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Abatement of Inhibitors in Recycled Process Water from Biomass Fermentations Relieves Inhibition of a *Saccharomyces cerevisiae* Pentose Phosphate Pathway Mutant †

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Received: 30 September 2020; Accepted: 6 November 2020; Published: 10 November 2020

Abstract: Understanding the nature of fermentation inhibition in biomass hydrolysates and recycled fermentation process water is important for conversion of biomass to fuels and chemicals. This study used three mutants disrupted in genes important for tolerance to either oxidative stress, salinity, or osmolarity to ferment biomass hydrolysates in a xylose-fermenting *Saccharomyces cerevisiae* background. The *S. cerevisiae ZWF1* mutant with heightened sensitivity to fermentation inhibitors was unable to ferment corn stover dilute-acid hydrolysate without conditioning of hydrolysate using a fungal strain, *Coniochaeta ligniaria*, to consume inhibitors. Growth of two other strains, a salt-sensitive *HAL4* mutant and a *GPD1* mutant sensitive to osmotic stress, was not negatively affected in hydrolysate compared to the parent xylose-metabolizing strain. In recycled fermentation process water, inhibition of the *ZWF1* mutant could again be remediated by biological abatement, and no effect on growth was observed for any of the mutants compared to the parent strain.

Keywords: biomass; fermentation; inhibitors; ethanol

1. Introduction

Conversion of lignocellulosic biomass to value-added products entails physical-chemical pretreatment to release sugar monomers, locked within the plant cell wall, as substrates. In addition to extracted sugars, hydrolysates contain other compounds formed under the harsh mechanical, thermal, and chemical conditions used to hydrolyze hemicellulose and make cellulose fibrils accessible to cellulase [1]. Many of these are aromatic and/or reactive compounds, and as such they are inhibitory to both saccharifying enzymes and fermenting microbes [2–4]. Monosaccharides released during the pretreatment process can undergo a dehydration reaction to form furfural and 5-hydroxymethylfurfural (HMF), cyclic aldehydes that are potent inhibitors that negatively impact cell membranes, enzymes, and growth [5–7]. Hemicellulose pretreatment releases organic acids and lignin depolymerization liberates phenolic compounds, both of which act synergistically with furans in affecting cell growth and viability [4,8,9].

The complexity of inhibitor mixtures is compounded when process water is recycled. For first generation ethanol, manufactured from corn, use of stillage as makeup water for the fermenting tanks is common and necessary to reduce the water footprint. This makeup water is termed backset and

the practice builds up inhibitors. Research on using backset in the context of cellulosic ethanol is limited, at least for laboratory studies [10–12]. Measuring "toxicity" is challenging because mixtures are complex, their effects interact, and "back-setting" allows new actors to accumulate to toxic levels. Yet, simply noting if a fermenting yeast grows or not is not very helpful either. In this study, we used phenotypically sensitive mutant yeast strains to probe for common causes of inhibition: chemical, saline, and osmotic stresses.

To demonstrate this sensor technology, we have applied it to hydrolysate that has been conditioned for fermentation following pretreatment using biological abatement, a method developed in this lab. A soil fungus, *Coniochaeta ligniaria* NRRL30616, which metabolizes furfural and HMF as well as numerous aromatic and aliphatic compounds as carbon sources for growth [13,14] is being investigated for use in biological abatement. Bioabatement has been shown to improve conversion of glucose as well as xylose, conversion of which is particularly sensitive to the presence of fermentation inhibitors [15–17], possibly due to redox imbalance [18,19].

More recently, using *Escherichia coli* as the fermenting microorganism, we showed that bioabated process water can be successfully used as backset at laboratory scale [20]. Process water recycling is a conservation strategy that will affect environmental impact by reducing both water consumption for the portion of water used for the process (as opposed to cooling and energy use) and wastewater discharge [21,22]. In cellulosic ethanol fermentations, recycling of water will depend on inhibitor load because fermentation inhibitors will accumulate with each backset of process water. In light of the need to reuse water in cellulosic ethanol fermentations, an understanding of whether inhibitor abatement can facilitate process water reuse is requisite. Here, we used *S. cerevisiae* mutants that are phenotypically sensitive to either fermentation inhibitors (*ZWF1*), salinity (*HAL4*), or osmolarity (*GPD1*) to probe fermentations of biomass hydrolysates. The *S. cerevisiae ZWF1* mutant with heightened sensitivity to fermentation inhibitors was impaired in fermentations, and bioabatement restored the strain's growth. Furthermore, bioabatement enabled the fermentation of recycled process water.

2. Materials and Methods

2.1. Strains, Media, Culture Conditions

Microbial strains and oligonucleotide primers are described in Table 1. *C. ligniaria* was maintained by subculturing at 30 °C in mineral medium [13] containing 10 mM furfural and was precultured for bioabatement in YPD medium (10 g yeast extract, 20 g peptone, and 20 g glucose (sterilized and added separately) per liter). *S. cerevisiae* was grown in YPD medium at 30 °C. For genetic manipulations, solid SD medium which contained 6.7 g/L yeast nitrogen base (United States Biological, Marblehead, MA, USA) supplemented with amino acids [23] was used. Synthetic medium was sterilized by filtration and sterile glucose was added separately (20 g/L). Solid media contained 15 g/L agar. Media components were obtained from Becton-Dickinson (Sparks, MD, USA) and sugars and inhibitors were purchased from Sigma-Aldrich (St. Louis, MO, USA). Oligonucleotides were synthesized by IDT (Coralville, IA, USA).

2.2. Yeast Growth Assays

Yeast growth was measured in microwell assays using a Bioscreen C Automated Growth Curve Analysis System (Growth Curves USA, Piscataway, NJ, USA). Unless otherwise stated, growth curves were carried out at least in quadruplicate as follows. Individual colonies were each inoculated into 1.0 mL of mineral medium and mixed by vortexing. Then, 10 µL was transferred to 290 µL of the experimental medium in 400 µL capacity sterile honeycomb wells in a 100-well plate. Hydrolysates were diluted to one-half strength to allow optical density measurements and were supplemented with 2 g/L yeast extract and 4 g/L peptone, in order to facilitate growth. Perimeter wells of the plate contained water to minimize evaporation during incubation. Plates were incubated at 30 °C with intermittent shaking. Optical density (420–580 nm Wideband setting) was recorded at 30 min. intervals via the

manufacturer's EZExperiment software and exported to a spreadsheet (Microsoft Excel, Redmond, WA, USA) for analysis. Optical density was converted to OD (600 nm) using the equation OD_{600} equivalent = $0.6748X^4 - 0.749X^3 + 0.5352X^2 + 1.0739X$ ($r^2 = 0.9989$, where X is the background-substracted wideband OD).

