Expression of the *Aspergillus niger InuA* gene in *Saccharomyces cerevisiae* permits growth on the plant storage carbohydrate inulin at low enzymatic concentrations

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The plant storage carbohydrate inulin represents an attractive biomass feedstock for fueling industrial scale bioconversion processes due to its low cost, ability for cultivation on arid and semi-arid lands, and amenability to consolidated bioprocessing applications. As a result, increasing efforts are emerging towards engineering industrially relevant microorganisms, such as yeast, to efficiently ferment inulin into high value fuels and chemicals. Although some strains of the industrially relevant yeast model *Saccharomyces cerevisiae* can naturally ferment inulin, the efficiency of this process is often supplemented through expression of exogenous inulinase enzymes that externally convert inulin into its more easily fermentable component monomeric sugars. Here, the effects of overexpressing the *Aspergillus niger* InuA inulinase enzyme in an *S. cerevisiae* strain incapable of endogenously fermenting inulin were evaluated to determine their impact on growth. Expression of the *A. niger* InuA inulinase enzyme permitted growth on otherwise intractable inulin substrates from both Dahlia tubers and Chicory root. Despite being in the top 10 secreted proteins, growth on inulin was not observed until 120 h post-inoculation and required the addition of 0.1 g fructose/l to initiate enzyme production in the absence of endogenous inulinase activity. High temperature/pressure pre-treatment of inulin prior to fermentation decreased this time to 24 h and removed the need for fructose addition. The pre-growth lag time on untreated inulin was attributed primarily to low enzymatic efficiency, with a maximum value of 0.13 ± 0.02 U InuA/ml observed prior to the peak culture density of 2.65 ± 0.03 g/l. However, a minimum excreted enzymatic activity level of only 0.03 U InuA/ml was found to be required for sustained growth under laboratory conditions, suggesting that future metabolic engineering strategies can likely redirect carbon flow away from inulinase production and reorient it towards product production or cellular growth in order to optimize strain development.

Keywords: *Aspergillus niger*; fermentation; inulin; inulinase; pre-treatment; *Saccharomyces cerevisiae*.

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Introduction

Inulin, a polyfructan of β-2,1-linked fructosyl units with a terminal α-1,2-linked glucose residue [1], functions as a carbohydrate storage molecule in more than 30,000 species of plants [2, 3]. However, unlike cellulose, these inulin reserves do not exist within a complex matrix of secondary components, and can therefore be broken down via a single enzymatic step performed under relatively mild conditions. Furthermore, this process can be easily performed extracellularly, making the resulting monomeric sugars accessible either directly as end products, as building blocks for the assemblage of more complex target chemicals, or as carbon sources for any number of microorganisms that can convert the sugars to high value products.

While this ability to break down inulin is spread across many different microorganisms, none of them have enjoyed the large-scale industrial use or ease of genetic manipulation of the model eukaryote *Saccharomyces cerevisiae* [4]. Previous evaluations of inulin utilization by *S. cerevisiae* have employed strains such as JZ1C that have endogenous inulinase activity [5]. The natural inulinase activity of these strains has been shown to be supplemented through expression of the *Aspergillus niger* InuA inulinase enzyme, but the strains background inulinase activity has made it difficult to determine if enzymatic production could be scaled back in order to increase metabolic flux towards product production [6].

To investigate the effects of inulinase expression in a non-inulinase-producing strain, and to estimate the minimal inulinase enzymatic activity required to permit growth on inulin, the *A. niger inuA* gene is here expressed in *S. cerevisiae* W303a, a strain which lacks the ability to endogenously grow on inulin as a sole carbon source. This approach ensures that any observed growth necessarily results from InuA activity. An increased understanding of the effects of exogenous inulinase expression on *S. cerevisiae* is warranted given this strategy's widespread application to the production of high fructose corn syrup [7-9], inulo-oligosaccharides used in the production of confections and baked goods [10], and bioethanol fuels [11-13].

This study evaluates the effectiveness of *A. niger inuA*-based inulin degradation by a non-naturally-inulin-degrading *S. cerevisiae* strain and provides insight towards its potential use in this role by providing a point of contrast for comparison with other inulinase-expressing microorganisms and alternative *S. cerevisiae* strains engineered to express inulinase enzymes. The growth of InuA-expressing *S. cerevisiae* on differentially sourced inulin feedstocks is presented and the effectiveness of inulin pretreatment is evaluated. The enzymatic efficiency of this strain is similarly quantified to provide a direct comparison with alternative systems.

Materials and Methods

Strains and growth conditions

E. coli 5α (New England Biosciences, Ipswich, MA, USA) was grown in LB broth at 37°C and 225 rpm or on LB plates containing 15 g agarose/l as required. Selection was performed by supplementing with 100 μg ampicillin/ml and growing under the same conditions for 24 h. *S. cerevisiae* W303a was obtained from the American Type Culture Collection (Manassas, VA, USA) and cultured in defined medium consisting of 6.7 g yeast nitrogen base/l (Sigma Aldrich, St. Louis, MO, USA), 1.92 g yeast synthetic drop-out medium supplement without uracil/l (Sigma Aldrich, St. Louis, MO, USA) for auxotrophic selection, and 2 g/l of a carbon source consisting of either a mixture of glucose and fructose or inulin as noted. Growth of wild type *S. cerevisiae* was supported through the addition of 76 mg uracil/l to complement the uracil auxotrophy.

All yeast strains were grown at 30°C and 225 rpm.

