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High-Level Expression of β-Glucosidase in *Aspergillus niger* ATCC 20611 Using the *Trichoderma reesei* Promoter P*cdna1* to Enhance Cellulose Degradation

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Abstract: β-glucosidase is a key component of cellulase for its function in hydrolyzing cellobiose to glucose in the final step of cellulose degradation. The high-level expression of β-glucosidase is essential for cellulose conversion. *Aspergillus niger* ATCC 20611 has the potential for efficient protein expression because of its ability to secret enzymes for the industrial production of fructooligosaccharides, but it lacks robust promoters for high-level protein expression. Here, the development of *A. niger* 20611 as a powerful protein expression system exploited the conserved constitutive promoter P*gpd1* of the glyceraldehyde-3-phosphate dehydrogenase-encoding gene from *Trichoerma reesei* to drive the expression of the enhanced green fluorescent protein in *A. niger* ATCC 20611. The mycelium of the transformant AGE9 exhibited intense fluorescence. Then, the promotor P*gpd1* was used to drive the expression of β-glucosidase and the enzyme activity of transformants AGB1 and AGB33 were 1.02 and 0.51 U/mL, respectively. These results demonstrate that the promotor P*gpd1* from *T. reesei* was applicable for *A. niger* ATCC 20611. Furthermore, the *T. reesei*-specific robust promoter P*cdna1* was used to drive the expression of β-glucosidase. The β-glucosidase exhibited a high-level expression with a yield of 15.2 U/mL, which was over 13.9 times higher than that driven by the promoter P*gpd1*. The β-glucosidase was thermally stable and accounted for 85% of the total extracellular proteins. Subsequently, the fermentation broth including β-glucosidase was directly added to the cellulase mixture of *T. reesei* for saccharification of the acid-treated corncob residues and the delignified corncob residues, which increased the saccharification efficiency by 26.21% and 29.51%, respectively. Thus, β-glucosidase exhibited a high level of expression in *A. niger* ATCC 20611 and enhanced cellulose degradation by addition in vitro. In addition, the robust promoter P*cdna1* of *T. reesei* could drive the high-level expression of protein in *A. niger* ATCC 20611. These results demonstrate that the promoters in filamentous fungi could be employed across species in *A. niger* ATCC 20611 and further facilitated the efficient expression of β-glucosidase to optimize cellulases for efficient cellulose transformation.

Keywords: β-glucosidase; *A. niger* ATCC 20611; promoter; cellulose degradation; *T. reesei*

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1. Introduction

The filamentous fungus *Aspergillus niger* ATCC 20611 is an industrial strain for fructooligosaccharides production, which can grow rapidly with sucrose as a carbon source and express β-fructofuranosidase at a high level [1]. The β-fructofuranosidase catalyzes the generation of fructooligosaccharides from sucrose and it is located in the cell wall. Thus, *A. niger* ATCC 20611 has a relatively low extracellular protein background, which is suitable for heterologous protein expression [2]. Because the regulation of protein expression mainly occurred at the transcriptional level, appropriate promoters were crucial for the efficient expression of heterologous proteins [3]. The promoter P*fopA* of the β-fructofuranosidase-encoding gene was sucrose-inducible and its strength enhanced with

the increase in sucrose concentration. So far, several heterologous proteins have been successfully expressed in *A. niger* ATCC 20611, including glucose oxidase, peroxidase, chitinase, β-N-acetylglucosaminidase and so on [4,5]. Nevertheless, when employing the promoter P*fopA* to drive heterologous protein expression, sucrose induction caused an abundant expression of β-fructofuranosidase, which would occupy the resources and space of heterologous protein expression. It is known that constitutive promoters can spontaneously drive continuous and stable transcription of genes without relying on carbon or nitrogen sources or other inducers [6]. The mostly used constitutive promoter is the promotor P*gpdA* of the glyceraldehyde-3-phosphate dehydrogenase-encoding gene (*gpd*) from *Aspergillus nidulans*, which has been successfully used in *A. niger* ATCC 20611 for expression of the enhanced green fluorescent protein and amino-acid-substitution variant FopA [1,2]. The promotor P*gpd* is from the glycolytic pathway, which was widely distributed and relatively conserved in different species. Currently, *A niger* ATCC 20611 lacks constitutive promoters with strong driving ability for the construction of a heterologous protein expression cassette [5]. It was reported that the specific promoter P*cdna1* in *T. reesei* was a strong constitutive promoter, which was identified through screening cDNA libraries and much stronger than the commonly used glycolysis pathway promoters P*gpd1* and *Ptef1* [7]. The expression of CBHI and EGI in *T. reesei* using P*cdna1* produced 10–50 times more cellulase than that of using P*tef1* and de-repressed P*cbh1* [8]. Currently, P*cdna1* is regarded as the strongest constitutive promoter and the optimal choice for constitutive overexpression or recombinant protein production in *T. reesei*.

Lignocellulose is the largest renewable biomass resource in the world and its effective treatment and utilization are of great importance for the sustainable development of society. The cellulase mixtures secreted by *T. reesei* can effectively degrade cellulose. Cellulase 1 (CBH1) and endoglucanase (EGs) jointly hydrolyze cellulose to produce cellulose oligosaccharide and cellulose disaccharide and then the product is further degraded to glucose by β-glucosidase (BGL) [9,10]. However, in the cellulase mixture of *T. reesei*, the BGL accounts for only about 1%, which is a rate-limiting step for effective hydrolysis of the cellulose substrate [11]. The addition of purified β-glucosidase enzyme in vitro can effectively improve the efficiency of cellulose hydrolysis, but the enzyme purification increases the time and cost [12,13]. Therefore, the utilization of an expression host with low-protein background to express β-glucosidase can effectively address the challenge of protein purification and enhance the efficiency of cellulose degradation.

In this study, we first used the species-conserved promoter P*gpd1* from *T. reesei* expressing the enhanced green fluorescent protein and β-glucosidase in *A. niger* ATCC 20611 to validate the feasibility of heterologous promoters. Then, the unique strong promoter P*cdna1* from *T. reesei* was used to drive the efficient expression of β-glucosidase in *A. niger* ATCC 20611 and the addition of the crude fermentation broth of transformants to cellulase mixtures of *T. reesei* SN1 improved the saccharification efficiency of different pretreated corncob residues. Briefly, the unique strong constitutive promoter P*cdna1* from *T. reesei* was used to establish a heterologous protein expression system in *A. niger* ATCC 20611 and achieve a high level of β-glucosidase expression for enhancing the rate of cellulose degradation.

2. Materials and Methods

2.1. Strains and Culture Conditions

A. niger ATCC 20611, an industrial strain for fructooligosaccharides production, was used as the host strain for transformation. *A. niger* C112 was used to obtain the β-glucosidase-encoding gene (*bglA*). *T. reesei* QM9414 was used to amplify genes, containing the promoter of *gpd1* (P*gpd1*), the promoter of *cdna1* (P*cdna1*), the terminator of *cbh1* (T*cbh1*). The plasmid T-ptrA was used to access resistance genes *ptrA*. The plasmid pIG 1783 was used to access the enhanced green fluorescent protein-encoding gene (*egfp*). *T. reesei* SN1, a hypercellulytic strain, was used to express cellulases for cellulose hydrolysis [14].

