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Bioabatement to Remove Inhibitors from Biomass-Derived Sugar Hydrolysates

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Abstract

Bioabatement is a potential method to remove inhibitory compounds from lignocellulose hydrolysates that could be incorporated into a scheme for fermentation of ethanol from cellulose. *Coniochaeta ligniaria* NRRL30616, an Ascomycete that metabolizes furfural and 5-hydroxymethylfurfural, is a unique strain that may be useful for detoxifying biomass sugars. NRRL30616 and 23 related fungal strains were screened for the ability to metabolize furans and grow in dilute-acid hydrolysate of corn stover. NRRL30616 was the best strain for removal of inhibitors from hydrolysate, and abatement of hydrolysate by inoculation with the strain allowed subsequent yeast fermentation of cellulose to ethanol.

Index Entries: Biomass; lignocellulose; inhibitor abatement; bioremediation; fermentation; detoxification; ethanol.

Introduction

The use of agricultural residues as feedstock for production of value-added products requires one or more pretreatment steps in order to release free sugar monomers from the lignocellulose matrix. The dilute-acid pretreatment method efficiently hydrolyzes hemicellulose to arabinose, xylose, and glucose and enables enzymatic digestion of cellulose to glucose. However, the dilute-acid process also releases furan derivatives, phenolic compounds, and acetate, which are toxic to fermenting microorganisms and may hinder or stall fermentation *(1)*. More than 35 potentially inhibitory compounds have been identified in acid hydrolysates *(2)*.

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†Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

Biologic abatement is potentially an effective and inexpensive method for eliminating unwanted compounds from biomass hydrolysates. Bioremediation is used for environmental cleanup *(3,4)* and wastewater treatment *(5–7)*, but has not been developed for biomass inhibitor abatement. Instead, various physical and chemical methods are used to reduce the toxicity of dilute-acid hydrolysates *(8)*. Earlier, we described an enrichment scheme used to isolate suitable organisms for bioabatement of hydrolysate *(9)*. From that screen, *Coniochaeta ligniaria* NRRL30616 was identified as the best candidate for inhibitor abatement based on substrate range and inhibitor chemical tolerance. *C. ligniaria* NRRL30616, a member of the *Coniochaeta/Lecythophora* family of Ascomycetes *(10,11)*, was enriched from a soil sample and observed to tolerate the toxic milieu of corn stover hydrolysate. Strain NRRL30616 metabolizes furfural, 5-hydroxymethylfurfural (HMF), and ferulic acid as sources of carbon and energy. Here, *C. ligniaria* NRRL30616 and related fungal strains were compared for the ability to grow on furfural, HMF, and other compounds as carbon sources, and for their potential for bioabatement of biomass-derived sugar streams. A yeast simultaneous saccharification and fermentation (SSF) scheme was developed that incorporates the use of *C. ligniaria* for inhibitor abatement.

Materials and Methods

Strains and Growth Conditions

Saccharomyces sp. LNH-ST, obtained from Nancy Ho (Purdue University, West Lafayette, IN), is a recombinant yeast that has genes for xylose metabolism integrated on its chromosome *(12)*. *C. ligniaria* NRRL30616 was isolated from furfural-contaminated soil *(9)*. Table 1 provides information regarding fungal strains related to NRRL30616.

Saccharomyces sp. LNH-ST was grown at 30°C in liquid YP medium (10 g of yeast extract/L and 20 g of peptone/L) containing 2% glucose. Solid YP medium contained 2% agar. Other fungal strains were propagated at 30°C in the same YP-glucose medium or in defined mineral medium, which contained 12.5 m*M* each of Na_2HPO_4 and KH_2PO_4, 0.1% (w/v) $(NH_4)_2SO_4$, and 1 mL/L of Hutner's mineral solution *(13)*. Solid mineral medium contained 1.5% Noble agar (Becton Dickinson, Sparks, MD), which was sterilized separately in water and added to the buffer and mineral solution. *C. ligniaria* NRRL30616 was routinely maintained on solid defined mineral medium containing a mixture of 5 m*M* furfural, 4 m*M* HMF, and 3 m*M* ferulic acid. Furfural and HMF were added to media from stock solutions prepared in methanol. Other carbon sources were dissolved in water and filter sterilized before adding to media. Glucose and glycerol were added to defined mineral medium at a final concentration of 0.2%, and other carbon sources were provided at 10 m*M* except where stated otherwise. Generation times were determined for NRRL30616 grown on

