Unraveling substrate dynamics and identifying inhibitors in hydrolysates of lignocellulosic biomass by exometabolomics

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CHAPTER 4

Inhibitory Compounds in Lignocellulosic Biomass Hydrolysates during Hydrolysate Fermentation Processes

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Abstract

To compare the composition and performance of various lignocellulosic biomass hydrolysates as fermentation media, 8 hydrolysates were generated from a grass-like and a wood biomass. The hydrolysate preparation methods used were (1) dilute acid, (2) mild alkaline, (3) alkaline/peracetic acid, and (4) concentrated acid. These hydrolysates were fermented at 30°C, pH 5.0, using *Saccharomyces cerevisiae* CEN.PK 113-7D as model strain. The growth in different hydrolysates varied in the aspects of lag-phase, growth rate, glucose consumption rate and ethanol production rate. Subsequently, 11 potential hydrolysate inhibitors were selected, their concentrations in the time-samples of the 8 fermentations were determined using a novel analytical method, ethyl-chloroformate derivatization-GC-MS. Some of these compounds, e.g. furfural, decreased during the fermentation process, while others, such as formic and benzoic acid, remained almost constant. The 11 compounds were tested individually for their inhibitory effects on the model yeast, the results showed that most of the compounds exhibited little effect at their concentrations detected in biomass hydrolysates. Only furfural and benzoic acid clearly affected the growth of the model yeast: furfural elongated the lag-phase, while benzoic acid reduced the growth rate and biomass yield.

Introduction

Lignocellulosic biomass is the feedstock for the production of 2nd generation biofuel. The biomass, such as bagasse, corn stover, wheat straw and willow wood, is structurally composed of cellulose, hemicellulose and lignin [1,2]. To transform the biomass to liquid fermentation medium, a pretreatment and a hydrolysis step are required to break down the biomass structure and to form monomer sugars, such as glucose and xylose [3,4]. The composition of this liquid medium, named hydrolysate, is determined by the biomass type and the pretreatment-hydrolysis method used.

During the pretreatment process, various degradation products of both sugar and lignin are formed, among which are some inhibitory compounds. These compounds negatively influence the hydrolysis as well as fermentation process [5,6]. Acetic acid, furfural and 5-hydroxymethyl furfural are the most studied inhibitory compounds in hydrolysates. These compounds were also used for toxicity studies in different microorganisms [1,7,8]. The other compounds that were reported inhibitory are mainly weak acids, phenolic and aromatic species. These compounds, for example, vanillin and syringic acid, are less well studied concerning their concentrations in hydrolysate and their effects [1,5].

The effects of inhibitory compounds in a fermentation process were shown as longer lag-phase, slower growth, lower cell density and decreased ethanol productivity [9,10]. To be able to use hydrolysate for biofuel production on an industrial level, these effects need to be reduced. Several detoxification methods have been developed and applied to different hydrolysates. Activated carbon, organic solvent absorbing and extracting inhibitory compounds were proven to be effective physical detoxification methods [11,12]. The chemical detoxification methods include over-liming, reacting with reducing agent and peroxide treatment [13,14].

Since hydrolysates were made from natural materials and the preparation methods are various, the composition and performance of different hydrolysates differ. These differences are of importance for both inhibitory compounds studies and detoxification method development. Therefore, studying the similarity and difference of various hydrolysates on their composition and fermentation performance is of considerable interest. The results of these studies will provide information to analyze the relationship between hydrolysate composition and its fermentation performance as medium. For a proper study design, the selected hydrolysates should be different in their fermentation performance. This can be achieved by using different biomass types and diverse pretreatment-hydrolysis methods to

prepare the hydrolysates. In this study, we generated 8 different hydrolysates from bagasse and oak sawdust to compare their performance as fermentation media. Hydrolysates and their fermentation time-series samples were taken to study their composition and dynamics during the fermentation process. These samples were analyzed with EC-GC-MS method. This analytical method was developed to remove the sugar content in the hydrolysates and detect sugar and lignin degradation products. Among all the compounds detected, 11 were selected to be quantified in hydrolysates and their fermentation time-series samples. These selected compounds are formic acid, acetic acid, levulinic acid, furfural, furfuryl alcohol, 2-furanmethanol acetate, HMF, vanillin, syringic acid, benzoic acid and 4-hydroxybenzaldehyde. They were chosen because they were either reported as inhibitory compounds [15,16] or belong to the categories of potential inhibitors [17,18]. The concentrations of these compounds detected in the hydrolysates were used to analyze their dynamics during the fermentation process, and test their inhibitory effects individually using a screening method.

Materials and Methods

Biomass

Sugar cane bagasse (Bag) was a kind gift from ZILOR, Brazil, and oak sawdust (Oak) was obtained from ESCO, the Netherlands, a wood-flooring supplier. Both types of biomass were pre-dried at 80°C for 5 hours when received, and stored at room temperature. Sugar cane bagasse was ground to pieces with average length of 3 mm. Prior to pretreatment, the biomass was dried again at 80°C for minimum 16 hours.

Hydrolysate preparation method

Four pretreatment methods were used to prepare bagasse and oak sawdust for hydrolysis, namely dilute acid (DA) (2% H_2SO_4), mild alkaline (MA) (3% $Ca(OH)_2$), alkaline/peracetic acid (PAA) [19], and concentrated acid (CA) (72% H_2SO_4). The biomass pretreated with the first three methods was hydrolyzed enzymatically while the concentrated acid pretreated biomass was hydrolyzed in acid. The detailed steps of these methods are described in Zha *et al.* [20].

Strain and preculture

Saccharomyces cerevisiae CEN.PK 113-7D (CBS8340) was used as model strain in this hydrolysate study. The strain was obtained from CBS Utrecht, the Netherlands. The preculture for both fermentation and Bioscreen test was prepared in a 500 ml Erlenmeyer

shake flask with 100 ml mineral medium and 20 g/l glucose. The mineral media was prepared according to van Hoek *et al.* [21]. The preculture was inoculated with 1 ml *S. cerevisiae* CEN.PK 113-7D glycerol stock, and incubated at 30°C, 200 rpm for 20 hours.

Fermentation setup

The batch fermentation was carried out in a 2 l New Brunswick fermentor with working volume of 1 l. The fermentor, filled with 1 l demineralized water, was sterilized at 121°C. After sterilization, the fermentor was connected to the console, emptied and filled with 950 ml filter-sterilized hydrolysate. For each hydrolysate, 1 fermentation run was conducted. The fermentation temperature was set at 30°C, pH at 5.0 by adding 2 M KOH or 1 M H_2SO_4, dissolved oxygen at 0 by flushing 0.5 l/min N_2 continuously. The fermentation began at the point of inoculation. The inoculum was prepared by harvesting the cells from 50 ml preculture and re-suspending the cells in 50 ml hydrolysate. Together with inoculum, 2 ml Tween 80-Ergosterol stock were added into the fermentor. The Tween 80-Ergosterol stock contained 5.0 g/l Ergosterol and 210.0 g/l Tween 80, which were dissolved in 95% ethanol. The whole fermentation process was monitored by continuously measuring the CO_2 percentage in the off-gas. The fermentation was considered finished when the CO_2 percentage value is 0 for 10 hours. During the fermentation process, samples were taken every 60 min or 99 min. The auto-samples were directly cooled to 4°C and later stored at 0°C.

