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

Zha, Y.

CHAPTER 2

Preparation and Evaluation of Lignocellulosic Biomass Hydrolysates for Growth by Ethanologenic Yeasts

Ying Zha, Ronald Slomp, Johan van Groenestijn, and Peter J. Punt

This chapter is published with minor modification in:
Microbial Metabolic Engineering: Methods and Protocols, 2012, 245-259

Abstract

Lignocellulosic biomass is a potential feedstock for bioethanol production. Biomass hydrolysates, prepared with a procedure including pretreatment and hydrolysis, are considered to be used as fermentation media for microorganisms, such as yeast. During the hydrolysate preparation procedure, toxic compounds are released or formed which may inhibit the growth of the microorganism and thus the product formation. To study the effects of these compounds on fermentation performance, the production of various hydrolysates with diverse inhibitory effects is of importance. A platform of methods that generates hydrolysates through four different ways and tests their inhibitory effects using Bioscreen C Analyzer growth tests is described here. The four methods, based on concentrated acid, dilute acid, mild alkaline and alkaline/oxidative conditions, were used to prepare hydrolysates from six different biomass sources. The resulting 24 hydrolysates showed great diversity on growth rate in Bioscreen C Analyzer growth tests. The approach allows the prediction of a specific hydrolysate's performance and helps to select biomass type and hydrolysate preparation method for a specific production strain, or vice versa.

Introduction

Lignocellulosic biomass, such as sugar cane bagasse, wheat straw and willow wood, are potential feedstocks for bioethanol production. They are inexpensive, abundant and not competitive with world food resources. Generally, lignocellulosic biomass is composed of cellulose, hemicellulose and lignin. To use lignocellulosic biomass as fermentation feed, a pretreatment and a hydrolysis step need to be carried out, which breaks the structure of the biomass and releases the sugar monomers, respectively. The resulting mixture after removing the lignin fraction, the lignocellulosic biomass hydrolysate, is then used as fermentation medium.

One of the drawbacks of the lignocellulosic biomass hydrolysate, when used as feedstock for bioethanol production, is its inhibitory effect on the growth of microorganisms [1,2]. The inhibitory effects are caused by the release of toxic compounds during the hydrolysate preparation process, since conditions like high temperature, high pressure, and acid/alkaline environment are used for the pretreatment. Growth inhibition mainly results in a longer lag-phase, a reduced growth rate and a lower ethanol productivity; and was found in both laboratory and industrial strains [3-5].

To restrict the inhibitory effect of the hydrolysate, identifying inhibitory compounds is a crucial step. In the last several decades, studies on hydrolysate inhibitory compounds have been conducted; the results showed that the potential inhibitors are aliphatic and aromatic acids, aldehydes and ketones from the degradation products of cellulose and hemicellulose, as well as phenolic compounds from lignin [6,7]. Among all the potential inhibitors, formic acid, acetic acid, furfural and hydroxymethylfurfural (HMF) were considered as the representatives, and were used to test the inhibition resistance of different microorganisms [8,9]. Heer *et al.* reported the identification of furfural as a key inhibitor in the wheat and barley straw hydrolysates, using ethylacetate extraction GC-MS [10].

Due to the complexity and the variability of the lignocellulosic biomass hydrolysate composition, and the requirement of advanced tools, such as LC-MS, GC-MS and solid-phase microextraction (SPME), for chemical analysis, the identification of inhibitory compounds has not yet been carried out systematically. In our research, we aim to use a non-targeted metabolomics approach to elucidate the substrate inhibitory compounds. The first requirement of this approach is a high-quality experimental design [11,12]. Such an experimental design should include a variety of hydrolysates that hold diverse inhibitory

effects on fermentation. It is expected that some inhibitory compounds are universal, while the others are more specific to a particular hydrolysate.

In our study, six types of biomass are selected and prepared with four different methods to test the inhibitory effects of the resulting 24 hydrolysates on different microorganisms. The procedures of the four hydrolysate preparation methods, namely, concentrated acid, dilute acid, mild alkaline and alkaline/oxidative method, are described here. In addition, we describe the procedure of the growth test using Bioscreen C Analyzer [13]. The results show that there is a great diversity of inhibitory effects among the 24 hydrolysates, which is contributed by both biomass type and hydrolysate preparation method.

The method provides a platform for testing lignocellulosic biomass hydrolysates on various microorganisms, especially bacteria and yeasts. The results obtained with this analysis platform will help to select biomass type and hydrolysate preparation method for a specific production strain, or vice versa.

Materials

Lignocellulosic biomass

Sugar cane bagasse is a kind gift from Zilor, Brazil. Corn stover is from the University of Cape Town, South Africa. Wheat straw, barley straw and willow wood are purchased from Oostwaardshoeve, the Netherlands. Oak sawdust is from ESCO, the Netherlands, a wood-flooring supplier. All biomass is pre-dried at 80°C for 5 h when received, and stored at room temperature. All biomass, except oak sawdust, is ground to pieces with average length of 3 mm.

