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Flocculation of an Isolated Flocculent Yeast, *Candida tropicalis* HY200, and its Application for Efficient Xylitol Production Using Repeated-Batch Cultivation

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Abstract Flocculation of *Candida tropicalis* HY200 was systemically investigated to elucidate its mechanism, and used for cell cycles in repeated-batch cultivations for the production of xylitol from xylose. Flocculation occurred only after the late exponential phase of growth in the culture media and buffer within the narrow pH range of 3.0–5.0. The flocculation was completely inhibited by treatments of cells with proteases and partially reduced by treatments with carbohydrate-hydrolyzing enzymes and by the presence of mannose and glucose. The addition of calcium ions significantly enhanced the flocculation during cultivation, which was completely abolished by the addition of EDTA. The flocculent yeast HY200 provided repeated-batch cultivations employing cell recycles by flocculation over 6 rounds of cultivation for the production of xylitol from xylose, resulting in a relatively high productivity of averaged 4.6 g xylitol/l·h over six batches and maximal 6.3 g xylitol/l·h in the final sixth batch. Cell recycle by flocculation was fast and convenient, which could be applicable for the industrial scale of xylitol production.

Key words: *Candida tropicalis*, flocculation, repeated-batch cultivation, xylose, xylitol

Xylitol, a natural functional sweetener, is increasingly used in the food and pharmaceutical industries because of its attractive characteristics, such as high sweetening power, anticariogenicity, and its tolerance by diabetics [10]. The microbial production of xylitol has been extensively studied mainly using *Candida* species, such as *C. guilliermondii* [5], *C. parapsilosis* [25], and *C. tropicalis* [32]. Among many critical factors for efficient xylitol production, improvement of volumetric productivity and yield of xylitol from xylose are important, which can be achieved by increasing cell mass in a bioreactor by cell recycles for fed-batch and repeated-batch operations [2, 3, 6, 16–18, 20]. Cell recycles have been achieved by employing extra processes, such as filtration [3, 6, 20] and centrifugation [18], which are complicated, time-consuming, and expensive processes. As an alternative approach, flocculation is an attractive property that can provide a convenient means of cell recycles by separating cells from culture broth after *in-situ* sedimentation of cells in the bioreactor.

Yeast flocculation can be defined as an asexual aggregation process observed for certain yeasts into cell flocs, with subsequent fast sedimentation in the medium [27, 29]. Flocculation of yeast cells has been considerably important in the brewing industry because it provides an effective, simple, and cheap separation process of yeast cells from culture broth at the end of fermentation [13, 33]. Thus, yeast flocculation has been extensively studied mainly for the Brewer's yeasts; *i.e.*, *Saccharomyces* species. Flocculation has also been observed in other yeast genera, such as *Kluyveromyces* [1, 9], *Hansenula* [28], *Schizosaccharomyces* [9, 14], *Kloeckera* [11], and *Candida* [7, 19].

The mechanism of flocculation, however, is not yet completely understood and has been proposed by several different hypotheses. The most generally recognized mechanism is the lectin-like model [21, 22], based on the observations that flocculation is reversibly inhibited by free sugars in the medium [29]. According to the model, specific lectin-like proteins (zymolectins) present only in flocculent cells, and encoded from the *flo* gene [29, 30], are secreted from the cell wall, and the N-terminal part of this protein binds sugar

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residues present in cell walls. Thus, flocculation may occur through specific interactions between the zymolectins that stick out of the cell walls of flocculent cells and the sugar residues present in the cell walls of neighboring cells [13, 21, 22, 29, 31]. In this process, calcium ions are needed in order to activate the zymolectins, supported by reversible dispersion of floc cells by EDTA addition [21, 22, 29, 31].

Very recently, we isolated a novel strain of *Candida tropicalis*, HY200, which steadily flocculated at the end of cultivation and efficiently produced xylitol from xylose with a decent xylitol yield (~70%) [15]. In this study, we systemically characterized the flocculated of HY200 to elucidate its mechanism by investigating effects of various factors, such as growth phases, enzyme treatments, sugars, salt, calcium ions, and pH, on the flocculation. In addition, repeated-batch cultivations with cell recycling by flocculation were successfully performed for the establishment of an efficient xylitol production system from xylose.

MATERIALS AND METHODS

Chemicals and Enzymes

Culture media components were purchased from Becton Dickinson (MD, U.S.A.). D-Xylose, xylitol, mannose, glucose, lactose, sucrose, fructose, galactose, and rhamnose were purchased from Sigma (MO, U.S.A.). Cellulase (1,4-(1,3:1,4)-β-D-glucan 4-glucano-hydrolase, 1 U/mg) and hemicellulase (β-galactose dehydrogenase, 0.1 U/mg) were obtained from Sigma, an endoglycosidase of PNGase F (1.8 U/g) from New England Biolabs (MA, U.S.A.), protease mixtures [papain (30 U/mg), pepsin (250 U/mg), pronase (7 U/mg), and trypsin (110 U/mg)] from Roche (Mannheim, Germany), and proteinase K (30 U/mg) from Intron Inc. (Sungnam, Korea). All other reagents were of analytical grade.

