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Continuous Cultivation of Dilute-Acid Hydrolysates to Ethanol by Immobilized *Saccharomyces cerevisiae*

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Abstract

The continuous cultivation of immobilized *Saccharomyces cerevisiae* CBS 8066 on dilute-acid hydrolysates of forest residuals was investigated. The yeast cells were immobilized in 2–4% Ca-alginate beads. The 2% beads were not stable. However, the 3 and 4% beads were stable for at least 3 wk when an extra resource of calcium ions was available in the medium. The continuous cultivation of a dilute-acid hydrolysate by the immobilized cells at dilution rates of 0.3, 0.5, and 0.6 h^{-1} resulted in 86, 83, and 79% sugar consumption, respectively, and an ethanol yield between 0.45 and 0.48 g/g. The hydrolysate was fermentable at a dilution rate of 0.1 h^{-1} in a free-cell system but washed out at a dilution rate of 0.2 h^{-1}. The continuous cultivation of a more inhibiting hydrolysate was not successful by either free- or immobilized-cell systems even at a low dilution rate of 0.07 h^{-1}. However, when the hydrolysate was overlimed, it was fermentable by the immobilized cells at a dilution rate of 0.2 h^{-1}.

Index Entries: *Saccharomyces cerevisiae*; dilute–acid hydrolysate; ethanol; glucose; immobilization.

Introduction

Lignocellulosic materials are probably the most abundant raw materials from which to produce ethanol as a fuel. The process involves two major steps: hydrolysis and fermentation. Hydrolysis is carried out either enzymatically or chemically. Dilute-acid hydrolysis *(1)* is a favorable

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method for either pretreatment before enzymatic hydrolysis or conversion of lignocellulose to sugars *(2)*. It demands a few minutes to break down the lignocellulose to sugars. However, the fermentation time has a magnitude of 1 d. This means that every attempt to reduce the fermentation time with no or low cost would be advantageous.

The fermentation of hydrolysates has traditionally been investigated in batch processes. One of the major drawbacks of batch fermentation is the high initial concentration of the inhibitors in the medium, which results in a long lag phase or sometimes even failure of the fermentation *(3,4)*. The fed-batch process has been developed successfully to overcome this problem *(5–7)*. In spite of several advantages, continuous fermentation has not really been developed in the fermentation of acid hydrolysates. The major drawback of the continuous fermentation relates to the presence of inhibitors in the hydrolysates. The inhibitors decrease the growth rate of the cells, which results in washout of the fermentor, unless a very low dilution rate is applied, which is not economically feasible. Furthermore, if a very low dilution rate is applied to ferment severe hydrolysates, the conversion rate of the inhibitors decreases with the decreased growth of the biomass. This results in washout of the fermentor, even at a very low dilution rate. There are some methods to avoid washout such as retaining the biomass by filtration, recirculation *(8)*, or immobilization of the biomass. In the current study, we consider the possibility and advantages of immobilization of the biomass in order to efficiently produce ethanol from dilute-acid hydrolysates.

Production of ethanol by an immobilized cell system from different raw materials, such as glucose *(9)*, sucrose *(10,11)*, xylose *(12)*, cellulose *(13)*, sugarcane baggase hydrolysate *(14)*, starch *(15)*, starch hydrolysate *(16)*, pineapple cannery waste *(17)*, hardwood hemicellulose of acid hydrolysate *(18)*, wood hydrolysate *(19)*, carob pod extract *(20)*, and molasses *(21,22)*, has been of interest. The application of the immobilized cell system for ethanol production has usually been motivated by higher ethanol yield and higher ethanol productivity. Higher overall ethanol yield compared to free cells was reported when *Saccharomyces cerevisiae* was immobilized in Ca-alginate to produce ethanol *(20,23)*. Similar effects have also been reported for some other microorganisms. The immobilization of *Pinus radiata* in Ca-alginate enhanced the ethanol yield from wood hydrolysate by 13% in a continuous cultivation *(19)*. Immobilized *Candida shehatae* also produced more ethanol and less byproduct compared to free cells *(24)*. The increase in yield was probably owing to a higher activity of hexokinase, pyruvate decarboxylase, and alcohol dehydrogenase (ADH) in immobilized cells compared with suspended cells *(25)*. Immobilization increases the volumetric ethanol productivity by several times compared to free cells. The volumetric ethanol productivity is not always a general factor and should only be considered in a specific system. It usually depends on the type of microorganism, immobilization materials, sugar concentration in the feed, percentage of substrate conversion, dilution rate, and type of

