

NATURAL VARIATION OF QUANTITATIVE TRAITS FOR CONSOLIDATED
BIOPROCESSING OF CELLULOSIC ETHANOL IN *NEUROSPORA CRASSA*

By

JOSHUA CAIN WATERS

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Dr. Kwangwon Lee

And approved by

Dr. Kwangwon Lee

Dr. Eric Klein

Dr. Desmond Lun

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THESIS ABSTRACT:

Natural Variation of Quantitative Traits for Consolidated Bioprocessing of Cellulosic Ethanol in *Neurospora crassa*

By JOSHUA C WATERS

Thesis Director:

Kwangwon Lee

Current methods for converting plant biomass to value-added products such as ethanol are expensive and time consuming, requiring thermochemical pretreatment, enzymatic hydrolysis, microbial fermentation, and product recovery. These steps are classically performed separately with different organisms used for enzyme production, hexose fermentation, and pentose fermentation, which further increases production costs. To achieve cost-effective conversion of lignocellulose to ethanol these steps must be consolidated into a one-step reaction where biomass is hydrolyzed and fermented directly. This consolidated bioprocessing (CBP) requires an organism capable of hydrolytic enzyme production and fermentation of hexose and pentose sugars. The model filamentous fungus *Neurospora crassa* possesses all of these capabilities, making it a strong candidate for CBP. Therefore, we sought to characterize natural variation among populations of *N. crassa* and assess if selective breeding would provide a reliable route to generating elite strains for bioethanol production. We observed significant variation in natural and lab generated strains, and demonstrated improvements in fermentation in a single generation. Quantitative trait loci (QTL) analysis pointed to genomic locations underlying the observed phenotypic variance in saccharification of cellulose and

fermentation of ethanol within one of the populations. Finally, we demonstrated direct fermentation of untreated biomass (*Miscanthus giganteus*) by *N. crassa*, highlighting its potential for CBP and demonstrating that natural strains are more proficient at utilization of biomass than the laboratory reference strain.

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Chapter 1 - Introduction

Filamentous fungi for bioethanol production

Lignocellulose

Lignocellulose is the most abundant biopolymer on Earth and has great potential to serve as a renewable feedstock for fuels and chemicals. Lignocellulose is the main constituent of plant cell walls that provides rigidity, stability, and protection that the cells require. It is a complex extracellular matrix whose primary components are cellulose, hemicellulose, and lignin, making lignocellulose a promising source of polysaccharides for biofuels and chemicals production. Cellulose is an unbranched homopolymer of glucose with β -(1,4)-glycosidic linkages that can be hydrolyzed to fermentable glucose for ethanol production. In plant cell walls, cellulose chains align to form cellulose fibrils that aggregate into crystalline and amorphous cellulose fibers held together by additive hydrogen bonding and Van-der Waals interactions [1]. Cellulose fibers are insulated by hemicellulose fibers which contain highly branched heteropolymers of pentose sugars, mainly xylose and arabinose, which can also be fermented to ethanol by select organisms [1]. The cellulose-hemicellulose complex (holocellulose) is further insulated by lignin, a sturdy aromatic heteropolymer of phenolic derivatives, crosslinked with hemicellulose to form a lignin-carbohydrate complex [1]. Interspersed within this matrix are other polysaccharides (mainly pectin) and proteins that further add to the stability of lignocellulose [2]. The multiplicity of interactions among and within chains of lignocellulose make it highly stable and resistant to deconstruction (recalcitrant), therefore there have been many attempts to find efficient fractionation methods to

separate the soluble polysaccharide components from the insoluble lignin. To date the most effective fractionation methods range from strong acid or base treatments, organic solvent treatments, and ionic-liquid treatments, however chemical fractionation methods have proven to produce byproducts that are inhibitory to enzymatic hydrolysis and fermentation [3, 4].

The complexity and stability of the lignin-carbohydrate complex that provide utility to plants hinder the processing of lignocellulose into its usable components. A major obstacle for generating economically viable bio-products, such as ethanol, from plant biomass is the complexity and cost of pretreatment and fractionation processes, which are intended to depolymerize lignocellulose, increase pore sizes, and make the polysaccharides available for hydrolysis [1, 5]. A large amount of recent research has been aimed at understanding how lignocellulose composition relates to structural stability to find more efficient fractionation and hydrolysis methods while reducing loss of materials to waste streams [6]. Unfortunately, the relative composition of cellulose : hemicellulose : lignin is highly variable among plant types, making identification of a single efficient fractionation and hydrolysis method unlikely [7]. Therefore, increasing focus has turned toward enzymatic hydrolysis as an effective method for pretreatment of biomass for fermentation to ethanol, since enzymes have evolved to effectively degrade lignocellulose in its natural state.

Cellulosic Ethanol

Enzymatic conversion of lignocellulosic biomass to ethanol is suggested to have great potential for production of renewable energy, and as a result an increasing amount of

research has been directed toward cellulolytic enzyme production and lignocellulose hydrolysis. Cellulases are the set of enzymes that collectively hydrolyze cellulose polymers into glucose monomers, which along with hemicellulases (i.e. Xylanases), are able to convert the polysaccharide components of plant cell walls to fermentable sugars [8]. Cellulase production is a large, fast-growing global industry with established applications beyond fuels production including food processing, animal feed processing, textiles, and the pulp and paper industries, and if cellulosic ethanol proves viable the demand for cellulase and cellulase producers could increase dramatically [9].

Cellulase and hemicellulase are broad terms that encompass a variety of catabolic enzymes that act on cellulose and hemicellulose, respectively. In general, cellulase refers to 3 classes of hydrolytic enzymes; endoglucanases, which cleave within cellulose polymers to create new chain ends, exoglucanases, such as cellobiohydrolases, which cleave cellobiose disaccharide units from the ends of cellulose chains, and β -glucosidases, which cleave cellobiose into glucose monomers or release glucose monomers from the ends of cellulose chains [10, 8]. A fourth class of newly defined cellulase, lytic polysaccharide monooxygenases (LPMOs), use oxidative mechanisms to depolymerize cellulose instead of hydrolysis [8]. Within each class there are multiple enzymes and isoforms, and separate classifications of processive and non-processive isoforms. Processive, or progressive, enzymes remain in contact with their substrate after catalysis for further depolymerization, whereas non-progressive enzymes release from their substrate after catalysis [10]. The situation is similar for hemicellulases, requiring the different classes of hydrolytic enzymes, although more complex due to the variety of monomeric subunits and bonding configurations occurring in branched hemicellulose

[11]. Hemicellulose is also extensively modified by acetylation requiring auxiliary enzymes for removing modifications [11].

Tailored enzyme cocktails from industrial enzyme producers, such as *Trichoderma reesei* and *Aspergillus niger*, are used for the conversion of biomass to simple sugars for fermentation in a variety of ways [12]. During separate hydrolysis and fermentation (SHF), fermentable hydrolysates from enzymatic degradation of lignocellulose are recovered and separated into C6 (glucose) and C5 (mainly xylose and arabinose) product streams for fermentation by yeasts (*Saccharomyces* or *Scheffersomyces*) or bacteria (*E. coli* or *Zymomonas mobilis*) [13–16]. However, SHF has proven to involve too many steps (pretreatment, enzyme production, hydrolysis, sugar recovery, and fermentation) to be economically viable on industrial scales. Therefore, multiple attempts have been made to consolidate the steps involved in bioethanol production. Simultaneous saccharification and fermentation (SSF) is an attempt to reduce production costs by introducing fermenting yeasts into the hydrolysis reactions, alleviating the need for sugar recovery and purification prior to fermentation [17]. However, while excellent fermenters of C6 sugars, *Saccharomyces* strains are not naturally equipped for fermentation of the C5 sugars present in lignocellulose [18]. In an attempt to remedy this problem, investigators have sought to engineer *Saccharomyces* strains with xylose metabolism pathways from other organisms, or optimizing co-fermentation cultures with pentose-utilizing strains (also known as simultaneous saccharification and co-fermentation (SSCF)) [19–23].

While SSF has greatly reduced the costs of lignocellulosic ethanol production, the process still lacks economic viability on an industrial scale. The limitations of SSF and

SSCF include the separate requirements for substrate pretreatment, exogenous enzyme production, and product recovery. Furthermore, researchers suggest that commercial cocktails have sub-optimal concentration ratios and lack ancillary proteins and enzymes indirectly involved in conversion of lignocellulose, which limits the amount of hydrolysis that can be achieved when compared with direct hydrolysis [24, 25]. Therefore, investigators have turned their attention toward engineering organisms capable of producing hydrolytic enzymes in high quantities which are capable of directly fermenting the hydrolysates in a single fermentation reaction. This process, referred to as consolidated bioprocessing (CBP), is considered to be the best route toward economic viability of lignocellulosic ethanol.

Consolidated Bioprocessing

In its SSF, the hydrolysis of cellulosic biomass into fermentable sugars is performed with tailored enzyme cocktails derived mainly from the cellulolytic fungus *Trichoderma reesei*, however current interest is turning toward direct hydrolysis by fermentative fungi *in situ* [26]. However, due to its slow growth rate, poor hexose fermentation, and inability to ferment pentose sugars, *T. reesei* is an unlikely candidate for consolidated bioprocessing [26]. Instead, most investigations have been aimed toward modifying efficient fermenters to include hydrolytic pathways for CBP. *Saccharomyces cerevisiae* has been the main focus in these experiments, and has been successfully transformed to incorporate xylose transporters and xylose metabolic pathways from both fungi and bacteria to convert D-xylose to D-xylulose-5-phosphate, which can then be processed through the pentose-phosphate pathway to fructose-6-phosphate and glyceraldehyde-3-phosphate for fermentation [18, 19, 27].

