Engineering *Neurospora crassa* for cellobionate production directly from cellulose without any enzyme addition

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Running title: cellobionate production by *N. crassa*

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Abstract

In a cellulosic biorefinery, the cellulase enzymes needed for hydrolysis are one of the major contributors to high processing costs, while the hydrolysis product, cellobiose, has strong inhibition to the cellulases. In this study, we report engineering recombinant *Neurospora crassa* strains which are able to produce cellobionate, an organic acid, from cellulose without any enzyme addition. Recombinant strains were constructed by heterologously expressing laccase genes from different sources under different promoters in *N. crassa* F5 Δ *mus-51* Δ *ace-1* Δ *cre-1* Δ *ndvB* which has six out of seven β -glucosidase (*bgl*), two transcription factor (*cre1* and *ace-1*), and the cellobionate *phosphorylase* (*ndvB*) genes deleted. The strain expressing laccase from *Botrytis aclada* under a copper metallothionein promoter (HL10) produced the highest laccase activity. *N. crassa* HL10 produced 47.4 mM cellobionate from cellulose without any enzyme

addition. The yield of cellobionate from hydrolyzed cellulose was about 94.5 %. Conversion of cellobiose to cellobionate improved cellulose conversion and increases product yield.

1. Introduction

The microbiological production of organic acids is an attractive alternative to chemical synthesis. The native and genetically engineered microorganism microbial have been widely used in the production of a variety of organic acids such as acetic acid, gluconic acid, lactic acid etc [1]. In order to compete with petroleum-based processes for the production of carboxylic acids, the development of microbial processes utilizing low-cost substrates is essential [1]. Cellulosic biomass is a sustainable, low cost, and abundant feedstock for organic fuels and chemicals production. However, the major impediment for the commercialization of cellulosic bioprocessing is the lack of a low-cost processing technology [2]. Most of cellulosic biorefineries for organic acids production need cellulases addition to hydrolyze cellulosic biomass to fermentable sugars, which presents a substantial processing cost.

In this study, we report engineering a filamentous fungus, *Neurospora crassa*, for the production of a specialty organic acid, cellobionic acid, directly from cellulose without any enzyme addition. The chemical structure of cellobionic acid comprises a glucose moiety linked to a gluconic acid molecule. It is an aldonic acid obtained from the oxidation of cellobiose. Although there are few reports in literature exploring the application of cellobionic acid, its stereoisomer lactobionic acid has numerous applications in pharmaceutical, food, and cosmetics industries due to its unique physiochemical properties [3]. Cellobionic acid is expected to have similar applications due to the similar physiochemical properties.

In a prior study, the strain *N. crassa* F5 Δ *mus-51* was constructed by deleting six out of seven of its β -glucosidase genes and a gene involved in non-homologous end joining from the wild type *N. crassa* FGSC 2489 that produces a full array of cellulases and cellobiose dehydrogenases. The strain *N. crassa* F5 Δ *mus-51* was able to produce about 20 mM (7 g/L)

cellobiose and less than 1 mM of cellobionic acid from 20 g/L Avicel in 4 days [4]. In order to further improve the production of cellobiose and its derivatives, we deleted two transcription factor genes (*cre-1* and *ace-1*) involved in carbon catabolite repression, and a gene encoding a cellobionate phosphorylase gene (*ndvB*), which is necessary for cellobionate utilization, resulting strain *N. crassa* F5 $\Delta mus-51\Delta ace-1\Delta cre-1\Delta ndvB$. This strain was able to produce 20 mM (7 g/L) cellobiose and 10 mM cellobionate (3.5 g/L) from 20 g/L Avicel in 7 days [5].