Table 1
Fungal Strains Studied

Organism[a]	NRRL	Other ID	Original substrate
Coniochaeta ligniaria	30616	C8[b]	Industrially contaminated soil
Lecythophora hoffmannii	31961	DSM2693[c]	Treated pine stake
Lecythophora mutabilis	31962	DSM10716[c]	Test of wood preservatives
Lecythophora lignicola	31963	DSM63551[c]	Soil
Coniochaeta ligniaria	32068	95.605[d]	Windblown pine branch
Coniochaeta ligniaria	32069	98.1105[d]	Windblown pine branch
Coniochaeta ligniaria	32070	98.1126[d]	Windblown pine branch
Coniochaeta ligniaria	32071	F3331[d]	Windblown pine branch
Coniochaeta ligniaria	32072	F3343[d]	Windblown pine branch
Phialophora decumbens	32073	CBS153.42[e]	Strawberry
Phialophora fasciculatus	32074	CBS205.38[e]	Butter
Lecythophora hoffmannii	32075	CBS245.38[e]	Butter
Lecythophora hoffmannii	32076	CBS140.41[e]	Sewage water
Lecythophora lignicola	32077	CBS267.33[e]	Unknown
Phialophora luteoviridis	32079	CBS206.38[e]	Butter
Lecythophora mutabilis	32080	CBS157.44[e]	River water
Lecythophora mutabilis	32081	CBS303.62[e]	Soil
Coniochaeta ligniaria	32082	CBS620.69[e]	Wood
Coniochaeta ligniaria	32083	CBS178.75[e]	Decaying cherry bark
Coniochaeta malacotricha	32084	CBS323.72[e]	Pine log
Coniochaeta subcorticalis	32085	CBS551.75[e]	Pine wood
Coniochaeta velutina	32086	CBS176.59[e]	Humic soil
Coniochaeta velutina	32087	CBS948.72[e]	Decaying wood
Coniochaeta velutina	32088	CBS981.68[e]	Waste stabilization pond

[a]*Lecythophora* and *Coniochaeta* are the names for the asexual and sexual states, respectively, of the genus. *Phialophora* is a synonym for *Lecythophora*.
[b]Lopez et al. *(9)*.
[c]Deutsche Sammlung von Mikroorganismen und Zellkulturen.
[d]Weber et al. *(11)*.
[e]Centraalbureau voor Schimmelcultures.

2% glucose and 10 m*M* furfural in three experiments, each performed in duplicate.

Preparation of Corn Stover Hydrolysate

Ground corn stover was suspended in 0.7% sulfuric acid at a 10% (w/w) loading in a 2-in. Schedule 80316 stainless steel pipe reactor with threaded end caps. The mixture was heated to 180°C, incubated for 10 min in a fluidized heating bath, and quickly cooled in a water bath. Solids were removed by centrifuging for 20 min at 15,000*g*, washed with a 10% vol of sterile water, and stored at –20°C. Wash liquid was combined with the original supernatant. The pH was adjusted with $Ca(OH)_2$

to 6.5 except where noted otherwise, and the hydrolysate was sterilized by filtration.

Bioabatement of Hydrolysate to Remove Inhibitory Compounds

Corn stover hydrolysate was supplemented with 0.1% $(NH_4)_2SO_4$ as a nitrogen source and inoculated with the cell pellet from a 10% vol of an overnight YP-glucose culture of *C. ligniaria* NRRL30616 or a related strain from Table 1. Cultures were incubated with shaking at 30°C and sampled periodically for analysis of furfural and HMF content.