Hydrolysate preparation

H_2SO_4, purity 95–97%, stored at room temperature. NaOH, purity ≥ 98%, stored at room temperature. $Ca(OH)_2$ purity ≥ 96%, stored at room temperature. Acetic acid, purity 100%, stored at room temperature. 30% H_2O_2, stored at 4°C. Penicillin:streptomycin (pen:strep) solution: 10000 unit penicillin and 10 mg streptomycin/ml. Used at 1:100 (v:v) dosage, stored at −20°C. Enzyme cocktail Accellerase 1500 (a gift from GENENCOR): endoglucanase activity 2200–2800 CMC U/g; beta-glucosidase activity 525–775 pNPG U/g.

Rotation is carried out on a two-deck modular roller apparatus (348971-C) from Wheaton Roller. All filtrations, unless otherwise stated, use Grade GF/D filter purchased from

Whatman. Concentration of H_2SO_4 is tested with HACH LANGE SO_4^{2-} testing kit (LCK353). Anion exchange is carried out with a MP-Cell from ElectroCell A/S (Tarm, Denmark), which includes two PVDF fluid distribution frames, four EPDM gaskets and two PVDF end frames. Anion exchange membranes are Neosepta AFN membranes from Eurodia Industrie SA, France. The ElectroCell equipment is assembled with stainless steel end plates and hardware, indicated as "EC" in Figure 1 . Liquid in both donor and acceptor compartment are pumped in a circuit over a glass bottle, a pump and the cell and back to the bottle again. The liquid volume of the circuit is 0.45 l. The flow direction over the two cells was counter current at a rate of 0.5 l/min.

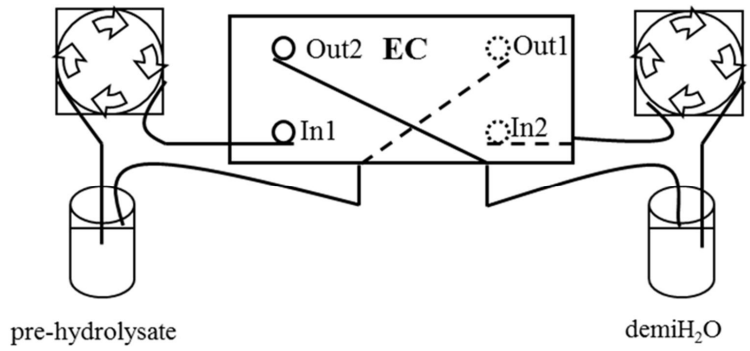

Figure 1 Ion exchange membrane facility for neutralization. EC: ElectroCell.

Yeast strains

Saccharomyces cerevisiae CEN.PK 113-7D is purchased from CBS Yeast Collection (CBS 8340). Ethanol Red yeast is a gift from Fermentis, France. Both strains are stored as 1 ml glycerol stocks at −80°C.

Bioscreen C Analyzer growth study

Bioscreen C Analyzer, Labsystems OY, Helsinki, Finland. In the Bioscreen C Analyzer, two honeycomb plates, each contains 100 wells, can be incubated at a preset temperature, and the growth of the microorganisms can be monitored by measuring optical density (OD 420–580) at a preset time interval, in our case, 15 min. The honeycomb plates can be shaken linearly at a preset speed. The Bioscreen C Analyzer is connected to a computer, which records all the measured optical density values.

Mineral medium (MM) is prepared according to the composition as described by van Hoek *et al.* [14]. Per liter: 5.0 g $(NH_4)_2SO_4$, 3.0 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 1 ml 1000x trace elements solution, 1 ml 1000x vitamin solution and 80 ml 250 g/l glucose solution.
Per liter 1000x trace element: 15.0 g EDTA, 4.5 g $ZnSO_4 \cdot 7H_2O$, 0.3 g $CoCl_2 \cdot 6H_2O$, 1.0 g $MnCl_2 \cdot 4H_2O$, 0.3 g $CuSO_4 \cdot 5H_2O$, 4.5 g $CaCl_2 \cdot 2H_2O$, 3.0 g $FeSO_4 \cdot 7H_2O$, 0.44 g $Na_2MoO_4 \cdot 2H_2O$, 1.0 g H_3BO_4 and 0.1 g KI.
Per liter 1000x vitamin solution: 0.05 g biotin, 1.0 g calcium pantothenate, 1.0 g nicotinic acid, 25.0 g inositol, 1.0 g thiamine·HCl, 1.0 g pyridoxine·HCl, and 0.2 g *para*–aminobenzoic acid.
250 g/l glucose solution is sterilized by autoclaving at 110°C, 1000x vitamin solution is sterilized by filtering through 0.2 µm filter and 1000x trace element solution and the $(NH_4)_2SO_4$, KH_2PO_4 , $MgSO_4 \cdot 7H_2O$ solution are sterilized by autoclaving at 121°C.