Product inhibition of cellulases by its hydrolysis product cellobiose has been known to significantly decrease the rates of enzymatic hydrolysis and limits the sugar yields in cellulase mediated hydrolysis processes [5, 6]. Alleviating product inhibition is therefore essential for improving the process efficiency. Since cellobionic acid is a much weaker inhibitor to cellobiohydrolase I as compared to cellobiose[5], conversion of cellobiose to cellobionic acid could alleviate product inhibition on cellulases, therefore, increasing cellulose hydrolysis rate and improving product yield. In the native system of *N. crassa*, cellobiose dehydrogenase (CDH) catalyzes the oxidation of cellobiose to cellobionate. However, the re-oxidization of the reduced CDH by a polysaccharide monooxygenase and eventually oxygen was the rate limiting step, which led to slow conversion of cellobiose to cellobionate [7]. One strategy to increase the rate of cellobiose oxidation by CDH with oxygen as the final electron acceptor is to employ catalytic amounts of redox mediator with *in situ* regeneration of the redox mediator by laccase using the CDH-redox-laccase system [8]. In such a system, cellobiose is oxidized to cellobionic acid, in turn, the reduced CDH is re-oxidized with the help of a redox mediator, which is then regenerated through oxidation by laccase. Lastly, laccase is regenerated when the electrons are passed to oxygen, the final electron acceptor. The addition of catalytic amount of laccase (0.05 U/mL) and 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid (ABTS) (0.01 mM) as the

redox mediator to the fermentation of Avicel (20 g/L) by F5 Δ *mus-51* Δ *ace-1* Δ *cre-1* Δ *ndvB* strain produced about 40 mM cellobionate after 120 hours. Whereas, the fermentation using the same strain without laccase and ABTS addition only 10 mM cellobionate and about 20 mM of cellobiose remain un-converted [8].

Since exogenous enzymes addition remains one of the most expensive components of a biochemical conversion process, it is desirable to develop *N. crassa* strains that do not require any exogenous enzyme addition for the production of cellobionate directly from cellulose. In this study, the *N. crassa* laccase gene (*Nclac*) and a codon optimized *Botrytis aclada* laccase gene (*sBalac*, GenBank: JN559771) were expressed under the control of the constitutive *Aspergillus nidulans* glyceraldehyde-3-phosphate dehydrogenase (*gpdA*) promoter, a long copper metallothionein promoter fragment (*cmt*), and a short copper metallothionein promoter fragment (*cmt-1*) in the *N. crassa* F5 Δ *mus-51* Δ *ace-1* Δ *cre-1* Δ *ndvB* strain [9]. The strain showed the highest laccase activities when *cmt* or *cmt-1* was used as the promoter to express *sBalac*. Cellobionate was produced from cellulose by the strain HL10, which expresses *sBalac* under the control of *cmt-1*, without any enzyme addition.

2. Material and Methods

2.1 Strains and materials

Strains used in this study and their sources are listed in Table 1. Primers used in strain construction and verification are listed in Table 2. The strain F5 Δ *mus-51* Δ *ace-1* Δ *cre-1* Δ *ndvB* was constructed in a previous study [10]. The methods for constructing AH003, HL 04, 05, 10, 11 and 13 and verifying them are described in the following sections. ABTS was purchased from

Roche Diagnostics (Indianapolis, IN). All other chemicals were purchased from Sigma-Aldrich.
All the primers used for strain construction are shown in Table 1S.

2.2Construction of vectors

Plasmid pCSR1-gpdA-Nclac was constructed to express *N. crassa* laccase under the *Aspergillus nidulans* glyceraldehyde-3-phosphate dehydrogenase (*gpdA*) promoter in *N. crassa* *F5Δmus-51Δcre-1Δace-1ΔndvB*. The 1917 bp *N. crassa* laccase gene *Nclac* was amplified with primers (Nclac-F and Nclac-R contained PmlI and SnaBI restriction site, respectively) from the *N. crassa* FGSC2489 genomic DNA. Then, the PCR product was digested by PmlI and SnaBI and cloned into the pCSR1-cdh1 vector with the *cdh1* gene cut out by the same two enzymes [11] [12], building the plasmid *pCSR1-gpdA-Nclac*. The constructed plasmid contained the *Nclac* gene flanked by a *gpdA* promoter and an *A. nidulans trpC* terminator.

Plasmid pCSR1-gpdA-sBalac was constructed with a similar method in order to express laccase from *B. aclada* under the *gpdA* promoter. The fragment containing the codon optimized *B. aclada* laccase gene, a 6-His tag (*sBalac*, GenBank: JN559771), and PmlI and SnaBI restriction sites was synthesized by Invitrogen (Thermo Fisher Scientific, CA). The product was then digested by PmlI and SnaBI and cloned into pCSR1-cdh1 vector with *cdh-1* gene cut out, building the plasmid pCSR1-gpdA-sBalac.