Fermentation sample analysis

The monomer sugar concentrations in the hydrolysates were determined with DIONEX ICS 3000, equipped with CarboPac PA20 carbohydrate column and plused amperometric detector. The column was operated at 30°C, with 7.5 mM NaOH as eluent, and the flow rate was 0.5 ml/min.

The optical density (OD), glucose and ethanol concentrations of the fermentation auto-samples were determined using ROCHE Cobas Mira Plus. Vortex was performed to each individual sample to reach a homogeneous cell distribution before measuring optical density at wavelength 600 nm. After optical density measurement, the samples were centrifuged at 4000 rpm for 15 min and the suspension was used for glucose and ethanol measurements. Glucose concentration was determined enzymatically, by adding reagent Glucose HK CP, purchased from ABX Pentra, and measuring formed NADH amount at wavelength 340 nm. The ethanol assay was performed by using NAD and aldehyde dehydrogenase in 0.4 M KH_2PO_4 buffer as the first reagent and alcohol dehydrogenase as the second reagent, and measuring NADH concentration at wavelength 340 nm (adapted from BIOCHEMICA © protocols).

For each fermentation, 5 auto-samples were selected, representing the following time points: directly after inoculation, end of lag-phase, growth phase, end of growth phase and stationary phase. The concentrations of formic acid and acetic acid of these samples were measured with DIONEX ICS 3000, equipped with IonPac ICE-AS6 ion-exclusion column and suppressed conductivity detector. The column was operated at 30°C, with 1.6 mM perfluorobutyric acid as eluent, and the flow rate was 1.0 ml/min.

The concentrations of furfural, furfurylalcohol, 2-furanmethanol acetate, levulinic acid, benzoic acid, syringic acid, HMF, 4-hydroxybenzaldehyde and vanillin were analyzed with EC-GC-MS method. The method was conducted as follows. NaOH solution was added to 0.5 ml hydrolysate to bring the mixture pH above 10. Into the mixture, the labeled internal standard containing leucine-D3, succinic acid-D4 and cinnamic acid-D5 in pyridine was added. 300 µl ethanol and the injection standard containing difluorobiphenyl and dicyclohexylphtalate in pyridine were also added. The formation of the ethylesters was done by two rounds of adding 40 µl ethyl chloroformate then shaking vigorously by hand for 15 seconds. The reaction was stopped by adding 750 µl dichloromethane and 500 µl of 1 M bicarbonate buffer. The formed derivates were extracted to the dichloromethane phase by shaking the mixture for 20 seconds. The dichloromethane phase was then transferred to another vial and dried with sodium sulfate. The dried dichloromethane phase was transferred to an auto-sample vial. The measurement was carried out by 1 µl splitless injection in the PTV injector of the AGILENT 7890A GC with AGILENT 5975C mass spectrometer as detector. A DB-1 30 m x 0.32 mm x 1 µm analytical column was used for the separation of the analytes.

Inhibitory effects test

The inhibitory effects of the selected compounds were examined by using growth tests in BIOSCREEN C Analyzer, LABSYSTEMS OY, Helsinki, Finland, as described in Zha *et al*. [20]. The compounds were added into mineral medium with 20 g/l glucose and 2 different hydrolysates, Oak-PAA and Bag-CA. The concentrations added were based on the highest levels detected in all hydrolysates, which are marked in bold in Table 5. The media pH was adjusted to 5.0±0.5 with either 3 M H_2SO_4 or 6 M KOH before inoculation. The tests were carried out in triplicates.

Results and Discussion

Biomass hydrolysates composition

Sugar cane bagasse and oak saw dust were chosen as the biomass for this study because they represent two distinct categories of biomass type, namely grass like and wood. More importantly, in a previous study, where the growth of the model yeast was screened in 24 different hydrolysates, bagasse and oak hydrolysates showed the largest diversity [20].

Both bagasse and oak were treated with the 4 different hydrolysate preparation methods. The resulting 8 hydrolysates were analyzed on their monosaccharide compositions, as shown in Table 1. Glucose and xylose were the major monomer sugars in all 8 hydrolysates, and glucose had an approximately two fold higher concentration compare to xylose. Small amounts of galactose and arabinose were detected in both bagasse and oak hydrolysates, while in oak hydrolysates, also low levels of mannose were found.

Table 1 Monomer sugar concentrations of the 8 hydrolysates (g/l)

Hydrolysates	glucose	xylose	galactose	arabinose	mannose
Bag-MA	57.9	33.6	0.1	3.3	0.0
Bag-DA	66.5	29.7	0.7	2.0	0.0
Bag-PAA	67.8	31.2	0.1	0.7	0.0
Bag-CA	107.3	62.9	1.5	4.2	0.0
Oak-MA	47.4	24.6	0.2	0.1	0.0
Oak-DA	42.1	25.0	1.0	0.6	1.3
Oak-PAA	61.0	28.1	0.2	0.1	0.0
Oak-CA	90.9	42.3	2.5	1.8	4.2

Hydrolysate fermentation

For each of the 8 hydrolysates in Table 1, a batch fermentation was carried out with the model yeast *S. cerevisiae* CEN.PK 113-7D. The fermentation performance was determined by measuring the optical density, glucose concentration and ethanol concentration of the samples taken during the whole fermentation process. The growth of the yeast varied in these 8 hydrolysates, as shown in Figure 1, as well as the glucose consumption (Figure 2) and the ethanol production (Figure 3).

Growth characteristics

The growth of the yeast cells in a fermentation process was monitored by measuring the optical density of the time samples at wavelength 600 nm. The optical density of a time sample was calculated by deducting the measured optical density value by the time-0 optical density value: $OD_{t\text{-}s}=OD_{t\text{-}m} - OD_{t\text{-}0}$.

The growth curves of the model yeast in 8 different hydrolysates and in mineral medium are shown in Figure 1. The growth of the model yeast in mineral medium in this study was highly comparable to the growth reported by Kuyper *et al.* [22]. By comparing the growth in hydrolysates and in mineral medium, it can be seen that the growth in all hydrolysates were negatively affected. This was mainly shown as slower growth, longer lag-phase and lower OD yield. It can be seen that the growth of the model yeast was similar in the hydrolysates prepared with the same method, indicating that the hydrolysate performance was mainly dependent on the pretreatment-hydrolysis method. The hydrolysates prepared by mild alkaline method resulted in the shortest lag-phase and relatively high growth rate, while the concentrated acid method prepared hydrolysates had the longest lag-phase and slower growth. The performance of the hydrolysates made by dilute acid and peracetic acid methods was in between the other two (Figure 1).

