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Effect of Furfural, Vanillin and Syringaldehyde on *Candida guilliermondii* Growth and Xylitol Biosynthesis

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Abstract Xylitol is a five-carbon sugar alcohol with established commercial use as an alternative sweetener and can be produced from hemicellulose hydrolysate. However, there are difficulties with microbiological growth and xylitol biosynthesis on hydrolysate because of the inhibitors formed from hydrolysis of hemicellulose. This research focused on the effect of furfural, vanillin, and syringaldehyde on growth of *Candida guilliermondii* and xylitol accumulation from xylose in a semi-synthetic medium in microwell plate and bioreactor cultivations. All three compounds reduced specific growth rate, increased lag time, and reduced xylitol production rate. In general, increasing concentration of inhibitor increased the severity of inhibition, except in the case of 0.5 g vanillin per liter, which resulted in a faster late batch phase growth rate and increased biomass yield. At concentrations of 1 g/l or higher, furfural was the least inhibitory to growth, followed by syringaldehyde. Vanillin most severely reduced specific growth rate. All three inhibitors reduced xylitol production rate approximately to the same degree.

Keywords Xylitol · Inhibitor · Furfural · Vanillin · Syringaldehyde · Toxicity · Hemicellulose hydrolysate

Introduction

Lignocellulose, consisting of lignin, hemicellulose, and cellulose, is the major structural component of woody and non-woody plants and represents a major source of renewable

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organic matter. Large amounts of lignocellulosic by-products are generated through forestry and agricultural practices, paper-pulp industries, timber industries, and many agroindustries that can potentially be converted into various different value added products including biofuels, chemicals, and inexpensive energy sources for fermentation and improved animal feeds. Lignocellulosic raw materials are inexpensive and abundant, and hydrolysis of hemicellulose, a component of lignocellulose, for microbiological feedstock is currently an important and active area of research. However, there are many difficulties with microbiological growth on hydrolysate because of the inhibitors formed from hydrolysis of hemicellulose.

D-Xylose is one of the primary sugars derived from the hydrolysis of hemicellulose. One of the organisms that most efficiently produces the sweetener xylitol is *Candida guilliermondii* [1], which is used in this study. Xylitol is a five-carbon sugar alcohol with established commercial use as an alternative sweetener and has possible uses in adhesives. The unique properties of xylitol include its being an insulin-independent sweetener and its being not utilized by acid producing oral cavity bacteria, thereby reducing dental caries. Xylitol is currently produced on an industrial scale by the chemical reduction of xylose. The xylose is obtained by hydrolysis of wood [2]. However, the cost of purification of xylose from the hydrolysate makes xylitol one of the more expensive sweeteners. Xylitol is used as a sweetener in food products such as chewing gum, candy, soft drinks, and personal health products such as mouth wash and toothpaste. A process to more economically produce xylitol would result in increased xylitol use in the confectionary industry.

C. guilliermondii produces the enzyme D-xylose reductase which catalyses a reaction where the proton carrier NADPH donates a hydrogen atom to D-xylose, and D-xylose is converted to xylitol as seen in Fig. 1. The xylitol can then be converted to D-xylulose, catalyzed by xylitol dehydrogenase, which is utilized in central metabolism [3]. Under semi-aerobic conditions, xylitol accumulation is favored compared to anaerobic or aerobic conditions. Under anaerobic conditions, the ratio of NAD(P)H to $NAD(P)^+$ is low, and NAD(P)H is required for xylitol production. Under aerobic conditions, excess oxygen allows oxidation of NADH to NAD^+, and a resulting high NAD^+/NADH ratio results in a faster xylitol conversion rate to D-xylulose, eliminating the accumulation of xylitol [4, 5].

Many factors impact the xylose to xylitol fermentation process, including oxygen delivery rate and concentration, pH, temperature, and the presence of other sugars (in addition to xylose), nutrients, and inhibitors [4, 6]. The least studied of these factors is the effect of specific inhibitors derived from biomass pretreatment on the growth of xylitol-