After engineering *Saccharomyces* strains for xylose utilization, investigators turned toward their attention toward cellulose metabolism. Yeast surface display techniques, which rely on fusion of proteins to glycosylphosphatidylinositol (GPI) in the outer membrane, have been used to anchor extracellular cellulase enzymes, including β -D-endoglucanase, cellobiohydrolase, and β -glucosidase, in *Saccharomyces cerevisiae* for cellulose depolymerization [28–31]. To further enhance cellulose fermentation, cellodextrin transporters have also been incorporated into the yeast genome [32]. Likewise, *Saccharomyces cerevisiae* has been transformed to co-display hemicellulase enzymes, including xylanase, β -xylosidase, and xylose reductase for depolymerization of hemicellulose, however, there have been no strains developed that produce both cellulase and hemicellulase enzymes for efficient conversion of lignocellulose to ethanol [22, 33]. The major drawbacks associated with these engineering approaches are growth deficits and increased fermentation times due to the excessive physiological burdens from incorporation of too many exogenous genes, which often requires further engineering to attempt to supplement these deficiencies [4, 22, 34]. There is also concern that the genetic manipulations that are easily performed in laboratory strains may not be as successful in more robust industrial organisms [34].

Some researchers have begun to look away from *Saccharomyces* in attempts to identify or engineer superior strains for CBP. Other yeasts, including *Kluyveromonas* and *Scheffersomyces*, have been investigated and developed for increased fermentation of lignocellulosic biomass [14, 35]. Attention has also begun to move beyond yeasts to include filamentous fungi and bacteria as potential candidates for bioconversion of lignocellulose in CBP. Industrial bacteria, such as *Escherichia coli* and *Zymomonas*

mobilis, have been previously employed in biotechnology and represent obvious candidates for bioprocessing. *E. coli* has been engineered for cellulase and hemicellulase production and fermentation of the products to ethanol, 2,3-propanediol, or other value-added products [15, 36–40]. CBP with *E. coli* is unlikely though, due to low cellulolytic activities requiring exogenous cellulase mixtures and low ethanol tolerance, preventing efficient conversion and product recovery [38, 41]. *Zymomonas mobilis* circumvents the problems of low ethanol tolerance, but still requires engineering for cellulase and hemicellulase production and fermentation of pentose sugars [16, 42]. The extensive engineering requirements for utilizing industrial bacteria presents similar limitations as those seen previously with yeasts: limited cellulolytic potential, long fermentation times, product inhibition, and growth deficits [41, 43]. In response to these issues, some researchers have begun evaluating naturally occurring cellulolytic bacteria, including *Clostridium cellulolyticum*, *Bacillus licheniformis*, and *Paenibacillus polymyxa*, in hope of limiting the extent of engineering required, focusing mainly on fermentation pathways [44–47]. Similarly, many investigators have turned their attention to naturally cellulolytic filamentous fungi.

Filamentous saprotrophic fungi are ideal candidates for conversion of lignocellulosic biomass considering that the main industrial cellulase producers are filamentous fungi. Filamentous saprotrophs are excellent candidates for direct conversion of biomass to ethanol in CBP due to their ability to effectively penetrate through particulate biomass and their saprotrophic nature, in which they secrete extracellular metabolic enzymes to digest complex natural substrates externally and transport hydrolyzed substrates into the cell to be further processed for metabolic needs.

Saprotrophic fungi have a major role in carbon cycling through decomposition of lignocellulose in plant litter, and therefore poses robust endogenous systems for enzymatic deconstruction of plant biomass. Furthermore, some filamentous saprotrophs also poses all of the requisite pathways for fermentation of hexose and pentose sugars, making them excellent candidates for strain improvement for CBP. Industrial filamentous fungi involved in cellulase production have been obvious initial candidates for CBP, however slow growth rates and poor fermentation ability in *T. reesei* and *Aspergillus* have made them poor candidates for CBP [26, 48]. Therefore, attention has again moved beyond industrial strains to naturally occurring cellulolytic fungi for evaluation of potential for bioethanol production through CBP. White-rot fungi, such as *Phanerochaete cryosporium*, which produce high levels of lignin peroxidase enzymes, have been investigated as potential pretreatments for lignin degradation, however they lack robust cellulolytic systems requiring additional cellulase and co-culture with fermenting strains [49]. Similarly, brown-rot fungi, which degrade cellulose but not lignin have been investigated, but poor fermentation ability requires co-culture with fermenting yeasts or separate fermentations [50, 51]. Many of these investigations have preceded based on the notion that a successful candidate must stand out as having superior capacity in one of the four major pathways required for CBP; cellulase production, hemicellulase production, hexose fermentation, or pentose fermentation. However, current understandings of metabolism and physiology indicate that balance and coordination among these pathways is vital for an organism to be successful in CBP.

N. crassa and Natural Variation

Neurospora crassa, the model filamentous fungus, is representative of an ideal candidate for lignocellulosic bioethanol production via CBP. *N. crassa* is a naturally occurring cellulolytic fungi with all of the requisite pathways and enzymes for bioethanol production from lignocellulosic biomass [52, 53]. In fact, *N. crassa* has twice the number of predicted cellulase enzymes within its genome than *T. reesei* [26]. *N. crassa* is commonly found growing on burnt trees after wildfires or controlled burns, however it has also been isolated from unburnt soil, grass, and sugarcane, along with a variety of burnt plant substrates [54]. A robust catabolic system underlies *Neurospora*'s ability to thrive on such varied lignocellulosic substrates.

Similar to other cellulolytic fungi, the cellulolytic system of *N. crassa* is repressed through carbon catabolite repression (CCR) when preferred substrates are available. In the presence of glucose, CreA/CRE-1 the analog of Mig1 in *S. cerevisiae*, directly and indirectly represses cellulase, xylanase, and ethanol metabolism genes in filamentous fungi by repressing transcription of major degradation regulators, such as the xylose degradation regulator (Xlr-1), or by blocking interactions of regulators with targets through competition in binding structural gene promoters [55]. Deletion of cre-1 in *N. crassa* results in increased production of cellulase and xylanase when grown in cellulose, however cellulase and xylanase are not significantly increased in glucose or sucrose, illustrating that induction by cellulose or its metabolites is also required for cellulase and xylanase production [55].

In the absence of easily digestible substrates, CCR is relieved and basal expression of catabolic enzymes for alternative substrates, including cellulase enzymes, are secreted for detection of nutrients. Upon detection of cellulose, short-chain

cellodextrins or cellobiose are hydrolyzed and transported into the cell to induce the full cellulolytic response. In this respect, cellodextrin transporters serve as an important intermediary in initiating cellulose metabolism; however recent evidence has also illustrated that cellodextrin transporter, *cdt-2*, serves as a transceptor that coordinates signaling for cellulase production [56]. The transceptor signaling function is thought to be mediated through cAMP-PKA dependent signaling pathway, which has been implicated in signaling lignocellulose enzyme production in *N. crassa* and other filamentous fungi [2, 56, 57]. After import into the cell, cellulose metabolites interact with the major degradation regulators, Clr-1 and Clr-2 for cellulose and Xlr-1 for hemicellulose degradation, to induce gene expression for the full lignocellulolytic response [58, 59]. Together Clr-1/2 and Xlr-1 induce the full metabolic response to lignocellulose, leading to production of endoglucanase, exoglucanase, β -glucosidase, xylanase, β -xylosidase, and auxiliary cellulose and hemicellulose metabolism enzymes, cellodextrin transporters, such as *cdt-1* and *cdt-2*, and major facilitator superfamily (MFS) sugar transporters [58–60]. Endoglucanase enzymes hydrolyze cellulose chains internally to create new chain ends for hydrolysis by exoglucanases (cellobiohydrolases), which produce cellobiose disaccharides [8, 26]. Cellobiose that is released can be imported into the cell where it acts as a main inducer of cellulase, or it can be hydrolyzed to glucose by (intracellular or extracellular) β -glucosidase for the cell's metabolic needs [8, 26]. Similar functions are performed by the hemicellulase enzymes to produce xylose, arabinose, or mannose from hemicellulose chains [26].

After deconstruction of cellulose and hemicellulose to simple sugars, *Neurospora* has the capacity to ferment the hexose and pentose monomers to ethanol under anaerobic

conditions [52, 53]. Hexose fermentation in *Neurospora* follows the classical route through the Embden-Meyerhof pathway of glycolysis to generate pyruvate, which is decarboxylated to acetaldehyde by pyruvate decarboxylase and converted to ethanol by alcohol dehydrogenase. *Neurospora* is also capable of fermenting xylose to ethanol through a pathway involving xylose reductase, which converts xylose to xylitol, xylitol dehydrogenase, which converts xylitol to xylulose, and xylulokinase which converts xylulose to xylulose-5-phosphate [18, 19]. Xylulose-5-phosphate can then be processed through the pentose-phosphate pathway to produce fructose-6-phosphate and glyceraldehyde-3-phosphate which can then be fermented to ethanol through the conventional route [18, 19].

Although the metabolic response to lignocellulose has been well classified, these investigations have involved the use of gene deletion strains or transformants with constitutively active expression of transcriptional regulators or structural genes [55, 58, 59, 61]. However, there has been no effort to evaluate natural variation in cellulose metabolism arising from allelic variation among these genes. Adaptation of *Neurospora* to various lignocellulosic substrates provides insight to the flexibility of the system for plant cell wall deconstruction and provides potential for different allelic combinations to be enriched after successful colonization of new substrates with varying lignocellulose composition [54]. Therefore, it should be possible to exploit allelic variation through selective breeding to identify hyper-cellulolytic or hyper-fermenting strains of *N. crassa* for industrial bioethanol production, or to develop lines with increased conversion of target substrates based on their lignocellulose composition. A molecular breeding

approach should alleviate the physiological burdens and occurrence of growth deficiencies commonly encountered in mutagenesis and engineering approaches.