bioreactor. Volumetric ethanol productivity between 3.5 and 15.3 g/(L·h) has been reported *(21,22,26–28)*.

Although several carriers have been applied for cell immobilization, alginate is the most widely used *(29)*. It is a natural cheap polymer that performs under mild conditions. The immobilized microorganisms retain their ability to produce ethanol for a number of days and even months, such as 9 d *(12)*, 10 d *(20)*, 23 d *(30)*, 30 d *(31)*, and 6 mo *(32)*. It is not quite clear whether the reported periods were limited by a decrease in the ability of the immobilized cells or some other experimental factors. However, some changes in enzyme activities probably can occur due to an increase in the age of the immobilized cells, which reduces the capacity of the cells. For instance, a decrease in NAD-specific ADH activity and an increase in NADP-specific ADH activity during prolonged production of ethanol by immobilized yeast has been reported *(25)*. Immobilization of *S. cerevisiae* into alginate beads has been successful in continuous fermentation of molasses. Sanchez et al. *(22)* obtained 97 and 48% of sugar conversion with the dilution rates of 0.97 and 5.22 h^{-1}, respectively. Nagashima et al. *(32)* successfully ran a pilot plant to produce ethanol from cane molasses with immobilized cells for 4000 h with a stable conversion rate of 95%, ethanol yield of 0.48 g/g, and productivity of 20 g/(L·h). Immobilization of *S. cerevisiae* provides a broad range of medium pH that gives an optimal growth and ethanol production *(14)*. The internal pH of immobilized cells is maintained at a fairly constant value throughout the fermentation, whereas it decreases steadily in freely suspended cells.

In the present study, we investigated ethanol production from two different dilute-acid hydrolysates by immobilized *S. cerevisiae*. Herein, we compare the results with cultivation by free cells of the yeast in both batch and continuous modes of operations. In addition, we highlight the advantages and drawbacks of immobilization on both the fermentability and fermentation rate of the hydrolysates.

Materials and Methods

Wood Material and Hydrolysis

The applied hydrolysates were produced in two-stage dilute-acid hydrolysates from Swedish forest residuals. Hydrolysis of the wood chips was carried out in a 350-L rebuilt masonite gun batch reactor. In the first stage of the hydrolysis process, water, H_2SO_4, and wood chips were added to obtain 5 g/L of the acid and 30% (w/w) of solid concentration. The impregnated wood was charged into the reactor and the reaction was started by direct steam injection, with a heat-up time of <1 min. After the heat-up period, the reactor was held at 12 bar for 7–10 min, followed by a rapid decompression and discharge of the material into a collecting vessel. The solid residue was separated from the liquid hydrolysate by filtration. Further hydrolysis of the remaining solid was carried out at a pressure of 21 bar for 7 min. The hydrolysate liquids from the two stages were then

Table 1
Composition of Two Different Hydrolysates from Swedish Wood Residues

Component	Hydrolysate I (g/L)	Hydrolysate II (g/L)
Glucose	10.2	19.1
Mannose	19.8	15.9
Xylose	11.0	7.2
Galactose	6.7	3.2
Furfural	0.8	1.0
HMF	1.8	1.3
Acetic acid	3.5	2.8

mixed and stored at 4°C before use. Although an almost identical procedure was applied for the hydrolysis of two batches of the forest residuals, it resulted in different fermentability of the batches at different seasons. The compositions of the hydrolysates are presented in Table 1.

