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Reusable Floating Beads with Immobilized Xylose-Fermenting Yeast Cells for Simultaneous Saccharification and Fermentation of Lime-Pretreated Rice Straw

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Abstract: Novel bioreactor beads for simultaneous saccharification and fermentation (SSF) of lime-pretreated rice straw (RS) into ethanol were prepared. Genetically modified *Saccharomyces cerevisiae* cells expressing genes encoding xylose reductase, xylitol dehydrogenase, and xylulokinase were immobilized in calcium alginate beads containing inorganic lightweight filler particles to reduce specific gravity. For SSF experiments, the beads were floated in slurry composed of lime-pretreated RS and enzymes and incubated under CO_2 atmosphere to reduce the pH for saccharification and fermentation. Following this reaction, beads were readily picked up from the upper part of the slurry and were directly transferred to the next vessel with slurry. After 240 h of incubation, ethanol production by the beads was equivalent to that by free cells, a trend that was repeated in nine additional runs, with slightly improved ethanol yields. Slurry with pre-saccharified lime-pretreated RS was subjected to SSF with floating beads for 168 h. Although higher cell concentrations in beads resulted in more rapid initial ethanol production rates, with negligible diauxic behavior for glucose and xylose utilization, no improvement in the ethanol yield was observed. A fermentor-scale SSF experiment with floating beads was successfully performed twice, with repeated use of the beads, resulting in the production of 40.0 and 39.7 g/L ethanol. There was no decomposition of the beads during agitation at 60 rpm. Thus, this bioreactor enables reuse of yeast cells for efficient ethanol production by SSF of lignocellulosic feedstock, without the need for instruments for centrifugation or filtration of whole slurry.

Key words: immobilization, inorganic lightweight filler particles, genetically modified yeast, lime-pretreated rice straw, simultaneous saccharification and fermentation

INTRODUCTION

With the increasing need for limited fossil fuels, lignocellulosic biomass, such as agricultural and forestry residues, is attracting more interest as a renewable source of the second-generation fuel ethanol (bioethanol).[1] Main components of lignocellulosic biomass are two kinds of polysaccharides, i.e., cellulose and xylan, which can be hydrolyzed to produce glucose and xylose, respectively. In addition to the requirement of saccharification of these polysaccharides, efficient fermentation of glucose and xylose to ethanol is also regarded as a key step in bioethanol production.[2)3)] *Saccharomyces cerevisiae,* the most commonly used microorganisms for ethanol production, can efficiently

ferment glucose, but cannot utilize xylose for ethanol production.[4] Therefore, co-fermentation of glucose–xylose mixtures using two kinds of ethanol-producing microorganisms[5] or a genetically engineered yeast strain capable of fermenting both sugars[6)7)8)] has been extensively investigated.

Simultaneous saccharification and fermentation processes (SSF) involve hydrolysis of polysaccharides to monosaccharides and their conversion to ethanol by microorganism(s) in the same reactor vessel. This type of process has several advantages over the use of separate hydrolysis and fermentation processes, such as increased rate of saccharification because of lower inhibition by saccharification products, reduced total conversion time for saccharification and fermentation, and lower capital costs for equipment.[9]

To improve the efficiency of ethanol production, reuse of microbial cells represents a potentially useful technique because reused cells adapt to the fermentation conditions and achieve higher ethanol production efficiency than the corresponding fresh cells.[10] The current reuse methods, however, cannot easily be applied to SSF of lignocellulosic materials because of inefficient recovery of cells in a slurry of partially degraded lignocellulosic materials. Many attempts to integrate a cell reuse step with SSF have been reported, and Matano *et al.*[11] have recently demonstrated that free

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cells can be collected and reused by a two-phase separation process comprising rough removal of lignocellulosic residue by low-speed centrifugation followed by cell recovery using high-speed centrifugation. This type of system could be demonstrated by integration of a centrifugation facility with SSF equipment.

Another integration strategy employing immobilized cells for continuous use or reuse has been demonstrated previously with the immobilized cell system retaining high cell density and exhibiting prolonged cellular stability. This system exhibited an increased yield of ethanol, improved tolerance to highly concentrated substrate, and reduced end-product inhibition.[12)13)14)15)] Immobilized yeast beads have also been employed for a repeated-batch SSF process using rice straw (RS) for hexose fermentation.[16)] In this process, a mesh sieve was used to recover the beads by separation from the filtrate with smaller residues in a slurry.

In this study, we have proposed a new cell reuse system using floating beads with immobilized yeast cells for efficient SSF production of ethanol from alkali pretreated RS. A genetically modified strain of *S. cerevisiae,* capable of xylose fermentation, was immobilized in alginate beads. After fermentation of glucose and xylose, the floating beads settle in the top layer of the slurry and can be readily scooped out. The reusability of the beads was also evaluated in this study by repeating the recovery–reuse steps.

