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Ethanol Production by Repeated Batch and Continuous Fermentations by *Saccharomyces cerevisiae* Immobilized in a Fibrous Bed Bioreactor

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In this work, a fibrous bed bioreactor with high specific surface area and good adsorption efficacy for *S. cerevisiae* cells was used as the immobilization matrix in the production of ethanol. In batch fermentation, an optimal ethanol concentration of 91.36 g/l and productivity of 4.57 g l^{-1} h^{-1} were obtained at an initial sugar concentration of 200 g/l. The ethanol productivity achieved by the immobilized cells was 41.93% higher than that obtained from free cells. Ethanol production in a 22-cycle repeated batch fermentation demonstrated the enhanced stability of the immobilized yeast cells. Under continuous fermentation in packed-bed reactors, a maximum ethanol concentration of 108.14 g/l and a productivity of 14.71 g l^{-1} h^{-1} were attained at 35°C, and a dilution rate of 0.136 h^{-1} with 250 g/l glucose.

Key words: Immobilization, ethanol fermentation, fibrous bed bioreactor

Ethanol is a specialty chemical with many applications in the chemical industry as well as in foodstuffs and medical treatments. It can also be used as a renewable and alternative form of bioenergy and is currently under intensive study for this purpose [11]. Traditional ethanol fermentation industries produce ethanol by batch or fed-batch processes. Compared with suspended-cell fermentation, the immobilization of microbial cells has many attractive advantages, such as increasing the biomass in the reactor and the productivity of fermentation, preserving the activity of cells, and affording the possibility of continuous fermentation, among others [10, 20]. However, conventional immobilized-cell fermentation usually suffers from productivity loss due to toxicity or poor stability of carriers such as calcium polyurethane foam, alginate, gelatin, and κ-carrageenan [1, 14].

Recently, a fibrous bed bioreactor (FBB) with cells immobilized in a fibrous matrix packed in the reactor has been successfully used to produce several organic products, such as lactic acid [28], butyric acid [34], butanol [12], and mycophenolic acid [32], due to its high specific surface area, high porosity, low cost, strong stability, and good adsorption efficacy. In addition, a FBB can also offer protection of the cells against mechanical shear stress [27] and continuously renew the cell population [8]. Kilonzo *et al*. [15, 16] observed a production of glucoamylase obtained by immobilizing recombinant *Saccharomyces cerevisiae* in a FBB using a cotton fabric. However, only a few articles have referred to the use of immobilized yeast in a FBB for ethanol production.

This study on ethanol fermentation has been focused primarily on comparing the fermentation performances of immobilized and free cells in batch culture and ascertaining the stability of the immobilized cells in repeated batch fermentation. Continuous fermentation in packed-bed reactors was also investigated.

Materials and Methods

Microorganism and Culture Medium

Industrial *Saccharomyces cerevisiae* 1308 was supplied by Tian-Guan Group (Henan, China). It was stored on a malt juice agar slant that contained 250 g/l malt and 20 g/l agar at 4°C and cultured on malt juice agar slant for 2 days at 30°C before use.

Starter cultures were prepared by transferring a loopful of the stock culture to 200 ml of the sterilized preculture medium, which

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was composed of 10 g/l yeast extract, 20 g/l peptone, 20 g/l glucose, 1.5 g/l KH_2PO_4, 4 g/l $(NH4)_2SO_4$, and 0.5 g/l $MgSO_4$. The cell cultivation was carried out on a shaker at 150 rpm and 30°C for about 18 h.

The fermentation medium used for microorganism culture contained (per L) 200 g glucose, 4 g peptone, 3 g yeast extract, 4 g $(NH_4)_2SO_4$, 3 g KH_2PO_4, 0.05 g $FeSO_4 \cdot 7H_2O$, 0.05 g $ZnSO_4 \cdot 7H_2O$, and 0.5 g $MgSO_4$. The initial pH was 5.17 without adjustment, and the medium was sterilized at 115°C for 15 min.