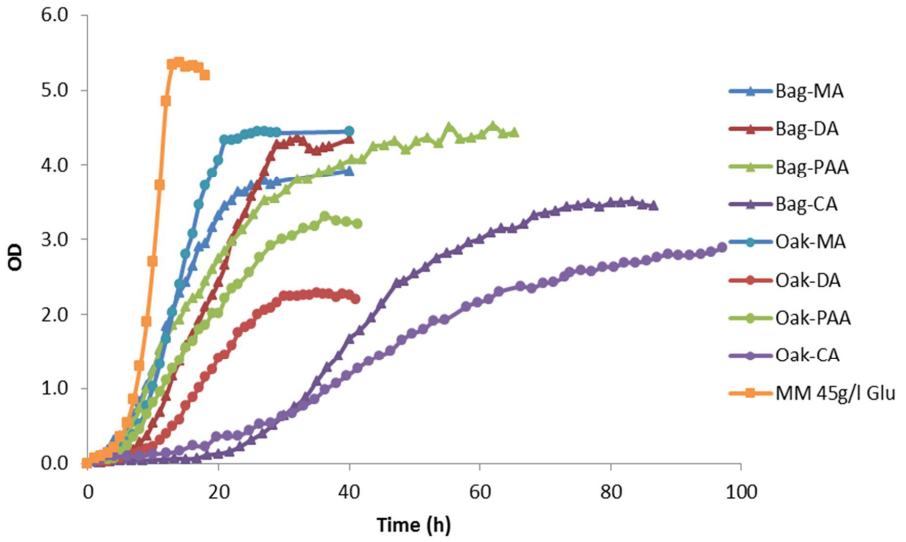


Figure 1 Growth curves of the model yeast, *Saccharomyces cerevisiae* CEN.PK113-7D, in 8 different hydrolysates. Bag: bagasse, Oak: oak sawdust; MA: mild alkaline, DA: dilute acid, PAA: alkaline/peracetic acid, CA: concentrated acid.

To quantitatively compare these 8 fermentations, lag-phase, growth rate and OD yield were defined as parameters to describe the characteristics of the fermentation performance (Table 2). By comparing the growth rates in bagasse and oak hydrolysate prepared with the same method, it is noticed that only when prepared with mild alkaline method, oak hydrolysate was with a higher growth rate than bagasse. This indicates that hydrolysates prepared from bagasse are, in general, less inhibitory than those prepared from oak. Probably, if the pretreatment method was mild and the biomass structure was relatively more difficult to break down, such as oak, there would be little inhibitory compounds released or formed. In this case, the generated hydrolysate would be less toxic. As shown, different from the growth in mineral medium, the growth in some hydrolysates slowed down several hours after the growth started (Figure 1). This phenomenon is most illustrative in PAA hydrolysates, the model yeast started with fast growth, but the growth rate dropped at a specific point, 14 h for Bag-PAA and 20 h for Oak-PAA. The 2 different growth rates shown in Table 2 are before and after the rate drop, respectively. A possible explanation for the phenomenon is that the amount of essential nutrients in these hydrolysates was limited, which could only support the growth in the first several hours. To continue growth, the yeast had to use different nutrients that were less efficient, which caused the growth rate to slow down. This explanation was consistent with the fact that the growth slowed down particularly in PAA hydrolysates. As during PAA pretreatment, 2 washing steps were involved, which removed dissolved nutrients at that moment. This possibly caused nutrient limitation in PAA hydrolysates, which lead to the reduction of the growth rate. In agreement with this, the analysis results of the hydrolysate fermentation time samples revealed that most of the amino acids present in the hydrolysates were consumed during the fermentation process (see Chapter 5).

As far as the OD yield is considered, it seems that it was related to lag-phase and growth rate, namely, long lag-phase and/or slow growth corresponded to low OD yield. For instance, the lowest OD yield was of Bag-CA and Oak-CA hydrolysate fermentations, which had the longest lag-phase (17 h) and lowest growth rate (0.035), respectively (Table 2). The differences in OD yield indicate that the yeast cells spent a higher percentage of the total energy on maintenance in hydrolysates, which maybe the result of overcoming inhibitory effect and/or using less efficient nutrients.

Table 2 Growth characteristics of the model yeast in the 8 hydrolysates

Hydrolysates	lag-phase[1] (h)	growth rate[2]	OD yield[3] (OD/ g glucose)
Bag-MA	1	0.169	0.067
Bag-DA	6	0.187	0.069
Bag-PAA	4	0.243 / 0.052	0.064
Bag-CA	17	0.085	0.033
Oak-MA	2	0.200	0.100
Oak-DA	5	0.125	0.060
Oak-PAA	4	0.190 / 0.058	0.057
Oak-CA	10	0.035	0.032
MM 45 g/l Glucose	1	0.306	0.132

[1]: lag-phase is defined as the time needed to reach 2% of the maximum OD;
[2]: growth rate is calculated as the slope of the linear part of the *logOD vs. time* plot;
[3]: OD yield is calculated by dividing maximum OD by the amount of glucose consumed in the whole fermentation process.

Table 3 Glucose consumption and ethanol production results of the 8 fermentations

Hydroly sates	initial glucose concentration[1] (g/l)	maximum ethanol concentration[2] (g/l)	ethanol yield[3] (g/g)	maximum glucose consumption rate[4] (g/l/h)	maximum ethanol production rate[5] (g/l/h)
Bag-MA	58.8	22.7	0.39	3.3	1.3
Bag-DA	63.3	24.2	0.38	3.1	1.2
Bag-PAA	69.8	24.0	0.34	1.6	0.6
Bag-CA	104.4	34.9	0.33	2.3	0.9
Oak-MA	44.4	19.5	0.44	3.1	1.4
Oak-DA	38.2	15.3	0.40	2.0	0.8
Oak-PAA	58.0	24.2	0.42	2.4	1.0
Oak-CA	88.9	22.0	0.35	1.1	0.4
MM 45 g/l Glucose	42.5	16.0	0.38	5.5	2.0

[1]: the glucose concentration of the time-0 fermentation sample;
[2]: the highest ethanol concentration among all fermentation samples;
[3]: maximum ethanol concentration divided by the total amount of glucose consumed;
[4]: the slope of the linear part of the *glucose concentration vs. time* plot;
[5]: the slope of the linear part of the *ethanol concentration vs. time* plot.

Glucose consumption and ethanol production profile

The 8 different hydrolysates differ in their initial glucose concentrations due to the diverse biomass types and hydrolysate preparation methods used, see Table 3. To analyze the effect of initial glucose concentration on growth, mineral medium with glucose concentration 20 g/l, 40 g/l, 60 g/l and 80 g/l were used to test the model strain in Bioscreen. The results showed that with glucose concentration at this range, the model yeast did not show any difference in their growth, in terms of the 3 parameters listed in Table 2 (data not shown). So it was assumed that the performance differences of the model strain in these hydrolysates were not caused by the variation of initial glucose concentration.

To present the glucose consumption and ethanol production of the 8 hydrolysate fermentations in a comparable manner, both glucose and ethanol concentrations were expressed as a percentage, with maximum value set as 100% and 0 g/l set as 0%, as shown in Figure 2. It can be seen that the hydrolysates prepared with the same method had similar pattern in both glucose consumption and ethanol production curves. This is consistent with the observation of growth curves, confirming that the hydrolysate performance was mainly determined by pretreatment-hydrolysis method rather than biomass type.

