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Engineering *Neurospora crassa* for Improved Cellobiose and Cellobionate Production

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We report engineering *Neurospora crassa* to improve the yield of cellobiose and cellobionate from cellulose. A previously engineered strain of *N. crassa* (F5) with six of seven β-glucosidase (*bgl*) genes knocked out was shown to produce cellobiose and cellobionate directly from cellulose without the addition of exogenous cellulases. In this study, the F5 strain was further modified to improve the yield of cellobiose and cellobionate from cellulose by increasing cellulase production and decreasing product consumption. The effects of two catabolite repression genes, *cre-1* and *ace-1*, on cellulase production were investigated. The F5 Δ*ace-1* mutant showed no improvement over the wild type. The F5 Δ*cre-1* and F5 Δ*ace-1* Δ*cre-1* strains showed improved cellobiose dehydrogenase and exoglucanase expression. However, this improvement in cellulase expression did not lead to an improvement in cellobiose or cellobionate production. The cellobionate phosphorylase gene (*ndvB*) was deleted from the genome of F5 Δ*ace-1* Δ*cre-1* to prevent the consumption of cellobiose and cellobionate. Despite a slightly reduced hydrolysis rate, the F5 Δ*ace-1* Δ*cre-1* Δ*ndvB* strain converted 75% of the cellulose consumed to the desired products, cellobiose and cellobionate, compared to 18% converted by the strain F5 Δ*ace-1* Δ*cre-1*.

Cellulosic biomass is an attractive resource for the development of biofuels and chemicals due to its widespread abundance, low cost, and distinction from food crops (1, 2). The recalcitrance of cellulosic biomass (converting the biomass into fermentable sugars) is the predominant obstacle to commercialization of this technology, as the associated processing steps (pretreatment, cellulase production, and enzymatic hydrolysis) comprise about 40% of the overall production cost (3, 4). Consolidating the process into fewer processing steps is one way to improve the overall economics (5–7). Utilizing lignocellulolytic microorganisms to directly hydrolyze the cellulose into reactive intermediates such as sugar or sugar-like intermediates for subsequent conversion to fuels or chemicals is one such way to consolidate the process (8). The major limitation of such a process is carbon loss due to the consumption of the hydrolysis products by the lignocellulolytic organism. A metabolic engineering strategy can be employed to direct the carbon flow toward products, thereby preserving those products for the subsequent conversion to fuels and chemicals.

Neurospora crassa is a model microorganism, and its genetics, biochemistry, and fungal biology have been extensively studied for many years (9). The tools for genetic manipulation are readily available (9). The sequenced genome and functional sexual crossing made it possible to construct strains with multiple mutations in a relatively short time (10, 11). *N. crassa* is also a proficient plant cell wall degrader, and it produces a wide spectrum of cellulases and hemicellulases. Additionally, it produces cellobiose dehydrogenase (CDH) under cellulolytic conditions, which oxidizes cellobiose to its aldonic acid, cellobionic acid (12, 13). Efficient hydrolysis of cellulose requires several cellulase enzymes, including endoglucanases (EGs) (EC 3.2.1.4) and cellobiohydrolases (CBHs) (EC 3.2.1.91). Endoglucanases hydrolyze cellulose internally, while exoglucanases hydrolyze cellulose at the reducing and nonreducing ends. Cellobiose is the primary product of this hydrolysis, which can be further hydrolyzed by β-glucosidase (BGL) (EC 3.2.1.21) to form glucose (14). In previous studies, we engineered *N. crassa* for direct cellobiose and cellobionate production from cellulose (8, 15). By knocking out six of the seven *bgl* genes in *N. crassa* (designated strain F5), more than 7 g/liter of cellobiose was produced as the primary cellulose hydrolysis product without any cellulase addition in 96 h. A small amount (less than 1 g/liter) of cellobionate was produced along with cellobiose due to cellobiose oxidation by CDH. The hydrolysis products of cellobionate are glucose and gluconate, both of which could be substrates for the production of fuels and chemicals (15). Of the hydrolyzed cellulose, about 50% was directed toward the production of cellobiose and cellobionate in the F5 strain (8, 15). The unmodified wild-type strain consumed all the cellulose for cell growth without any cellobiose or cellobionate accumulation. The deletion of six of seven *bgl* genes did not negatively affect the rate of cellulose hydrolysis, and the cellulose conversion achieved by the F5 strain increased compared to that of the wild type. The level of cellulase production was comparable to that of the wild type (8).