Yeast Strain and Medium

The yeast *S. cerevisiae* CBS 8066, obtained from Centraalbureau voor Schimmelcultures (Delft, the Netherlands), was used in all experiments. The strain was maintained on agar plates made from 10 g/L of yeast extract (Merck, Germany), 20 g/L of soy peptone (Merck), and 20 g/L of agar with 20 g/L of D-glucose as an additional carbon source. Inoculum cultures were grown in 300 mL of cotton-plugged conical flasks on a shaker at 30°C for 24 h. The liquid volume was 100 mL. The growth medium was a defined synthetic medium as previously reported *(33)*.

Immobilization Procedure

A 100-mL inoculum culture with 5 g/L of biomass was mixed with a 400-mL sterilized solution of Na-alginate to give 500 mL of 2–4% alginate. The inoculum-alginate mixture was then dropped into a sterilized solution of 30 g/L of $CaCl_2$ in the fermentor and mixed gently. The formed beads of the alginate were kept in the fermentor overnight to complete the hardening process. All solutions and equipment were sterilized in an autoclave at 121°C for 20 min.

Cultivation Conditions

Anaerobic batch cultivations were carried out in a Biostat-A bioreactor (B. Braun Biotech, Germany) with a working volume of 1 L at a temperature of 30°C and a stirring rate of 250–300 rpm. The pH value in the medium was controlled by the addition of a slurry of 1 *M* $Ca(OH)_2$ in all experiments, unless otherwise stated. Nitrogen was continuously sparged through the reactor at a flow rate of 0.8 L/min (at 0°C and 1 atm) controlled by a mass flow controller (Hi-Tech, Ruurlo, the Netherlands). A peristaltic pump (Watson-Marlow Alitea AB, Sweden) was applied to feed the bioreactor.

The beads of immobilized inoculum were first subjected to a completely defined medium *(33)* with a glucose concentration of 50 g/L to prepare a high concentration of biomass inside the beads. The continuous cultivation was then carried out at different dilution rates by adding the hydrolysate and the mineral solution separately. The mineral solution contained all necessary chemicals for growth *(33)* except glucose. Continuous cultivation with free cells was also performed accordingly to make a comparison with the cells immobilized in the alginate beads.

Detoxification

Overliming was used to partially detoxify the hydrolysates *(34)*. A suspension of calcium hydroxide was used to increase the pH of the hydrolysate to 10.0, followed by an adjustment to 5.0 by adding sulfuric acid. The hydrolysate was subsequently centrifuged and autoclaved.

Analytical Methods

The carbon dioxide and oxygen content in the outlet gas were continuously measured with an acoustic gas monitor (model 1311; Innova, Denmark). Samples for high-performance liquid chromatography analysis were withdrawn from the bioreactor and then centrifuged. Glucose, xylose, galactose, and mannose were analyzed on an Aminex HPX-87P column (Bio-Rad, Hercules, CA) at 85°C. Ultrapure water was used as the eluent at a flow rate of 0.6 mL/min. Ethanol, acetic acid, succinic acid, pyruvic acid, glycerol, furfural, and hydroxymethylfurfural (HMF) concentrations were determined by an Aminex HPX-87H column (Bio-Rad) at 60°C eluted by 5 m*M* H_2SO_4 at 0.6 mL/min. A refractive index detector (Waters 410; Millipore, Milford, MA) and an ultraviolet (UV) absorbance detector (Waters 486) were used in series. Concentrations of all mentioned metabolites were determined from the refractive index chromatograms except pyruvic acid, furfural, and HMF, which were determined from the UV chromatograms at 210 nm.