Simultaneous Saccharification and Fermentation

Hydrolysate that had undergone bioabatement with *C. ligniaria* NRRL30616 was remixed with washed solids, so that the original liquid:solid ratio was restored. Then, SSF was initiated without removal of *C. ligniaria* cells from the hydrolysate. A cell pellet of *Saccharomyces* sp. LNH-ST was collected by centrifugation from an overnight culture grown on YP-glucose medium and suspended in each flask of hydrolysate, to achieve a 10% (v/v) inoculum. To catalyze saccharification of cellulose, cellulase (15 filter paper units/g of cellulose) was added as a 1:1 mixture of cellulase and β-glucosidase (Novozyme Celluclast 1.1 and Novozyme 188, respectively) at the time of inoculation with the fermenting yeast strain LNH-ST. Fermentations (10 mL) were carried out in 20-mL Erlenmeyer flasks that were each capped with a rubber stopper and vented with a needle. Cultures were incubated at 30°C with gentle mixing and sampled periodically for measurement of sugar and ethanol content.

Analytical Methods

Optical densities (ODs) (550 nm, 1-cm path length) were determined using a Beckman DU640 spectrophotometer (Beckman Coulter, Fullerton, CA). Sugar, acetate, and ethanol concentrations were determined using a high-performance liquid chromatography (HPLC) system equipped with refractive index detection *(14)*. Furfural and HMF were quantitated using reverse-phase HPLC with ultraviolet detection at 277 nm *(9)*.

Results

Characterization of Related Strains

Twenty-three strains related to soil isolate NRRL30616 (Table 1) were screened for their ability to metabolize furfural, HMF, and other inhibitory compounds (Table 2). Strains tested included seven additional *C. ligniaria* isolates and three other *Coniochaeta* species, as well as three species each of *Lecythophora* and *Phialophora*, names that designate the asexual state of *Coniochaeta*. Nine strains grew in mineral medium with furfural or HMF or both as sole sources of carbon and energy, and seven of these also grew on

Table 2
Growth of *Coniochaeta/Lecythophora* Strains on Selected Compounds[a]

	Carbon source[b]			
NRRL strain	Furfural	HMF	Levulinic acid	*p*-Hydroxybenzaldehyde
30616	0.605	0.090	—	0.081
31961	0.560	0.148	—	0.086
32068	0.278	—	0.308	—
32069	0.577	0.166	0.145	0.063
32070	0.640	0.101	0.073	0.196
32071	0.005	0.104	ND	0.136
32072	0.723	0.214	0.148	0.073
32081	—	—	ND	0.090
32083	0.186	0.071	0.088	—

[a]Growth in defined mineral medium containing each carbon source was determined by measuring OD (550 nm) after 6 d of incubation at 30°C in a shaking incubator.

[b]—, Final densities of 0.05 or lower were considered as no growth; ND, not done. Strains chosen for further study (Fig. 1) are in boldface. All of the strains from Table 1 grew on ferulic acid, acetate, glucose, glycerol, 4-hydroxybenzoate, catechol, gallic acid, syringaldehyde, coniferyl alcohol, vanillin, and vanillic acid. NRRL strains 31962, 31963, 32073, 32074, 32075, 32076, 32077, 32079, 32080, 32081, 32082, 32084, 32085, 32086, 32087, and 32088 did not grow on furfural, HMF, and *p*-hydroxybenzaldehyde.

levulinic acid or *p*-hydroxybenzaldehyde. The strains from Table 2 that could metabolize furfural were tested for their ability to abate furfural and HMF in hydrolysate (Fig. 1). A subset of those that metabolize furfural and HMF were able to grow and remove inhibitors from hydrolysate, although none performed as well as NRRL30616. As shown in Fig. 1A, B, NRRL30616 was the only strain that completely consumed furfural and HMF from corn stover hydrolysate. Four additional strains abated the inhibitors in hydrolysate that was diluted to three-quarter strength (0.75X; Fig. 1C,D), and all eight strains in Fig. 1 metabolized the furfural and HMF in 0.5X hydrolysate (not shown).

Physiology of Furfural and HMF Removal by C. ligniaria NRRL30616

C. ligniaria NRRL30616 was previously shown to utilize furfural and HMF as sole sources of carbon and energy *(9)*. The generation time of NRRL30616 grown on 10 m*M* furfural as the carbon source in mineral medium is 10.0 ± 0.9 h. By comparison, the strain's generation time on the same basal medium supplemented with glucose is 2.7 ± 0.2 h. In light of this comparison, we sought to determine whether the presence of additional nutrients would promote or hinder consumption of inhibitors. Figure 2 presents the results of strain NRRL30616 grown in mineral medium containing a mixture of furfural (15 m*M*), HMF (5 m*M*), and ferulic acid (2.5 m*M*). Inhibitors were consumed more quickly when glucose was included to the media. Furfural was consumed within 30 h when 0.1% glucose (Fig. 2) or glucose plus yeast extract (not shown) was included in the medium,