The Potato Dextrose Agar plates (PDA) were used for spore production of strains, which included 200 g/L peeled potato extraction, 20 g/L glucose and 2% agar. The minimal medium (MM) with 0.3% PT was used for selection of transformant strains, including 20 g/L glucose, 5 g/L $(NH_4)_2 \cdot SO_4$, 15 g/L KH_2PO_4, 0.6 g/L $MgSO_4 \cdot 7H_2O$, 0.6 g/L $CaCl_2$, 2 g/L peptone and 100 μL/L trace elements ($FeSO_4 \cdot 7H_2O$, $MnSO_4 \cdot H_2O$, $ZnSO_4 \cdot 7H_2O$, $CoCl_2 \cdot 2H_2O$). In addition, 2% agar was added to obtain solid MM plates and 0.5% Triton was added to inhibit mycelium growth. The fermentation medium of *A. niger* strains was used to obtain fermentation supernatant for enzyme assay, which consisted of 20 g/L glucose, 2 g/L $NaNO_3$, 0.5 g/L KCl, 5 g/L K_2HPO_4, 0.5 g/L $MgSO_4 \cdot 7H_2O$ and 15 g/L yeast extract. The CMC-esculin plates were used for detection of the β-glucosidase activities of transformants, which contained 3 g/L esculin, 2 g/L $(NH_4)_2SO_4$, 0.5 g/L $MgSO_4 \cdot 7H_2O$, 0.5 g/L ferric citrate, 1 g/L KH_2PO_4,1 g/L yeast extract, 2‰ Triton X-100 and 2% agar. *A. niger* strains were cultured at 30 °C, 200 rpm for 5 days. To obtain cellulase, the spores of *T. reesei* were inoculated into 50 mL of seed culture medium (except for the glucose content of 10 g/L, the other components were the same as those of the minimal medium) at a concentration of 10^6 spores/mL and cultured at 30 °C, 200 rpm for 1.5 days to obtain seed liquid. Then, according to the inoculation amount of 10%, the seed liquid was added to 100 mL of *T. reesei* fermentation medium and cultured at 30 °C, 200 rpm for 7 days. The *T. reesei* fermentation medium contained 20 g/L corn steep liquor, 20 g/L microcrystalline cellulose, 5 g/L KH_2PO_4, 5 g/L $(NH_4)_2SO_4$, 0.6 g/L $MgSO_4 \cdot 7H_2O$, 1 g/L $CaCl_2$ and 100 μL/L trace elements ($FeSO_4 \cdot 7H_2O$, $MnSO_4 \cdot H_2O$, $ZnSO_4 \cdot 7H_2O$, $CoCl_2 \cdot 2H_2O$).

2.2. Construction of Expression Strains

The *gpd1* promoter-driven enhanced green fluorescent protein (EGFP) expression cassette was constructed as follows. Firstly, the promoter of *gpd1* (1.4 kb) and the terminator of *cbh1* (1.7 kb) were amplified from the genome of *T. reesei* QM9414 using the primer pairs gpd1-UF/gpd1-UR and cbh1-1DF/cbh1-1690DR, respectively. The complete fragment of *ptrA* (2.1 kb, containing its own promoter and terminator) with the *gpd1* promoter homologous sequence at the 3′ end was amplified from the plasmid T-ptrA using the primer pairs ptrA-F/ptrA-R (gpd1-UF). The *egfp* gene (0.7 kb) with the homologous sequence of the *gpd1* promoter at the 5′ end and of the *cbh1* terminator at the 3′ end was amplified from the plasmid pIG 1783 using the primer pairs egfp-1F (gpd1-UR)/egfp-720R (cbh1-1DF). Then these fragments were fused together by double-joint PCR. The *gpd1* promoter-driven EGFP expression cassette (5.6 kb) was amplified from the fused production using the primer pairs ptrA-F/cbh1-1690DR.

The *gpd1* promoter-driven β-glucosidase expression cassette was constructed as follows. Firstly, the promoter of *gpd1* (1.4 kb) and the terminator of *cbh1* (1.7 kb) were amplified from the genome of *T. reesei* QM9414 using the primer pairs gpd1-UF/gpd1-UR (SPcbh1) and cbh1-1DF/cbh1-1690DR, respectively. The complete fragment of *ptrA* (2.1 kb, containing its own promoter and terminator) with the *gpd1* promoter homologous sequence at the 3′ end was amplified from the plasmid T-ptrA using the primer pairs ptrA-F/ptrA-R (gpd1-UF). The *bglA* gene (3.0 kb) with the homologous sequence of the *cbh1* signal peptide at the 5′ end and of the *cbh1* terminator at the 3′ end was amplified from *A. niger* C112 using primer pairs bglA-F (SPcbh1)/bglA-R (cbh1-1DF). Then these fragments were fused together by double-joint PCR. The *gpd1* promoter-driven β-glucosidase expression cassette (8.2 kb) was amplified from the fused production using the primer pairs ptrA-F/cbh1-1690DR.

The *cdna1* promoter-driven β-glucosidase expression cassette was constructed as follows. Firstly, the promoter of *cdna1* (1.0 kb) with the homologous sequence of the *cbh1* signal peptide at the 3′ end and the terminator of *cbh1* (1.7 kb) were amplified from the genome of *T. reesei* QM9414 using the primer pairs gpd1-UF/gpd1-UR (SPcbh1) and cbh1-1DF/cbh1-1690DR, respectively. The complete fragment of *ptrA* (2.1 kb, containing its own promoter and terminator) with the *gpd1* promoter homologous sequence at the 3′ end was amplified from the plasmid T-ptrA using the primer pairs ptrA-F/ptrA-R (gpd1-UF).

The *bglA* gene (3.0 kb) with the homologous sequence of the *cbh1* signal peptide at the 5′ end and of the *cbh1* terminator at the 3′ end was amplified from *A. niger* using primer pairs bglA-F (SPcbh1)/bglA-R (cbh1-1DF). Then these fragments were fused together by double-joint PCR. The *cdna1* promoter-driven β-glucosidase expression cassette (7.8 kb) was amplified from the fused production using the primer pairs ptrA-F/cbh1-1690DR.

The primers used in this study are listed in Table S1. The expression cassettes were transformed into *A. niger* ATCC 20611 using polyethylene glycol (PEG)-mediated protoplast transformation system [2].

2.3. Fluorescence and Light Microscopy

For the detection of EGFP expression, the spores of the transformant strains were planted on MM solid medium with coverslips and incubated for 48 h at 30 °C. Fluorescence and light microscopy (Nikon Eclipse 80i fluorescence microscope, Tokyo, Japan) were used to observe the fluorescence of the mycelia on the coverslips [15].

2.4. RNA Extraction and RT-qPCR Analysis

RNA was extracted from the transformant strains of *A. niger* on the first day of fermentation using RNAiso™ reagent (TaKaRa, Dalian, China). According to the manufacturer's protocol, RNA was converted into cDNA using the PrimeScript RT Kit (TaKaRa, Dalian, China). Then, quantitative real-time PCR was performed using a LightCycler 480 System (Roche Diagnostics, Mannheim, Germany) using SYBR Premix Ex Taq™ (Tli RNaseH Plus) Kit (Takara, Osaka, Japan). The CT value was calculated by LightCycler 480 software version 1.5.1.62 (Roche, Vienna, Austria). The transcript levels of *egfp*, *bgl*A, *bip1*, *pdi1*, *yos9*, *der1* were normalized using the *actin* gene as the endogenous control. The primers used in this study for qRT-PCR are listed in Table S1.

2.5. Enzyme Assay and SDS PAGE

The spores of the transformed strains were inoculated into the fermentation medium with 2% glucose as a carbon source at a concentration of 10^6 spores per mL. The fermentation supernatant was collected for enzyme activity analyses and protein detection. The activity of β-glucosidase was determined using p-Nitrophenyl-β-d-glucopyranoside (pNPG) as the substrate. Specifically, 50 μL of 10 mM pNPG solution, 150 μL of citric acid buffer (pH 4.8) and 100 μL of appropriately diluted enzyme solution were mixed and incubated at 50 °C for 30 min. Then,150 μL10% Na_2CO_3 solution was used to terminate the reaction. Subsequently, absorbance was measured at 420 nm. One enzyme unit (U) was defined as the amount of enzyme liberating 1 μmol p-nitrophenol per min at 50 °C and pH 4.8.