Plasmids pCSR1-pcmt-Nclac and pCSR1-pcmt-sBalac were constructed to express laccase from *N. crassa* or *B. aclada* under the copper metallothionein (*cmt*) promoters [13]. The 4480 bp copper metallothionein (*cmt*) promoter was amplified from the genomic DNA of *N.*

crassa FGSC 2489 through PCR using primers (pcmt-slic-F and pcmt-slic-NcR, or pcmt-slic-F and pcmt-slic-sBaR). The pCSR1-gpdA-Nclac and pCSR1-gpdA-sBalac vector were amplified through PCR using primers (NcLac_F and 3flank_slic_R, and sBalacF and 3flank_slic_R, respectively), and then *pcmt* PCR product was ligated into the vector PCR product by sequence and ligation independent cloning (SLIC) [14], building the plasmid *pCSR1-pmct-Nclac* and *pCSR1-pcmt-sBalac*.

Plasmids pCSR1-pmct1-Nclac and pCSR1-pcmt1-sBalac were constructed using similar methods in order to express laccase from *N. crassa* or *B. aclada* under a truncated copper metallothionein (*cmt*) promoter [13]. The 511 bp copper metallothionein (*cmt-I*) promoter was amplified with primers (pcmt1-slic-F and pcmt-slic-NcR, or pcmt1-slic-F and pcmt-slic-sBaR) from *N. crassa* FGSC2489 genomic DNA. The pCSR1-gpdA-Nclac and pCSR1-gpdA-sBalac vector was amplified through PCR using primers (NcLac_F and 3flank_slic_R, and sBalacF and 3flank_slic_R, respectively), and then the *pcmt* PCR product was ligated into the vector PCR product by sequence and ligation independent cloning (SLIC), building the plasmid pCSR1-pmct1-Nclac and pCSR1-pcmt1-sBalac.

2.3. Transformation and diagnosis

Transformation of DNA containing the laccase genes into *N. crassa* F5 Δ *mus-51* Δ *ace-1* Δ *cre-1* Δ *ndvB* and screening of the transformants were performed following the protocols reported by Bardiya and Shiu [12]. Plasmids were amplified in *E. coli* Top10, and purified using a Zypzy™ Plasmid Miniprep Kit. The plasmids were double digested by PciI and PsiI, purified by agarose gel and extracted by NucleoSpin Gel Clean-up Kit (MACHEREY-NAGEL,

Germany). 90 μ L conidia suspension (2.5×10^9 conidia/mL) and 10 μ L DNA solution (100 ng/ μ L) were mixed in microcentrifuge tubes (1.5 mL) on ice for 5 min, then transferred to pre-chilled 0.2-cm electroporation cuvette (BioRad, Richmond, CA). After shock at 1.5 kV/cm; 25 μ FD and 600 ohms, 1 mL pre-chilled recovery solution containing 1 M sorbitol, 2% yeast extract and 20 g/L sucrose was immediately added in each cuvette, and transferred into a 15 mL conical tube. The cells were cultured at 30 °C and shaken at 150 rpm for 6 hours, then spread on selection plates (1 $\times$ Vogel's, 2% yeast extract, 20 g/L sorbose, 0.5 g/L fructose, 0.5 g/L glucose, 5 μ g/mL cyclosporine A, 2% agar). The positive transformants resistant to cyclosporine A were screened by diagnostic PCR.

2.4. Fermentation experiments

50 mL 1x Vogel's media containing glucose (40g/L) or Avicel (20g/L) and CuSO₄ (0.8 mM) in 250 mL Erlenmeyer flasks were inoculated by 10-14 day old conidia suspensions of the recombinant *N. crassa* strains at a final OD₄₂₀ of 0.05 [15]. Flasks were incubated at 28 °C and shaken at 200 rpm with constant light. At the beginning of each fermentation, 3 g/L of glucose was added to initiate conidial germination when Avicel was used as the substrate, and the pH of the cultures was adjusted to around pH 5.0-5.5 by NaOH (1 M). Samples were taken at various time intervals for enzyme activity analysis and compositional analysis.