CHAPTER 2

Developing elite *Neurospora crassa* strains for cellulosic ethanol production using fungal breeding

Joshua C. Waters¹, Andrew Nixon², Morgan Dwyer¹, Justin C. Biffinger³, Kwangwon Lee^{1,2*}

¹ Department of Biology, Rutgers, The State University of New Jersey, Camden NJ 08103, USA

² Center for Computational and Integrative Biology, Rutgers, The State University of New Jersey, Camden NJ 08103, USA

³ Chemistry Department, US Naval Research Laboratory, Washington DC., 20375, USA

* Corresponding Author: Kwangwon Lee, Ph.D., Waterfront Technology Center, Room 510, Camden, NJ 08103, Phone: 856-225-2624, e-mail: kwangwon.lee@rutgers.edu

Abstract

The demand for renewable and sustainable energy has generated considerable interest in the conversion of cellulosic biomass into liquid fuels such as ethanol using a filamentous fungus. While attempts have been made to study cellulose metabolism through the use of knockout mutants, there have been no systematic effort to characterize natural variation for cellulose metabolism in ecotypes adapted to different habitats. Here, we characterized natural variation in saccharification of cellulose and fermentation in 73 ecotypes and 89 laboratory strains of the model fungus *Neurospora crassa*. We observed significant

variation in both traits among natural and laboratory generated populations, with some elite strains performing better than the reference strain. In the F1 population N345, 15% of the population outperformed both parents with the top performing strain having 10% improvement in ethanol production. These results suggest that natural alleles can be exploited through fungal breeding for developing elite industrial strains for bioethanol production.

Key Words

natural variation, cellulosic ethanol, cellulase, fungal breeding, strain improvement

Introduction

Depletion of global resources and increasing environmental concerns have illustrated the need for the development of renewable and sustainable resources. Cellulosic biomass, the most abundant resource on the planet, is capable of being converted into fuels or chemicals after the plant biopolymers are degraded to fermentable sugars. One of the important bio-products that can be obtained from cellulosic biomass is ethanol for liquid transportation fuels. The successful implementation of scalable bioethanol production as an alternative fuel has been demonstrated in Brazil [62]. However, the recalcitrance (resistance to degradation) of lignocellulose presents a large hurdle toward efficiently utilizing the abundant carbohydrates in plant biomass [1].

Current methods for converting plant biomass to value-added products are expensive and inefficient [63]. Lignocellulose is decomposed to simple sugars through

the use of enzyme cocktails derived from industrial fungi, such as *Trichoderma reesei* [63]. The liberated sugars then need to be recovered and fermented to final products by various other species of fungi [12, 44]. These steps are traditionally carried out separately, due to high energy demands for enzyme production and energy limiting conditions of fermentation. An alternative approach, consolidated bioprocessing (CBP), attempts to combine these steps to reduce the overall costs of production, however there are further technical drawbacks to this process from an engineering standpoint when considering implementation of CBP on an industrial scale and economically viable methods for the recovery of dilute ethanol from large fermentation cultures. Perhaps the most important limitation is the lack of a single organism that is equipped to efficiently perform all of the requisite processes for CBP; enzyme production, substrate hydrolysis, and fermentation of the liberated hexose and pentose sugars. Before tackling the technical limitations of scalability, identifying organisms that are efficient in all of the requisite physiological processes is paramount.

Although yeasts (especially *Saccharomyces*) are the most efficient fermenters of hexose sugars, many are poorly adapted to fermentation of pentose sugars (a large component of plant biomass) and are unequipped for decomposition of cellulose to fermentable sugars [64, 65]. On the other hand, the main fungus used to generate the exoenzymes responsible for decomposition of cellulose to simple sugars, *T. reesei*, is ill equipped to ferment the sugars it releases from cellulose through the process of saccharification [66]. In attempts to overcome these hurdles researchers have attempted to engineer organisms that are capable of robust cellulase expression and efficient fermentation of both hexose and pentose sugars to consolidate the production stages into

one-stage CBP [15, 17, 19, 35, 67]. There are, however, filamentous fungi capable of producing the exoenzymes to decompose cellulosic plant biomass to simple sugars, along with the full sets of enzymes required to ferment both the hexose and pentose sugars of plant biomass to ethanol; among them is the model filamentous fungus *Neurospora crassa* [2, 52, 60, 68].

Although the cellulolytic system of *N. crassa* has been characterized and investigations have been carried out to assess the potential of laboratory strains to decompose a variety of pure and natural substrates, there have been no systematic efforts to characterize the variation within and across populations arising from allelic variation [2, 69–72]. The genetic malleability, fast growth, and the well-developed tools and protocols for working with *N. crassa* make it a prime organism for studying the combined process of decomposing and fermenting cellulosic substrates simultaneously. In addition to the plethora of available resources and tools, over 2,000 *Neurospora* ecotypes are available in the Fungal Genetics Stock Center. We reasoned that these genetic resources are valuable for developing a strain for industrial usage. To achieve this goal, first, we characterized the natural variation in saccharification and fermentation that exists among ecotypes. Second, we generated a population by crossing the two top performing ecotypes to test if we can generate better performing strains in saccharification and fermentation. This process we call ‘fungal breeding’ in this study; developing an elite strain with desirable traits by crossing two related or unrelated parental strains. We have observed a significant variation for both traits in the natural and laboratory populations. Furthermore, a correlation was observed among the ecotypes abilities to ferment hexose and pentose sugars, suggesting that those ecotypes are highly

capable of fermenting hexose sugars and may also be well equipped for fermenting pentose sugars. Interestingly, there was no correlation observed between the amount of total exoenzyme produced and saccharification capability, suggesting that there are qualitative differences in exoenzyme production that underlie the observed high saccharification potential in some of the elite strains.

Materials and methods

Strains, propagation, and crosses

73 natural isolates of *N. crassa* and the wild type reference strain (FGSC2489) were ordered from the Fungal Genetics Stock Center (FGSC)[54, 73][53, 72] [54, 73], and grown from long-term stock prior to each experiment on Vogel's minimal media slants (1X Vogel's Salts, 2% Sucrose, 1.5% Agar, pH 5.8), whereas a mutant strain carrying a deletion of an extracellular β -Glucosidase (Δ Bgl, FGSC18387; NCU08755) from the FGSC was grown up on Horowitz Complete Media (1X complete salts, 1.6% glycerol, 5% casein hydrolysate, 0.5% yeast extract, 0.5% malt extract, 1.5% agar, pH 5.8). *T. reesei* (QM9414) Simmons Anamorph ATCC26921 was ordered from American Type Culture Collection and the freeze-dried pellet was propagated on Potato Dextrose Agar (2% potato dextrose broth, 1.5% agar, pH 5.8) according to the supplier's instructions. Spore suspensions in High Glucose Liquid Media (HGLM) (1X Vogel's salts, 2% glucose, 0.5% L-Arginine, pH 5.8) were used to generate mycelial mats in petri plates, which were used to generate replicate mycelial pads for each experiment using a bore punch.

Top performing ecotype strains from saccharification and fermentation screens

were each crossed against strains of both mating types (FGSC2489, Mat A and FGSC18387, Mat a) on crossing media slants (1X Westergaard's salts, 2% Sucrose, 5% Biotin, 1.5% Agar, pH 5.8). Successful crosses were used to type each strain against the mating type it was able to cross with. The two best strains with opposite mating types (JW220, Mat A and JW228, Mat a) were successfully crossed to generate the N345 population. Individual spores were picked, heat-shocked at 60°C for 30 min, and germinated on minimal media slants. A total of 89 germinated progeny of the cross was studied in this study.

Plate clearing assay

Plate clearing assays were performed using Congo Red indicator as described by Meddeb-Mouehli et al., in which 4 gauge mycelial pads from spore suspension in HGLM were inoculated onto Carboxymethylcellulose (CMC) / Sorbose agar (1X Vogel's salts, 0.5% CMC, 2% Sorbose, 1.5% Agar) and plates were flooded 3 days post-inoculation with 0.1% Congo Red for 20 min, and washed sequentially with 1M NaCl for 15 min [74]. All samples were performed in biological triplicates. Images of the zone of hydrolysis and fungal growth were captured with a Nikon D7000 and analyzed with ImageJ software. Plate clearing assays revealed differential growth and zones of hydrolysis among the natural isolates screened in this study. Despite the addition of sorbose to the medium to restrict lateral growth isolates demonstrated varied responses to sorbose. Therefore, since some strains were able to accumulate more cell mass, and therefore secrete more protein, an index was created in an attempt to normalize enzyme secretion to the amount of cell mass increase. A Cell Mass Increase (CMI) index was

created to determine how much cell mass was accumulated during growth on the cellulose substrate. However, it is important to note that measuring lateral growth does not account for how dense the cell mass may be, and is therefore not a precise measurement of the total increase in cell mass. Nevertheless, it was considered useful in assessing how much of the consumed substrate was utilized for growth. A Cellulase Production Index (CPI) was created in an attempt to normalize the area of hydrolysis to the total cell mass capable of producing enzyme. Finally, a Substrate Utilization Index (SUI) was created as an attempt to score individuals based on their cellulase production relative to how much hydrolyzed substrate was diverted toward growth, with high scores representing strains with large areas of hydrolysis and little utilization of substrate for growth. Individuals with high CPI are of interest for their ability to hydrolyze more substrate with less cell mass required, while individuals with high SUI are of interest for ethanol production since less of the hydrolyzed substrate is deferred to growth and is available for fermentation. The indices were calculated as follows: Area of Cellulase Activity (ACA) = Area of clearing; Cell Mass Increase (CMI) = Area of growth_{final} – Area of pad_{initial}; Cellulase Production Index (CPI) = ACA/Area of growth_{final}. A Substrate Utilization Index (SUI) = CPI/CMI.

Enzyme activity assays

A modified FPA assay was performed using 96-well plates as described by Camassola and Dillon, in which secreted protein extracts were taken from wells in 6-well plates containing 1% CMC broth (1X Vogel's salts, 1% CMC) 4 days after inoculation with 10 gauge mycelial pads [75]. Culture broth containing secreted enzyme was filtered and

centrifuged at 13.2k rpm to remove any fungal cells or debris. 50 μ L of supernatant was added to 100 μ L of 50 mM sodium acetate buffer pH 5.6 in a 96-well deep-well plate, which was then equilibrated to 50°C for 5 min in a hot-water bath. A 5mg strip of Whatman Grade No. 1 filter paper was submerged in the solution, and the plate was incubated at 50°C for 60 min. After 60 min, 300 μ L of DNS Reagent was added to stop the enzymatic reaction and visualize glucose equivalents. The plate was incubated at 100°C for 10 min to develop color, then transferred to an ice bath to stop color development. 100 μ L of Enzyme/DNS mixture was transferred to a clear-bottom assay plate, diluted with 200 μ L of diH₂O, and absorbance was measured at 545 nm. The concentration of reducing equivalents released was determined with standard curves generated with glucose standards. All samples were performed in biological triplicate. Due to the relatively low concentration of secreted enzymes, activity could not be quantified in conventional Filter Paper Units, which requires the production of at least 2mg/ml of glucose equivalents, therefore results were presented in the form of the concentration of reducing equivalents released by each aliquot. Similarly, a modified carboxymethylcellulase (CMCase) assay was performed using soluble 2% CMC as the reaction substrate instead of Whatman filter paper. The enzymatic reaction was carried out for 30 min instead of 60 min, however the rest of the procedure was carried out as it was in the FPA assay.