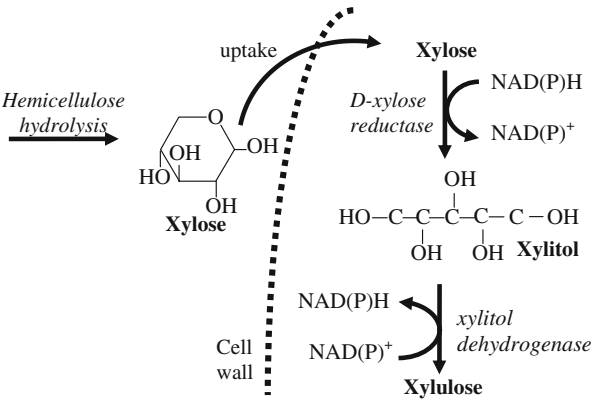


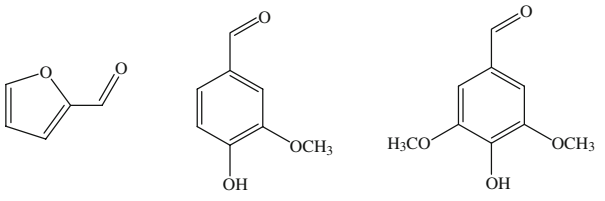
Fig. 1 Xylitol biosynthesis [3–5]

producing yeast and the effect on xylitol production. Xylose can be obtained from hydrolysis of hemicellulose after pretreatment of the lignocellulosic biomass via dilute acid hydrolysis or steam explosion. Under the high temperature and pressure conditions employed, a range of compounds are produced that are inhibitory to yeasts. Hydrolysate inhibitors include weak acids, furan derivatives, and phenolic compounds. In this study, the effects of furfural, vanillin, and syringaldehyde were investigated. Furfural is a degradation product of xylose. Vanillin and syringaldehyde are derived from the lignin fraction of lignocellulose. The chemical structures of these compounds are indicated in Fig. 2. Furfural, vanillin, and syringaldehyde can be assimilated or metabolized by the yeast *Saccharomyces cerevisiae* [7].

The xylitol production rate is highest at very high xylose concentrations (80 g/l) [8, 10]. However, concentrating the hemicellulose hydrolysates using vacuum evaporation to achieve high xylose concentrations also concentrates the non-volatile inhibitors [11]. At these inhibitor concentrations, volumetric productivity actually declines [12–14]. Many studies have demonstrated the inhibitory effect of these hydrolysis-derived compounds on growth and production of products, such as ethanol and xylitol [15–16]. Concentrated hydrolysate requires detoxification for optimum xylitol production.

Efforts to decrease the effects of inhibitors on yeast fermentations include decreasing the temperature, pressure, and residence time during dilute acid hydrolysis to minimize inhibitor formation, although decreases in xylose yield also result [17]. A number of methods have also been developed for concentrating and pretreating the hydrolysate to increase the xylose concentration and reduce the concentration of inhibitors, including precipitation by overliming, adsorption on activated carbon, removal with ion-exchange resins, and vacuum evaporation. A third approach is to adapt the yeast to the inhibitors by sequential culture and selection with increasingly concentrated hydrolysate and inhibitors [18–21].

The identity and effects of hemicellulose hydrolysis-derived inhibitors on potential ethanol and xylitol production processes have been investigated using both hemicellulose hydrolysates, followed by different detoxification processes and semi-synthetic media spiked with known concentrations of toxicants. The use of complex hemicellulose hydrolysates provides practical information on technological aspects of potential industrial designs, and when coupled with chemical analyses, also assists in identification of the major inhibitors. Semi-synthetic media can provide exposure of strains to known inhibitor concentrations and can be used to provide less ambiguous information on the microbial physiology and toxicology of specific inhibitors or combinations of inhibitors. These experiments are typically performed in shake flasks or bench-scale fermentors. In cases where it is desirable to investigate many different inhibitor concentrations and combinations microwell scale (200 μl), experiments may be useful. This approach has been employed for investigating the enzymatic hydrolysis of lignocellulosic feedstocks [22]. Although the



Furfural Vanillin Syringaldehyde

Fig. 2 Furfural, syringaldehyde, and vanillin chemical structures

scalability of the information obtained must ultimately be verified when making extrapolations to industrial processes, these approaches may prove useful in providing rapid comparison of the effects of different types and combinations of inhibitors, growth substrates, and production strains.

The yeast *C. guilliermondii* efficiently converts xylose in sugarcane bagasse to xylitol and is commonly considered for potential commercial production processes [1]. Lignin and xylose-derived degradation products are commonly found at inhibitory levels in concentrated hemicellulose hydrolysates from sugarcane bagasse [23, 16–17], although the concentrations of specific chemical species may vary widely depending on the feedstock and pretreatment conditions. The objectives of this research were to experimentally examine the effects of the inhibitors furfural, syringaldehyde, and vanillin on *C. guilliermondii* growth (low cell density) and xylitol production (high cell density) using semi-synthetic medium in microtiter plates and bench-scale fermentors, respectively. Quantitative analysis of the effects of hydrolysate inhibitors on growth and xylitol production in a semi-synthetic medium with known toxicant concentrations may contribute to further studies on the optimization of biomass pretreatment methods, thereby minimizing costs and ancillary environmental impacts.

