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Alcohol Consumption and Tolerance of *Neurospora crassa*

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Abstract

The alcohol consumption and tolerance of the ascomycete *Neurospora crassa* was investigated in this study. This fungus is able to utilize both native alcohol and non-native alcohols as carbon sources, yet little is known about the enzymes involved in these processes. The deletion of alcohol dehydrogenase 1 gene (*adh-1*) from the genome can efficiently prohibit both ethanol and isobutanol metabolism, while the deletion of the alcohol dehydrogenase 3 gene (*adh-3*) does not have an observable effect on the prevention of alcohol consumption. Both wild type *N. crassa* and the *N. crassa* Δ*adh-1* strain can tolerate up to 48 g/L ethanol and 8.5 g/L isobutanol when grown on glucose or Avicel.

Keywords: Ethanol consumption; Isobutanol consumption; Alcohol tolerance; *Neurospora crassa*

Introduction

The use of biofuels produced from renewable lignocellulosic biomass has been proposed as a potential solution to worldwide challenges related to a rapidly diminishing fossil fuel supply and global climate change. Although lignocellulosic biomass is available in large abundance and at low cost, the emergence of cellulosic biorefineries is hindered by the lack of low cost processing technologies [1,2]. The canonical conversion process for biofuels production from lignocellulosic biomass consists of five steps: pretreatment, cellulase production, hydrolysis, fermentation, and product recovery. Consolidated bioprocessing (CBP), the conversion of lignocellulosic biomass into products (such as ethanol) in one step without added enzymes, is a transformative technology, offering potentially breakthrough solutions to these current high cost methods. The CBP enabling microorganism can be constructed via either a native or recombinant strategy [3,4]. The native strategy of CBP microbial construction begins with a naturally efficient cellulase producer (category I CBP microorganism) and entails engineering the ability to produce designated biofuels or chemicals effectively within the organism. The recombinant strategy starts with a powerful biofuels producer (category II CBP microorganism), and consists of conveying the ability to utilize cellulose via cellulase production.

Cellulolytic bacteria and fungi are the main candidates for category I CBP microorganisms [5]. Compared to cellulolytic bacteria, cellulolytic fungi can naturally produce cellulases in the quantity and with the quality suitable for complete saccharification of pretreated lignocellulose, as well as have better tolerance toward inhibitors from lignocellulosic biomass pretreatment. They have great potential to be used as CBP enabling microorganisms either alone or in cooperation with other microorganisms, and they have been used previously to produce alcoholic biofuels [6,7]. *N. crassa* is amongst the most attractive candidates for CBP application either as the sole producer or a member in a microbial consortium for producing native or non-native alcoholic biofuels directly from cellulosic biomass [5,8].

N. crassa is a model filamentous fungus, with its genetics, biochemistry, and biology having been extensively studied for more than 70 years [9]. It is an efficient plant cell wall degrader, because of its wide array of cellulases and hemi-cellulases [10]. *N. crassa* produces ethanol as a fermentation product and has two major predicted alcohol dehydrogenase genes (*adh-1* and *adh-3*) present in the genome [11]. The ADH1 and ADH3 peptide sequences showed 65% identity and 79% similarity [12]. Both have >50% identity to *S. cerevisiae ADH-1* and ADH-2, which are responsible for ethanol degradation and synthesis, respectively (Xie et al., 2004). Xie et al. suggested that *N. crassa ADH-1* would be responsible for ethanol consumption, and *ADH-3* would be responsible for ethanol production due to their homology to the *S. cerevisiae* alcohol dehydrogenases and their regulatory response to glucose. Subsequently, *N. crassa ADH-1* and *ADH-3* were hetero-expressed in *E. coli* and the recombinant *ADH-1* gave a specific activity of 283.8 ± 8.9 mU/mg toward ethanol as a substrate, while *ADH-3* only gave a specific activity of 4.1 ± 0.1 mU/mg. The 70-fold higher specific activity toward using ethanol provides *in vitro* evidence that *ADH-1* in *N. crassa* may be responsible for ethanol consumptio. However, *in vivo* evidence for the function of these enzymes has not been previously explored [12].

Alcohol consumption and tolerance levels are important issues that need to be addressed if *N. crassa* is used as a CBP host for alcohol production. Herein, we studied the consumption of a native alcohol (ethanol) and a non-native alcohol (isobutanol) using different ADH knock out strains of *N. crassa*. Thus, we provide the first *in vivo* evidence for the roles of *ADH-1* and *ADH-3* in *N. crassa*. The alcohol tolerance of the *N. crassa* strains are also investigated.