MATERIALS AND METHODS

Microorganism and culture conditions. Genetically modified yeast (ScX), expressing three genes encoding xylose reductase, xylitol dehydrogenase, and xylulokinase, from *Pichia stipitis* was prepared according to a method by Matsushika *et al.*[6)] using the *S. cerevisiae* strain Inv*Sc* 1 (Invitrogen Japan K.K., Tokyo, Japan) as the host for transformation. The ScX cells were maintained at 4 °C on YPD agar (20 g/L peptone, 10 g/L yeast extract, 10 g/L glucose, and 20 g/L agar) plates supplemented with 0.5 μg/L aureobasidin A.

The ScX cells from a single colony on a YPD agar plate were pre-cultured at 30 °C at a speed of 250 rpm for 24 h in YPM [5 g/L peptone, 3 g/L yeast extract, 3 g/L malt extract, 0.2 g/L CaCl$_2$, 2.5 g/L KH$_2$PO$_4$, 0.5 g/L MgSO$_4$ 7H$_2$O, and 1 g/L (NH$_4$)$_2$SO$_4$] medium supplemented with 20 g/L glucose. The grown cells were collected by centrifugation at 3,000 × G for 5 min at 4 °C and suspended in autoclave-sterilized distilled water. The cell concentration was adjusted to 10, 40, and 100 g dry cells/L with autoclave-sterilized distilled water (these ScX suspensions were designated as ScX10, ScX40, and ScX100, respectively).

Yeast cell immobilization. Inorganic lightweight filler particles (Fuji Balloon, Fuji Silysia Chemical Ltd., Aichi, Japan) were mixed with filter-sterilized 1.5 % sodium alginate (500–600 cP, Wako Pure Chemical Industries, Ltd., Osaka, Japan) solution at a particle concentration of 10 % (w/v of total suspension). To this mixture, the ScX cell suspension (ScX10, ScX40, or ScX100) was added at a volume ratio of 1:1, and the aliquots of the mixture (1.2 mL) were dropped into cold, autoclave-sterilized 0.2 M CaCl$_2$

solution through a syringe to prepare 28 beads with a diameter of 4.4 mm. The immobilized yeast beads (designated as ScX10B, ScX40B, and ScX100B, which were prepared from ScX10, ScX40, and ScX100, respectively) were stabilized by settling them in CaCl$_2$ solution at 4 °C for 1.5 h, washed three times with 20 mL cold autoclave-sterilized distilled water, and resuspended in water for storage at 4 °C until use. For a large-scale experiment (2-L fermentor test) and an SSF experiment with pre-saccharified RS, a slight modification of the above procedure was performed for preparation of ScX100B; ScX100 cell slurry was mixed with autoclave-sterilized 2 % sodium alginate solution (High G series I-3G, Kimica Corporation, Tokyo, Japan) containing 10 % lightweight filler particles.

Small-scale simultaneous saccharification and fermentation (S-SSF). Sun-dried RS (cv. *Koshihikari*) was provided by NARO Agricultural Research Center (Ibaraki, Japan) as a by-product of rice grain harvesting. RS was oven-dried at 65 °C to decrease the water concentration below 5 %. It was then coarsely chopped (HM-40, Orient, Chiba, Japan), followed by further milling with a high-speed milling machine (PM-2005, Osaka Chemical Co., Ltd., Osaka, Japan) to pass through a 0.5-mm mesh sieve. RS powders in this study contained 32.6 % glucan and 13.4 % xylan of the total biomass dry weight. RS powders were pretreated with Ca(OH)$_2$, according to the alkali pretreatment method in the calcium capturing by carbonation (CaCCO) process[17)] with the following modifications. RS powders (5 g) were mixed with Ca(OH)$_2$ (0.5 g) and distilled water (11.7 g) in a 50-mL glass vial [(No. 7), Maruemu Corporation, Osaka, Japan]. After sealing with a butyl rubber septum and an aluminum cap, the vial was autoclaved in a high-pressure steam sterilizer (TOMY LSX-700, Tomy Seiko Co., Ltd., Tokyo, Japan) at 120 °C for 1 h. After the vial was cooled to room temperature (25 °C), the mixture was neutralized by injecting filtered CO$_2$ through an inlet needle and releasing the gas in the vial through an outlet needle. The pressure was maintained at 1.5 atm for 2 min, followed by two repeated CO$_2$ treatments to ensure that neutral pH was achieved. After neutralization, the vial was stored overnight at room temperature and used for subsequent fermentation tests.