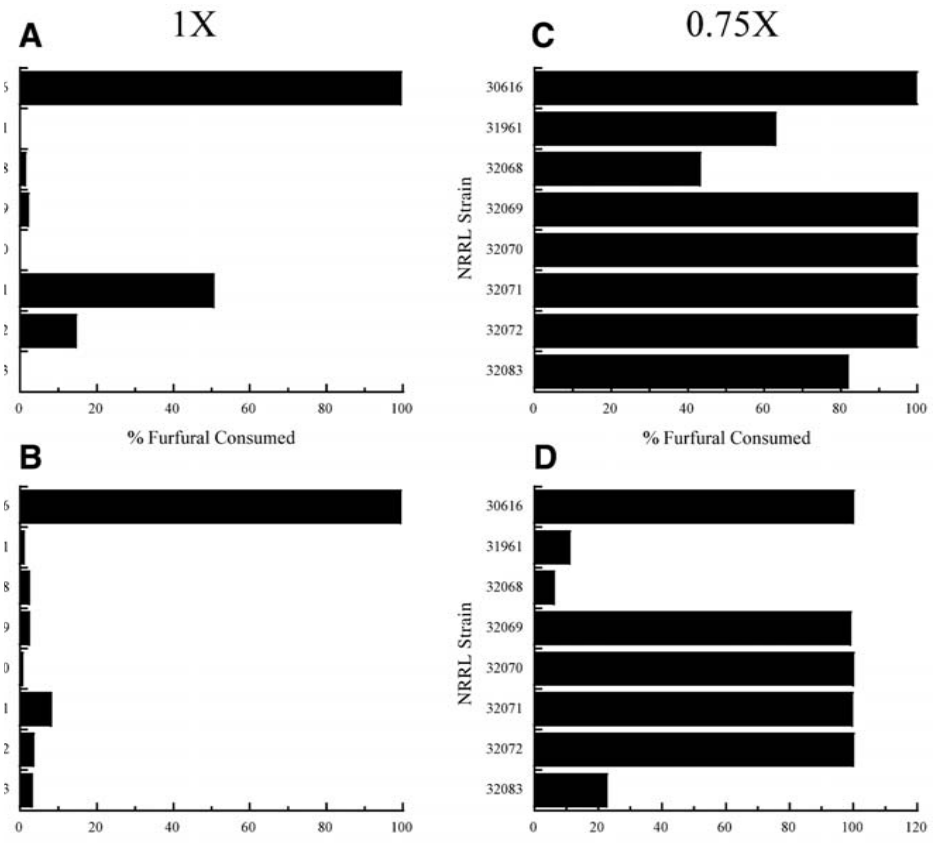


Fig. 1. Comparison of inhibitor consumption by *Coniochaeta* strains. Furfural and HMF consumed is shown for **(A, B)** 1X and **(C, D)** 0.75X corn stover hydrolysate. Results are shown as a percentage of the values obtained for untreated hydrolysate, which contained (in 1X hydrolysate) 16.8 m*M* furfural and 3.4 m*M* HMF initially and 6.1 m*M* furfural and 3.2 m*M* HMF after incubation for 7 d at 30°C.

compared with 48 h when only yeast extract was added (Fig. 2) and approx 60 h (*9*) when no additional nutrients were added to the medium.

Next, NRRL30616 was grown in rich YP medium containing 7.5 m*M* furfural, 0.5% glucose, or both. In this experiment, furfural was consumed before metabolism of glucose commenced. Utilization of furfural began immediately and occurred at the same rate whether glucose was present (Fig. 3) or absent (not shown) in the YP medium. Utilization of glucose was similarly deferred when *C. ligniaria* was grown in corn stover hydrolysate (Table 3): HMF and furfural were metabolized prior to consumption of glucose.