In this study, we aimed to further modify the *N. crassa* F5 strain to improve the yield of cellobiose and cellobionate from cellulose

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TABLE 1 Strains used in this study

Strain	Genotype	Source
F5	*bgl-1*::*hph bgl-2*::*hph bgl-3*::*hph bgl-4*::*hph bgl-6*::*hph bgl-7*::*hph matA*	8
F5-25a	*bgl-1*::*hph bgl-2*::*hph bgl-3*::*hph bgl-4*::*hph bgl-6*::*hph bgl-7*::*hph mata*	8
F5 Δ*ace-1*	F5 *ace-1*::*six*	This study
F5 Δ*mus-51* Δ*cre-1*	F5 *mus-51*::*six cre-1*::*six*	27
F5 Δ*cre-1*	F5 *cre-1*::*six*	27
F5 Δ*ace-1* Δ*cre-1*	F5 *ace-1*::*six cre-1*::*six*	This study
F5 Δ*mus-51* Δ*ace-1* Δ*cre-1*	F5 *mus-51*::*six ace-1*::*six cre-1*::*six*	This study
F5 Δ*ace-1* Δ*cre-1* Δ*ndvB*	F5 *mus-51*::*ace-1*::*six cre-1*::*six*	This study

TABLE 2 Primers used in strain construction and verification

Primer	Sequence[a]
ES034JF	AATTCGAGCTCGGTACtttaaaGAAGTCGACTGCATCAGG
ES035JF	GGACCTGAGTGAgatGTTTGCTGAGTTGTGTGGAG
ES036JF	TGGTCCATCTAGTgatGACAAGTTGGGGAGAACGC
ES037JF	GCCAAGCTTGCATGCCtttaaaGGCAGATTCAATAACGACC
ES038JF	AGCGTTTGTTGTCGAACCC
ES039JF	GATGAGGAAGCAAGCAGAGG
ES056JF	CTCTCCCACTACTCCAGCC
ES057JF	AGATGTCCTGAGATGATGGC
ES058JF	CCAAGCCTGAATACCAACCC
ES059JF	AAATGAACAATATCAGCAAGGG
ES113JF	AATTCGAGCTCGGTACgaattcTATTTAGGATACAGTAGC AGCG
ES114JF	GGACCTGAGTGAtttTTGGTTGTGTGTGAAGTTGAG
ES115JF	TGGTCCATCTAGTtttAACCTTACAGTGACTATTCCG
ES116JF	GCCAAGCTTGCATGCCgaattcGGATGTTGAGCACCTTGACG
ES117JF	ACTACGTTCTTCGATAGTAGG
ES118JF	TTCTTGACCACCGTGTGAC
ES119JF	GCTACGAAATCACCAACCC
ES120JF	GATGTCCTTGACGTGAGGC
Sv739	ACAAATAAGTATACTCTATTGACC
Sv740	AGAGTAGGTCATTTAAGTTGAGC

[a] Lowercase indicates a restriction site (see Materials and Methods).

by promoting cellulose hydrolysis via improved cellulase production and decreasing product consumption. Specifically, we aimed to improve cellulase production by knocking out a carbon catabolite repression (CCR) gene, *cre-1*, and a cellulase repressor gene, *ace-1*. The zinc finger transcription factor CRE1/CRE-1/CreA is a known carbon catabolite repressor for many plant-degrading fungi, including *N. crassa*, aspergilli, and *Trichoderma reesei* (16–22). Deletion of the *cre-1* (NCU08807) gene in *N. crassa* and the *cre1* gene in *T. reesei* led to improved cellulase production (16–18). Additionally, another zinc finger transcription factor, encoded by *ace1*, in *T. reesei* was found to repress cellulases and hemicellulose gene expression (23). This gene alone is subjected to CRE-1-dependent CCR (24). An *ace1* orthologue (NCU09333) has been identified in *N. crassa*, and the effects of such a mutation on the homologous gene in *N. crassa* are currently unknown (25). Since *ace-1* derepresses some of the same cellulases as *cre-1* in other filamentous fungi, this opens the possibility of derepressing cellulase expression in *N. crassa* by knocking out the *ace-1* gene and possibly obtaining a synergistic effect with a double knockout of the *ace-1* and *cre-1* genes. Furthermore, the *ndvB* gene (NCU09425), encoding a cellobionate phosphorylase enzyme, was previously shown to phosphorylate cellobionic acid to α-D-glucose 1-phosphate and D-gluconic acid, both of which can be metabolized by *N. crassa* (26). In this study, we also analyzed the effects of an *ndvB* knockout strain on the hydrolysis of cellulose for the production of cellobiose and cellobionate.

