometry data were identified using the Andromeda engine integrated with MaxQuant.

Analysis of the induction effect of sophorose and laminaribiose

The Δ*bgl1B* strain was used to evaluate the possible induction effect of sophorose and laminaribiose on lignocellulose-degrading enzymes' synthesis. An equal amount of mycelium of Δ*bgl1B* strain collected from the growth medium was transferred into Mandel's medium supplemented with various saccharides (10 g/L sucrose, 1 g/L sophorose, or 1 g/L laminaribiose), respectively. The mixtures were incubated in the rotator at 28 ℃ for 6 h and 24 h, respectively, for assays of gene expression level, enzyme activities, and secreted proteome.

Statistical analysis

All results are presented as the mean of triplicates with standard deviation significance set as $p < 0.05$. A p value of less than 0.05 (*) indicates a statistically significant difference. The data were initially subjected to an analysis of variance (ANOVA) and then graphed using Prism 9 software (GraphPad).

Results

Effects of knockout and overexpression of *bgl1B* on the growth of *A. niger*

The *bgl1B* knockout strain Δ*bgl1B* was obtained by homologous recombination, and the overexpression strains OE-7 and OE-10 were obtained by introducing the strong promoter of *gpdA* gene from *A. nidulans ans* for *bgl1B*. To verify the effectiveness of Δ*bgl1B*, the relative expression level of *bgl1B* was detected when Δ*bgl1B* was cultured in the growth medium with sucrose and wheat straw as carbon sources, respectively (Fig. 2a). Taking the expression level of *bgl1B* in the starting strain (noted as WT) cultured on sucrose as 1, it could be seen that when cultured on sucrose, *bgl1B* in Δ*bgl1B* had a similar expression level to that in WT. In contrast, when cultured on wheat straw, *bgl1B* in Δ*bgl1B* showed a significantly lower expression level than in WT. The expression level of *bgl1B* in Δ*bgl1B* on wheat straw condition was kept the same as that in WT on sucrose condition, demonstrating that *bgl1B* in Δ*bgl1B* could not be induced normally and *bgl1B* was successfully knocked out. To verify the effectiveness of the overexpression strains OE-7 and OE-10, the two strains were cultured in the growth medium with wheat straw as a carbon source, and the expression of *bgl1B* was analyzed in the same way as above (Fig. 2b). The results showed that compared with the expression of *bgl1B* in WT, the expression of *bgl1B* in OE-7 showed a 300-fold increase. In contrast, the expression of *bgl1B* in OE-10 showed a 795.5-fold increase, indicating that OE-10 was a better candidate for the overexpression strain than OE-7. As a result, OE-10 was selected for further detection of the intracellular BGL1B's protein level. As shown in Fig. 3c, OE-10's intracellular proteins showed an extra band with the size of approximately 55 kDa compared to that of WT. Since the theoretical molecular weight of BGL1B was 54.3 kDa, it was very likely that the extra band was the overexpressed BGL1B. Therefore, OE-10 was taken as the *bgl1B* overexpressed strain, noted as OE::*bgl1B*.

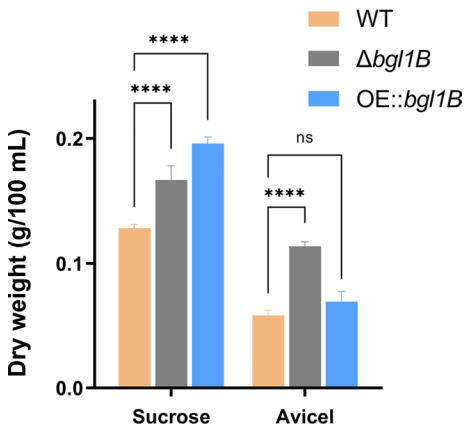

Fig. 3 Mycelium dry weights of the three strains after cultured on sucrose or Avicel carbon source for 72 h. Asterisks indicate significant differences (****$p < 0.0001$). ns: no significance

To determine the effect of *bgl1B* on the growth of *A. niger*, the biomass of the three strains WT, Δ*bgl1B,* and OE::*bgl1B* were examined after cultured in the growth medium with sucrose or microcrystalline cellulose Avicel as carbon source for 72 h (Fig. 3). Unsurprisingly, all three strains showed higher mycelium dry weight on the sucrose carbon source than Avicel carbon source. However, it was unexpected that both Δ*bgl1B* and OE::*bgl1B* showed a higher mycelium dry weight than that of WT, no matter on sucrose carbon source or Avicel carbon source. It was noticeable that the biomass difference between OE::*bgl1B* and WT on Avicel carbon source did