The influence of pH on inhibitor abatement was also evaluated. The pH of batches of corn stover hydrolysate was adjusted between 5.0 and 7.0, and the batches were inoculated with NRRL30616. Bioabatement was strongly influenced by pH and was optimal at near-neutral pH. Furfural

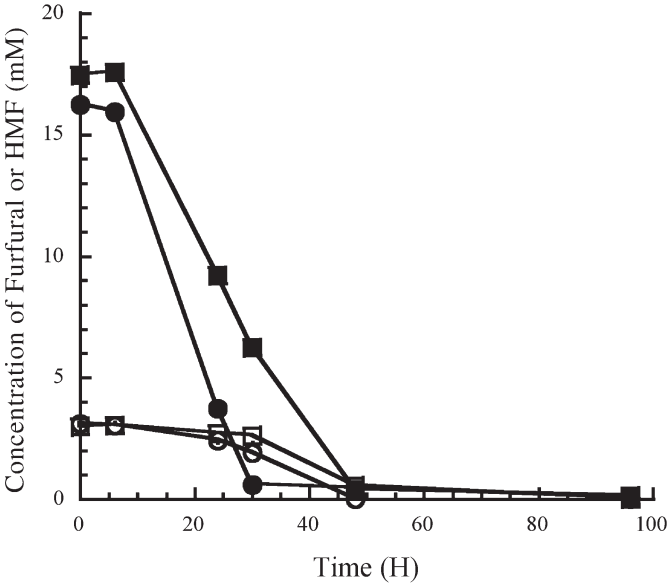


Fig. 2. Depletion of furfural (solid symbols) and HMF (open symbols) by *C. ligniaria* NRRL30616 grown in mineral medium also containing (●) 0.1% glucose or (■).0.1% yeast extract.

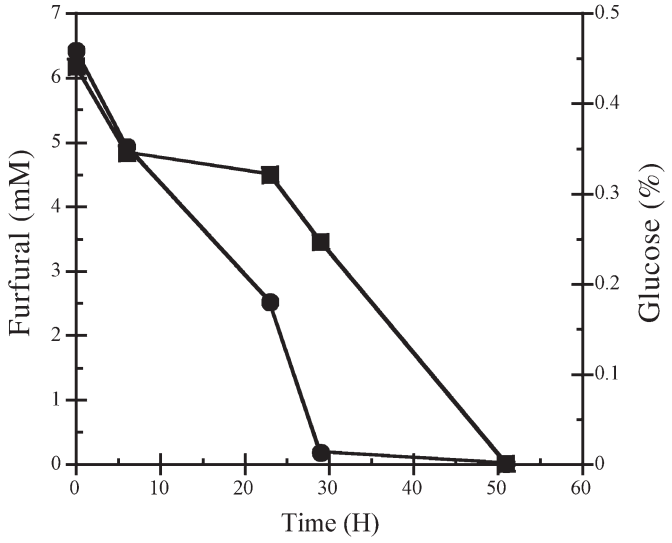


Fig. 3. Metabolism of furfural (●) by *C. ligniaria* NRRL30616 in presence of glucose (■). YP medium contained 7.5 m*M* furfural and 0.5% glucose.

and HMF were consumed from hydrolysate at pH 6.5 and 7.0, but not at pH 6.0 or lower. In defined mineral medium, NRRL30616 could grow on glucose, but not furfural, as the sole carbon and energy source when the pH of the medium was adjusted to 5.7 (not shown).

Table 3
Concentration of Inhibitors and Sugars in Corn Stover Hydrolysate Before and After Bioabatement with *C. ligniaria* NRRL30616[a]

Time (h)	Furfural (m*M*)	HMF (m*M*)	Acetate (%)	Glucose (%)	Xylose (%)	Arabinose (%)
0	13.1	2.4	0.26	0.35	1.81	0.27
17	0.02	0.38	0.20	0.19	1.47	0.23

[a]Hydrolysate was sampled at the time of inoculation with *C. ligniaria* and after 17 h of incubation.