Results

Stability of Ca-Alginate Beads

The stability of Ca-alginate beads was examined within repeated batch and continuous cultivation on glucose. Different concentrations of sodium alginate solution (2–4%) were mixed with inoculum and dropped into the solution of $CaCl_2$ for the preparation of gel beads. The beads were then exposed to culture medium in the presence or absence of an additional source of calcium ion. When 2% of sodium alginate was applied, the beads were not sufficiently stable. The stability of the beads in this case was shorter than 5 d in continuous cultivation, even after further addition of calcium ion to the solution. Using 3% Ca-alginate and no addition of extra calcium, the beads were stable for only 4 d, when sequential batch fermentations of

50 g/L of glucose solution were performed. The stability of the beads was also considered in continuous cultivation, in which the beads were not stable for more than 4 d. Subsequently, we have tried to improve the stability of the beads using calcium ions by adding 3 g/L of $CaCl_2$ to the medium. However, the method was not fully successful, because calcium was precipitated with phosphate. Then, the base was changed from NaOH to $Ca(OH)_2$ to provide a higher concentration of Ca^{2+}. This resulted in a good stability of the beads for at least 3 wk, where 30 g/L glucose was continuously fermented at a dilution rate 0.4 h^{-1}. Consequently, $Ca(OH)_2$ was used as a base for pH adjustment in the bioreactor for all other experiments. The beads were also prepared by a solution of 4% sodium alginate. However, no significant improvement in the stability of the beads was observed within 3 wk, in comparison to the 3% alginate. Therefore, we decided to use a 3% alginate solution for bead preparation in all other experiments.

Fermentation of Glucose by Immobilized Cells

The performance of the immobilized *S. cerevisiae* was investigated when cultivated in the synthetic medium with glucose as the carbon and energy sources. An initial biomass concentration of 0.1 g/L was immobilized in Ca-alginate and transferred to the culture. The profiles for the glucose assimilation, carbon dioxide evolution rate (CER), and ethanol production from batch cultivation are presented in Fig. 1. Ethanol was the major metabolite together with glycerol, with yields of 0.39 (±0.01) and 0.093 (±0.003) g/g, respectively. Acetic, pyruvic, and succinic acids were also detected. The yields of these carboxylic acids were approx 4, 3, and 3 mg/g, respectively. The batch cultivation was repeated six times, and the beads were separated and exposed to the medium. Since high concentrations of the yeast cells were produced in the beads by the first batch, the repeated-batch cultivations were performed in a much shorter time. More than 99% of sugars in each cultivation was assimilated in <5 (±1) h, and the ethanol productivity increased to 9 g/(h·L) of bead volume. The glycerol yield was slightly decreased to 0.088 (±0.002) g/g, but no significant changes in the yields of ethanol and the other carboxylic acids were obtained.

The performance of the immobilized cells was also investigated during continuous cultivation with glucose as the carbon and energy sources. The fermentation was carried out at dilution rate of 0.4 h^{-1} for 300 h, and 50% of the liquid volume in the bioreactor was filled with beads. In the steady-state condition, 90% (±2%) of the glucose was consumed. This resulted in a yield of 0.47 (±0.01) g/g of ethanol and 0.055 (±0.005) g/g of glycerol. The yield of acetic, pyruvic, and succinic acids was 0.005 (±0.001) g/g. A similar experiment was also carried out at a dilution rate of 0.2 h^{-1}. No significant changes in the yield of the metabolites were obtained, and the ethanol and glycerol yields were 0.47 and 0.055 g/g, respectively. However, the sugar conversion was higher (99.5%), and the concentration of sugar in the medium was <0.2 g/L.

