Appl Biochem Biotechnol (2008) 145:59–67
DOI 10.1007/s12010-007-8054-6

Bioethanol Production from Uncooked Raw Starch by Immobilized Surface-engineered Yeast Cells

Jyh-Ping Chen · Kuo-Wei Wu · Hideki Fukuda

Received: 9 May 2007 / Accepted: 14 September 2007 /
Published online: 4 October 2007

Abstract Surface-engineered yeast *Saccharomyces cerevisiae* codisplaying *Rhizopus oryzae* glucoamylase and *Streptococcus bovis* α-amylase on the cell surface was used for direct production of ethanol from uncooked raw starch. By using 50 g/L cells during batch fermentation, ethanol concentration could reach 53 g/L in 7 days. During repeated batch fermentation, the production of ethanol could be maintained for seven consecutive cycles. For cells immobilized in loofa sponge, the concentration of ethanol could reach 42 g/L in 3 days in a circulating packed-bed bioreactor. However, the production of ethanol stopped thereafter because of limited contact between cells and starch. The bioreactor could be operated for repeated batch production of ethanol, but ethanol concentration dropped to 55% of its initial value after five cycles because of a decrease in cell mass and cell viability in the bioreactor. Adding cells to the bioreactor could partially restore ethanol production to 75% of its initial value.

Keywords Bioethanol · Immobilized cells · Recombinant cells · Bioreactor

Introduction

Bioethanol produced from biomass resources by fermentation is the most promising biofuel and the starting material of various chemicals. Starch is a cheap, clean, nontoxic, renewable carbon source for bioethanol production [1]. In the process currently employed for industrial-scale ethanol production from starchy materials, starch is first hydrolyzed by adding a liquefying enzyme, α-amylase (EC 3.2.1.1), to avoid gelatinization and then

J.-P. Chen (✉) · K.-W. Wu
Department of Chemical and Materials Engineering, Chang Gung University, Kwei-San, Taoyuan 333 Taiwan, Republic of China
e-mail: jpchen@mail.cgu.edu.tw

H. Fukuda
Division of Molecular Science, Graduate School of Science and Technology, Kobe University, Nada-ku, Kobe 657-8501, Japan

cooked at high temperature (140–180 °C). The liquefied starch is then hydrolyzed to glucose with a saccharifying enzyme, glucoamylase (EC 3.2.1.3). Finally, the glucose is converted to ethanol by yeast cells [2]. Traditional processes for bioethanol production from starch are expensive. There are two main reasons for the high costs, one being that as the yeast *Saccharomyces cerevisiae* cannot utilize starchy materials, large amounts of amylolytic enzymes need to be added. On the other hand, the starchy materials need to be cooked at a high temperature to obtain a high ethanol yield.

To improve the conventional high-temperature-cooking fermentation system, efficient processes for one-step bioconversion of starch into ethanol have been developed. Starch-utilizing yeast displaying amylolytic enzymes on the cell surface have been constructed for producing ethanol from soluble starch at high yield using a flocculating *S. cerevisiae* strain displaying *Rhizopus oryzae* glucoamylase on the cell surface [3–5]. Further improved ethanol productivity was reported by developing yeast strains that display *R. oryzae* glucoamylase and codisplay or secrete *Bacillus stearothermophilus* α-amylase [6]. Direct ethanol production in a single step was also performed using low-temperature-cooked corn starch as the sole carbon source instead of soluble starch using a yeast strain displaying only glucoamylase on the cell surface or yeast strains displaying glucoamylase and either codisplaying or secreting α-amylase [7]. These strains cannot, however, ferment raw starch to ethanol. Recently, instead of the *B. stearothermophilus* α-amylase, α-amylase from the lactic acid bacterium *Streptococcus bovis* 148 was codisplayed with *R. oryzae* glucoamylase on the surface of a yeast strain by using the C-terminal half of α-agglutinin and the flocculation functional domain of Flo1p as anchor proteins [8]. Extracellular α-amylase secreted from *S. bovis* 148 is known to have a strong ability to hydrolyze and be adsorbed onto raw corn starch [9]. This recombinant yeast, YF207/pGA11/pUFLA, not only can hydrolyze raw starch and ferment ethanol in a short time but also keep the flocculation ability for convenient immobilization in a porous carrier.