Methods

To investigate the inhibitory effects of the lignocellulosic biomass hydrolysates using exo-metabolomics, generating a diverse collection of hydrolysates is crucial [11,12]. Biomass type and hydrolysate preparation method are the sources for creating such diversity. In particular, the hydrolysate preparation method is a target of optimization. Therefore, establishing various hydrolysate preparation methods and applying them on different biomass types is of importance to allow a meaningful experimental design for process improvement.

The four hydrolysate preparation methods selected in our study are (1) concentrated acid, (2) dilute acid, (3) mild alkaline, and (4) alkaline/oxidative. Each method composes of a pretreatment step, which breaks down the structure of the biomass, and a hydrolysis step, which hydrolyzes the cellulose and hemicellulose into sugar monomers. Dilute acid, mild alkaline and alkaline/oxidative are relatively mild methods; they employ acidification, alkalization and oxidation for pretreatment, respectively. Dilute acid pretreatment takes place under high pressure for several minutes; alkaline/oxidative pretreatment is carried out at high temperature for many hours; while mild alkaline pretreatment undergoes high temperature for multiple days. All three methods apply enzymatic hydrolysis, which can be conducted in a fed-batch manner to reach high glucose concentration in the hydrolysate. Concentrated acid is more aggressive, it uses concentrated sulfuric acid for both pretreatment and hydrolysis. This method is more suitable for preparing hard wood hydrolysate than the three mild methods.

The detailed procedure of these four hydrolysate preparation methods are described in the following two sections, separating pretreatment and hydrolysis.

Pretreatment

To prepare 1 l lignocellulosic biomass hydrolysate, 500 g dried biomass is used for concentrated acid method, while 300 g is used for the other three methods. The pre-dried biomass contains 5–10% free water, indicating that the initial biomass amount before drying should be above 550 and 330 g. The drying takes place in an oven at 80°C, for at least 16 h.

Concentrated acid

500 g dried biomass is impregnated with 72% H_2SO_4 in a 5 l Duran laboratory glass bottle. The biomass dry matter to H_2SO_4 ratio is 1:2 (w:w), the concentration of 72% H_2SO_4 is 1155 g/l, so the amount of 72% H_2SO_4 added is 866 ml. The biomass is impregnated by rotating the bottle on the two-deck modular roller at room temperature for 24–48 h, rotating speed 10 rpm. The pretreated biomass is stored at 4°C before hydrolysis.

Dilute acid

300 g dried biomass is soaked in 2% (20.28 g/l) H_2SO_4, at a ratio of 8 g dried biomass/100 ml 2% H_2SO_4, in a 5 l polypropylene (PP) bucket, i.e. in total 3750 g 2% H_2SO_4 is used. The biomass is impregnated in an oven at 60°C for minimum 3 h, with the bucket lid closed. The impregnated biomass is cooled down to room temperature, and the free liquid in the wet biomass is partially removed by vacuum filtration. The wet biomass is transferred onto an aluminum dish, which is then autoclaved at 160°C for 3.5 min. After the wet biomass is cooled down to room temperature, its liquid content is determined by measuring the total wet biomass weight. If the liquid content in the biomass exceeds 1000 g, i.e. if the biomass dry matter content is below 23%, the wet biomass is air dried at room temperature (20°C) until the liquid content drops below 1000 g (see Note 1). The wet biomass is stored at 4°C before enzymatic hydrolysis.

Mild alkaline

A $Ca(OH)_2$ suspension is added to the dried biomass at 0.15 g $Ca(OH)_2$ /g biomass and at a 20% biomass dry matter content in a 5 l PP bucket: 300 g biomass is mixed with 48 g $Ca(OH)_2$ suspended in 1500 g demineralized water. The biomass is impregnated by placing the bucket in an oven at 80°C for 3–5 days, with the bucket lid closed. After the wet biomass is cooled down to room temperature, its liquid content is determined by measuring the total wet biomass weight. The wet biomass is air dried at room temperature (20°C) to reduce its

liquid content to below 1000 g, i.e. biomass dry matter content increases to 23% (see Note 1). Store the biomass at 4°C before enzymatic hydrolysis.

Alkaline/Oxidative

This pretreatment method is adapted from a method described by Zhao *et al.* [15]. 300 g dried biomass is mixed with 3.3% NaOH at a ratio of 1:3 (w:v) in a 5 l Duran laboratorial glass bottle. The biomass is impregnated by placing the bottle in a water bath at 90°C for 90 min (see Note 2). The impregnated biomass is cooled down to room temperature and washed with demineralized water, using vacuum filtration, to remove the detached and dissolved lignin (see Note 3). Vacuum filtration is continued at the end of the washing to increase the dry matter content of the wet biomass.