Materials and Methods

Strain and Medium

The xylitol producing yeast *C. guilliermondii* (ATTC No. 201935) was cultivated in a semi-synthetic basal salt medium (BSM) for experiments investigating cell growth and xylitol production. This medium consisted of 40 g/l xylose (EMD Chemicals, Darmstadt, Germany) as the carbon source, 5 g/l ammonium sulfate as the nitrogen source, 0.5 g/l magnesium sulfate, 0.1 g/l calcium chloride, 1 g/l potassium sulfate, and 1 g/l yeast extract (Fisher Scientific, Rochester, NY). The medium was prepared separately for each experiment and autoclaved for 20 min at 121 °C to assure sterility.

Cultivation

Multiwell plates, shake flasks, and bioreactors were used to cultivate the *C. guilliermondii* yeast. The multiwell plates contained 96 wells each with 200-μl liquid working volume. For toxicity experiments in the multiwell plates, 0.022 g of the inhibitors vanillin, syringaldehyde (Alfa Aesar, Karlsruhe, Germany), and furfural (TCI America, Portland, OR) were each added separately to 10 ml BSM medium. The syringaldehyde solution was heated in a hot tap water bath to increase the rate of dissolution. All toxicant solutions were then filter-sterilized (0.22 μm) before preparing a series of dilutions in BSM medium. *C. guilliermondii* inoculum cultures were grown in BSM in shake flasks at 30 °C, centrifuged, and resuspended in BSM medium to an OD_{600nm} of 0.7. Multiwell plate cultivation experiments were performed by combining 180 μl BSM medium containing the appropriate concentration of inhibitor with 20 μl of the appropriate density of exponential phase *C. guilliermondii* cells in each well. Triplicates microwells were prepared and analyzed for each experimental condition. The plates were covered with plastic lids and incubated at 30 °C.

Bioreactor experiments were conducted in 3-l New Brunswick Bioflow 110 bioreactors (New Brunswick Scientific, Edison, NJ) equipped with pH, temperature,

and dissolved oxygen control. The bioreactors were autoclaved with 1 l of medium, allowed to cool, and then inoculated with 50 ml of overnight cultures of *C. guilliermondii*. The experiments consisted of two phases: batch growth and resuspension xylitol biosynthesis. During both phases, the reactor temperature was maintained at 30 °C and the pH at 6.0. The batch phase was aerated at 1.5 vvm (volume of air per volume of reactor per minute) air introduced through a sparger beneath the axial impellor, and the stirrer speed was constant at 500 rpm. At the completion of batch phase (about 30 h), the reactor contents were centrifuged and the cell pellet resuspended in new BSM medium, with or without inhibitor, and returned to the bioreactor vessel. The inhibitor was added as a dry powder (or liquid) to the BSM medium after autoclaving but before resuspending the yeast cells. The aeration rate was reduced to 0.28 vvm and introduced to the headspace (not the sparger). The agitation rate was reduced to 200 rpm. These conditions resulted in a oxygen mass transfer coefficient ($k_L a$) of ~9 h^{-1}, as measured by the gassing out method, to facilitate xylitol accumulation. Samples were taken for cell density and xylitol measurements from the bioreactor contents for about 24 h during this resuspension phase. Samples for xylitol were centrifuged and the supernatant frozen for later analysis. Individual batch fermentations were conducted for each inhibitor concentration. The inhibitor concentrations and fermentor operating conditions were chosen based on previous results using the same strain and medium [24]. Analyses were performed on triplicate samples from each bioreactor

Cell Density

Cell density was assessed using absorbance at a 600 nm wavelength ($Abs_{600\ nm}$). For the bioreactor experiments, samples were diluted to the linear range of the assay, transferred to a 1-ml cuvette, and the absorbance was measured using a Spectronic 20 Genesys spectrophotometer. In the multiwell plate experiments, the plate cover was removed, and absorbance was measured using a Perkin Elmer Victor^3 V 1420 multilabel counter. A second-order polynomial was fit (R^2=0.97) to describe the relationship between absorbance and dry mass of cells per liter (Eq. 1), and all data are reported as cell density (g/l).