The maximum ethanol concentration and ethanol yield of the 8 different hydrolysates are listed in Table 3. The highest ethanol concentration in all fermentations was 34.9 g/l of Bag-CA hydrolysate, while the highest ethanol yield was of Oak-MA hydrolysate, 0.44 g ethanol per g glucose. This yield was 86% of the theoretical ethanol yield on glucose [23]. Furthermore, also the maximum glucose consumption rate and the maximum ethanol production rate of the 8 fermentations are compared in Table 3. It can be seen that these two rates were closely related, in general, the faster the glucose was consumed the quicker the ethanol was produced, in other words, the ethanol yields of these 8 fermentations were quite similar. Additionally, these ethanol yields were not only similar to each other, but also comparable to the one of mineral media fermentation. This suggests that ethanol yield was only slightly influenced by the inhibitory compounds in the hydrolysates, which agrees with the effect of furans and phenols on yeasts performance [17]. Since the effects of inhibitory compounds in hydrolysates were mainly on growth rate, OD yield and glucose consumption rate, it is practical to use these parameters as indicators for studying the hydrolysate inhibitory effect.

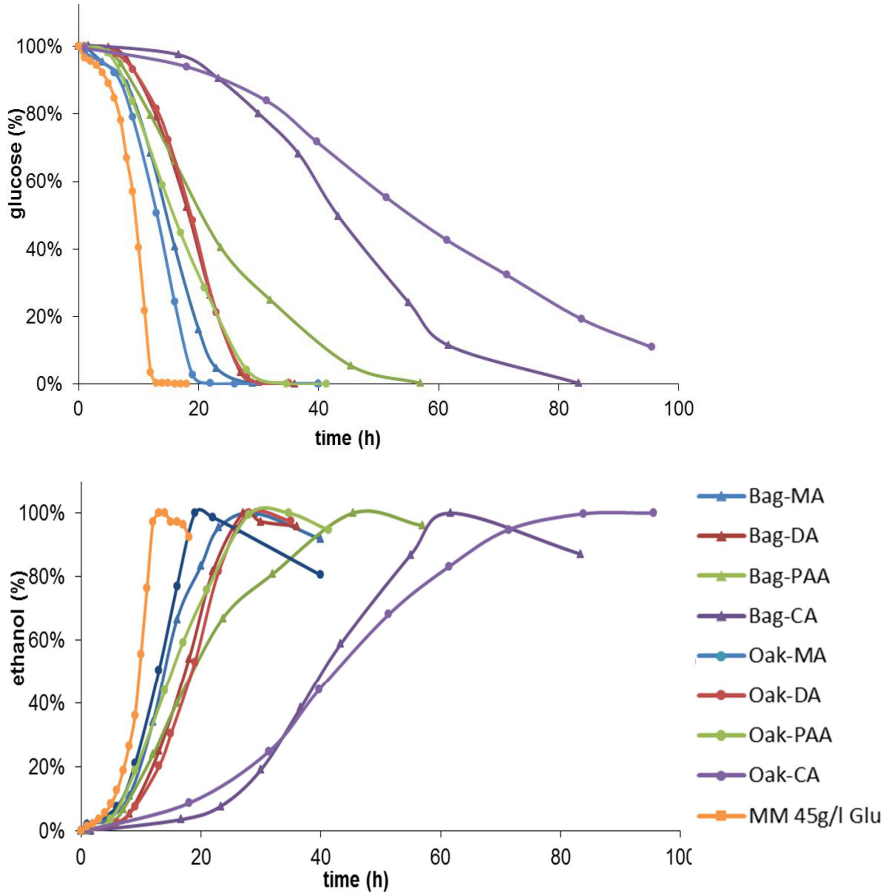


Figure 2 Glucose consumption (top) and ethanol production (bottom) curves of the 8 fermentations in percentage. Bag: bagasse, Oak: oak sawdust; MA: mild alkaline, DA: dilute acid, PAA: alkaline/peracetic acid, CA: concentrated acid.

Selection and quantification of inhibitory compounds in hydrolysate samples

To identify the role of specific hydrolysate inhibitors on fermentation performance, exo-metabolomics analysis was carried out. As a first step in interpreting this type of analysis, a group of 11 compounds were selected and quantified in their hydrolysate fermentation samples. Base on quantification results, the dynamics of these compounds during a fermentation process could be determined. This analysis will also allow tests of inhibitory effects of these compounds at concentrations present in the hydrolysates. The selected compounds were formic acid, acetic acid, levulinic acid, furfural, furfurylalcohol, 2-

furanmethanol acetate, HMF, vanillin, syringic acid, 4-hydroxybenzaldehyde and benzoic acid. The selection was made based on data reported in literature and observations made in our preliminary studies (Table 4).

Furfural and HMF are both furan compounds, and were identified as potential inhibitors in biomass hydrolysates [17,24,25]. Furfural was pointed to be the key inhibitor in hydrolysates by Heer *et al.* in 2008 [15]. It was known that furfural was converted to furfurylalcohol by yeast as a detoxification mechanism [26]. The inhibitory effect of furfural was reported as increasing lag-phase [15] and reducing specific growth rate [6].

Formic, acetic and levulinic acid are the weak acids formed in most of the biomass hydrolysis preparation process [1,5] and their inhibitory effects and mechanism on yeasts have been studied in the past several years [6,27]. It was suggested that these weak acids reduce yeast growth and ethanol yield by causing intracellular anion accumulation, which is pH dependent [1,16]. Recently, Sanda *et al*. reported that both formic acid and acetic acid affect the utilization of xylose in recombinant xylose-fermenting strain [27].

Vanillin, syringic acid and 4-hydroxybenzoic acid were characterized phenolic compounds in hydrolysates [5,17]. The inhibitory effects of 4-hydroxybenzoic acid have been studied with several different yeast strains [18], the study concluded that the compound showed little effects on the yeasts used. In this study, the two closely related compounds, benzoic acid and 4-hydroxybenzaldehyde were chosen to be quantified in hydrolysates and tested on their effects on the growth of the model yeast.

As summarized in Table 4, formic acid, acetic acid, levulinic acid, furfural, HMF, vanillin, syringic acid, 4-hydroxybenzaldehyde and benzoic acid are the characterized degradation products in biomass hydrolysates; while furfurylalcohol and 2-furanmethanol acetate are the possible conversion products of furfural and/or HMF.

These selected compounds were analyzed and quantified in both hydrolysates and their fermentation samples. For each fermentation, 5 samples were chosen according to the following criteria: (1) directly after inoculation, (2) end of lag-phase, (3) growth phase, (4) end of growth phase, (5) stationary phase. The concentrations of these selected compounds in the fermentation samples are listed in Table 5.

Formic acid and acetic acid were detected in all hydrolysates and their fermentation samples. In general, acetic acid concentrations were 10-15 times higher than that of formic acid. The

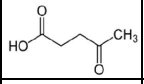
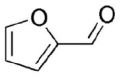
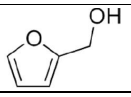
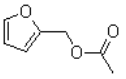
highest concentrations of these two acids were found in CA hydrolysates, 0.57 g/l of formic acid and 8.0 g/l of acetic acid. These concentrations are comparable with the ones detected in acid pretreated spruce and bagasse hydrolysates [14]. During fermentation processes, no obvious consumption of either acid was observed, though both fluctuated slightly. Unlike formic acid and acetic acid, levulinic acid was only present in CA hydrolysates with a concentration of 1.2 g/l, without a decrease during fermentation.