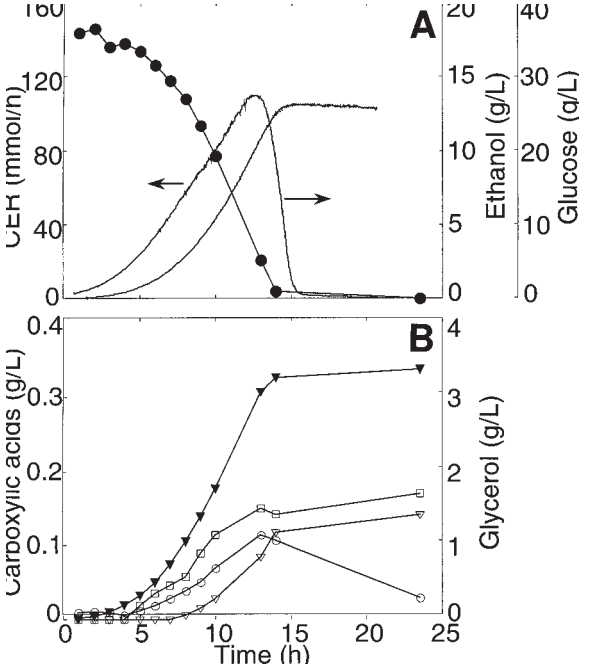

Fig. 1. Batch cultivation of glucose by immobilized *S. cerevisiae*. **(a)** CER, ethanol (solid line, right-hand scale), and glucose (●) concentrations; **(b)** glycerol (▼) and carboxylic acids including succinic acid (▽), pyruvic acid (○), and acetic acid (□).

Fermentation of Hydrolysate I

Free-Cell System

Fermentability of the hydrolysate I (Table 1) was investigated by continuous cultivation of the hydrolysate by freely submerged *S. cerevisiae*. The steady-state results of continuous cultivation at a dilution rate 0.1 h^{-1} are presented in Table 2. The yeast was able to ferment hydrolysate and washout did not occur. However, consumption of the sugars, especially mannose, was low (Table 2). No furfural was detected in the medium, but the HMF was partially converted. The ethanol yield was in a reasonable range, whereas the glycerol yield was lower than similar values for growth on glucose (Table 2). No xylose and galactose could be fermented by the applied strain of *S. cerevisiae*. The dilution rate was then increased to 0.2 h^{-1}. However, in that case the hydrolysate was not sufficiently fermentable, and the cells washed out (Fig. 2).

Immobilized-Cell System

The forest residual hydrolysate I could be fermented continuously with immobilized *S. cerevisiae* in Ca-alginate. The most important fermentation results at different dilution rates of 0.3, 0.5, and 0.6 h^{-1} are presented in Table 2. The yeast was able to ferment sugars to ethanol for all these dilution rates, although the consumption of sugars was different at various

Table 2
Results of Continuous Cultivation of Hydrolysate (I)
at Steady-State Conditions by Free or Immobilized *S. cerevisiae* at Different Dilution Rates

Parameter	Free cells	Free cells	Immobilized cells	Immobilized cells	Immobilized cells[a]	Immobilized cells
Dilution rate (h^{-1})	0.1	0.2	0.3	0.5	0.5	0.6
Glucose consumption (%)	92	Washout	86	83	83	79
Residual glucose (g/L)	0.60		1.4	1.7	1.8	2.2
Mannose consumption (%)	63		72	67	67	55
Furfural conversion (%)	100		98	97	97	97
HMF conversion (%)	66		60	66	68	60
Ethanol yield (g/g)[b]	0.44		0.45	0.47	0.48	0.47
Glycerol yield (g/g)[b]	0.035		0.04	0.05	0.03	0.04

[a]Furfural concentration in the hydrolysate was manually increased to 2.5 g/L.
[b]All yields are reported in grams per gram of consumed glucose and mannose.

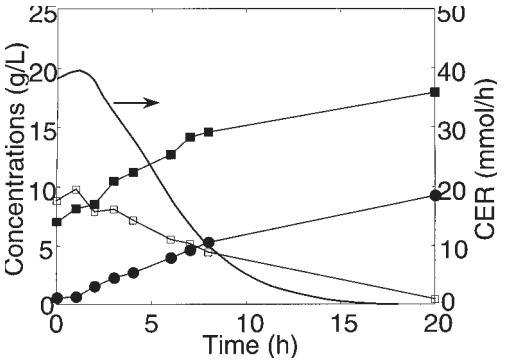

Fig. 2. CER (solid line) and glucose (●), mannose (■), and ethanol (□) (left-hand scale) during washout of the culture of free *S. cerevisiae* when the dilution rate was increased from the steady-state conditions at 0.1 to 0.2 h^{-1} at time zero.

dilution rates. The yeast preferred glucose rather than mannose regardless of the dilution rate. The conversion of furfural was almost complete, whereas the cells were not able to convert HMF by more than 68% (Table 2). The ethanol yield was higher than the yield obtained from free-cell experiments. The values of the glycerol yield (Table 2) were clearly lower than previously reported yields on glucose by free and immobilized cells.