$$\text{Cell Density}\left[\frac{\text{g}}{\text{L}}\right] = 3.44(\text{abs})^2 + 0.374(\text{abs}) \quad (1)$$

Xylitol

To quantify the amount of xylitol produced during each bioreactor run, the Megazyme D-sorbitol/xylitol assay kit (Wicklow, Ireland) was used. Xylitol (Calbiochem, Darmstadt, Germany) was used as a standard. In the assay, xylitol reacts with NAD^+ in the presence of sorbitol dehydrogenase to form D-xylulose and NADH. This NADH is then reacted with iodonitrotetrazolium chloride (INT) in the presence of diaphorase to form INT-formazan. The amount of INT-formazan is quantified by measuring absorbance at 492 nm. The amount of INT-formazan formed is in stoichiometric proportion to the amount of xylitol. The original assay protocol was adapted so that the assay could be performed using a microplate reader. The volumes of sample and reagents were reduced 20-fold so that the reaction mixes would fit into 96-well assay plate. In addition, sorbitol dehydrogenase was added 4 min after diaphorase because no reducing substances are present in the samples.

Results and Discussion

The effects of furfural, vanillin, and syringaldehyde as individual inhibitors and in combination on cell growth were investigated in microwell plate cultivations. At inhibitor concentrations of 1 g/l, vanillin most severely inhibited *C. guilliermondii* growth, followed by syringaldehyde. Furfural was the least inhibitory of the three compounds (Fig. 3). Each inhibitor caused a concentration-dependant decrease in growth rate, with the exception of vanillin at low concentration (Fig. 4). The initial growth rate of cells exposed to vanillin at 0.5 g/l was suppressed, but then increased dramatically after about 20 h of cultivation (Fig. 4). The biomass yield on xylose was unaffected by furfural or syringaldehyde at concentrations up to 2 g/l, whereas no growth was observed in the presence of 2 g/l vanillin. However, at 0.5 g/l vanillin, biomass yield was higher than the no inhibitor control, perhaps indicating metabolism of vanillin.

Duarte et al. [25] found that 0.5 g/l furfural decreased the specific growth rate of *Debaryomyces hansenii* CCMI 941, and at concentrations above 3.5 g/l furfural or 1.5 g/l syringaldehyde, no growth was observed. Olsson and Hahn-Hagerdal [26] reported that 1 g/l furfural resulted in a 47% inhibition of growth of *P. stipitis*, and 2 g/l furfural resulted in 99 and 90% inhibition in *P. stipitis* and *S. cerevisiae*, respectively. Syringaldehyde (0.75 g/l) and vanillin (0.5 g/l) resulted in approximately 50–88% inhibition of growth in the same strains. Generally, it is observed that log $P_{o/w}$ is a good indicator of toxicity. However, in the microtiter assays, syringaldehyde was less inhibitory to the yeast than vanillin, although it is more hydrophobic. Fitzgerald et al. [27] have shown that the toxicities of six structural analogues of vanillin are not correlated with log $P_{o/w}$, and the solubilities and availabilities of these compounds can vary between actual hemicellulose hydrolysates and defined media [7]. *S. cerevisiae* has been shown to assimilate vanillin and syringaldehyde during fermentation [28]. It is unknown whether *C. guilliermondii* has similar metabolic capabilities that may affect inhibitor toxicity.

The inhibitor compounds can affect cell growth by increasing the adaptation lag time and decreasing or altering the growth rate. The cells not exposed to inhibitor and the cells exposed to syringaldehyde exhibited a ~2-h lag time after introduction to the wells, whereas the vanillin caused a 5-h lag at all concentrations, and the furfural resulted in a concentration-dependant lag time from 0 to 15 h. Duarte et al. [25] also found a concentration-dependant lag time in response to furfural with the xylitol-producing yeast *D. hansenii*. At a furfural concentration of 2 g/l, a 13-h lag time was observed.