SSF was initiated by adding the enzyme mixture and immobilized yeast beads to the neutralized pretreated slurry of RS powder. The enzyme mixture (1.5 mL) was composed of Celluclast 1.5 L (Novozymes Japan Co. Ltd., Chiba, Japan) as cellulase/hemicellulase [12.0 filter paper unit (FPU) and 10 U xylanase/g dry RS powders at pH 5], Novozyme 188 (Sigma-Aldrich Japan K.K., Chiba, Japan) as β-glucosidase [43.4 cellobiase unit (CbU)/g dry RS powders at pH 5], and Ultraflo L (Novozymes Japan) as hemicellulase/β-glucosidase (16.8-U xylanase and 0.5 CbU/g dry RS powders at pH 5). Enzyme activities were measured according to IUPAC recommendations.[18)] The free ScX cell suspension or cell-immobilized beads were added so as to reach cell concentrations of 1.2, 4.8, and 12 g dry cells/kg dry RS powders. The concentration of dry RS powders in the whole suspension in each vial was adjusted to 20 % (weight/weight of the whole suspension) with autoclave-

sterilized distilled water. CO_2 gas was injected into the vial at 1.5 atm for 2 min, and the gas in the vial was released through an outlet needle to remove dissolved oxygen. SSF was performed at 30 °C with a stirring speed of 50 rpm for 240 h. After each SSF test, the beads were manually recovered from the suspension using a spatula under sterilized atmosphere, the beads were then directly added to another vial with a suspension of pretreated RS powders with enzymes, and the vial was sealed with a septum and aluminum cap for reuse in the next SSF experiment. The bead reuse experiment was repeated nine times in series. During the SSF test, aliquots (0.5 mL) of the slurry were periodically taken using a syringe with a needle. Each slurry sample was centrifuged at 10,000 × G for 3 min, and the supernatant was used for determining amounts of glucose, xylose, and ethanol.

High-pressure pre-saccharification and small-scale SSF (HS-SSF). Pre-saccharified RS slurry was prepared according to our previously reported method.[19] Chopped RS (10 kg) was mixed with 1 kg of $Ca(OH)_2$ and 23 L of distilled water, and the mixture was crushed using a wet-milling machine (Shokusenki, Shinko Engineering Co. Ltd., Gifu, Japan). The milled mixture was placed in an autoclave bag (Thermo Fisher Scientific Inc., Pittsburgh, USA) prior to heating at 95 °C for 1 h in a high-pressure steam sterilizer (KS-323, Tomy Seiko Co. Ltd.). The bag was cooled to ambient temperature, and the pretreated RS was saccharified by adding enzyme Cellic CTec2 (Novozymes Japan) as cellulose/hemicellulose (12 FPU/g dry RS), under a pressurized condition at 9 atm with CO_2 gas at 15–30 rpm, 40 °C for 72 h. After pre-saccharification, the slurry was manually divided into aliquots, which were put in bags and stored in a freezer until use. Each bag containing an aliquot of the slurry [the concentration of RS: 28.5 % (w/w of slurry)] was thawed in water and used for SSF by adding the enzyme mixture and immobilized yeast beads in a 50-mL glass vial [(No. 7), Maruemu Corporation]. The enzyme mixture was composed of Novozyme 188 as β-glucosidase (2.6 CbU/g dry RS at pH 4.8) and Ultraflo L (Novozymes Japan) as hemicellulose/β-glucosidase (0.5-U xylanase and 0.02 CbU/g dry RS at pH 4.8). Immobilized yeast beads were added to obtain a series of cell concentrations of 4.8, 12, 24, 36, and 48 g dry cells/kg dry RS. Distilled water was added to adjust the concentration of RS to 20 % (w/w), and CO_2 was injected at 1.5 atm for 2 min to ensure anaerobic conditions. An antibiotic, ampicillin sodium salt (Nacalai Tesque, Inc, Kyoto, Japan), was added at a concentration of 120 μg/g slurry to prevent contamination. Total amount of the reaction mixture was 25 g. Fermentation was performed at 30 °C with a stirring speed of 100 rpm for 168 h. After each fermentation test, the beads were manually recovered from the suspension under sterilized atmosphere using a spatula, and all beads were directly added to the next HS-SSF process. The reuse of the beads was repeated one additional time.