MATERIALS AND METHODS

Fungal strains. The *N. crassa* strains used in this study and their sources are given in Table 1. The F5 Δ*cre-1*, F5 Δ*cre-1* Δ*ace-1*, and F5 Δ*cre-1* Δ*ace-1* Δ*ndvB* strains were constructed in this study.

Construction of the F5 Δ*ace-1* and F5 Δ*cre-1* Δ*ace-1* strains. To construct the F5 Δ*ace-1* and F5 Δ*cre-1* Δ*ace-1* strains, 1.5-kb-long 5′ and 3′ flanking regions of the *ace-1* gene (NCU09333) were amplified from the wild-type genomic DNA using Phusion high-fidelity DNA polymerase (ThermoScientific, Waltham, MA) and primer pair ES034JF and ES035JF or ES036JF and ES037JF, respectively (see Fig. S1A in the supplemental material). Unique DraI restriction sites were introduced at the ends of the deletion construct in the primers ES034JF and ES037JF (shown in lowercase in Table 2) to facilitate release of the fragment from the pUC19 vector. Using the GeneArt Seamless Cloning & Assembly kit (Invitrogen), a *xylP*(p)β-rec/*six*(*bar*) recyclable marker cassette was incorporated to create a deletion cassette, as previously described (27). The *ace-1* deletion cassette was released from the plasmid using DraI and was used to transform the F5 Δ*mus-51* Δ*cre-1* using the standard transformation method (27). Twelve transformants resistant to phosphinothricin were screened by diagnostic PCR using primer pairs ES038JF-Sv739 and ES039JF-Sv740. Ten transformants showed correct *ace-1* replacement (see Fig. S1B in the supplemental material). Lack of the *ace-1* gene was confirmed with primers ES056JF and ES057JF (see Fig. S1C). The marker cassette was removed as described before (27), and marker excision was confirmed by PCR using primer pair ES058JF and ES059JF (see Fig. S1D). One of the resulting transformants was crossed with strain F5-25a to obtain the homokaryotic F5 Δ*cre-1*, F5 Δ*ace-1*, and F5 Δ*cre-1* Δ*ace-1*strains.

Construction of the F5 Δ*cre-1* Δ*ace-1* Δ*ndvB* strain. To construct the F5 Δ*cre-1* Δ*ace-1* Δ*ndvB* strain, 1-kb-long 5′ and 3′ flanking regions of the *ndvB* gene (NCU09425) were amplified from wild-type genomic DNA using Phusion high-fidelity DNA polymerase and primer pair ES113JF and ES114JF or ES115JF and ES116JF, respectively (see Fig. S2A in the supplemental material). Unique EcoRI restriction sites were introduced at the ends of the deletion construct in primers ES113JF and ES116JF (shown in lowercase in Table 2) to facilitate release of the fragment from the pUC19 vector. Using the GeneArt Seamless Cloning & Assembly kit, a *gh10-2*(p)β-rec/*six*(*bar-tk*) recyclable marker cassette was incorporated to create a deletion cassette, as described by Szewczyk et al. (28). The 3′ flanking region of the *ndvB* gene incorporated in the cassette was sequenced to ensure correct amplification of the hypothetical gene NCU09424 located only 559 bp downstream of *ndvB*. The *ndvB* deletion cassette was released from the plasmid using EcoRI restriction enzyme digestion. The cassette was then used to transform the F5 Δ*mus-51* Δ*cre-1* Δ*ace-1* strain. The positive transformants resistant to phosphinothricin were screened by diagnostic PCR using primer pairs ES117JF-Sv739 and ES118JF-Sv740 (see Fig. S2A in the supplemental material). Twenty of 24 screened *ndvB* transformants showed correct *ndvB* replacement (see Fig. S2B). Lack of the *ndvB* gene was confirmed by PCR with primer pair ES119JF and ES120JF (see Fig. S2C). The marker cassette was removed as described before (18), and marker excision was confirmed by PCR using primer pair ES117JF and ES118JF (see Fig. S1D).