Cell Immobilization

Cells were immobilized as follows: 15 g of fibrous matrix (described in the section below) and 200 ml of culture medium were added to a 500 ml Erlenmeyer flask and autoclaved for 15 min at 115°C. Then, 20 ml of stock cell suspension was added to the sterilized culture medium and incubated at 100 rpm and 30°C to induce natural cell adhesion for 48 h, when the OD value declined to 1 in the solution. The fermented liquid was decanted and the support was wrung out gently and then used for ethanol production.

Batch Fermentation

Batch fermentations were performed in 500 ml flasks containing 200 ml of fermentation medium and the immobilized cells or free cells (free cells were incubated as long as immobilized cells and were then collected by centrifuging at 5,000 ×*g* for 10 min and washed twice with sterile water). The flasks were then inoculated in a rotary shaker at 150 rpm and 35°C. All experiments were carried out in triplicate.

Repeated Fermentation

The repeated batch fermentations were first carried out in batch mode. At the end of each batch (namely, no glucose was determined), the fermented broth was drained and only the carrier with immobilized cells was retained in the flask. Then, the same amount of the fermentation medium was immediately replaced to start the next cycle. Twenty-two cycles were performed, and all experiments were also carried out in triplicate.

Continuous Fermentation

Fibrous bed bioreactor. The fibrous materials used in this study were the woven cotton fabric. The cotton matrix (thickness of 0.30 cm, specific surface area of 42 m^2/m^3, and porosity of 96%) was bought from Weifang Hao Embellish Textile Co., Ltd, China. A stainless steel wire cloth (SUS 304; porosity 0.97, mesh no. 50; Hisin Wire Mesh Products Co., Ltd, China) topped with the fibrous cotton sheets (60.00 cm × 30.50 cm × 0.30 cm and 120 g) was wound into a spiral configuration along the vertical axis, just as described by Kilonzo [16].

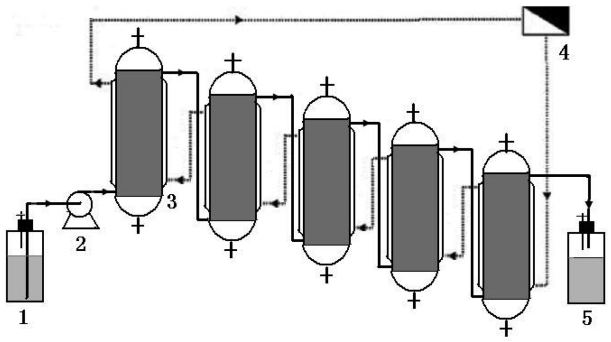

Fig. 1. Schematic diagram of the immobilized-cell packed-bed reactors: 1, feed tank; 2, pump; 3, bioreactor; 4, water bath; 5, product tank.

Fig. 1 shows the experimental setup of the continuous FBB system. The FBB system was made of five stainless steel fermentation tanks (each of working volume 1.6 L) that were linked in series and packed with spiral wound cotton matrix. Before use, the reactor was sterilized at 121°C for 1 h and then cooled to room temperature. The stock cell suspension was then introduced at the bottom of the first reactor and returned from the top outlet of the last reactor to the culture vessel for re-aeration at a flow rate of 10 ml/min. After about 24 h of continuous circulation, most of the cells were immobilized, and the cell density declined quite slowly. Then, the flow was stopped and the medium was replaced by the fermentation medium, which was continuously fed at a dilution of 0.5 h^{-1} to allow the medium to fill all of the reactors quickly until they were full.

Fermentation kinetics study. The fermentation kinetics, mainly in the solution phase, was determined with the FBB fed continuously with fermentation medium. The effect of the dilution rate on the fermentation was first examined at 25°C and 200 g/l glucose by varying the dilution rate. The effect of temperature was then investigated for three different temperatures at a dilution rate of 0.136 h^{-1} and 200 g/l glucose. Finally, measurements were made for a high initial concentration of glucose under conditions of 35°C and a dilution rate of 0.136 h^{-1}. In all cases, the conditions were not changed until the fermentation had reached a steady state.

Analytical Methods

At specified time intervals, 2 ml of sample was collected. The free cells were determined by measuring the optical density (OD) at 660 nm using a UV-visible spectrophotometer (CA, USA) and by applying the gravimetric method (dry cell weight). The immobilized cells in the FBB were removed from the fibrous matrix by vortexing the matrix and then estimated by measuring the OD value. The values of the absorbance were converted to the cell dry weight (DCW) using a calibration curve: 1 OD = 0.81 g DCW/l.