Fermentation

To characterize fermentation among the top ecotypes (JW-16, JW-60, JW-160, JW-161, JW-176, JW-220, JW-228, JW-234) based on their performance in preliminary

experiments investigating cellulolytic potential, the top 9 candidates, along with wild type *N. crassa* strain (FGSC2489), and commercial brewer's yeast were screened for variation in fermentation of glucose. Mycelial mats of *Neurospora* were generated from spore suspensions in HGLM. Mycelial pads were punched from the mats with a 10 gauge punch, rinsed in sterile deionized water, and inoculated into 15ml conical tubes containing 15ml of 2% Glucose, 2% Xylose, or 2% CMC. 500 ul of yeast suspension was inoculated into 15 ml of corresponding substrate as positive control for glucose fermentation. The tubes were tightly sealed with screw-on caps to ensure anaerobic conditions. Cultures were incubated at room temp for 9 days, after which they were centrifuged at 2,500 rpm for 10 min. at 4°C. The supernatant was recovered and filtered through grade No. 1 Whatman filter paper, centrifuged at 5000rpm for 5min and the supernatant was recovered for analysis. All samples were performed in biological quadruplicate. The recovered supernatant was aliquoted into HPLC vials for ethanol analysis by HPLC. HPLC quantitation was performed using a Varian ProStar HPLC with a Varian ProStar Autosampler and a Varian 356-LC Refractive Index Detector. An isocratic elution was used with an Agilent Hi-Plex H⁺ (300 mm x 7.7 mm ion-exchange column) with 5mM H₂SO₄ at a flow rate of 0.7 ml/min at 60°C. The concentration of ethanol present was determined from a standard curve based on ethanol standards with a known concentration.

A 96-well format for fermentation was carried out in deep-well plates to characterize the amount of fermentation among the lab generated N345 first filial (F1) generation. Replicates for each strain were collected from mycelial mats with a 6 gauge punch and inoculated into 750ul of HGLM (2% glucose), sealed with aluminum

Thermowell™ seals, and allowed to ferment for 7 days in 12:12 LD conditions at 25°C. All samples were performed in biological quadruplicate. After fermentation, 600 µL of media was recovered and cell debris was removed by sequential centrifugation at 13.2k rpm for 5 min. Recovered supernatant was analyzed at NRL as previously described.

Finally, to characterize direct fermentation of cellulosic biomass, 6 gauge mycelial pads of FGSC2489, JW220 and JW228 (cross parents), and N345-2 (best cross progeny) were inoculated into 750uL of 2% *Miscanthus giganteus* broth and incubated at ambient room temperature for 7 days. Culture broth was recovered and sequentially centrifuged at 13.2k rpm to remove fungal debris and residual plant matter. Supernatants were transferred to HPLC vials and analyzed for ethanol content as previously described.

Statistical analysis

T-tests and Single Factor ANOVA were performed in Microsoft Excel using the data analysis tool-pack. Prism Graph Pad was used to test for Pearson correlations and for construction of graphs.

Results

Natural variation in saccharification and fermentation among *Neurospora* ecotypes

To quantitatively measure the level of cellulase expression by different ecotypes of *Neurospora*, we have used several indices, Cell mass increase (CMI), Area of Cellulase Activity (ACA), and Cellulase Production Index (CPI) (Materials and methods). We reasoned that a desirable strain for ethanol production is the strain that can hydrolyze the largest amounts of available cellulose without diverting too much of the liberated sugars

toward its own cellular growth. Thus, Substrate Utilization Index (SUI) was defined as the ratio of CPI to CMI, with larger values indicative of high levels of hydrolysis with minimal growth. In general, the observed data for ACA, CMI, and CPI appeared to be normally distributed, however, the data for SUI shows a strong positive skew (Fig. 1a, b).

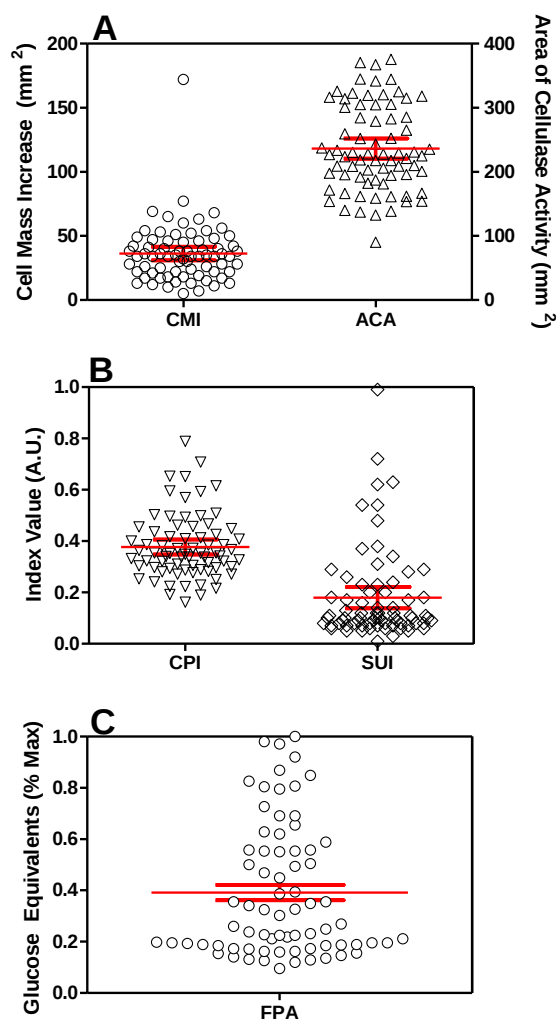


Fig. 1 Natural variation in cellulolytic activity among natural isolates. A & B) Significant variation was observed among 73 natural strains in the indices analyzed in plate clearing experiments. The area of cellulase activity (ACA), cell mass increase (CMI), and cellulase production index (CPI) demonstrated normal distributions, while substrate utilization index (SUI) was negatively skewed. The skew of SUI is expected to arise from variation in sorbose resistance opposed to cellulolytic activity, as sorbose was used to restrict lateral growth of hyphae. C) Significant variation was observed in FPA assay among natural strains, presented as percentage glucose equivalents released by top

performer since values fall outside the range of the standard curve used. Red bars represent mean and 95% CI

The activity of secreted enzyme from each isolate was determined using a modified filter paper activity (FPA) assay as previously described (Materials and methods and [75]). An almost 10-fold difference was observed among the activity of the natural isolates tested (Fig. 1c). The top performing strains from preliminary FPA screens were selected for comparison based on their cellulolytic enzyme production. Glucose equivalents released during FPA and CMCase assays among the top ten strains showed minor variation (Fig. 2a) with QM9414 generating almost twice the concentration of glucose/mL compared to the rest of the strains tested in the current study. There was no correlation between secreted protein and cellulolytic activity of the top performing strains in either assay, suggesting that the key catalytic enzymes for degradation of cellulose, cellobiohydrolases and endo-glucanases, represent only a small fraction of secreted protein (Fig. 2b, c). All strains secreted proteins to a concentration of 35-50 μ g/mL in the course of the experiments, resulting in approximately 1.2 $\pm$ 0.2 mg/mL of glucose except for QM9414 which produced twice the concentration of glucose.

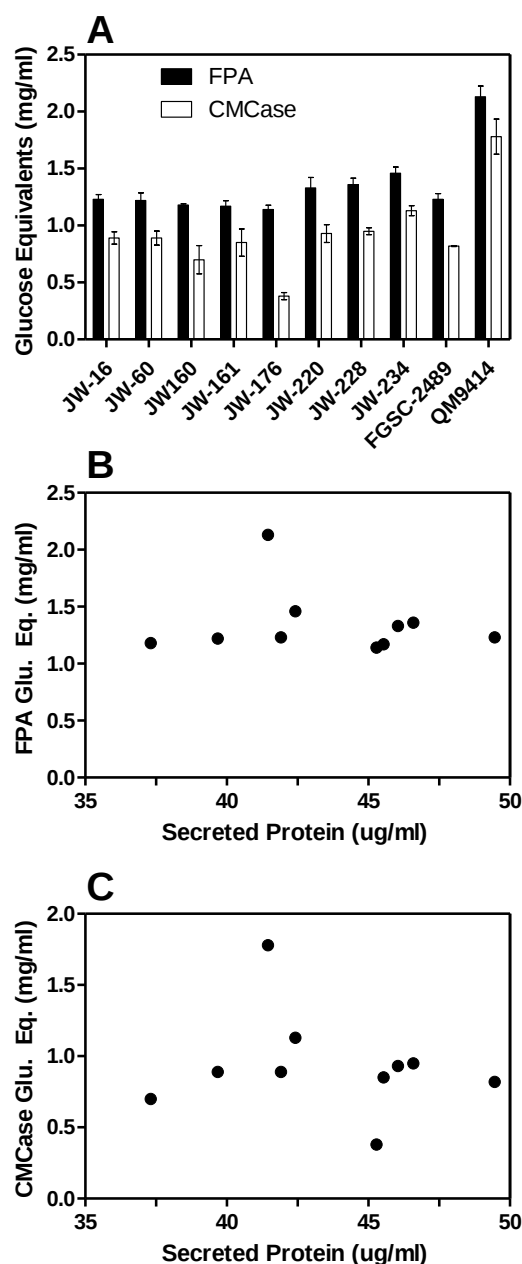


Fig. 2 Natural variation in cellulolytic activity among top performing natural ecotypes. Variation was observed among the top performing strains in activity in the FPA assay and CMCase assay (A). No correlation was observed between the amount of protein secreted and the level of cellulase activity measured by FPA assay (B) or CMCase assay (B) ($R^2=0.02285$, $p=0.6768$ and $R^2=0.03114$, $p=0.6258$ respectively). QM9414 (A) is *T. reesei*, used as a standard for cellulase activity. Error bar represents one standard deviation

These same strains were then compared based on the concentration of ethanol that resulted from the fermentation of glucose, xylose, and CMC as substrates. The top performing strains from the combined cellulase production screens were selected for this analysis. An approximately 6-fold difference was observed among the selected strains for fermentation of glucose, however commercial brewer's yeast proved superior (Fig. 3a). There was no significant difference between the same strains when fermenting the pentose sugar xylose, while a 2-fold difference was observed for the selected strains when fermenting the more complex polysaccharide CMC (Fig. 3b). Brewer's yeast was unable to ferment xylose or CMC since it is lacking the enzymes for conversion of xylose to fermentable substrates and hydrolysis of cellulose to fermentable glucose (Fig. 3b). A correlation was observed between fermentation of glucose and xylose among strains (Fig. 3c), while a weaker correlation was observed between fermentation of glucose and CMC, ($R^2=0.7511$, $p=0.0025$ and $R^2=0.6711$, $p=0.0069$ respectively) (Fig. 3d).