To increase the toxicity of the hydrolysate, the furfural concentration of the hydrolysate was increased to 2.5 g/L. The hydrolysate was then cultivated by immobilized *S. cerevisiae* at a dilution rate of 0.5 h^{-1}, and the results were summarized in Table 2. The addition of furfural did not affect the fermentability of the hydrolysate by the immobilized cells. While the system reached steady-state conditions, the residual furfural was 0.09 g/L. The glucose and mannose consumption rates were similar to the cultivation of the hydrolysate without the addition of furfural at a similar dilution rate (Table 2). The only significant difference was the glycerol yield, which decreased from 0.05 to 0.03 g/g because of the addition of furfural. This is probably one of the main reasons that a higher yield of ethanol was achieved.

Fermentation of a More Inhibiting Hydrolysate (II)

Although the concentrations of furfural, HMF, and acetic acid of the hydrolysate II were not significantly different from those of hydrolysate I (Table 1), the fermentability was quite different. We tried to cultivate the hydrolysate in continuous mode of operation by *S. cerevisiae* at dilution rates of 0.2, 0.1, and 0.07 h^{-1}. However, the hydrolysate II was so inhibiting that none of the trials were successful. Performance of the immobilized-cell system on the cultivation of this inhibiting hydrolysate was then investigated. The yeast grew first in Ca-alginate beads in a synthetic medium containing 30 g/L of glucose. The continuous feeding of the hydrolysate started at a dilution rate of 0.2 h^{-1}, when the cells were growing exponentially and had already consumed half of the glucose (Fig. 3). Although there

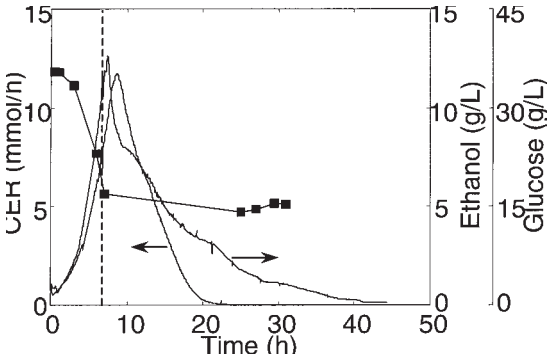

Fig. 3. CER (solid line), ethanol (solid line, right-hand scale), and glucose (■) during continuous cultivation of hydrolysate II by the immobilized yeast. The cells were grown in a batch phase in synthetic medium and then exposed to the hydrolysate at the point indicated by the dashed line at a dilution rate of 0.2 h^{-1}.

was enough sugar, the CER decreased significantly after the addition of 110 mL of the hydrolysate to the 1 L of medium in the bioreactor. The CER decreased by 99% within 14 h and could not be recovered within 40 h after starting the feeding (Fig. 3). Similar experiments were carried out at different dilution rates down to 0.07 h^{-1}, but the cells failed to produce ethanol and carbon dioxide. The yeast was not able to ferment this hydrolysate in either batch or continuous cultivations with the free-cell system.

The hydrolysate II was then detoxified by overliming with calcium hydroxide. The pH was increased to 10.0 and then readjusted to 5.0. The hydrolysate was centrifuged to separate the precipitant. The hydrolysate was applied as the feed for continuous cultivation with the immobilized yeast at a dilution rate of 0.2 h^{-1}. The cultivation was carried out successfully, and the concentration of residual glucose and mannose were both <1 g/L at steady-state conditions. This resulted in a yield of 0.44 g/g of ethanol and 0.047 g/g of glycerol. The detoxification process decreased the furfural concentration from 1.0 to 0.35 g/L, which was further decreased to 0.08 g/L during cultivation. Detoxification had no effect on HMF concentration, but it was decreased during the cultivation to 0.2 g/L. No significant changes in the concentration of acetic acid were detected in either detoxification or cultivation.

