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On-line estimation of sugar concentration for control of fed-batch fermentation of lignocellulosic hydrolyzates by *Saccharomyces cerevisiae*

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Abstract A feed control strategy, based on estimated sugar concentrations, was developed with the purpose of avoiding severe inhibition of the yeast *Saccharomyces cerevisiae* during fermentation of spruce hydrolyzate. The sum of the fermentable hexose sugars, glucose and mannose, was estimated from on-line measurements of carbon dioxide evolution rate and biomass concentration by use of a simple stoichiometric model. The feed rate of the hydrolyzate was controlled to maintain constant sugar concentration during fed-batch fermentation, and the effect of different set-point concentrations was investigated using both untreated and detoxified hydrolyzates. The fed-batch cultivations were evaluated with respect to cellular physiology in terms of the specific ethanol productivities, ethanol yields, and viability of the yeast. The simple stoichiometric model used resulted in a good agreement between estimated sugar concentrations and off-line determinations of sugar concentrations. Furthermore, the control strategy used made it possible to maintain a constant sugar concentration without major oscillations in the feed rate or the sugar concentration. For untreated hydrolyzates the average ethanol productivity could be increased by more than 130% compared to batch fermentation. The average ethanol productivity was increased from 0.12 to 0.28 g/g h. The productivity also increased for detoxified hydrolyzates, where an increase of 16% was found (from 0.50 to 0.58 g/g h).

Keywords Ethanol, *Saccharomyces cerevisiae*, Dilute acid hydrolyzate, Inhibitors, Fed-batch control

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List of symbols

CER	carbon dioxide evolution rate (mmol/h)
F	feed rate (l/h)
k	constant used in Eq. (11) (h^{-1})
K	empirical constant, defined in Eq. (1)
n	molar flow rate (mol/h)
q	volumetric production rate (mol/l h)
r	specific reaction rate (mol/g biomass h)
S	concentration of fermentable sugar (g/l)
t	time (h)
V	volume (l)
x	mole fraction in liquid phase
y	mole fraction in gas phase
Y_{SI}	yield (mol/mol substrate) for product *I*

Subscripts

tot	total
E	ethanol
G	glycerol
S	fermentable sugar
X	biomass
CO_2	carbon dioxide
in	inlet conditions

1 Introduction

Fuel ethanol produced from lignocellulose contributes only to a little net carbon dioxidie production [1]. An increased use of ethanol as a fuel, or as a fuel additive, is therefore one option to help fulfill the commitments of the 1997 Kyoto protocol [2]. Acid or enzymatic hydrolysis of the lignocellulosic material can be used to convert the cellulose and the hemicellulose into monomeric sugars. These fermentable sugars can thereafter be anaerobically converted to ethanol by the yeast *Saccharomyces cerevisiae*. A number of by-products are, however, formed during the hydrolysis, and these compounds, e.g. furfural and hydroxymethyl furfural (HMF), may inhibit yeast metabolism [3, 4, 5, 6, 7]. Inhibition can be avoided either by detoxification prior to fermentation (e.g. by over-liming) [8], or by in situ detoxification by the yeast itself [9, 10, 11]. It has been shown for both untreated and detoxified hydrolyzate that the ethanol productivity is increased in fed-batch fermentation compared to batch fermentation [12, 13], probably because high concentrations of inhibitors can be avoided in fed-batch fermentation. It is, however, crucial to control the feed rate carefully in order to avoid inhibition effects.

Different strategies for control of the feed rate have previously been developed for fermentation of lignocellulosic

hydrolyzates. Feed rate control based on measured changes of carbon dioxide evolution rate (CER) from step changes of feed rate was used by Taherzadeh et al. [14]. The best fermentation performance in terms of specific fermentation rate was obtained when the feed rate was increased in a stepwise manner as long as the relative increase of CER was at least 50% of the relative increase of feed rate. Alternatively, feed rate control based on the derivative of CER after a step change in feed rate was used by Nilsson et al. [13]. In both of the previous studies, the fermentation strategies in which the concentration of sugar was maintained at a low level throughout the fermentation were the most successful. This could be because a complete sugar conversion also gives a minimum increase in the concentration of inhibitors, whereas accumulation of sugar in the reactor is accompanied by accumulation of inhibitors, which affects the ethanol productivity negatively. No conclusion concerning the effect of the ratio between sugar concentration and inhibitor concentration per se could be drawn from the previous experiments.

The objective of the present work was to develop a feed strategy based on maintaining a constant concentration of fermentable sugars. Since no reliable on-line sensors allowing the direct measurement of glucose and mannose in hydrolyzates are currently available, it was necessary to use model-based on-line estimation of the sugar concentration. The total concentration of fermentable sugars in the reactor was estimated based on the carbon dioxide evolution rate (CER) and the cell-mass concentration. The effect of using different concentration set-points was investigated with both untreated and detoxified hydrolyzates. The fed-batch cultivations were evaluated with respect to specific ethanol productivities, ethanol yields, and viability of the yeast.

2
Material and methods

2.1
Cultivation procedures

Wood material and hydrolysis The hydrolyzate used was produced from forest residues using a two-stage dilute-acid hydrolysis process. The forest residue originated mainly from spruce. Hydrolysis of the untreated material was carried out as described elsewhere [13], and the composition of the hydrolyzate is given in Table 1. The hydrolyzate was stored at 8°C until used. The pH of the hydrolyzate was adjusted to 5.0 prior to fermentation in two cases. This was done to minimize the potential carbon dioxide released from sodium hydroxide used to control the pH, which could otherwise affect the carbon mass balance.

Detoxification Both untreated and partially detoxified hydrolyzates were used in the study. Partial detoxification of the hydrolyzate was carried out by over-liming, in which calcium hydroxide was used to increase the pH of the hydrolyzate to 10. After incubating overnight, the pH was decreased to 5.0 (using concentrated sulfuric acid), and the hydrolyzate was centrifuged. The composition of the detoxified hydrolyzate is given in Table 1.