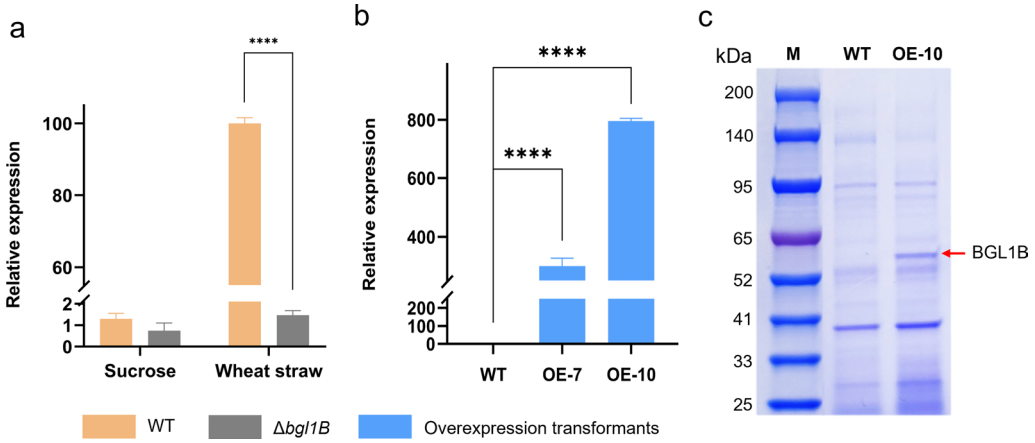

Fig. 2 Quantitative PCR detection of relative expression of *bgl1B* and SDS-PAGE detection of intracellular BGL1B. **a** Quantitative PCR of knockout strain with sucrose or wheat straw as carbon source; **b** quantitative PCR of overexpression strains with wheat straw as carbon source; **c** SDS-PAGE detection of OE-10 overexpression strain. Asterisks indicate significant differences (****$p < 0.0001$)

not show statistical significance. The results indicated that knockout of *bgl1B* benefited *A. niger*'s growth on Avicel carbon source better than overexpression of *bgl1B*.

Effects of knockout and overexpression of *bgl1B* on the activities of lignocellulose-degrading enzymes of *A. niger*

To explore how *bgl1B* affects the ability of *A. niger* to produce lignocellulose-degrading enzymes, the WT, Δ*bgl1B*, and OE::*bgl1B* strains were cultured in the growth medium with sucrose or microcrystalline cellulose Avicel as a carbon source for 72 h, and the activities of the main lignocellulose-degrading enzymes were measured, including endoglucanase (EG), xylanase (Xyn), cellobiohydrolase (CBH) and β-glucosidase (BGL). As shown in Fig. 4, when cultured on sucrose, there was no significant difference in the activities of the main lignocellulose-degrading enzymes among the three strains, suggesting that BGL1B did not affect the constitutive expression of lignocellulose-degrading enzymes' genes of *A. niger*. However, when cultured on Avicel, the activities of four lignocellulose-degrading enzymes of Δ*bgl1B* were significantly higher than those of WT. OE::*bgl1B* also showed slightly higher activities of lignocellulose-degrading enzymes than WT. It showed that the knockout of *bgl1B* could improve the activities of *A. niger*'s main lignocellulose-degrading enzymes and hence enhance the strain's ability to utilize Avicel. Interestingly, the overexpression strain OE::*bgl1B* could also enhance the ability of *A. niger* to produce lignocellulose-degrading enzymes, although the enhancement effect was not as significant as that of the knockout strain Δ*bgl1B*. The improvement of lignocellulose-degrading enzymes' activities of Δ*bgl1B* and OE::*bgl1B* was in accordance with the increased biomass of these mutant strains on the Avicel carbon source, shown in Fig. 3.

Transcriptome and proteome analysis of *bgl1B* knockout and overexpression strains

To further explore the involvement of BGL1B in the synthesis of lignocellulose-degrading enzymes in *A. niger*, the mycelia of WT, Δ*bgl1B*, and OE::*bgl1B* were incubated at Mandel's medium supplemented with 10 g/L of microcrystalline cellulose Avicel for 6 h, and the