After removing the cells by centrifuging at 12,000 ×*g* for 3 min, the clear fermentation broth was used to analyze for residual glucose, ethanol, and glycerol by high-performance liquid chromatography (HPLC) with a BIO-Rad HPX-87H organic acid analysis column at 25°C, using 0.05 N H_2SO_4 as the eluent at a flow rate of 0.6 ml/min.

Results and Discussion

Batch and Repeated Batch Fermentation

Comparisons of free and immobilized cells in the batch fermentation. Preliminary studies have shown typical kinetics for batch fermentations with free cells and immobilized cells in the fibrous bed bioreactor. As seen in Fig. 2, the OD value of and ethanol produced by the free cell system increased as glucose decreased, and the former remained constant after 20 h. The OD value of and ethanol produced by the immobilized cell system also increased as glucose decreased at the initial stage of fermentation. The

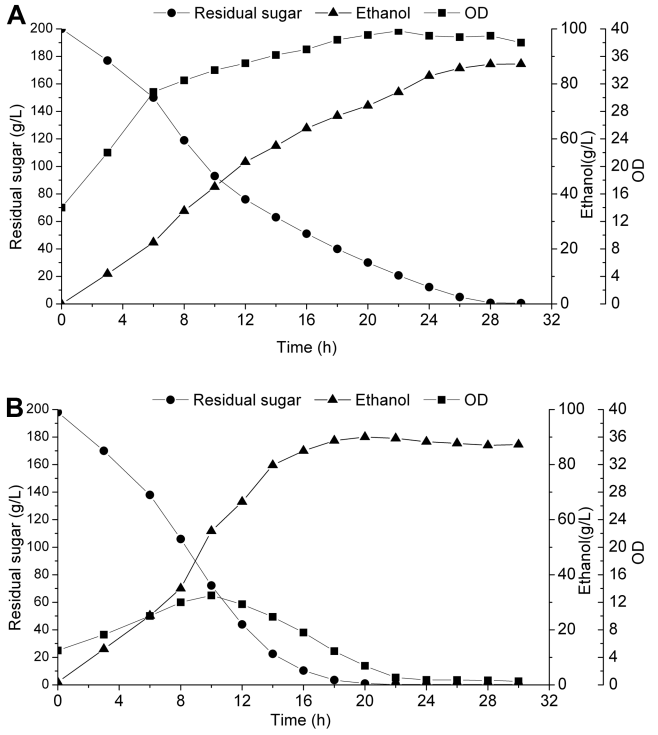

Fig. 2. Comparisons of the fermentation performances of free and immobilized cells in batch culture.
(**A**) Free-cell fermentation. (**B**) Immobilized-cell fermentation in the FBB.

OD reached a maximum value at 10 h, but then decreased because of decreasing nutrition and adsorption by the FBB. However, the final total biomass concentration of the FBB system was much higher than that of the free system (Table 1). This result indicated that cell immobilization could increase the total cell concentration. In the FBB system, yeast cells attached to the support and may form thick layers of cells known as "biofilm." The biofilm cells produce extracellular polymeric substances (EPS) that provide protection by providing a diffusive barrier to any toxic compounds that could harm the cells, which results in cell densities in the support increasing [9, 23]. Meanwhile, from the table, we can see that the final ethanol concentration and the yield (grams of ethanol produced per gram of glucose consumed) obtained from FBB-immobilized cells (FBB-cells) were similar to those from the free cells. However, the ethanol productivity (concentration of ethanol

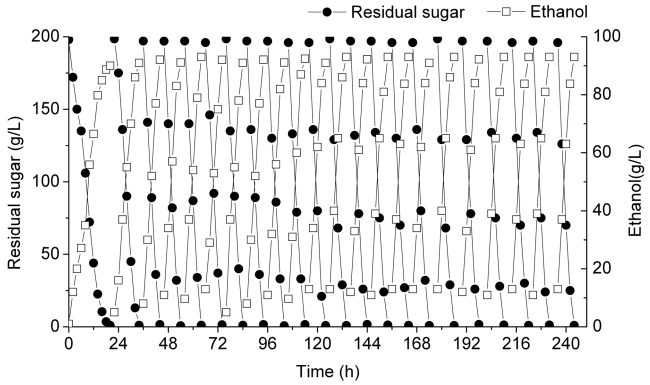

Fig. 3. Stabilization of the immobilized yeast cells by the FBB in repeated batch fermentation.

produced per hour) of the immobilized cells was 41.93% higher than that of the free cells, which may be due to the increased cell concentration.