Prepare peracetic acid (PAA) by adding acetic acid, 30% H_2O_2 and 96% H_2SO_4 at a ratio of 60:40:1 (v:v:v) together, and allowing to react at room temperature for 72–96 h before use. As soon as the PAA solution is ready for use, add it to the washed biomass at a ratio of 1:1 (w:w) with respect to the initial dried biomass amount, i.e. 300 g, in a 2 l Duran laboratory glass bottle. The mixture is impregnated in an oven at 70°C for 2 h, while the bottle is rotated at a speed of 10 rpm on the two-deck modular roller apparatus. After the impregnation, the biomass is washed with demineralized water to remove PAA. The washing is facilitated by vacuum filtration, and PAA is considered as removed when washing water pH reaches 3.5. The washed biomass is mixed with 3–4 l demineralized water, and the slurry pH is adjusted to 5.0 using 6 M NaOH. The free liquid in the mixture is then partially removed by vacuum filtration. The liquid content of the vacuum filtrated biomass is determined by measuring the total wet biomass weight. If liquid content of the wet biomass exceeds 1000 g, i.e. if the biomass dry matter content is below 23%, the wet biomass is air dried at room temperature (20°C) till the liquid content drops below 1000 g (see Note 1). Store the biomass at 4°C before enzymatic hydrolysis.

Hydrolysis

Two different hydrolysis methods are applied to the pretreated biomass, namely acid hydrolysis and enzymatic hydrolysis. The biomass pretreated with concentrated acid is hydrolyzed with acid, while the biomass pretreated with either dilute acid, mild alkaline, or peracetic acid method is hydrolyzed enzymatically.

Acid hydrolysis

At the end of the impregnation, 900 ml demineralized water is added to the impregnated biomass to reduce SO_4^{2-} concentration to around 42% (570 g/l). The slurry is then pre-hydrolyzed in the bottle in an incubator at 60°C for 8 h, with shaking speed 100 rpm (see Note 2). The pre-hydrolyzed slurry is cooled down to room temperature and vacuum filtrated to separate the liquid and the solid phase. The filter cake, which has around 50% biomass dry matter content, is collected and stored at 4°C, while the filtrate, the pre-hydrolysate, is neutralized by using anion exchange membranes to reduce SO_4^{2-} concentration to a required level (see Note 4). The anion exchange process is facilitated with ElectroCell (EC) equipment and two pumps, as shown in Figure 1. The donor compartment is filled with pre-hydrolysate, while the acceptor compartment contains 5 l demineralized water. In about 5 days, the SO_4^{2-} concentration of the pre-hydrolysate is reduced to the required level (see Note 4); during which the acceptor, demineralized water, is refreshed daily, and the SO_4^{2-} concentration is checked daily with HACH LANGE LCK 353 kit (see Note 5).

The neutralized pre-hydrolysate is then combined with the filter cake from the pre-hydrolysis in a 2 l Duran laboratory glass bottle. The slurry is hydrolyzed in a second round by placing the bottle in a water-bath at 95°C for 3 h (see Note 2). After the hydrolysis, the slurry is cooled down to room temperature and vacuum filtrated. The filter cake, mainly composed of lignin, is discarded. The filtrate, the hydrolysate, is again neutralized with the anion exchange membranes facilitated with EC equipment, as described before. In about 5 days, the SO_4^{2-} concentration is reduced to below 15 g/l (1.5%); during which the acceptor, demineralized water, is refreshed daily, and the SO_4^{2-} concentration is checked every other day (see Note 5). After anion exchange neutralization, the hydrolysate pH is adjusted to 5.0 using $Ca(OH)_2$ (see Note 6). The hydrolysate is sterilized by using 0.2 µm filters (see Note 7) and stored at −20°C before use.

Enzymatic hydrolysis

The free liquid content of the wet biomass after pretreatment, W_{FL} , is determined by measuring the total wet biomass weight (see (1)).

$$W_{FL}\,(g) = W_{WB} - W_{DB} \qquad (1)$$

Where, W_{WB} is the total wet biomass weight (g)

W_{DB} is the initial dried biomass weight (300 g)

W_{H2O} amount of demineralized water is added to a 2 l Duran laboratorial glass bottle (see

Note 8 and (2)).

$$W_{H2O}\ (g) = 1200\ g - W_{FL} \quad (2)$$

Wet biomass is added into the same bottle until a mixable slurry is formed (see Note 9). Record the amount of wet biomass added as W_{PWB} and calculate the added amount of initial dried biomass, W_{PDB} , using (3).

$$W_{PDB}\ (g) = \frac{W_{PWB}}{W_{WB}} \times 300\ g \quad (3)$$

Enzyme Accellerase 1500 and pen:strep are then added into the slurry according to the mixing ratio, 0.5 ml Accellerase 1500/g initial dried biomass, and 1 ml pen:strep/100 ml slurry.