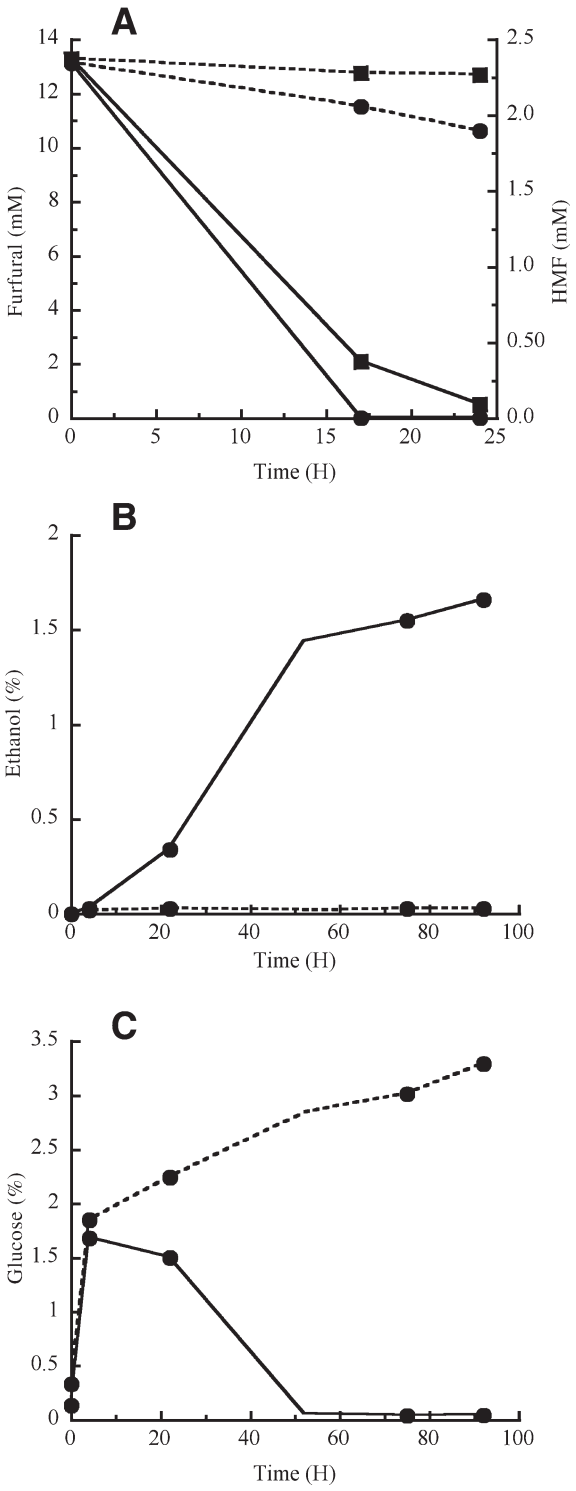
Integration of Bioabatement with Yeast SSF

Judging isolate NRRL30616 to be the most promising strain for inhibitor removal, we sought to incorporate bioabatement into a model scheme for production of ethanol from lignocellulosic biomass (Fig. 4). Corn stover was pretreated with dilute acid, the solids were removed and washed, and the wash liquid was added to the hydrolysate. Neutralized hydrolysate was treated with NRRL30616 and recombined with the solids. The abated complete hydrolysate was fermented with *Saccharomyces* in the presence of cellulase enzymes. Bioabatement with NRRL30616 allowed subsequent fermentation of cellulose to ethanol (Fig. 4). SSF of treated hydrolysate yielded 1.66% ethanol within 80 h. By contrast, no ethanol was obtained in fermentations of untreated hydrolysate.

Discussion

Additional value could be obtained from agricultural biomass if lignocellulose were efficiently converted by microbial processes into products such as fuel ethanol *(15)*. One factor constraining the use of lignocellulosic sugars is the severe inhibitory effect of furans, phenolics, and organic acids on fermenting microorganisms *(16,17)*. We developed a fermentation scheme for converting corn stover into ethanol that integrates the fungal strain *C. ligniaria* NRRL30616 as a biologic means of inhibitor abatement. A biologic method would potentially have a relatively low cost, generate little or no chemical waste, and allow decreased water use if the wash liquid were added to the fermentation. In addition to the fungus described here, Schneider *(18)* proposed the use of a mutant yeast to remove acetic acid from hemicellulose hydrolysates. Treatment of hydrolysates with laccase and peroxidase enzymes has also been described *(19)*.