Cell immobilization is an effective method of improving the efficiency of substrate utilization and productivities of various fermentation processes. The concept of cell immobilization provides a promising strategy for the use of recombinant yeast cells in a bioreactor for easy scale up and industrial production of bioethanol. However, to use this technology in fuel ethanol production, the immobilized carrier must be cheap, and cell immobilization should be achievable with minimal additional cost. In addition, the carrier should preferentially be also biodegradable and renewable considering the nature of intended purpose of its fermentation product. Loofa sponge is available in abundance at lower prices and is an environment friendly material with sustainable sourcing. This natural plant sponge consists of a fibrous network and can be obtained from the dried fruit of *Luffa cylindrica*. It was found to be a very economical and excellent support matrix for immobilization of both nonflocculating and flocculating cells because of its high porosity, high specific pore volume, stable physical properties, nontoxicity, and low cost [10, 11]. This could be promising for achieving large-scale and economical production of bioethanol by immobilized yeast cells.

Although genetically engineered yeast cells has been used for direct production of ethanol from raw starch, only free cell systems have been studied with no data on reusability, and few immobilized cell systems have been reported [12]. In the present study, we have developed a process for direct production of bioethanol from insoluble raw starch using surface-engineered *S. cerevisiae.* The flocculating recombinant yeast strain codisplaying α-amylase and glucoamylase on its surface could be conveniently immobilized within the loofa sponge at high cell density for direct production of bioethanol in a packed-bed bioreactor.

Materials and Methods

Microorganism, Media, and Free Cell Fermentation

A genetically engineered *S. cerevisiae* strain YF207/pGA11/pUFLA coexpressing the glucoamylase from *R. oryzae* and α-amylase from *S. bovis* on cell surface was used in the experiment [8]. Yeast cells were aerobically grown in 100 mL of SDC medium (6.7 g/L yeast nitrogen base without amino acids [Difco] with appropriate amino acids and nucleotides, 20 g/L glucose, and 20 g/L casamino acids) for cultivation under selective condition at 30°C and 150 rpm for 48 h. The cell pellet collected by centrifugation at 6,000×*g* for 10 min was used to inoculate 100 mL YPS medium (10 g/L yeast extract, 20 g/L polypeptone, and 200 g/L insoluble raw starch). Ethanol fermentation from uncooked insoluble starch was carried out at 30 °C under anaerobic condition with agitation at 150 rpm for 240 h. For repeated batch fermentation, the flocculated cells were separated from the culture broth by centrifugation at 6,000×*g* for 10 min and replaced with 100 mL fresh YPS medium. The initial cell density was adjusted to 10 to100 g cell dry weight (CDW) per liter. The dry weight of cells corresponds to 0.15 times the wet weight of cells.

Fermentation with Immobilized Cells in a Bioreactor

Loofa sponge was cut from the peripheral part of the dried fruit of *L. cylindrica* with 1×1×12 cm size and soaked in deionized (DI) water for 24 h followed by extensively washing under running DI water. Three pieces of loofa sponge were weighed (6 g) after drying at 70 °C for 24 h and spirally packed into a column (diameter=3 cm, length= 15 cm). For cell immobilization, 50 g CDW/L yeast cells in 200 mL YP medium (10 g/L yeast extract and 20 g/L polypeptone) was placed in a 250-mL flask, and the cell-containing medium was pumped through the column by a peristaltic pump. The void in the reactor was calculated to be 71.4±7.2%. The recirculation flow rate was adjusted from 10 to 35 mL/min. Cell concentration in the flask was monitored with time to determine the extent of immobilization and the immobilization efficiency (defined as the mass of cells immobilized divided by that of cells added). For fermentation, 200 mL YPS medium was pumped through the column containing the immobilized cells at 20 mL/min after loading the column at the optimum flow rate for immobilization. For repeated batch fermentation with the immobilized cells, the whole culture broth was removed and replaced with 200 mL fresh YPS medium every 7 days. The YPS medium bottle was stirred at 150 rpm to avoid settling of raw starch, and fermentation was carried out anaerobically at 30 °C.

