A novel biochemical route for fuels and chemicals production from cellulosic biomass.

Permalink
https://escholarship.org/uc/item/0s7787ph

Journal
PloS one, 7(2)

ISSN
1932-6203

Authors
Fan, Zhiliang
Wu, Weihua
Hildebrand, Amanda
et al.

Publication Date
2012

DOI
10.1371/journal.pone.0031693

Peer reviewed
A Novel Biochemical Route for Fuels and Chemicals Production from Cellulosic Biomass

Zhiliang Fan1*, Weihua Wu1, Amanda Hildebrand1, Takao Kasuga2,3, Ruifu Zhang1,4, Xiaochao Xiong1

1 Biological and Agricultural Engineering Department, University of California Davis, Davis, California, United States of America, 2 Department of Plant Pathology, University of California Davis, Davis, California, United States of America, 3 Agricultural Research Service, United States Department of Agriculture, Davis, California, United States of America, 4 College of Resources and Environmental Sciences, Nanjing Agricultural University, Nanjing, Jiangsu, People’s Republic of China

Abstract

The conventional biochemical platform featuring enzymatic hydrolysis involves five key steps: pretreatment, cellulase production, enzymatic hydrolysis, fermentation, and product recovery. Sugars are produced as reactive intermediates for subsequent fermentation to fuels and chemicals. Herein, an alternative biochemical route is proposed. Pretreatment, enzymatic hydrolysis and cellulase production is consolidated into one single step, referred to as consolidated aerobic processing, and sugar aldolates are produced as the reactive intermediates for biofuels production by fermentation. In this study, we demonstrate the viability of consolidation of the enzymatic hydrolysis and cellulase production steps in the new route using Neurospora crassa as the model microorganism and the conversion of cellulose to ethanol as the model system. We intended to prove the two hypotheses: 1) cellulose can be directed to produce cellobionate by reducing β-glucosidase production and by enhancing cellobiose dehydrogenase production; and 2) both of the two hydrolysis products of cellobionate—glucose and gluconate—can be used as carbon sources for ethanol and other chemical production. Our results showed that knocking out multiple copies of β-glucosidase genes led to cellobionate production from cellulose, without jeopardizing the cellulose hydrolysis rate. Simulating cellobiose dehydrogenase over-expression by addition of exogenous cellobiose dehydrogenase led to more cellobionate production. Both of the two hydrolysis products of cellobionate: glucose and gluconate can be used by Escherichia coli KO 11 for efficient ethanol production. They were utilized simultaneously in glucose and gluconate co-fermentation. Gluconate was used even faster than glucose. The results support the viability of the two hypotheses that lay the foundation for the proposed new route.

Introduction

Cellulosic biomass is a sustainable source for organic fuels, chemicals, and materials; and is available at low cost and in large abundance [1,2,3]. The central obstacle impeding the widespread utilization of cellulosic biomass is the absence of a low-cost processing technology [3]. The conventional biochemical platform featuring enzymatic hydrolysis involves five key steps: pretreatment, cellulase production, enzymatic hydrolysis, fermentation, and product recovery, as shown in Figure 1. Sugars are produced as reactive intermediates for subsequent fermentation to fuels and chemicals. The steps involved in overcoming the recalcitrance of cellulosic biomass (making sugars)—pretreatment, cellulase production and enzymatic hydrolysis—are the three costliest steps in the entire process. Lowering the processing cost of these three steps is essential in order to make cellulosic biofinereries economically viable [3,4,5].

One strategy to reduce the processing cost is through process consolidation. Herein, we propose an alternative route for fuels and chemical production, which combines cellulase production and enzymatic hydrolysis into a single biological step and produces sugar aldolates instead of sugars as the reactive intermediates. As shown in Figure 2, this new route will utilize microorganism(s) that secrete all of the enzymes needed to solubilize lignin and hydrolyze cellulose and hemicellulose to the resulting sugars, despite the presence of associated lignin. Once formed, the majority of the sugars will be oxidized to the corresponding sugar aldonic acids, such as celllobionic acid or xylobionic acid, thereby preventing sugar utilization by microorganisms. A small fraction of the sugars will be available to support cell growth and enzyme production. In the second step, sugar aldolates (instead of sugars) will be utilized as the reactive intermediates for production of biofuels and other chemicals.