Cloning and transformation of the *A. niger inuA* gene

The *A. niger inuA* gene sequence (NCBI accession number: AF369388) was codon optimized for expression in *S. cerevisiae* by increasing the incorporation frequency of highly utilized codons, removing interfering secondary structures and motifs, and more closely matching the GC content level of the *S. cerevisiae* genome. This optimized sequence was then synthetically assembled and cloned into the pEX-K vector for propagation (Operon, Huntsville, AL, USA). The pEX-K:InuA vector was transformed into *E. coli* and grown for 16 h before it was isolated and purified using a Pure Yield Plasmid Midiprep System (Promega, Madison, WI, USA). The isolated plasmid DNA was used as a template sequence to PCR amplify the codon optimized *inuA* gene, which was then cloned into an Electra Mother vector and subcloned into the pD1214 yeast expression vector using the Electra Cloning System (DNA 2.0, Menlo Park, CA, USA). The pD1214:InuA vector was then selected in *E. coli*, purified, and confirmed via restriction digest and Sanger sequencing of the *inuA* gene open reading frame. Verified pD1214:InuA was transformed into *S. cerevisiae* using a PEG/lithium acetate procedure [14] and selected by complementation of the yeast's uracil auxotrophy. Restriction digest and Sanger sequencing of the *inuA* gene open reading frame were used to verify successful transformation.

Verification of *inuA* gene expression in transformed yeast

Total mRNA was isolated using a Direct-Zol RNA Miniprep kit (Zymo Research, Irvine, CA, USA) and converted to cDNA using a Power SYBR Green RNA-to-C_T 1-Step Kit (Applied Biosystems, Grand Island, NY, USA). Relative transcript levels of the *inuA* gene were then interrogated via rtPCR and normalized to expression of the endogenous β-Actin gene. Amplification of InuA cDNA was performed using the primers $InuA_{RTf}$ (5′-TGTTGACACATG TTCGGTTG-3′) and $InuA_{RTr}$ (5′-AAACGGATCTGA CATCGACA-3′) and amplification of β-Actin cDNA was performed using the $BAct_{RTf}$ (5′-CCACCACTGCTGAAAGAGAA-3′) and $BAct_{RTr}$ (5′-AGAAGATTGAGCAGCGGTTT-3′) primers, respectively. Similarly processed wild type *S. cerevisiae* was employed as negative controls for each stage of the analysis. Wild type and *inuA*-expressing *S. cerevisiae* were prepared for proteomic analysis by growing for 48 h, pelleting the cells at 3,000 × *g* for 5 min, and filtering to remove any remaining cells. 40 ml of supernatant was concentrated to 500 µl via 5 kDa MWCO spin filtration, adjusted to 2% (v/v) SDS (1:1 addition by volume of 4% (v/v) SDS in 100 mM Tris-HCl, pH 8.0), and boiled. Denatured supernatant proteins were precipitated by TCA, pelleted, washed with ice-cold acetone, and air-dried. The protein pellet was then resuspended in 8 M urea, 5 mM dithiothreitol, 100 mM Tris-HCl, pH 8.0, adjusted to 15 mM iodoacetamide to block disulfide bond formation, and digested with trypsin. Digested peptides were analyzed by tandem mass spectrometry using a hybrid LTQ-Orbitrap-XL mass spectrometer (Thermo Scientific, Pittsburgh PA, USA).

Growth curve analysis

Cells were grown in triplicate in individual Balch tubes. OD_{600} values were recorded at the indicated intervals using a Spectronic 200 spectrophotometer (Thermo Scientific, Pittsburgh PA, USA) and converted to dry cell weights through the generation of a dry cell weight to OD_{600} standard curve as determined following two washes with $_{di}H_2O$ and drying at 80°C until consistent weights were obtained. All cultures were maintained in the incubator between readings and were only removed long enough to obtain culture density measurements.

HPLC analysis

Fermentation broth samples were analyzed for residual carbohydrates (fructose and glucose) using a LaChrome Elite HPLC (Hitachi) with a refractive index detector. All carbohydrates were separated on an Aminex HPX-87H column (BioRad, Hercules, CA, USA) at a flow rate of 0.5 ml/min in 5 mM sulfuric acid at a temperature of 60°C.

Enzymatic analysis

Inulinase activity was calculated using a 3,5-dinitrosalicylic acid (DNS) reducing sugar assay [15]. 50 μl of culture was removed at the indicated intervals and mixed with 50 μl of a 10 g inulin/l, 0.1 M sodium acetate buffer solution at pH 5.0. Assays were run for 30 min at 50°C before the addition of DNS reagent (10 g 3,5-dinitrosalicylic acid/l and 300 g sodium potassium tartrate tetrahydrate/l in 400 mM NaOH) and 10 min incubation at 94°C. The resulting color change catalyzed by the presence of free fructose was measured using absorbance at 540 nm on a $Synergy_{MX}$ plate reader (BioTek, Winooski, VT, USA) and converted to liberated fructose in g/l through comparison with a standard curve generated using similarly processed fructose controls at known concentrations. All values were normalized to enzyme-free blanks via background absorbance subtraction. One unit of enzyme activity was defined as the amount of enzyme required to liberate 1 μmol of fructose per min.

Enzymatic treatment

Crude extracts of the InuA enzyme were obtained directly from the culture medium of cells expressing pD1214:InuA. To obtain the extracts, cells expressing pD1214:InuA were grown for 48 h at 30°C and 225 rpm. The cells were then centrifuged at 3,000 × *g* for 5 min and the medium was filtered to ensure no cells were retained. The total enzymatic activity level of the medium extract was determined by DNS assay as described above and the results were background subtracted from cell free medium to control for the presence of any free fructose present. For comparison purposes, a purified extract of *A. niger* inulinase was purchased (Sigma Aldrich, St. Louis, MO, USA). The activity level of this extract was similarly confirmed via DNS assay prior to use. For supplementation, centrifuged, filtered culture extracts were added directly to cellular medium as described. Commercial inulinase extracts were first diluted in 100 mM Sodium Acetate buffer (pH 5.0) to match the activity level of the crude extracts, and then similarly applied.

Inulin pre-treatment

Inulin from either Dahlia tubers or Chicory root was dissolved in standard defined yeast medium to a concentration of 2 g/l as indicated. The inulin/medium mixture was then pressurized to 110 kpa and rapidly heated to 121°C for 20 min before being cooled to room temperature. The cooled, pre-treated inulin medium was then used directly for culture inoculation.