Fermentation experiments. *N. crassa* strains were grown on agar with 1× Vogel's salts and 1.5% sucrose in an incubator at 30°C with light (29). After 3 days, flasks were removed from the incubator, and the strains were grown for 7 days at room temperature. After a total of 10 days of growth, the conidia were harvested in water and filtered through eight layers of

cheesecloth. Fermentation experiments were conducted in 250-ml Erlenmeyer flasks with a 50-ml working volume, 1× Vogel's salts medium, and 20 g/liter Avicel unless otherwise noted. At the beginning of each fermentation, 0.5 g/liter of glucose was added to initiate cell growth. Conidia were inoculated at a volume to yield a final optical density at 420 nm (OD_{420}) of 0.15. Flasks were incubated at 28°C in a rotary shaker at 200 rpm with light. Samples were taken at various time intervals for enzyme activity analysis and compositional analysis.

Sample analysis. Concentrations of sugars (glucose, cellobiose, and cellobionate) were analyzed using a Shimadzu high-pressure liquid chromatograph (HPLC) equipped with a refraction index detector, photodiode array (PDA) detector, and CARBOSep Coregel-87C (Transgenomic, San Jose, CA, USA) column. Four millimolar calcium chloride at a flow rate of 0.6 ml/min was used as the mobile phase.

Enzyme assays. CDH activity was determined by following the decrease in absorbance of 2,6-dichlorophenolindophenol (DCPIP) at 520 nm according to previously established methods, using cellobiose as the substrate (30).

Exoglucanase activity was measured similarly, with the following modifications. One hundred microliters of 1 mg/ml *p*-nitrophenyl-β-D-lactopyranoside in 50 mM citric acid buffer (pH 5.0) was incubated at 37°C for 15 min. Eighty microliters of sample was added to the substrate and incubated for 15 min at 37°C. The reaction was stopped with 120 μl of 1 M NaOH. Absorbance was immediately measured at 405 nm and compared to that of *p*-nitrophenol standards (0 to 10 mM) in citric acid buffer (pH 5.0). One unit of enzyme activity is defined as the amount of enzyme required to liberate 1 μmol of *p*-nitrophenol per minute under the given assay conditions.

Endoglucanase activity was measured as described by King et al. in a 96-well plate with minor modifications (31). One hundred microliters of 2% carboxymethyl cellulose in 50 mM sodium citrate buffer (pH 5.0) was incubated at 37°C for 15 min. Eighty microliters of sample was added to the substrate and incubated for an additional 15 min at 37°C, after which the reaction was stopped by adding 120 μl of 3,5-dinitrosalicylic acid (DNS) reagent (0.01 g DNS, 3 g sodium potassium tartrate, 4 ml 1 M NaOH, with a total volume of 10 μl made with deionized [DI] water). The reaction mixture was transferred to PCR plates and incubated in a thermocycler at 95°C for 5 min, followed by 1 min at 4°C. The absorbance was measured at 540 nm and compared to that of glucose standards (0 to 1.0 g/liter). One unit of activity corresponds to the release of 1 μmol of reducing sugar per minute.

Mycelial biomass measurements. The dry weight of mycelia contained in the fermentation samples was measured by extracting ergosterol from the mycelia and measuring the amount by HPLC (32). The fermentation residue was collected by filtration through a 0.8-μm membrane. All the residue, including the mycelia, was harvested and frozen in liquid nitrogen for 1 h, and ethanol (6 ml) was added to the frozen sample and incubated at 37°C for 2 h with shaking. An aliquot of KOH solution (60% [wt/vol], 0.8 ml) was added to the mixture, which was then heated to 97°C for 20 min. This sample was cooled and neutralized with HCl (36.5%, ~0.7 ml). The solution was extracted three times with 5 ml hexane, the hexane fractions were combined, and air was used to evaporate the solvent. The residue was dissolved in ethanol (1 ml), filtered through a 0.22-μm membrane filter, analyzed by HPLC with a PDA detector on a reverse-phase column (Zorbax Eclipse Plus C_{18} [4.6 by 250 mm, 5-μm particle size]; Agilent), and eluted at 1.0 ml/min with methanol-water (97:3, vol/vol). The amount of biomass was quantified using a standard curve prepared with known *N. crassa* dry biomass.