Lignocellulolytic fungi have remarkable potential for cellulosic biomass pretreatment, cellulase production, and direct enzymatic hydrolysis. Biological pretreatment is an attractive alternative to thermal chemical pretreatment due to its low energy requirements, mild reaction conditions, and environmental benefits [4,6]. Most biological pretreatment studies have been focused on use of lignocellulolytic microorganisms such as brown-, white- and soft-rot fungi or cellulolytic bacteria to selectively remove lignin in lignocellulosic materials, either to increase the digestibility of lignocellulose or as a pretreatment reagent for bio-pulping [6,7,8,9]. Of these options, white rot fungi are the preferred
microorganisms for the pretreatment tasks due to their ability to degrade lignin. However, two factors impede the practical application of biological pretreatment: carbohydrate loss during the treatment process and the slow reaction rate. To solve the carbon loss problem, alternative strategies have been proposed to selectively delignify lignin and suppress carbon-degrading enzymes [10,11,12,13]. Research has been conducted to select for naturally-occurring white rot fungi that preferentially attack lignin [14,15,16], selecting cellulase-less mutants [8,17,18], or repressing enzymes that degrade wood carbohydrates [19]. Limited success was achieved in all of these cases. Because lignin does not provide a carbon source for microbial growth of white rot fungi, repressing carbohydrate hydrolysis enzymes limits the reactivity of these fungi and leads to even longer processing times. There is no efficient strategy to tackle the slow growth problem of the biological pretreatment, and the genetic modification of white rot fungi is rather difficult. Lignocellulolytic fungi remain the main workhorse for cellulase production in industry. However, they have not been directly used in conversion of cellulose to sugars, also due to the fact that the fungi consume sugars for their own growth, which leads to severe carbohydrate loss during the process.

In the proposed new route, “consolidated aerobic bioprocessing,” intends to consolidate the pretreatment, cellulase production, and enzymatic hydrolysis steps in the conventional platform into a single microbiological step. Hence, we need to solve the same two problems associated with the utilization of fungi in biological pretreatment and direct enzymatic hydrolysis: slow growth rates and carbohydrate loss. The strategy we adopt to tackle the slow growth problem is to use the fast-growing fungus such as *Neurospora crassa*, which produces powerful cellulases, hemicellulases, and a variety of oxidases and laccases that are involved in phenol degradation, and potentially in lignin modification [20,21,22]. It is a genetically-tractable microorganism and tools for its genetic engineering are readily accessible [23,24,25]. Although *N. crassa* does not produce as powerful ligninases as white rot fungi, it has the potential for improvement of its lignin degrading ability by engineering the lignin degrading enzymes. Moreover, we will adopt a metabolic engineering strategy to solve the carbohydrate loss problem. Specifically, carbohydrate loss will be controlled by modifying the fungus’ metabolic pathway so that sugar aldonates instead of sugars will be produced from cellulose. In a separate step, sugar aldonates will be converted to fuels and chemicals by fermentation.

In this study, we aim to demonstrate the feasibility of consolidation of the enzymatic hydrolysis and cellulase production steps in the new route using *N. crassa* as the model microorganism and conversion of cellulose to ethanol as the model conversion system. The feasibility of consolidation of the cellulase production and enzymatic hydrolysis steps is based on the following two hypotheses:

Hypothesis 1: Cellulose can be directed to cellobionic acid production by reducing β-glucosidase production and by enhancing cellobiose dehydrogenase production.

Figure 3 shows the cellulose degradation mechanism for some cellulolytic microorganisms, including white rot fungi, brown rot fungi, and *Neurospora* [14,26,27,28,29,30]. Cellulose is hydrolyzed by endoglucanases and exoglucanases to cello-oligosaccharides, with cellobiose as the main component. Cellobiose and other cello-oligosaccharides are hydrolyzed by extracellular β-glucosidase (BGL) to form glucose or will be dehydrogenated by cellobiose dehydrogenase (CDH) to produce celllobionolactone, which reacts spontaneously with water to form cellobionic acid [31,32,33]. Cellobiose and other cello-oligosaccharides can also be transported intracellularly and be hydrolyzed by intracellular BGL to form glucose. Cellobionic acid and cello-oligosaccharide aldonic acids also can be hydrolyzed by intracellular or extracellular BGL to form glucose and gluconic acid, both of which can be metabolized by fungi [34].