Estimate the H_2SO_4 or the $Ca(OH)_2$ content, W_{PH2SO4} or $W_{PCa(OH)2}$, in the slurry if dilute acid or mild alkaline pretreated biomass is used, with (4a) and (5a). Pre-neutralize the slurry by adding either $Ca(OH)_2$ powder or pure H_2SO_4, $W_{ACa(OH)2}$ or W_{AH2SO4} , according to (4b) and (5b).

$$W_{PH2SO4}\ (g) = (W_{PWB} - W_{PDB}) \times 0.0208\ g/g \quad (4a)$$

$$W_{ACa(OH)2}\ (g) = 95\% \times \frac{W_{PH2SO4}}{98\ g/mol} \times 74\ g/mol \quad (4b)$$

$$W_{PCa(OH)2}\ (g) = W_{PDB}\ \text{x}\ 0.15\ g/g \quad (5a)$$

$$W_{AH2SO4}\ (g) = 105\% \times \frac{W_{PCa(OH)2}}{74\ g/mol} \times 98\ g/mol \quad (5b)$$

Where, 0.0208 g/g is the concentration of 2% H_2SO_4

0.15 g/g is the adding ratio of $Ca(OH)_2$ in mild alkaline pretreatment

98 g/mol is the molar mass of H_2SO_4

74 g/mol is the molar mass of $Ca(OH)_2$

95 and 105% are rough compensation factors for reaching pH 5.0 instead of pH 7.0

The hydrolysis is conducted in an incubator at 50°C with shaking speed 150 rpm. After about 1 h, the slurry pH is measured with pH electrode and re-adjusted to 5.0 using either 6 M NaOH or 3 M H_2SO_4. The hydrolysis is then continued under the same condition till the slurry is liquefied (see Note 10). The pH of the liquefied slurry is adjusted with either 6 M NaOH or 3 M H_2SO_4 to 5.0, before the next round of wet biomass addition. Step 3–7 are repeated until all the wet biomass is used, the hydrolysis slurry is then incubated at 50°C with shaking speed 150 rpm for another 48–72 h. After the enzymatic hydrolysis, the slurry is cooled down

to room temperature and vacuum filtrated or centrifuged to separate the hydrolysate from the remaining solids. The hydrolysate is stored at −20°C before use.

Bioscreen C Analyzer evaluation

The inhibitory effects of the generated hydrolysates are examined with growth tests, measuring growth rates. The growth tests were conducted in 100-well honeycomb plates in a Bioscreen C Analyzer [13], which monitors the growth curves by measuring optical density in a preset time interval.

Preculture preparation

The preculture of the growth test is conducted in a 500 ml shake flask with 100 ml MM. The preculture is inoculated with 1 ml glycerol stock and cultivated at 30°C for about 24 h with shaking speed 200 rpm. After the incubation, the pre-culture optical density (OD600) is measured, and if it reaches approximately 3.5, 5 ml pre-culture is centrifuged at 15000 rpm, 4°C, for 20 min to separate the cells from the medium. After the centrifugation, the medium is discarded and the cells are resuspended in sterilized demineralized water to reach OD600 1.5–2.0, which is used as inoculum for Bioscreen C incubation.

Bioscreen C incubation

The growth test is conducted in triplicate in the 100-well honeycomb plates. The Bioscreen C Analyzer can cultivate two plates at the same time. To minimize medium evaporation, the wells that are on the outside border are filled with 400 µl demineralized water each, leaving 72 wells to be used for growth tests on one plate. Before filling the wells, Bioscreen C Analyzer is set at a temperature of 30°C, a detecting wavelength of 420–580 nm, a measuring interval of 15 min and no shaking (see Note 11) [13]. The experimental duration is set long enough to allow the growth in all wells to complete, e.g. 5 days. Fill each of the 72 wells with either 400 µl medium or hydrolysate according to the experimental design, and inoculate with 4 µl prepared inoculum. Place the inoculated plate(s) in the Bioscreen C Analyzer and start the experimental run. When all growth curves are complete, stop the experimental run and collect the measured optical density data from the computer. Calculate the $\ln(OD_{measure}-OD_{blank})$ value for each measurement point and plot these values against time (hours). Determine the slope of the linear part of the plot and use it as measure for growth rate.