Table 1. Strains and oligonucleotides used in this study.

Strain	Description	Source or Reference
S. cerevisiae CEN.PK2-1C	*MAT***a** *ura3-52 trp1-289 leu2-3,112 his3*Δ1 *MAL2-8*^{C} *SUC2*	EUROSCARF
S. cerevisiae YRH388	CEN.PK2-1C *ho*Δ::(KanMX4; P_{PGK1}-*XYL1*-T_{PGK1}; P_{ADH1}-*XYL2*-T_{ADH1}; P_{HXT7}-*XKS1*-T_{HXT7})	[17]
S. cerevisiae YRH512	CEN.PK2-1C *zwf1*Δ::*LEU2*	This work
S. cerevisiae YRH1511	YRH388 *zwf1*Δ::*LEU2*	This work
S. cerevisiae YRH1512	YRH388 *hal4*Δ::*LEU2*	This work
S. cerevisiae YRH1513	YRH388 *gpd1*Δ::*LEU2*	This work
C. ligniaria NRRL 30616	Bioabatement strain	[13]
Oligonucleotide	Sequence	
229	GGCAGTGTCTAAGACTTTACAGGGA	
675	gtacggaAAACTCGCCAAGGCTATCC	
676	CCAAAATGTCACTGACCGCGGCTAAA	
677	AAAAAAAAGTAATCAGATTTTATTTTATTTCGACATTACCCCTC AAATATAGATTGTACTGAGAGTGCAC	
678	AATACCGAATTGTTCCAAAATATCGTGATATTTGAAAGTGAAG TACTCGTCTGTGCGGTATTTCACACCG	
679	TATATTGTACACCCCCCCCCTCCACAAACACAAATATTGATAAT ATAAAGAGATTGTACTGAGAGTGCAC	
680	CCTCGAAAAAAGTGGGGGAAAGTATGATATGTTATCTTTCTCC AATAAATCTGTGCGGTATTTCACACCG	
681	ATGTGGGATTTTTGGCTCAAGGTGT	
682	TTTCCTGTCGGTTTCG	
683	TGCCGGATAACAAAGC	
684	CCGCACAACAAGTATCAGAATGGG	
685	AAATGCGGAAGAGGTGTACAGC	

2.3. Construction of S. cerevisiae Mutants

Construction of the *HAL4* and *GPD1* deletions in the xylose-metabolizing *S. cerevisiae* strain YRH388 to create YRH1512 and YRH1513 was performed by gene replacement with a selectable marker as described in Hector et al. [24]. Gibson assembly primers 677 and 678 were used for *HAL4* and primers 679 and 680 were used for *GPD1*. A *ZWF1* deletion was similarly constructed in *S. cerevisiae* strain CEN.PK2-1C, to create YRH512. For the construction of YRH1511, genomic DNA was isolated from *S. cerevisiae* YRH512 using the YeaStar kit (Zymo Research, Irvine, CA, USA) and used as template to amplify the *zwf1*Δ::*LEU2* insertion fragment using primers 675 and 676 and Phusion HFII polymerase (NEB, Ipswich, MA, USA). *S. cerevisiae* YRH1511 was then constructed by transforming YRH388 with the *zwf1*Δ::*LEU2* PCR product through homologous recombination. Transformants were selected on SD medium without leucine and DNA from resulting colonies [25] was screened for the presence of the insertion using PCR with primers 229 and 681 for *zwf1*Δ::*LEU2*, primers 682 and 683 for *hal4*Δ::*LEU2*, and primers 684 and 685 for *gpd1*Δ::*LEU2*.

2.4. Preparation and Analysis of Hydrolysate

Corn stover was air-dried and milled using a Wiley Model 4 mill (Thomas Scientific, Swedesboro, NJ, USA) to pass through a 2 mm screen. Milled stover was pretreated in 0.5% (*w*/*v*) H_2SO_4 in 50 rpm rotating stainless steel reactors with infrared heating (Labomat BFA-12 v200, Werner Mathis, Concord, NC, USA). Reactors were incubated for 10 min at 160 °C with heating and cooling times of approximately 45 and 35 min, respectively. Solids were removed by filtration through a buchner funnel lined with Whatman #1454 110 filter paper and washed with a 10% volume of sterile water. The supernatant was combined with wash liquid, pH-adjusted to 6.5 with $Ca(OH)_2$, and sterilized through a 0.2 µm filter. Switch grass dilute-acid hydrolysate was a gift from ICM (Colwich, KS, USA).

Sugars and fermentation products were quantitated using HPLC and refractive index detection [26]. Furfural and HMF in hydrolysate were measured with ultraviolet detection at 277 nm in reverse phase HPLC [13]. Total phenolics were assayed using a spectrophotometric method [27].

2.5. Inhibitor Abatement

Bioabatement of inhibitors in dilute-acid corn stover hydrolysate has been described previously [20]. In brief, precultured *C. ligniaria* NRRL 30616 equal to a 10% (*v*/*v*) inoculum of *C. ligniaria* NRRL 30616 cells was washed and incubated in hydrolysate for 15–18 h at 30 °C with aeration by shaking at 225 rpm. Unabated (i.e., uninoculated) controls were incubated in parallel with each bioabatement experiment. Hydrolysate was sampled to determine the concentration of inhibitors and sugars.

2.6. Ethanol Fermentations and Recycle of Hydrolysate

Fermentations of dilute-acid pretreated CSH were initiated with a 5% (*v*/*v*) inoculum of *S. cerevisiae* YRH388 or YRH1511 and addition of 1% (*w*/*v*) yeast extract, 2% (*w*/*v*) peptone, and 0.1% $(NH_4)_2SO_4$. Fermentations were carried out and monitored using Ankom (Macedon, NY, USA) RF gas production modules to measure accumulation of pressure due to CO_2 production as previously described [20]. Samples were collected for HPLC analysis of inhibitors and fermentation products at the beginning and end of incubation. Re-use of fermentation process water has been previously described by our group [20]. In brief, post-fermentation hydrolysate was heated to remove ethanol by evaporation. Volume lost to evaporation was replaced with distilled water and the hydrolysate was diluted with an equal volume of fresh hydrolysate that had been treated by bioabatement to mitigate fermentation inhibitors. The reconstituted hydrolysate was filter-sterilized prior to fermentation. The fermentation-evaporation-reconstitution cycle was repeated four times.