Measurement of Starch and Ethanol Concentration

Cells were separated from the fermentation broth by centrifugation at 6,000 rpm for 10 min. The supernatant was removed and determined for ethanol concentration by gas chromatograph (model GC-8A; Shimadzu Seisakusho, Kyoto, Japan), fitted with a flame-ionization detector and a glass column (3.0 mm×3.1 m) packed with Unisole 3000 (GL Science, Tokyo, Japan). The conditions for analysis are: column temperature 210 °C, temperature of injector and detector 270 °C, and nitrogen carrier gas flow rate 25 mL/min.

For determination of starch concentration, starch was first hydrolyzed with glucoamylase to glucose. Glucoamylase from *Aspergillus niger* (6,100 U/mL, Sigma) was used for measurement of starch concentration after diluted 100 times with distilled water. Culture broth of 0.1 mL and 0.8 mL DI water were preincubated at 30 °C for 5 min before adding

0.1 mL enzyme solution, and the mixture was incubated at the same temperature for 30 min by shaking at 150 rpm. The reaction was stopped by boiling the mixture for 10 min and centrifuged at×10,000 rpm for 1 min, and the concentration of glucose was determined using a glucose test kit from Human (Glucose Liquicolor). Glucose concentration in the culture broth was similarly determined using the glucose test kit.

Results and Discussion

Fermentation with Free Yeast Cells

The direct fermentation using surface-engineered recombinant *S. cerevisiae* strain YF207/pGA11/pUFLA, which coexpresses the glucoamylase from *R. oryzae* and α-amylase from *S. bovis*, can directly produce ethanol from raw starch. To study the influence of cell concentration on the production of ethanol, fermentation runs were carried out with cell concentrations ranging from 10 to 100 g/L. As shown in Table 1, ethanol concentration increases with increasing cell concentration until reaching 50 g/L, after which no statistical difference ($p<0.05$, one-way analysis of variance) in ethanol concentrations was found between runs carried out with higher cell concentrations. The starch consumption rate and ethanol productivity also shows similar dependence on cell concentrations. However, the ethanol yield from sugar consumed ($Y_{P/S}$) was independent of cell concentration ranging from 0.39 to 0.49, which corresponds to 80 to 96% of the theoretical yield from sugar (0.51 g of ethanol produced per g of sugar consumed). Taken together, 50 g/L was chosen as the best cell density under the experiment conditions, and this value was used in the following studies.

To test the stability of the ethanol production capability of recombinant yeast cells, the cells were recovered by low-speed centrifugation after 7 days and reused for fermentation for up to seven cycles. As shown in Fig. 1, the concentration of ethanol in each consecutive cycle shows no statistical difference ($p<0.05$) between each repeated runs and ranging from 48 to 58 g/L (day 7) for each fermentation cycle.

Cell Immobilization

With the excellent reusability of free recombinant yeast cells, using immobilized cells in a bioreactor could facilitate the reuse of cells and scale up the process. The immobilization of

Table 1 Effects of cell density on ethanol fermentation with free recombinant yeast cells at day 10.

Cell density (g CDW/L)	Starch consumption rate (g starch/h)	Ethanol concentration (g/L)	$Y_{P/S}$[a]	R_P (g h^{-1} L^{-1})[b]
100	0.741	57.12±4.12*	0.40	0.238
75	0.765	55.43±3.45*	0.39	0.231
50	0.741	56.11±4.60*	0.41	0.234
25	0.680	49.68±1.21	0.39	0.207
10	0.383	34.97±4.68	0.49	0.146

[a] $Y_{P/S}$ Product yield (g ethanol produced/g sugar consumed)

[b] R_P Productivity (g ethanol produced h^{-1} L^{-1})