Yeast strain and medium *Saccharomyces cerevisiae* CBS 8066 (Centralbureau voor Schimmelcultures, the Netherlands) was used in all experiments. The strain was maintained on agar plates made from yeast extract 10 g/l, soy peptone 20 g/l, and agar 20 g/l with D-glucose 20 g/l as additional carbon source. Inoculum cultures were grown in 300-ml cotton-plugged conical flasks on a rotary shaker at 30°C for 24 h. The liquid volume was 100 ml and the shaker speed was 150 rpm. The growth medium was a defined medium according to Taherzadeh et al. [15], with the exception of the glucose concentration, which here was 15 g/l.

Table 1. Composition of the hydrolyzate before and after detoxification by over-liming. The pH of the hydrolyzate was increased to 10.0 with calcium hydroxide and maintained at this value overnight. Subsequently, the pH was decreased to 5.0 with concentrated sulfuric acid. Precipitates were removed by centrifugation

Concentration (g/l)	Untreated hydrolyzate	Detoxified hydrolyzate
Glucose	17.38	17.05
Mannose	17.46	17.58
Xylose	7.92	7.52
Galactose	3.80	3.50
Arabinose	1.90	1.96
Acetic acid	2.81	2.47
Furfural	0.39	0.23
HMF	1.85	1.00

Cultivation conditions Cultivations were made in a 3.3-l bioreactor (BioFlo III, New Brunswick Scientific, USA) at a temperature of 30°C and a stirring rate of 450 rpm. The pH value in the medium was controlled at 5.0 by the addition of 2-M NaOH or 2-M H_2SO_4. Anaerobic conditions were maintained by continuous sparging with nitrogen at a flow rate of 600 ml/min (or 1000 ml/min). The oxygen content in the gas was less than 5 ppm (ADR class 2, 1(a), AGA, Sweden). The reactor was initially filled with 1.0 l of defined medium according to Taherzadeh et al. [15], containing 50 g glucose. However, the concentrations of mineral salts and vitamins in the original medium were tripled in order to compensate for the dilution that occurred during the feed of the hydrolyzate. At the start of each experiment, 20 ml of inoculum was added to the reactor. When the glucose in the batch had been consumed, indicated by a CER value less than 1 mmol/h, 20 ml of a glucose solution was added to give the desired initial set-point. Immediately thereafter the fed-batch phase was automatically started.

On-line measurements and control The software used for data acquisition and feed rate control was developed locally in Visual Basic. Analog signals from on-line measurements were translated to digital signals using a 12-bit A/D board. Feed rate control, according to a developed model presented in Sect. 3, was obtained by manipulating the speed of a peristaltic pump (Watson-Marlow Alitea, Sweden). The medium bottle was placed on a balance connected to the computer to allow calculation of the amount of added hydrolyzate. The fed-batch operation was stopped after the addition of 1500 ml of hydrolyzate.

2.2
Analytical methods

Biomass concentration A flow-injection analysis (FIA) system [16] was used to provide measurements of optical

density every hour. Cell dry weight was determined from duplicate 10-ml samples taken three times during each fermentation. The samples were centrifuged, washed with distilled water, and dried for 24 h at 105°C. The optical density measured by the FIA system was calibrated against the dry weight samples.

Gas analysis The carbon dioxide, oxygen, and ethanol content of the outlet gas were continuously measured with an acoustic gas monitor (Innova model 1311, Denmark) described by Christensen et al. [17]. Gas measurement signals were averaged for 30 s. The gas analyzer was equipped with three channels, one for oxygen, one for carbon dioxide, and a third channel for analysis of hydrocarbons. In the present work the third channel was used for on-line estimation of ethanol concentration in the medium. Calibration was done using a gas containing 20.0% oxygen and 5.0% carbon dioxide. The ethanol signal was calibrated against ethanol concentrations measured by HPLC. The amount of carbon dioxide formed was calculated based on the measured mass flow of nitrogen into the reactor and the content of carbon dioxide in the exit gas from the reactor. Using these values, the carbon recovery in an anaerobic batch cultivation was 101–102%. To further increase the precision for on-line estimation, the gas calibration was normalized to reach 100% carbon recovery in the batch phase.

Metabolite analysis Samples for HPLC analysis were withdrawn from the reactor, centrifuged, and filtered through 0.2-μm filters. The concentrations of glucose, xylose, galactose, and mannose were determined using a polymer column (Aminex HPX-87P, Bio-Rad, USA) at 85°C. The concentrations of ethanol, glycerol, furfural, 5-hydroxymethyl furfural (HMF), and acetic acid were determined on an Aminex HPX-87H column (Bio-Rad, USA) at 65°C. All the compounds were detected on a refractive index detector, except furfural and HMF, which were detected on a UV detector at 210 nm.

It is very difficult to quantitatively recover ethanol in a condenser, and produced ethanol therefore continuously evaporates from the reactor. To correct for this evaporation in the calculation of ethanol productivity and ethanol yield, the evaporated ethanol amount was estimated according to [18]. The following equations were used:

$$y_E = K_E x_E, \tag{1}$$

$$n_E = n_{tot} \cdot y_E, \tag{2}$$

where y_E is the mole fraction of ethanol in the gas phase, x_E is the mole fraction of ethanol in the liqiud phase, and K_E is an empirical constant with the estimated value of 0.52. The molar flow rate of ethanol is given by F_E, and F_{tot} is the total molar gas flow rate in the reactor.

Determination of yeast viability The viability of the yeast was measured by determination of colony forming units (CFU). Measurements were done three times during each fermentation, except for the fastest fermentation (set-point 10 g/l with detoxified hydrolyzate), during which the viability was measured only twice. To prepare samples, 0.1 ml of different dilutions (triplicates) from the reactor were spread on agar plates (with the same composition as above). After two days the number of colonies on plates with 30–300 colonies were counted. The total cell number was determined by microscopic counting using a Bürker chamber. The viability is here given as the ratio between number of cells able to form colonies and the total cell number.