Stability of immobilized yeast cells in repeated batch fermentation. To investigate the stability of the FBB as a support material for cell immobilization and the ability of the immobilized cells to produce ethanol, a 22-cycle repeated batch fermentation with *S. cerevisiae* 1308 immobilized on FBB was carried out. As shown in Fig. 3, the residual glucose was almost completely consumed by immobilized cells within 8 h after the second batch. The ethanol production and yield were found to be almost the same (91.57 ± 1.71 g/l, 0.457 ± 0.009 g/g) in the 22-cycle fermentation. The productivity was improved from 4.57 to 11.31 g l^{-1} h^{-1} in the first 3 cycles of batch fermentation, due to the increased biomass attached to the FBB. After the third cycle of fermentation, because the FBB was saturated with cells, the productivity reached a steady state, and the average value was 11.75 g l^{-1} h^{-1}, which was much higher than the productivities reported by others (Table 2).

These results suggest that the FBB enhanced the productivity and provided sufficient stability for immobilized-cell fermentation. The increased ethanol productivity was consistent with an increase of yeast cells adsorbed on the inner/outer surfaces during the process, because ethanol is a typical primary metabolite during which cell growth also occurs. In addition, the time reduction during the fermentation of successive batches process may be due to the diffusion limitation having been overcome by surface

Table 1. Comparison of the final parameters of the free and immobilized yeast cells in batch fermentation.

	Residual sugar (g/l)	Ethanol (g/l)	Total biomass (g/l)	Productivity (g l^{-1} h^{-1})	Yield (g/g)
Free fermentation	0.80	90.14	30.80	3.22	0.453
Immobilized fermentation	0.93	91.36	41.52[a]	4.57	0.459

[a]41.52 represents the value of the total cell concentration in the FBB system, which includes free and immobilized cells. The methods of detection and calculation are described in the Materials and Methods section.

Table 2. Comparison of repeated batch ethanol fermentation with yeast cells immobilized on various carriers.

Carrier	Initial sugar (g/l)	Residual sugar (g/l)	Batches	Ethanol (g/l)	Yield (g/g)	Productivity (g l^{-1} h^{-1})
Cashew apple bagasse [22]	83.29	3.30	10	37.83	0.454	6.28
Silk cocoons [24]	240.00	52.12	5	88.30	0.470	1.84
Sweet sorghum stalk [4]	230.00	8.17	8	104.26	0.470	1.43
Corncob [18]	230.00	27.52	8	97.19	0.480	2.02
Sorghum bagasse [33]	200.00	0	21	96.00	0.480	5.72
Calcium alginate [13]	150.00	4.00	7	73.50	0.50	6.10
Fibrous bed reactor (this work)	200.00	0	22	91.57	0.458	11.75

growth of cells [3]. In the repeated batch process, we found no lag time for ethanol production at the start of each batch, indicating that the immobilized cells were vigorous throughout the experiment, just as reported by Watanabe *et al*. [31]. The enhanced cell stability of the immobilized cell culture may imply that an accumulation of saturated fatty acids, which might be gradually increased with cell reuse, leads to greater ethanol tolerance by increasing the fluidity of the plasma membranes [33]. Moreover, the carrier may have protected the yeast cells from toxic conditions, such as acetic acid [17], during the fermentation of successive batches.