Statistical analysis

All experiments were performed in triplicate (n = 3) unless otherwise noted, standard deviations were calculated for each replicate trial, and errors are expressed as ± the standard error of the mean. Student's t-tests were used for the comparison of means, with significance defined as $p < 0.05$. Relative gene expression levels in a given sample, or compared between two samples, were analyzed using ΔC_T or $\Delta\Delta C_T$ methods, respectively.

Results

Expression of *inuA* in *Saccharomyces cerevisiae*

When normalized to the endogenous β-Actin gene, expression of the codon optimized *inuA* gene was significantly up regulated. An analysis of wild type negative controls indicated that there were no similar transcript sequences in the native transcriptome,

Table 1. Transcriptional analysis of *inuA* gene expression demonstrated successful transcription in the pD1214:InuA-expressing strain

Sample Name	Target Gene	Mean Cт	Standard Deviation of Cт	Mean ΔCт	Standard Error of ΔCт	ΔΔCт	Relative Quantification
S. cerevisiae + pD1214:InuA	β-Actin	24.46	0.42	-	-	-	-
	inuA	22.03	0.06	-2.43	0.24	-15.41	43403.41
Wild Type *S. cerevisiae*	β-Actin	23.52	0.15	-	-	-	-
	inuA	36.49	0.49	12.97	0.29	< 0.01	1

Means, standard deviations, and standard errors were calculated from triplicate replicates

suggesting that the expression strategy was successful (Table 1). Proteomic assessment of culture medium following 48 h of growth supported these results and indicated that translation was similarly efficient. Subsequent identification of the InuA enzyme as the 9th most abundant protein in the culture medium demonstrated that the mature protein was being successfully secreted from the cells (Table 2). However, this high level of constitutive expression was found to impact growth, with the InuA-expressing strain growing at a lower rate and reaching an overall lower density than the wild type strain when grown on a mixture of 1.9 g fructose and 0.1 g glucose/l that mimics the complete breakdown of 2 g inulin/l to its component monomeric sugars [16] (Figure 1).

Growth of InuA-expressing strains on inulin

Despite the expression of the *inuA* gene, transformed strains did not grow in medium containing inulin as a sole carbon source (Figure 2), even though an analysis of enzyme activity during the growth period showed that the enzymatic activity increased with time consistent with the constitutive expression imparted by the TEF promoter in the pD1214:InuA vector (Table 3). When supplemented with 0.1 g fructose/l to initiate cellular growth, cells demonstrated a larger enzymatic activity maximum than when grown on inulin alone (0.13 ± 0.02 U/ml versus 0.05 ± 0.02 U/ml) and, as a result, increased in growth rate beginning at 120 h post inoculation (96 h post fructose-supported growth) through the utilization of inulin as a carbon source (Figure 3). HPLC analysis of the culture medium indicated that this fructose loading was fully consumed within 24 h post inoculation. Analysis of growth patterns supported this result, with growth rates declining for both wild type and *inuA*-expressing strains beyond the 24 h time point (Figure 3). Despite the increased time required to reach stationary phase, cultures grown on inulin as a sole carbon source reached similar final culture densities relative to cells grown on monomeric sugars representative of fully degraded inulin, suggesting that full inulin breakdown was achieved by the end of the growth period (Figures 1 and 3).

Table 2. Proteomic analysis of culture medium was performed to detect the successful translation and secretion of InuA. Peptide-spectrum matches resulting from mass spectral analysis were filtered and assigned by matching to a database of *S. cerevisiae* protein sequences amended to include the *A. niger* InuA sequence. Relative rank abundance was assigned based on the number of observed spectral counts and demonstrated that InuA was the 9th most abundant excreted peptide in each replicate. Results are representative of duplicate replicates

Relative Rank Abundance	Protein Name	Accession Number	Filtered Spectra
1	Uth1	Z28267	809
2	Hsp150	Z49434	483
3	Pir1	Z28164	471
4	Exg1	AY693069	418
5	Ygp1	Z71436	387
6	Eno1	Z73039	385
7	Eno2	NM_001179305	373
8	Pst1	Z74351	350
9	InuA	AF369388	329
10	Pgk1	NM_001178725	293

Table 3. Enzymatic activity levels from the secreted InuA enzymes of pD1214:InuA-expressing strains grown on inulin as a sole carbon source continuously increased across time, consistent with the constitutive expression of the TEF promoter in the pD1214 plasmid. Enzymatic activity became significantly detectable ($p < 0.05$) following 96 h in culture, but did not reach a sufficient level to permit strain growth at any point throughout the assay.

Time (h)	Enzyme Activity (U/ml)
24	ND
48	ND
72	ND
96	0.018 (± 0.016)
120	0.023 (± 0.016)
144	0.027 (± 0.017)
168	0.035 (± 0.016)
192	0.046 (± 0.017)

Data reported as triplicate means ± standard error of the mean
ND = No detectable activity

Effects of pre-treatment on inulin accessibility

Pre-treatment of a 2 g inulin/l solution by incubation at 110 kpa and 121°C for 20 min eliminated the lag time observed in non-pre-treated inulin-containing medium (Figure 4) while maintaining a similar ($p = 0.34$) peak culture density (2.58 ± 0.05 g/l in the pre-treated cultures versus 2.65 ± 0.03 g/l in the fructose-supplemented cultures), indicating that it allowed for full inulin breakdown in a more rapid fashion under similar culture conditions. However, the time required to reach stationary phase growth using pre-treated inulin was still prolonged relative to growth on monomeric sugars (Figures 1 and 4). This pre-treatment procedure was also observed to allow limited growth of wild type *S. cerevisiae* negative control strains. HPLC analysis of cell-free medium containing pre-treated inulin identified free fructose at a concentration of 0.1 g/l, which likely contributed to this observation.