2.5. Sample analysis

Concentrations of sugars (glucose, cellobiose, cellobionate) were analyzed using a Shimadzu HPLC equipped with a refraction index detector (RID), PDA detector, and a

CARBOSep COREGEL-87C (Transgenomic, San Jose, CA, USA) column. 4 mM calcium chloride at a flow rate of 0.5 mL/min was used as the mobile phase.

2.6. Enzyme activity assay

Laccase activity assays were performed in 96-well plate following previous protocol with minor modifications [8]. The reaction mixture contained 180 μ L of 1 mM ABTS in 100 mM sodium acetate buffer, pH 4.5. One unit of laccase activity is defined as the amount of enzyme oxidizing 1 μ mol of ABTS per minute under the above reaction conditions.

Exoglucanase activity assay was performed using *p*-nitrophenyl- β -D-lactopyranoside (pNPL, Sigma) as the substrate following previous a protocol [9]. The reaction mixtures containing 100 μ L of 1 mg/mL pNPL in 50 mM citric acid buffer (pH 5.0) and 80 μ L of sample were incubated for 15 minutes at 37 °C. The amount of released *p*-nitrophenol was measured at 405 nm after addition of 120 μ L of 1 M NaOH. One unit of enzyme activity is defined as the amount of enzyme required to release 1 μ mole of *p*-nitrophenol per minute.

2.7. Mycelial biomass measurements

The mycelia mass in the fermentation samples was measured by extracting ergosterol from the mycelia and quantification by HPLC following a protocol described by Hildebrand et al [8]. The amount of biomass was quantified using a standard curve prepared with known *N. crassa* dry mycelia.

3. Results

3.1. Laccase over-expression in *N. crassa* F5 Δ *mus-51* Δ *ace-1* Δ *cre-1* Δ *ndvB*

Six expression cassettes carrying the *A. nidulans* *gpdA* promoter, the *N. crassa* FGSC2489 *cmt* (copper metallothionein, 4.5 kb fragment) promoter, or the *N. crassa* FGSC2489 *cmt1* (copper metallothionein, 511 bp fragment) promoter; the *N. crassa* FGSC2489 laccase gene (*Nclac*), or the synthesized codon-optimized *B. aclada* laccase gene (*sBalac*); and the *A. nidulans* *trpC* terminator were knocked in the *csr-1* site of *N. crassa* F5 Δ *mus-51* Δ *lace-1* Δ *cre-1* Δ *ndvB* for laccase over-expression. Correct transformants were confirmed by diagnostic PCR.

3.2. Laccase production by recombinant strains on glucose

The laccase activities produced by the recombinant strains were characterized after 3 days' culture in Vogel's media containing 40 g/L glucose and 0.8 mM CuSO₄. As shown in **Figure 1**, *N. crassa* HL10, expressing *sBalac* under *cmt-1* promoter, and *N. crassa* HL13, expressing *sBalac* under *cmt* promoter, were the most successful laccase producers. Both strains secreted up to 0.59 U/mL laccase in the broth at day 3. The strain AH003, which expressed *Nclac* under the *gpdA* promoter, did not show obvious activity. Other strains produced detectable laccase activity (0.03-0.08 U/mL). Overall, *Nclac* or *sBalac* expressions under *N. crassa* *cmt1* or *cmt* promoter achieved 10-fold higher activities than those under the *A. nidulans* *gpdA* promoter. Strains expressing *sBalac* produced higher laccase activity as compared to the strain expressing *Nclac* when the same promoter was used. The two *N. crassa* recombinants (HL10 and HL13) that express the codon optimized *B. aclada* laccase gene under the 4.5 kb fragment or 511 bp fragment of *cmt* promoter were the best laccase producers. They were chosen for further study.