Continuous fermentation

The effects of dilution rate. Ethanol production using FBB cells was further examined by continuous fermentation in a series-wound bioreactor. The reactors were operated by gradually decreasing the dilution rate from 0.136 h^{-1} initially to 0.091 h^{-1} at 52 h and 0.045 h^{-1} at 88 h. As can be seen in Fig. 4, the OD value and outlet concentrations of substrate and products changed as the dilution rate changed and, in general, they achieved a steady state at the end of each period of constant operating conditions. In the early stage, the OD reached the maximum value, due to suspended cells that had leaked from the carrier by oscillations during which the fresh medium been fed, and

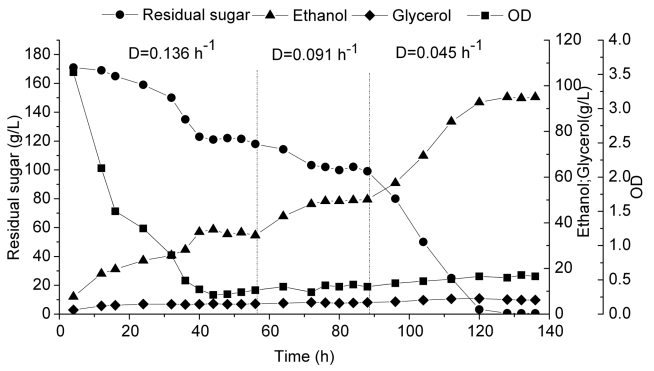

Fig. 4. Kinetics of continuous ethanol fermentation at 25°C, 200 g/l glucose and various dilution rates.

then rapidly decreased because of adsorption and loss. Besides this, we found the outlet concentrations of ethanol and glycerol increased gradually with glucose decreased, and after 40 h, all concentrations became almost constant. When the dilution rate decreased from 0.136 to 0.091 h^{-1}, the OD value had no increase, and this may be because the new growth of cells was compensated for adsorption by the carriers. The outlet concentrations of glucose, ethanol, and glycerol changed as before. When the dilution rate was adjusted to 0.045 h^{-1}, the OD value had a little improvement because the growth rate was faster than the adsorption rate. The highest concentrations of ethanol and glycerol produced in the fermentation were 95.0 and 6.23 g/l, respectively, when the glucose was exhausted after 128 h. In contrast, low ethanol and glycerol concentrations of 35.9 and 4.33 g/l were obtained at the higher dilution rate of 0.136 h^{-1}. This may be explained by the fact that, at a higher dilution rate, the medium had a shorter contact time with the cells [12] and, in addition, the biomass concentration was reduced by wash-out due to the higher dilution rate [25] so that less glucose was consumed and less products were produced in the fermentation.

The reactor volumetric productivities (and yields as analyzed below) at 25°C and various dilution rates were estimated based on the time course data in the steady state and are shown in Table 3. In general, the productivities for ethanol and glycerol decreased as the dilution rate decreased from 0.136 to 0.045 h^{-1}, which is in agreement with data published by others [12, 21, 20]. This phenomenon could be explained by a decline in the effect of mass transfer and the inhibition of cell activity by the higher ethanol concentration [2, 25] as the dilution rate decreased. At the same time, less oxygen was conveyed to the bioreactors from the feed pump at the lower dilution rate, which exacerbated the inhibition by ethanol [26]. Therefore, the maximum ethanol (4.88 g l^{-1} h^{-1}) and glycerol (0.59 g l^{-1} h^{-1}) productivities were obtained at a dilution rate of 0.136 h^{-1}, whereas lower productivities for ethanol and glycerol were obtained at the lower dilution rate.

In terms of the effect of dilution rate on ethanol yield, the lower dilution rate gave a higher yield than the high dilution rate owing to a more complete reaction [12],

Table 3. Effects of dilution rate on fermentation parameters at 25°C and 200 g/l glucose.

Dilution (h^{-1})	Residual sugar (g/l)	Ethanol (g/l)	Glycerol (g/l)	Productivity (g l^{-1} h^{-1})		Yield (g/g)	
				Ethanol	Glycerol	Ethanol	Glycerol
0.136	122.10	35.90	4.33	4.88	0.59	0.460	0.056
0.091	94.80	49.40	4.99	4.50	0.45	0.470	0.047
0.045	0.40	95.0	6.23	4.27	0.28	0.476	0.031

which was in contrast to the trend for the glycerol yield. As can be seen from Table 3, the maximum value of the ethanol yield was 0.476 g/g at a dilution rate of 0.045 h^{-1}, and the minimum value was 0.460 g/g at a dilution rate of 0.136 h^{-1}, whereas the maximum and minimum of glycerol yields were 0.056 g/g at a dilution rate of 0.136 h^{-1} and 0.031 g/g at a dilution rate of 0.045 h^{-1}, respectively.