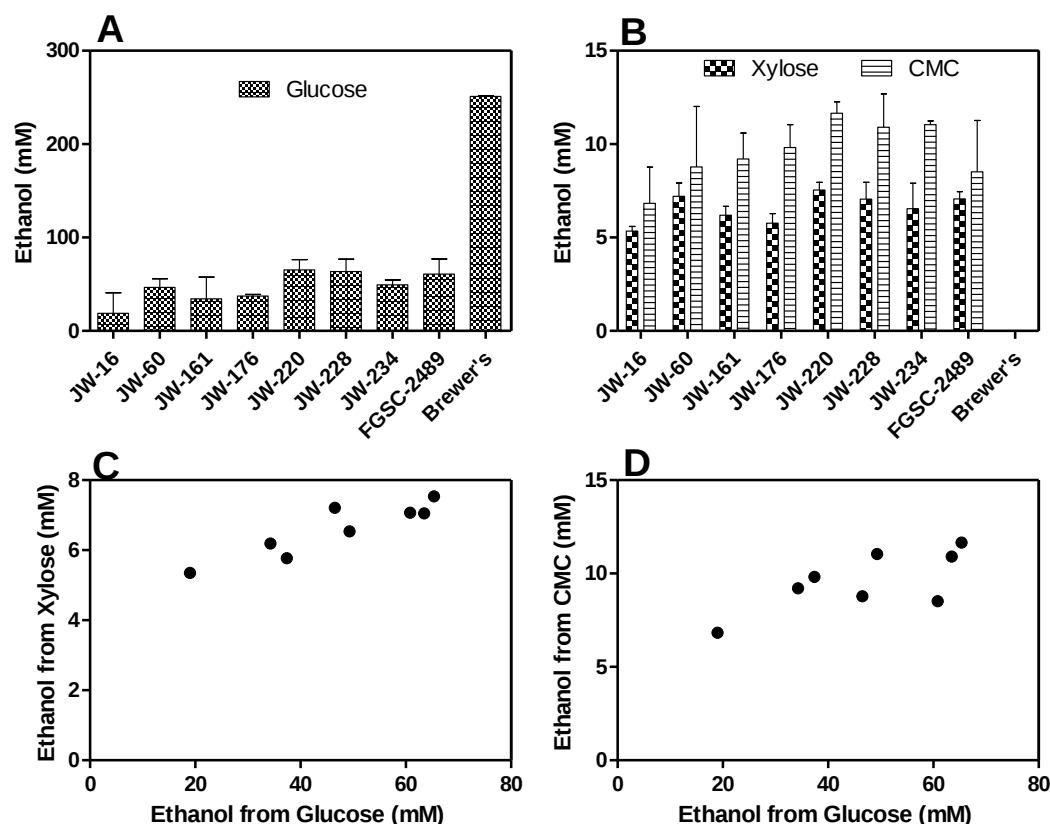


Fig. 3 Natural variation in fermentation among top performing ecotypes. A) Ethanol produced from fermentation of glucose. B) Ethanol produced from fermentation of 2% Xylose or 2% CMC. C& D) Correlations were observed between ethanol produced from fermentation of glucose and xylose (C), and between fermentation of glucose and CMC (D) ($R^2=0.7511$, $p=0.0025$ and $R^2=0.6711$, $p=0.0069$ respectively). Error bars represent one standard deviation

N345 cross population

To explore the potential of fungal breeding as an approach to enrich cellulase production and fermentation, the top performing natural strains of opposite mating types were crossed and the progeny were tested for saccharification of cellulose and fermentation of glucose. Although significant variation was observed in the FPA assay, only one strain (N345-2) was able to outperform both parents, and only four strains were able to outperform the lesser parent (ANOVA p -value = 1.67×10^{-22}) (Fig. 4). We also sought to assess the potential of fungal breeding as a route to enrich fermentation. Therefore, the

progeny obtained from crossing the top performing parents were also screened for fermentation of glucose. Significant variation was observed among the 87 progeny tested (ANOVA p-value = 5.15×10^{-7}). Importantly, 13 of the progeny were able to outperform both parents demonstrating the ability of fungal breeding to enrich fermentation capacity in a single generation.

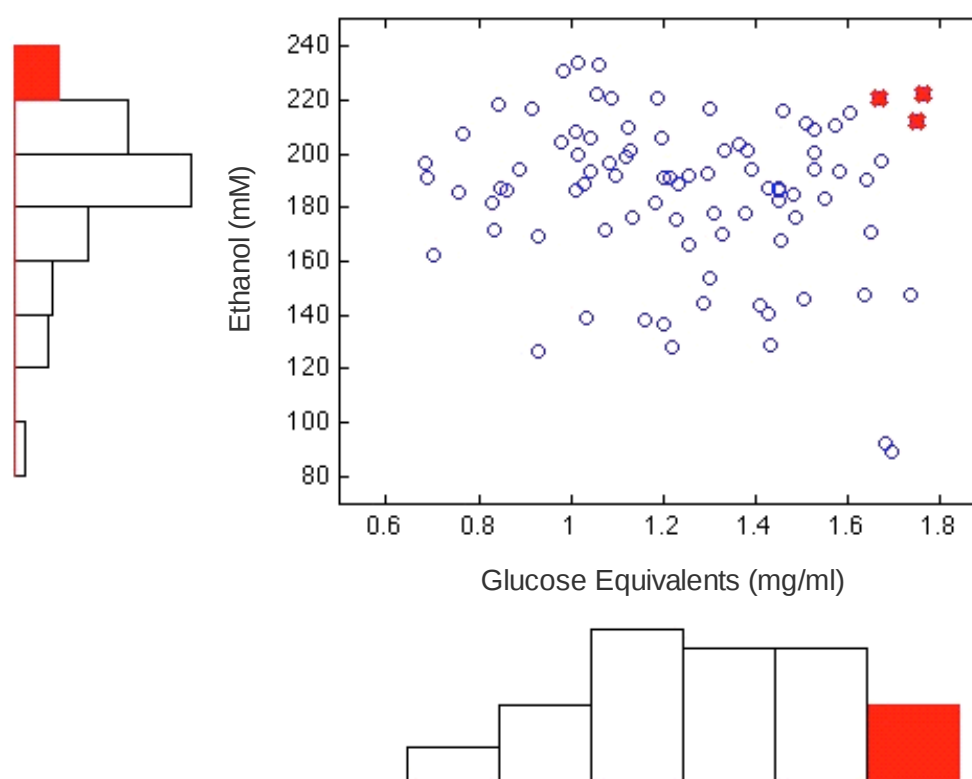


Fig. 4 Natural variation in cellulolytic activity and fermentation among a laboratory population (N345 population). A 2-dimensional scatterhist plot illustrating distributions for saccharification of cellulose (x-axis) and fermentation of glucose (y-axis). Red dots represent strains with the highest potential for both traits

Biomass fermentation

Finally, to validate our hypothesis that strains capable of decomposing pure defined cellulose substrates should also be able to decompose more complex natural substrates,

fermentations were performed with FGSC2489 and elite ecotypes JW220, JW228, and N345-2 using the high-energy crop *Miscanthus giganteus* as the sole carbon source.

While all strains were able to directly ferment lignocellulosic biomass to ethanol, the elite ascensions chosen from preliminary screenings were able to produce almost twice as much ethanol as the reference strain (Fig. 5). This result supports the hypothesis that elite ecotypes exist that are better adapted for direct fermentation of lignocellulosic substrates, and that natural variation could be exploited through selective fungal breeding or genetic engineering to enrich for traits involved in cellulosic biofuel production.

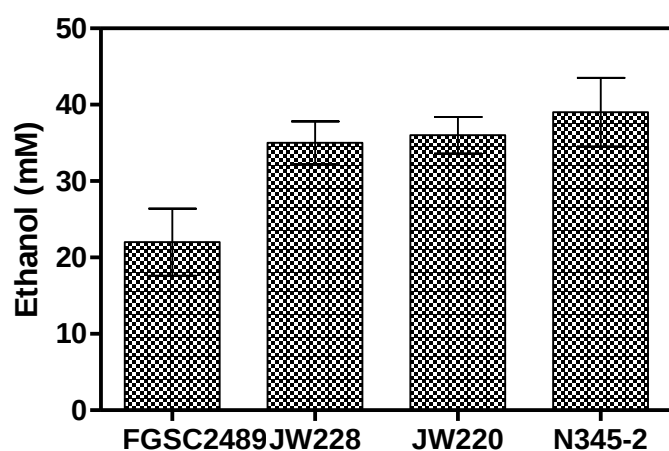


Fig. 5 Fermentation of a High Energy crop by elite ascensions. Elite ascensions (JW220, JW228, and N345-2) were able to produce more ethanol from a 2% *Miscanthus* culture than the sequence strain (FGSC2489) which serves as a wild type reference. Significant difference was observed between each strain and reference strain using 2-tailed t-test (JW220 $p = 0.000005$, JW228 $p = 0.000005$, N345-2 $p = 0.00007$). Error bars represent one standard deviation

Discussion

Various attempts have been made to reduce costs and labor of cellulosic ethanol production via CBP, however these attempts have utilized organisms engineered to incorporate cellulolytic or fermentation pathways from other species [16, 17, 19, 32, 35]. While investigations aimed at understanding cellulolytic and fermentative systems, and

even attempts at CBP have been made utilizing wild type and gene deletion mutants of *N. crassa*, none have investigated natural variation due to allelic effects as a source of elite strains for CBP [55, 76–79]. Here, we investigated variation in saccharification of cellulose and fermentation among natural and laboratory populations using model cellulose substrates to identify elite strains for CBP. Although model cellulose substrates, such as CMC and Whatman filter paper, are not as complex as natural cellulosic substrates, they have proven to be effective tools for screening endoglucanase and total cellulase activity respectively [75, 80–84].