We mixed the equal volume of fermentation supernatant with loading buffer and boiled the mixed solution at 100 °C for 5–10 min to denature the protein. Then, the proteins were loaded on a 12% sodium dodecyl sulfate–polyacrylamide separating gel and were separated electrophoretically at a constant voltage of 90 V. The gel was stained using fast protein staining buffer for 30 min, then recorded. And the predicted bands were confirmed to be β-glucosidase by MS identification with the Orbitrap Fusion Tribrid (Thermo Fisher Scientific, Waltham, U.S.) in the Core Facilities for Life and Environmental Sciences of Shandong University. The total protein concentration in the fermentation fluid was determined by Bradford's method.

2.6. Enzymatic Characterization

The optimum temperature of β-glucosidase was determined by measuring activities in the temperature range of 30–80 °C at pH 4.8. To assay thermostability, the residual activities were determined at pH 4.8 and 50 °C after preincubation of the fermentation broth without substrate at 50, 60 and 70 °C for 0.5 h to 10 h and the initial activity at pH 4.8 and 50 °C was defined as 100%. The optimum pH of BGLA was determined by measuring activities in the pH range 3–8 at 50 °C. To assay pH stability, the residual activities were determined at pH 4.8 and 50 °C after preincubation of the fermentation broth without substrate in citric

acid buffer of different pH for 24 h in 4 °C and the initial activity at pH 4.8 and 50 °C was defined as 100%. The relative enzyme activity was the ratio between the residual enzyme activity after pretreatment and the initial enzyme activity.

2.7. Saccharification of Different Pretreated Corncob Residues

The fermentation supernatant of *T. reesei* SN1 and *A. niger* ACB8 were collected and detected FPA and β-glucosidase activity, respectively. Acid-pretreated corncob residues and delignified corncob residues were used as substrate for saccharification and their components were different [16]. The saccharification reaction system was 30 mL, including 5% (*w*/*v*) pretreated corncob substrate, the fermentation broth of *T. reesei* SN1 with the FPA activity (10 U/g substrate), the fermentation broth of *A. niger* ACB8 with the β-glucosidase activity (equal to the β-glucosidase enzyme activity of SN1), 30 μL proclin 300 and with the addition of citric acid buffer (pH 4.8) to make up to 30 mL. Saccharification by adding fermentation broths of *A. niger* ACB8 was used as a control. The saccharification system proceeded at 50 °C and 200 rpm for 48 h. Then, the amount of glucose released from saccharification was measured with an SBA-40C biological sensor analyzer (BISAS, Jinan, China). Cellulose conversion was calculated according to Chen's method [9].

3. Results

3.1. The T. reesei gpd1 Promoter-Driven EGFP Expression in A. niger ATCC 20611

In filamentous fungi, the promoter of glyceraldehyde-3-phosphate dehydrogenase-encoding gene (P*gpd*) has usually been applied as an efficient constitutive promoter for the expression of heterologous proteins [17,18]. Here, we selected the promoter (P*gpd1*) from *T. reesei* to express heterologous proteins in *A. niger* ATCC 20611. Firstly, the enhanced green fluorescent protein (EGFP) as a reporter protein was expressed under the control of the promoter P*gpd1*. The *gpd1* promoter-driven EGFP expression cassette was constructed by double-joint PCR, which contained the *gpd1* promoter, the *egfp* gene and the *trpC* terminator (Figure 1A). The resistance gene *ptrA* was used as the selectable marker for transformation. Then, the expression cassette and the resistance gene were co-transformed into *A. niger* ATCC 20611. The transformant strains were selected on an MM plate with 0.3% pyrithiamine (Figure 1B). Subsequently, one of the putative transformants validated by PCR was named AGE9, which was used for the next analysis. RT-qPCR was used to detect the transcript level of *egfp* in AGE9 (Figure 1C). It was found that the gene of *egfp* was successfully transcribed in AGE9, while its transcription could not be detected in the parental strain *A. niger* ATCC 20611. Then, the spores of AGE9 were planted on the MM plates with coverslips and grown at 30 °C for 48 h to observe the fluorescence. It was shown that the transformant AGE9 was able to emit intense green fluorescence, while no fluorescence was detected in the parental strain (Figure 1D). These results demonstrate the adaptability of the *T. reesei* promoter P*gpd1* for protein expression in *A. niger* ATCC 20611.

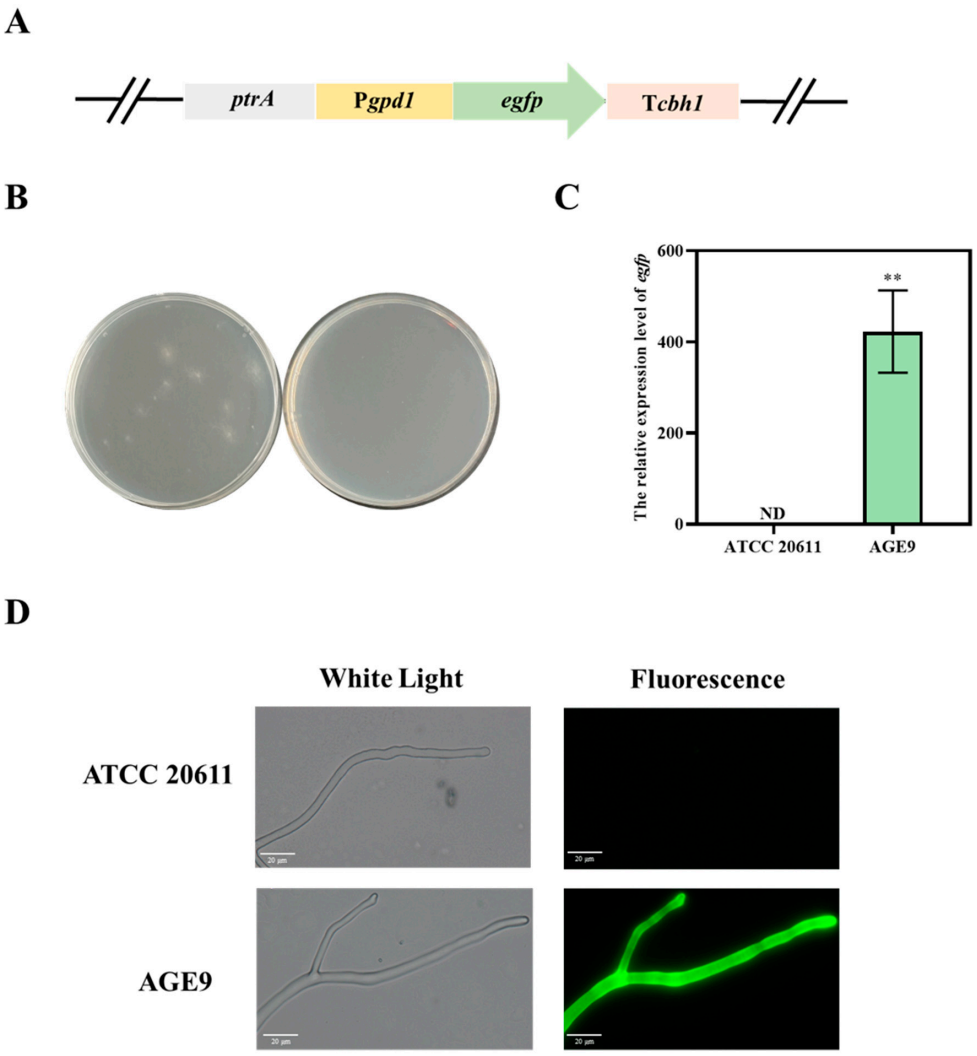

Figure 1. Construction of the *gpd1* promoter-driven EGFP expression strains of *A. niger* ATCC 20611: (**A**) Schematic representation of the *egfp* expression under the control of *gpd1* promoter. The resistance gene *ptrA* was used as the reporter gene. (**B**) The transformant strains expressing EGFP under the control of *gpd1* promoter were selected on an MM plate with 0.3% PT (the left). The plate diagram on the right showed the growth of *A. niger* ATCC 20611 on a MM plate with 0.3% PT as the control. (**C**) The transcript levels of *egfp* in the transformant AGE9 and the parental strain *A. niger* ATCC 20611 were detected by RT–qPCR at 24 h. The relative expression was the level of transcripts normalized to that of *actin* gene. (**D**) Detection of the fluorescence in the mycelium of *A. niger* AGF9-1 and the parental strain ATCC 20611 at 48 h by light and fluorescent microscopy. Error bars indicate the standard deviation (SD) of three biological replicates. Student's *t*-test: ** $p < 0.01$. ND, not detected.