Effect of enzyme loading on inulin degradation

To determine the minimum level of InuA activity required to sustain growth, cultures of wild type *S. cerevisiae* were supplemented with between 0.0005 U InuA/ml and 0.05 U InuA/ml cell-free enzymatic extracts sourced from pD1214:InuA-expressing strains in the presence of non-pre-treated inulin medium to control for the availability of free fructose that could mimic enzymatic effects. Following inoculation, wild type cells treated with 0.03 and greater U InuA/ml demonstrated significant increases in culture density beginning within 24 h, while cells treated with less than 0.03 U InuA/ml did not show significant growth at any time throughout the course of the assay (Figure 5). Because previous reports have demonstrated that enzyme purification can increase inulinase efficiency [17], this treatment process was repeated using purified *A. niger* inulinase extracts. Using purified inulinase extracts, significant increases in culture density were achieved at 24 h with all treatment levels at or above 0.01 U/ml. Growth was also observed at the 0.005 U/ml treatment level, however, no significant change in culture density was observed at this treatment level until 48 h post-treatment (Figure 6). The fact that the wild type *S. cerevisiae* cells used in this study cannot endogenously utilize inulin as a carbon source due to their lack of native inulinase genes, combined with a lack of demonstrated growth by any of the yeast strains in this study when excreted enzymatic activity levels were lower than 0.03 U/ml, suggests that this is the minimum enzymatic activity that must be achieved for *S. cerevisiae* to sustain growth on inulin as a sole carbon source under the growth conditions employed. However, while the use of purified inulinase extracts can reduce this required enzymatic activity level, this reduction necessitates a requirement for increased enzymatic processing prior to deployment.

Growth of InuA-expressing stains on an alternative inulin source

Inulin sourced from either Dahlia tubers or Chicory root was pre-treated as previously described and introduced as a sole carbon source in the yeast medium at 2 g/l. Cultures

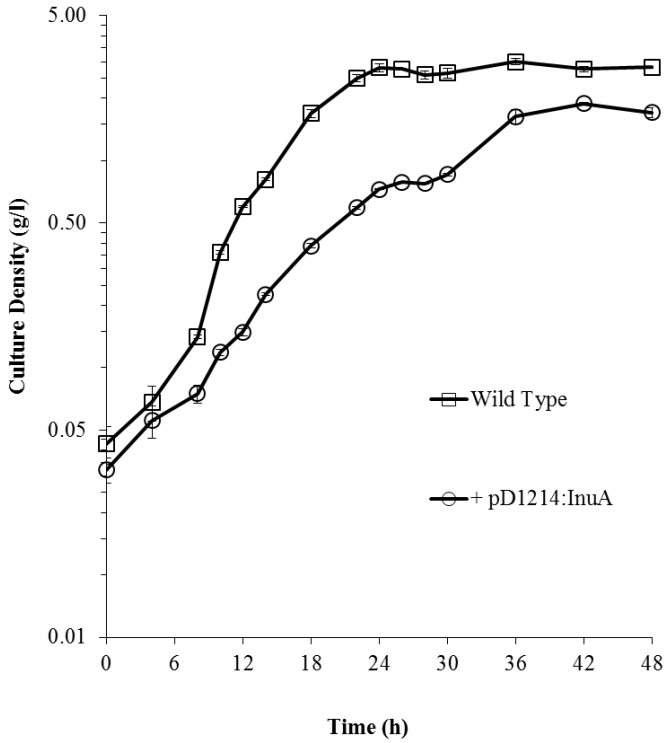

Figure 1. *S. cerevisiae* strains expressing the pD1214:InuA vector (open circles), which constitutively transcribes the *A. niger inuA* gene, reached lower peak culture densities and displayed reduced growth rates relative to wild type *S. cerevisiae* strains (open boxes) when grown on a 1.9 g fructose/l, 0.1 g glucose/l carbon mixture that mimicked fully degraded inulin. Values are the means of three replicates, with error represented as ± the standard error of the mean

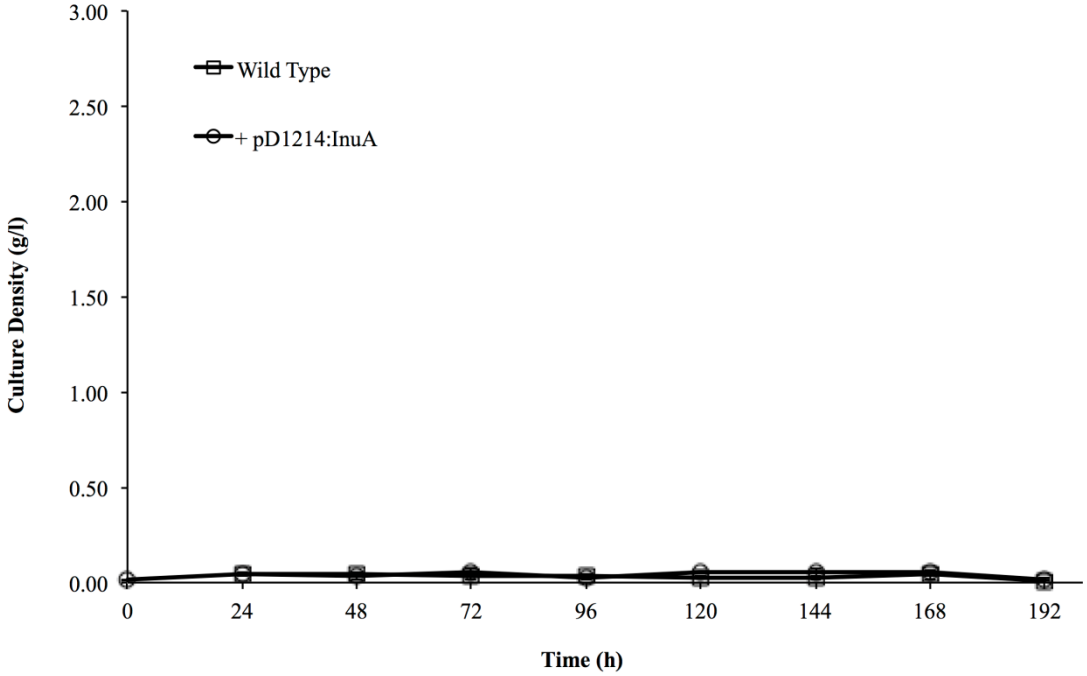

Figure 2. Despite the expression of *inuA*, pD1214:InuA-expressing *S. cerevisiae* strains (open circles) were unable to grow more efficiently on inulin as a sole carbon source relative to wild type *S. cerevisiae* negative controls (open boxes) and neither stain exhibited significant changes in culture density over a one week growth period. Values are the means of three replicates, with error represented as ± the standard error of the mean.