3. Results and Discussion

An understanding of process water reuse and inhibitor abatement is needed for lignocellulosic biofuel fermentations. Continued reuse of process water could result in accumulation of initially low-concentration components, causing concentrations to increase with each backset and eventually reach inhibitory levels if they are not adequately cleared during fermentation. It is therefore important to address fermentation inhibition in both fresh biomass hydrolysates and in recycled fermentation process water. This study employed yeast strains, with heightened sensitivity to specific stresses, to probe the source of toxicity in biomass hydrolysates and the utility of bioabatement for growth of the strains.

Strain YRH388 is a *S. cerevisiae* CEN.PK2 derivative, engineered to metabolize xylose by the addition of xylose reductase and xylitol dehydrogenase genes from *Scheffersomyces stipitis* and overexpression of the *S. cerevisiae* xyulokinase gene [17]. Three isogenic mutants of YRH388 were constructed: *ZWF1*, a pentose phosphate pathway mutant sensitive to oxidative stress [28]; *HAL4*, a serine/threonine protein kinase mutant sensitive to salt [29]; and *GPD1*, the glycerol-6-phosphate dehydrogenase gene mutant affecting yeast tolerance to osmolarity [30]. Mutants in these three genes were used to assess the fermentability of hydrolysates in the YRH388 xylose-fermenting genetic background. The mutants were also assayed for growth in recycled hydrolysate in order to detect any sensitivity that would indicate a particular problematic factor arising from recycle of fermentation process water. Corn stover dilute-acid hydrolysates were prepared and inhibitors were mitigated using bioabatement (Table 2), and growth of the four strains in corn stover hydrolysate was measured (Table 3). On average, bioabatement reduced HMF and furfural concentrations by 42% and 78%, respectively. Phenolics content decreased by half. Because cellulose was not saccharified, the hydrolysate contained only a small amount of glucose, which decreased by 0.08% (*w*/*v*) during bioabatement; little to no xylose, galactose, and arabinose were consumed during bioabatement.

Table 2. Composition of corn stover dilute-acid hydrolysate and abatement of inhibitors.

Hydrolysate [1]	HMF (mM)	Furfural (mM)	Phenolics (AU)	Glucose (% *w*/*v*)	Xylose (% *w*/*v*)	Arabinose (% *w*/*v*)	Galactose (% *w*/*v*)
No abatement	0.60 ± 0.07	12.72 ± 0.84	167 ± 28	0.32 ± 0.00	1.82 ± 0.02	0.29 ± 0.02	0.14 ± 0.00
Bioabated	0.35 ± 0.13	2.86 ± 2.38	82 ± 73	0.24 ± 0.06	1.78 ± 0.02	0.28 ± 0.01	0.14 ± 0.00
Recycled	0.00	0.00	88 ± 9	0.00	0.38	0.15	0.04

[1] Unabated and bioabated hydrolysates reflect the average of three hydrolysate preparations, sampled in duplicate. Bioabated hydrolysates were sampled after 15–18 h incubation. Recycled hydrolysate was sampled after four cycles of reuse as described in the methods.

Table 3. Effect of bioabatement on growth of YRH388 isogenic mutants in corn stover hydrolysate.

S. cerevisiae Strain	Relevant Mutation	Phase of Growth	Doubling Time (h)		Maximum OD	
			Unabated CSH	Bioabated CSH	Unabated CSH	Bioabated CSH
YRH388	—	Early	4.5 ± 0.4	3.7 ± 0.4		
YRH1511	*ZWF1*	Early	19.5 ± 1.7	3.4 ± 0.2		
YRH1512	*HAL4*	Early	4.8 ± 0.4	3.4 ± 0.7		
YRH1513	*GPD1*	Early	5.7 ± 0.2	4.6 ± 0.4		
YRH388	—	Late	90.1 ± 8.9	98.0 ± 6.7	1.19 ± 0.06	1.15 ± 0.06
YRH1511	*ZWF1*	Late	138.8 ± 13.6	123.9 ± 19.5	0.66 ± 0.04	0.76 ± 0.29
YRH1512	*HAL4*	Late	108.7 ± 13.3	91.5 ± 9.1	1.14 ± 0.04	1.14 ± 0.05
YRH1513	*GPD1*	Late	128.8 ± 16.0	49.6 ± 1.9	1.12 ± 0.05	1.56 ± 0.03

Hydrolysates were diluted two-fold. Resulting sugar concentrations (% *w*/*v*) were in unabated hydrolysate: 0.18% glucose, 0.89% xylose: 0.06% galactose, 0.14% arabinose. Bioabated hydrolysate contained 0.18% glucose, 0.92% xylose, 0.08% galactose, 0.15% arabinose.

Growth of *S. cerevisiae* in hydrolysate containing glucose and xylose is biphasic, consistent with preferential metabolism of glucose followed by a switch to metabolism of xylose. Compared to the parent strain, xylose-metabolizing strain YRH388, only the *ZWF1* mutant showed impaired growth in unabated corn stover hydrolysate, as evidenced by increased doubling times and decreased cell density. The *HAL4* mutant, YRH1512, did not markedly differ from the parent strain YRH388, while the *GPD1* mutant, YRH1513, had normal early growth and somewhat slower later growth, compared to YRH388 (Table 3). The sum of these results suggests that neither high salt nor osmolarity is a significant source of microbial inhibition in these biomass hydrolysates. The maximum growth in corn stover hydrolysate (optical density expressed as A600 in microwell assays) achieved by YRH1511 was roughly 60% of the other strains (Table 3). In comparison, the maximum A600 in anaerobic batch fermentations of YP medium containing 1% glucose and xylose was 4.24 ± 0.25, 3.62 ± 0.06, 3.56 ± 0.01, and 4.12 ± 0.14 for YRH388, YRH1511, YRH1512, and YRH1513, respectively. These higher final ODs reflect the higher glucose concentration and lack of inhibitors in YP medium; YRH1511 had only a 15% decrease in final OD compared to the 40% decrease observed in hydrolysate, highlighting the increased toxicity that appears to be specific for the *ZWF* mutant strain.