3.2. The gpd1 Promoter-Driven β-Glucosidase Expression in A. niger ATCC 20611

β-glucosidase could hydrolyze cellobiose into glucose, which is a rate-limiting step in the hydrolytic saccharification of cellulose [19]. Meanwhile, β-glucosidase is a large molecular weight secreted protein with glycosylation sites, which could be used as a reporter protein [20]. Here, the *T. reesei* P*gpd1* promotor was used to drive the expression of β-glucosidase in *A. niger* ATCC 20611. Firstly, the *gpd1* promoter-driven β-glucosidase expression cassette was constructed by double-joint PCR, which contained the *gpd1* promoter, the signal peptides of *cbh1* (SP), the *bglA* gene and the *trpC* terminator (Figure 2A). The *ptrA* gene was also used as the selectable marker. After genetic transformation, the transformants were selected on the MM plates with 0.3% pyrithiamine and validated by PCR. The putative transformants were inoculated on the CMC-esculin plates to detect the β-glucosidase secretion. The esculin could be degraded by β-glucosidase to glucose and

aesculetin, which would interact with Fe^{3+} to form a black halo. It was found that the transformants AGB1 and AGB33 displayed wider black halos, while the parental strain *A. niger* ATCC 20611 showed a much smaller halo (Figure 2B,C). Moreover, RT-qPCR was used to detect the transcript level of *bglA* (Figure 2D). It was found that the gene of *bglA* was successfully transcribed in AGB1 and AGB33, while it could not be detected in *A. niger* ATCC 20611. The β-glucosidase activities of transformants were further measured after 4-day fermentation (Figure 2E). It was found that the β-glucosidase activity of AGB1 and AGB33 were 1.02 and 0.51 U/mL, respectively, while it was barely detectable in the parental strain *A. niger* ATCC 20611. These results suggest that the *gpd1* promotor from *T. reesei* could successfully drive the β-glucosidase expression in *A. niger* ATCC 20611.

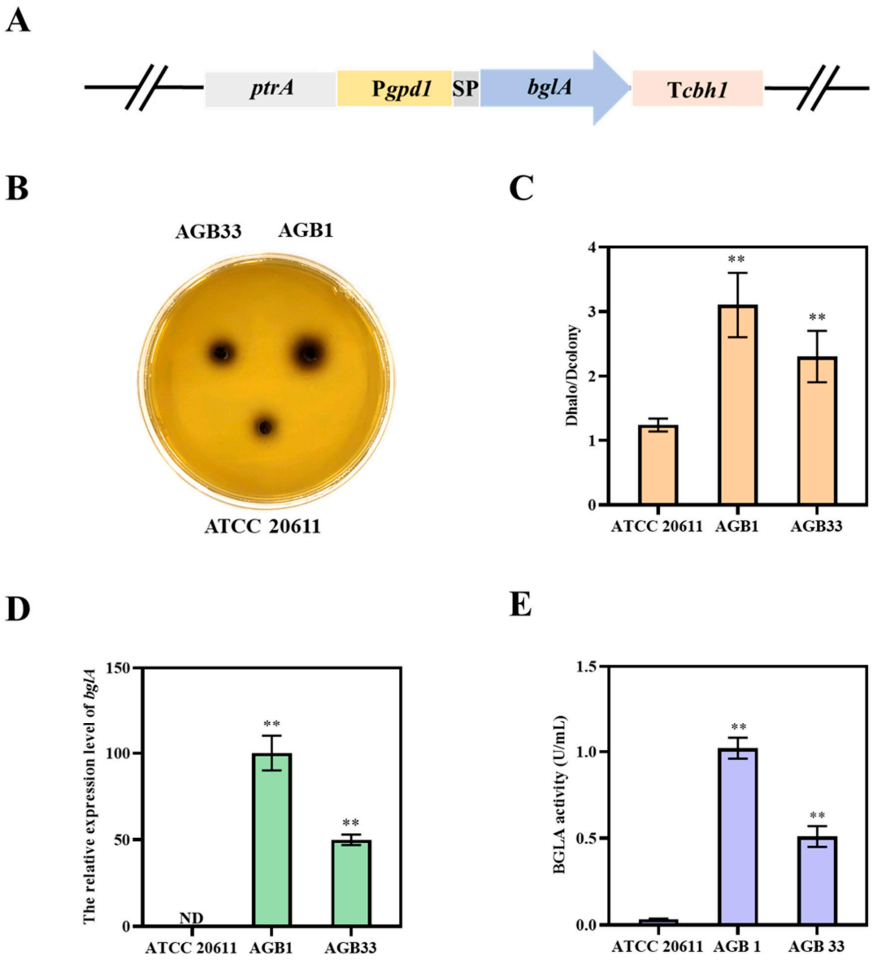

Figure 2. Construction of the *gpd1* promoter-driven β-glucosidase (BGLA) expression strains of *A. niger* ATCC 20611: (**A**) Schematic representation of the *bglA* expression cassette, which contained the promotor of *gpd1* (P*gpd1*), the signal peptides of *cbh1* (SP), the β-glucosidase-encoding gene (*bglA*) and the terminator of *cbh1* (T*cbh1*). (**B**) Detection of the β-glucosidase activities of transformants on the CMC-esculin plates. The parental strain ATCC 20611 was used as control. (**C**) The ratios of halo diameter to colony diameter of the transformants in (**B**). (**D**) The transcript levels of *bglA* in transformants AGB1, AGB33 and the parental strain ATCC 20611 at 24 h by RT–qPCR. The relative expression was the level of transcripts normalized to that of *actin* gene. (**E**) Detection of β-glucosidase activity using p-Nitrophenyl-β-d-glucopyranoside (pNPG) as the substrate of the *cdna1* promoter-driven β-glucosidase (BGLA) expression strains and the parental strain ATCC 20611 after 4-day fermentation. Error bars indicate the standard deviation (SD) of three biological replicates. Student's *t*-test: ** $p < 0.01$. ND, not detected.

3.3. Detection of the ER Pressure in the gpd1 Promoter-Driven β-glucosidase Expression Strains

The production of heterologous proteins could lead to the accumulation of a large number of unfolded proteins in the endoplasmic reticulum, which exceeds the processing capacity of the endoplasmic reticulum and then trigger the UPR response to alleviate the stress and damage to a certain extent, restore the homeostasis of the endoplasmic reticulum [21]. Therefore, to investigate whether the expression of *bglA* in *A. niger* caused the ER pressure, the transcript levels of the genes encoding the protein folding molecular chaperone gene (*bip1*) and the disulfide bond isomerase (*pdi1*), which indicated the degree of the UPR, were detected by RT-qPCR (Figure 3A). Meanwhile, the transcript levels of the genes encoding the ubiquitin ligase enzymes (*yos9* and *der1*), which indicated the degree of the ERAD, were also detected (Figure 3B). It was found that there were no remarkable differences in the transcript levels of *pdi1*, *yos9* and *der1* between the transformants and *A. niger* ATCC 20611, only that the transcript level of *bip1* slightly increased. The results show that the *T. reesei gpd1* promoter driven-β-glucosidase expression could not cause intense ER pressure, indicating the strength of the *gpd1* promoter was not enough to accumulate β-glucosidase in the ER.