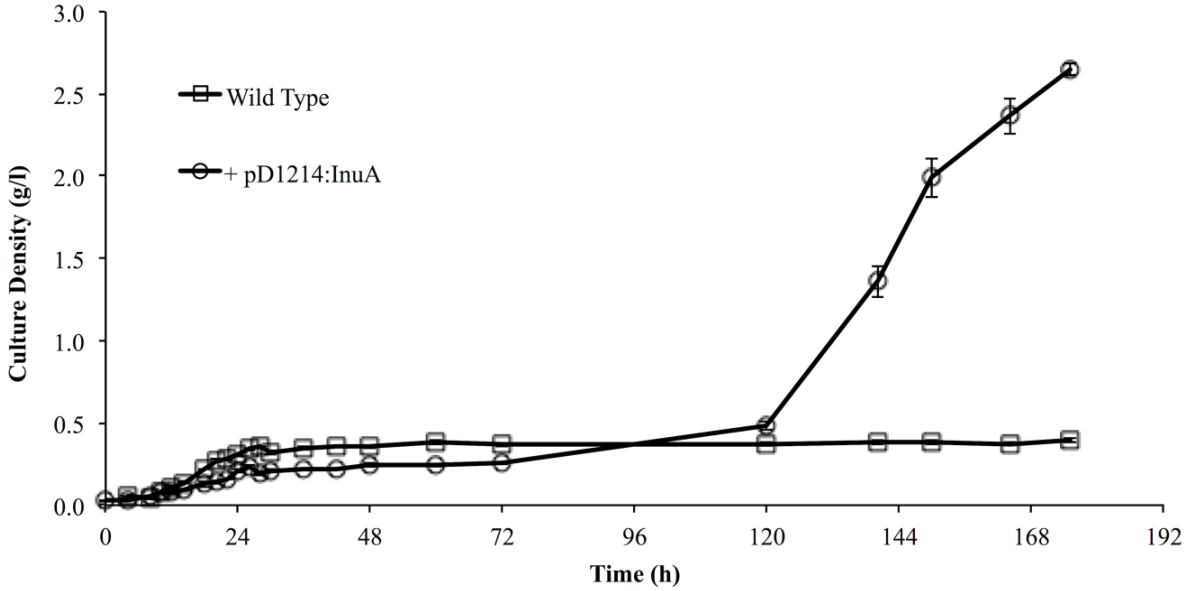

Figure 3. When grown on a mixture of 2 g inulin/l and 0.1 g fructose/l, both the wild type (open boxes) and pD1214:InuA-expressing (open circles) *S. cerevisiae* strains demonstrated increases in culture density for the first 24 h. HPLC analysis confirmed that this period was sufficient to consume all free fructose, leading to a decrease in growth rate for both strains following this time point. Beginning at 120 h post inoculation (96 h following the end of fructose-supported growth) the InuA-expressing strains demonstrated a secondary increase in culture density concurrent with an increase in InuA enzymatic activity. Values are the means of three replicates, with error represented as ± the standard error of the mean.

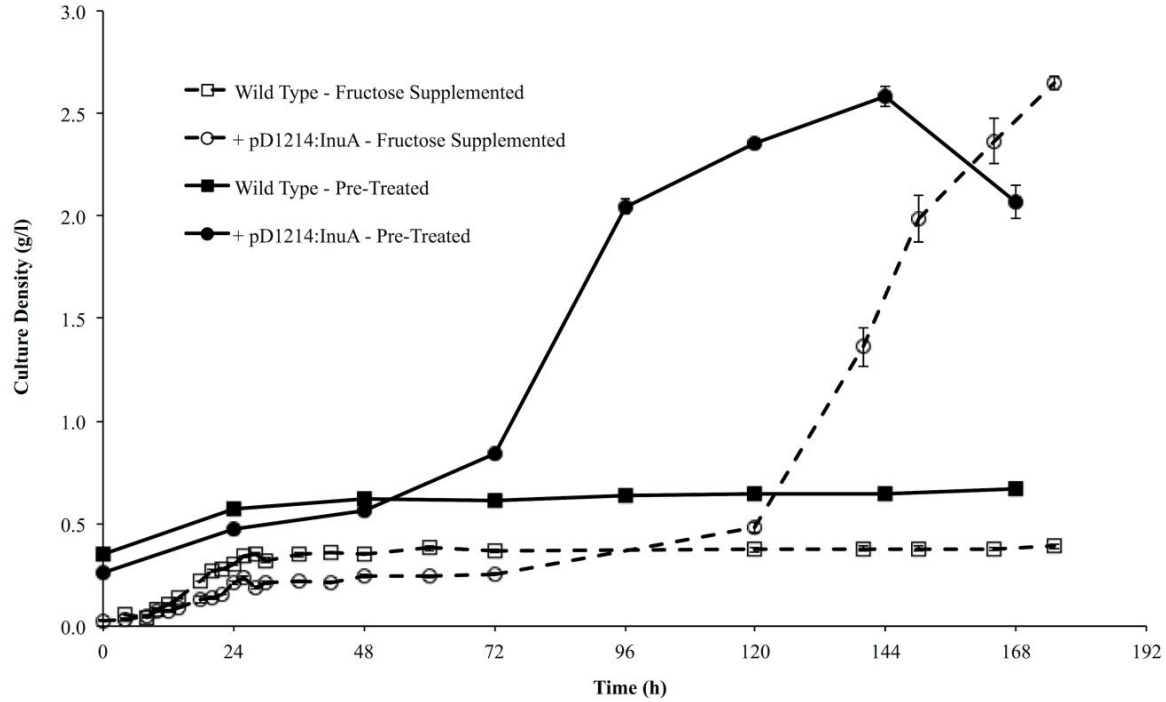

Figure 4 pD1214:InuA-expressing strains fermented on pre-treated inulin (closed circles) began to utilize inulin as a carbon source at an earlier time point than when grown on non-pre-treated inulin and supplemented with 0.1 g fructose/l to support initial expression of the InuA enzyme (open circles). Concurrent enzymatic analysis suggested this was due to increased accessibility of the inulin following pre-treatment, with greater enzymatic activity levels observed at earlier time points in the pre-treated samples. Wild type negative controls were able to utilize the free fructose released from the pre-treatment process (closed boxes) or supplemented in the medium (open boxes) to support growth for the first 24 h, but were unable to utilize inulin as a carbon source following fructose depletion. Values are the means of three replicates, with error represented as ± the standard error of the mean.