3 Control strategy

3.1 Estimation of sugar concentration

The main products formed by *S. cerevisiae* during anaerobic fermentation of hexoses, such as glucose or mannose, are carbon dioxide, ethanol, biomass, and glycerol. The overall macroscopic reaction involving these products can conceptually be divided into one "anabolic" reaction and one or more "catabolic" reactions. The division into anabolism and catabolism is, to some extent, arbitrary. For practical reasons, the overall anabolic reaction is here taken to also include part of what is sometimes referred to as catabolic reactions (cf. e.g. [19]), namely the necessary regeneration of NAD^+ by glycerol formation, and the necessary generation of NADPH by the oxidative operation of the pentose phosphate pathway. With this division, the overall anabolic reaction can be written:

$$\begin{aligned} C_6H_{12}O_6 + Y^a_{SN}NH_3 \\ \Rightarrow Y^a_{SX}CH_{1.76}N_{0.168}O_{0.56} + Y^a_{SG}C_3H_8O_3 + Y^a_{SC}CO_2 \\ + Y^a_{SW}H_2O, \end{aligned} \tag{3}$$

where Y_{SI} represents the yield (mol/mol glucose) for the product I (N is ammonium; X, biomass; G, glycerol; C, carbon dioxide; and W, water). It was furthermore assumed that NADPH was generated from glucose in the PPP by a complete oxidation of glucose. The elemental composition of *S. cerevisiae* was assumed to be that given by Verduyn et al. [20]. The catabolic reaction, here taken as simply the ethanol forming reaction, is given by

$$C_6H_{12}O_6 \Rightarrow 2C_2H_5OH + 2CO_2. \tag{4}$$

The reactions are schematically shown in Fig. 1. By combining Eqs. (3) and (4), the relation between the volumetric production rates q (mol/l h) and the specific rates of the reaction r (mol/g biomass h) given by Eq. (5) can be derived.

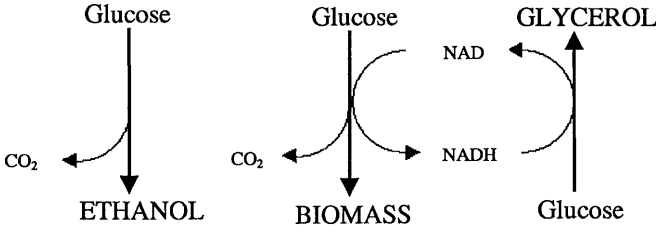

Fig. 1. Schematic picture of the reactions used in the model for on-line estimation of sugar concentration

$$\begin{bmatrix} q_{CO_2} \\ q_X \\ q_E \\ q_G \\ q_S \end{bmatrix} = \begin{bmatrix} Y^a_{SC} & 2 \\ Y^a_{SX} & 0 \\ 0 & 2 \\ Y^a_{SG} & 0 \\ -1 & -1 \end{bmatrix} \begin{bmatrix} r_1 X \\ r_2 X \end{bmatrix}. \quad (5)$$

Note that the reaction rate of reaction (4) has been normalized with respect to X, although no biomass is formed in reaction (4). The overall biomass yield Y_{SX} can be calculated, where the ratio between r_2 and r_1 will depend on the biomass yield on ATP

$$Y_{SX} = \frac{Y^a_{SX}}{I + (r_2/r_1)}. \quad (6)$$

Only the volumetric production rate of biomass q_X and carbon dioxide q_{CO2} were measured on-line in the present work. However, the nonmeasured volumetric production rates can readily be calculated from

$$\begin{bmatrix} q_E \\ q_G \\ q_S \end{bmatrix} = \begin{bmatrix} 0 & 2 \\ Y^a_{SG} & 0 \\ -1 & -1 \end{bmatrix} \begin{bmatrix} Y^a_{SC} & 2 \\ Y^a_{SX} & 0 \end{bmatrix}^{-1} \begin{bmatrix} q_{CO_2} \\ q_X \end{bmatrix}. \quad (7)$$

The value of q_S can therefore be calculated on-line, provided that the yield coefficients of the anabolic reaction are known. For this purpose, a batch fermentation was performed using synthetic medium. The yield coefficients in the anabolic reaction were calculated from this experiment and were subsequently used in the on-line calculations. The concentrations of sugars in the feed were known from off-line measurements prior to fed-batch fermentation. A dynamic mass balance therefore allowed estimation of the concentration of sugar in the reactor:

$$\frac{d(SV)}{dt} = FS_{in} - q_S V, \quad S(t=0) = S_0, \quad (8)$$

where S is the substrate concentration (mol/l), V volume (l), t is time (h), F is feed rate (l/h), S_{in} is the inlet substrate concentration (mol/l), and q_S is the volumetric substrate consumption rate (mol/l h).

Since the volume changes during the experiments, it is necessary to carefully record the reactor volume and make proper adjustments for substrate feeding, and for on-line and off-line sampling. The increase of volume from hydrolyzate feeding was calculated from on-line balance signals as previously described. Decreases in volume originating from sampling for on-line optical density analysis by FIA, for HPLC analysis, and for dry-weight samples were logged on the computer, and the decrease in volume was taken into account when the volume in the reactor was calculated. The amounts of glucose, mannose, and biomass were also corrected for sampling losses. Evaporation of ethanol was calculated from Eqs. (1) and (2). Reported ethanol concentrations were calculated from the model with the evaporation taken into account.

3.2 Control of feed rate

One option to obtain set-point control is to use the feed rate as a manipulated variable and to control the feed rate by a straightforward PID controller, in which the desired set-point concentration is compared to the estimated sugar concentration. However, such a system does not work satisfactorily because of its slow dynamics, and oscillations in sugar concentrations easily occur [21]. A somewhat different controller that takes into account not only the error between estimated the sugar concentration and the set-point but also the sugar consumption rate and the liquid volume in the reactor was therefore developed. The accumulation of substrate is described by Eq. (8). The left side of Eq. (8) can be expanded into two terms according to Eq. (9)

$$\frac{d(SV)}{dt} = V\frac{dS}{dt} + S\frac{dV}{dt} = V\frac{dS}{dt} + SF. \quad (9)$$

Combining Eqs. (9) and (8), the mass balance over the reactor can be written

$$V\frac{dS}{dt} + SF = FS_{in} - q_S V \Rightarrow F = \frac{\frac{dS}{dt}V + q_S V}{(S_{in} - S)}. \quad (10)$$