The effects of temperature. After 136 h, the dilution rate was increased from 0.045 to 0.136 h^{-1}, and ethanol decreased and glucose increased. When the temperature was adjusted to 30°C at 184 h, the OD value and the concentration of ethanol increased and glucose obviously decreased. After the temperature was adjusted to 35°C, ethanol increased to 93.6 g/l, while glucose declined to about 4 g/l by 240 h (Fig. 5). However, the concentration of glycerol was evidently not affected throughout the process. From the kinetics, we know that increasing the temperature not only improves the enzyme activity, but also promotes new cell growth.

Table 4 shows the productivities and product yields that were estimated at the dilution rate of 0.136 h^{-1} and various temperatures. In general, the productivity for ethanol increased with increasing temperature and reached a maximum value of 12.78 g l^{-1} h^{-1} at 35°C, while the productivity for glycerol increased slowly and the maximum value was 0.93 g l^{-1} h^{-1}. Furthermore, when the temperature was adjusted to 30°C from 25°C, it affected the productivities more than when the temperature was adjusted from 30°C to 35°C, because the high ethanol concentration produced at 35°C enhanced the inhibitory effect. The effect of temperature on the ethanol yield was also obvious. The maximum value was 0.480 g/g, acquired at 35°C, which was far higher than that at 25°C. However, the glycerol yield showed the opposite tendency in the range studied.

The effects of high initial concentration of glucose. High ethanol concentrations have been continuously pursued in the industry because they reduce the energy consumed during distillation and waste distillage treatment [5, 6]. It has been possible to make the final ethanol concentration increase dramatically owing to the high ethanol tolerance of *S. cerevisiae* [30]. Laopaiboon *et al*. [19] attained an ethanol concentration of 120 g/l by fermenting sweet

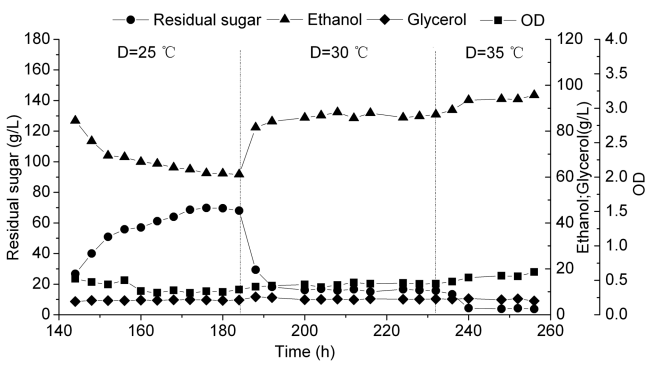

Fig. 5. Kinetics of continuous ethanol fermentation at a dilution rate of 0.136 h^{-1}, 200 g/l glucose, and various temperatures.

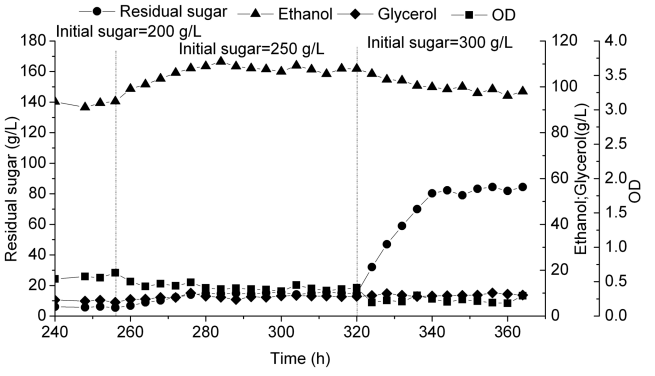

Fig. 6. Kinetics of continuous ethanol fermentation at 35°C, dilution rate of 0.136 h^{-1}, and various high initial sugar concentrations.