The *ZWF1* gene is known to be important for furfural tolerance [31], and in this work, sensitivity was likewise established for the *ZWF1* mutant, YRH1511. Figure 1A confirms the presence of the ZWF1 phenotype and visually demonstrates the impact of furfural on growth. In YP medium containing glucose, the YRH388 and YRH1511 strains had roughly equal doubling times when growth media contained up to 7.5 mM furfural. At 10 mM furfural, growth of YRH1511 was more variable, and at 15 mM YRH1511 failed to grow (Figure 1B). Pronounced sensitivity for xylose metabolism was also observed. With no furfural and up to 2.5 mM furfural present, the two strains had approximately equal generation times in YP medium containing xylose (Figure 1B); however, at 2.5 mM furfural, the lag time was more than four-fold longer for YRH1511 compared to YRH388 (not shown). At 5 mM furfural, three out of four YRH1511 cultures failed to grow, whereas YRH388 grew in YP medium containing xylose with up to 10 mM furfural.

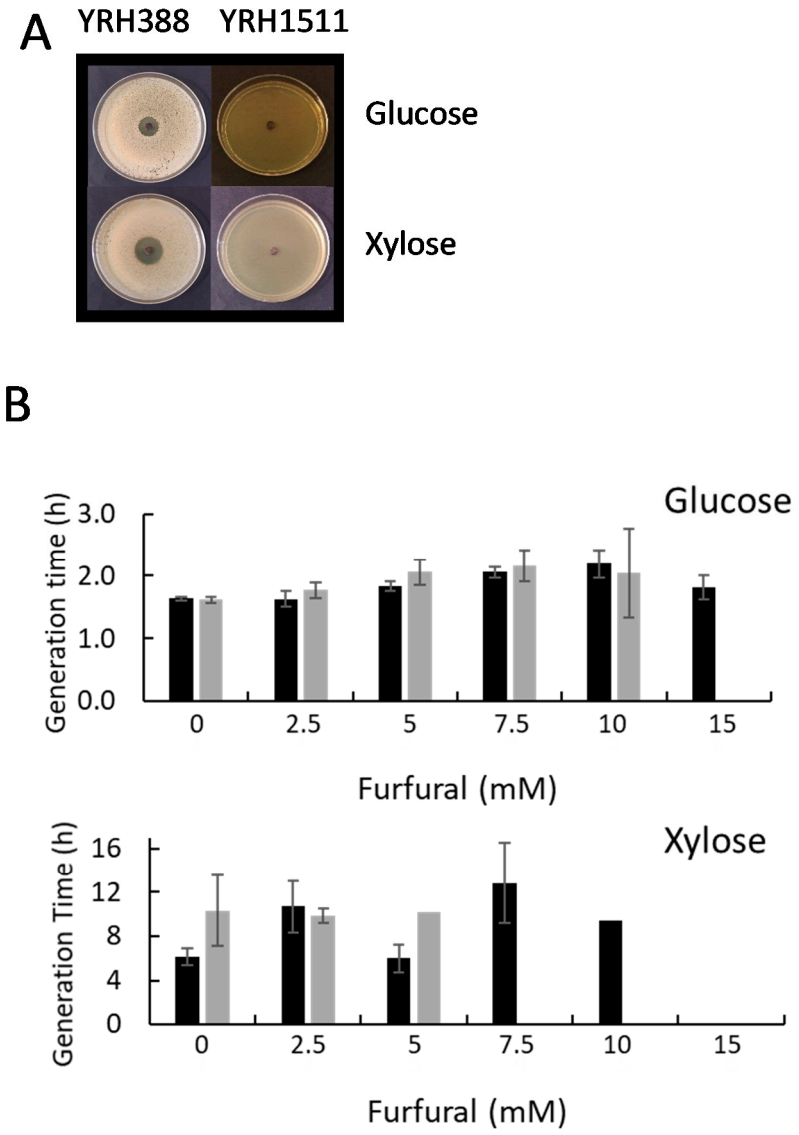


Figure 1. Inhibition by furfural. Comparison of inhibition of parental strain *S. cerevisiae* YRH388 and *zwf1*-mutant YRH1511. (**A**). A filter disk saturated with liquid furfural (8 µL) was placed in the center of a lawn of plated cells on YP medium containing 2% *w/v* glucose or xylose. Representatives of triplicate plates are shown. A small zone of inhibition was observed for YRH388, compared to nearly complete inhibition of YRH1511 after incubation for 96 h. Glucose plates were inoculated with 10^6 cfus; xylose plates were inoculated with 10^7 cfus. (**B**). Doubling times of YRH388 (black) and YRH1511 (gray) on YP medium containing glucose (top) or xylose (bottom) and furfural at varying concentrations. YRH1511, growing on glucose, did not grow when more than 10 mM furfural was present. In medium containing xylose, YRH1511 did not grow when greater than 5 mM furfural was present. Error bars show standard deviation of four replicates.

After the furfural-sensitive phenotype of *S. cerevisiae* YRH1511 was confirmed, the ability of bioabatement to rescue growth was tested. The inhibitor-resistant soil isolate *C. ligniaria* has been previously shown to improve fermentability of biomass hydrolysates fermented with *E. coli* [32] and *S. cerevisiae* [33]. As shown in Figure 2, the parent strain, YRH388, grew in both unmitigated and inhibitor-abated hydrolysate. The lag phase observed for YRH388 in unabated hydrolysate was eliminated in the bioabated samples. In contrast, YRH1511 exhibited an extended delay before growth in unabated hydrolysate. In bioabated hydrolysate, the lag phase was eliminated and the *ZWF1* mutant grew similarly to the parent strain over approximately the first 24 h of incubation.