The values of S, q_S, and V can be measured (or estimated) on-line. Thus, the derivative of the substrate concentration can be predicted for any given feed rate F. It is therefore possible to choose the derivative of the substrate concentration (i.e. how fast the set-point concentration will be reached), and from this value calculate a desired feed rate. In the present work, the derivative of the substrate concentration was chosen to be proportional to the difference between the set-point concentration S_S and the estimated concentration of substrate S

$$\frac{dS}{dt} = k(S_S - S). \quad (11)$$

The value of F was determined by combining Eqs. (10) and (11)

$$F = \frac{Vk(S_S - S) + q_S V}{(S_{in} - S)}. \quad (12)$$

The value of k was chosen to be 1 1/h in all experiments. A new value for q_S was calculated every 15 min immediately after biomass concentration had been measured by the FIA system. The feed rate was updated every 5 min based on the sugar concentration in the reactor, the liquid volume, and the sugar consumption rate. The calculation of the sugar concentration was based on the carbon dioxide produced since the start of the fed-batch mode and an approximative value of the biomass content in the reactor. The amount of biomass was calculated using a linearization of four earlier measurements as long as the yeast was growing (resulting in a positive slope in the linearization). When the yeast growth stopped, an average of eight earlier measurements was used as an approximate biomass content in the reactor.

4 Results

4.1 Determination of yield coefficients

A batch fermentation was run in order to calculate the anabolic yield coefficients to be used in the model. Carbon dioxide and biomass formations were measured on-line, and samples for off-line analysis by HPLC were taken regularly. The yield coefficients in the anabolic reaction could then be calculated by elemental balancing, which resulted in the following stoichiometry:

$$C_6H_{12}O_6 + 0.451NH_3 \Rightarrow 2.687CH_{1.76}N_{0.168}O_{0.56} + 0.92C_3H_8O_3 + 0.552CO_2. \quad (13)$$

As a comparison, the stoichiometry given in Eq. (14) obtained for *S. cerevisiae* grown in a chemostat at D=0.1 h^{-1} during anaerobic conditions can be calculated from Verduyn et al. [20]

$$C_6H_{12}O_6 + 0.495NH_3 \Rightarrow 2.943CH_{1.76}N_{0.168}O_{0.56} + 0.865C_3H_8O_3 + 0.459CO_2. \quad (14)$$

There is a difference in the yield coefficients, with a somewhat lower biomass yield in the present work. It is well known that the cellular composition in terms of, for example, protein and RNA content varies depending on specific growth rate [20]. This difference in composition will also give different anabolic yield coefficients.

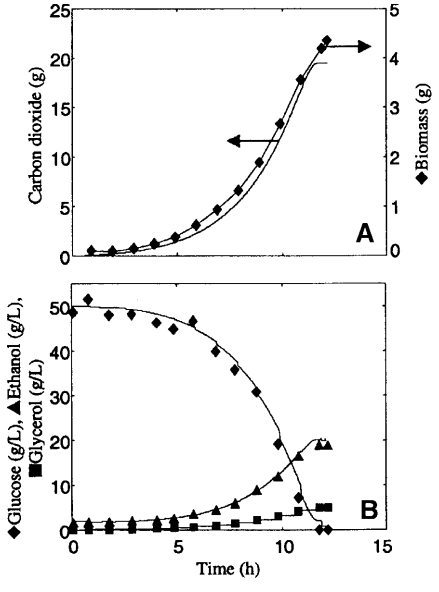

Fig. 2A, B. Batch cultivation of *S. cerevisiae* on a synthetic medium. The initial concentration of glucose in the synthetic medium was 50 g/l, and the initial volume was 1 liter: **A** formation of carbon dioxide and biomass was measured on-line; **B** off-line HPLC analysis of glucose (◆), ethanol (▲), and glycerol (■) could be compared with the estimated concentrations from the model (*solid lines*). The model takes into account changes in concentrations of glucose, ethanol, and glycerol that are due both to consumption/production and sampling. Ethanol evaporation is also included in the model

The observed yield difference here may thus be caused by the higher specific growth rate in the present work (μ=0.4 h^{-1}). The consistency of the model described by Eq. (7) is seen in Fig. 2, where model predictions as well as HPLC measurements of glucose, ethanol, and glycerol are shown for the batch cultivation.

4.2 Batch fermentation

The composition of hydrolyzate before and after detoxification can be seen in Table 1. Two batch fermentations were performed to test the toxicity of the hydrolyzates before and after detoxification (Fig. 3). In these experiments, the feeding of 1500 ml untreated hydrolyzate or detoxified hydrolyzate was started (solid line in Fig. 3A1 and 3B1) at the maximal feeding rate (approximately 1900 ml/h) immediately after the glucose in the synthetic medium initially present in the bioreactor had been consumed (CER decreased to less than 1 mmol/h). The yeast culture was clearly unable to consume all fermentable sugar present in the untreated hydrolyzate, and 14.9 g/l of sugar (corresponding to 58% added fermentable sugars) remained in the reactor after the fermentation activity had stopped. CFU measurements showed that the yeast viability was drastically lowered, and yeast samples taken 3 h after the hydrolyzate had been added were unable to form any colonies. In contrast, all fermentable