Significant variations in cellulase secretion (Fig. 1, 2) and in fermentation (Fig. 3) were observed among natural strains adapted to different habitats, as well as the lab generated N345 population (Fig. 4). The lack of correlation between total exoenzymes production and enzyme activity suggests that qualitative differences in the enzymes produced may explain the observed variation. Whether the observed differences in cellulase activity are the result of differential expression of cellulolytic enzymes among strains or differences in relative abundances of cellulase enzymes present remains to be determined. In the progeny, we could identify multiple strains with increased fermentation capacity, however, only one strain was able to outperform the parents in cellulase activity assay and fermentation (Fig. 4). Future studies should investigate the richness of enzyme production and how enzyme diversity affects saccharification.

It was particularly interesting that we have observed a significant variation in fermentation, with a five-fold difference in fermentation of glucose among ecotypes (Fig. 3a). Although there was minimal variation in fermentation of xylose, there was a correlation in general fermentation ability, with strains more adept at fermenting glucose

also producing more ethanol from xylose (Fig. 3c). There was also a weaker correlation observed between fermentation of simple sugars and fermentation of cellulose (Fig. 3d). This is likely the result of the underlying, rate-limiting step of saccharification, which also varied among strains. Therefore, the potential for developing an industrial fungal strain for ethanol production from plant polysaccharides lies in exploring the elite alleles in the ecotypes responsible for converting complex sugars to monosaccharides, and their fermentation to ethanol. As a proof of the concept, we found that there exists almost 2-fold difference in ethanol production from the natural ecotypes (JW228, JW220, and N345-2) over the reference strain (FGSC2489) (Fig. 5).

Conclusion

The successful enrichment of fermentation ability in the first F1 generation from a cross between top performing ecotypes illustrates the potential for fungal breeding to generate elite strains with improved traits for industrial purposes. Further investigation into the molecular mechanisms underlying variation should be able to identify specific allelic combinations for further strain optimization through breeding and genome-wide metabolic engineering. We illustrate here that *N. crassa* is a strong candidate for consolidated bioprocessing of biomass to ethanol. In addition to the previous efforts in screening knock-out strains to understand a transcriptional responses in cellulose metabolism, characterizing allelic effects in ecotypes will provide novel insights in designing industrial *N. crassa* strains.

Acknowledgements

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CHAPTER 3

Quantitative trait loci underlie phenotypic variation in bioethanol-related processes in
Neurospora crassa

Abstract:

Bioethanol production from lignocellulosic biomass has received increasing attention over the past decade. Many attempts have been made to reduce the costs of bioethanol production by combining the separate steps of the process into a single-step process known as consolidated bioprocessing. Consolidation requires identification of organisms that can efficiently decompose lignocellulose to simple sugars and ferment the pentose and hexose sugars liberated to ethanol. While many have tried to engineer laboratory strains to have the capacity for all of the requisite processes, there have been no attempts to understand natural variation in these processes that arises from allelic variation. In this study, we sought to identify genomic loci contributing to variation in saccharification of cellulose and fermentation of glucose in the fermenting cellulolytic fungus *Neurospora crassa* through quantitative trait loci (QTL) analysis. We identified one major QTL contributing to fermentation of glucose and multiple putative QTL's underlying saccharification.

Introduction:

Fermentation of cellulosic biomass by microorganisms is a complex process requiring coordinated regulation of multiple pathways, including production and secretion of at least 3 distinct classes of catabolic enzymes (cellulases, hemicellulases, and lignin peroxidases), transport of substrates into the cell, and fermentation of the resultant

monomeric C5 and C6 sugars. Identification of organisms efficient in all requisite processes has been a major limitation toward realizing the vision of consolidated bioprocessing (CBP) of biomass to ethanol for fuels [3, 34, 48, 70]. While many researchers attempt to modify existing strains by introducing the genes for pathways lacking in the given organisms, success has been limited due to the increased physiological burden imposed by genetic manipulation, and there is concern that genetic manipulations that are effective in laboratory strains may not be translatable to the more robust strains used in industry [22, 34]. There are, however, organisms that already possess the genetic elements needed to perform all of the requisite processes, such as the model filamentous fungus *Neurospora crassa*.

Although far from superior in their natural states, these organisms present excellent potential sources of useful industrial organisms. Breeding has proven to be a reliable method for manipulating genomes to select for desired traits, as evidenced by the successes of plant breeding. Selective mating has long been used in plant breeding to improve traits ranging from fruit size and yield to appearance or flavor [85, 86]. The advent of computational tools such as genome-wide association studies (GWAS) and quantitative trait loci (QTL) analysis have had drastic impacts on how plant geneticists design new lineages with improved traits and qualities [87]. GWAS and QTL analysis are computational tools for investigating the genetic elements underlying quantitative traits. Unlike qualitative traits such as color, which are inherited discretely and exhibit discontinuous variation, quantitative traits exhibit continuous variation and cannot be discretely grouped [87]. GWAS and QTL use polymorphic genetic markers to predict the

extent to which a given genetic locus contributes to observed variation in quantitative traits, such as plant height.

QTL analysis and marker assisted selection (MAS) have revolutionized plant genetics and should be equally useful for improvement of quantitative traits in fungi, such as saccharification and fermentation of cellulose. While a wealth of information is available on the effects of gene deletions or overexpression of cellulolytic enzymes or fermentation pathways, there have been no studies to characterize the variation resulting from allelic variation in populations [2, 55, 59, 77–79, 88, 89, 89, 90]. While gene deletions and overexpression studies can implicate genetic elements in these processes, these manipulations increase cellular burden due to unknown pleiotropic effects, and multiple manipulations can result in compounding deficits [22, 34, 43]. The inherent complexity of lignocellulose metabolism allows for abundant sources of variation that will effect performance. Understanding how allelic variation attenuates performance will lead to a more robust understanding of cellulose metabolism and identify superior combinations of alleles for trait enhancement for CBP. To this end, we chose to perform QTL analysis on a laboratory generated population of the model filamentous fungus *Neurospora crassa*. 111 offspring and two parental strains were genotyped by sequencing, evaluated for their ability to decompose cellulose and ferment glucose, and subjected to QTL analysis. A major QTL was identified for fermentation and multiple putative QTL's were identified for saccharification of cellulose.

Materials and methods:

Strains

Parental strains (FGSC2223 and FGSC4825) from the Fungal Genetics Stock Center (FGSC) [73], and their cross progeny (designated as the N6 population) were grown from long-term stock prior to each experiment on Vogel's minimal media slants (1X Vogel's Salts, 2% Sucrose, 1.5% Agar, pH 5.8). Spore suspensions in High Glucose Liquid Media (HGLM) (1X Vogel's salts, 2% glucose, 0.5% L-Arginine, pH 5.8) were used to generate mycelial mats in petri plates, which were used to generate replicate mycelial pads for each experiment using a bore punch.

Enzyme activity assay

A modified FPA assay was performed using 96-well plates as described by Camassola and Dillon, in which secreted protein extracts were taken from wells in 6-well plates containing 1% CMC broth (1X Vogel's salts, 1% CMC) 4 days after inoculation with 10 gauge mycelial pads [75]. Culture broth containing secreted enzyme was filtered and centrifuged at 13.2k rpm to remove any fungal cells or debris. 50 μ L of supernatant was added to 100 μ L of 50mM sodium acetate buffer pH 5.6 in a 96-well deep-well plate, which was then equilibrated to 50°C for 5 min in a hot-water bath. A 5mg strip of Whatman Grade No. 1 filter paper was submerged in the solution, and the plate was incubated at 50°C for 60 min. After 60 min, 300 μ L of DNS Reagent was added to stop the enzymatic reaction and visualize glucose equivalents. The plate was incubated at 100°C for 10 min to develop color, then transferred to an ice bath to stop color development. 100 μ L of Enzyme/DNS mixture was transferred to a clear-bottom assay plate, diluted with 200 μ L of diH₂O, and absorbance was measured at 545 nm. The

concentration of reducing equivalents released was determined with standard curves generated with glucose standards. All samples were performed in biological triplicate. Due to the relatively low concentration of secreted enzymes, activity could not be quantified in conventional Filter Paper Units, therefore results were presented in the form of the concentration of reducing equivalents released by each aliquot.

Fermentation

To characterize fermentation of glucose among the N6 population and the parental strains (FGSC 2223 and FGSC4825), fermentation was carried out in a 96-well format in deep-well plates to characterize ethanol fermentation among the lab generated N6 first filial (F1) generation. Replicates for each strain were collected from mycelial mats grown from spores suspension in High glucose liquid media (HGLM) with a 6 gauge punch and inoculated into 750ul of HGLM (2% glucose), sealed with aluminum ThermowellTM seals, and allowed to ferment for 7 days in 12:12 LD conditions at 25°C. All samples were performed in biological quadruplicate. After fermentation, 600 µL of media was recovered and cell debris was removed by sequential centrifugation at 13.2k rpm for 5 min. The recovered supernatant was aliquoted into HPLC vials for ethanol analysis by HPLC. HPLC quantitation was performed using a Varian ProStar HPLC with a Varian ProStar Autosampler and a Varian 356-LC Refractive Index Detector. An isocratic elution was used with an Agilent Hi-Plex H⁺ (300 mm x 7.7 mm ion-exchange column) with 5mM H₂SO₄ at a flow rate of 0.7 ml/min at 60°C. The concentration of ethanol present was determined from a standard curve based on ethanol standards with a known

concentration.