Materials and Methods

Fungal strains

Wild type *N. crassa* FGSC 2489, *N. crassa* Δ*adh-1* (ΔNCU01754, FGSC 12935), *N. crassa* Δ*adh-3* (ΔNCU02476, FGSC 12920) were obtained from the Fungal Genetics Stock Center. Strain *N. crassa* Δ*adh-1*Δ*adh-3* was constructed through genetic crossing following a standard mating protocol [13,14]. The single knockout strains and resulting double knockout strains were verified by PCR genotyping. The three engineered *N. crassa* strains have similar growth rate to the wild type strain in the Vogel's media contained sucrose.

N. crassa strains' growth conditions for alcohol consumption

N. crassa strains were grown in 250 mL Erlenmeyer flasks on 1x Vogel's solid medium supplemented with 15 g /L sucrose at 30 °C with constant light for 3 days [15]. They were then moved to the bench in room temperature for an additional 7 to 10 days. Conidia were subsequently harvested and re-suspended in sterile water (20 mL). Fermentation experiments were conducted in 250 mL Erlenmeyer flasks with a 50 mL working volume, containing 1x Vogel's salts medium and 0.5 g /L glucose to initiate growth. Ethanol or isobutanol was added to a final concentration of 20 g/L or 5 g/L respectively. 50 mL water in 250 mL Erlenmeyer flasks contained same amount of ethanol or isobutanol without any conidia inoculated were used as control. Flasks were incubated at 28 °C in a rotary shaker at 200 rpm in constant light. Samples were taken out after 5 days for measurement of the alcohol concentrations via High Performance Liquid Chromatography (HPLC).

Alcohol tolerance of *N. crassa* with glucose and Avicel as carbon sources

1x Vogel's media containing 40 g/L glucose or 20 g/L Avicel were inoculated with conidial suspensions of *N. crassa* FGSC 2489 and *N. crassa* Δ*adh-1* to a final OD420 of 0.1. They were then were cultured at 28 °C for 12 h. Varying amounts of ethanol or isobutanol were added at 12 h. Samples were taken out at 0, 12, 18, 24, 36, 48, and 60 h time points to analyze the concentrations of glucose and alcohol. A duplicate experiment was conducted identically to the above experiment, in which cultures were harvested for mycelial biomass quantification.

Quantification of mycelial biomass

The mycelial biomass of the fungus grown in glucose medium was harvested by filtration through filter paper, washed with 50 mL distilled water, then dried at 105°C overnight and quantified by weighing the dried residues. The dry weight of the mycelial biomass of the fungus grown in the Avicel medium was measured by extracting ergosterol from the mycelia and measuring the amount by HPLC [16,17].

Sample analysis

Concentrations of glucose, ethanol, and isobutanol were analyzed using a Shimadzu LC-20AD HPLC equipped with a refraction index detector and a Transgenomic ICSep ION-300 column (Transgenomic, San Jose, CA, USA) at 80°C. 5 mM sulfuric acid at a flow rate of 0.6 mL/min was used as the mobile phase.

Results

Deletion of *ADH-1* can efficiently prevent ethanol and isobutanol utilization

N. crassa FGSC 2489, *N. crassa* Δ*adh-1*, *N. crassa* Δ*adh-3*, and *N. crassa* Δ*adh-1*Δ*adh-3* strains were used to characterize the consumption of ethanol and isobutanol as the carbon source in flasks. As shown in Figure 1, the wild type *N. crassa* FGSC 2489 strain consumed about 12.7 g/L ethanol or 3.2 g/L isobutanol in 5 days. The strain *N. crassa* Δ*adh-3* consumed a similar amount of alcohols compared to the wild type stain. However, the ethanol and isobutanol concentrations in the flasks containing *N. crassa* Δ*adh-1* or *N. crassa* Δ*adh-1*Δ*adh-3* only decreased approximately 4.0 g/L and 1.8 g/L, respectively.

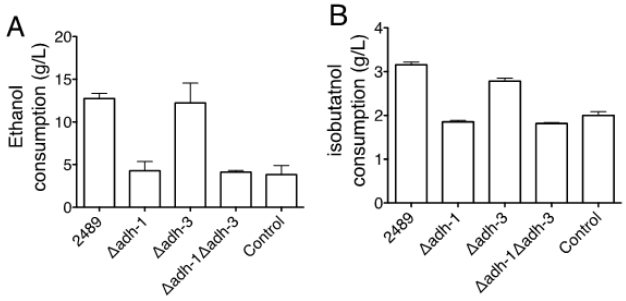

Figure 1: Ethanol (a) and isobutanol (b) consumption by N. crassa strains in 5 days.