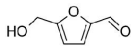
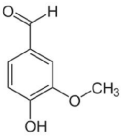
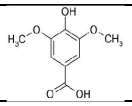
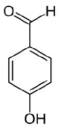
Vanillin, syringic acid and 4-hydroxybenzaldehyde were present mainly in MA and DA hydrolysates although in rather low amounts, 30-50 mg/l. These concentrations are similar with those detected previously [1,5]. In contrast to syringic acid, both vanillin and 4-hydroxybenzaldehyde decreased during fermentation, suggesting their conversion or consumption. Due to its presence as a preservative in the enzyme cocktails of about 2.0 g/l [28], benzoic acid was detected in all enzymatic hydrolyzed hydrolysates, namely MA, DA and PAA hydrolysates, with a similar concentration of 150 mg/l. During the whole fermentation process, the level of benzoic acid did not change (Table 5). Surprisingly, in PAA treated bagasse hydrolysate, benzoic acid was apparently converted into its corresponding ethanol before the starting of the fermentation. It is unclear why this conversion took place specifically in Bag-PAA hydrolysate.

It can be seen in Table 5 that furfural was found at considerable levels in CA hydrolysates, and at low amounts in DA hydrolysates. In both DA and CA hydrolysate fermentations, the furfural concentration rapidly decreased at the onset of the fermentation until levels of about 30 mg/l, with exception of Bag-DA hydrolysate. Correspondingly, the concentration of furfurylalcohol increased in the same time frame. This suggests that furfural was converted to furfurylalcohol in the lag-phase of the fermentation, which agrees with the report of Palmqvist *et al.* [26]. Different from the observation in *A. niger* [29], furfurylalcohol was not further converted into furoic acid. Furfural was also found in Bag-PAA hydrolysate at 30 mg/l, but it was not converted during the whole fermentation process. Similar to furfural, HMF was also found in DA and CA hydrolysates, but with a much lower amount. The HMF concentration reduced gradually in both lag-phase and growth-phase of these hydrolysate fermentations.

Interestingly, 2-furanmethanol acetate showed similar pattern as furfurylalcohol, the compound increased with the decrease of furfural and HMF in the fermentation lag-phase (Table 5). Based on the structure of 2-furanmethanol acetate, it is suspected that the compound was the reaction product of furfurylalcohol and acetic acid. From this result, we suggest that furfurylalcohol was possibility partially converted to 2-furanmethanol acetate by reacting with acetic acid.

Table 4 A summary of the selected compounds: their concentrations detected in various hydrolysates, and the concentrations at which inhibitory effects were shown on *S.cerevisiae*.

compound	structure	hydrolysates		concentrations in hydrolysates		concentrations showed effects on *S.cerevisiae*	
		biomass	preparation method	mg/l	ref	mg/l	ref
formic acid		Corn stover	Acid/temperature	130-310	[12]	4000	[16]
		Spruce/ bagasse	Acid/temperature	600-800	[14]	2700	[30]
		Hardwood chips	Autohydrolysis/ temperature	4000-4600	[31]		
		Corn stover	Steam explosion	6800	[16]		
acetic acid		Bagasse	Acid hydrolysis	2400	[32]		
		Bagasse	Enzyme hydrolysis	2100	[32]	5000	[32]
		Wheat straw	Acid hydrolysis	1300	[32]	6000	[16]
		Wheat straw	Enzyme hydrolysis	900	[32]	7500	[30]
		Corn stover	Acid hydrolysis	2300	[32]	>10000	[33]
		Willow wood	Acid hydrolysis	2200	[32]		
		Yellow polar wood	Organosolv	900-4900	[34]		
		Spruce/ bagasse	Acid/temperature	3100-5200	[14]		
		Corn stover	Acid/temperature	2270-3740	[12]		
		Hardwood chips	Autohydrolysis/ temperature	4500-5800	[31]		
		Corn stover	Steam explosion	7800	[16]		
levulinic acid		Spruce/ bagasse	Acid/temperature	200-300	[14]		
		Corn stover	Acid/temperature	130-410	[12]		
furfural		Yellow polar wood	Organosolv	0.2-35.2	[34]	1000	[32]
		Wheat straw	Alkaline/oxidation	0-146*	[5]	>800	[33]
		Bagasse	Acid hydrolysis	410	[32]		
		Wheat straw	Acid hydrolysis	270	[32]	>4000	[16]
		Corn stover	Acid hydrolysis	510	[32]		
		Willow wood	Acid hydrolysis	500	[32]		
		Hardwood chips	Autohydrolysis/ temperature	510-780	[31]		
		Corn stover	Acid/temperature	570	[12]		
		Corn stover	Steam explosion	710	[16]		
		Wheat straw	Acid steam explosion	480-680	[15]		
		Spruce	Acid steam	1100	[15]		
		Barley straw	Acid steam	2880	[15]		
		Spruce/ bagasse	Acid/temperature	600-1200	[14]		
furfuryl alcohol		N.A.					
2-furan methanol acetate		N.A.					

HMF		Wheat straw	Alkaline/oxidation	0-16*	[5]	1000	[32]
		Yellow polar wood	Organosolv	0-56.5	[34]	>3000	[33]
		Hardwood chips	Autohydrolysis/ temperature	80-130	[31]	>4000	[16]
		Bagasse	Acid hydrolysis	70	[32]		
		Wheat straw	Acid hydrolysis	60	[32]		
		Corn stover	Acid hydrolysis	100	[32]		
		Willow wood	Acid hydrolysis	140	[32]		
		Corn stover	Acid/temperature	50-140	[12]		
		Wheat straw	Acid steam explosion	177-277	[15]		
		Spruce	Acid steam	2140	[15]		
		Barley straw	Acid steam	996	[15]		
		Corn stover	Steam explosion	560	[16]		
		Spruce/ bagasse	Acid/temperature	1600-3400	[14]		
vanillin		Wheat straw	Alkaline/oxidation	8-96*	[5]	4000	[16]
		Wheat straw	Acid steam explosion	91-122	[15]		
		Spruce	Acid steam	152	[15]		
		Barley straw	Acid steam	106	[15]		
		Willow	Dilute acid	430	[1]		
		Corn stover	Steam explosion	4000	[16]		
syringic acid		Wheat straw	Wet oxidation	22	[1]		
		Wheat straw	Alkaline/oxidation	6-52*	[5]		
4-hydroxy benzaldehyde		Wheat straw	Wet oxidation	21	[1]		
		Willow	Dilute acid	10	[1]		
		Wheat straw	Alkaline/oxidation	12-59*	[5]		
benzoic acid		Corn stover	Steam explosion	900	[16]	2000	[16]

*: these values are expressed as g/100g straw; N.A.: Not Available.