Table 4. Effects of temperature on the fermentation parameters at a dilution rate of 0.136 h^{-1} and 200 g/l glucose.

Temperature (°C)	Residual sugar (g/l)	Ethanol (g/l)	Glycerol (g/l)	Productivity (g l^{-1} h^{-1})		Yield (g/g)	
				Ethanol	Glycerol	Ethanol	Glycerol
25	69.00	60.50	6.40	8.23	0.87	0.462	0.049
30	16.90	86.90	6.75	11.82	0.92	0.475	0.037
35	4.10	94.00	6.81	12.78	0.93	0.480	0.035

Table 5. Effects of initial sugar concentration on the fermentation parameters at 35°C and a dilution rate of 0.136 h^{-1}.

Initial sugar (g/l)	Residual sugar (g/l)	Ethanol (g L^{-1})	Glycerol (g/l)	Productivity (g l^{-1} h^{-1})		Yield (g/g)	
				Ethanol	Glycerol	Ethanol	Glycerol
200	4.10	94.00	6.81	12.78	0.93	0.480	0.035
250	14.80	108.14	8.53	14.71	1.16	0.460	0.036
300	82.20	98.31	9.23	13.37	1.26	0.451	0.042

sorghum juice. However, the productivity was very low, which limited the feasibility of industrial production.

In this paper, in order to improve the high-gravity fermentation by immobilized yeast cells, different high initial sugar concentrations (200, 250, and 300 g/l) were analyzed (Fig. 6). It can be seen that the ethanol concentration increased as the initial sugar concentration increased from 200 to 250 g/l, reaching a value of 108.14 g/l, but decreased at the initial sugar concentration of 300 g/l. The initial sugar utilization was improved by 25% from 200 g/l, but the practical utilized sugar was improved by 20%, which was less than the theoretical value. In addition, when the initial sugar concentration was adjusted to 300 g/l, the practical utilized sugar was improved by 11%, which was much less than when the initial concentration was 200 g/l. This might be due to the osmotic pressure as described by Laopaiboon *et al*. [19], which causes inhibition of cell activities and biomass. In addition, the high concentration of glucose led to a high ethanol concentration and increased the amount of CO_2 generated, which may be another inhibiting factor. Beyond the above, Bai *et al*. [6] found that the negative impact of high temperature on the ethanol fermentation performance was much worse under the VHG conditions than the regular fermentation.

Table 5 indicates that a high initial sugar concentration had significant effects on the main fermentation parameters. When the initial sugar concentration increased from 200 to 250 g/l, the productivity correspondingly increased, and when it was adjusted to 300 g/l, the productivity declined. However, the glycerol concentration and productivity always exhibited rising trends. The maximum values of the ethanol and glycerol productivities were 14.71 g l^{-1} h^{-1} at 250 g/l glucose and 1.26 g L^{-1} h^{-1} at 300 g/l glucose, respectively.

The yield of ethanol obviously decreased as the initial sugar concentration increased. The maximum and minimum values were 0.480 g/g at 200 g/l and 0.451 g/g at 300 g/l, respectively. This may be explained by the fact that glucose was used to form by-products such as glycerol, acetaldehyde, and acetate, and some of these have inhibitory effects on ethanol fermentation [6].

In conclusion, we have demonstrated the potential use of *S. cerevisiae* 1308 immobilized by FBB for repeated batch and continuous ethanol fermentations. Many advantages, such as reusability, good adsorption efficacy, and high cell density, were achieved and resulted in stable operation with a high ethanol productivity and high yield [10]. A comparison of the batch and repeated batch fermentations showed that the FBB system was faster and more stable than the conventional free fermentation system. A further study of the continuous fermentation in a series of five reactors showed that progressively decreasing dilution rates can enhance the efficiency of ethanol production in terms of ethanol concentration and product yield; however, low productivity was obtained. Glycerol, which acts as a by-product, was also affected greatly by the dilution rate. The temperature and high initial sugar concentration mainly affected the activities of the cells and biomass and, finally, had an evident effect on the concentrations of products, productivities, and yields.

Acknowledgments

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