3.3. The effects of copper concentrations and addition time on laccase production

Since laccases are copper-containing enzymes and copper is the inducer for the *cmt* or *cmtI* promoter, copper ions likely play an important role in laccase activity and expression [13, 16]. Vogel's medium contains only 1 μ M copper, and it was unknown if this was high enough to induce the *cmt* or *cmtI* promoter and express functional laccase. Here, the effects of copper concentrations and its addition time on laccase production of *N. crassa* HL10 and HL13 were investigated (**Figure 2** and **Figure 3**). CuSO₄ was added to a final concentration of 0.5 mM at 0, 12, 24, or 36 hours for inducing the *cmt* or *cmtI* promoter and functional laccase expression (**Figure 2**). The laccase production profiles were not affected by the copper addition time. When copper was added at 0, 12, or 24 hours, all strains produced the maximum laccase activities at 60 hours (**Figure 2**). However, a little less laccase was produced when CuSO₄ was added at 36 hours as compared to the cases when the copper solution was added earlier.

The effects of copper concentrations on laccase expression in *N. crassa* HL10 and HL13 were also investigated. As shown in **Figure 3**, no obvious laccase production was detected (<0.03 U/mL) by either HL10 or HL13 when the culture contained less than 0.1 mM CuSO₄ (**Figure 3**). When the copper concentrations in media were increased to 0.2 mM or more, HL10 and HL13 produced obvious laccase activity in the media. Laccase production by HL10 and HL13 were tightly related to the copper concentrations in the media. Both strains were responsive to copper concentrations in the broth and their response patterns are similar to each other. Strain *N. crassa* HL10 was chosen for further study.

3.4. The effect of temperature on laccase production

Temperature is an important factor in *N. crassa* protein expression [17]. To evaluate the effect of temperature on laccase production by the recombinant strains, *N. crassa* HL10 was cultured on glucose (40 g/L) in Vogel's media with 0.8 mM CuSO₄ at different temperatures. As

shown in **Figure 4**, very little laccase was produced when *N. crassa* HL10 was cultured at 34 °C. When the culture temperature lowered to 24 or 26 °C, the laccase production reached the highest level of about 0.98 U/mL. Lower temperatures seem to favor the laccase production, which is a phenomena seen in laccase heterologous expression in *Pichia* as well [18]. Whether the temperature affect the protein expression seems to be protein specific [18].

3.5. The effect of temperature on cellobionate production

The effect of temperature on cellobionate production by *N. crassa* HL10 was tested and the results are shown in **Figure 5**. *N. crassa* HL10 produced the highest concentration of cellobionate (48 mM) when the culture was incubated at 30 °C, while the concentration of cellobionate produced was lowest (30 mM) when the culture was incubated at 24 °C. The trend was opposite that of laccase production when the strain was grown on glucose.

3.6 *N. crassa* F5Δ*mus-51Δace-1Δcre-1ΔndvB* and *N. crassa* HL10 fermentation on Avicel

N. crassa F5Δ*mus-51Δace-1Δcre-1ΔndvB* and *N. crassa* HL10 were grown on Avicel in Vogel's medium. ABTS (0.1 mM) was added into the fermentations at day 3. *N. crassa* HL10 produced higher exoglucanase activities than those of *N. crassa* F5Δ*mus-51Δace-1Δcre-1ΔndvB* cultures in the fermentation (**Figure 6A**). Cellobionate production by *N. crassa* HL10 increased over the course of 8 days of fermentation, and cellobionate concentration reached 47.4 mM, while the parent strain F5Δ*mus-51Δace-1Δcre-1ΔndvB* only produced about 17.1 mM cellobionate (**Figure 6B**). There was only about 0.6 mM cellobiose remaining in the fermentation broth for the HL10 strain, compared to 20.3 mM cellobiose for the F5Δ*mus-51Δace-1Δcre-1ΔndvB* strain. The HL10 strain produced 28.3% more of combined cellobiose

and cellobionate (48 mM) from 20 g/L Avicel than that produced by the F5 Δ *mus-51* Δ *ace-1* Δ *cre-1* Δ *ndvB* strain (37.4 mM).

In a separate experiment conducted identically to the above experiment, flasks were harvested after an 8-day fermentation and analyzed for residual Avicel and mycelium amount. As shown in Table 3, HL10 has higher cellulose conversion as compared to *N. crassa* F5 Δ *ace-1* Δ *cre-1* Δ *ndvB*. However, it produced slightly less mycelial biomass.