Pre-saccharification and fermentor-scale SSF (PF-SSF). RS powders (2 kg) were mixed with 200 g of $Ca(OH)_2$ and 4.67 L of distilled water, and the mixture was autoclaved at 120 °C for 90 min. After the mixture was cooled to room temperature, distilled water (8 L) was added. pH of the pretreatment slurry was lowered to 5 with 5 M HCl, and the neutralized slurry was allowed to settle at room temperature overnight. Solids in the neutralized slurry were recovered with a horizontal belt filter (HBF-04-0.24-S4, Mitsubishi Kakoki Co., Ltd., Kanagawa, Japan), then dried in an oven at 65 °C for 3 days. The dried solids were milled with a hammer mill (PM-2005, Osaka Chemical) to pass through a 0.5-mm sieve to obtain dried, pretreated RS powders (DPRS). In a 2-L fermentor (MDL.N-2L, B. E. Marubishi Co., Ltd., Tokyo, Japan), DPRS (200 g) was mixed with 740 mL of 50 mM sodium acetate (pH 5.0) for pH control and 24 mL of enzyme cocktail composed of Celluclast 1.5 L (12 FPU/g DPRS powders at pH 5), Novozyme 188 (43 CbU/g dry DPRS powders at pH 5), and Ultraflo L (16.8-U xylanase and 0.5 CbU/g DPRS powders at pH 5). Preliminary enzymatic saccharification was performed by mixing the slurry at 200 rpm at 40 °C for 24 h. The temperature of the slurry was then lowered to 30 °C, and immobilized yeast beads (ScX100B, 4.8 g dry cell/kg DPRS) were added to the fermentor for large-scale fermentation. The SSF test was performed at 30 °C with a stirring speed of 60 rpm for 264 h. After the fermentation run, the slurry was statically maintained at 30 °C for 3 h, and the floating beads in the slurry were scooped out and transferred to new slurry, which had been pre-saccharified at 40 °C for 24 h. The second SSF test was performed under the same conditions, except that distilled water was used instead of 50 mM sodium acetate. Aliquots of the slurry (2 mL) were sampled at regular intervals and used for component analyses.

Analytical methods. Amounts of glucose and xylose in RS were determined by the sulfuric acid hydrolysis method described previously.[20] Quantification of amounts of glucose, xylose, and ethanol was performed with high-performance liquid chromatography equipped with a refractive index detector (RID-10A, SHIMADZU Co., Tokyo, Japan), using an Aminex HPX-87 ion exclusion column (Bio-Rad Laboratories, Inc., Hercules, USA) maintained at 50 °C, eluted using a 5 mM sulfuric acid solution at a flow rate of 0.6 mL/min. During each fermentation test, aliquots (0.5 mL for small-scale tests and 2 mL for large-scale tests) of the slurry were periodically taken using a needle and syringe.

Ethanol yield was calculated using Equation (1):

$$\text{Ethanol yield}(\%)=\frac{\text{Produced ethanol(g)}}{\text{The sum of amount of glucose and xylose in RS(g)}\times0.511}\times100 \quad (1)$$

Yeast cell counting. Beads with immobilized yeast cells were placed in 2 % citrate buffer (pH 5) in a test tube, and the tube was shaken to dissolve calcium alginate at 30 °C, with a stirring speed of 250 rpm for 30 min. The number of cells in the suspension was measured with a hemocytometer (DigitalBio, Nano EnTek Inc., Seoul, Korea) using a microscope (Olympus BX51, Olympus Corporation, Tokyo, Japan). Cell survival ratios were determined by the method used by Sami et al.,[21] modified such that the cell suspension was mixed with 0.01 % methylene blue at a ratio of 1:1 (v/v) for 5 min before the ratio of unstained cells was determined.
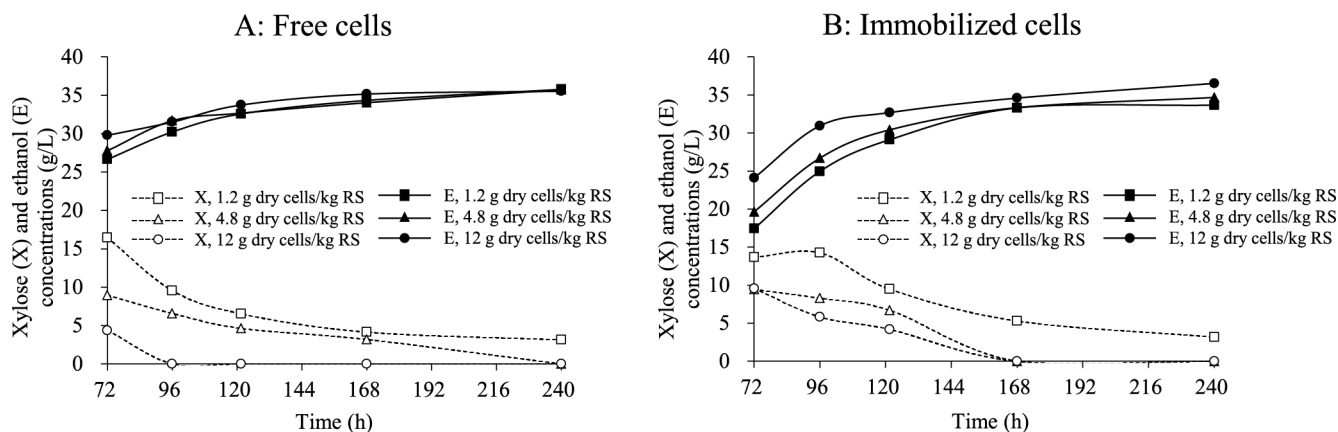
Fig. 1. Change in concentrations of xylose and ethanol in a slurry of lime-pretreated rice straw (RS) for small-scale simultaneous saccharification and fermentation (S-SSF) with free (a) and immobilized (b) yeast cells.