Furfural and HMF are two of the most problematic compounds in acid hydrolysates, because of their high toxicity and ubiquitous presence *(20,21)*. Consequently, any method for abatement of lignocellulosic inhibitors must effectively remove these two furans. *C. ligniaria* NRRL30616 was isolated from furfural-contaminated soil by enrichment in mineral medium containing


A
Furfural (mM)
HMF (mM)
Time (H)
B
Ethanol (%)
Time (H)
C
Glucose (%)
Time (H)

a mixture of furfural, HMF, and ferulic acid as carbon sources, followed by selection in sterilized, pH-neutralized corn stover hydrolysate *(9)*. Accordingly, the strain metabolizes both furfural and HMF. As shown in Table 2, strain NRRL30616 and a group of related strains also metabolize a number of other compounds; thus, it is possible that the bioabatement strategy employed here results in removal of additional inhibitory substances, such as aromatic molecules derived from lignin *(22)*.

The glucose present in corn stover hydrolysate was not completely consumed during bioabatement (Fig. 2, Table 3), even though NRRL30616 grows faster on glucose than on furfural when each is supplied as the sole carbon source. Therefore, it is likely that the ordered consumption of furfural before glucose reflects a cellular mechanism for dealing with a toxic compound, rather than a true preference for furfural as a growth substrate. Corn stover hydrolysate contains only a small amount of glucose, compared to the pentose content. However, the use of *C. ligniaria* for remediation of inhibitors from biomass sugars may warrant the use of a mutant that is blocked for metabolism of glucose, in order to reserve all of the hydrolysate sugars for fermentation to ethanol.

Treatment with *C. ligniaria* NRRL30616, the best strain identified here (Fig. 1), showed that bioabatement may be an efficient method for removal of inhibitors from corn stover hydrolysate (Fig. 4). Hydrolysate that had been conditioned by the growth of *C. ligniaria* supported fermentation of ethanol from cellulose, whereas no ethanol was produced from untreated hydrolysate. In addition, 35% of the xylose present in the treated hydrolysate was depleted during the fermentation (data not shown). It should be noted that *Saccharomyces* sp. LNH-ST is a recombinant strain, engineered to metabolize xylose *(12)*.

Additional strains related to NRRL30616 were evaluated for their ability to metabolize furans and other inhibitory compounds (Table 2), and for use in bioabatement (Fig. 1). The strains could be separated into two classes, based on their ability to metabolize four compounds: furfural, HMF, levulinic acid, and *p*-hydroxybenzaldehyde. A subset of strains including NRRL30616 was distinguished from other candidate strains by the ability to both metabolize those inhibitory compounds (Table 2) and abate hydrolysate (Fig. 1). The best strain was NRRL30616, followed by its closest relatives (phylogenetic analysis not shown). In fact, five of the *C. ligniaria* strains in Fig. 1 are identical to NRRL30616, to the extent that their large subunit ribosomal RNA genes have been sequenced *(9,11)*.

Fig. 4. *(Fig. 4 on previous page)* SSF of corn stover hydrolysate after bioabatement with *C. ligniaria* NRRL30616. Solid lines represent the results for *C. ligniaria*–treated hydrolysate, and dashed lines represent results for the negative control (hydrolysate that was not abated with *C. ligniaria* prior to SSF). **(A)** Removal of (●) furfural and (■) HMF from corn stover hydrolysate prior to SSF; **(B)** successful ethanol fermentation by *Saccharomyces* sp. LNH-ST in treated but not untreated hydrolysate; **(C)** accumulation of glucose from cellulose in treated and untreated hydrolysate.

Thus, the superior performance of *C. ligniaria* NRRL30616 is likely a result of a combination of the innate metabolic capacity of the species and an adaptive tolerance for inhibitors.

The results described here for bioabatement of corn stover hydrolysate compare favorably with overliming, a widely applied method for detoxification in which calcium hydroxide is used after dilute-acid pretreatment to adjust the hydrolysate to pH 10.0. The mixture is heated, with sulfite also sometimes added, resulting in a hydrolysate with reduced furan and phenolic levels and improved fermentability *(8,23,24)*. Similarly, bioabatement yielded a detoxified (fermentable) corn stover hydrolysate. The furans present in hydrolysate were completely removed, but the effect of bioabatement on other inhibitory compounds including phenolic compounds has not yet been determined. Further work will be required to determine the utility of bioabatement with hydrolysates obtained from a variety of hemicellulosic substrates.

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