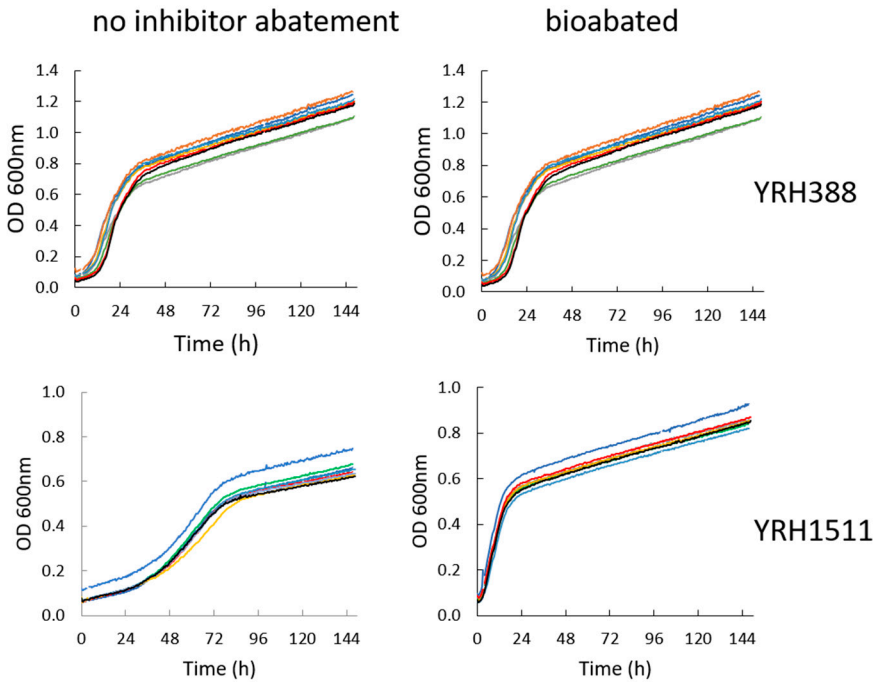
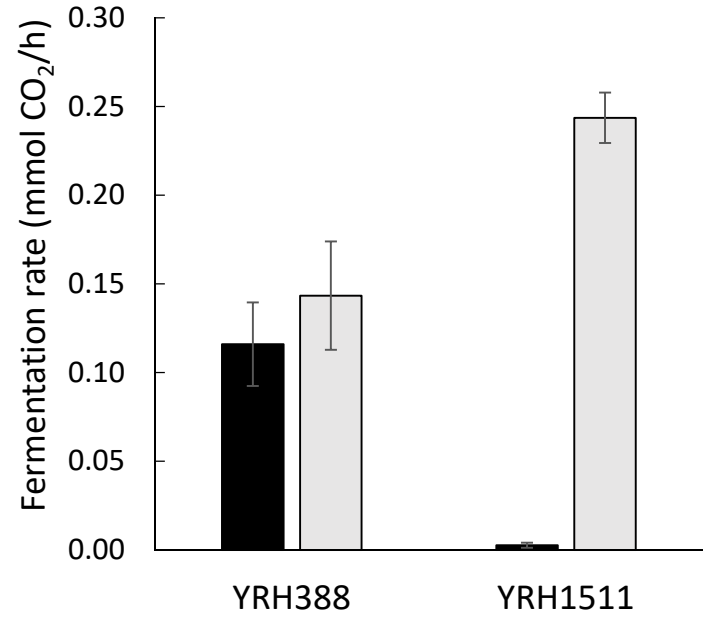


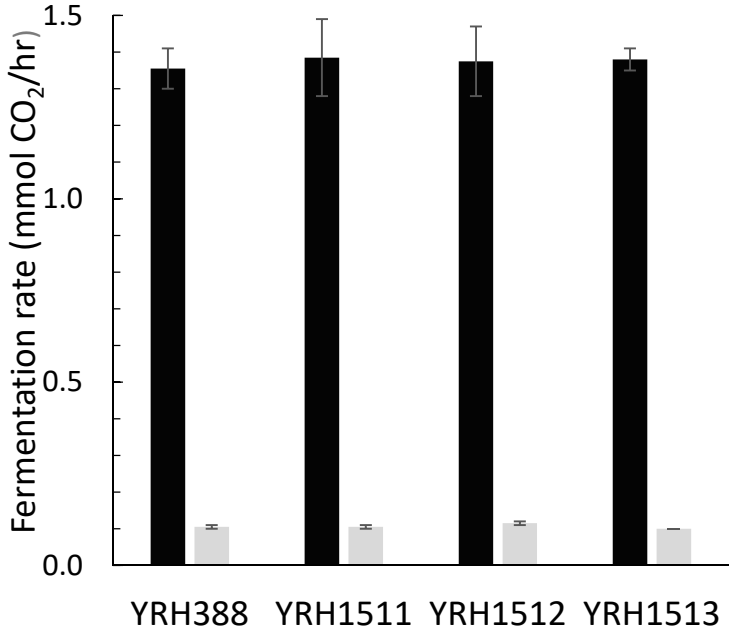
Figure 2. Microwell growth assays of YRH388 and PPP mutant YRH1511 in unabated and bioabated corn stover dilute-acid hydrolysate. Panels show seven or eight replicates (shown as individual lines of various colors) for the indicated strain and hydrolysate. Sugar concentrations are stated in Table 3.

Notable for both strains is the marked biphasic nature of growth. Both strains grow more slowly in the second phase, consistent with glucose being a preferred carbon source and with the afore-mentioned sensitivity of xylose metabolism to inhibitors. For YRH1511, bioabatement of hydrolysate led to recovered early phase growth and partially-recovered late phase growth as compared to YRH388 (Table 3). The early phase (glucose) doubling time for YRH1511 was greater than four times higher than that of YRH388 in unabated hydrolysate, and essentially the same as that of YRH388 in bioabated hydrolysate. The late phase doubling times were also similar for YRH388 in unabated and bioabated hydrolysates. In contrast, the late-phase growth of YRH1511 in unabated CSH again reflected the increased inhibitor sensitivity when fermenting xylose in this strain; the doubling time for YRH1511 in the late phase of growth was 1.5 times that of YRH388 in unmitigated corn stover hydrolysate. With bioabatement, the late phase doubling time of YRH1511 was reduced by 11%. For YRH1512 and YRH1513, comparison of growth rates (Table 3) revealed that bioabatement restored growth to rates that equaled growth of YRH388. These results demonstrate that for cases in which growth of a mutant, particularly the *ZWF1* mutant, was impaired in corn stover hydrolysate, aerobic growth could be rescued using bioabatement to remove inhibitory compounds.