As shown in Figure 3, cellobiose and cello-oligosaccharides can be diverted to the production of cellobionic acid and other cello-oligosaccharide aldonic acids if all the BGL productions are blocked. In practice, some BGL activity must be maintained for
cellulase induction and glucose generation. However, it is still possible to divert the majority of the carbon flow toward aldonic acid formation by over-expressing CDH, and lowering the activity of BGL. The added advantage is that organic acids are produced as products and methodologies for organic acid recovery such as calcium hydroxide precipitation can be adopted to remove them from the fermentation broth continuously so that they will not accumulate at high enough concentrations if they are inhibitory to the enzymes and the microorganisms [35]. However, detailed studies on the inhibitory effect of aldonate are still to be carried out.

Meanwhile, blocking gluconic acid utilization by inactivating the gluconokinase gene (gnk), which encodes the enzyme that is responsible for the first step in the gluconic acid utilization pathway, will improve the cellobionic acid yield from cellulose. As shown in Figure 3, gluconic acid is the end product of the reaction of cellobionic acid hydrolysis. Gluconic acid was found to be a non-competitive product inhibitor to the BGL from Aspergillus niger and its presence could prevent any noticeable hydrolysis of cellobionic acid by BGL [36].

**Hypothesis 2:** Both of the two hydrolysis products of cellobionate—glucose and gluconate—can be used as carbon sources for ethanol production.

Cellobionate and other cello-oligosaccharide aldonic acids can be easily converted to glucose and gluconate by adding exogenous β-glucosidase or by engineering in the fermenting microorganisms the ability to produce β-glucosidase in the subsequent fermentation step. Glucose is easily utilized by various microorganisms to produce ethanol and other chemicals. Theoretically, sugar aldonates can be utilized via the Entner-Doudoroff pathway to produce a wide variety of fermentation products including ethanol. From one mole gluconic acid, 1.5 moles of ethanol, 0.5 mole of acetic acid and 1.5 moles of ATP will be generated. If starting from the substrate cellobionic acid (Equation 2), 1.75 moles of ethanol, 0.25 mole of acetate and 1.75 mole of ATP are produced from sugar acids on a per glucose equivalent basis.

\[
\begin{align*}
C_6H_{12}O_7 & \rightarrow 1.5C_2H_5O_2 + 0.5C_2H_4O_2 + 2CO_2 + 0.5H_2O + 1.5ATP \\
0.5C_{12}H_{22}O_{12} + 0.25H_2O & \rightarrow 1.75C_2H_6O + 0.25C_2H_4O_2 + 2CO_2 + 1.75ATP 
\end{align*}
\]

Results and Discussion

**Cellobionate production by the multiple bgl knockout strain with or without exogenous CDH addition**

The hextuple bgl knockout strain F5 was able to produce about 0.4 g/L and 6.5 g/L cellobionate and cellobiose from cellulose, while the wild type strain did not produce any detectable amount of cellobionate or cellobiose. As shown in Figure 4, addition of the exogenous CDH increased the concentration of cellobionate produced by strain F5 from 0.4 g/L to 0.6 g/L. Strain F5 converted cellulose faster and produced more CDH and less cell mass as compared to the wild type. As shown in Table 1, the cellulose conversion achieved with F5 was 64%, as compared to that of wild type (55%). The mycelium yields from Avicel were about 22% and 52% for strain F5 and for the wild type, respectively. As shown in Figure 3, the highest CDH detected in the broth of F5 was 0.79 IU/L, as compared to 0.18 IU/L for the wild type. About 52% of Avicel was converted to cellobiose and cellobionate by strain F5, while no Avicel has been diverted to cellobiose and cellobionate production by the wild type strain.

Our results clearly showed that cellobionate was produced from cellulase by a mutant N. crassa strain with six out of seven copies of bgl knocked out. Simulating CDH over-expression by adding exogenous CDH produced more cellobionate. Substantial amount of cellobiose (about 6 g/L) was accumulating in the broth, which is an indication that the conversion of cellobiose to cellobionate is
Figure 4. The cellobionate, cellobionate and CDH production by wild type and strain F5 with or without CDH addition.
doi:10.1371/journal.pone.0031693.g004
limiting in the system. It seemed that knocking out six copies of bgl not only did not affect the cellulose utilization by this strain.