In both, S-SSF tests with three different initial cell concentrations, 1.2, 4.8, and 12 g dry cells/kg dry RS, were performed. All experiments were replicated, and the average values are presented.
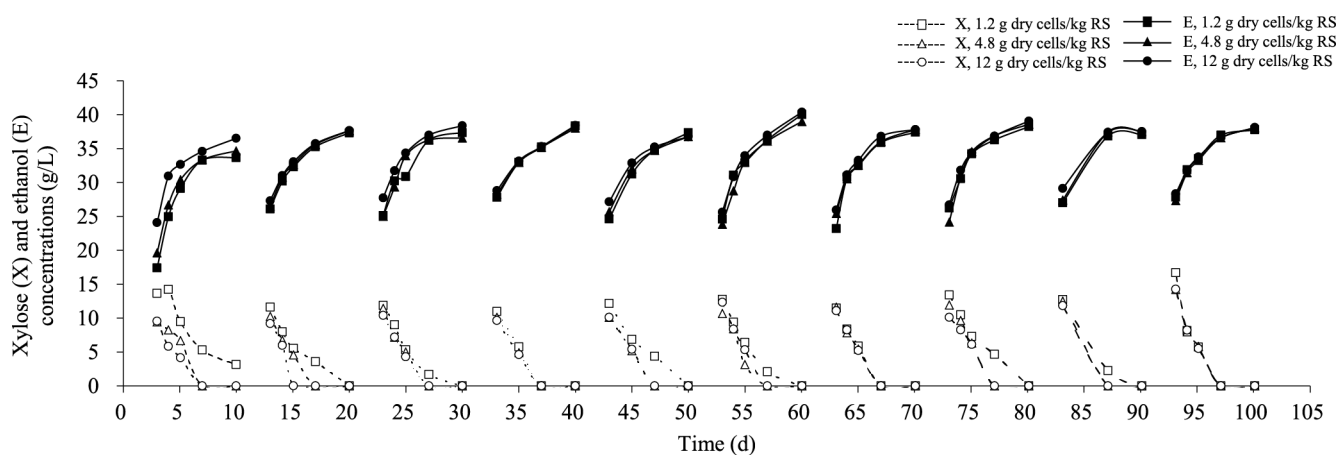

Fig. 2. Change in concentrations of xylose and ethanol in a slurry of lime-pretreated rice straw (RS) for repeated small-scale simultaneous saccharification and fermentation (S-SSF) by multiple-time uses of floating immobilized cells beads.

S-SSF tests were performed with three different initial cell concentrations, 1.2, 4.8, and 12 g dry cells/kg dry RS. Data shown for the first run is the same as that depicted in Fig. 1b. All experiments were replicated, and the average values are presented.

RESULTS AND DISCUSSION

S-SSF experiments.

Figure 1 shows time courses (from 72 h after fermentation to 240 h) of SSF of lime-pretreated RS using xylose-fermenting recombinant yeast cells in the free cell (Fig. 1A) and immobilized cell forms (Fig. 1B). In both figures, glucose was completely consumed before 72 h after fermentation (data not shown). Meanwhile, xylose was consumed faster as the initial cell density increased. The ethanol production rate using immobilized cells was affected to a greater extent by the cell density than that using free cells, whereas the ethanol concentration reached a similar plateau at 240 h after fermentation under all conditions.

Methods for neutralizing the pretreatment slurry and controlling pH for SSF (Fig. 1) have been established as the CaCCO process,[17] and immobilized yeast cells can successfully convert pretreated RS to ethanol in CO_2 atmosphere. The CaCCO process enables omission of a washing step for the pretreated feedstock and a solid–liquid separation step prior to enzymatic saccharification. This minimizes the risk of microbial contamination, which could be a

major problem if bead reactors are repeatedly applied over a long time.