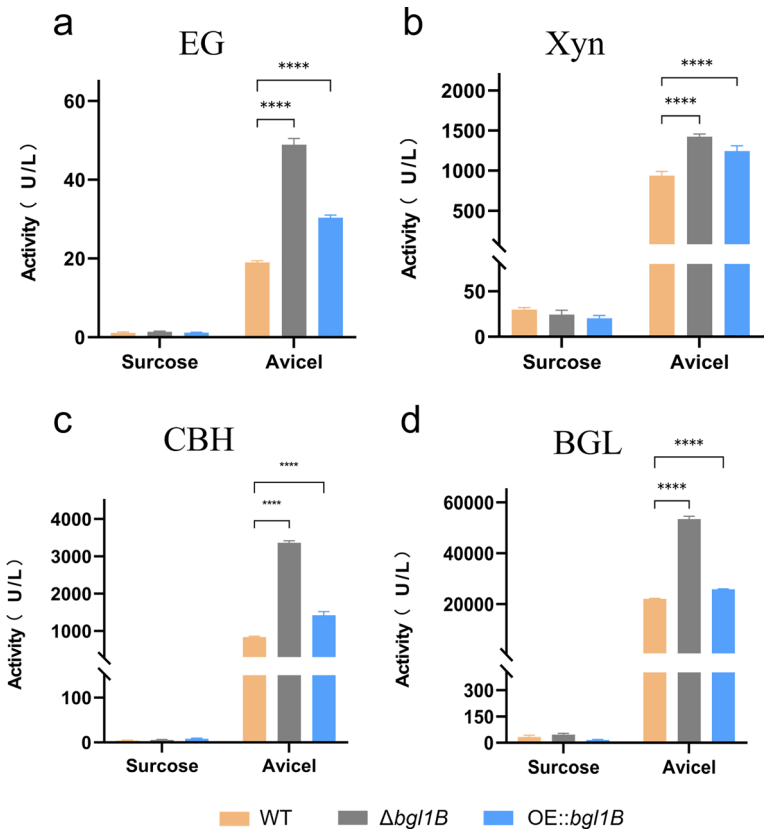

Fig. 4 The main lignocellulose-degrading enzymes' activities of the three strains after cultured with sucrose or Avicel as a carbon source for 72 h. **a** Endoglucanase activity. **b** Xylanase activity. **c** Cellobiohydrolase activity. **d** β-Glucosidase activity. Asterisks indicate significant differences (**** $p < 0.0001$)

transcriptomes were analyzed. For secreted proteome analysis, the above incubations were kept for 24 h, and the supernatants of the mixtures were subject to mass spectrometry analysis.

The RNA-seq results of the 3 strains showed significant differences in the expression level of lignocellulose-degrading enzyme genes (Table 1). The strain Δ*bgl1B* had 6 lignocellulose-degrading enzyme genes with significantly increased expression levels compared with those in WT, including 4 cellulase genes *gun4* (An14g02670), *gun4* (An04g08550), *bglB* (An06g02040), and putative β-glucosidase (An15g01890), as well as a hemicellulase gene *xynA* (An01g14600) and a cellobiose dehydrogenase gene *cdh* (An02g09270). All these genes in OE::*bgl1B* also showed increased expression levels compared with those in WT, although the increase was not as remarkable as that in Δ*bgl1B*. It indicated that *bgl1B* did not exhibit a typical negative regulatory effect on lignocellulose-degrading enzyme genes' expression, that lignocellulose-degrading enzyme genes' expression increased when *bgl1B* was knocked out while decreased when *bgl1B* was overexpressed. The enhanced effect that both Δ*bgl1B* and OE::*bgl1B* showed on the expression level of lignocellulose-degrading enzyme genes compared to WT was precisely the same as that on activities of lignocellulose-degrading enzymes.

At the same time, RNA-seq showed that Δ*bgl1B* caused the upregulation of some transcriptional activators, such as XlnR, ClrA, and AraR, and the downregulation of transcriptional repressor CreA compared to WT. In OE::*bgl1B*, the transcription activators XlnR and ClrA also showed upregulated expression compared to WT.

The secreted proteome of the three strains was additionally analyzed (Table 2). A total of 22 lignocellulose-degrading enzymes were found to have peptides matched in mass spectra. All the detected lignocellulose-degrading

Table 1 Differential gene expression of lignocellulose-degrading enzymes in 3 strains under Avicel carbon source

Locus	Gene name	Gene annotation	WT Average FPKM	Δ*bgl1B* Average FPKM	OE::*bgl1B* Average FPKM
An14g02670	*gun4*	Endoglucanase	2.643	9.433	3.510
An04g08550	*gun4*	Endoglucanase	0.137	0.443	0.250
An02g09270	*cdh*	Cellobiose dehydrogenase	4.297	13.293	5.173
An04g10400	*abr2*	Laccase abr2	0.013	0.040	0.013
An15g01890	–	Putative β-glucosidase	8.713	22.787	13.857
An01g00330	*abfA*	α-L-Arabinofuranosidase	11.100	6.510	7.223
An07g09760	*bglD*	β-Glucosidase	1.877	1.080	1.617
An12g05810	*lac1*	Laccase ARB	0.113	0.063	0.040
An07g08950	*eglB*	Endoglucanase	121.940	65.417	117.603
An05g02540	*abr2*	Laccase abr2	0.060	0.027	0.087
An12g05010	*axeA*	Acetylxylan esterase	185.057	84.710	121.027
An08g05230	-	Putative endoglucanase	6.217	3.523	3.283
An05g02340	*lac2*	Laccase 2	0.140	0.060	0.067
An14g02670	-	Putative endoglucanase	9.433	3.510	2.643
An01g09960	*xlnD*	Xylosidase	67.590	28.153	40.960
An14g07390	*xyn5*	β–1,4-Endo-xylanase	72.457	29.490	35.130
An15g04550	*xynB*	β–1,4-Endo-xylanase	567.910	232.683	327.447
An08g01760	*cbhC*	β–1,4-Cellobiohydrolase	51.973	20.923	27.493
An01g00780	*xynA*	β–1,4-Endo-xylanase	148.387	56.103	108.920
An09g00880	*abfA*	α-L-Arabinofuranosidase	0.053	0.013	0.013
An07g09330	*cbhA*	β–1,4-Cellobiohydrolase	31.673	7.767	7.347
An12g02220	*cbhC*	β–1,4-Cellobiohydrolase	31.627	6.447	14.287
An12g04610	*gun4*	Endoglucanase	954.653	191.647	378.207
An03g00940	*xynF1*	β–1,4-Endo-xylanase	6.247	0.900	1.830
An01g14600	*xynA*	β–1,4-Endo-xylanase	0.110	0.183	0.373
An06g02040	*bglB*	β-Glucosidase	1.670	3.033	3.443
An09g00790	*abfA*	Acetylxylan esterase	1.140	1.097	0.573
An01g13660	*abr2*	Laccase abr2	0.850	0.537	0.293