RESULTS

Effects of *cre-1* and *ace-1* gene deletions on enzyme production. Strains with single and double knockouts of the *ace-1* and *cre-1* genes were evaluated for cellulase production in shake flask fermentations with 20 g/liter Avicel. Exoglucanase, endoglucanase, and CDH activities were analyzed at 3 and 4 days of culture. The enzyme activity data normalized to mycelial biomass are shown in Fig. 1. Production of exoglucanase and CDH was improved for strains containing the *cre-1* deletion. While the F5 Δ*ace-1* single-knockout strain was indistinguishable from the parent strain, F5 Δ*ace-1* Δ*cre-1* had exoglucanase production that was improved by 26% and CDH production that was improved by 50% compared to those of the F5 parent strain. All of the strains showed similar endoglucanase activity (see Fig. S3 in the supplemental material) and rate of biomass production (see Fig. S4 in the supplemental material).

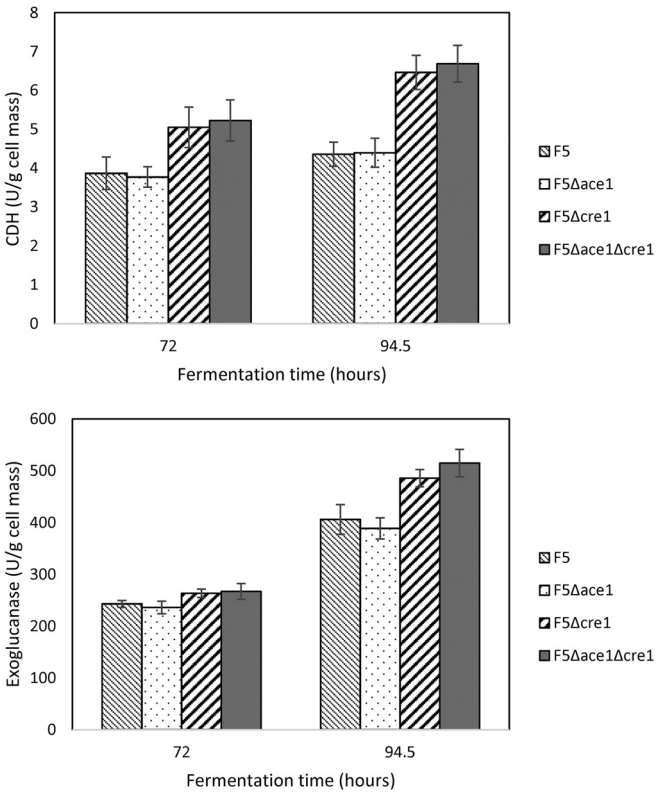

FIG 1 CDH and exoglucanase activities for *ace-1* and/or *cre-1* knockout strains at selected time points. The values shown are the means for biological triplicates. Error bars are the standard deviations.

Effects of *cre-1* and *ace-1* gene deletions on cellobiose and cellobionate production. Shake flask experiments in Vogel's medium with 20 g/liter Avicel were analyzed for the production of cellobiose. No difference in cellobiose production was observed for the *ace-1* and *cre-1* knockout strains (see Fig. S5 in the supplemental material). Interestingly, despite the presence of a high level of CDH, no cellobionate was detected over the time course of the fermentation. This prompted the investigation of the gene(s) responsible for cellobionate and/or cellobiose metabolism in *N. crassa*, as discussed below.