Qualitative Trait Loci (QTL) Analysis

To investigate the underlying sources of variation in saccharification and fermentation, a qualitative trait loci (QTL) analysis was performed using R to identify candidate genes contributing to the observed variation. 113 strains from a previously genotyped population (N6 population) were screened for saccharification and fermentation as previously described and single nucleotide polymorphisms (SNP's) were used to generate a linkage map for QTL Analysis. Briefly, 50,000 SNP markers were filtered to 146 evenly distributed informative markers using Python and later Excel. First, all markers that were not polymorphic between parents were removed, followed by any markers in which one of the two parents was missing data. The resulting 4900 markers were formatted for Excel for further filtering. Chi-square tests were performed for each marker and those markers with unequal segregation among progeny (>20% disparity) were removed, followed by markers with >10% missing data among progeny. The filtered genotype data was combined with phenotype data from FPA and glucose fermentation assays and formatted for R-QTL. The formatted data was imported into R-QTL as and the create map function was used to generate a linkage map from a physical map based on recombination frequencies. Markers were then hand curated to generate a linkage map for all 7 chromosomes (Linkage groups) of *Neurospora crass* with approximately 22 evenly spaced markers per chromosome.

After generating a linkage map for the N6 population, QTL scans were performed for each phenotype. Composite interval mapping (CIM) was used to scan for single

QTL's using the maximum likelihood (em) , Haley-Knott regression (hk), and multiple imputation (imp) methods in the R-QTL package, allowing for up to 4 covariate markers within a 20 marker window. Likelihood odds ratio (LOD) thresholds were generated for 95% and 90% confidence intervals for each method based on 1000 permutations of the data. Peak marker positions for each chromosome (based on LOD score) were used to identify linkage blocks with flanking markers to identify chromosomal regions containing the suspected QTL, according to their physical positions. These chromosomal regions were searched in the Fungi Database (FungiDB.org) to identify all genes within the region to find candidate genes contributing to the observed variation.

Results:

N6 cross population and QTL Analysis:

Significant variation was observed among the N6 population for both saccharification of cellulose and fermentation of glucose (Fig.6). Both traits demonstrated transgressive segregation, with 21 or 58 of 111 offspring outperforming the more proficient parent and 66 or 33 of 111 underperforming the lesser parent in FPA assay or fermentation, respectively.

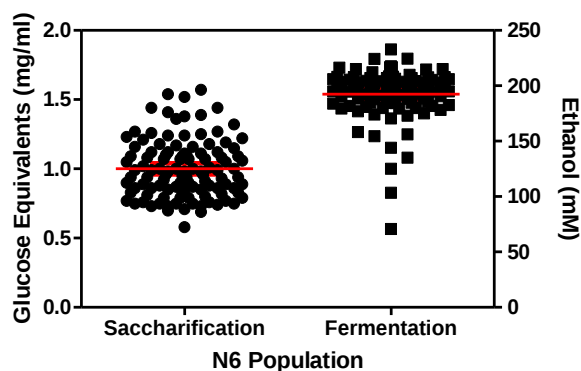


Fig.6. Natural variation in cellulolytic activity and fermentation among a sequenced laboratory population (N6 population). Significant variation was observed for saccharification and fermentation among the N6 population ($p=3.99 \times 10^{-33}$ and $p=8.75 \times 10^{-17}$ respectively). Red bars represent mean and 95% CI.

Due to the low level of recombination from only 1 meiotic event in a single cross, generating a map with evenly spaced markers required filtering a large amount of polymorphic markers reducing the power of QTL analysis. Ultimately, a linkage map based on recombination frequencies was generated from a physical map of SNP markers with approximately 22 evenly spaced markers on each of *N. crassa*'s 7 chromosomes (Fig.7).

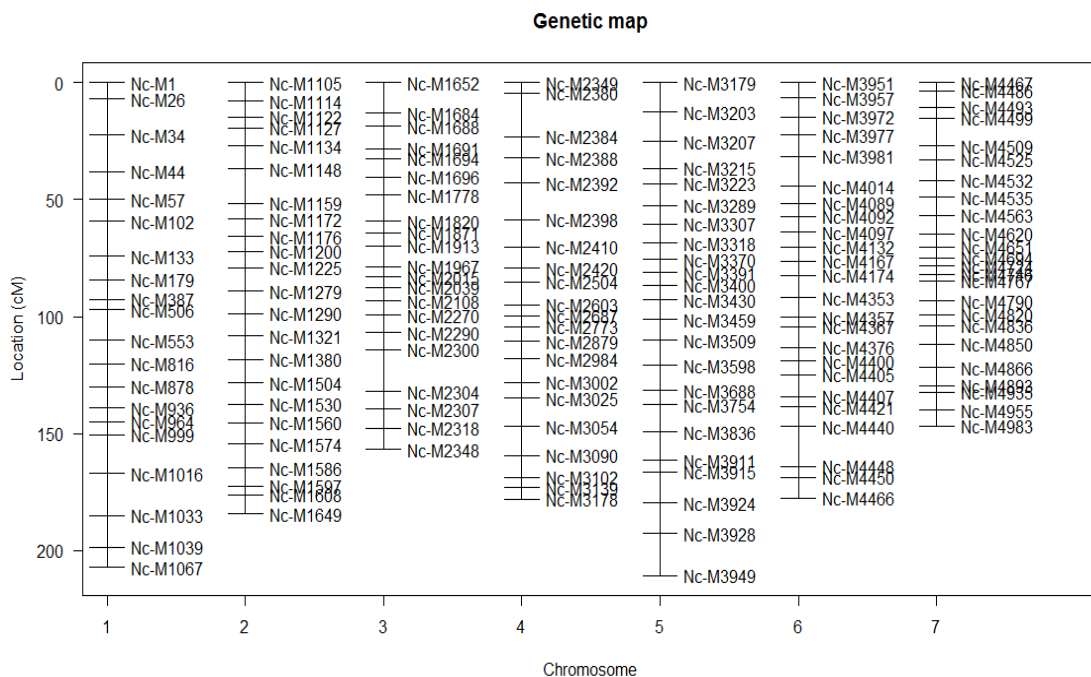


Fig.7. Linkage map of SNP markers for the N6 population. A linkage map created in R-QTL from physical marker positions, with approximately 22 evenly distributed markers per chromosome.

A major QTL region contributing to variation in fermentation was identified on Linkage Group (LG) I (361 Genes - 160 Annotated, 142 Hypothetical, 59 Unspecified)

within a 90% confidence interval (CI) (p -value=0.075) (Fig.8A, 9A). Additionally, putative QTL's were observed for fermentation on LG IV (344 Genes - 158 Annotated, 134 Hypothetical, 52 Unspecified), LG VI (62 Genes - 28 Annotated, 32 Hypothetical, 2 Unspecified), and LG VII (77 Genes - 30 Annotated, 37 Hypothetical, 10 Unspecified), however, none were above the LOD threshold for 90% CI. Of particular interest in the linkage block in LG I, indicated by QTL analysis for fermentation, was a cluster of glycolytic enzymes, sugar transporters, and enzymes involved in alternate fates of pyruvate, including specifically D-lactate dehydrogenase (lactate dehydrogenase-4), short-chain alcohol dehydrogenase, and tca-14 (L-lactate dehydrogenase).

Similarly, putative QTL's were observed for saccharification on LG II (184 Genes - 84 Annotated, 64 Hypothetical, 36 Unspecified), LG IV (184 Genes - 74 Annotated, 72 Hypothetical, 38 Unspecified), LG VI (144 Genes - 44 Annotated, 54 Hypothetical, 16 Unspecified), and LG VII (62 Genes - 22 Annotated, 36 Hypothetical, 4 Unspecified), although none were above the LOD threshold for 90% CI (Fig.8B, 9B). Among those genes within the regions pointed to by the QTL analysis were some xylanase enzymes, cellodextrin transporters, and a number of transcription factors. A major gene of interest for saccharification of cellulose indicated by QTL analysis is the Xylose degradation regulator (Xlr-1) located on LG IV, as it is involved in induction of the hemicellulose response, and regulation of cellodextrin transporters and sugar transporters, some of which were identified in the putative QTL on LG II.

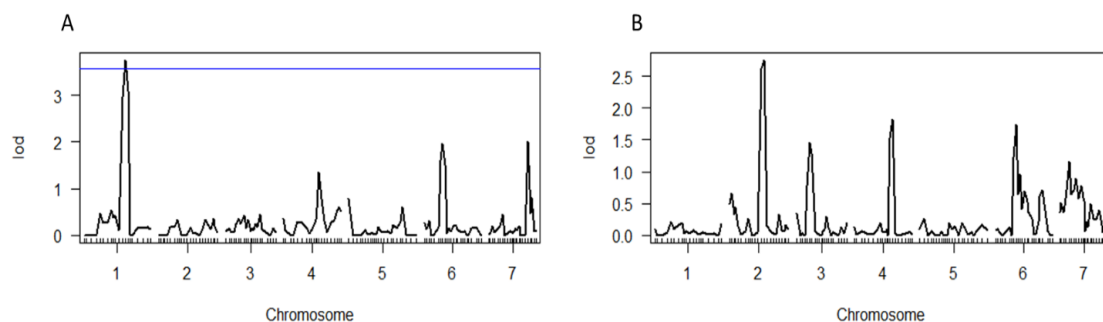


Fig.8. LOD plots for QTL analysis of the N6 population. A) Composite Interval Mapping single-QTL scan for fermentation of glucose using Haley-Knott regression. B) Composite Interval Mapping single-QTL scan for saccharification of cellulose in FPA assay using Haley-Knott regression. Blue line represents threshold for 90% confidence interval.