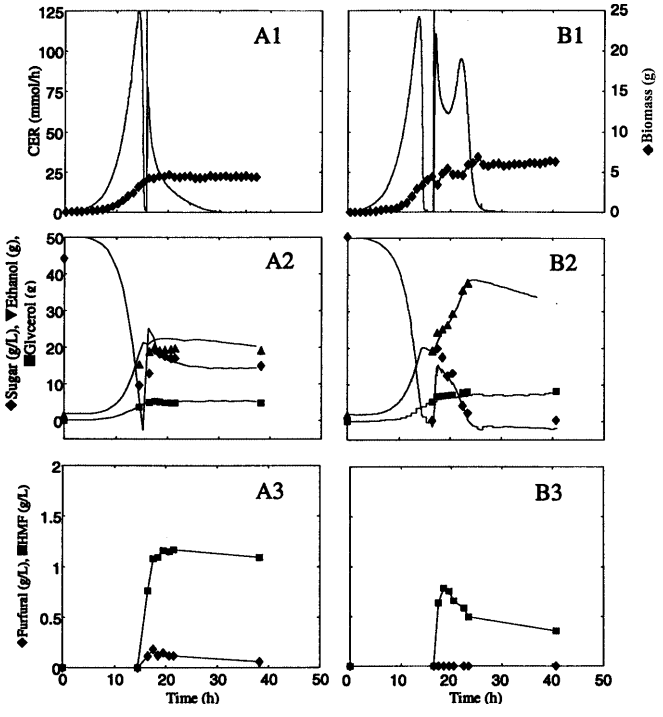

Fig. 3. Fermentation of untreated hydrolyzate (**A**) and detoxified hydrolyzate (**B**). The cultivations were started with 1 l of synthetic medium containing 50 g of glucose. When CER decreased to less than 1 mmol/h, 1500 ml of hydrolyzate was pumped into the reactor at maximum feeding rate (1900 ml/h) (*solid vertical line* in A1 and B1). *Top row*: measured CER (*left scale*), biomass (◆, *right scale*). *Middle row*: Amount of fermentable sugar (◆), ethanol (▲), and glycerol (■) present in the reactor, measured by HPLC. *Solid lines*: model estimations of fermentable sugar, ethanol, and glycerol in the reactor. *Bottom row*: measured concentrations of furfural (◆) and HMF (■)

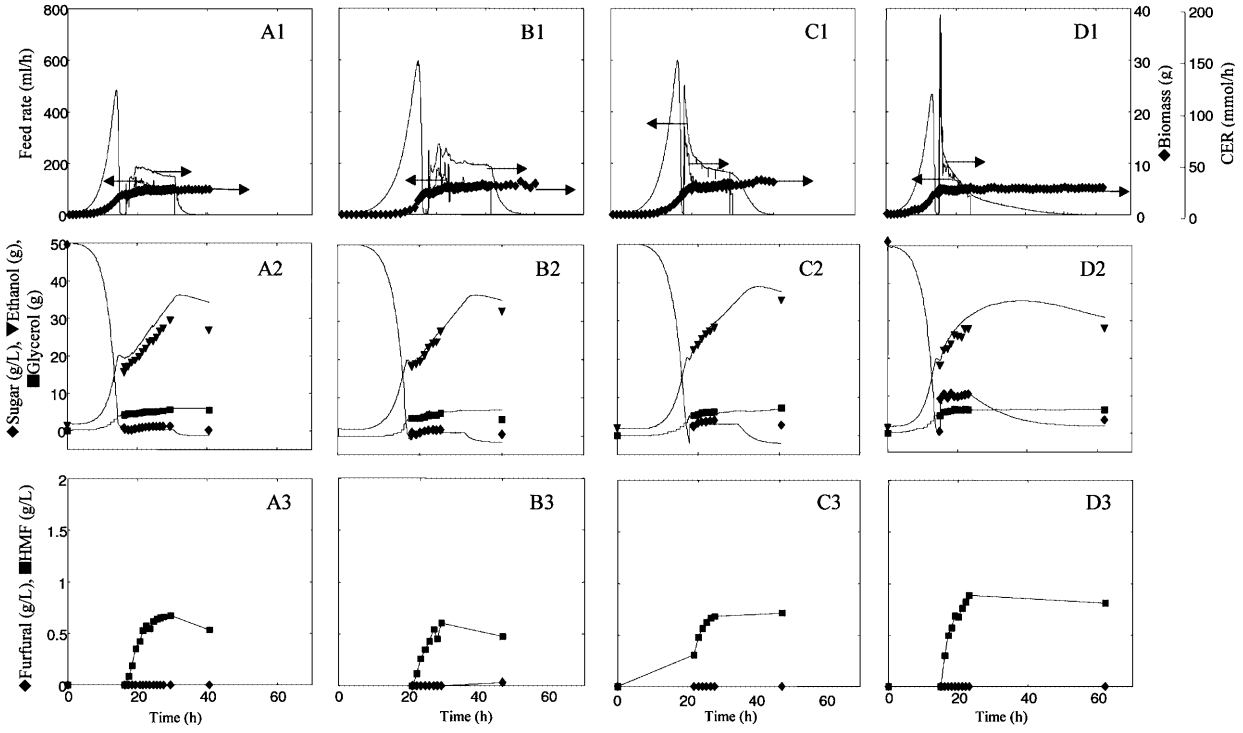

Fig. 4. Fed-batch fermentation of untreated hydrolyzates using different sugar concentration set-points: 0.5 g/l (**A**), 1 g/l (**B**), 3 g/l (**C**) and 10 g/l (**D**). When glucose (50 g/l) present in the 1 l of synthetic medium initially present in the reactor had been consumed (CER decreased to less than 1 mmol/h), the fed-batch mode was started. Initially 20 ml of a glucose solution immediately reached the set-point. *Top row*: feed rate (*left scale*), measured CER (*right scale*), biomass (◆, *right scale*). *Middle row*: Amount of fermentable sugar (◆), ethanol (▼), and glycerol (■) present in the reactor, measured by HPLC. *Solid lines*: model estimations of fermentable sugar, ethanol, and glycerol in the reactor. *Bottom row*: measured concentrations of furfural (◆) and HMF (■)

sugars in the detoxified hydrolyzate were consumed, and 2.1 g biomass was formed during after feeding of the detoxified hydrolyzate. The average ethanol productivity was 0.37 g/g h during the fermentation of the detoxified hydrolyzate, and the viability was maintained close to 100% during the entire fermentation.

4.3
Controlled fed-batch fermentation

The developed control strategy was tested using four different set-points: 0.5, 1, 3, and 10 g/l with untreated hydrolyzate as the substrate (Fig. 4). The feed rate control indeed made it possible to control the estimated sum of glucose and mannose concentration in all four cases. However, the measured sugar concentrations were slightly higher than those estimated from the model. Furthermore, somewhat less ethanol was found than predicted by the model. In the experiments with set-points 1 and 3 g/l, the pH of the hydrolyzate was set to 5.0 prior to fermentation. This was done to minimize possible errors in the CER measurements from carbon dioxide dissolved in the sodium hydroxide solution.

The chosen set-point had a significant effect on the fermentation performance. The changes in average ethanol productivity and the final viability of the cells can be seen in Table 2. Cell growth stopped shortly after the feed start in all cases. However, the viability of the cells was different for different set-points. At the end of the experiments the viabilities were 95, 100, 100, and 6.5 for set-points 0.5, 1, 3, and 10 g/l, respectively. In all experiments, furfural was completely consumed, whereas HMF was only partly consumed. In the experiments with set-points 3 and 10 g/l, the remaining concentrations of HMF were higher than at lower set-points (Fig. 4).