Gluconate can be utilized as the substrate for ethanol production by recombinant E. coli KO 11 strain

Glucose and gluconate were investigated as the sole carbon source for ethanol production by E. coli KO 11, which was engineered for efficient ethanol production from sugars [37]. Both glucose and gluconate were used for ethanol production. As shown in Figure 5, ethanol was the main fermentation product and very little acetate and lactate was produced when glucose was utilized as the substrate. About 163.6 mM of ethanol was produced from 87.1 mM glucose, reaching 94% of the theoretical yield. When gluconate was utilized as the substrate, ethanol and acetate were found to be the two main products, along with a small amount of lactate. About 113 mM ethanol and 46.3 mM of acetate were produced from 88.5 mM of gluconate. The stoichiometry of ethanol and acetic acid produced from gluconate follows Equation 1. Ethanol yield reached 85% of the theoretical yield, while acetate reached 105% of the theoretical yield. The gluconate was metabolized faster than glucose.

Since one mole of glucose and one mole of gluconate will be generated from cellobionate hydrolysis, we also studied the glucose and gluconate co-utilization by E. coli KO11. Glucose and gluconate co-fermentation was conducted starting with about 100 mM of glucose and 100 mM of gluconate. It was found that glucose and gluconate were utilized simultaneously. Ethanol and acetate were the two main products and the amounts produced follow the stoichiometry of equation 2. Produced ethanol and acetate reached about 80.7% and 99.6% of the theoretical yields, respectively. Gluconate was, again, found to be utilized faster than glucose (Figure 6).

E. coli was found to be able to metabolize gluconate aerobically [38,39]. According to our knowledge, our study is the first to report that gluconate is able to be metabolized for the production of fermentation products by E. coli under anaerobic conditions. Gluconate seems to be an excellent substrate for fermentation. It was utilized faster than glucose when they were used separately or in a co-culture. The same trend was found in E. coli JM 101 when glucose and gluconate were used as the carbon source in an aerobic culture [39]. The reason why gluconate is utilized faster than glucose is still to be elucidated. It is likely due to the different efficiency of their transporters and glucose and gluconate are transported by different transporters in E. coli [39,40,41]. Glucose and gluconate were also found to be utilized by E. coli KO 11 simultaneously when both of them were supplied as the carbon source, which indicated that the catabolite repression effect of glucose on gluconate was not obvious.

The proposed new route represents a substantial different route for fuels and chemicals production from cellulosic biomass. Sugar aldonates were produced as the reactive intermediates for the subsequent fermentation to fuels and chemicals. Since sugar aldonates are more reduced than glucose, a small amount of acetate has to be produced along with glucose, which led to lower yield of ethanol. However, the loss due to the production of acetate is relative small. Taking the production of cellobionic acid as an

Table 1. Summary of cellulose conversion and mycelium cell mass production.

|               | Starting Avicel (g) | Residual Avicel (g) | Cellulose Conversion (%) | Mycelium produced (g) | Yield of celllobiose and cellobionate from consumed Avicel (mol/mol×100%) | Yield of mycelium mass from consumed Avicel (g/g×100%) |
|---------------|--------------------|---------------------|--------------------------|-----------------------|---------------------------------------------------------------------------------|-----------------------------------------------------|
| wild type     | 1.0                | 0.47±0.05           | 53±5%                    | 0.28±0.02             | 52±5%                                                                            |                                                     |
| wild type+CDH | 1.0                | 0.40±0.03           | 60±3%                    | 0.31±0.04             | 51±7%                                                                            |                                                     |
| F5            | 1.0                | 0.36±0.01           | 64±1%                    | 0.14±0.02             | 52±7%                                                                            | 22±2%                                               |
| F5+CDH        | 1.0                | 0.35±0.0002         | 65±0.02%                 | 0.16±0.01             | 49±2%                                                                            | 24±1%                                               |

doi:10.1371/journal.pone.0031693.t001

Figure 5. Ethanol production from glucose and gluconate by E. coli KO11.
doi:10.1371/journal.pone.0031693.g005
example, the yield of ethanol from cellobionic acid is about 87.5\% of that from glucose on per glucose equivalent basis and the ratio of acetate produced versus ethanol is one to seven. The ethanol yield on a per glucose basis will increase when the chain length of cellooligosaccharide aldonates increases. Since the amount of acetate is relatively small, it is probably not economical to recover it as an individual product from the fermentation broth. However, in a biorefinery, most of the acetate will remain in the wastewater and can be easily converted to methane in the wastewater treatment step and the energy contained in acetate can be recovered.