These consumptions show no statistical difference compared to control flasks without any inoculation where ethanol and isobutanol are lost solely through evaporation. The results provide *in vivo* evidence that *ADH-1* is responsible for the alcohol consumption in *N. crassa* FGSC 2489 and *N. crassa* Δ*adh-3*, and the deletion of *adh-1* can partially eliminate this metabolic pathway. Contrarily, *ADH-3* appears to have little contribution to the alcohol consumption. Since the *adh-3* deletion in *N. crassa* did not affect alcohol consumption, the Δ*adh-1* strain was used for further study.

Alcohol tolerance of wild type and Δ*adh-1* deficient *N. crassa* strains on glucose as a carbon source

The ethanol and isobutanol tolerance of *N. crassa* FGSC 2489 and strain Δ*adh-1* were analyzed (Figure 2). As shown in Figures 2a and 2c, the biomass accumulation and rate of glucose utilization decreased with increasing concentration of alcohol in the culture for both strains. When 32 g/L ethanol was added at 12 h, biomass produced at 60 h was about 55% of that of without any ethanol addition, and the glucose utilization rate was about 62% of the control. When the added ethanol reached 56 g/L, the growth and glucose utilization for both strains ceased as there was no obvious increase of biomass or decrease of glucose concentration since the time of alcohol addition (Figure 2c). Our results indicate that *N. crassa* FGSC 2489 and *N. crassa* Δ*adh-1* can tolerate up to 48 g/L ethanol in the medium, respectively. The growth phenotypes of *N. crassa* Δ*adh-1* with different concentrations of alcohols are similar to those of *N. crassa* FGSC 2489.

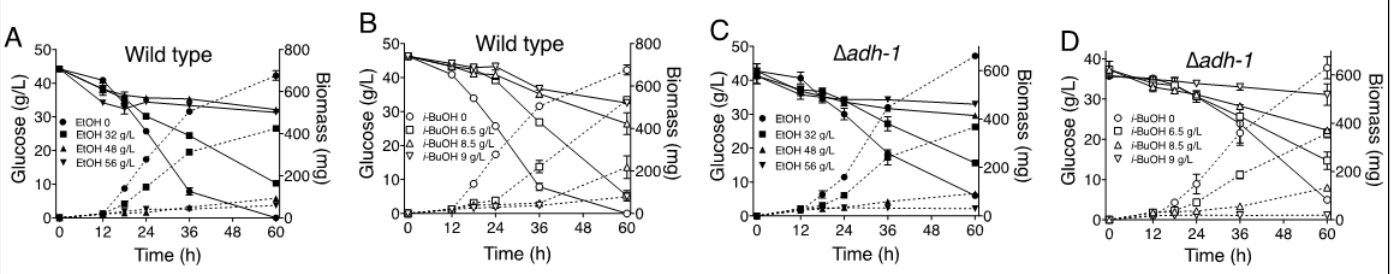

Figure 2: Glucose consumption (solid lines) and biomass accumulation (dotted lines) of wild type *N. crassa* FGSC 2489 cultures with different concentrations of ethanol (a) or isobutanol (b); and the *N. crassa* Δ*adh-1* cultures with different concentrations of ethanol (c) or isobutanol (d).

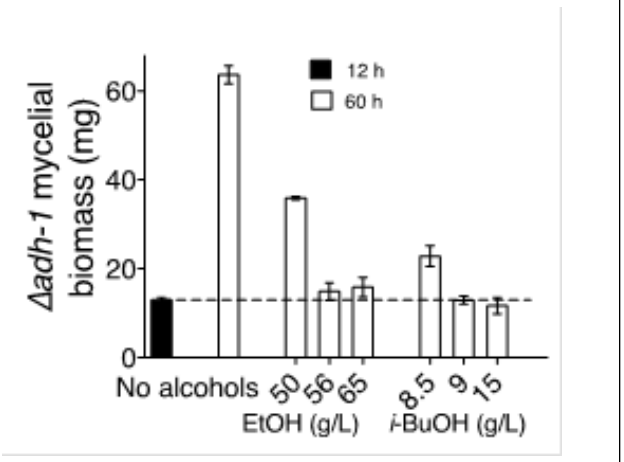

Figure 3: The mycelial biomass amount of *N. crassa* Δ*adh-1* in Avicel media at 60 h after alcohol addition at 12 h.

Results of isobutanol tolerance of *N. crassa* FGSC 2489 and *N. crassa* Δ*adh-1* are shown in Figures 2b and 2d, respectively, which demonstrated similar isobutanol tolerance between the strains. Comparing a culture containing 6.5 g/L isobutanol to the cultures containing no added isobutanol the biomass and glucose utilization rate of *N. crassa* FGSC 2489 and *N. crassa* Δ*adh-1* decreased 56% and 63%, respectively. When 8.5 g/L isobutanol was added, approximately 10 g/L glucose was consumed after the addition of isobutanol at 12 h, and 100 mg of additional mycelial biomass was produced. However, there was neither detectable glucose utilization nor biomass production when 9.0 g/L isobutanol was added. These results indicate that the isobutanol g/L tolerance of both *N. crassa* FGSC 2489 and *N. crassa* Δ*adh-1* is about 8.5 g/L when grown on glucose.