In light of the pronounced sensitivity to furfural observed for growth by the pentose phosphate pathway mutant YRH1511, we also tested fermentations with this strain under anaerobic conditions where ATP limitation and redox imbalance markedly increase the negative effect of inhibitors. The effect of inhibitor abatement was examined by monitoring CO_2 production as a measure of ethanol fermentation (Figure 3A) and by quantitating fermentation end products (Table 4). For comparison, growth and CO_2 production for all four strains in rich medium is shown in Figure 3B and Table 4. Maximum fermentation rates (Figure 3B) were higher in rich medium than in hydrolysate due to increased glucose concentration and absence of inhibitors, and the rates were similar among all strains in rich conditions. The difference between the parent strain YRH388 and the *ZWF1* mutant YRH1511 is, however, evident in fermentations of unconditioned hydrolysate, where YRH388 fermented glucose and xylose to ethanol and YRH1511 essentially failed to ferment. This difference reflects the enhanced sensitivity to fermentation inhibitors affecting YRH1511 compared to the other mutant strains.



(**A**)



(**B**)

Figure 3. (**A**)**.** Fermentation of corn stover dilute-acid hydrolysate by YRH388 and *ZWF1* mutant YRH1511. Bars show rate of accumulation of CO_2 in unabated (black) or bioabated (gray) hydrolysates. Values are the average of three experiments each carried out with two or three replicates for fermentations of bioabated hydrolysate and one unabated control. Plots are shown with error bars representing the standard error. (**B**). Fermentation of YP medium containing 1% each glucose and xylose. Bars show the maximum rate of accumulation of CO_2 during growth on glucose (black) and xylose (gray).

Table 4. A: Fermentation of corn stover hydrolysate by *S. cerevisiae* YRH388 and YRH1511; B: Fermentation of rich medium containing glucose and xylose.

		Glucose Consumed [1]	Xylose Consumed	Residual Glucose	Residual Xylose	Ethanol	Glycerol	Xylitol	Ethanol Yield [2]
					A				
Unabated	YRH388	0.240 ± 0.079	0.192 ± 0.011	0.010 ± 0.003	1.548 ± 0.006	0.172 ± 0.011	0.010 ± 0.008	0.040 ± 0.023	0.398
	YRH1511	0.066 ± 0.059	0.020 ± 0.006	0.224 ± 0.034	1.726 ± 0.008	0.030 ± 0.031	0.000 ± 0.000	0.000 ± 0.000	0.349
Bioabated	YRH388	0.220 ± 0.025	0.317 ± 0.016	0.011 ± 0.001	1.415 ± 0.024	0.159 ± 0.015	0.066 ± 0.005	0.068 ± 0.001	0.296
	YRH1511	0.224 ± 0.023	0.160 ± 0.035	0.010 ± 0.002	1.568 ± 0.042	0.171 ± 0.017	0.008 ± 0.001	0.007 ± 0.010	0.445
					B				
	YRH388	0.956 ± 0.057	0.832 ± 0.059	0.000 ± 0.000	0.150 ± 0.014	0.544 ± 0.034	0.062 ± 0.003	0.350 ± 0.020	0.304
	YRH1511	0.970 ± 0.048	0.670 ± 0.096	0.000 ± 0.000	0.326 ± 0.059	0.564 ± 0.023	0.052 ± 0.011	0.192 ± 0.028	0.344
	YRH1512	0.942 ± 0.054	0.792 ± 0.071	0.000 ± 0.000	0.206 ± 0.020	0.562 ± 0.025	0.066 ± 0.008	0.302 ± 0.037	0.324
	YRH1513	0.976 ± 0.011	0.880 ± 0.034	0.000 ± 0.000	0.164 ± 0.006	0.556 ± 0.023	0.064 ± 0.006	0.388 ± 0.000	0.300

[1] Concentrations are % (*w*/*v*); [2] g ethanol/(g glucose plus g xylose) consumed.

Saccharomyces cerevisiae engineered to ferment xylose to ethanol via the xylose reductase-xylitol dehydrogenase pathway converts xylose to xylulose, which enters the pentose phosphate pathway after phosphorylation by xylulokinase. The xylose conversion pathway oxidizes primarily NADPH to NADP+ in the first enzymatic step and generates NADH in the second step, thereby disrupting cytoplasmic availability of NADPH and likewise depleting NAD^+ [17]. The pentose phosphate pathway is a major source of biosynthetic reducing equivalents in the form of NADPH. The first and rate-limiting enzyme of the pentose phosphate pathway, glucose-6-phosphate dehydrogenase (E.C. 1.1.1.49), is encoded in *S. cerevisiae* by *ZWF1*. The ZWF1 enzyme is important for cellular adaptation to oxidative stress [34], and thus a *ZWF1* mutant is markedly sensitive to furfural in biomass liquors [31]. When growing on xylose, gluconeogenesis is the source of glucose for ZWF1 [35].

Mitigation of inhibitors by bioabatement dramatically improved the fermentation of corn stover hydrolysate by YRH1511 (Figure 3A). YRH1511 and the parent strain, YRH388, ended fermentations with approximately equal accumulation of ethanol, but differed in production of fermentation side-products (Table 4). At the end of fermentations, cell density of YRH388 and YRH1511 in bioabated hydrolysates was 3.7×10^7 and 4.6×10^7 cfu/mL, respectively, while unabated hydrolysates contained 3.4×10^7 and 2.2×10^7 cfu/mL, respectively. Improved consumption of xylose by YRH388 in hydrolysate treated to mitigate inhibitors was offset by increased production of xylitol, with the result that ethanol concentration was roughly equal in bioabated hydrolysates compared to unabated hydrolysate. Xylose consumption was likewise increased in YRH1511 when grown in hydrolysate mitigated with bioabatement, although the strain produced less glycerol and xylitol and consequently had higher ethanol yield. The highest ethanol yield (g ethanol produced/g sugar consumed) was observed for strain YRH1511 in hydrolysate that was mitigated by bioabatement. The difference in fermentations between the two strains likely reflects the difference in cofactor balance in the two strains influenced by disruption of the oxidative portion of the pentose phosphate pathway. The production of xylitol by YRH388 is a consequence of a NADPH imbalance produced by metabolism of xylose [36]. YRH1511 consumed less than half as much xylose as YRH388 in bioabated corn stover hydrolysate (Table 4). The final ethanol concentration for the two strains was therefore similar, and as reported by Jeppsson et al. [34] the metabolic yield of ethanol was higher for YRH1511 due to lower production of reduced fermentation products, xylitol and glycerol.