Effects of the *ndvB* gene deletion on cellobiose, cellobionate, and cell mass production. The F5 Δ*ace-1* Δ*cre-1* Δ*ndvB* knockout strain was compared to the parent strain (F5 Δ*ace-1* Δ*cre-1*) in a shake flask fermentation experiment in Vogel's medium with 0.5 g/liter glucose to initiate cell growth and 5 g/liter cellobionate to evaluate cellobionate consumption. The results are shown in Fig. 2. Glucose was completely utilized within 11.5 h for both strains. For the F5 Δ*ace-1* Δ*cre-1* strain, cellobionate consumption began

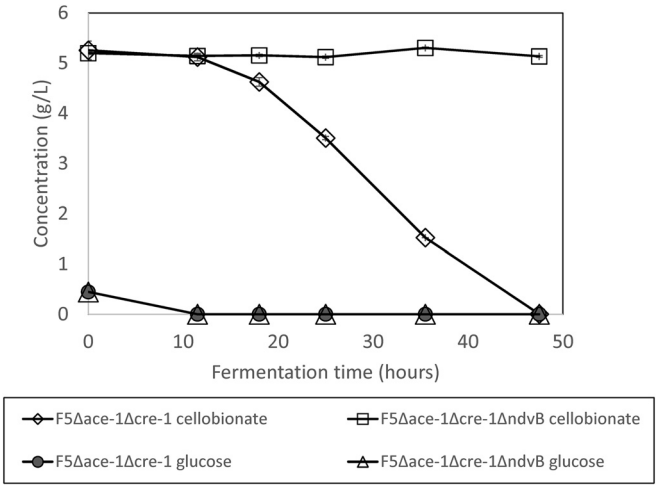

FIG 2 Cellobionate consumption by the *ndvB* knockout F5 Δ*ace-1* Δ*cre-1* Δ*ndvB* strain compared to the parent F5 Δ*ace-1* Δ*cre-1* strain. The values shown are the means for biological triplicates for each strain. Error bars are the standard deviations for the biological triplicates.

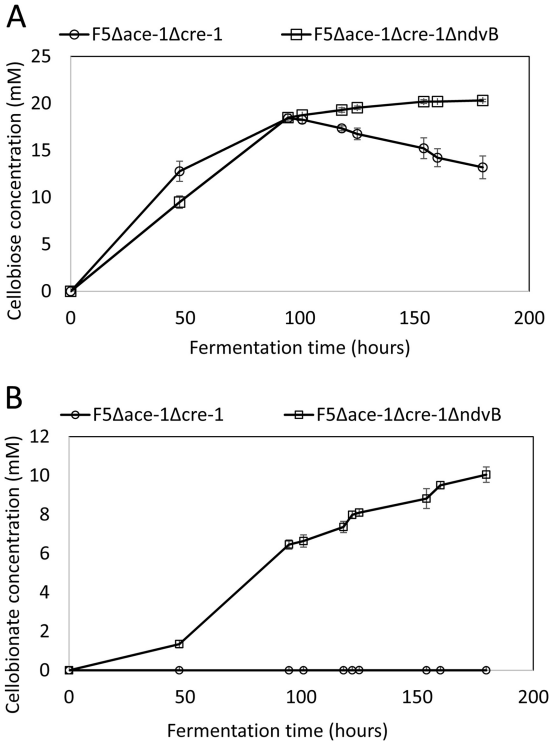

FIG 3 Cellobiose (A) and cellobionate (B) production by the F5 Δ*ace-1* Δ*cre-1* and F5 Δ*ace-1* Δ*cre-1* Δ*ndvB* strains grown on 20 g/liter Avicel. The values shown are the means for biological triplicates. Error bars are the standard deviations.

after glucose was utilized, and all cellobionate was completely used up in 48 h. In contrast, no cellobionate was consumed for the F5 Δ*ace-1* Δ*cre-1* Δ*ndvB* knockout strain, confirming that the *ndvB* gene was responsible for cellobionate metabolism.

The F5 Δ*ace-1* Δ*cre-1* and F5 Δ*ace-1* Δ*cre-1* Δ*ndvB* strains were subsequently evaluated in a shake flask fermentation in Vogel's medium with 20 g/liter Avicel. The concentrations of cellobiose and cellobionate production along the time course are presented in Fig. 3. Cellobiose production increased over the course of 167 h of fermentation time for the F5 Δ*ace-1* Δ*cre-1* Δ*ndvB* strain, with a maximum concentration of just over 20 mM, whereas the parent strain reached a similar maximum of 18.4 mM cellobiose at 95 h, followed by a steady decrease in concentration over the remainder of the fermentation. There was no detectable cellobionate production for the F5 Δ*ace-1* Δ*cre-1* strain, whereas the F5 Δ*ace-1* Δ*cre-1* Δ*ndvB* strain accumulated 10 mM over the course of the fermentation. The combined cellobiose and cellobionate production from 20 g/liter of cellulose was 10.5 g/liter for the F5 Δ*ace-1* Δ*cre-1* Δ*ndvB* strain.