Conclusion

Our results showed that knocking out multiple copies of β-glucosidase genes led to cellobionate production from cellulose, without jeopardizing the cellulose hydrolysis rate. Simulating cellbiose dehydrogenase over-expression by addition of exogenous cellbiose dehydrogenase led to more cellobionate production. Both of the two hydrolysis products of cellobionate: glucose and gluconate can be used by 

*Escherichia coli* KO 11 for efficient ethanol production. They were utilized simultaneously in glucose and gluconate co-fermentation. Gluconate was used even faster than glucose. Our preliminary results support the viability of the two hypotheses that lay the foundation for the proposed new route.

Materials and Methods

**Microbial strains and culture conditions**

All the single *bgl* knockout strains were from Fungal Genetic Stock Center (FGSC). The spores were stocked in glycerol at 

\(-80^\circ\text{C}\). *E. coli* KO11 (ATCC 29191) was purchased from ATCC and was stocked in the 

\(-80^\circ\text{C}\).

**Construction of hextuple *bgl* knock out strain**

Multiple *bgl* knockout strains were constructed through genetic crossing following a standard mating protocol [42]. Knockout strains were selected using a PCR-genotyping method. Since each single *bgl* knockout mutant from the FGSC contains a hygromycin resistance gene (*hph") inserted within a specific *bgl* locus, reverse primers were designed based on the *hph* open reading frame and forward primers were designed based on the flanking sequence of the knockout loci. The double knockout strains should produce two PCR products corresponding to two replaced genes. Two single knockout strains were crossed to generate the double knockout strain. Quadruple knockout strains were constructed by crossing two double knockout strains. The procedures were repeated until obtaining hextuple knockout strains. The *bgl* loci have been successfully knocked out in a mutant strain designated F5, and the diagnostic primer pairs for PCR genotyping are listed in Table 2.

![Figure 6. Ethanol and acetic acid production from glucose and gluconate co-fermentation.](https://doi.org/10.1371/journal.pone.0031693.g006)

**Table 2. The primers used for PCR geno-typing.**

| Locus Number | Primers | Sequence            |
|--------------|---------|---------------------|
| NCU00130     | left primer | 5'-ACACACCAAGCACAAGGAAACA-3' |
| NCU04952     | left primer | 5'-GAAGGACCAACCCATCTCCAC-3' |
| NCU05577     | left primer | 5'-TATTCGTTACCCACTCGGCA-3' |
| NCU07487     | left primer | 5'-AGGACGCTCTCAACACCTTCG-3' |
| NCU08755     | left primer | 5'-TCTCGCGACCTTCTCCTTCC-3' |
| NCU03641     | left primer | 5'-TATCGGAAAGACCTGCGAAACCT-3' |
| hph           | right primer | 5'-AGAGCCTGTGTAGCGGAATTTCC-3' |

[doi:10.1371/journal.pone.0031693.t002]
Preparation of crude CDH and CDH activity assay
The crude CDH was produced from a recombinant strain of *P. pastoris*, which heterologously expresses CDH from *N. crassa* following the production and concentration protocols as described by Zhang et al [43]. CDH activity was measured using 2,6-dichlorophenol-indophenol (DCPIP) as the electron acceptor as described in Zhang et al [43]. One international unit (IU) was equivalent to the reduction of 1 μmol of DCPIP per min at 30°C.

Cellubionate production by F5 and the wild type strains
The wild type and the hextupl knockout strain F5 were cultured in Vogel's medium with Avicel and glucose supplied at 20 g L⁻¹ and 0.8 g L⁻¹, respectively, as the carbon source. Fermentation was initiated by inoculating 10-day-old conidia to the flasks at the initial concentration of 10⁶ mL⁻¹. The fermentation was carried on a rotary shaker at 200 rpm and at 27°C for 100 hours with the light on. Samples were taken at various time intervals to monitor the concentrations of cellubiose, 2,6-dichlorophenol-indophenol (DCPIP) as the electron acceptor as described in Zhang et al [43]. CDH activity was measured using 2,6-dichlorophenol-indophenol (DCPIP) as the electron acceptor as described in Zhang et al [43]. One international unit (IU) was equivalent to the reduction of 1 μmol of DCPIP per min at 30°C.