FPKM: fragments per kilobase per million mapped fragments; differential genes shown in the table referred to genes with $|\log2(FC)| \geq 1.5$ (here FC meant fold change of gene expression)

Table 2 Mass spectrometry detection of lignocellulose-degrading enzymes in different strains incubated on Avicel for 24 h

Protein ID	Protein annotation	WT PSM	Δ*bgl1B* PSM	OE::*bgl1B* PSM
A0A2H4YI50	β−1,4-Endoglucanase	9	72	43
A0A100I3X0	β−1,4-Endoglucanase	ND	25	5
A0A100IID9	β−1,4-Endoglucanase	61	142	81
A0A100IKG6	β−1,4-Endoglucanase	ND	63	29
A0A100IKJ9	β−1,4-Endoglucanase	ND	15	ND
A0A117E1I3	Putative β−1,4-endoglucanase	44	99	43
A0A1V1G264	β-Glucosidase	24	185	101
A0A100IJJ3	β-Glucosidase	ND	23	ND
A0A370BY04	β-Glucosidase	ND	99	10
A0A370C0H7	Putative β-glucosidase	16	112	35
A0A370CH70	Putative β-glucosidase	ND	256	ND
A0A124BXC2	Putative β-glucosidase	ND	6	ND
A0A0S2CW27	β−1,4-Endo-xylanase	61	514	249
Q9C1G6	β−1,4-Endo-xylanase	184	4652	1390
A0A223GCX3	β−1,4-Endo-xylanase	ND	23	10
U6C0N2	β−1,4-Endo-xylanase	ND	23	5
U6C186	Putative β−1,4-xylanase	21	340	177
A0A319A8S1	α-L-Arabinosidase	135	199	129
A0A060IP77	α-L-Arabinofuranosidase	ND	17	ND
A0A100I6G0	α-L-Arabinofuranosidase	70	219	77
A0A0S2CVZ9	α-L-Arabinofuranosidase	310	572	360
A0A2Z4FSY9	α-L-Arabinofuranosidase	10	63	34

PSM represents the number of peptide spectrum matches detected in each 100 mL of the supernatant of the incubation mixture

ND: not detected

enzymes in Δ*bgl1B* showed higher peptide spectrum match (PSM) values than those in WT, among which β−1,4-endo-xylanase (Q9C1G6) had the highest PSM value with a 25.3-fold increase compared to that in WT. Similarly, nearly all of the detected lignocellulose-degrading enzymes in OE::*bgl1B* showed higher PSM values than those in WT, and β−1,4-endo-xylanase (Q9C1G6) had a 7.5-fold PSM value compared to that in WT. The results of the mass spectrometry analysis of the 3 strains were consistent with the activity analysis of lignocellulose-degrading enzymes.

The gene expressions and the peptide mass spectra of lignocellulose-degrading enzymes of Δ*bgl1B* and OE::*bgl1B* showed an increased trend compared to WT's. The results could not be explained by simply regarding *bgl1B* as a negative control factor for the synthesis of lignocellulose-degrading enzymes. We then speculated that some positive factors in OE::*bgl1B* might be relevant to lignocellulose-degrading enzymes' synthesis. Given that the intracellularly located BGL1B could form transglycosylation products sophorose and laminaribiose, it is reasonable to presume that the overexpression of *bgl1B* in OE::*bgl1B* may produce sophorose or laminaribiose which acts as an inducer of lignocellulose-degrading enzymes' synthesis and promotes OE::*bgl1B* to produce more enzymes than WT. Therefore, the induction effect of sophorose and laminaribiose on lignocellulose-degrading enzymes' synthesis will be investigated.

Analysis of the induction effect of sophorose and laminaribiose

We used Δ*bgl1B* as the experimental strain to avoid any possible influence of intracellular BGL1B's hydrolysis and transglycosylation activities. An equal amount of mycelium of Δ*bgl1B* was transferred into Mandel's medium supplemented with various saccharides (10 g/L sucrose, 1 g/L sophorose, or 1 g/L laminaribiose), respectively. After incubation for 6 h, the mycelium was collected for transcriptome sequencing. After incubation for 24 h, the mixture supernatant was collected for enzyme activity assay and secreted proteome analysis.

Overall, the activities of the 4 lignocellulose-degrading enzymes of Δ*bgl1B* in the sophorose and laminaribiose groups were higher than those in the sucrose group (Fig. 5). Especially for CBH and BGL, the activities in the sophorose group and laminaribiose group were significantly increased, with at least 7.5-fold higher compared with those in the sucrose group, suggesting that both sophorose and laminaribiose promoted the production of lignocellulose-degrading enzymes of Δ*bgl1B* strain.