Alcohol tolerance of wild type and *N. crassa* Δ*adh-1* strains on Avicel as a carbon source

As we would like to use *N. crassa* as a candidate of CBP host, we are also interested in investigating the alcohol tolerance of *N. crassa* Δ*adh-1* when Avicel, a commonly used model cellulose product representing cellulosic biomass, is used as the carbon source. As shown in Figure 3, growth was readily detected when 50 g/L ethanol was added, or 8.5 g/L isobutanol was added. However, the fungal growth diminished when the ethanol or isobutanol were added at higher concentrations. Therefore, the ethanol and isobutanol tolerance of N. crassa Δ*adh-1* when grown on Avicel were 50 g/L and 8.5 g/L, respectively, comparable to the data acquired when strains are grown on glucose media (Figures 2 & 3).

Discussion

The two major alcohol dehydrogenases (*ADH-1* and *ADH-3*) of *N. crassa* FGSC 2489 were previously hetero-expressed in *E. coli* [12]. In this study it was shown that *ADH-1* had much higher *in vitro* specific activity toward ethanol conversion than *ADH-3*, indicating *ADH-1* in *N. crassa* may be responsible for ethanol consumption. Our results provide *in vivo* experimental verification that *ADH-1* in *N. crassa* is the primary alcohol dehydrogenase responsible for alcohol metabolism (>99%). The presence of *ADH-1* enables *N. crassa* to use a native alcohol (ethanol), and a non-native alcohol (isobutanol) as the carbon source. The deletion of *adh-1* in the genome thus leads to the elimination of >99% of the total alcohol consumption, while the deletion of *adh-3* has no effect on alcohol utilization. The deletion of *adh-1* does not lead to an obvious growth phenotype in *N. crassa* (data not shown).

High concentrations of alcohols inhibit microbial growth by inactivating the cytosolic enzymes and damaging cell membranes [16]. This intolerance to high concentrations of alcohol in microorganisms limits the final titer and the productivity in fermentation, both of which have significant impacts on the economics of a bio-refinery system. Most current designs for production methods of ethanol from lignocellulosic substrates featuring the enzymatic hydrolysis step aim for ethanol concentrations of at least 50 g/L due to the constraints associated with slurry handling [18]. Both *N. crassa* FGSC 2489 and *N. crassa* Δ*adh-1* can tolerate ethanol 48 g/L. *ADH-1* is the enzyme responsible for alcohol consumption, but it did not seem to affect the ethanol tolerance of *N. crassa* FGSC 2489. Although the ethanol tolerance level of *N. crassa* is far inferior to that of Saccharomyces cerevisiae and Zymomonas mobilis (Category II CBP microorganisms, tolerate 150-200 g/L ethanol), it is higher than those of naturally occurring cellulolytic bacteria (e.g. Clostridium thermocellum tolerates 10 ethanol g/L), and in the range of what could be achieved from the adapted or engineered cellulolytic bacteria [19]. The ethanol tolerance level is suitable for industrial ethanol production from cellulosic biomass.

Isobutanol is a non-native alcohol, and it was able to be produced by only a handful of recombinant hosts in recent studies [20]. The responses to isobutanol and ethanol vary depending on the microorganism. Z. mobilis, for example grows at about 60% of its maximal growth rate when the strain was grown on 12 g/L isobutanol [20,21]. In contrast, *S. cerevisiae*, which has high tolerance to ethanol, was not be able to grow when 8 isobutanol g/L was added. Thermoanaerobacterium saccharolyticum could not grow when ethanol concentration was as high as 20 g/L, while it maintained about half the growth rate when isobutanol concentration was as high as 12 g/L. Both *N. crassa* Δ*adh-1* and *N. crassa* FGSC 2489 can tolerate up to 8.5 g/L isobutanol, which is in a similar range as naturally occurring *E. coli* which has a tolerance level of 8 g/L and *S. cerevisiae*, as mentioned above [22-24].

Conclusion

N. crassa is able to metabolize both a native alcohol, ethanol, and a non-native alcohol, isobutanol, as a carbon source under aerobic conditions. Deletion of the *adh-1* gene in *N. crassa* can efficiently prevent alcohol consumption under aerobic conditions. *N. crassa* Δ*adh-1* can tolerate up to 48 g /L ethanol and 8.5 g /L isobutanol when grown on glucose or Avicel as the carbon source.

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