In a separate experiment conducted identically to the above-described experiment, flasks were harvested at 167 h into the fermentation and analyzed for cell mass production. Based on the cell mass percentage in the insoluble fraction, the amounts of utilized and remaining Avicel were calculated, including the amount of Avicel directed toward cellobiose and cellobionate. The results are shown in Table 3. The F5 Δ*ace-1* Δ*cre-1* strain hydrolyzed approximately 76% of the Avicel. However, only 18% of the hydrolyzed Avicel remained as the desired products, cellobiose and cellobionate, at the end of the fermentation. In comparison, the F5 Δ*ace-1* Δ*cre-1* Δ*ndvB* strain hydrolyzed only 62% of the Avicel, but 75% of this Avicel was directed toward cellobiose and cellobionate. Substantially less biomass was produced with the F5 Δ*ace-1* Δ*cre-1* Δ*ndvB* strain than with the parent strain.

DISCUSSION

Previous studies have shown that the *cre-1*/*creA* genes encode a carbon catabolite repressor which regulates plant cell wall-degrading enzyme production in many filamentous fungi, such as *T. reesei*, *Aspergillus niger*, and *N. crassa* (16–18). CRE1/CreA regulate the production of enzymes associated with alternative carbon source utilization, including plant cell wall-degrading enzymes, in a double-lock manner (33–36). For example, CRE1 in *T. reesei* is known to bind to the promoters of the structure genes such as *cbh1* and *xyn1*, which encode a key cellulase and a key hemicellulase (37, 38). It also represses the expression of a regulatory gene, *xyl1*, which encodes the main activator protein for cellulase and hemicellulase enzyme production in *T. reesei* (39, 40). The deletion of *cre-1* in *T. reesei* led to enhanced cellulase and hemicellulase production (41, 42). The *cre-1* gene in *N. crassa* also represses the production of the plant cell wall-degrading enzymes (16). More than 100 genes in the Δ*cre-1* mutant showed more than a 2-fold increase over levels in the wild-type strain under Avicel growth conditions. Among them are 16 cellulase genes and 7 predicted hemicellulase genes (16). Δ*cre-1* mutants of *N. crassa* grew faster on Avicel than the wild type (16).

In addition to *cre-1*, *ace-1* is another important repressor for cellulase and hemicellulase gene expression (23). It was reported that it repressed cellulase gene expression (including that of *cbh1* and *xyn1*) in *T. reesei* grown on cellulose (23, 43). The expression of *ace-1* is also subject to the global CCR regulated by *cre-1* in *T. reesei* (18). An orthologue of *ace-1* was identified in *N. crassa* (25). However, its function was not yet characterized. In another study, it was found that mutations in cellulase genes, such as *cbh1*, could alter the effect of mutations of other regulatory genes in the zinc finger family on cellulase activity (25). Therefore, it is interesting to characterize the effect of *cre-1* and/or *ace-1* on cellulase expres-

TABLE 3 Percentages of Avicel hydrolyzed and directed toward fermentable products for the F5 Δ*ace-1* Δ*cre-1* and F5 Δ*ace-1* Δ*cre-1* Δ*ndvB* strains grown on 20 g/liter Avicel[a]

Strain	Amt (g) of Avicel		Cellulose conversion (%)	Mycelium produced (g)	Yield (%) from consumed Avicel	
	Starting	Residual			Cellobiose and cellobionate (mol/mol)[b]	Mycelium mass (g/g)
F5 Δ*ace-1* Δ*cre-1*	1.00	0.24 ± 0.02	76 ± 2	0.12 ± 0.003	18 ± 2	15.7 ± 0.6
F5 Δ*ace-1* Δ*cre-1* Δ*ndvB*	1.00	0.38 ± 0.002	62 ± 0.2	0.02 ± 0.001	75 ± 2	3.3 ± 0.2

[a] Errors are calculated based upon standard deviations and error propagation theory.
[b] The molecular mass of Avicel was assumed to be 324 g/mol.

sion with our mutant strain that contains deletions of six of seven *bgl* genes. Our data suggest that the F5 Δ*ace-1* single deletion strain has no improvement of CDH and exoglucanases expression compared to the parent F5 strain. In contrast, the F5 Δ*cre-1* and F5 Δ*ace-1* Δ*cre-1* double-knockout strains show an increase in exoglucanase and CDH expression compared to the parent strain. While CDH and exoglucanase expression increased for the F5 Δ*cre-1* and F5 Δ*ace-1* Δ*cre-1* deletion strains, this did not lead to an increase in cellobiose concentration under the fermentation conditions tested.