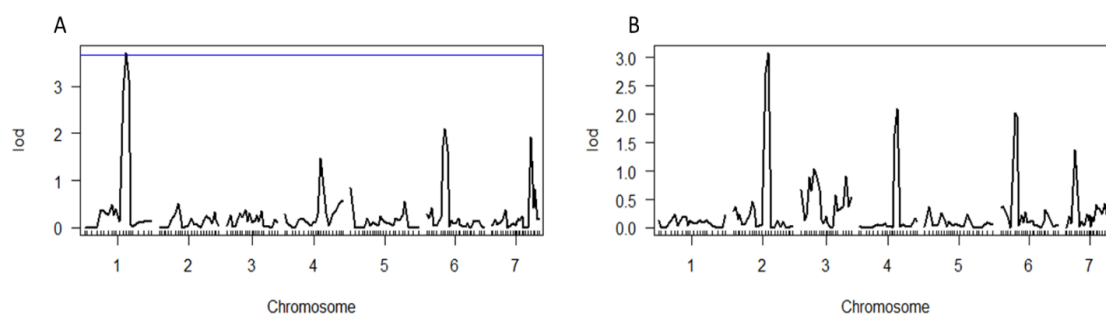


Fig.9. LOD plots for QTL analysis of the N6 population. A) Composite Interval Mapping single-QTL scan for fermentation of glucose using EM Maximum likelihood algorithm. B) Composite Interval Mapping single-QTL scan for saccharification of cellulose in FPA assay using EM Maximum likelihood algorithm. Blue line represents threshold for 90% confidence interval.

Discussion:

Here, our QTL analysis was limited due to low number of markers, resulting in large linked regions containing large numbers of candidate genes, as well as small population size, and small effect size of the investigated traits (especially saccharification). Nevertheless, some candidates were identified that may contribute strongly to the

observed variation, including the clustered glucose metabolism genes on LG I for fermentation and Xlr-1 on LG IV for saccharification. The clustering of glucose metabolism genes on LG I makes identification of candidate genes for further analysis more complicated, requiring RNA-seq expression analysis for identification of those genes within the region that are differentially regulated and contributing to the observed variation in fermentation potential. However, the identification of a major regulator of the lignocellulose response, Xlr-1, on LG IV presents a more reasonable candidate for further analysis.

Although Xlr-1 has not been shown to directly bind to promoters for cellulase enzymes, it has been implicated in cellulase production. In transcriptional analyses, two independent groups demonstrated that Xlr-1 knockouts have reduced hemicellulase and cellulase production [59, 79]. ChIP-seq experiments further revealed that although Xlr-1 does not directly bind to cellulase promoters it does bind to the promoter of transcription factor Clr-1, a major regulator of cellulase expression, and can be found bound to targets in conjunction with Clr-1 and/or Clr-2 [59]. Similarly, Cai et al., demonstrated that Xlr-1, not Clr-1/2, is required for expression of the major cellodextrin transporter Cdt-2 [56]. Cdt-2 has been implicated as a major cellodextrin importer for facilitating induction of the cellulase response, as cellulase production and growth on cellulose are significantly reduced in Cdt-2 knockouts [32, 56]. Overexpression of Cdt-2 has also been shown to increase cellulase and hemicellulase production under cellulose and xylan conditions [56]. These data suggests pleiotropic and epistatic roles for Xlr-1 as a key component for cellulase production through its role in regulation of Cdt-2 for carbon sensing and cellulase induction, and co-regulation of genes targeted by Clr-1/2.

Allelic variation in lignocellulose degradation regulators and their target genes may provide unique insights into the lignocellulose response of *N. crassa*. Transcriptomic profiling and expression analysis may reveal differential interactions between Xlr-1 and its' targets, resulting in differential enzyme induction and degradation of cellulosic substrates. Furthermore, expression QTL (eQTL) analysis would be important for discerning the pleiotropic effects of Xlr-1 from its epistatic effects. Understanding the implications of allelic effects on lignocellulose metabolism should allude to elite genotypes for further enhancement and possible industrial applications in bioethanol production.

Chapter 4 – Discussion:

Significant variation in saccharification of cellulose and fermentation of glucose was observed among natural and lab generated populations, with a 10-fold difference in saccharification observed among natural strains (Fig.1c,4,6). The large fold difference observed in the natural populations may be due to the types of substrates the strains had adapted to, with those adapted to sugarcane having less robust cellulolytic systems due to adaptation to substrates with high starch content. Within the F1 population derived from high performing natural strains (N345), there was only 2-fold difference in cellulolytic activity and saccharification did not show transgressive segregation among the progeny (Fig.4). Transgressive segregation was, however, observed within the lab generated N6 population, which exhibited an approximately 3-fold difference in cellulolytic activity (Fig.6). It is unknown whether this disparity between segregation among the lab generated populations is the result of similarity in genotype among the N345 parental strains, or an upper threshold for cellulolytic activity in natural populations of *Neurospora*, as no strains were capable of reaching the 2mg/ml of glucose equivalents required for calculating activity in conventional filter paper units (FPU).

The variation that was observed in saccharification among the populations could not be explained based solely on the total amount of secreted protein, as no correlation was observed between total protein and FPA or CMCase activity in the natural population (Fig.2b,c) or the N6 population (data not shown). This finding suggests that there may be qualitative differences in the types of cellulase enzymes produced, or quantitative differences in the amounts of cellulase enzymes produced among strains. Further investigations into the specific enzymes produced by each strain, and their

relative, and absolute, abundances is required to fully understand natural variation in cellulolytic potential among strains. Proteomics and biochemical approaches will prove beneficial in elucidating the functional differences among strains.

Although saccharification was not significantly enriched in the N345 population, with only 1 progeny strain outperforming both parents, there was significant improvement over the parental strains in the N6 population, with 21 strains outperforming the superior parent. Although promising, it is important to note that no strain surpassed the 2mg/ml of glucose equivalents required for FPU determination in the FPA assay, so it is still unclear if fungal breeding is a viable route for improving cellulolytic ability in *Neurospora crassa*. On the other hand, breeding was successful in enriching fermentation in both lab generated populations, with 13 N345 progeny and 58 N6 progeny able to outperform the superior parent in ethanol production from glucose. Further breeding experiments are required to determine if there is a similar threshold capacity for increasing fermentation in *N. crassa*, however increases in fermentation will also be limited by ethanol toxicity, which must also be improved for efficient fermentation of lignocellulose.

While ethanol production from glucose by *Neurospora* was inferior to that of a commercial *Saccharomyces* strain, the *Neurospora* isolates from natural populations were able to ferment the pentose sugar xylose and the cellulosic substrate CMC, while *Saccharomyces* and *Trichoderma* were not (Fig.3a,b). This finding, in conjunction with the ability to improve fermentation through breeding illustrates the potential for improving lignocellulose utilization through breeding as well. Further screening of cross progeny will be required to demonstrate the ability to increase pentose or cellulose

fermentation in *Neurospora* through breeding. However, it is important to recognize that the ability to ferment cellulose will be directly affected by cellulolytic ability, so it may be possible to overlook improvements in individual traits by screening only cellulose fermentation ability. Furthermore, it may be possible that crossing strains with high cellulolytic potential to those with high fermentation potential may be a superior method compared to screening for enrichment of both traits simultaneously in each generation, since strains with high cellulolytic potential but poor fermentation, or vice versa may be discarded as poor candidates due to low yield in fermentations of cellulose.

Direct fermentation of lignocellulosic biomass by *Neurospora* has been previously described, however this is the first time that it has been shown that natural strains are superior fermenters of biomass than the WT reference strain (FGSC2489) and the first example of ethanol production by *Neurospora* from untreated biomass (Fig.5) [76]. Importantly, the increases in saccharification and fermentation observed in the candidate N345 progeny (N345-2) were reflected by increased ethanol production from the high energy crop *Miscanthus giganteus*. This finding further supports the notion that breeding can be a viable route for improving fermentation of lignocellulosic biomass. Further understanding of the coordinated processes of biomass degradation and fermentation and the contributions of allelic variations in underlying genes should provide insight into designing the ideal candidates for lignocellulosic ethanol production via CBP.

Although QTL analysis has demonstrated considerable power in plant genetics, the QTL analysis was limited in this study due to limitations in recombination events in a single generation, small sample size, and small effect size for any of the given genes

contributing to the observed phenotypes. Despite these limitations, a major QTL located on Linkage Group I (LG I) was observed for fermentation of glucose. Unfortunately, the QTL region covered approximately 1Mb of LG I and contained 360 genes, of which only 160 have annotated functions. This region can be reduced in size through progressive backcrosses to reduce the number of candidate genes and identify the QTL allele. A similar approach could be concurrently applied to the putative QTL's for saccharification on LG II and LG IV, however the presence of the xylose degradation regulator on LG IV provides an interesting candidate to pursue. Among other filamentous fungi, Xlr-1 analogs (XlnR) have been shown to directly regulate cellulase expression alongside hemicellulase, however this role has been lost in *Neurospora* [59, 79, 88, 89, 91]. However, a vestige of this role may be evident in Xlr-1's indirect role in the response to cellulose through its regulation of critical genes involved in sensing cellulose in the environment, especially through *cdt-2* and major facilitator superfamily (MFS) sugar transporters. While RNA-seq and ChIP-seq experiments have previously been employed to decipher the role of Xlr-1 in *N. crassa* these studies have used knockout and overexpression strains that share the same genetic scaffold as the wild type reference. Similar studies using diverse natural strains may demonstrate that differential expression of Xlr-1 leads to differences in cellulolytic potential, or illustrate potentially disparate roles arising from allelic variation at the Xlr-1 locus or its targets.

While further improvements are needed to derive strains of *N. crassa* capable of efficient ethanol production from lignocellulose on an industrial scale, the data presented here provides evidence of *Neurospora*'s potential as a candidate for CBP. Perhaps the most critical finding of this study was the observation that natural strains were able to

produce twice as much ethanol from natural lignocellulosic substrates as the WT reference. Therefore, approaches that have used gene deletions to increase cellulolytic enzyme production in the WT genetic background could also be applied to natural strains that have been bred for efficient conversion of lignocellulose to ethanol after reaching the point where no further improvements can be made through breeding alone.

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