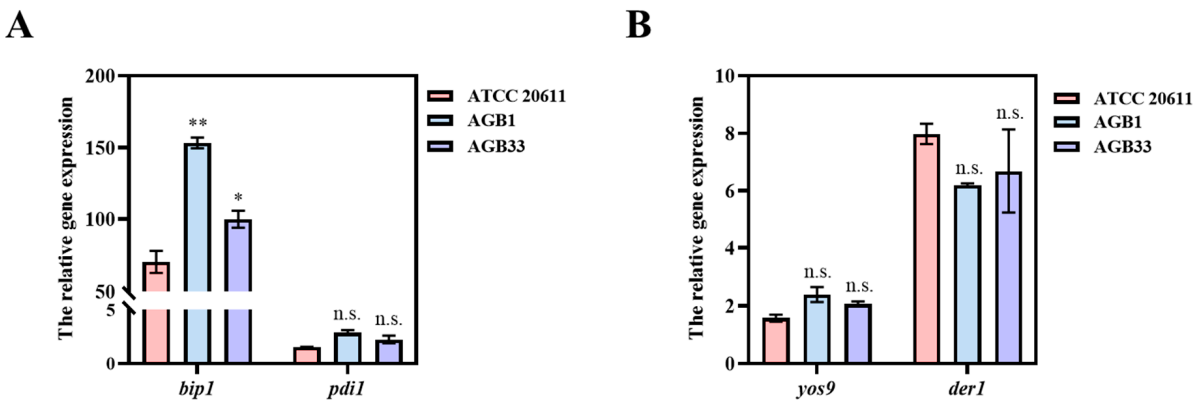

Figure 3. Transcript analysis of endoplasmic reticulum-associated genes (*bip1*, *pdi1 yos9* and *der1*) by RT-qPCR. The transcript levels of UPR—(**A**) and ERAD—(**B**) related genes of transformants AGB1, AGB33 and the parental strain ATCC 20611. The relative expression was the level of transcripts normalized to that of the *actin* gene. Error bars indicate the standard deviation (SD) of three biological replicates. Student's *t*-test: ** $p < 0.01$, * $p < 0.05$. n.s., no significant differences.

3.4. The cdna1 Promoter-Driven β-Glucosidase Expression in A. niger ATCC 20611

The use of strong promoters is an effective means of increasing the expression of heterologous proteins [22]. It was reported that the promoter P*cdna1*, screened from the cDNA library of *T. reesei*, was stronger than the commonly used promoters P*gpd1* and P*tef1* [22]. And it has been used to drive the expression of uronate dehydrogenase, β-mannanase, SARS-CoV-2 neutralizing nanobody Nb20 in *T. reesei* [23–25]. Here, the promoter P*cdna1* was selected to drive β-glucosidase expression in *A. niger* ATCC 20611. Firstly, the P*cdna1*-driven β-glucosidase expression cassette was constructed by double-joint PCR, which contained the *cdna1* promoter, the signal peptides of *cbh1* (SP), the *bglA* gene and the *trpC* terminator (Figure 4A). The resistance gene *ptrA* was used as the selectable marker. After transformation, the transformant strains were selected on the MM plates with 0.3% pyrithiamine. Then, the complete expression cassette was validated by PCR and the putative transformants were inoculated on the CMC-esculin plates to detect the β-glucosidase secretion. It was shown that the transformants ACB8 and ACB11 displayed wider black halos than the parental strain *A. niger* ATCC 20611 (Figure 4B,C). Furthermore, RT-qPCR was used to detect the transcript level of *bglA* (Figure 4D). It was found that the gene of *bglA* was successfully transcribed in ACB8 and ACB11, while it could not be detected in *A. niger* ATCC 20611. Moreover, the β-glucosidase activities of transformants were further measured after 4-day fermentation (Figure 5A). It was found that the β-

glucosidase activity of ACB8 and ACB11 were 15.2 U/mL and 5.6 U/mL, respectively, while it was barely detectable in *A. niger* ATCC 20611. These results suggest that the *cdna1* promotor from *T. reesei* could successfully drive the high-level expression of β-glucosidase in *A. niger* ATCC 20611. Subsequently, the fermentation supernatant was determined by SDS-PAGE assay (Figure 5B). The transformants ACB8 and ACB11 showed clear and specific bands at around 120 kDa, consistent with the theoretical size of β-glucosidase. And this band was further confirmed to be β-glucosidase by MS identification. Moreover, the total protein concentration in the fermentation supernatant was measured by Bradford's method (Figure 5C). It was found that the total extracellular protein content of ACB8 and ACB11 were 1.2 and 0.9 mg/mL, respectively, while that of *A. niger* ATCC 20611 was 0.18 mg/mL. It was estimated that β-glucosidase secreted represented more than 85% of the total extracellular protein. Sun et al. in 2021 reported the production of β-glucosidase using the promoter P*cdna1* in *T. reesei*, in which the specific enzyme activity of β-glucosidase in the seventh-day fermentation broth of *T. reesei* QVB-1 was 18.6 U/mg, with the enzyme production rate reached up to 2.66 U/mg/d [20]. In this study, the specific enzyme activity of β-glucosidase in the fourth day of fermentation broth of *A. niger* ACB8 was 12.7 U/mg (the enzyme activity was 15.2 U/mL and the extracellular protein content was 1.2 mg/mL), with the enzyme production rate reached up to 3.17 U/mg/d. These results demonstrate that the promoter P*cdna1* could drive β-glucosidase expression not only in *T. reesei* but also in *A. niger* and that *A. niger* ACB8 had a faster growth rate and higher β-glucosidase production efficiency compared with *T. reesei* QVB-1, indicating the superiority of *A. niger* ATCC 20611 as a protein expression system.