Reusability of immobilized cells under the conditions shown in Fig. 1 was examined for an additional 90 days (Fig. 2). The duration of each SSF was 10 days (240 h), and SSF was performed 10 times in total. The low initial rate of ethanol production using immobilized cells in the first run increased after the second use of the beads, such that ethanol production rates of ScX10B, ScX40B, and ScX100B became equivalent. Ethanol concentration obtained after 240-h fermentation in the initial run was 33.7–36.5 g/L, whereas that in the 2nd–10th runs were slightly higher (37.3–37.7 g/L). The slight increase in fermentation efficiency suggests that immobilized cells adapted to the reaction conditions. Observation of improved ethanol production with reused cells could be explained by improved substrate infiltration and consumption in swollen alginate beads after fermentation.[16)22)23)] Watanabe *et al.*[16] reported that reused cells stably produced ethanol with higher yield than cells in the initial run probably because of better adaptation of the yeast cells to inhibitory compounds after long-term exposure to the slurry.

In this study, beads with three initial cell concentrations, i.e., 1.2 g dry cell/kg dry RS, 4.8 g dry cell/kg dry RS, and 12 g dry cell/kg dry RS, showed equivalent ethanol productivities after reuse, suggesting that high cell concentrations in the beads are unnecessary for ethanol production. Under these three conditions, differences in cell concentrations decreased, and approximately 5.3 g dry cells/kg dry RS (6.6 × 10^7 cells/bead) were observed under all three conditions after 10 runs.

In a model fermentation test for 48 h using immobilized yeast beads and YPM supplemented with 5 % glucose and 2 % xylose, 16.4, 6.4, and 4.0 % of immobilized yeast cells were released into the medium from ScX10B, ScX40B, and ScX100B, respectively (data not shown). The low numbers of released cells indicate that immobilized cells can be used as a stable system for efficient fermentation. It is notable that 10 rounds of serial fermentations can be efficiently performed without supplementation with new yeast preculture.

Before starting SSF, floating beads were observed to be mixed throughout the slurry. However, following incubation of the slurry for 168 h, the amount of liquid in the slurry was found to increase because of enzymatic liquefaction of RS fibers, and the beads were then found in the uppermost part of the slurry. It is anticipated that centrifugation- or filtration-assisted separation of the beads from solids in the slurry can be omitted when transferring the beads to the next reaction vessel because they could be easily removed and transferred by manual use of a spatula in our reuse experiment (Fig. 2). Beads made of calcium alginate were fragile under harsh conditions, such as high-speed agitation of the slurry; the beads were stable at 50 rpm, whereas most of them were found to be broken at 150 rpm causing precipitation (data not shown). If the beads had not floated, they would have dispersed or precipitated in the slurry, making recovery of the beads by filtration of the whole slurry challenging because of the presence of other insoluble matter. These manipulations could adversely affect mechanical stability of the beads. Using our methods, the calcium alginate beads appeared to be stable, without any visible decomposition after 10 runs for total 100 days (Fig. 2). This suggests that re-stabilization of alginate might be achieved in the pretreatment slurry using calcium ions.

HS-SSF experiments.

High-pressure pre-saccharified RS slurry was used for ethanol production with the floating beads to evaluate the effect of cell concentration on the ethanol production profile (Fig. 3). A two-step process was adopted for rapid fermentation of monomeric sugars (glucose and xylose) by immobilized yeast cells with various cell concentrations: first, a 72-h pre-saccharification reaction step, followed by a 168-h SSF step under CO_2 atmosphere. Pre-saccharification with a commercial cellulase preparation was performed in the first step to provide the yeast cells with sufficient amount of fermentable glucose at the initial stage of fermentation in the second step; in this condition with sufficient sugars it is expected that the yeast cell concentrations would significantly affect the fermentation profiles. At lower cell concentrations of 4.8 and 12 cells/kg RS, slower

initial ethanol production rates were observed with diauxic behavior[24] in which xylose consumption becomes slow until most of the glucose is consumed (Figs. 3A and 3B). At higher cell concentrations of 24–48 g cells/kg RS, the diauxic behavior became negligible and faster initial ethanol production rates were observed (Figs. 3C, 3D, and 3E). The reaction using a high cell concentration is ideal for rapid ethanol production. In addition, efficiencies of fermentation with reused beads were excellent in all cases, suggesting that combination of a sufficient supply of fermentable sugars and relatively short-duration SSF reaction used in this experiment enabled the cells to remain viable. Differences in ethanol concentrations after 168 h of SSF were not observed, suggesting that larger quantities of cells would simply result in consumption of more sugars for cell growth without increasing the ethanol yield.[15]