4. Discussion

Wild type *N. crassa* contains a laccase gene in its genome, which is transcribed only during sexual differentiation stage [19]. During vegetative growth, the laccase gene is repressed unless some protein synthesis inhibitors such as cycloheximide are added to trigger its expression [20]. Homologous expression of laccase in *N. crassa* under a 3.6 kb *cmt* promoter to enable its expression in the vegetative growth phase was achieved by Schilling et al [16]. We achieved the expression of *Nclac* and *sBalac* under the control of a 4.5 kb *cmt* promoter which contains the 3.6 kb *cmt* promoter and enabled laccase production under vegetative growth phase as well. The transgenes in the strains prepared by Schilling et al., are most likely to be ectopically integrated in a random genome location through non-homologous recombination [16]. In contrast, our engineered strains were prepared by integrating a single copy of a laccase expression cassette at the *csr-1* locus through homologous recombination and the resulting strain is a homokaryotic thanks to improved genetic engineering tools. Despite of this difference, our strain and the strain reported by Schilling et al. behaved similarly in response to available copper ions. Laccase production was only achievable when copper was added. The extracellular

laccase levels became detectable 36 hours after inoculation and the maximal laccase activities were detected around 60 hours.

The *cmt* promoter is an ideal promoter for regulated protein expression in *N. crassa* due to its high inducibility, ease of use, and economic applicability [21]. Kupper et al. compared a full length 3.6 kb and a truncated 400 bp promoter to express tyrosinase in *N. crassa* to enable the production of tyrosine under vegetative growth phase [13]. Recombinant strains using both promoters successfully produced tyrosinase activities. They reported that the full length 3.6 kb promoter was found to be inducible, while the 400 bp version was constitutive. In this study, we achieved the expression of laccases under the control of a long 4.5 kb *cmt* promoter and a shorter 511 bp *cmt-1* promoter. The 4.5 kb *cmt* promoter used in our study contains the 3.6 kb promoter reported by Tupper et al. Likewise, our 511 bp promoter contains their 411 bp promoter. They were built a little longer for the convenience of cloning. The 511 bp promoter is located upstream (from -511 to -1) of the copper metallothionein gene NCU05561. To our surprise, the 511 bp promoter was found to be inducible instead of constitutive. The strains containing either the long or the short promoters behave very similar to each other. The 511 bp promoter is likely to contain all the key elements to function as efficiently as the 4.5 kb one. It contained a typical TATA box at site -176 [22], and two putative CCAAT boxes at position -473 and -375 [23].

We also tested a constitutive promoter from *A. nidulans*'s D-glyceraldehyde-3-phosphate dehydrogenase gene (*gpdA*) for laccase production in *N. crassa*. Although the *gpdA* promoter has been successfully used as a strong promoter for heterologous gene expression in fungi [24], including expression of cellobiose dehydrogenase in *N. crassa* by our lab[11], we observed laccase (*lac*) expression under the *gpdA* promoter in *N. crassa* F5 Δ mus-51 Δ ace-1 Δ cre-1 Δ dvB was inferior to those under *cmt* or *cmt-1* control.

Under the same *cmt-1* promoter, *N. crassa* HL05 which expresses *Nclac* showed 10-fold less laccase activity than *N. crassa* HL10 which expresses *sBalac*. A possible reason is that *N. crassa* laccase has lower catalytic activity than that of *B. aclada* laccase on ABTS [9, 20] or the expression level of *N. crassa* laccase was lower.

The laccase production decrease when the culture temperature increases in the range of 24-30 °C when the recombinant strains were grown on glucose. However, the cellobionate production was highest at 30°C in the Avicel fermentation. Since the cellobionate production from Avicel needs the coordination of cellulases, cellobiose dehydrogenase, and laccases, the overall reaction seemed to favor a high temperature instead of a low temperature. Since cellobiose was present in the whole process, it indicates that the oxidation reaction was the rate limiting step.