Two different set-points (1 and 10 g/l) were tested using hydrolyzates detoxified by over-liming as described in Sect. 2.1. Estimated concentrations from the model agreed well with the results of the off-line analyses (Fig. 5). With the detoxified hydrolyzate the fermentation performance appeared to be somewhat enhanced with a higher set-point. The average ethanol productivity was 16% higher for a set-point of 10 g/l than for 1 g/l. The viability did not

Table 2. Average ethanol productivity for the different experiments and final percentage of CFU

Set-point/batch	Hydrolyzate	Average ethanol productivity[a] (g/g h)	Final CFU (%)
Batch	Untreated	0.12	0
0.5	Untreated	0.23	95
1	Untreated[b]	0.24	>100
3	Untreated[b]	0.28	>100
10	Untreated	0.10	6.5
Batch	Detoxified	0.50	>100
1	Detoxified	0.50	94
10	Detoxified	0.58	99

[a]Average ethanol productivity was calculated for the time period from the start of feed until CER had decreased to less than 1 mmol/h
[b]pH of the hydrolyzate was adjusted to 5.0 with 2-M sodium hydroxide before fermentation

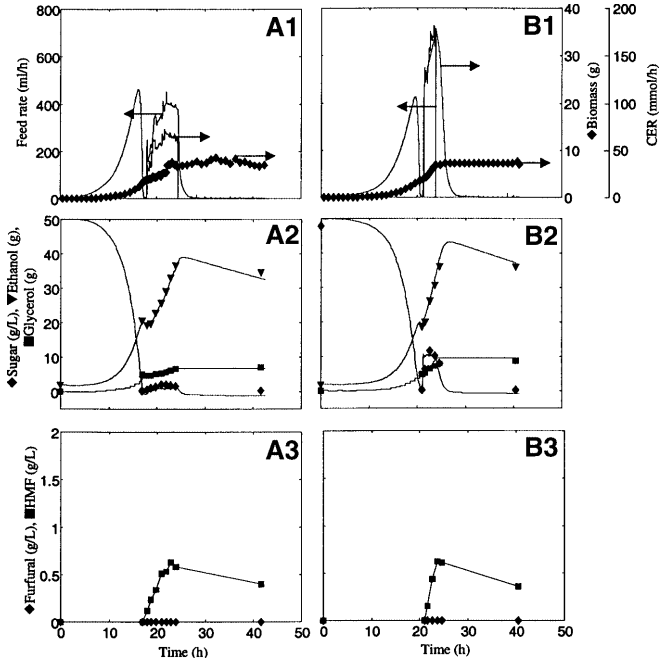

Fig. 5. Fed-batch fermentation of detoxified hydrolyzate: 1 g/l (**A**), 10 g/l (**B**). Cultivations were started with 1 l of synthetic medium containing 50 g glucose. When CER had decreased to less than 1 mmol/h the control phase was started by addition of 20 ml of a glucose solution that resulted in an immediate rise in sugar concentration up to the set-point concentration. *Top row*: feed rate (*left scale*), measured CER (*right scale*), biomass (◆, *right scale*). *Middle row*: Amount of fermentable sugar (◆), ethanol (▼), and glycerol (■) present in the reactor, measured by HPLC. *Solid lines*: model estimations of fermentable sugar, ethanol, and glycerol in the reactor. *Bottom row*: measured concentrations of furfural (◆) and HMF (■)

decrease during the fermentation but was maintained at a value between 80 and 90%. Furthermore, growth did not stop after the start of feeding. The levels of HMF were somewhat lower than found in fed-batch experiments with untreated hydrolyzates.

4.4
Sugar concentrations

The objective here was to maintain the sum of concentration of fermentable sugars at a constant level during the fed-batch phases. The fermentable sugars were glucose and mannose, and the hydrolyzates contained approximately equal amounts of these sugars (Table 1). However, the measured concentrations of these sugars in the broth were not equal during fed-batch operation (Fig. 6). Glucose was, as expected, taken up with a higher specific uptake rate than mannose, which resulted in a higher concentration of mannose during the fed-batch phase. Glucose and mannose were, however, taken up simultaneously, although mannose was taken up at a lower specific uptake rate.

5
Discussion

5.1
Fed-batch control

Most often fed-batch cultivation is made with a low concentration of sugar in the broth. In the present study,

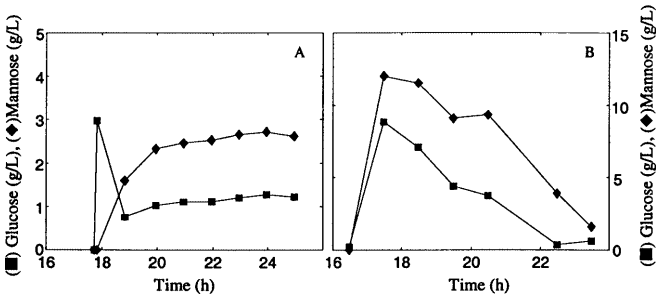

Fig. 6A, B. Concentrations of glucose (■) and mannose (◆) measured during the fed-batch phases of: **A** fed-batch experiment with set-point 3 g/l and untreated hydrolyzate as the substrate; and **B** batch experiment with detoxified hydrolyzate. The feeding started when CER decreased to less than 1 mmol/h, after the initial batch phase with 1 l of synthetic medium containing 50 g glucose. In experiment A, 20 ml of glucose solution was added at the start of the feeding phase to attain a glucose concentration of 3 g/l. In experiment B, the hydrolyzate was pumped into the reactor with the maximum feed rate (1900 ml/h)

however, it was of interest to also control the sugar concentration at a high level during the fed-batch cultivation. This seemingly modest desire gave rise to several non-trivial experimental problems. For the medium used in this study – a complex mixture containing many sugars as well as lignocellulose-derived breakdown product – reliable on-line sensors were not to be found. Therefore, the sugar concentration needed to be estimated from available on-line measurements. By a macroscopic modelling approach involving just two reactions – one anabolic and one catabolic reaction – it was possible to estimate the sum of glucose and mannose from on-line measurements of carbon dioxide and biomass. The model could then be used for on-line control of the feed rate of hydrolyzate in order to maintain a constant concentration of fermentable sugar. Considering the simplicity of the model and the complexity of the medium, the estimated sugar concentration agreed well with off-line HPLC analyses. Furthermore, with the control strategy used it was possible to maintain a fairly constant sugar concentration in the reactor, without any major oscillations in the feed rate or the sugar concentration. It deserves to be pointed out that a simple proportional control method will not work satisfactorily. This was originally tried, but resulted in major oscillations in the sugar concentration, even when a low value of the proportionality constant was used.