Inhibitory effect of the selected compounds tested in mineral medium

The quantification results of the selected compounds in hydrolysates provided reference concentrations to test their inhibitory effects. For each compound, the highest concentration detected among all samples, marked as bold in Table 5, was used as the initial testing value. Based on initial test results, the concentrations were increased or decreased up to 5-10 folds for the actual test. The medium used here was mineral medium with 20 g/l glucose.

Table 5 The concentrations of the selected 11 compounds in the samples of the 8 hydrolysate fermentations (mg/l)

fermentation samples	formic acid	acetic acid	levulinic acid	furfural	furfuryl alcohol	2-furanmethanol acetate	HMF	vanillin	syringic acid	4-hydroxybenzaldehyde	benzoic acid
Bag-MA 1	93	1342	<25	<10	<10	<1	<8	**34**	7	**42**	139
Bag-MA 2	83	1190	<25	<10	<10	<1	<8	26	6	40	143
Bag-MA 3	77	1164	<25	<10	<10	<1	<8	3	6	5	143
Bag-MA 4	63	1026	<25	<10	<10	<1	<8	3	6	5	140
Bag-MA 5	56	978	<25	<10	<10	<1	<8	3	6	5	143
Bag-DA 1	184	1816	<25	30	21	<1	11	19	8	23	141
Bag-DA 2	173	1750	<25	<10	42	1	10	5	9	13	141
Bag-DA 3	165	1661	<25	<10	44	1	<8	4	9	6	145
Bag-DA 4	147	1535	<25	<10	46	1	<8	5	9	10	149
Bag-DA 5	153	1520	<25	<10	48	2	<8	5	9	10	153
Bag-PAA 1	16	241	<25	27	<10	<1	<8	3	<5	4	<10*
Bag-PAA 2	16	273	<25	27	<10	<1	<8	3	<5	4	<10*
Bag-PAA 3	0	128	<25	28	<10	<1	<8	3	<5	6	<10*
Bag-PAA 4	0	28	<25	29	<10	<1	<8	3	<5	9	<10*
Bag-PAA 5	0	48	<25	30	<10	<1	<8	3	<5	10	<10*
Bag-CA 1	**568**	7234	1148	**579**	97	12	**57**	<1	<5	<1	<10
Bag-CA 2	528	7049	1159	32	**750**	98	29	<1	<5	<1	<10
Bag-CA 3	552	6922	1206	28	730	99	<8	<1	<5	<1	<10
Bag-CA 4	533	6460	1297	30	739	97	<8	<1	<5	<1	<10
Bag-CA 5	534	6469	1314	29	747	99	<8	<1	<5	<1	<10
Oak-MA 1	133	1198	<25	<10	<10	<1	<8	13	7	<1	128
Oak-MA 2	135	1310	<25	<10	<10	<1	<8	3	7	<1	130
Oak-MA 3	173	1679	<25	<10	<10	<1	<8	2	6	<1	129
Oak-MA 4	151	1547	<25	<10	<10	<1	<8	2	7	3	132
Oak-MA 5	154	1562	<25	<10	<10	<1	<8	2	7	3	130
Oak-DA 1	330	3490	<25	60	30	1	17	14	46	<1	157
Oak-DA 2	318	3420	<25	28	94	6	14	4	44	<1	159
Oak-DA 3	302	3228	<25	26	106	7	8	3	46	<1	163
Oak-DA 4	282	3003	<25	26	107	7	<8	3	**47**	<1	162
Oak-DA 5	278	3051	<25	27	112	7	<8	3	45	<1	**164**
Oak-PAA 1	36	560	<25	<10	<10	<1	<8	4	<5	<1	144
Oak-PAA 2	39	592	<25	<10	<10	<1	<8	3	<5	<1	139
Oak-PAA 3	33	603	<25	<10	<10	<1	<8	3	<5	<1	141
Oak-PAA 4	34	474	<25	<10	<10	<1	<8	3	<5	<1	144
Oak-PAA 5	37	500	<25	<10	<10	<1	<8	3	<5	<1	141
Oak-CA 1	492	**7994**	1082	431	95	12	55	<1	7	<1	<10
Oak-CA 2	454	7877	1119	50	603	84	37	<1	6	<1	<10
Oak-CA 3	499	7869	1198	34	640	93	10	<1	7	<1	<10
Oak-CA 4	479	7591	1324	33	684	97	<8	<1	7	<1	<10
Oak-CA 5	509	7901	**1360**	34	698	**100**	<8	<1	7	1	<10

*: Instead of benzoic acid, benzylalcohol peak was found in Bag-PAA hydrolysate samples. Since benzylalcohol and several unknown peaks that may relate to benzoic acid were unique to Bag-PAA samples, it is possible that the benzoic acid presented in Bag-PAA hydrolysate was converted to several related compounds.

Furfural and benzoic acid

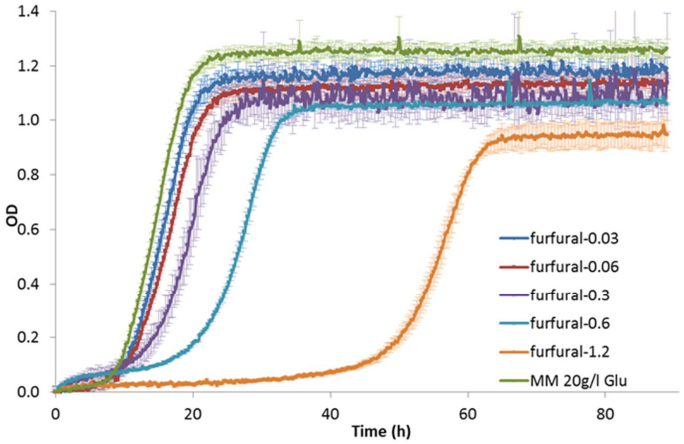
Furfural and benzoic acid clearly affected the growth of the model yeast at concentrations as were present in the hydrolysates, see Figure 3. The inhibitory effect of furfural displayed mainly as longer lag-phase. The lag-phase started to elongate already at a very low furfural concentration, 0.06 g/l, and increased from about 5 hours to 15 hours at a concentration of 0.6 g/l, which was about the concentration in CA hydrolysates (Table 5). It was observed that furfural concentration reduced mainly in the lag-phase during the fermentation process. This suggests that the presence of furfural obstructed the growth of the model yeast, and only when its concentration in the medium dropped below a threshold, the growth could start. It is suspected that this threshold was 0.03 g/l, as the growth commenced in most hydrolysates at this furfural concentration.

Unlike furfural, the inhibitory effect of benzoic acid was lowering the growth rate and final optical density level of the model yeast, as shown in Figure 3. At the concentration of 0.16 g/l, which was also the highest benzoic acid concentration detected in the hydrolysates, the growth rate decreased more than 60% compared to the reference medium, and the final optical density level dropped from 1.28 to 0.65. It seems that the inhibitory effect of benzoic acid was closely related to its concentration present in the medium.

The combination effect of furfural and benzoic acid on the model yeast seems to be addable, as shown in Figure 4. That is to say, the lag-phase and the growth rate in the medium with both furfural and benzoic acid were very similar to which in the medium with furfural and with benzoic acid, respectively. Apparently, the inhibition by furfural and benzoic acid takes place at different stages of the growth process, namely, furfural before growth started and benzoic acid after. This indicates the inhibitory mechanisms of furfural and benzoic acid were different.