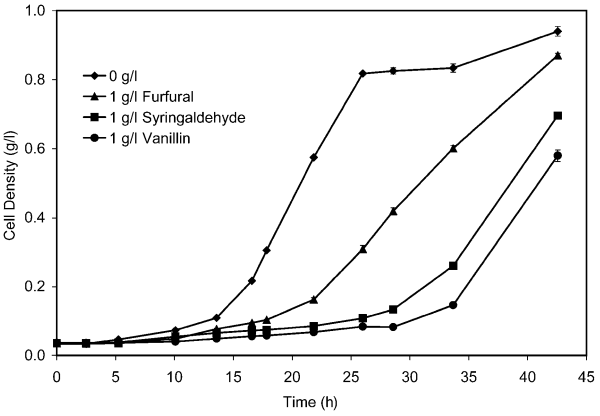


Fig. 3 Effect of inhibitor type on *C. guilliermondii* cell growth in microwell plates with a 200-µl cultivation volume and inhibitor concentrations of 1 g/l. *Filled diamond*, 0 g/l inhibitor; *filled triangle*, 1 g/l furfural; *filled square*, 1 g/l syringaldehyde; *filled circle* , 1 g/l vanillin. *Error bars* (sometimes obscured by the datum symbol) are ±standard error of the mean

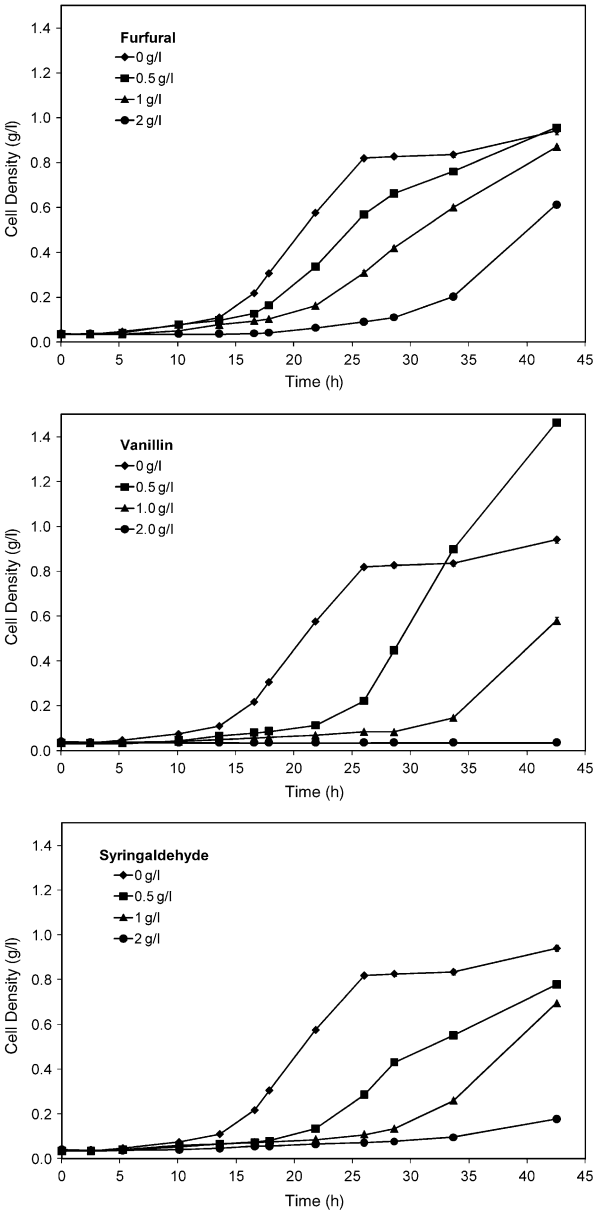


Fig. 4 Effect of inhibitor concentration on *C. guilliermondii* cell growth in microwell plates with a 200-µl cultivation volume. *Filled diamond*, 0 g/l; *filled triangle*, 0.5 g/l; *filled square*, 1.0 g/l; *filled circle*, 2.0 g/l. *Error bars* (sometimes obscured by the datum symbol) are ±standard error of the mean

The average specific growth rate of the *C. guilliermondii* cultures was determined by calculating the slope of time versus $\ln(X/X_o)$ line for each treatment, where X is cell density and X_o is initial cell density (Fig. 5). The plots for specific growth rate calculation for the 1-g/l treatments are shown in Fig. 5, and the same method was used to determine specific growth rates for inhibitor concentrations of 0.5 and 2 g/l. For the case of cultures with no inhibitor and with furfural present, *C. guilliermondii* grew with a constant growth rate. All three concentrations of furfural examined (0.5, 1 and 2 g/l) resulted in a moderately decreased specific growth rate compared to the cells not exposed to an inhibitor (Fig. 6).