To allow estimation of the sugar concentrations in a batch or fed-batch operation, high accuracy of all measurements is needed. This is particularly important since measurement errors tend to accumulate because of the integration made of CER, giving a gradually increasing difference between the estimated and actual sugar concentrations. In the current work, the estimated sugar concentration was systematically somewhat lower than the measured values. The estimated sugar concentration after complete fermentation of remaining sugars was in fact found to be slightly negative. One possible source of error, which may easily be overlooked, is the carbon dioxide release from carbon dioxide that may be dissolved in the sodium hydroxide used to control the pH in the reactor. This will result in an overestimation of the ethanol

production and an overestimation of sugar consumption. Apart from measurement errors, the model used is clearly a considerable simplification of the true metabolic network. Basing the stoichiometric coefficients on values from growth on a synthetic medium probably contributes to the error between estimated and measured sugar concentrations. It is known that the conversion of furfural (which is present in both untreated and detoxified hydrolyzate) to furfuryl alcohol leads to a concomitant decrease in glycerol yield (I. Sárvári Horváth, submitted article). However, since the amount of biomass formed in the fed-batch operation is small, this should only contribute marginally to the observed offset. Another factor to consider is the presence of intracellular storage carbohydrates. Stored carbohydrates, in the form of trehalose or glycogen, can potentially be used after the exhaustion of fermentable carbon sources. The trehalose and glycogen content can be as high as 32% of the dry weight and typically occurs during nutrient limitation by nutrients other than the carbon source [22]. However, during the batch phase in our experiments glucose is the limiting substrate, and therefore storage carbohydrates only represent a small fraction of the dry weight (typically less than 3 wt%) at the end of the batch phase. Storage carbohydrates therefore are not the explanation for the estimated negative sugar concentration at the end of the fed-batch.

5.2 Fermentation performance

As previously reported in other studies [12, 13, 14], much higher average ethanol productivity (133% increase) could be obtained using fed-batch compared to batch fermentation. Even more important is, however, the fact that the yeast is unable to consume all sugar present in the untreated hydrolyzate in a batch fermentation. Also, for detoxified hydrolyzates fed-batch operation gave an increased productivity (up to 16% higher) than batch fermentation. The detoxification of the hydrolyzate in itself resulted in an average ethanol productivity up to twice that obtained with untreated hydrolyzate (Table 2). This increase must, however, be weighed against the increased costs for detoxification.

In the different fed-batch fermentations the choice of set-point concentrations had a significant effect on fed-batch performance. The average ethanol productivity during fermentation of untreated hydrolyzate increased when the set-point was increased from 0.5 to 3 g/l, but decreased to 36% of the productivity for set-point 10 g/l (Table 2). It was also seen that the CER (and consequently also the ethanol productivity) decreased during the whole fed-batch phase when set-points of 3 or 10 g/l were applied. It is very important to avoid this decrease if the fed-batch phase is to be prolonged or if the yeast is to be reused. Reuse of the yeast is clearly desirable for a large-scale process.

Severely inhibited yeast cells (as in e.g. the experiment with set-point 10 g/l and untreated hydrolyzate as the substrate) appear to be almost irreversibly inhibited. Even if the feed rate is decreased for a short while after CER has started to decrease, the feed rate will continue to decrease during the rest of the fed-batch operation. This suggests that the first contact with the inhibitors triggers further inhibition. The yeast was not able to consume all sugars present in the medium for a set-point of 10 g/l (the remaining sugar concentration was 3.5 g/l). The irreversibility of inhibition was tested also in batch fermentation with untreated hydrolyzate. Shortly after the addition of 1.5 l of hydrolyzate, the broth was centrifuged, and the cells were resuspended in 1 l of a purely synthetic medium. The specific ethanol productivity of the yeast (g/g h) in the synthetic medium was almost exactly the same as that in the hydrolyzate medium for as long as 20 h and increased first thereafter.

Growth stopped during the fed-batch experiments with untreated hydrolyzate (e.g. Fig. 4D). It is known that catabolite inactivation of sugar uptake occurs in yeast cells exposed to nitrogen starvation in the presence of a fermentable carbon source, and this results in a gradual breakdown of glucose transporters [23]. One could suspect that similar mechanisms may be involved in the gradually decreased sugar transport found in batch fermentation of untreated hydrolyzate, and possibly in the gradually decreased sugar transport seen in some of the fed-batch experiments (cf. Figs. 3Aand 4C–D). Although not caused by nitrogen limitation, the cell growth has certainly stopped and fermentable sugars are present in the medium. A few measurements of the uptake rate of glucose (measured as the initial uptake rate for 10 s of radioactively labeled sugar – data not shown) on yeast samples taken from a batch experiment with untreated hydrolyzate also showed a decreased maximal glucose uptake rate (measured for a glucose concentration of 90 g/l). The uptake rate decreased from 0.86 g glucose/g h shortly after start of the feeding to 0.44 g glucose/g h 5 h later. This decrease could, of course, be caused by a direct inhibition effect on the transport proteins, but could also be caused by a catabolite inactivation mechanism. There are 20 different genes encoding known or putative hexose transporters in *S. cerevisiae* [24]. The high-affinity glucose transporters Hxt6p and Hxt7p are repressed by glucose, whereas the low-affinity transporter Hxt1p and Hxt3p are nonrepressed. Possibly, the repression of the high-affinity transport system may be avoided by maintaining a very low glucose concentration during fed-batch operation, which provides an alternative explanation for the better performance at a low sugar set-point concentration.