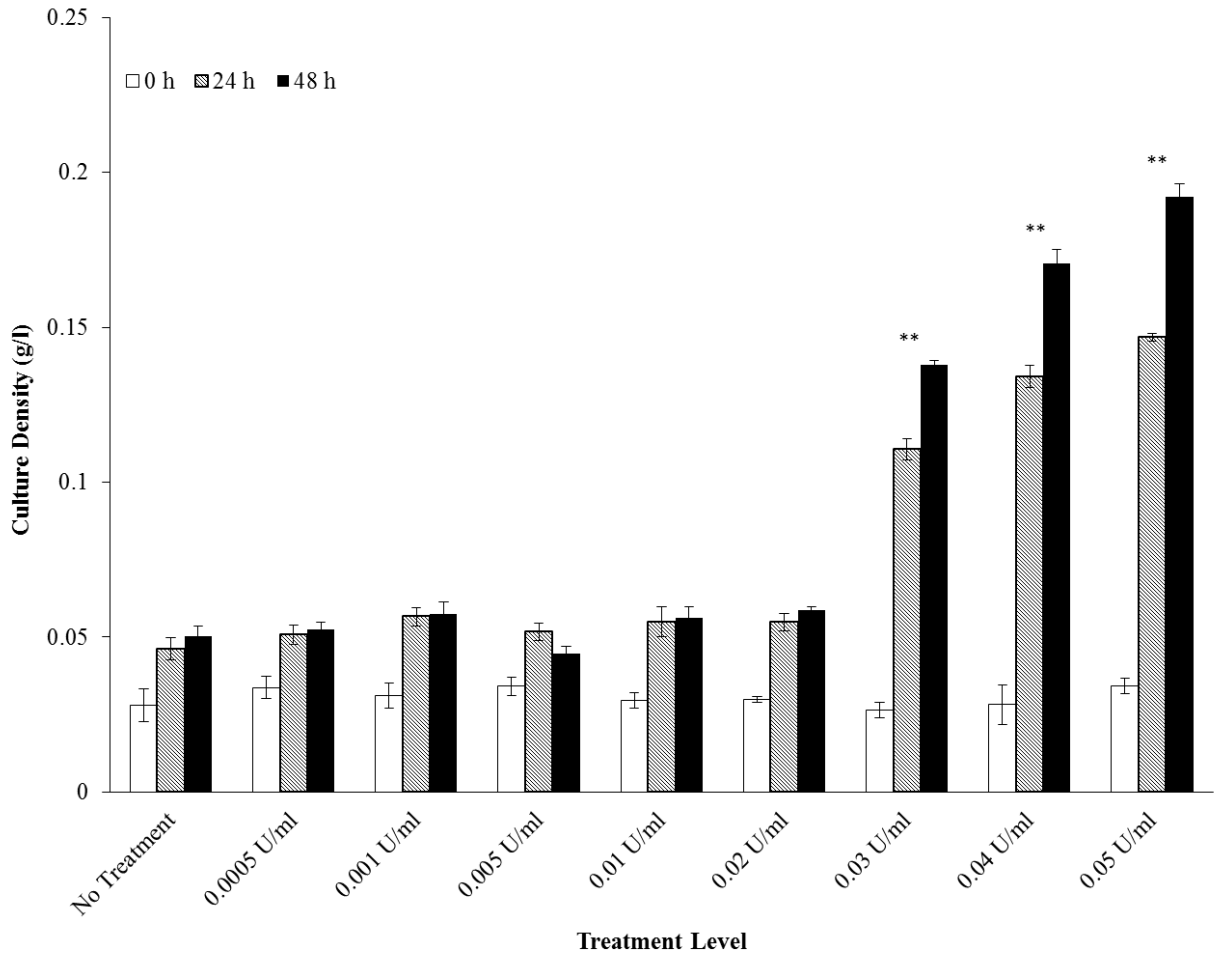

Figure 5. Supplementation with crude extracts of the InuA inulinase enzyme sourced from pD1214:InuA-expressing strains enabled the growth of wild type *S. cerevisiae* at enzymatic activity levels of 0.03 U InuA/ml and greater. Values are the means of three replicates, with error represented as ± the standard error of the mean

* = Significant ($p < 0.05$) growth relative to untreated controls beginning at 48 h post treatment

** = Significant ($p < 0.05$) growth relative to untreated controls beginning at 24 h post treatment

grown under these conditions produced similar growth curves (Figure 7), with each demonstrating initial growth on pre-treatment liberated fructose followed by growth on inulin. Similar times (120 h) were observed for the onset of stationary phase, and similar (p = 0.12) final culture densities were observed (2.07 ± 0.08 g/l in Dahlia-sourced cultures and 2.26 ± 0.05 g/l in Chicory-sourced cultures). These results indicate that the InuA enzyme is not significantly more efficient in processing inulin from either of these two sources when expressed in *S. cerevisiae*.