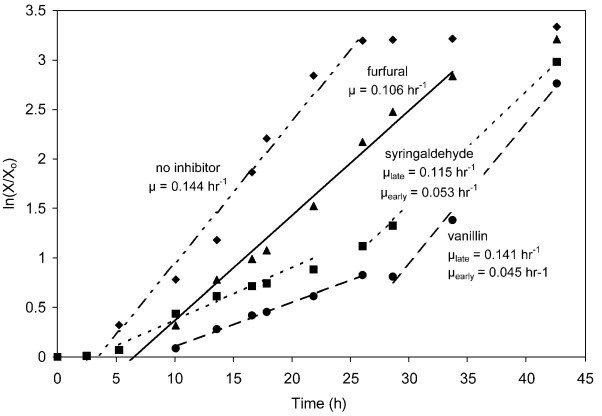


Fig. 5 Determination of specific growth rates in the presence of 1 g/l of individual inhibitors. *Lines* are linear regression, *symbols* are experimental data. Slope of the line is the specific growth rate. *Filled diamond*, no inhibitor; *filled triangle*, furfural; *filled square*, syringaldehyde; *filled circle*, vanillin

Syringaldehyde and vanillin both caused significant reductions in specific growth rate in the first 20 to 30 h of cultivation, followed by increased growth rates for late cultivation times. However, at 2 g/l vanillin, the cells did not grow in the 42 h of cultivation. Similarly, Duarte et al. [25] found no correlation between furfural concentration and specific growth rate in *D. hansenii* at concentrations above 0.5 g/l, and syringaldehyde resulted in a concentration-dependant decrease in specific growth rate in the range of 0.25 to 1.5 g/l.

In a separate multiwell plate investigation, the effects of combinations of inhibitors on *C. guilliermondii* growth were investigated. Treatments consisted of single inhibitors at 1 and 2 g/l, double inhibitor combinations of 0.5 and 1 g/l of each inhibitor, and triple inhibitor combinations of 0.33 and 0.66 g/l of each inhibitor. Figures 7 and 8 present the growth curves for the cultivations with a total of 1 and 2 g inhibitor/l, respectively. The results from this study support the single inhibitor experimental findings in that (1) the inhibition of growth was more severe with higher inhibitor concentrations, (2) vanillin at a low concentration increases biomass yield, and (3) at a high concentration, vanillin causes the most severe inhibition to *C. guilliermondii* growth rate. In addition, single inhibitors seem to be more inhibitory than multiple inhibitors at the same total concentration.

To investigate the effect of furfural, syringaldehyde, and vanillin on xylitol biosynthesis, as opposed to *C. guilliermondii* growth rate, the cells were grown to about 10 O.D in batch

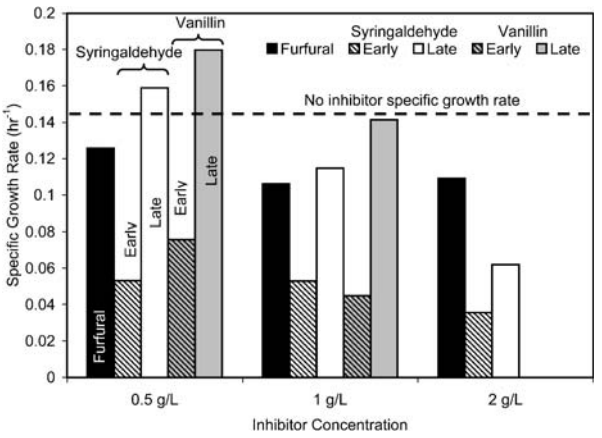


Fig. 6 Specific growth rates in the presence of individual inhibitors. Overall specific growth rate presented for furfural and two specific growth rates presented for syringaldehyde and vanillin: and early phase and late phase. The specific growth rate of *C. guilliermondii* with no inhibitor is represented by the *horizontal dashed line*

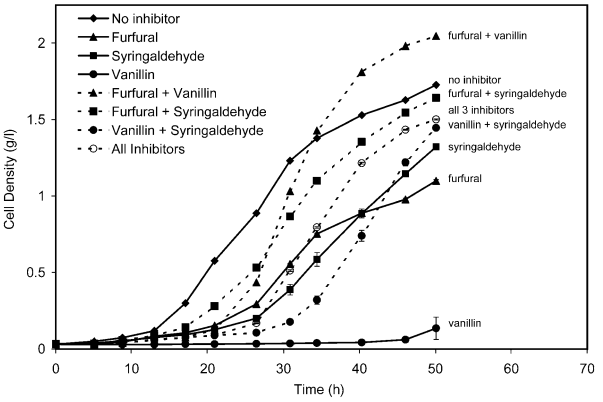


Fig. 7 Effect of 1 g/l multiple inhibitors on *C. guilliermondii* cell growth in microwell plates with a 200-µl cultivation volume. In each well the total inhibitor concentration was 1 g/l. Single inhibitor concentrations are 1 g/l, double inhibitor concentrations are 0.5 g/l each, and the triple inhibitor concentration is 0.33 g/l each. *Error bars* (sometimes obscured by the datum symbol) are ±standard error of the mean