The gene expression also showed significant differences between the sophorose or laminaribiose and sucrose groups (Table 3). 17 lignocellulose-degrading enzyme genes were upregulated in the sophorose group compared to those in the sucrose group. The significantly upregulated genes were β-glucosidase genes *bgl1* (An18g03570), *bglB* (An06g02040), and *bglM* (An14g01770), and hemicellulose genes *xynB* (An15g04550), *abfB* (An15g02300), and *abnC* (An02g10550). Similarly, 14 genes were upregulated in the laminaribiose group compared to those in the sucrose group. At the same time, the expression levels of related transcriptional activators (XlnR, AraR) were upregulated in both the sophorose and laminaribiose groups. It indicated that both sophorose and laminaribiose could induce the expression of a large amount of lignocellulose-degrading enzyme genes.

As for the secreted proteome analysis, the PSM values of 16 lignocellulose-degrading enzymes in the sophorose group were found to increase compared to those in the sucrose group, including 7 cellulases and 9 hemicellulases (Table 4). Similarly, the laminaribiose group had 19 lignocellulose-degrading enzymes with increased PSM values compared to those in the sucrose group, including 9

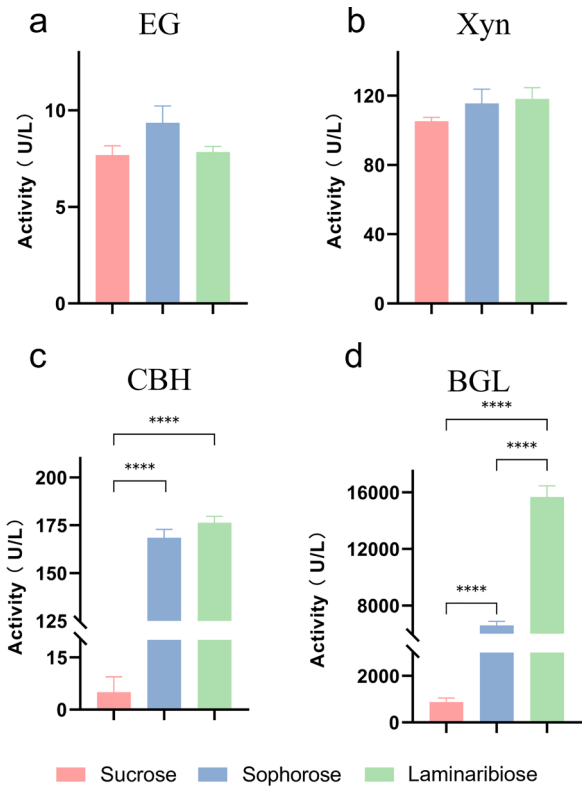

Fig. 5 Activities of the main lignocellulose-degrading enzymes of Δ*bgl1B* after incubation on different saccharides. **a** Endoglucanase activity. **b** Xylanase activity. **c** Cellobiohydrolase activity. **d** β-Glucosidase activity. Asterisks indicate significant differences (****$p < 0.0001$)

cellulases and 10 hemicellulases. Overall, most of the lignocellulose-degrading enzymes of Δ*bgl1B* in sophorose and laminaribiose groups showed significantly increased production compared to those in sucrose group, along with the above enzyme activity and gene expression results in these 3 groups, strongly suggesting that BGL1B transglycosylation products sophorose and laminaribiose are involved in inducing the synthesis of lignocellulose-degrading enzymes in *A. niger*.

The function of BGL1B in the expression of lignocellulose-degrading enzyme genes in *A. niger*

The analyses of biomass and lignocellulose-degrading enzymes' activity of WT, Δ*bgl1B*, and OE::*bgl1B* strains cultured on Avicel carbon source, as well as the transcriptome and secreted proteome analyses clearly showed that the intracellular β-glucosidase BGL1B of *A. niger* was involved in the regulation of lignocellulose-degrading enzyme genes expression, the mechanism of which seemed to be intriguing. By comparing Δ*bgl1B* with WT when cultured on an Avicel carbon source, we can see that knockout of *bgl1B* promoted the expression of lignocellulose-degrading enzyme genes. Thus, BGL1B might be a negative regulatory factor in the synthesis of lignocellulose-degrading enzymes. However, the lignocellulose-degrading enzyme genes in overexpression strain OE::*bgl1B* did not exhibit the repressed expression, which was not a typical mode for negative regulation. OE::*bgl1B* showed the promoted expression of lignocellulose-degrading enzyme genes compared to WT. The subsequent induction experiment of BGL1B transglycosylation products sophorose and laminaribiose confirmed that both sophorose and laminaribiose had an induction effect on lignocellulose-degrading enzyme production. That helped explain why OE::*bgl1B* had a higher expression level of lignocellulose-degrading enzyme genes and higher enzyme activity than WT. Although the overexpression of *bgl1B* in OE::*bgl1B* could promote the hydrolysis of cellodextrin into glucose, causing CCR on the synthesis of lignocellulose-degrading enzymes, the cellular environment in OE::*bgl1B* might also be beneficial to the production of sophorose and laminaribiose through BGL1B's transglycosylation action, and the overproduction of sophorose and laminaribiose induced the expression of lignocellulose-degrading enzyme genes. In OE::*bgl1B*, the induction effect of sophorose and laminaribiose outperformed the CCR effect caused by BGL1B's hydrolysis product glucose. Eventually, OE::*bgl1B* showed a high expression of lignocellulose-degrading enzyme genes. Therefore, we suggest that the intracellular β-glucosidase BGL1B plays dual roles in regulating the synthesis of lignocellulose-degrading enzymes in *A. niger*, that is, the repressive role caused by BGL1B's hydrolysis product glucose and the induction role caused by BGL1B's transglycosylation products sophorose and laminaribiose. The potency of each role depends on the conditions under which *A. niger* cells grow, and ultimately BGL1B may display a different regulation effect in different strains.