5-hydroxymethyl furfural (HMF)

HMF was frequently mentioned as a inhibitor next to furfural in hydrolysates [24,35], but seemed to have a milder inhibitory effect [7,23]. The highest HMF concentration present in the 8 hydrolysates in this study was 0.06 g/l, which did not give any effect on growth when added into mineral medium (data not shown). The inhibitory effect of HMF only became visible when its concentration reached 0.6 g/l and enhanced strongly when it was increased to 1.2 g/l, see Figure 5. In contrast to furfural, the inhibitory effect of HMF was mainly shown as slower growth and lower final optical density, next to elongated lag-phase.



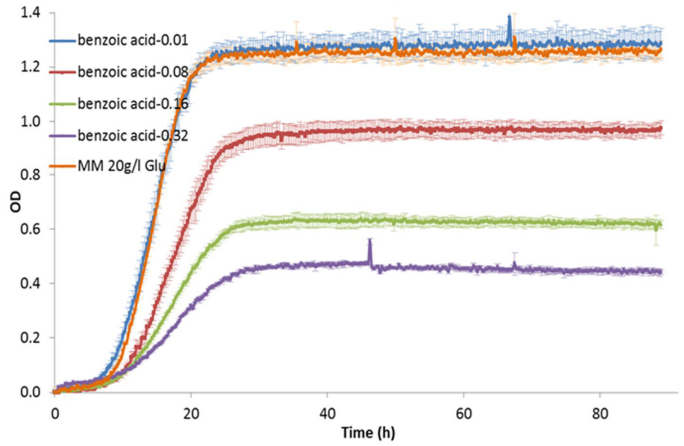


Figure 3
Inhibitory effect of furfural (top) and benzoic acid (bottom) on the model yeast in MM with 20 g/l glucose.
The values in the label are the compound concentrations in media (g/l).

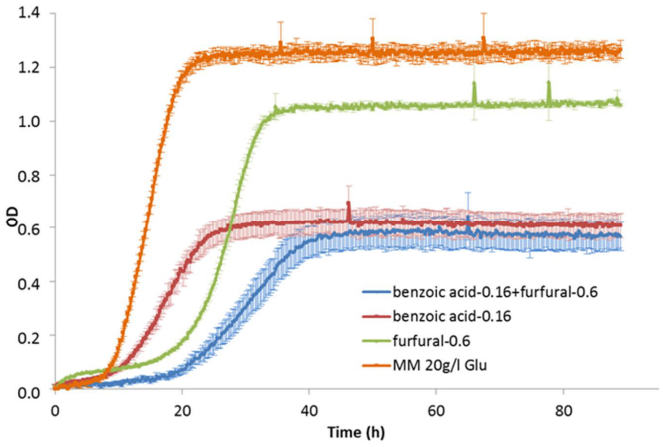


Figure 4
Combined inhibitory effects of furfural and benzoic acid on the model yeast in MM with 20 g/l glucose.
The values in the label are the compound concentrations in media (g/l).

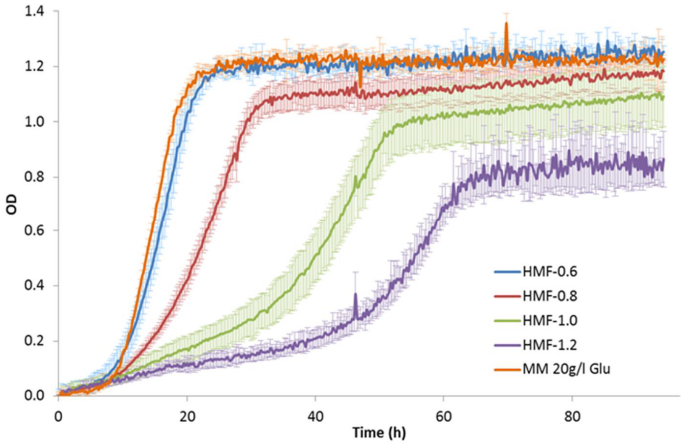
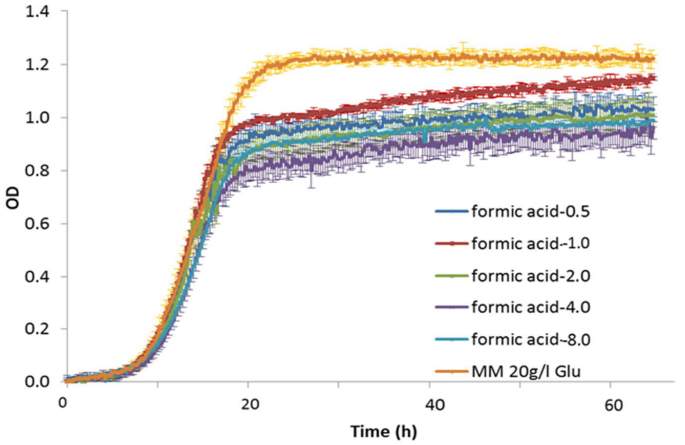


Figure 5
Inhibitory effects of HMF on the model yeast in MM with 20 g/l glucose.
The values in the label are the compound concentrations in media (g/l).



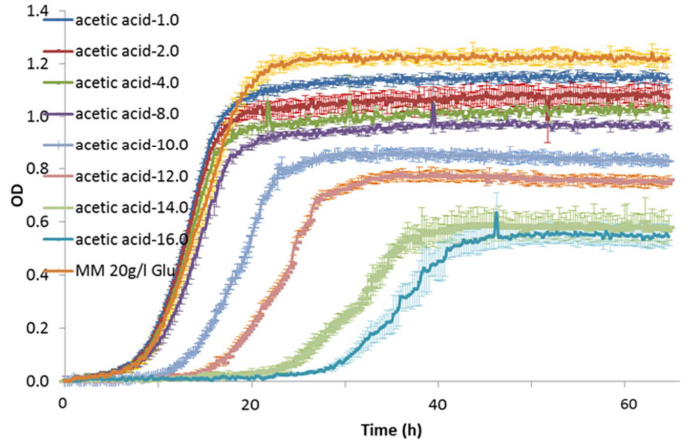


Figure 6
Inhibitory effects of formic acid (top) and acetic acid (bottom) on the model yeast in MM with 20 g/l glucose.
The values in the label are the compound concentrations in media (g/l).

Formic acid and acetic acid

The presence of formic acid at 0.5 g/l had little effect on the growth rate of the model yeast, but reduced final optical density slightly, see Figure 6. Increasing the formic acid concentration in mineral medium from 0.5 g/l to 8.0 g/l hardly enhanced this effect. The influence of acetic acid on the growth of the model yeast was similar to formic acid up to 8.0 g/l. Only when acetic acid concentration exceeded 8.0 g/l, both growth rate and final optical density were reduced significantly, and the lag-phase was clearly elongated, similar as described previously [16,36]. The highest concentrations of formic acid and acetic acid found in hydrolysates were 0.6 g/l and 8.0 g/l, respectively (Table 5). At these concentrations, the inhibitory effects of both acids were only marginal. To reach severe inhibitory effect, the level of formic acid needs to be enhanced by more than 13 folds, while the acetic acid levels are close to the inhibiting concentration. From this point of view, acetic acid is more likely to be an inhibitor in hydrolysates than formic acid.