*No statistical difference, $p<0.05$

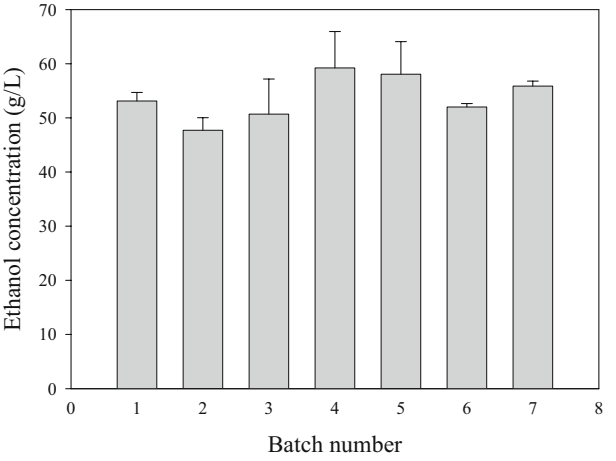

Fig. 1 Repeated batch fermentations with free recombinant yeast cells. Fermentation time for each batch=7 days. Initial cell density=50 g CDW/L

flocculation yeast in a porous carrier is a challenging task considering the size of the flocculated mass. Traditional method for cell immobilization by entrapment in polymeric gel beads is not suitable for construction of fixed-bed bioreactors with the constraints of physical properties for these gel matrices. Alternatively, for cell immobilization by adsorption, the carrier must be highly porous with large pore size to accommodate the flocculated cells. However, the carrier must also have good mechanical strength to withstand the high pressure drop associated with a packed-bed bioreactor. The loofa sponge with pore size around 800 μm was chosen in this study. During the immobilization step, cell suspension was continuously circulated through the column from the top for cell attachment to the loofa sponge. As can be seen from Fig. 2, the efficiency of cell immobilization could reach 100% within 120 min irrespective of flow rates. However, for flow rates above 30 mL/min, cell immobilization could be completed within 20 min. This flow rate was thus chosen as the optimum flow rate for cell immobilization and used in all future fermentation studies.

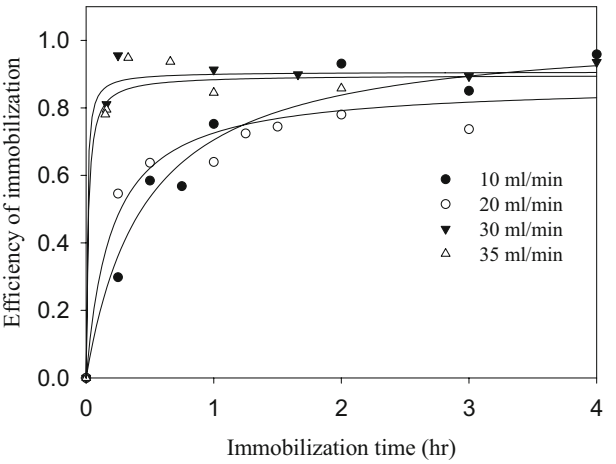

Fig. 2 The effects of recirculation flow rate on cell immobilization efficiency in a packed-bed bioreactor. Cell density=50 g/L

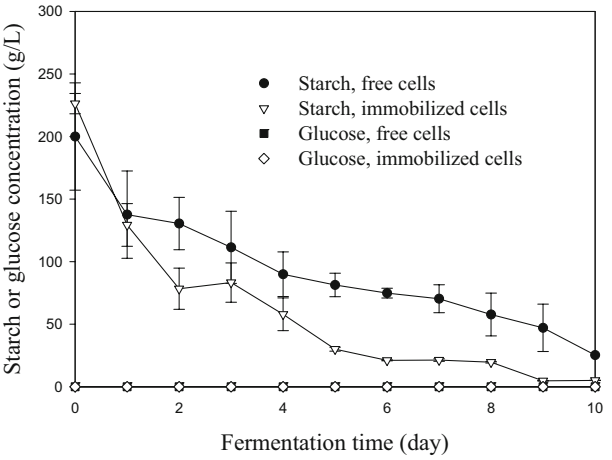

Fig. 3 Starch and glucose concentrations during batch fermentation with free and immobilized recombinant yeast cells. Cell density=50 g/L