Growth test results of 24 hydrolysates prepared from six biomass types

The hydrolysate preparation methods were applied to 6 different biomass, namely, sugar cane bagasse, corn stover, wheat straw, barley straw, oak sawdust and willow wood chips. The resulting 24 hydrolysates were used as media of the Bioscreen C Analyzer incubation to test their inhibitory effects on two model strains, *S. cerevisiae* CEN.PK 113-7D and Ethanol Red Yeast. The results are presented as growth rates in Figure 2 and 3. The reproducibility of the Bioscreen C Analyzer growth tests is high. The experiments were carried out in triplicate, of which the standard deviations of calculated growth rates were between 0.5 and 15%, with an average of 2.6%. An example of the Bioscreen C growth test results is shown in Figure 4. In general it can be seen that the optical density readings are stable for all growth curves. Occasional unstable readings are discarded for further growth rate calculations. The results show that the growth rate among different hydrolysates was diverse. This diversity was caused by both biomass type and hydrolysate preparation method, though the latter seems to be the major influencer, indicating that the forming or/and releasing of the inhibitory compounds tightly relates to the pretreatment method. As expected, the growth rates in all hydrolysates were lower than those in MM.

It can be seen from Figure 2 and 3 that both yeasts performed best in mild alkaline prepared hydrolysates, independent of the biomass type, suggesting that least inhibitory compounds were released or formed when mild alkaline was used as pretreatment method. However, the hydrolysis efficiency of the alkaline pretreated biomass was lower compare to the other 3 methods, especially when wood is used (results not shown). This makes mild alkaline a less attractive pretreatment method on industrial scale. On the contrary, the hydrolysates prepared with concentrated acid method had much stronger inhibitory effect on both yeasts. Particularly, when corn stover or oak sawdust was used, little growth was observed. Concentrated acid was a much severer method, which resulted in hydrolysates with high glucose concentration (80–95 g/l) but also strong inhibitory effects. This makes this method, because of its high efficiency, interesting for industrial use, valuable for studying the effects of inhibitory compounds in the hydrolysate.

As far as biomass type is concerned, the highest average growth rate among all four hydrolysate preparation methods was in wheat straw hydrolysates, considering concentrated acid and dilute acid methods particularly. The performance of wheat straw and barley straw hydrolysates were quite similar (see Figure 2 and 3). This observation is different from the result published by Almeida *et al*. [1], which indicated that barley straw hydrolysate is much more inhibitory than wheat straw hydrolysate. Bagasse and oak hydrolysates had the lowest average growth rate, especially in dilute acid prepared hydrolysates (see Figure 2 and 3).

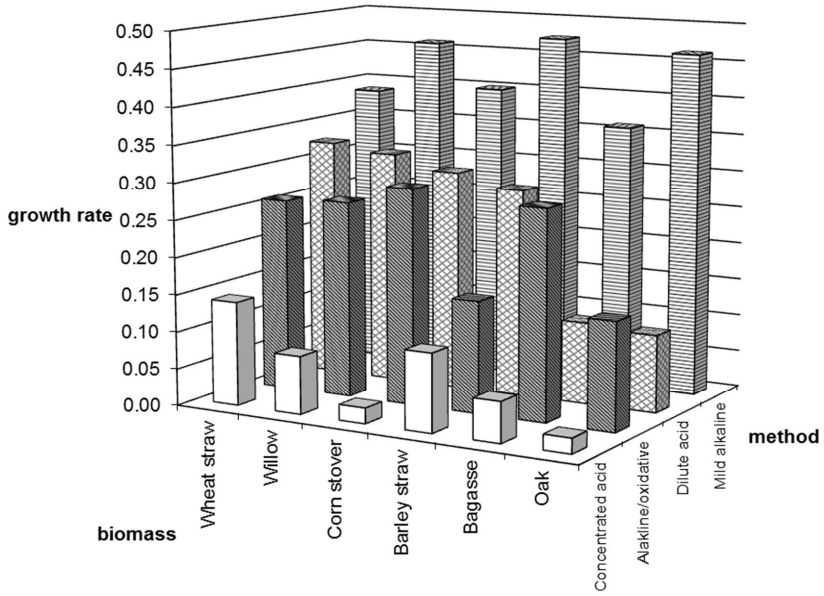

Figure 2 Growth rates of *S. cerevisiae* CEN.PK 113-7D in 24 different hydrolysates.