When *N. crassa* HL10 and *N. crassa* F5 Δ *mus-51* Δ *ace-1* Δ *cre-1* Δ *ndvB* both grew on 20 g/L Avicel in Vogel's medium containing 3 g/L glucose, 0.1 mM ABTS and 0.8 mM CuSO₄, HL10 produced cellobionate as the main product (47.4mM) with only about 0.6 mM of cellobiose unconverted, while the strain F5 Δ *mus-51* Δ *ace-1* Δ *cre-1* Δ *ndvB* produced cellobiose (55%) as the major product and cellobionate as the minor product (45%). The *N. crassa* HL10 achieved higher cellulose conversion than that of *N. crassa* F5 Δ *mus-51* Δ *ace-1* Δ *cre-1* Δ *ndvB*, which indicated that accelerating of the conversion of cellobiose to cellobionate is an effective strategy to alleviate cellobiose inhibition on cellulases and to improve cellulobionate yield.

Filmentous fungi have been widely known for their ability to degrade cellulosic biomass and for their ability to convert sugars to organic acids. To our knowledge, this study was the first report to exploit both the cellulose degradation and organic acid production properties of a single filamentous fungi. It is worth noting that cellobionate could potentially be a high value chemical

with wide application itself. It could also be alternative carbon source instead of cellobiose or glucose for fuel and chemical production [5]. It can be directly used by engineered *Escherichia coli* for isobutanol production and an engineered *Saccharomyces cerevisiae* for ethanol production [25, 26]. Both of its hydrolysis products, glucose and gluconate, could be used for ethanol and acetic acid production by *E. coli* [4, 27].

5. Conclusions

In conclusion, we created an engineered *N. crassa* strain, HL10, for conversion of cellulose to cellobionate without any enzyme addition. *N. crassa* HL10 achieved higher cellulose conversion and higher product yield than those of its parent strain, which illustrates the advantages of relieving product inhibition by conversion of cellobiose to cellobionate in cellulose hydrolysis. This strain has the potential to be used for homo-cellobionate production from cellulosic biomass.

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383

384 Table 1. Strains used in this study.

Strain	Genotype	Source
FGSC 2489	Wild type	FGSC
F5 $\Delta ace-1$ $\Delta ndvB$	$\Delta mus-51$ $\Delta cre-1$ <i>bgl-1::hph bgl-2::hph bgl-3::hph bgl-4::hph bgl-6::hph bgl-7::hph mus-51::six ace-1::six cre-1::six ndvB::six</i> matA	(Hildebrand, Szewczyk et al. 2015)
AH003	F5 $\Delta mus-51$ $\Delta ace-1$ $\Delta cre-1$ $\Delta ndvB$ <i>csr-1::gpdA-Nclac</i>	This study
HL04	F5 $\Delta mus-51$ $\Delta ace-1$ $\Delta cre-1$ $\Delta ndvB$ <i>csr-1::gpdA-sBalac</i>	This study
HL05	F5 $\Delta mus-51$ $\Delta ace-1$ $\Delta cre-1$ $\Delta ndvB$ <i>csr-1::pcmt-1-Nclac</i>	This study
HL10	F5 $\Delta mus-51$ $\Delta ace-1$ $\Delta cre-1$ $\Delta ndvB$ <i>csr-1::pcmt-1-sBalac</i>	This study
HL11	F5 $\Delta mus-51$ $\Delta ace-1$ $\Delta cre-1$ $\Delta ndvB$ <i>csr-1::pcmt-Nclac</i>	This study
HL13	F5 $\Delta mus-51$ $\Delta ace-1$ $\Delta cre-1$ $\Delta ndvB$ <i>csr-1::pcmt-sBalac</i>	This study

385

386

387 Table 2. Primers used in strain construction and verification.

Primer	Sequence (5' to 3')
NcLac-F	TCACCCacgtgATGAAATTCTTGGGCATTGCCG
NcLac-R	cctgcaggtacgtaCTACCACTTCATCTTCACCTCCCTCG
pcmt-slic-F	GTGTGCAGCGCAGGAAATCCAccgcggCATCATAAGATCATCACAAACCACCCA
pcmt-1-slic-F	GTGTGCAGCGCAGGAAATCCAccgcggTAGTGTACGATGAGGGTATTTCGCC
pcmt-slic-NcR	GCAATGCCCAAGAATTTcATcacgtgTTTGATAGAAGTGGTTGTTCTTTTGAGAGG
pcmt-slic-sBaR	AACACGGTAAAGTACTTCATcacgtgTTTGATAGAAGTGGTTGTTCTTTTGAGAGG
3flank_slic_R	TGGATTTCTGCGCTGCAC
NcLac-F	ATGAAATTCTTGGGCATTGCCG
sBaLac-F	ATGAAGTACTTTACCGTGTTTCACCGCC
sBaLac-R	TCAGTGGTGATGGTGGTGGTG