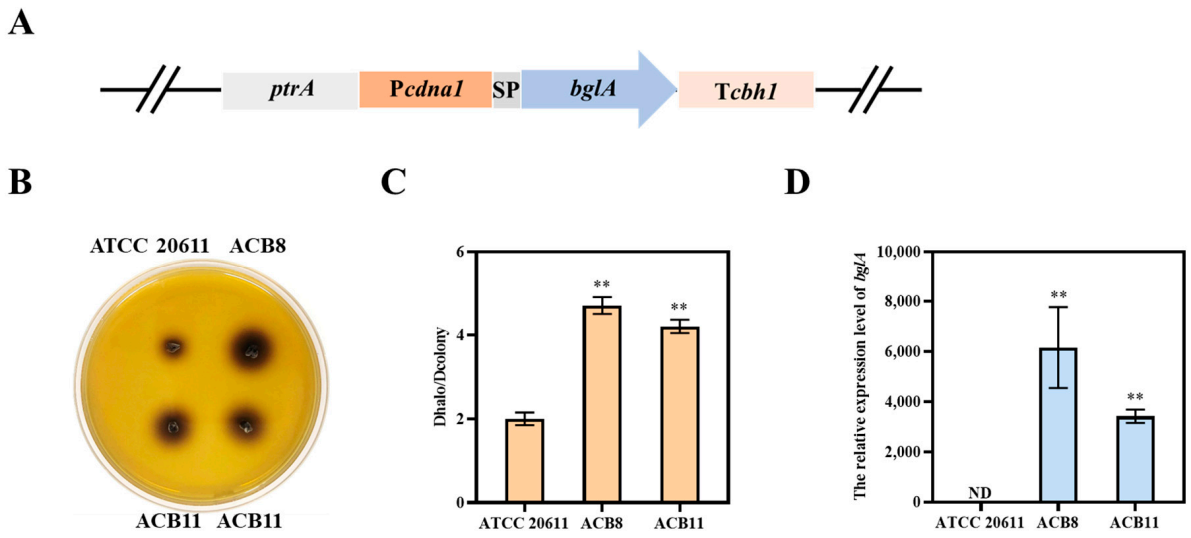

Figure 4. Construction of the *cdna1* promoter-driven β-glucosidase (BGLA) expression strains of *A. niger* ATCC 20611: (**A**) Schematic representation of the *bglA* expression under the control of *gpd1* promoter, which contained the promotor *gpd1* (P*cdna1*), the signal peptides of *cbh1* (SP), the β-glucosidase-encoding gene (*bglA*) and the terminator of *cbh1* (T*cbh1*). (**B**) Detection of β-glucosidase activities of transformant on the CMC-esculin plate. The parental strain ATCC 20611 as control. (**C**) The ratios of halo diameter to colony diameter. The data assessed the β-glucosidase production capacity. Analysis of the expression level of *bglA*. (**D**) The transcript levels of *bglA* in transformants ACB8, ACB11 and the parental strain ATCC 20611 at 24 h by RT–qPCR. The relative expression was the level of transcripts normalized to that of the *actin* gene. Error bars indicate the standard deviation (SD) of three biological replicates. Student's *t*-test: ** $p < 0.01$. ND, not detected.

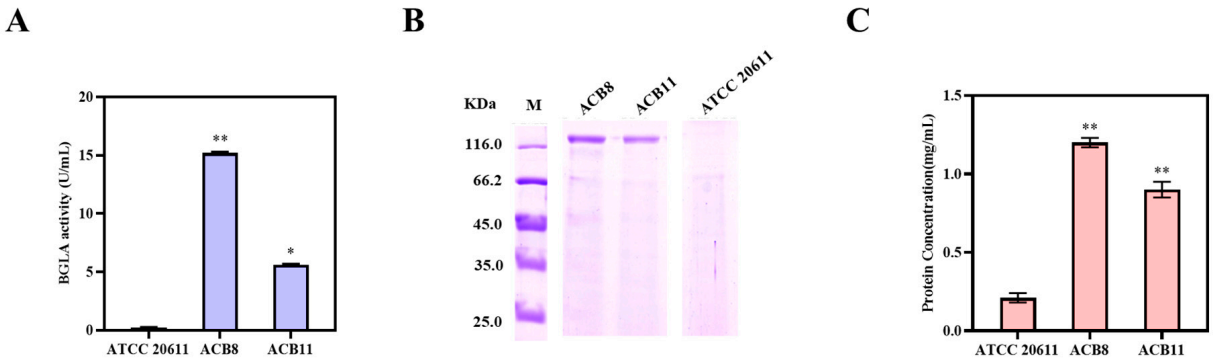

Figure 5. Analysis of the expression level of *bglA*: (**A**) Detection of β-glucosidase activity using p-Nitrophenyl-β-d-glucopyranoside (pNPG) as the substrate of the *cdna1* promoter-driven β-glucosidase (BGLA) expression strains and the parental strain ATCC 20611 after 4-day fermentation. (**B**) SDS PAGE showing secreted BGLA expression. (**C**) Detection of extracellular total protein concentration by Bradford's method. Error bars indicate the standard deviation (SD) of three biological replicates. Student's *t*-test: ** $p < 0.01$, * $p < 0.05$.

In addition, the transcript levels of the genes *bip1*, *pdi1*, *yos9* and *der1* were detected by RT-qPCR (Figure 6A,B). It was found that the transcript levels of *bip1*, *pdi1*, *yos9* and *der1* in the ACB8 and ACB 11 were all to a certain extent higher than those in *A. niger* ATCC 20611, indicating that the expression of β-glucosidase under the control of the promotor P*cdna1* caused the certain ER-pressure. These results demonstrate that the *T. reesei* promoter P*cdna1* could drive the high-level expression of β-glucosidase with a low background of protein secretion in *A. niger* and the strength of the promoter P*cdna1* was sufficient to saturate the expressed proteins in the ER.

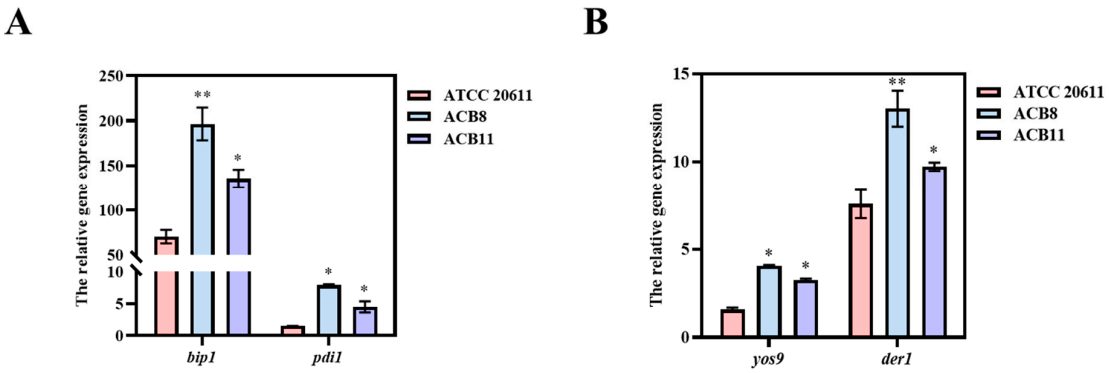

Figure 6. Transcript analysis of endoplasmic reticulum-associated genes (*bip1*, *pdi1 yos9* and *der1*) by RT-qPCR: The transcript levels of UPR—(**A**) and ERAD—(**B**) related genes of transformants ACB8, ACB11 and the parental strain ATCC 20611. The relative expression was the level of transcripts normalized to that of actin. Error bars indicate the standard deviation (SD) of three biological replicates. Student's *t*-test: ** $p < 0.01$, * $p < 0.05$.

3.5. Enzymatic Property of β-Glucosidase in A. niger ACB8

To test the property of the β-glucosidase heterologously expressed in *A. niger* ATCC 20611, we detected the effects of temperature and pH on the activity and stability of β-glucosidase in *A. niger* ACB8. *T. reesei* QVB-1. *T. reesei* QVB-1 expressed β-glucosidase under the control of the promoter P*cdna1*, as the control [20]. Firstly, β-glucosidase activity was measured at different temperatures (Figure 7A). It was found that the β-glucosidase in *A. niger* ACB8 showed the maximal activity at 60 °C and was active in a temperature range of 20–70 °C. After preincubation of the fermentation broth of *A. niger* ACB8 and the *T. reesei* QVB-1 at 50, 60 and 70 °C for 0.5 h to 10 h, the residual activities were determined at pH 4.8 and 50 °C (Figure 7B,C). It was found that the β-glucosidase in *A. niger* ACB8 was highly

stable at temperatures of 50 °C and 60 °C, which retained 85% relative activity at 50 °C after 10 h and 50% relative activity at 60 °C after 2 h. However, the enzyme was inactive at 70 °C after 0.5 h. Then, β-glucosidase activity was measured in different pH ranges of 3.0–8.0 (Figure 7D). It was found that the β-glucosidase of *A. niger* ACB8 had high stability in acid conditions and its optimum pH was 4.5. After preincubation of the fermentation broth of the *A. niger* ACB8 and the *T. reesei* QVB-1 without substrate in citric acid buffer of different pH in 4 °C for 24 h, the residual activities were determined at pH 4.8 and 50 °C (Figure 7E). It was found that the β-glucosidase in *A. niger* ACB8 was highly stable in acid conditions, which could remain at more than 44% relative activity after incubation in citric acid buffer of pH 3.0–6.0 for 24 h and maintain 31% in pH 8. The effects of temperature and pH on the activity and stability of β-glucosidase in *T. reesei* QVB-1 were essentially the same as that of ACB8. The results show that the activity and stability of the *cdna1* promoter-driven β-glucosidase in *A. niger* ACB8 were consistent with that of *T. reesei*, indicating that the heterologous promoter-driven protein expression in *A. niger* ATCC 20611 could not result in changes in enzymatic property. Meanwhile, the β-glucosidase under the control of the *T. reesei* promoter P*cdna1* was a moderately acidic β-glucosidase, suggesting that it had potential for application in cellulose hydrolysis.