Although a 50% increase in CDH activity was detected for the F5 Δ*ace-1* Δ*cre-1* strain, cellobionate production was still not detected. This led to the assumption that *N. crassa* must have the ability to metabolize cellobionate. Recent literature suggested that the *ndvB* gene encodes a cellobionate phosphorylase enzyme, which converts cellobionate to α-D-glucose 1-phosphate and D-gluconic acid in an energy-efficient manner, both of which can be metabolized by *N. crassa* (26). We tested the effect of the *ndvB* gene deletion on cellobionate consumption, and our data suggest that deletion of *ndvB* can eliminate cellobionate consumption completely. Knocking out this gene resulted in a strain (F5 Δ*ace-1* Δ*cre-1* Δ*ndvB*) which generated 10 mM cellobionate and 20 mM cellobiose over the course of a 167-hour fermentation on 20 g/liter of Avicel. In contrast, the F5 Δ*ace-1* Δ*cre-1* parent strain produced no detectable cellobionate and about 18 mM cellobiose at 96 h, and the cellobiose declined to about 13 mM by the end of the fermentation (180 h). While the cellulose conversion achieved was higher for the F5 Δ*ace-1* Δ*cre-1* strain (76%) than for the F5 Δ*ace-1* Δ*cre-1* Δ*ndvB* strain (62%), a smaller fraction of fermentable sugars was produced. With the F5 Δ*ace-1* Δ*cre-1* strain, only 18% of the utilized Avicel was converted to the desired products (cellobiose and cellobionate), compared to 75% for the F5 Δ*ace-1* Δ*cre-1* Δ*ndvB* strain, where only a small fraction of the Avicel is consumed for cell growth and maintenance. Additionally, we showed that the *ndvB* gene allows *N. crassa* to consume cellobiose in addition to cellobionate. The deletion of the *ndvB* gene effectively prevented the cellobiose concentration from declining after 96 h into the fermentation.

Over the course of the fermentation of the F5 Δ*ace-1* Δ*cre-1* Δ*ndvB* strain, cellobiose production reached a plateau while cellobionate production continuously increased, which indicates that the conversion of cellobiose to cellobionate was the limiting step. Cellobiose is a known inhibitor of cellulases, although there is much debate as to the mechanism of inhibition due to the complexity of the cellulase mixtures and hydrolysis of cellulose (44). Previous studies have shown that cellobiose is more inhibitory to CBHs than to EGs and BGLs and that the presence of CDH can alleviate the competitive inhibition of cellobiose on CBHs by oxidizing cellobiose to cellobionate (45, 46). Should the rate of cellobiose conversion to cellobionate be increased by means such as improving CDH production in the F5 Δ*ace-1* Δ*cre-1* Δ*ndvB* strain, more cellobiose could be directed to cellobionate, which can possibly relieve the inhibition of cellobiose on cellulase. Therefore, higher cellulase activities could be exploited for increased rates of hydrolysis of cellulosic substrates, a higher cellulose conversion, a shorter processing time, and a possible higher product yield. Improving the conversion of cellobiose to cellobionate will be one of the focuses of our future work.

In conclusion, knocking out the *cre-1* gene in an *N. crassa* mutant with six of seven *bgl* genes deleted increased expression of exoglucanases and CDH, while no effect was observed for endoglucanase expression. Furthermore, knocking out the *ndvB* gene (strain F5 Δ*ace-1* Δ*cre-1* Δ*ndvB*) prevented metabolism of cellobiose and cellobionate and resulted in a significant improvement of cellulose conversion toward fermentable sugar and sugar-like products. While *N. crassa* was studied here as a model organism, the results obtained here could be extrapolated to industrially relevant organisms such as *T. reesei* to improve cellulose conversion toward fermentable products.

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