A substantial part of the sugar in the hydrolyzate is mannose, which is believed to be transported by the same transporters as glucose [24]. From the sugar analyses (Fig. 6) one may conclude that the transport affinity for glucose is higher than for mannose (the concentrations of mannose and glucose in the medium are approximately equal). This is in agreement with previous reports [25].

The present work has shown how a simple stoichiometric model may be used for on-line control of sugar concentration during fed-batch operation. The performance of fermentation of dilute-acid hydrolyzates was found to be clearly affected by the sugar concentration set-point. Furthermore, the yeast cells appeared to be almost

irreversibly inhibited once they had entered an "inhibited state". Further work concerning, in particular, the sugar transport in hydrolyzates will most likely provide important information for continued improvement of the fermentation process.

References

1. Bergeron PW, Wright JD, Wyman CE (1989) Dilute acid hydrolysis of biomass for ethanol production. Energy Biomass Wastes 12:1277–1296
2. Oberthur S, Ott H (1999) The Kyoto protocol: international climate policy for the 21st century. Springer, Berlin Heidelberg New York
3. Banerjee N, Viswanathan L (1976) Effect of browning reaction products on the cell composition of *Saccharomyces cerevisiae* and *Aspergillus niger*. In: Proc Ann Conv Sugar Technol Assoc India 41, pp G75–G80
4. Chung IS, Lee YY (1985) Ethanol fermentation of crude acid hydrolyzate of cellulose using high-level yeast inocula. Biotechnol Bioeng 27:308–315
5. Sanchez B, Bautista J (1988) Effects of furfural and 5-hydroxymethylfurfural on the fermentation of *Saccharomyces cerevisiae* and biomass production from *Candida guilliermondii*. Enzyme Microbiol Tech 10:315–318
6. Taherzadeh M, Eklund R, Gustafsson L, Niklasson C, Lidén G (1997) Characterization and fermentation of dilute-acid hydrolyzates from wood. Ind Eng Chem Res 36:4659–4665
7. Larsson S, Palmqvist E, Hahn-Hägerdal B, Tengborg C, Stenberg K, Zacchi G, Nilvebrant N (1999) The generation of fermentation inhibitors during dilute acid hydrolysis of softwood. Enzyme Microbiol Tech 24:151–159
8. Larsson S, Reimann A, Nilvebrant NO, Jonsson LJ (1999) Comparison of different methods for the detoxification of lignocellulose hydrolyzates of spruce. Appl Biochem Biotechnol 38:27–40, 77–79, 91–103
9. Palmqvist E, Almeida J, Hahn-Hägerdal B (1999) Influence of furfural on anaerobic glycolytic kinetics of *Saccharomyces cerevisiae* in batch culture. Biotechnol Bioeng 62:447–457
10. Taherzadeh M, Gustafsson L, Niklasson C, Lidén G (1999) Conversion of furfural in aerobic and anaerobic batch fermentation of glucose by *Saccharomyces cerevisiae*. J Biosci Bioeng 87:169–174
11. Taherzadeh MJ, Gustafsson L, Niklasson C, Liden G (2000) Physiological effects of 5-hydroxymethylfurfural on *Saccharomyces cerevisiae*. Appl Microbiol Biotechnol 53:701–708
12. Taherzadeh M, Niklasson C, Liden G (1999) Conversion of dilute-acid hydrolyzates of spruce and birch to ethanol by fed-batch fermentation. Bioresource Technol 69:59–66
13. Nilsson A, Taherzadeh MJ, Liden G (2001) Use of dynamic step response for control of fed-batch conversion of lignocellulosic hydrolyzates to ethanol. J Biotechnol 89:41–53
14. Taherzadeh MJ, Niklasson C, Liden G (2000) On-line control of fed-batch fermentation of dilute-acid hydrolyzates. Biotechnol Bioeng 69:330–338
15. Taherzadeh M, Lidén G, Gustafsson L, Niklasson C (1996) The effects of pantothenate deficiency and acetate addition on anaerobic batch fermentation of glucose by *Saccharomyces cerevisiae*. Appl Microbiol Biotechnol 46:176–182
16. Björkqvist S, Ansell R, Alder R, Lidén G (1997) Glycerol-3-phosphate dehydrogenase mutants of *Saccharomyces cerevisiae* grown under aerobic and anaerobic conditions. Appl Environ Microbiol 63:128–132
17. Christensen L, Schulze U, Nielsen J, Villadsen J (1995) Acoustic off-gas analyser for bioreactors: precision, accuracy and dynamics of detection. Chem Eng Sci 50:2601–2610
18. Taherzadeh MJ, Gustafsson L, Niklasson C, Liden G (2000) Inhibition effects of furfural on aerobic batch cultivation of *Saccharomyces cerevisiae* growing on ethanol and/or acetic acid. J Biosci Bioeng 90:374–380
19. Battley EH (1995) A reevaluation of the thermodynamics of growth of *Saccharomyces cerevisiae* on glucose, ethanol and acetic acid. Can J Microbiol 41:388–398
20. Verduyn C, Postma E, Scheffers WA, van Dijken JP (1990) Physiology of *Saccharomyces cerevisiae* in anaerobic glucose-limited chemostat cultures. J Gen Microbiol 136:395–403
21. Ozturk SS, Thrift JC, Blackie JD, Naveh D (1997) Real-time monitoring and control of glucose and lactate concentrations in a mammalian cell perfusion reactor. Biotechnol Bioeng 53:372–378
22. Lillie SH, Pringle JR (1980) Reserve carbohydrate metabolism in *Saccharomyces cerevisiae*: response to nutrient limitation. J Bacteriol 143:1384–1394
23. Busturia A, Lagunas R (1986) Catabolic inactivation of the glucose transport system in *Saccharomyces cerevisiae*. J Gen Microbiol 132:379–385
24. Boles E, Hollenberg CP (1997) The molecular genetics of hexose transport in yeast. Fems Micobiol Rev 21:85–111
25. Larsson S, Cassland P, Jönsson L (2001) Development of *Saccharomyces cerevisiae* with enhanced resistance to phenolic fermentation inhibitors in lignocellulose hydrolysates by hetrologous expression of laccase. Appl Environ Microbiol 67:1163–1170