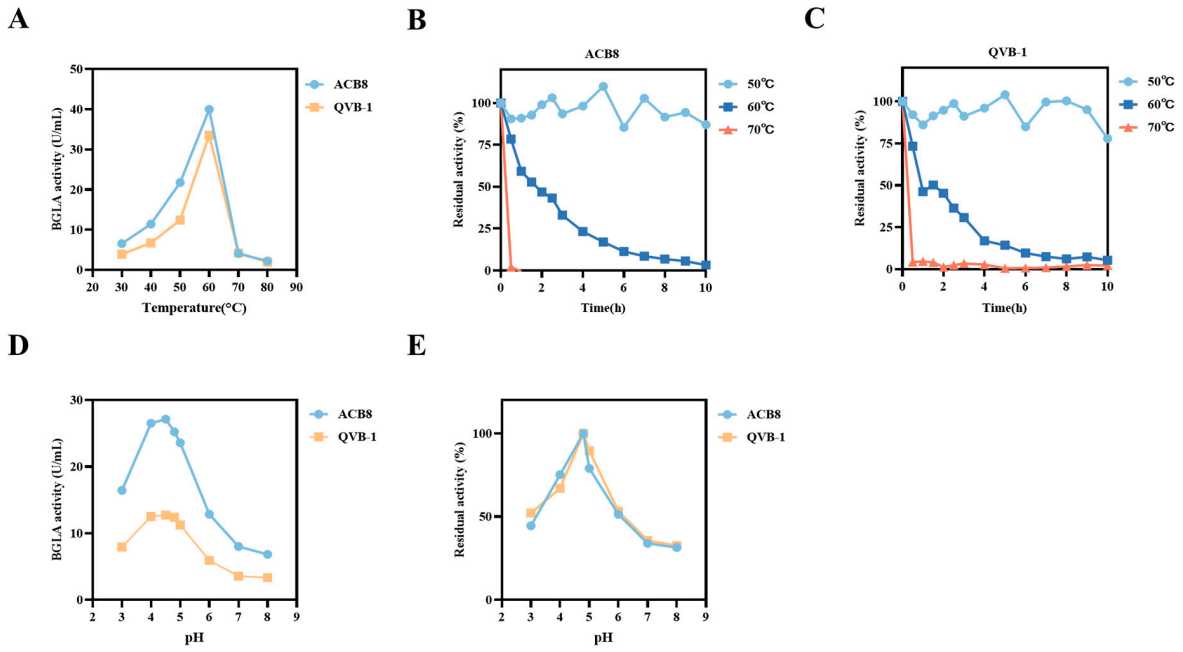

Figure 7. Enzymatic characterization of BGLA in *A. niger* ACB 8. The BGLA in *T. reesei* QVB-1 was the control: (**A**) Optimal temperature. The activity of BGLA was detected in the temperature range of 30–80 °C at pH 4.8. (**B**) and (C) Thermostability. After preincubation of the fermentation broths of the ACB8 and QVB-1 at 50, 60 and 70 °C for 0.5 h to 10 h, respectively, the residual activities were determined at pH 4.8 and 50 °C and the initial activity at pH 4.8 and 50 °C was defined as 100%. (**D**) Optimal pH. The activity of BGLA was detected at 50 °C in the pH range 3 to 8. (**E**) pH stability. After preincubation of the fermentation broths of the ACB8 and QVB-1 in citric acid buffer of different pH for 24 h in 4 °C, respectively, the residual activities were determined at pH 4.8 and 50 °C and the initial activity at pH 4.8 and 50 °C was defined as 100%.

3.6. Saccharification of the Corncob Residues by Supplementing the β-glucosidase to the Cellulase Mixture of T. reesei

The filamentous fungus *T. reesei* is widely used for the production of cellulases, in which the β-glucosidase represents only 1% of the cellulase mixture, resulting in the lack of BGL becoming the rate-limiting step for effective hydrolysis of the cellulose substrate [11]. To improve the saccharification ability of the cellulase mixture of *T. reesei*, the fermentation broth of *A. niger* ACB8, which contains β-glucosidase under the control of the *T. reesei*

promoter P*cdna1*, was added to the cellulase mixture of *T. reesei* SN1 according to the ratio of the FPA to β-glucosidase activity of 1:1 for saccharification of acid pretreated corncob residues (ACR) and delignified corncob residues (DCR). As shown in Figure 8A, in the saccharification of the acid-pretreated corncob residues, the glucose released by the cellulase mixture of *T. reesei* SN1 supplemented with the fermentation broth of *A. niger* ACB8 was 11.78 mg/mL (corresponding to 33.86% cellulose conversion), which was 26.21% higher than that of *T. reesei* SN1 (9.33 mg/mL, corresponding to 26.83% cellulose conversion) after 48 h. While in the saccharification of the delignified pretreated corncob residues (Figure 8B), the glucose released by the cellulase mixture of *T. reesei* SN1 supplemented with the fermentation broth of *A. niger* ACB8 was 27.64 mg/mL (corresponding to 75.74% cellulose conversion), which was 29.51% higher than that of *T. reesei* SN1 (21.35 mg/mL, corresponding to 58.48% cellulose conversion) after 48 h. And the saccharification with the addition of the equal volume of fermentation broth of *A. niger* ATCC 20611 as the control and the results show that the glucose released had no significant difference from that of *T. reesei* SN1 (Figure 8A,B). Taken together, the results demonstrate that the β-glucosidase of *A. niger* ACB8 significantly enhanced the ability of the cellulase system of *T. reesei* for highly efficient biomass hydrolysis.

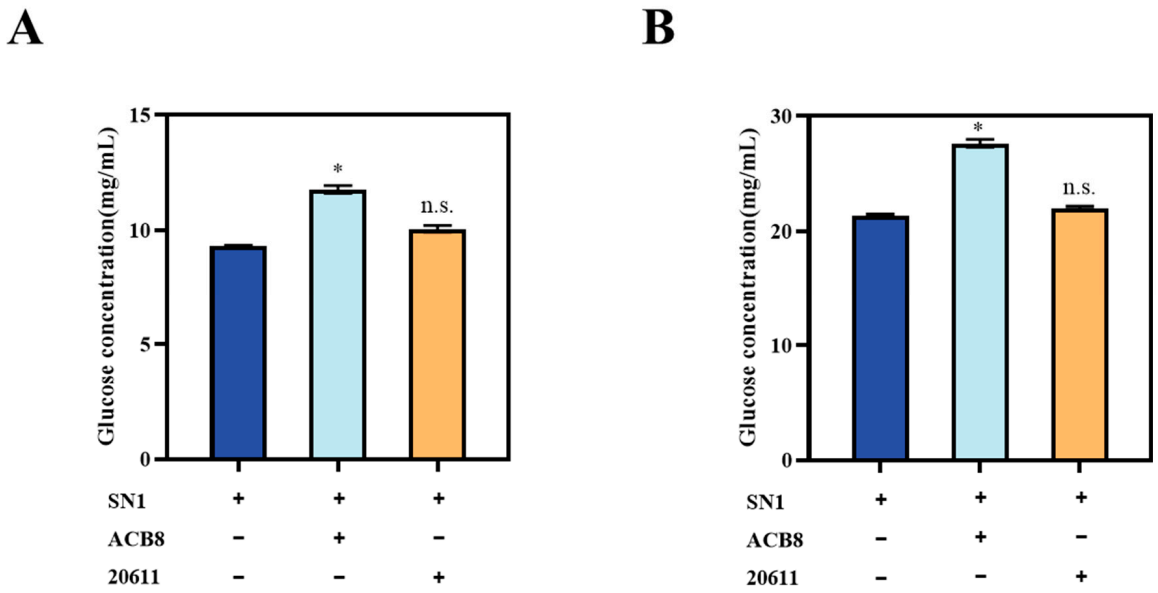

Figure 8. Saccharification of different pretreated corncob: (**A**) Glucose released from saccharification of acid-pretreated corncob residues by *T. reesei* SN1 with the addition of the fermentation broth of *A. niger* ACB8 for 48 h. (**B**) Glucose released from the saccharification of delignified corncob residues by *T. reesei* SN1 adding the fermentation broth of ACB8 for 48 h. Saccharification by adding the fermentation broth of the parental strain *A. niger* ATCC 20611 was used as the control. Error bars indicate the standard deviation (SD) of three biological replicates. Student's *t*-test: * $p < 0.05$. n.s., no significant differences.