388

389 Table 3. Percentage of Avicel hydrolyzed and the percentage directed toward fermentable
 390 products for the F5 Δ *mus-51* Δ *ace-1* Δ *cre-1* Δ *ndvB* and HL10 strains grown on 20 g/L Avicel.
 391 Errors are calculated based upon standard deviations and error propagation theory.

	Starting Avicel (mg)	Residual Avicel (mg)	Cellulose Conversion (%)	Mycelium produced (mg)	Yield of cellobionate and cellobiose from consumed Avicel (mol/mol $\times$ 100%)*	Yield of cellobionate from consumed Avicel (mol/mol $\times$ 1 00%)*
F5 Δ <i>mus-51</i> Δ <i>ace-1</i> Δ <i>cre-1</i> Δ <i>ndvB</i>	1000	305 $\pm$ 6	69.5 $\pm$ 0.6	90 $\pm$ 1	87.1 $\pm$ 0.6	39.9 $\pm$ 0.3
HL10	1000	188 $\pm$ 3	81.2 $\pm$ 0.3	81 $\pm$ 5	95.7 $\pm$ 1.1	94.5 $\pm$ 0.9

392

393 *Molecular weight of Avicel was assumed to be 324 g/mol.

394

Figure Captions

Figure 1. Laccase activities for the day 3 cultures of *N. crassa* recombinants with different promoter and laccase gene cassettes insertion. Conditions: *N. crassa* recombinants were cultured in 1x Vogel's media containing glucose (40 g/L) and CuSO₄ (0.5 mM) at 28 °C and shaken at 200 rpm for 3 days. The values shown are the means of biological triplicates for each strain, and the error bars are the standard deviations.

Figure 2. Laccase activities for the cultures of *N. crassa* HL10 (A) and *N. crassa* HL13 (B) different copper adding time. Conditions: *N. crassa* was cultured in 1x Vogel's medium containing glucose (40 g/L) at 28 °C and shaken at 200 rpm with CuSO₄ (0.5 mM) added at different time. The values shown are the means of biological triplicates for each strain, and the error bars are the standard deviations.

Figure 3. Laccase activities for the cultures of *N. crassa* HL10 (A) and *N. crassa* HL13 (B) at different copper concentrations. Conditions: *N. crassa* was cultured in 1x Vogel's medium containing glucose (40 g/L) and different concentration of CuSO₄ at 28 °C and shaken at 200 rpm. The values shown are the means of biological triplicates for each strain, and the error bars are the standard deviations.

Figure 4. Laccase activities for the cultures of *N. crassa* HL10 at different culture temperatures. Conditions: *N. crassa* was cultured in 1x Vogel's medium containing glucose (40 g/L) and CuSO₄ (0.8 mM) at different temperature and shaken at 200 rpm.

The values shown are the means of biological triplicates for each strain, and the error bars are the standard deviations.

Figure 5. Cellobiose (A) and cellobionate (B) production by *N. crassa* HL10 in Avicel Vogel's media at different fermentation temperature. Conditions: *N. crassa* was cultured in 1x Vogel's medium containing Avicel (20 g/L), CuSO₄ (0.8 mM) and ABTS (0.1 mM) at different temperatures and shaken at 200 rpm. The values shown are the means of biological triplicates for each strain, and the error bars are the standard deviations.

Figure 6. Exoglycanase activity (A), cellobiose and cellobionate production (B) by the F5Δmus-51Δace-1Δcre-1ΔndvB and HL10 strains grown on 20 g/L Avicel. The values shown are the means of biological triplicates for each strain, and the error bars are the standard deviations.

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433 Figure 1

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Figure 2.

Figure 3

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441 Figure 4.

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Figure 5.

Figure 6