Based on the interpretation above, the mechanism of BGL1B in the regulation of the expression of lignocellulose-degrading enzyme genes in *A. niger* was proposed (Fig. 6). When *A. niger* grows on a cellulosic substrate, a small amount of cellulase produced constitutively hydrolyzes cellulose to produce cellodextrins, which are transported into cells by cellodextrin transporter. Then BGL1B hydrolyzes cellodextrin to produce glucose, repressing the expression of lignocellulose-degrading enzyme genes through the transcriptional repressor CreA; at the same time, BGL1B can generate sophorose and laminaribiose through transglycosylation, which plays as inducers of transcription factors such as XlnR and ClrA through currently unknown pathways, thereby leading to the expression of lignocellulose-degrading enzyme genes. Both the repressive role and the induction role are involved in the

Table 3 Differential expression of lignocellulose-degrading enzyme genes of Δ*bgl1B* after incubated on different saccharides

Locus	Gene name	Gene annotation	Sucrose Average FPKM	Sophorose Average FPKM	Laminaribiose Average FPKM
An15g02300	*abfB*	α-L-Arabinofuranosidase	0.273	7.093	10.503
An18g03570	*bgl1*	β-Glucosidase	5.203	72.367	60.620
An07g07630	*bglG*	β-Glucosidase	0.100	1.037	0.827
An08g01710	*abfC*	α-L-Arabinofuranosidase	1.143	9.767	7.293
An15g04550	*xynB*	β−1,4-Endo-xylanase	31.940	130.650	20.720
An01g09960	*xlnD*	Xylosidase	0.607	2.290	1.270
An12g05010	*axeA*	Acetylxylan esterase	0.377	1.250	0.357
An14g01770	*bglM*	β-Glucosidase	1.353	3.783	2.883
An06g02040	*bglB*	β-Glucosidase	1.090	2.963	2.710
An12g04610	*gun4*	Endoglucanase	2.823	7.567	4.980
An11g06090	–	Putative β-glucosidase	1.077	2.827	1.527
An02g10550	*abnC*	α-L-Arabinosidase	54.017	138.320	50.587
An11g00200	*bglM*	β-Glucosidase	1.707	3.807	2.440
An01g14600	*xynA*	β−1,4-Endo-xylanase	0.823	1.803	0.210
An01g13660	*abr2*	Laccase abr2	3.120	6.037	2.123
An09g01010	–	Acetylxylan esterase	0.933	0.403	0.643
An08g08240	*bglD*	β-Glucosidase	0.053	0.020	0.040
An04g10400	*abr2*	Laccase abr2	0.040	0.013	0.027
An01g00780	*xynA*	β−1,4-Endo-xylanase	42.730	14.630	12.940
An05g02540	*abr2*	Laccase abr2	0.153	0.043	0.057
An12g05810	*lac1*	Laccase ARB	0.060	0.013	0.037
An01g11660	*cbhB*	β−1,4-Exoglucanase	8.240	1.780	4.080
An12g02220	*cbhC*	β−1,4-Exoglucanase	0.170	0.180	0.530
An07g09760	*bglD*	β-Glucosidase	0.833	0.747	1.740
An14g02670	*gun4*	Endoglucanase	6.297	4.640	12.280
An09g00880	*abfA*	α-L-Arabinofuranosidase	0.200	0.170	0.370
An16g06800	*eglB*	Putative endoglucanase	84.323	64.273	36.577
An10g00390	*cdh*	Cellobiose dehydrogenase	42.597	23.683	15.433
An01g01870	*xg74*	Xyloglucanase	0.450	0.407	0.153
An11g03580	*lac2*	Laccase ARB	0.027	0.030	0.000
An04g08550	*gun4*	Endoglucanase	0.787	0.510	0.213
An03g00940	*xynF1*	β−1,4-Endo-xylanase	7.700	13.153	1.253

FPKM: fragments per kilobase per million mapped fragments; differential genes shown in the table referred to genes with |log2(FC)|≥ 1.5 (here FC meant fold change of gene expression)

regulation of the synthesis of lignocellulose-degrading enzymes in *A. niger*, and the eventual amount of lignocellulose-degrading enzymes produced is dependent on the conditions cells are located in.