It was postulated that slow diffusion of a solution with glucose and xylose in immobilized yeast beads would cause rapid and selective consumption of glucose at the surface of the beads, where the resultant xylose-rich solution inside the beads would be further assimilated by the inner cell. Thus, immobilized cells could, in part, eliminate the diauxic behavior.[25] Our observations of simultaneous consumption of glucose and xylose (as denoted in Figs. 3C, 3D, and 3E) support this hypothesis. In our preliminary result, the concentration of glucose required to start xylose consumption was higher for immobilized yeast cells than for free cells (Fig. S1; see J. Appl. Glycosci. Web site). Further studies will be necessary to optimize the use of immobilized yeast beads for more efficient glucose and xylose fermentation.

PF-SSF experiments.

A pre-saccharification and fermentor-scale SSF (PF-SSF) experiment using the floating beads was also performed (Fig. 4). In this experiment, lime-pretreated, thoroughly washed RS was used as substrate for pre-saccharification at 40 °C for 24 h, without using CO_2 for neutralization and pH control for the CaCCO process. Pre-saccharification in the first step is expected not only to increase the amount of fermentable sugars in the initial stage of fermentation but also to liquefy solid biomass to less-viscous slurry so that the stirring blades could turn smoothly and keep the yeast-immobilized beads unbroken. In the first run, rates of glucose and xylose consumption as well as ethanol production were slightly lower than those observed during the second run. However, final ethanol concentrations after 264-h incubation were almost the same (40.0 g/L for the first run and 39.7 g/L for the second run corresponding to the ethanol yields of 61.4 and 60.9 %, respectively). Stirring blades in the fermentor (at 60 rpm) did not appear to break the floating beads under the incubation condition. Thus, our success in using floating beads for this fermentor-scale experiment confirms the potential of this system as a new biotechnology tool for efficient catalysis.

Further improvement of the bioreactor for industrial uses could be achieved using heat-stable, stress-tolerant microorganisms and co-immobilization of enzymes, such as β-glucosidases, β-xylosidases, and amyloglucosidases (for
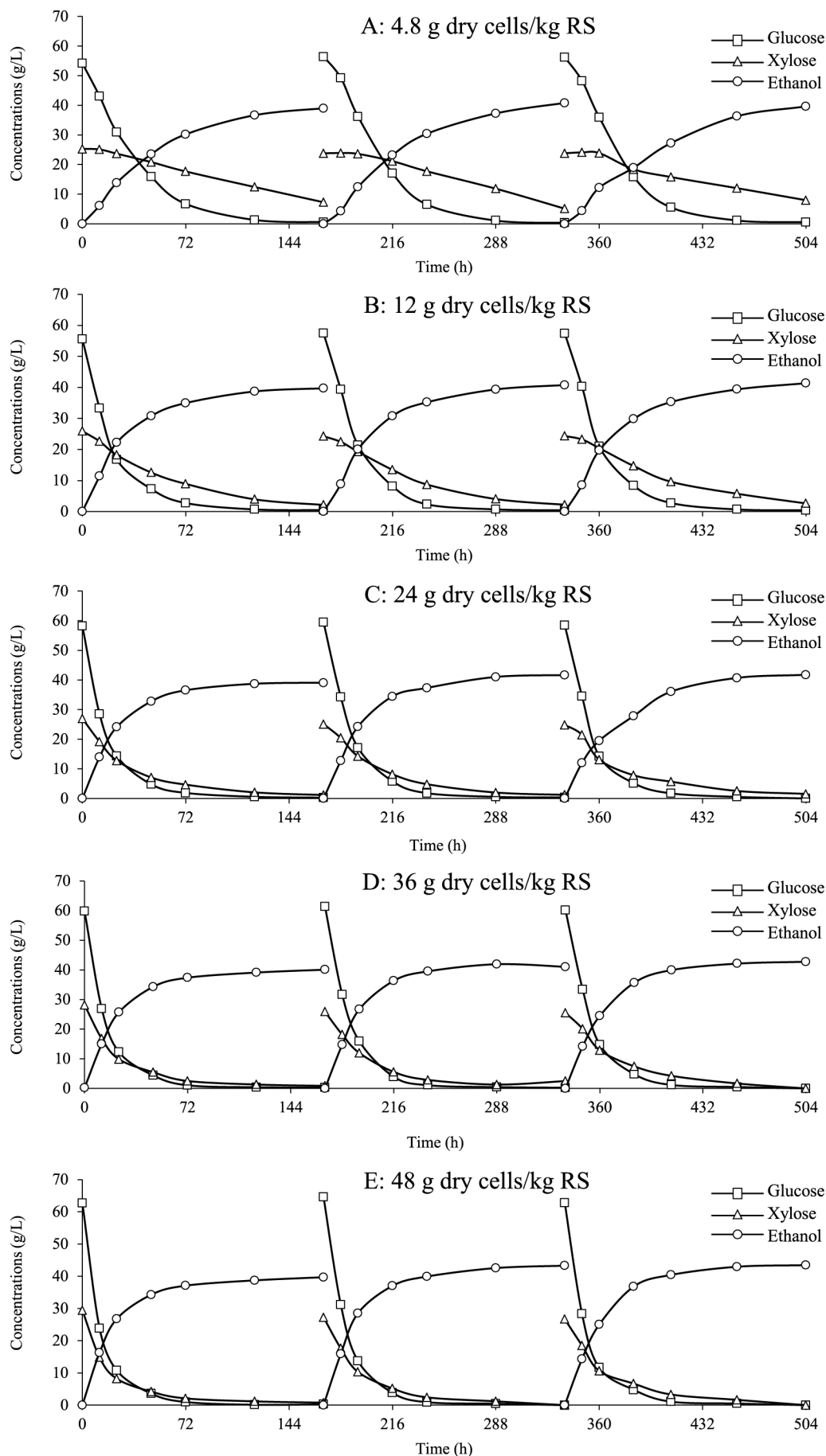
Fig. 3. Time courses of monomeric sugar consumption and ethanol production in repeated high-pressure pre-saccharification and small-scale simultaneous saccharification and fermentation (HS-SSF) tests with pre-saccharified lime-pretreated rice straw (RS) and immobilized yeast beads.