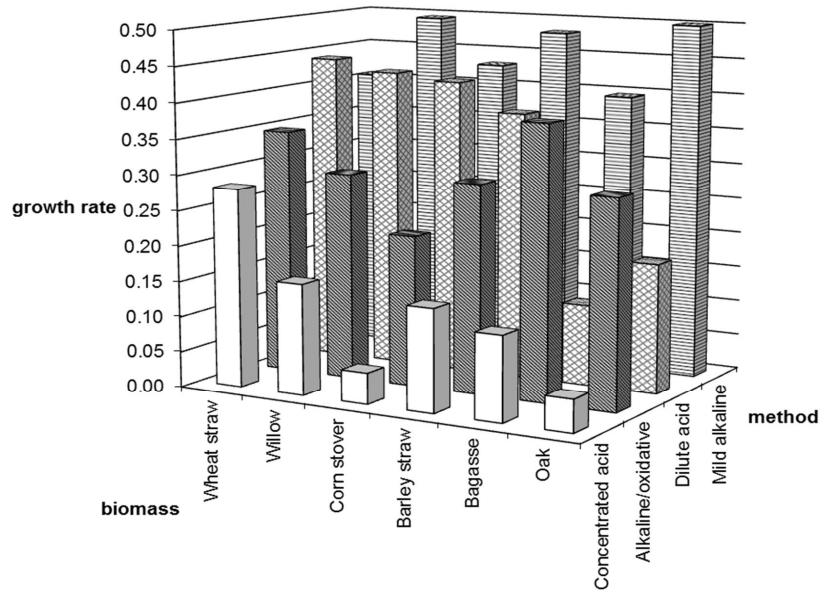

Figure 3 Growth rates of Ethanol Red Yeast in 24 different hydrolysates.

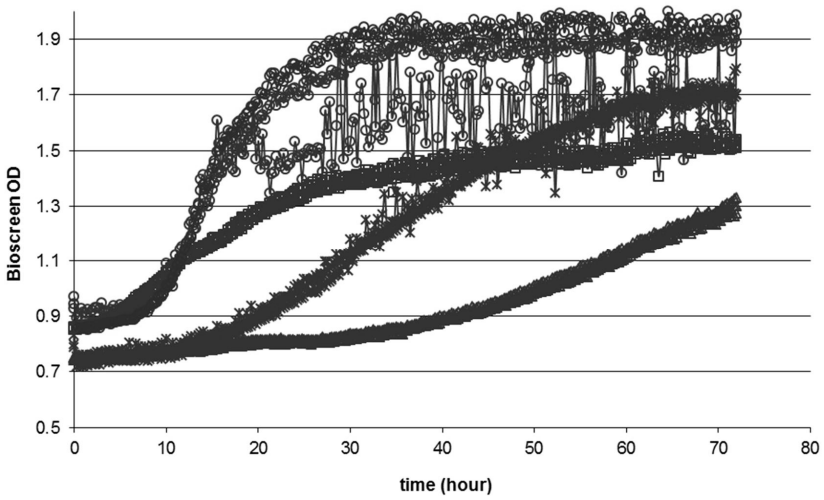

Figure 4 An example of Bioscreen C growth test results: the growth of S. cerevisiae CEN.PK 113-7D in hydrolysates prepared from bagasse. Circle, mild alkaline hydrolysate; star, dilute acid hydrolysate; square, alkaline/oxidative hydrolysate; triangle, concentrated acid hydrolysate.

The two microorganisms used in this experiment, *S. cerevisiae* CEN.PK 113-7D and Ethanol Red yeast, had similar performance in different hydrolysates, indicated by the comparable pattern of Figure 2 and 3. This may due to the close phenotype of the two microorganisms, both are ethanol production yeast strains. An overall comparison of the two strains shows that the average growth rate of Ethanol Red yeast is higher than CEN.PK, suggesting that the industrial strain is more resistant to the inhibitory compounds than the laboratorial one.

In conclusion, the method described here provides a platform for, on one hand, a quick and simple analysis of hydrolysate performance, which helps select the biomass type and the hydrolysate preparation method when a specific microorganism is used as production host. On the other hand, for screening of different microorganism strains in a particular hydrolysate to test their resistance on inhibitory compounds as well as other phenotypes, such as optimum growth pH and temperature.

Notes

1. To ensure the starting of enzymatic hydrolysis, a certain amount of water needs to be added to the pretreated biomass to form a mixable slurry. Limiting the free liquid content in the wet biomass to below 1000 ml allows the addition of minimum 200 ml demineralized water at the beginning of the hydrolysis.
2. To avoid pressure difference, don't completely close the bottle lid.
3. The washing process may cost 20–30 l demineralized water, depending on the biomass type. The detached and dissolved lignin is considered removed when the color of the washing water turns from dark black to light yellow.
4. The SO_4^{2-} concentration in the pre-hydrolysate should decrease to a specific level so that when it is mixed with the filter cake, the slurry has a SO_4^{2-} concentration of 150–200 g/l. This concentration range is optimum for the second round hydrolysis, which assists the releasing of the remaining sugars in the filter cake, while causes little sugar degradation in the hydrolysate.
5. SO_4^{2-} concentration testing kit, HACH LANGE LCK353, has a testing range of 150–900 mg/l. Therefore, the samples need to be diluted 100–1000 times before testing.
6. To avoid dilution, $Ca(OH)_2$ is added as dry powder into the hydrolysate, so pH is only measured after complete $Ca(OH)_2$ dissolution.
7. The filter sterilization is only suitable for small scale experiments. For industrial scale, contaminations may be eliminated by using antimicrobial compounds, such as antibiotics or antimicrobial plant (hop) extracts.
8. To make 1 l hydrolysate, there should be between 1300 and 1400 ml free liquid in the enzymatic hydrolysis slurry. Since, per 300 g dried biomass, in total 150 ml enzyme Accellerase 1500 will be added, 1200g - W_{FL} demineralized water is added here to make up the volume.
9. The slurry should be mixable to allow pH adjustment and to ensure the starting of enzymatic hydrolysis. For most biomass types, if 200 ml demineralized water was added firstly, the wet biomass adding amount is around 200 g.
10. It takes about 4 h to liquefy the slurry, which allows the proceeding to the next hydrolysis step.
11. Choosing no shaking is to avoid uneven cell distribution and to obtain steady optical density reading during the whole growth stage [13].