Discussion

β-Glucosidase, an important component of lignocellulose-degrading enzymes, degrades cellodextrin to glucose, the last step of cellulose degradation. It has been reported that β-glucosidase regulates lignocellulose-degrading enzymes' synthesis. Znameroski et al. [24] confirmed that β-glucosidases in *Neurospora crassa* masked the inducing role of cellobiose by the CCR effect. When the three β-glucosidases were deleted, cellobiose could function as an inducer of lignocellulolytic gene expression. Similarly, studies have shown that a constitutive extracellular β-glucosidase in *P. purpurogenum* and β-glucosidase BGL3I in *T. reesei* can also cause CCR by hydrolyzing inducers to produce glucose, thereby repressing the expression of cellulose-degrading enzyme genes [11, 25]. Contrary to the repressive effect of β-glucosidases, another type of β-glucosidase Cel1A, in *T. reesei* was confirmed to play an induction role in the expression of cellulose-degrading enzyme genes by

Table 4 Mass spectrometric detection of lignocellulose-degrading enzymes of Δ*bgl1B* after incubation on different saccharides

Protein ID	Protein annotation	Sucrose PSM	Sophorose PSM	Laminaribiose PSM
A0A100I3X0	β−1,4-Endoglucanase	1	37	18
A0A117E2M7	β−1,4-Endoglucanase	3	25	18
A0A2H4YI50	β−1,4-Endoglucanase	ND	ND	50
A0A319AEB8	β−1,4-Endoglucanase	ND	25	18
A0A100IID9	β−1,4-Endoglucanase	1	41	39
A0A117E1I3	Putative β−1,4-endoglucanase	26	57	46
A0A100IMP4	Putative β−1,4-endoglucanase	ND	ND	7
K7WQK7	β-Glucosidase	5	29	36
A0A505IG16	β-Glucosidase	2	ND	ND
A0A370C0H7	Putative β-glucosidase	ND	61	43
A0A0S2CW27	β−1,4-Endo-xylanase	ND	33	ND
A0A100I6U9	β−1,4-Endo-xylanase	ND	4	7
Q9C1G6	β−1,4-Endo-xylanase	6	110	110
A0A100IKK3	Acetylxylan esterase	58	2126	163
A0A117E0W8	α-L-Arabinosidase	3	ND	ND
A0A319A8S1	α-L-Arabinosidase	ND	ND	32
A0A0S2CWE4	α-L-Arabinofuranosidase	ND	4	21
A0A117DZC8	α-L-Arabinofuranosidase	ND	33	67
A0A0S2CVZ9	α-L-Arabinofuranosidase	ND	ND	14
A0A100I6G0	α-L-Arabinofuranosidase	8	65	39
A0A068FLM3	Ferulic acid esterase	ND	74	75
A0A100IRB1	Ferulic acid esterase	ND	25	53

PSM represents the number of peptide spectrum matches detected in each 100 mL of the mixture supernatant

ND: not detected

producing sophorose through Cel1A's transglycosylation [26]. *A. niger* is well-known for its ability to secret a variety of extracellular β-glucosidases. Seidle and Huber [27] reported that *A. niger*'s major secreted β-glucosidase, a Family 3 β-glycosidase could form gentiobiose from cellobiose through its transglycosylation activity. They proposed that gentiobiose might be an inducer of the *A. niger* cellulase system, but no further evidence followed. Since *A. niger* could produce abundant extracellular β-glucosidases, the intermediate products of cellulose hydrolysis, cellodextrins are typically considered to be fully degraded to glucose which is transported into cells. However, we believe that there is still another strategy for *A. niger* to use cellodextrins, a strategy to absorb cellodextrins into cells and degrade them intracellularly, given that the cellodextrin transporter CtA [28] as well as the intracellular β-glucosidase BGL1B in *A. niger* [14] have been identified. Therefore, we presumed that BGL1B might be involved in the regulation of the synthesis of lignocellulose-degrading enzymes in *A. niger*.

When Δ*bgl1B* and OE::*bgl1B* were compared with WT, we can see that both *bgl1B* knockout and *bgl1B* overexpression had an enhanced effect on expression of lignocellulose-degrading enzyme genes, which is not a typical mode for gene function identification through knockout and overexpression method. By proving that BGL1B's transglycosylation products sophorose and laminaribiose could induce the expression of lignocellulose-degrading enzyme genes individually, we propose that the intracellular BGL1B plays dual regulatory roles in the synthesis of lignocellulose-degrading enzymes in *A. niger*, the repressive role caused by BGL1B's hydrolysis product glucose and the induction role caused by BGL1B's transglycosylation products sophorose and laminaribiose. Further investigation should be done to confirm the induction effect of sophorose and laminaribiose in OE::*bgl1B*, such as analyzing the content of intracellular sophorose and laminaribiose in both OE::*bgl1B* and WT strains.

The involvement of the intracellular β-glucosidase BGL1B in the regulation of the synthesis of lignocellulose-degrading enzymes in *A. niger* is different from that in *T. reesei*. The intracellular β-glucosidases Cel1A and Cel1B in *T. reesei* play a key role in the induction of cellulose-degrading enzymes, especially Cel1A, which induces the expression of cellulose-degrading enzymes