Discussion

Inulin represents a highly available biomass feedstock that can be sourced from a wide variety of agricultural resources capable of growing non-competitively with existing food crops [2, 3]. Unlike cellulose, the inulin reserves within these resources do not exist in a complex matrix of secondary components, and can therefore be broken down into their basic components via a single enzymatic step performed under relativity mild conditions in consolidated bioprocessing applications. This has made inulin an increasingly attractive target as a renewable biomass substitute for

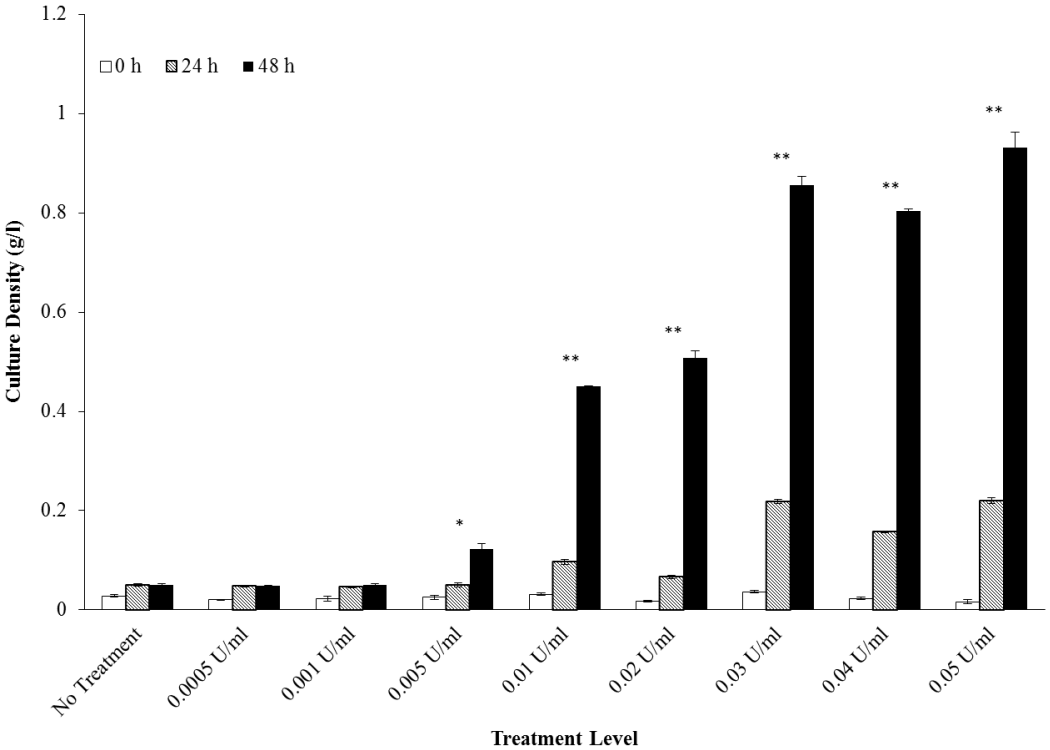

Figure 6. Supplementation with purified *A. niger* inulinase extracts enabled the growth of wild type *S. cerevisiae* at enzymatic activity levels of 0.005 U/ml and greater. This level was approximately 6.8-fold lower than when crude InuA extracts were employed, supporting previously published reports indicating that purified inulinase enzymes are significantly more efficient at liberating free sugar from inulin than those sourced from crude extracts [17]. Values are the means of three replicates, with error represented as ± the standard error of the mean
* = Significant ($p < 0.05$) growth relative to untreated controls beginning at 48 h post treatment
** = Significant ($p < 0.05$) growth relative to untreated controls beginning at 24 h post treatment

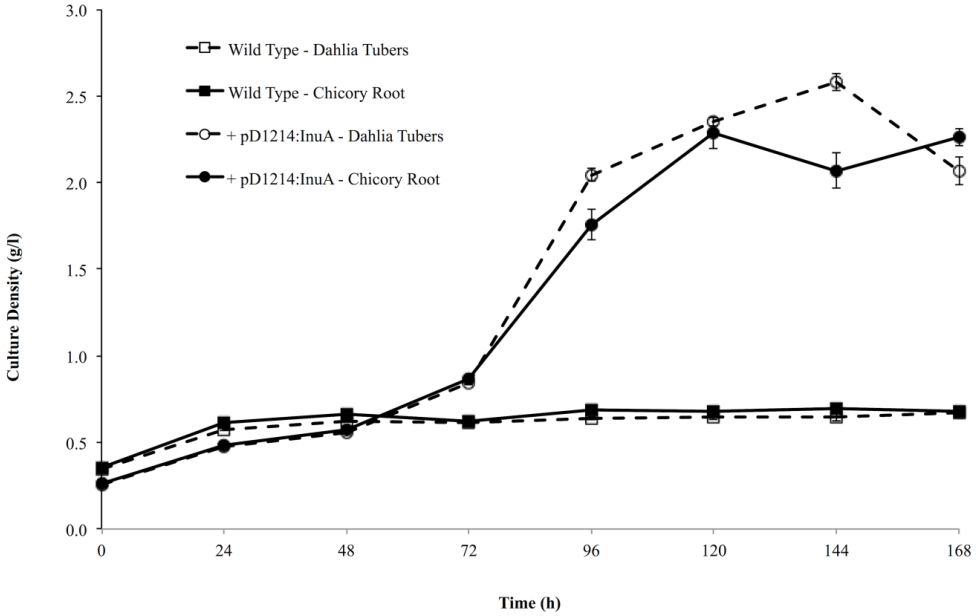

Figure 7. *S. cerevisiae* strains expressing the pD1214:InuA vector were able to grow similarly on pre-treated 2 g inulin/l sourced from either Dahlia tubers (open circles) or Chicory root (closed circles). Regardless of the inulin source, inulin-dependent increases in culture density occurred concurrently beginning at 72 h post-inoculation, and both the time required to reach stationary phase and the final peak culture densities were similar ($p > 0.05$). Wild type control strains were not able to utilize either the Dahlia (open boxes) or Chicory (closed boxes) sourced inulin as a carbon source. Values are the means of three replicates, with error represented as ± the standard error of the mean.

cellulosic or starch-based materials. An escalating focus on inulin over the past decade has begun to reveal not only its potential as an inexpensive and abundant biomass feedstock, but also its diverse product potential resulting from metabolism of its component monomeric sugars. Aside from the low hanging fruit of facile industrial scale high fructose syrup and inulo-oligosaccharide production for use in the food industry [4], recent work has suggested that inulin-based transportation fuel synthesis may be economically beneficial as well. Using inulin as a starting material, bioethanol yield can be doubled compared to corn-based processes [18] and biodiesel can be produced at nearly seven times the amount per hectare compared to soy [19]. Furthermore, inulin can be metabolized to a variety of high value intermediate chemicals such as 2,3-butanediol, lactic acid, butyric acid, butanol, acetone, succinic acid, acetic acid, and sorbitol at a lower cost relative to alternative carbon sources using single step consolidated bioprocessing strategies [20].