In addition to the differing ethanol yields between parent strain YRH388 and *ZWF1* mutant strain YRH1511, the production of fermentation side products by YRH388 differed when the strain was grown in unconditioned hydrolysate versus hydrolysate conditioned to remove inhibitors (Table 4). Production of glycerol in particular by YRH388 was higher in bioabated hydrolysates than in untreated samples. Glycerol production acts as a redox sink to oxidize excess NADH produced during growth, and decreased yield of glycerol in the presence of fermentation inhibitors has been described [8,36,37]. In hydrolysate containing furfural, NADP-linked conversion of dihydroxyacetone-phosphate to glyceraldehyde-3-phosphate leading ultimately to production of glycerol, is diverted instead to cellular reduction of furfural to furfuryl alcohol by NADH-dependent dehydrogenase. The measurable result is decreased glycerol production in the presence of furfural. Production of xylitol by YRH388 was also higher in the inhibitor-mitigated hydrolysates and reflects the greater amount of xylose consumed.

The CEN.PK2-1C parent strain used to construct mutations for this work was previously engineered to metabolize xylose (Table 1). The strain is not an efficient producer of ethanol from xylose [17]; a significant fraction (approximately half) of xylose is converted to xylitol rather than ethanol under fermentative conditions, resulting in markedly decreased ethanol yields when xylose consumption is factored into yield calculations (Table 4). However, the relative sensitivity of the strain to fermentation inhibitors serves as a useful platform for comparing the effect of fermentation inhibitors on the mutations introduced in this study.

After the utility of bioabatement to rescue the fermentation of fresh corn stover hydrolysate was established for YRH1511 as described above, growth of the strain, along with the *HAL4* and *GPD1* mutants, was measured in recycled process water, using switchgrass hydrolysate in microwell

plate assays (Figure 4). In these experiments, growth of the mutants was similar to that of the parent strain in switchgrass hydrolysate that was bioabated and recycled four times and had low concentrations of furfural and HMF. The data indicated no difference between growth of the strains in this switchgrass hydrolysate.

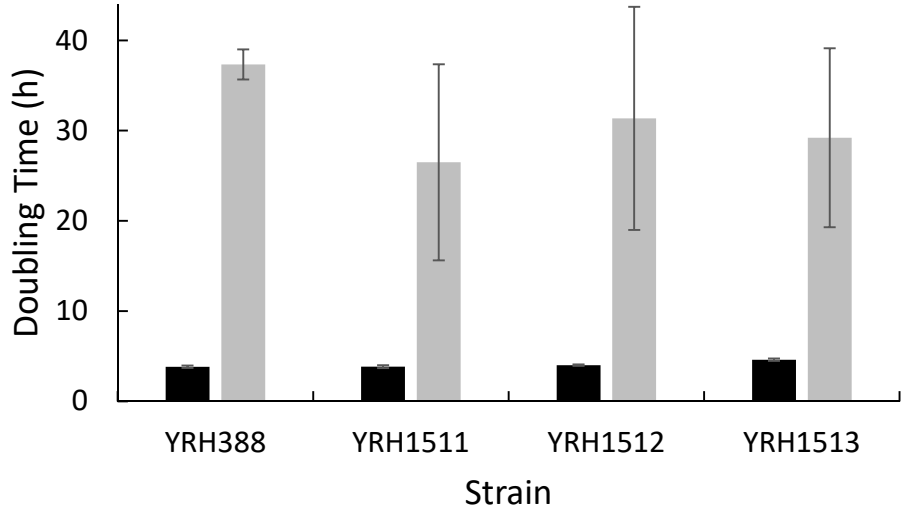


Figure 4. Growth of *S. cerevisiae* YRH388 and mutants in recycled switchgrass hydrolysate. Black bars: early growth stage; gray bars: late growth stage. Error bars indicate standard deviation for one experiment carried out with four replicates. After hydrolysate was recycled four times (see text), the hydrolysate contained 0.008% (*w*/*v*) glucose and 0.66% (*w*/*v*) xylose. Inhibitors were 0.08 M HMF and 0.09 mM furfural. 1 g/L yeast extract and 2 g/L peptone were added prior to this growth assay.

Next, we sought to use the inhibitor-sensitive phenotype of YRH1511 for growth in recycled corn stover hydrolysate. In these experiments, biomass hydrolysate was initially bioabated to remove inhibitors, then subjected to ethanol fermentation. Upon completion, the ethanol was stripped by evaporation and the liquor was mixed with 50% of the original bioabated hydrolysate and inoculated again for fermentation. Cycles of fermentation, ethanol removal, and addition of hydrolysate were repeated a total of four times, until the fermentation failed to progress after inoculation (described in [20]). The resulting "failed" hydrolysate was then finally subjected to inhibitor mitigation by bioabatement using *C. ligniaria*.

Subsequent experiments focused on growth of YRH388 and YRH1511 using recycled corn stover liquor (i.e., hydrolysate that had been recycled and then treated by bioabatement). Because the recycled medium was partially depleted of monosaccharides, either 0.2% (*w*/*v*) glucose or 1% (*w*/*v*) xylose was added to determine the ability of the two *S. cerevisiae* strains to grow (Figure 5). Growth was similar for YRH388 and YRH1511 and similar for hydrolysate that was bioabated. In the recycled liquor, without mitigation of inhibitors, YRH388 and YRH1511 did not grow (not shown). After inhibitor abatement, the growth rate of YRH1511 in four-times recycled process water (Table 5) was not reduced compared to its growth in fresh bioabated hydrolysate as reported in Table 3. The growth of YRH1511 at rates at least equal to YRH388 indicates that redox stress in recycled process water for fermentation can be mitigated by bioabatement for the pentose phosphate pathway *ZWF1* mutant YRH1511.