Discussion

Continuous cultivation of dilute-acid hydrolysates with the immobilized yeast turned out to be a promising method for ethanol production. The results of the current study show the capability of the immobilized cells to ferment hydrolysate I with a high dilution rate (e.g., 0.6 h^{-1}) and with a reasonable conversion of the sugars. The values should be compared with the continuous cultivation by free cells, where the maximum dilution rate was <0.2 h^{-1} for the fermentation of a similar hydrolysate. The yeast sur-

vived several weeks and the beads show good stability when an additional resource of calcium was present in the medium. The results are in agreement with the reported fermentation of the other sugar resources by immobilized cells *(9–22)*.

Neither the free nor the immobilized cells were able to ferment the more inhibiting hydrolysate II, even at very low dilution rates; this is definitely a drawback of continuous cultivation. The inhibitors in the hydrolysates decrease the viability of the yeast and some other microorganisms *(7,35,36)*. Since the hydrolysate inhibitors are continuously added to the culture in a continuous cultivation, the cells have no time to restore their decreasing viability. This results in eventual failure of the fermentation. Although the fermentation of hydrolysate II failed in the current study, the immobilized cells may perform better than the free cells in maintaining the viability *(37)*.

The high mass transfer resistance is probably one of the most important drawbacks in cultivation with the immobilized cells. The resistance could be owing to the transport of gases such as oxygen and carbon dioxide, sugars, and metabolites *(38,39)*. The production of ethanol in the current study was performed anaerobically, and, therefore, the transfer of oxygen from the gas phase to the beads and cells is not an issue. Furthermore, stirring makes the resistance of the external film around the beads negligible compared with its effect with, e.g., packed-bed bioreactors *(40)*. On the other hand, some transport resistance of sugars has been observed. Glucose needs a few minutes to penetrate the cells (data not shown). In addition, the profile of the CER and ethanol production in batch fermentation was not exponential in the growth phase (Fig. 1). Furthermore, at the end of the exponential growth phase, the decrease in CER (Fig. 1) was much slower than for corresponding experiments with free cells. These facts are probably the evidence for significant mass transfer resistance inside the beads. However, it cannot be a critical factor for the fermentation since the mass transfer rate is still higher than the fermentation rate.

Fermentation of glucose by immobilized cells resulted in a higher yield of ethanol (0.47 g/g) and a lower yield of glycerol (0.055 g/g), compared with the reported ethanol yield (0.39 g/g) and glycerol yield (0.085 g/g) by free cells of the same strain *(41)*. The formation of glycerol is mainly owing to reoxidation of NADH produced in relation to biomass production *(33)*. In the immobilized system, the formation of biomass is low. Consequently, the glycerol yield would also be low, which results in more carbon available to improve ethanol yield. In addition, the reported production of NADH per biomass *(41)* is not necessarily of the same stoichiometry as for the free cells owing to possible shifting between NADH and NADPH enzymes *(25)*. When the hydrolysate was fermented by the yeast, the glycerol yield was even lower at an average of 0.04 g/g (Table 2). This is probably the result of partial reoxidation of NADH by the reduction of furfural and HMF to their corresponding alcohols in the continuous cultivation *(42)*.

We can conclude that the application of immobilized-cell systems can increase the fermentation rate by several fold in continuous cultivation compared to free-cell systems. It can be successfully applied to dilute-acid hydrolysates. However, the continuous cultivation of more inhibiting hydrolysate with either free- or immobilized-cell systems was not successful. A suitable combination of detoxification and fermentation should probably be applicable to the toxic hydrolysates. Furthermore, if batch fermentation were more favorable compared to continuous, the immobilization could help decrease the total time of fermentation owing to the neglected lag phase and the high cell content within the beads in the bioreactor.

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