culture in 1-l bioreactors to establish cell mass. The broth was centrifuged and the cell pellet resuspended in fresh xylose-containing medium in the bioreactor. Aeration was reduced to promote xylitol accumulation. Six bioreactor experiments were performed, including no inhibitor, 1 g/l of each inhibitor (separately), and 2 and 3 g/l of furfural. During the resuspension (xylitol accumulation) phase, the cell density remained about constant, and the dissolved oxygen concentration was stable at less than 1% of saturation in air. Figure 9 presents the xylitol concentration in the broth during the resuspension phase for each experiment. Although the cell density was the same in all experiments, the cells in the presence of 1 g/l inhibitor produced xylitol at about 85% of the no inhibitor control (0.29 versus 0.25 g xylitol per liter per hour). Furfural at 2 and 3 g/l reduced the xylitol production rate even further to about 43% of the no inhibitor control (Fig. 9).

The results of toxicity experiments performed using *C. guilliermondii* in batch fermentations are generally similar to that observed in other studies using semi-synthetic or actual hemicellulose hydrolysates for xylitol or ethanol production. Dominguez et al. [29] treated sugarcane bagasse hemicellulose hydrolysate with activated carbon before fermentation with *Candida* sp. 11-2. Starting with an initial xylose concentration of 43 g/l, this yeast produced 10.5 g/l xylitol, corresponding to a xylitol productivity of 0.2 g xylitol per liter per hour. Higher productivities may be achieved by increasing the xylose concentration to approximately 80 g/l. However, the consequent increase in the concentration of inhibitors can cause a decrease in productivity. Other important factors

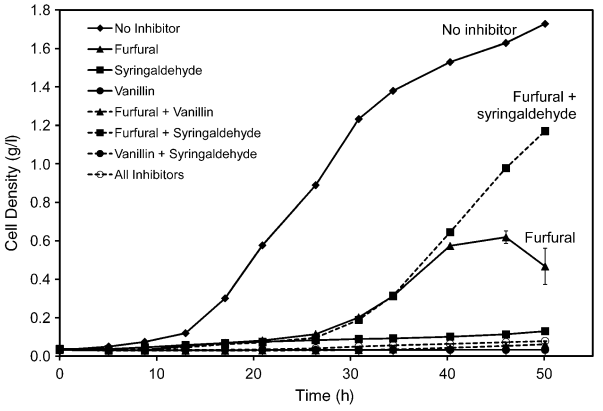


Fig. 8 Effect of 2 g/l multiple inhibitors on *C. guilliermondii* cell growth in microwell plates with a 200-µl cultivation volume. In each well, the total inhibitor concentration was 2 g/l. Single inhibitor concentrations are 2 g/l, double inhibitor concentrations are 1.0 g/l each, and the triple inhibitor concentration is 0.66 g/l each. *Error bars* (sometimes obscured by the datum symbol) are ±standard error of the mean

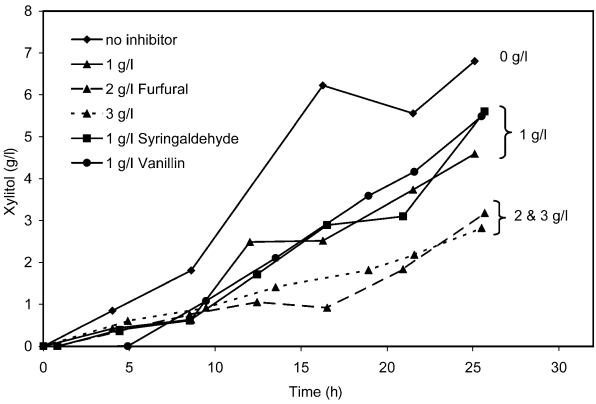


Fig. 9 Effect of inhibitors on xylitol biosynthesis in high cell density bioreactor cultivation during the resuspension phase. Time 0 is the start of resuspension. Cell density in the bioreactors was constant during the entire resuspension phase in all experiments at ~8 $OD_{600\ nm}$. *Filled diamond*, no inhibitor; *filled triangle*, furfural; *filled square*, syringaldehyde; *filled circle*, vanillin

affecting xylitol productivity include the concentrations of other growth substrates, previous adaptation of the strain, inoculum concentration, the oxygen transfer rate, the severity of the hydrolysis conditions, and the detoxification processes employed. Ding and Xia [30] found that a $k_L a$ of about 6 h^{-1} resulted in maximum xylitol accumulation during the production phase of a batch cultivation with *Candida* sp. ZU04, whereas in these experiments, the $k_L a$ was about 9 h^{-1}. Xylitol productivities of 0.66 g xylitol per liter per hour have been achieved under optimum conditions.