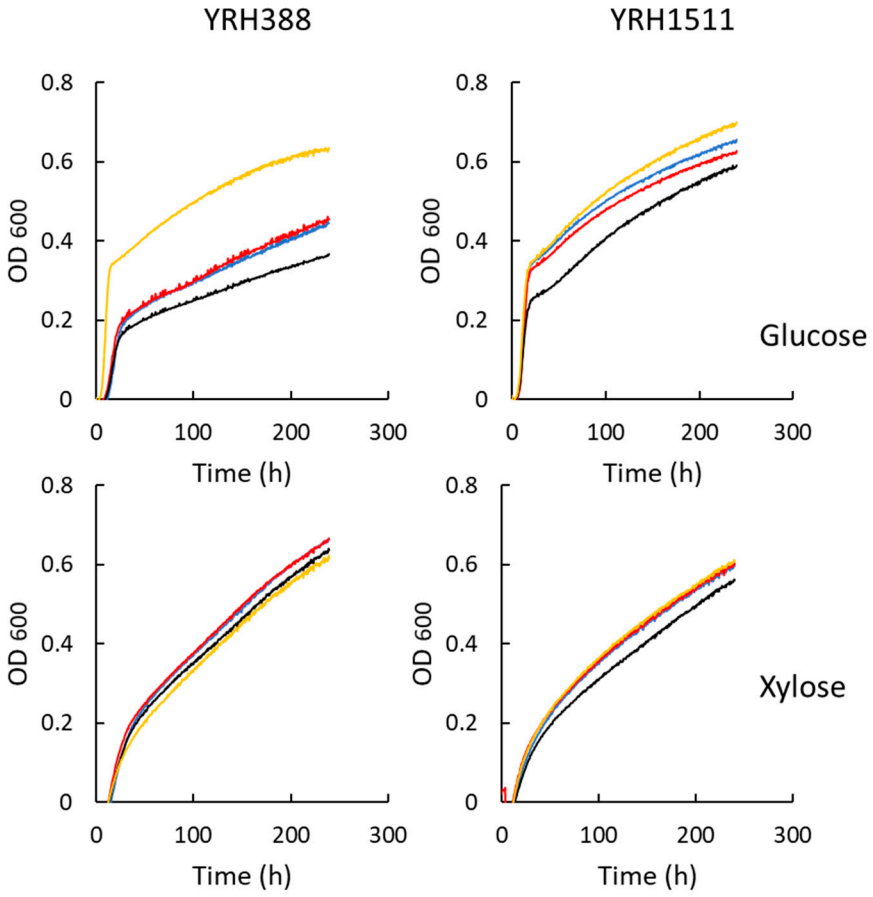


Figure 5. Growth of YRH388 and PPP mutant YRH1511 in recycled corn stover dilute-acid hydrolysate supplemented with glucose (top) or xylose (bottom). Due to carryover of unconsumed xylose in recycled hydrolysate, the hydrolysate shown in the top panels contained 0.20% (*w*/*v*) xylose along with 0.14% (*w*/*v*) added glucose. The bottom panels had no residual glucose and 0.52% added xylose. Each panel shows four replicates (shown as individual lines of various colors) for the indicated strain and hydrolysate in microwell growth assays.

Table 5. Growth of *S. cerevisiae* YRH 388 and YRH1511 in recycled corn stover hydrolysate.

		Doubling Time (h)		Maximum OD	
***S. cerevisiae* Strain**	**Phase of Growth**	**Bioabated + Glucose [1]**	**Bioabated + Xylose [2]**	**Bioabated + Glucose**	**Bioabated + Xylose**
YRH388	Early	1.1 ± 0.2	1.4 ± 0.1		
YRH1511	Early	1.6 ± 0.83	4.1 ± 3.9		
YRH388	Late	78.4 ± 37.9	62.8 ± 10.1	0.50 ± 0.11	0.75 ± 0.03
YRH1511	Late	94.3 ± 24.2	47.6 ± 17.4	0.67 ± 0.05	0.70 ± 0.02

[1] Sugar concentrations after addition of glucose (% *w*/*v*): 0.27% glucose, 0.38% xylose, 0.03% galactose, 0.13% arabinose; [2] Sugar concentrations after addition of xylose (% *w*/*v*): 0.00% glucose, 0.92% xylose, 0.05% galactose, 0.16% arabinose.

4. Conclusions

Biological conditioning of biomass fermentation liquor using *C. ligniaria* NRRL 30616 decreased inhibitors and allowed reuse of a portion of the fermentation process water. Improved understanding of yeast response to stress is important for enhancing fungal fermentations [38]. In summary, the pentose phosphate pathway mutant *S. cerevisiae* YRH1511, which is sensitive to the presence of fermentation inhibitors, could be rescued for growth by bioabatement of hydrolysate. Xylose conversion is more

sensitive to inhibition and the growth rate in bioabated hydrolysate was intermediate between that of the parent strain YRH388 and the mutant without bioabatement. In recycled process water, inhibitor abatement restored growth and there was little difference between YRH388 and YRH1511. Inhibition occurs in recycled media, as indicated by the eventual failure of fermentations after four or five cycles of re-use, but the inhibition could be remediated by inhibitor abatement and no increase in sensitivity of the *ZWF1* mutant YRH1511 was then observed compared to the parent strain YRH388. The *HAL4* mutant YRH1512 and *GPD1* mutant YRH1513 showed growth similar to that of YRH388. This study thus shows that inhibition is not due to salinity or osmolarity effects. Residual furans or some other cause of oxidative stress is experienced by the *ZWF1* (pentose phosphate pathway) mutant, and the inhibition can be remediated by bioabatement, Xylose metabolism is profoundly sensitive to disruption in this mutant and so the *ZWF1* mutant, xylose-metabolizing strain in particular may be viewed as a reporter of the fermentability of recycled process water.

Author Contributions: Conceptualization, N.N.N., R.E.H., J.A.M.; Methodology, N.N.N., S.E.F., R.E.H.; Data Analysis, N.N.N., R.E.H., J.A.M., S.E.F.; Writing—Original Draft Preparation, N.N.N.; Writing—Review & Editing, N.N.N., R.E.H., J.A.M., S.E.F. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Acknowledgments: This research was supported by the U.S. Department of Agriculture, Agricultural Research Service. We thank Katie Card for technical assistance and Ryan Hoefling (ICM, Inc.) for providing switchgrass hydrolysate.

Conflicts of Interest: The authors declare no conflicts.

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