HS-SSF tests using five different initial cell concentrations, 4.8, 12, 24, 36, and 48 g dry cells/kg dry RS, were performed with twice repeated uses of the beads (Figs. 3A, 3B, 3C, 3D, and 3E, respectively). All experiments were performed in triplicates, and the average values are presented.
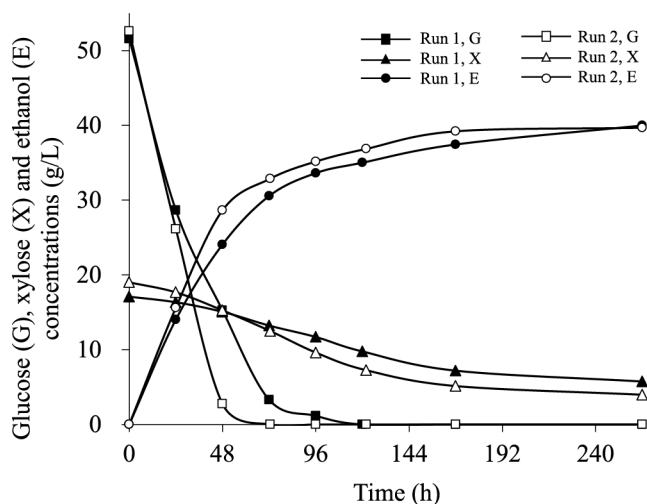
Fig. 4. Time courses of monomeric sugar consumption and ethanol production at pre-saccharification and fermentor-scale simultaneous saccharification and fermentation (PF-SSF) tests with pre-saccharified lime-pretreated rice straw (RS) and immobilized yeast beads.

PF-SSF tests were performed with an initial cell concentration of 4.8 g dry cells/kg of dried, lime-pretreated RS, and the beads from the first run were reused for the second run. All experiments were replicated, and the average values are presented.

starch digestion),[9] to the beads. Sakakibara *et al.*[26] recently developed heat-tolerant (at 40 °C), xylulose-fermenting yeast strains for efficient ethanol production from glucose and xylose. These yeasts are promising candidates for immobilizing in floating beads, which may be further improved by co-immobilization with xylose isomerases.

CONCLUSION

Floating beads containing immobilized xylose-fermenting *S. cerevisiae* cells were used for SSF of lime-pretreated RS, and their stable performance, from the 2nd run to the 10th run in a repeated-batch experiment, was confirmed. The challenge of slow saccharification rates in SSF was partially solved using pre-saccharified slurry for fermentation with floating beads, which resulted in a high fermentation performance starting with the initial run. Successful results of a fermentor-scale experiment with the floating beads will lead to further improvement of this new bioreactor for industrial applications. The beads can be readily prepared and also dissembled to components of calcium, alginate, inorganic filler particles for floating, and free yeast cells and this would save the cost of preparing beads as these parts could be reused. Thus, the reactor system represents an improved method for ethanol production from RS by simple bioprocesses for use in Japan and other countries.

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