Fermentation with Immobilized Yeast Cells

Fermentation by immobilized cells was studied in the fixed-bed bioreactor by circulating the starch-containing medium (YPS medium) through the column at 20 mL/min under anaerobic condition. As can be seen from Fig. 3, starch concentration decreases quickly to 0 after 9 days for the immobilized cell system, and the starch consumption rate for the immobilized cells system is 50% higher than that for the free cell system (1.116 vs 0.741) using the same cell density (50 g/L) for fermentation. Glucose concentration in the fermentation broth remained at close to 0 throughout the fermentation period for both systems (Fig. 3). Increase in ethanol concentrations also shows similar trends for both systems up to day 5 where ethanol concentration reaches 42 g/L, and there is no statistical difference in ethanol concentration for both systems up to day 7 ($p<0.05$). However, no further increase in ethanol concentration was found for the immobilized cell system after day 5. In contrast, the ethanol concentration continues to increase throughout the

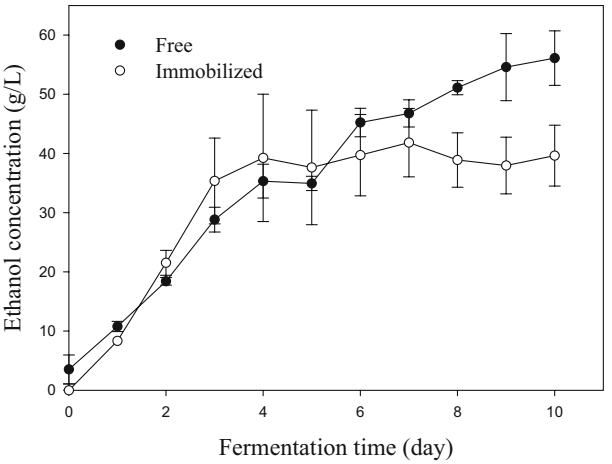

Fig. 4 Ethanol concentrations during batch fermentation with free and immobilized recombinant yeast cells. Cell density=50 g/L

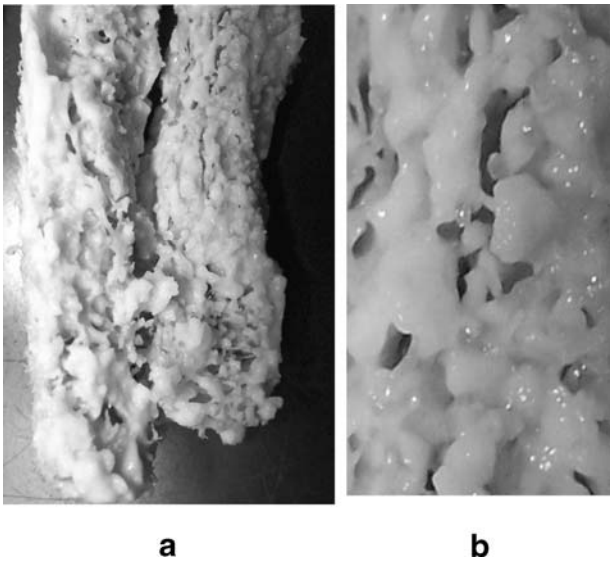

Fig. 5 Attachment of starch granules to the **a** center and **b** the surface of loofa sponge during fermentation with immobilized recombinant yeast cells

fermentation period for the free cell system, and the ethanol concentration reaches 53 g/L at day 7 (Fig. 4). The productivities of ethanol were 0.316 and 0.249 g h^{-1} L^{-1} for free and immobilized cells systems at day 7, respectively.