Acknowledgment

This project was co-financed by the Netherlands Metabolomics Centre (NMC) which is part of the Netherlands Genomics Initiative/Netherlands Organization for Scientific Research.

References

1. Almeida JRM, Karhumaa K, Bengtsson O, Gorwa-Grauslund MF: Screening of *Saccharomyces cerevisiae* strains with respect to anaerobic growth in non-detoxified lignocellulose hydrolysate. *Bioresource Technol.* **2009**, 100, 3674–3677.
2. Modig T, Almeida JRM, Gorwa-Grauslund MF, Lidén G: Variability of the response of *Saccharomyces cerevisiae* strains to lignocellulose hydrolysate. *Biotechnol. Bioeng*. **2008**, 100, 423–429.
3. Tofighi A, Azin M, Mazaheri Assadi M, Assadirad MHA, Nejadsattari T, Fallahian MR: Inhibitory effect of high concentrations of furfural on industrial strain of *Saccharomyces cerevisiae. Int. J. Environ. Res*. **2010**, 4, 137–142.
4. Albers E, Larsson C: A comparison of stress tolerance in YPD and industrial lignocellulose-based medium among industrial and laboratory yeast strains. *J. Ind. Microbiol. Biot.* **2009**, 36, 1085–1091.
5. Martín C, Jönsson LJ: Comparison of the resistance of industrial and laboratory strains of *Saccharomyces* and *Zygosaccharomyces* to lignocellulose-derived fermentation inhibitors. *Enzyme Microb. Tech.* **2003**, 32, 386–395.
6. Klinke HB, Thomsen AB, Ahring BK: Inhibition of ethanol-producing yeast and bacteria by degradation products produced during pre-treatment of biomass. *Appl. Microbiol. Biot.* **2004**, 66, 10–26.
7. Palmqvist E, Hahn-Hägerdal B: Fermentation of lignocellulosic hydrolysates. I: inhibition and detoxifi cation. *Bioresource Technol.* **2000**, 74, 17–24.
8. Almeida JRM, Modig T, Petersson A, Hahn-Hägerdal B, Lidén G, Gorwa-Grauslund MF: Increased tolerance and conversion of inhibitors in lignocellulosic hydrolysates by *Saccharomyces cerevisiae*. *J. Chem. Technol. Biot.* **2007**, 82, 340–349.
9. Nichols NN, Sharma LN, Mowery RA, Chambliss CK, van Walsum GP, Dien BS, Iten LB: Fungal metabolism of fermentation inhibitors present in corn stover dilute acid hydrolysate. *Enzyme Microb. Tech.* **2008**, 42, 624–630.
10. Heer D, Sauer U: Identification of furfural as a key toxin in lignocellulosic hydrolysates and evolution of a tolerant yeast strain. *Microb. Biotechnol.* **2008**, 1, 497–506.
11. Xia J, Psychogios N, Young N, Wishart DS: MetaboAnalyst: A web server for metabolomic data analysis and interpretation. *Nucleic Acids Res.* **2009**, 37, W652–W660.
12. Braaksma M, van den Berg RA, van der Werf MJ, Punt PJ: A Top-down Systems Biology Approach for The Identification of Targets for Fungal Strain and Process Development. In *Cellular and Molecular Biology of Filamentous Fungi*, Borkovich KA, Ebbole DJ, Ed., ASM Press: Washington, DC, 2010, pp. 25-35.
13. Franden MA, Pienkos PT, Zhang M: Development of a high-throughput method to evaluate the impact of inhibitory compounds from lignocellulosic hydrolysates on the growth of *Zymomonas mobilis. J. Biotechnol.* **2009**, 144, 259–267.
14. van Hoek P, van Dijken JP, Pronk JT: Effect of specifi c growth rate on fermentative capacity of baker's yeast. *Appl. Environ. Microbiol.* **1998**, 64, 4226–4233.
15. Zhao X, Peng F, Cheng K, Liu D: Enhancement of the enzymatic digestibility of sugarcane bagasse by alkali-peracetic acid pretreatment. *Enzyme Microb. Tech.* **2009**, 44, 17–23.