Anaerobic fermentation experiment
Anaerobic fermentations were carried out in 200 mL serum bottles with a working volume of 100 mL and a N₂ gas phase. Luria Bertani (LB) broth with glucose or gluconate or both as the carbon source was used as the cultural medium. The pH was

References
1. Lynd LR, van Zyl WH, McBride JE, Laser M (2005) Consolidated bioprocessing of cellulosic biomass: an update.Curr Opin Biotechnol 16: 577–583.
2. Lynd LR, Weimer PJ, van Zyl WH, Pretorius IS (2002) Microbial cellulose utilization: fundamentals and biotechnology. Microbiol Mol Biol Rev 66: 506–577.
3. Lynd LR, Wymann CE, Gurrnang TU (1999) Biocommodity Engineering. Biotechnol Prog 15: 777–793.
4. Wymann CE (2007) What is (and is not) vital to advancing cellulase technology. Trends Biotechnol 25: 153–157.
5. Aden A, Ruth M, Isen B, Jechura J, Neves K, et al. (2002) Lignocellulosic biomass to ethanol process design and economics utilizing co-current dilute acid prehydrolysis and enzymatic hydrolysis for corn stover. NREL/TP-510–32430.
6. Sun Y, Cheng J (2002) Hydrolysis of lignocellulosic materials for ethanol production: a review. Bioresour Technol 83: 1–11.
7. Kurakake M, Ide N, Komaki T (2007) Biological pretreatment with two bacterial strains for enzymatic hydrolysis of office paper. Curr Microbiol 54: 424–428.
8. Akin DE, Sethuraman A, Morrison WH, Martin SA, Eriksson KE (1993) Microbial delignification with white-rot fungi improves forage digestibility. Appl Environ Microbiol 59: 4274–4282.
9. Blanchette RA (1991) Delignification by wood-decay fungi. Annu Rev Phytopathol 29: 381–396.
10. Bushwell JA, Osler R (1987) Lignin biodegradation. Crc Critical Reviews in Biochemistry 6: 1–60.
11. Gold MH, Warish K, Walli K (1989) Extracellular peroxidases involved in lignin degradation by the white rot basidiomycete *Phanecrichia chrysosporium*. ACS Symposium Series 309: 127–140.
12. Gold MH, Ali M (1993) Molecular-biology of the lignin-degrading basidio- mycete *Phanecrichia chrysosporium*. Microbiol Rev 57: 605–622.
13. Kirk TK, Farrell RL (1987) Enzymatic combustion - the microbial-degradation of lignin. Annu Rev Microbiol 41: 463–505.
14. Ander P, Eriksson KE (1997) Selective degradation of wood components by white-rot fungi. Physiol Plant 41: 239–248.
15. Kirk TK, Moore WE (1972) Removing lignin from wood with white-rot fungi and digestibility of resulting wood. Wood and Fiber 4: 72–79.
16. Lee JW, Gwak KS, Park JY, Park MJ, Choi DH, et al. (2007) Biological pretreatment of softwood Pinus densiflora by three white rot fungi. J Microbiol 45: 485–491.
17. Rud K, Barnsou F, Eriksson KE (1981) Micro-morphological and ultrastructural aspects of spruce wood degradation by wild-type *Spontumicium paliuratum* and its celluase-less mutant s444. Holzforschung 35: 157–171.
18. Eriksson KE, Grunewald A, Nilson T, Vallander L (1980) A scanning electron-microscopy study of the growth and attack on wood by 3 white-rot fungi and their cellulase-less mutants. Holzforschung 34: 207–213.
19. Yang HH, Efland MJ, Kirk TK (1980) Factors influencing fungal degradation of lignin in a representative lignocellulosic, thermomechanical pulp. Biotechnol Bioeng 22: 65–77.
20. Luke AK, Burton SG (2001) A novel application for Neurospora crassa: Progress from batch culture to a membrane bioreactor for the bioconversion of phenolics. Enzyme Microb Technol 29: 348–356.
21. Romero MD, Aguado J, Gonzalez L, Ladano M (1999) Cellulase production by *Neurospora crassa* on wheat straw. Enzyme Microb Technol 25: 244–250.
22. Eberhart BM, Beck RS, Goolsby KM (1977) Cellulase of *Neurospora crassa*. J Bacteriol 130: 101–106.
23. Davis RH, Perkins DD (2002) Neurospora: a model of model microbes. Nature Reviews Genetics 3: 397–403.
24. Tiao C, Berson WT, Lavorino AT, Sun J, Marletta MA, et al. (2009) Systems analysis of plant cell wall degradation by the model filamentous fungus *Neurospora crassa*. Proc Natl Acad Sci U S A 106: 22157–22162.
25. Tiao CG, Kasuga T, Sachs MS, Glass NL (2007) Transcriptional profiling of cross pathway control in *Neurospora crassa* and comparative analysis of the Gcn4 and CPC1 regulons. Eukaryot Cell 6: 1018–1029.
26. Eriksson KE, Petersson B, Westermark U (1974) Oxidation - Important enzyme reaction in fungal degradation of cellulose. FEBS Lett 49: 282–285.
27. Westermark U, Eriksson KE (1975) Acidic cellulase degradation products from wood attacked by *Spontumicium paliuratum*. Svensk Papperstidn 78: 653–656.
28. Cullen D (1997) Recent advances on the molecular genetics of ligninolytic fungi. J Biotechnol 53: 273–289.
29. Streamer M, Eriksson KE, Petersson B (1975) Extracellular enzyme-system utilized by fungus *Spontumicium paliuratum* (*Chrysosporium lignenum*) for breakdown of lignin.
of cellulose - functional characterization of 5 endo-1,4-beta-glucanases and one exo-1,4-beta-glucanase. Eur J Biochem 59: 607–613.