It is not reasonable to expect a higher starch consumption rate for the immobilized cell system without a concomitant increase in ethanol concentration. Because raw starch granules will be trapped within the pores of the loofa sponge with time when circulated through the carrier, it can be inferred that the amount of starch consumed and rate of starch consumption observed for the immobilized system will be overestimated. During initial fermentation up to day 5, the immobilized cell system is as effective as the free cell system for producing ethanol from raw starch (Fig. 4). Nonetheless, limited diffusion of starch to the immobilized cells at the later stage of fermentation, where the cell surface was covered with stationary starch granules, will limit the supply of starch and hydrolysis reaction to

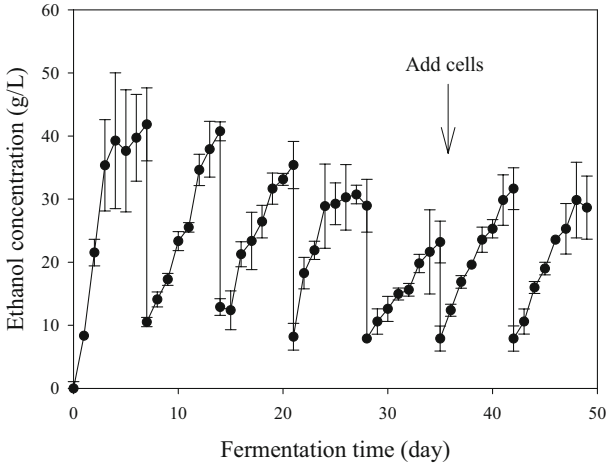

Fig. 6 Repeated batch fermentation with immobilized recombinant yeast cells. Fermentation time for each batch=7 days. Initial cell density=50 g/L. Cells were added at day 35

Table 2 Summary of repeated batch fermentation with immobilized recombinant yeast cells.

Batch number	Starch consumption rate (g starch/h)	Ethanol concentration (g/L)	$Y_{P/S}$[a]	R_P (g h^{-1} L^{-1})[b]
1	1.116	41.81±5.78	0.28	0.249
2	0.893	40.75±1.47	0.34	0.243
3	0.833	35.40±3.74	0.32	0.211
4	0.720	28.94±4.18	0.31	0.172
5	0.697	23.23±3.31	0.25	0.138
6	0.803	31.56±3.30	0.30	0.187
7	0.774	28.63±5.01	0.30	0.170

Fermentation time for each batch=7 days. Initial cell density=50 g/L. Cells were added after batch 5.

[a] $Y_{P/S}$ Product yield (g ethanol produced/g sugar consumed)

[b] R_P Productivity (g ethanol produced h^{-1} L^{-1})

produce glucose. This could be inferred from the appearance of the loofa sponge matrix during fermentation with immobilized cells where white starch granules could be seen to cover the immobilized cells layer in the loofa sponge (Fig. 5). A similar situation will not happen in the case of free cells where starch could be freely contacted with cells by shaking.

Repeated Batch Fermentation with Immobilized Cells

Figure 6 gives the results of repeated batch fermentation with immobilized cells in the bioreactor. Ethanol concentrations decrease with batch numbers, and the ethanol