However, while *S. cerevisiae* strains endowed with exogenous expression of an efficient inulinase enzyme, such as the InuA enzyme of *A. niger*, are commonly employed to breakdown and covert inulin [4], little work has been dedicated to understanding the effect of exogenous inulinase expression on strains lacking endogenous inulinase activity. Here, these effects were investigated through the expression of the *A. niger inuA* gene in a strain which lacks the ability to endogenously grow on inulin as a sole carbon source. This approach ensures that any observed growth or phenotypic effects necessarily result from InuA expression activity.

The *A. niger inuA* gene sequence was codon optimized prior to introduction in *S. cerevisiae* since transcriptional and translational efficiency are the two greatest hurdles for expression of any exogenous gene product and the codon optimization process has been shown to aid significantly in increasing the efficiency of both these processes [21]. This resulted in efficient expression (Tables 1 and 2) of the gene product and permitted growth on fermentation medium containing inulin as a sole carbon source. However, without the use of pre-treatment procedures that were demonstrated to release an initial 0.1 g/l concentration of free fructose, this growth was not observed without an initial exogenous addition of fructose to the medium. Because enzymatic activity from InuA-expressing cultures was observed to increase over time due to the constitutive expression of the *inuA* gene and the increasing number of cells attained with each doubling (Table 3), it is hypothesized that this initial supplementation was required to initiate culture growth and achieve a critical mass for enzymatic production at a rate capable of degrading enough inulin to support sustained growth.

To further investigate if a minimal enzymatic activity level required for sustaining culture growth could be identified, a range of both crude and purified exogenous enzyme loadings representative of activity levels from 0.0005 U/ml to 0.05 U/ml were spiked into wild type *S. cerevisiae* cultures in non-pre-treated medium. This evaluation suggested that, under the growth conditions employed, a minimum activity level of 0.03 U InuA/ml was required to permit culture growth when crude enzyme extracts were used (Figure 5). It was previously reported that purification of inulinase enzyme extracts resulted in a 6.8-fold increase in efficiency relative to crude extracts [17]. This increase in activity would place the theoretical threshold value for the application of purified enzyme extracts at ~0.004 U/ml, which was supported experimentally by our data. When purified inulinase enzymes were used in place of crude InuA extracts, growth was observed at treatment levels of 0.005 U/ml and greater, but was not observed below this value (Figure 6). This suggests that the minimal observed crude value of 0.03 U InuA/ml may be the lowest value of inulinase expression required to sustain growth under relevant fermentation

conditions without the need for external enzymatic purification.

Interestingly, while the commercial purification of the *A. niger* inulinase enzyme used to verify these results contains both the InuA and InuB inulinase enzymes, the presence of the InuB enzyme in the purified extract did not affect the theoretical difference in the enzymatic activity level threshold required for growth. However, treatment with the purified inulinase extract did result in the attainment of higher cell densities relative to treatment with identical levels of crude InuA extract. This difference is especially pronounced at 48 h post-inoculation, while minimal at 24 h. This may suggest that the purified enzyme is retaining its activity longer relative to the crude enzyme extract, but the possibility that the inclusion of the InuB enzyme results in a reduction to the overall degree of polymerization of the inulin to a level that can be utilized by the yeast without achieving full breakdown into fructose monomers cannot be ruled out. Indeed, if this is the case it can be concluded that the reduction in the degree of polymerization of inulin's oligo-fructosaccharide content afforded by low-level inulinase expression is sufficient to permit growth due to the native invertase activity of *S. cerevisiae* [17], albeit with an expected reduction in growth rate relative to the utilization of monomeric sugars.

Another relevant observation that can be drawn from these data is that the minimum enzymatic activities required to support cellular growth (0.005 U/ml using purified enzyme extracts and 0.03 U InuA/ml using crude extracts) are significantly lower than the enzymatic activities commonly reported in the literature [4, 7], and are also much lower than the native enzymatic activities of naturally inulinase-expressing yeast strains such as *S. cerevisiae* JZ1C [6]. While the JZ1C strain natively expresses inulinase activity at a rate of 2.1 U/ml [5], this is almost an order of magnitude greater than the total activity level that was observed to sustain growth using crude extracts in this study. Furthermore, when the *A. niger* InuA enzyme was expressed in the JZ1C strain, inulinase activity increased 148% to 3.1 U/ml [17]. This increase in enzymatic activity resulted in the liberation of additional fructose at each time point assayed, as well as a corresponding increase in product (ethanol) production, however, the total free sugar released and total ethanol yield were identical between the wild type and InuA-expressing strains after 3 days of growth [5]. This suggests that the increase in enzymatic activity induced through InuA expression in an already inulinase-expressing strain is primarily beneficial in increasing the rate of product formation, but may not be necessary for increasing total product yield.

Keeping in mind that the overexpression of exogenous enzymes has been shown to adversely impact both cellular growth and biomass degradation in yeast [22], these data indicate that inulinase expression levels may be depressed during the metabolic engineering of novel host strains in order to redirect carbon flow away from their sustained production and towards the development of alternative processes focused on product development or cellular growth, but that this strategy will be most beneficial when longer production times can be acceptably employed. By doing so it may be possible to better optimize strains for product production and increase the efficiency of inulin-based consolidated bioprocessing fermentations using yeast in the future.

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