30. Eberhart BM, Beck RS (1975) Induction of beta-glucosidases in Neurospora crassa. J Bacteriol 116: 295–303.

31. Cameron MD, Aust SD (2001) Cellobiose dehydrogenase - an extracellular fungal flavocytochrome. Enzyme Microb Technol 28: 129–138.

32. Harreither W, Sygmand C, Augustin M, Narciso M, Rabinovich ML, et al. (2011) Catalytic properties and classification of cellobiose dehydrogenases from Ascomycetes. Appl Environ Microbiol 77: 1084–1093.

33. Henriksson G, Johansson G, Pettersson G (2000) A critical review of cellobiose dehydrogenases. J Biotechnol 78: 93–113.

34. Baldrian P, Valaskova V (2008) Degradation of cellulose by basidiomycetous fungi. FEMS Microbiol Rev 32: 501–521.

35. Singh OV, Kumar R (2007) Biotechnological production of gluconic acid: future implications. Appl Microbiol Biotechnol 75: 713–722.

36. Brechman, Ee, Markiano, E (1969) Enzymatic cleavage of cellobionic acid. Hoppe-Seylers Zeitschrift Fur Physiologische Chemie 350: 554–558.

37. Ohta K, Beall DS, Mejia JP, Shanmugam KT, Ingram LO (1991) Genetic improvement of Escherichia coli for ethanol production: chromosomal integration of Zymomonas mobilis genes encoding pyruvate decarboxylase and alcohol dehydrogenase II. Appl Environ Microbiol 57: 893–900.

38. Fliege R, Tong S, Shibata A, Nickerson KW, Conway T (1992) The Entner-Doudoroff pathway in Escherichia coli is induced for oxidative glucose metabolism via pyrroloquinoline quinone-dependent glucose dehydrogenase. Appl Environ Microbiol 58: 3026–3029.

39. Ponce E, Garcia M, Munoz ME (2005) Participation of the Entner-Doudoroff pathway in Escherichia coli strains with an inactive phosphotransferase system (PTS- Glc) in gluconate and glucose batch cultures. Can J Microbiol 51: 975–982.

40. Klemm P, Tong S, Nielsen H, Conway T (1996) The gntP gene of Escherichia coli involved in gluconate uptake. J Bacteriol 178: 61–67.

41. Porco A, Peekhaus N, Bausch C, Tong S, Isturiz T, et al. (1997) Molecular genetic characterization of the Escherichia coli gntT gene of GntT, the main system for gluconate metabolism. J Bacteriol 179: 1504–1509.

42. Fungal Genetics Stock Center website. Available: http://www.fgsc.net/neurosporaprotocols/How%20to%20make%20a%20cross-2.pdf. Accessed 2011 Jan 26.

43. Zhang R, Fan Z, Kasuga T (2011) Expression of cellobiose dehydrogenase from Neurospora crassa in Pichia pastoris and its purification and characterization. Protein Expression Purif 75: 63–69.