In fermentation experiments employing semi-synthetic sugarcane bagasse hemicellulose hydrolysate containing 60–92 g/l xylose, vanillin inhibited the metabolism of the yeast *D. hansenii* at concentrations above 0.5 g/l, and the inhibitory effects on fermentation increased with vanillin concentration up to 3 g/l. Furfural was less inhibitory to batch fermentations, with concentrations between 1 and 5 g/l exhibiting similar effects [23]. From experiments with brewers spent grain hydrolysate (85 g/l xylose) pretreated in various manners (raw, treated with activated carbon, concentrated, or amended with xylose), it appears that total phenolics (3.93 g/l), including vanillin and syringaldehyde, are the most inhibitory compounds to *C. guilliermondii* fermentations. The presence of furfural (0.61 g/l) did not effect the fermentation. Sanchez and Bautista [31] found that 2 g/l furfural inhibits oxidative metabolism and fermentation by *C. guilliermondii*, and it has been previously observed that furfural concentrations below 1 g/l are not inhibitory [32–33]. Keating et al. [34] observed that furfural had a dose-proportional effect in the range of 0.8–1.6 g/l on sugar consumption rate and ethanol productivity. Although these studies have employed a wide array of experimental approaches and extrapolation is complicated by wide differences in concentrations of specific phenolic and furaldehyde compounds in different hydrolysates, inhibitory effects of furfural, vanillin, and syringaldehyde on fermentations appear to occur in the general range of 0.5–2 g/l for individual inhibitors.

In the microwell experiments, no mixing is provided. The yeast cells grow as a layer on the bottom of the well. The inhibitory effects on cell growth are clearly visible and easily measured. This format was chosen to investigate inhibitor effects at low cell densities. Although *C. guilliermondii* appears to grow well, and reproducibly, in this environment, growth rates may be lower than that achievable in well-aerated shake flasks because of oxygen limitation. However, effects such as increased lag time and decrease in specific growth rate occur during the period of lowest cell density and oxygen consumption. Also, the concentrations of inhibitors studied (0.5–2 g/l) are within the range of those typically found in untreated hemicellulose hydrolysates, and the inhibitor concentrations observed to

effect *C. guilliermondii* growth are consistent with those reported in other toxicity studies. The primary advantages of using microtiter plates for inhibitor studies is the large number of experimental variables and synergistic effects that can be investigated, and growth can be monitored in the clear 96-well microtiter plates without any sampling by using a standard microtiter plate reader.

The batch xylitol fermentations conducted in the bench-scale fermentors were more typical of studies on inhibitor toxicities at high cell densities. It is possible that the xylitol production phase could also be considered at the microscale (100- to 200-μl volume), as this is a microaerophilic process in which low oxygen transfer rates are desirable. While this may not provide an accurate measure of the maximum volumetric productivity for a particular hydrolysate, it may be useful for investigating fundamental toxicological effects and inhibitor synergies on xylitol production.

Conclusion

Furfural, syringaldehyde, and vanillin inhibited *C. guilliermondii* growth rate, increased lag time, and reduced xylitol production rate in microwell and bioreactor cultivations. In general, increasing concentration of inhibitor increased the severity of inhibition, except in the case of 0.5 g vanillin per liter which resulted in a faster late batch phase growth rate and increased biomass yield. At concentrations of 1 g/l or higher, furfural was the least inhibitory to growth, followed by syringaldehyde, and vanillin most severely reduced growth rate. There was no synergistic effect observed by combining inhibitors, and in contrast, combinations were slightly less inhibitory to growth than single inhibitors at the same mass concentration. All three inhibitors reduced xylitol production rate approximately to the same degree. These results indicate that concentrations of these inhibitors that are easily obtained in concentrated hydrolysates can have a significant effect on both *C. guilliermondii* growth and xylitol production. For an efficient process, measures should be taken to adapt the organism to the inhibitors or remove the compounds to non-inhibitory levels.

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