4. Discussion

A. niger ATCC 20611, as an industrial strain for fructooligosaccharides production, has its main enzyme protein β-fructofuranosidase localized in the cell wall, resulting in a pure protein background [2]. Therefore, *A. niger* ATCC 20611 is expected to be a potentially excellent protein expression host. Meanwhile, the use of robust promoters is essential for the construction of protein expression systems [3]. In this study, we established an *A. niger* ATCC 20611 protein expression secretion system driven by the strong constitutive promoter P*cdna1* from *T. reesei* for the high-level production of high-purity β-glucosidase, which was used to increase the rate of cellulose degradation.

The process of gene expression begins with transcription. And the transcription level of genes is largely determined by promoters, which determine the mode and level of expression of the recombinant protein. Therefore, it is crucial for the construction of protein expression systems by using robust promoters. The approaches obtaining robust promotors include screening by traditional biochemical and constructing synthetic hybrid promoters [26,27]. Erden et al. attempted to express recombinant proteins in *P. pastoris* using heterologous yeast promoters and the results demonstrate that the expression pattern of the heterologous promoters in *P. pastoris* was similar to that of their native host [28]. Based on RNA-seq data, Zhang et al. used 18 promoters selected from GS115 genome to drive the expression of reporter proteins in *P. pastoris* and found that the methanol-induced promoter pFDH1 was an alternative to pAOX1 [29]. It was reported that the constitutive promoter P*cdna1* was identified from the cDNA libraries in *T. reesei* [7]. It is generally considered the strongest constitutive promoter in *T. reesei* [8]. Additionally, it is only found in *T. reesei* and differs from the commonly used promoters of the gene encoding glyceraldehyde-3-phosphate dehydrogenase P*gpd1* and the gene encoding translation elongation factor P*tef1*. The promoters P*gpd1* and P*tef1* are relatively conservative and widely exist in different microorganisms. In our study, the β-glucosidase activity in the transformant ACB8 under the control of P*cdna1* was 15.2 U/mL, which was a 13.9-fold increase from that of the transformant AGB1 under the control of the promoter P*gpd1* (1.02 U/mL). The results demonstrate that using the P*cdna1* promoter of *T. reesei* could effectively enhance the expression level of heterologous proteins in *A. niger*, which indicated that the driving pattern of the *T. reesei*-specific promoter P*cdna1* is probably universal in filamentous fungi.

Lignocellulose biomass is the largest renewable resource on Earth and it can be converted into second-generation biofuel ethanol [30]. This renewable energy source is considered a promising substitute for fossil fuels [31]. The cellulase mixtures secreted by *T. reesei* can effectively degrade cellulose. The process of cellulase hydrolysis is characterized by a synergistic effect between different enzyme components. However, in the cellulase mixture, the β-glucosidase accounts for only about 1%, which is a rate-limiting step for effective hydrolysis of the cellulose substrate. Moreover, enzyme purification increases the time and cost [32,33]. Consequently, it is of great importance to adjust the content of key enzyme components in the cellulase mixture in order to improve the cellulose degradation rate. It was reported that the *T. reesei* Δ*vib1* demonstrated a low background of cellulase and protease secretion [20]. Then, the *T. reesei* QVB-1 was constructed to express β-glucosidase using the *T. reesei* Δ*vib1* as the host strain. The β-glucosidase activity in the seventh-day fermentation broth of *T. reesei* QVB-1 was 18.6 U/mg, with the enzyme production rate reaching up to 2.66 U/mg/d. In this study, the β-glucosidase secreted represented more than 85% of the total extracellular protein in the transformant *A. niger* ACB8. And the specific enzyme activity of β-glucosidase in the fourth day of fermentation broth of *A. niger* ACB8 was 12.7 U/mg, with the enzyme production rate reached up to 3.17 U/mg/d. These results demonstrate that *A. niger* ATCC 20611 has a fast growth rate and a low extracellular protein background and it had obvious advantages in pure β-glucosidase production to improve cellulose degradation rate when compared with *T. reesei*.

The β-glucosidase was stable at low pH, which makes it suitable for cellulose hydrolysis [34]. The saccharification reaction conditions for the pretreated corn cob residues were 50 °C and a pH of 4.8 [14]. In our study, the β-glucosidase in *A. niger* ACB8 was highly stable at temperatures of 50 °C, which retained 85% relative activity at 50 °C after 10 h and had high stability in acid condition, remaining more than 44% relative activity after incubation in citric acid buffer of pH 3.0–6.0 for 24 h (Figure 7D). To optimize cellulose hydrolysis, a fermentation broth of ACB8 was added to the cellulase mixture from *T. reesei* SN1. The glucose released from the saccharification system was 11.78 mg/mL and 27.64 mg/mL after 48 h when the acid-pretreated corncob residues and delignified pretreated corncob residues were used as the substrates for saccharification, respectively. And the saccharification efficiency increased by 26.21% and 29.51% compared with that of *T.*

reesei SN1 alone after 48 h, respectively (Figure 8A,B). Ma et al. expressed the β-glucosidase gene *bgl1* from *P. decumbens* in *T. reesei* Rut-C30 and purified β-glucosidase with a HiTrap column from the fermentation broth [13]. The addition of purified pBGL1 to *T. reesei* cellulase preparation could facilitate cellulose saccharification and the promotion was more remarkable with further adding pBGL1. Taken together, the successful expression of β-glucosidase under the control of the *cdna1* promoter P*cdna1* in *A. niger* ACB8 showed the applicability of heterologous promoter in *A. niger* ATCC 20611.

In conclusion, the robust promoter P*cdna1* from *T. reesei* was successfully applied in the construction of a protein expression system in *A. niger* ATCC 20611. And the production level of β-glucosidase driven by the promoter P*cdna1* in *A. niger* ATCC 20611 reached 15.2 U/mL, representing a 13.9-fold increase compared with that of the promotor P*gpd1*. Furthermore, the β-glucosidase in *A. niger* ACB8 exhibited high stability under the temperature and pH conditions of cellulose degradation. Moreover, the β-glucosidase accounted for 85% of the total extracellular protein in the fermentation broth of *A. niger* ACB8. The efficiency of saccharification of different pretreated corncob residues could be increased with the addition of the fermentation supernatant of *A. niger* ACB8 to the cellulase system of *T. reesei*. Therefore, the high-level expression of β-glucosidase in *A. niger* ATCC 20611 using the *T. reesei* promoter P*cdna1* has enhanced the efficiency of cellulose degradation and facilitated the effective utilization of lignocellulose, which was of great importance for the conversion of renewable biomass resources.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation10090461/s1, Table S1: Primers used in this study.

Author Contributions: J.C., J.W. and Y.Z. conceived the work and drafted the manuscript. J.W., J.C., Z.L., L.W. and P.L. performed the experiments and analyzed the data. Y.Z. and H.L. designed the work and revised the manuscript. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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