Levulinic acid, syringic acid, vanillin and 4-hydroxybenzaldehyde

Though reported as inhibitory compounds in the hydrolysates [17], the inhibitory effects of levulinic acid, syringic acid, vanillin and 4-hydroxybenzaldehyde were only marginal. The four compounds were tested by adding them individually into mineral medium according to their highest concentrations detected in the hydrolysates. Only at 10 fold increased levels, effects became visible for these compounds, although still mild, see Figure 7.

Among the 4 compounds, vanillin with concentration 0.5 g/l gave the most inhibitory effect, which was mainly on lag-phase. Levulinic acid showed similar effect on growth as formic and acetic acid, but at a much higher concentration, 15.0 g/l. Since these 4 compounds only started to affect the growth of the model strain at a 10-fold concentration compare to their highest concentrations in hydrolysates, they are thought to be none-inhibitory in the hydrolysates.

Inhibitory effect test in hydrolysates

The inhibitory effect tests of the selected compounds in mineral medium suggested that furfural and benzoic acid were the most important inhibitory compounds. They affected the growth of the model strain considerably at their concentrations presented in the hydrolysates. As hydrolysates have a total different matrix compare to mineral medium, it is interesting to test if these two compounds display similar inhibitory effect in hydrolysates.

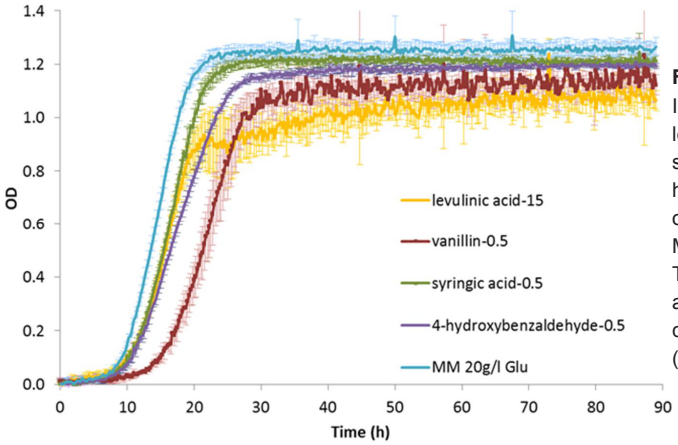
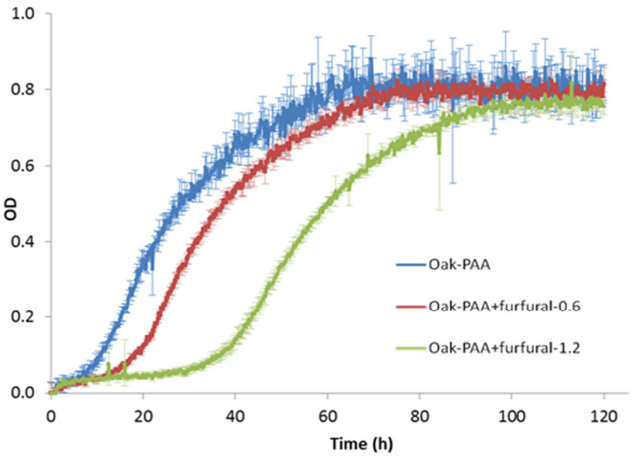


Figure 7
Inhibitory effects of levulinic acid, vanillin, syringic acid and 4-hydroxybenzaldehyde on the model yeast in MM with 20 g/l glucose. The values in the label are the compound concentrations in media (g/l).



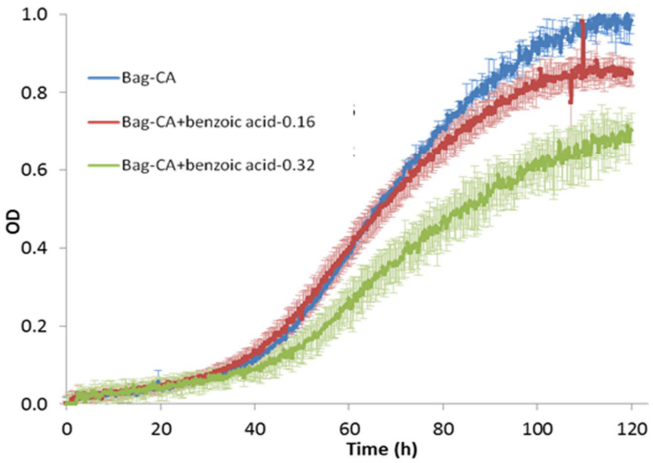


Figure 8
Inhibitory effects of furfural in Oak-PAA hydrolysate (top) and benzoic acid in Bagasse-CA hydrolysate (bottom), on the model yeast.
The values in the label are the compound concentrations in media (g/l).

Furfural and benzoic acid were tested in Oak-PAA and Bagasse-CA hydrolysate, respectively. The reason for using these two hydrolysates was that Oak-PAA was a furfural free hydrolysate and Bagasse-CA was benzoic acid free. The testing concentrations of both compounds were one and twofold of their highest levels in the hydrolysates, 0.6 g/l and 1.2 g/l of furfural, and 0.16 g/l and 0.32 g/l of benzoic acid (Figure 8).

As shown in Figure 8, the inhibition effects of both compounds were also observed in hydrolysates and similar to those seen in mineral medium. The presence of furfural lengthened the lag-phase of the growth in Oak-PAA hydrolysate, and benzoic acid affected the growth rate and the final optical density level in Bag-CA hydrolysate. However, these effects were milder than in mineral medium, as by adding 1.2 g/l furfural in mineral medium, the lag-phase increased to 40 h, while the lag-phase enhanced to only 30 h when added into hydrolysate. For benzoic acid, in mineral medium the optical density dropped to half of that in reference medium when 0.16 g/l was present, while in hydrolysate the optical density decreased less than 10% (Figure 2, 8). These results indicate that the hydrolysate matrix buffers inhibitory effects. It can also be seen in Figure 8 that the pattern of the growth curve of both hydrolysates changed little by adding either furfural or benzoic acid. This suggests that the growth curve pattern of a hydrolysate is determined by the combined structure of most or all the compounds present in it.

Conclusion

This study showed that the fermentation performance of different hydrolysates varied in lag-phase, growth rate and biomass yield, as well as their composition as far as the selected compounds are considered. These differences among hydrolysates seem to be caused mainly by hydrolysate preparation method, and secondly by biomass type. The detection of the 11 selected compounds in fermentation samples revealed that the levels of most compounds changed during fermentation process. Remarkably, furfural was converted to furfurylalcohol and possibly also 2-furan methanol acetate in the fermentation lag-phase. The toxicity test of the 11 selected compounds showed that furfural and benzoic acid exhibited clear inhibitory effects on model yeast *S.cerevisiae* CEN.PK 113-7D at their concentrations detected in hydrolysates, while the effects of acetic acid and HMF were minor, but enhanced dramatically at the increase of concentration.

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