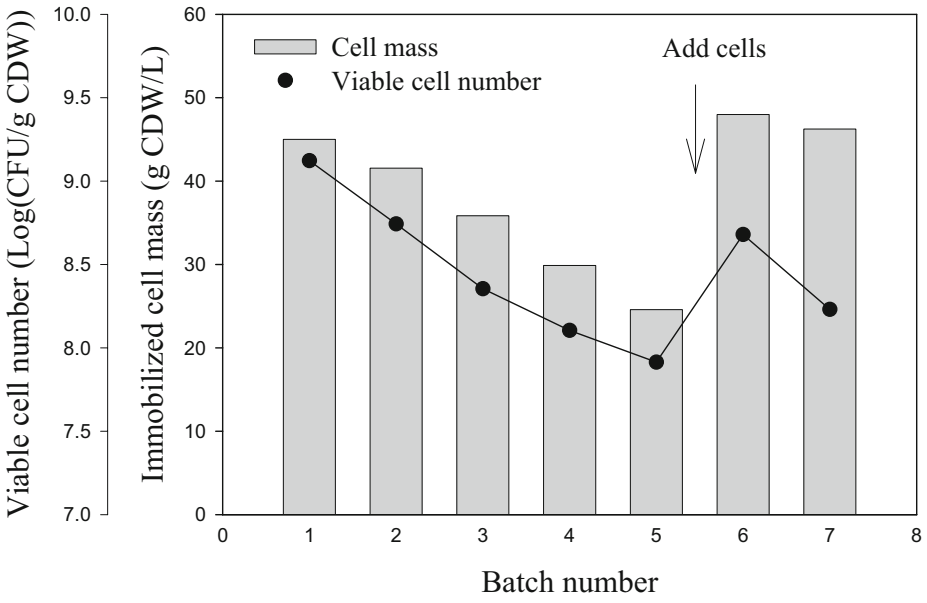

Fig. 7 Cell mass and viable cell number of immobilized recombinant yeast cells during repeated batch fermentation. Fermentation time for each batch=7 days. Initial cell density=50 g/L. Cells were added at day 35. *CDW* Cell dry weight, *CFU* colony-forming unit

concentration after batch 5 drops to 55% of its initial value. Starch consumption rate and ethanol productivity also show similar trends (Table 2). The reasons for diminished ethanol production may arise from loss in cell mass or cell viability. The cell density in the bioreactor after each batch was determined from the amount of cells lost from the carrier and recovered in the culture medium after each batch. The results in Fig. 7 indicates that about 10% cell mass was lost in each run with cell density decreased from 50 to 24.6 g/L at the end of cycle 5. In contrast, the cell viability decreased more than one order from $10^{9.1}$ to $10^{7.9}$ colony-forming unit per g CDW. To compensate for the lost cells, the cell mass was brought to initial value (50 g/L) by immobilizing new cells in the bioreactor at the end of cycle 5. However, the number of viable cells can only be raised to 42% of its initial value with the dead cells left in the bioreactor. Under such condition, ethanol production in cycle 6 could be restored to 75% of that in the first cycle (31.6 g/L; Fig. 6 and Table 2).

References

1. Spencer-Martins, I., & Van Uden, N. (1977). *European Journal of Applied Microbiology*, *4*, 29–35.
2. Knox, A. M., du Preez, J. C., & Kilian, S. G. (2004). *Enzyme and Microbial Technology*, *34*, 453–460.
3. Kondo, A., Shigechi, H., Abe, M., Uyama, K., Matsumoto, T., & Takahashi, S., et al. (2002). *Applied Microbiology and Biotechnology*, *58*, 291–296.
4. Murai, T., Ueda, M., Yamamura, Z. M., Atomi, H., Shibasaki, Y., & Kamasawa, N., et al. (1997). *Applied and Environmental Microbiology*, *63*, 1362–1366.
5. Murai, T., Ueda, M., Kawaguchi, T., Arai, M., & Tanaka, A. (1998). *Applied and Environmental Microbiology*, *64*, 4857–4861.
6. Shigechi, H., Uyama, K., Fujita, Y., Matsumoto, T., Uedac, M., & Tanaka, A., et al. (2002). *Journal of Molecular Catalysis B: Enzymatic*, *17*, 111–222.
7. Shigechi, H., Fujita, Y., Koh, J., Ueda, M., Fukuda, K., & Kondo, A. (2000). *Biochemical Engineering Journal*, *18*, 149–153.
8. Shigechi, H., Koh, J., Fujita, Y., Matsumoto, T., Bito, Y., & Ueda, M., et al. (2004). *Applied and Environmental Microbiology*, *70*, 5037–5040.
9. Satoh, E., Uchimura, T., Kudo, T., & Komagata, K. (1997). *Applied and Environmental Microbiology*, *63*, 4941–4944.
10. Ogbonna, J. C., Liu, Y. C., Liu, Y. K., & Tanaka, H. (1994). *Journal of Fermentation and Bioengineering*, *78*, 437–442.
11. Ogbonna, J. C., Mashima, H., & Tanaka, H. (2001). *Bioresource Technology*, *76*, 1–8.
12. Kobayashi, F., & Nakamura, Y. (2004). *Biochemical Engineering Journal*, *21*, 93–101.