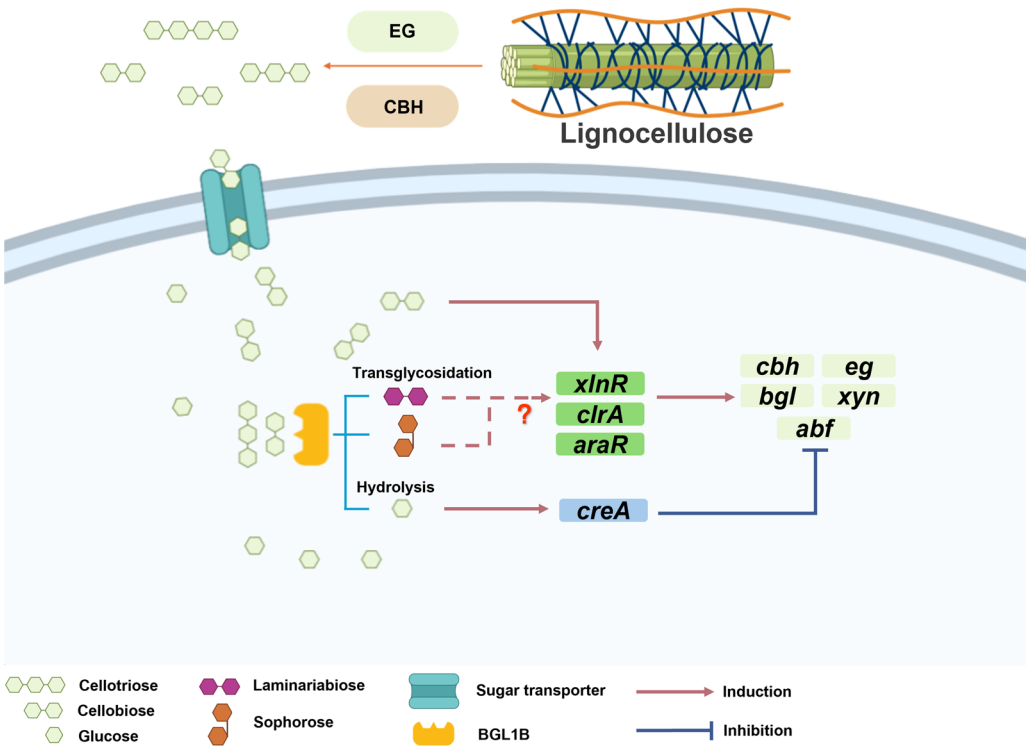

Fig. 6 Schematic diagram of the involvement mechanism of BGL1B in the regulation of lignocellulose-degrading enzymes' synthesis in *A. niger*

by generating sophorose through transglycosylation. Knockout of Cel1A and Cel1B significantly represses the expression of cellulose-degrading enzymes [29]. However, another intracellular β-glucosidase BGL3I can hydrolyze sophorose formed by Cel1A's transglycosylation and hence plays a negative role in the expression of cellulose-degrading enzyme genes by degrading the potential inducer sophorose [10, 26]. As such, different types of intracellular β-glucosidases in *T. reesei* are responsible for various regulatory roles. In contrast, BGL1B in *A. niger* itself showed dual roles in regulating the expression of lignocellulose-degrading enzyme genes through its hydrolysis and transglycosylation actions. Yet little is known currently about what conditions cause hydrolysis predominant or otherwise transglycosylation predominant. We assume that when cellobiose concentration in the cell is low due to a certain extracellular circumstance, the hydrolysis effect may be predominant while when cellobiose concentration in the cell is high, the transglycosylation may be predominant and consequently the transglycosylation products cause a positive regulation on lignocellulose-degrading enzyme synthesis.

From a practical perspective, the dual regulatory roles of the intracellular BGL1B in *A. niger* on the synthesis of lignocellulose-degrading enzymes provide a solution for creating a strain with high production of lignocellulose-degrading enzymes. Here, we found both knockout and overexpression of *bgl1B* could improve the production of lignocellulose-degrading enzymes. When these two kinds of modified strains were compared, the knockout strain Δ*bgl1B* showed a higher increase in activity of lignocellulose-degrading enzymes on cellulosic carbon source than the overexpression strain OE::*bgl1B*. As shown in Fig. 4, the EG activity of Δ*bgl1B* was increased by 157% while that of OE::*bgl1B* increased by 60%, and the CBH activity of Δ*bgl1B* was increased by 302% while that of OE::*bgl1B* increased by 70%, all compared with WT. Similar phenomena were also found in the activities of Xyn and BGL. Therefore, *bgl1B* knockout is a more promising way than *bgl1B* overexpression to enhance the capacity of *A. niger* to produce lignocellulose-degrading enzymes.

Conclusions

The intracellular β-glucosidase BGL1B in *A. niger* was proved to be involved in the regulation of the synthesis of lignocellulose-degrading enzymes. We proposed that BGL1B played dual regulatory roles: the repressive role caused by BGL1B's hydrolysis product glucose and the induction role caused by BGL1B's transglycosylation products sophorose and laminaribiose. This study broadens the understanding of the regulatory network of the

synthesis of lignocellulose-degrading enzymes in *A. niger* and also provides a strategy based on *bgl1B* to create an engineered strain with high production of lignocellulose-degrading enzymes.

Author contributions
KC and HC conceived and designed the research. ZZ, Hua Li and FD conducted experiments. Hui Lin and YL analyzed the data. ZZ and HC wrote the manuscript. All authors read and approved the manuscript.

Funding
This work was supported by the National Natural Science Foundation of China (Grant number 32171476).

Availability of data and materials
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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Received: 16 December 2024 Accepted: 15 January 2025
Published online: 27 January 2025

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