Microorganism and Flask Cultures

The strain used throughout this study was *Candida tropicalis* HY200, recently isolated from rice paddy in Korea [15] and deposited to Korean Culture Center of Microorganisms (Taejon, Korea). The yeast was maintained frozen at −70°C on 20% (v/v) glycerol stocks and subcultured every 6 months. A frozen cell suspension was inoculated into 250-ml Erlenmeyer flasks containing 50 ml of growth medium with 5 g/l yeast extract, 3 g/l malt extract, 3 g/l peptone, and 20 g/l xylose, and cultivated at 30°C and 200 rpm for 36 h in a shaker incubator. For the test of flocculation in media containing various carbon sources, xylose was replaced with the same amount of other carbon sources. For the preparation of culture media, carbon sources, nitrogen sources, and, if necessary, calcium chloride and EDTA were autoclaved separately and mixed together before the cell culture.

Flocculation Assay

The extent of flocculation was assayed directly in the culture media or in buffer, as described below. For the assay in buffer, yeast cells were harvested by centrifugation (4,000 rpm for 5 min), washed with double-distilled water, and resuspended in sodium acetate buffer (50 mM, pH 5.0). The extent of flocculation was quantified by measuring the total cell concentration and the non-flocculated fraction [8]. The flocculation ability (FA) was calculated in percentage as follows:

$$FA\ (\%)=[1-(A_2/A_1)]\times 100,$$

where A_1 is the optical density at 600 nm (A_{600}) of yeast suspension at time=0 in culture medium or in 50 mM sodium acetate buffer (pH 5.0) after vigorous shaking for 10 s, and A_2 is the A_{600} of the upper phases of yeast suspension after 5 min of standing at room temperature without shaking. The cell concentration used for the assay of flocculation was 5×10^7 cells/ml, unless otherwise specified.

Effect of Enzyme Treatments, Sugars, Triton X-100, Salt, and pH on Flocculation

Stationary phase cells grown on xylose-containing media for 36 h were harvested by centrifugation, washed twice with distilled water, and resuspended in 50 mM sodium acetate buffer (pH 6.5). For enzyme treatments, about 1×10^8 cells/ml were mixed with each enzyme and incubated at 37°C for 1 h with constant gentle mixing [11, 12]. The enzyme concentrations used were as follows: cellulase, 2 mg/ml; hemicellulase, 2 mg/ml; PNGase F, 0.2 mg/ml; proteases mixtures, 0.2 mg/ml; proteinase K, 0.2 mg/ml. After the enzyme treatments, samples were washed with the buffer and subjected to the flocculation assay as described above.

Effects of sugars, Triton X-100, and sodium chloride on flocculation were determined by addition to the resuspended cells in 50 mM sodium acetate buffer (pH 5.0). After incubation at 30°C for 30 min with constant gentle mixing, samples were subjected to the flocculation assay. The concentrations used were as follows: Triton X-100, 1% (v/v); sugars (xylose, mannose, glucose, lactose, sucrose, fructose, galactose, and rhamnose), 250 and 500 mM; NaCl, 0.25–1.0 M.

The flocculation of HY200 over various pH ranges was assessed by washing of the stationary phase cells with double-distilled water and then resuspending in the following buffers: 50 mM Na-acetate (pH 2–6), 50 mM Tris-Cl (pH 7–9), and 50 mM Na_2HPO_4-KOH (pH 10) [11, 35].

Effect of Calcium Ions and EDTA on Flocculation

Calcium chloride (7.5 mM) with or without EDTA (50 mM) were added to the initial flask growth medium. Cells were grown in 250-ml Erlenmeyer flasks at 30°C and 200 rpm for 36 h in a shaker incubator, and the extent of flocculation was monitored in the various growth phases.

Repeated-Batch Cultivations with Cell Recycles by Flocculation

A frozen cell suspension was precultured at 30°C and 200 rpm for 9 h in a 250-ml Erlenmeyer flasks containing 50 ml growth medium composed of 5 g/l yeast extract, 3 g/l malt extract, 3 g/l of peptone, and 20 g/l glucose. The seed culture was transferred to the 2.5-l jar bioreactor (KoBioTech, Korea) with 1-l working medium composed of 100 g/l xylose, 10 g/l yeast extract, 10 g/l bacto-peptone. The initial pH of the medium was about 6.4. The pH of the culture broth was monitored during cultivations, but not controlled. Temperature was controlled at 30°C and the rates of agitation and aeration were 360 rpm and 1.0 vvm or 420 rpm and 0.5 vvm [15]. Foams were controlled by the addition of an antifoam agent (antifoam 289, Sigma). At the end of each batch culture, monitored by the concentration of xylose below 5 g/l, agitation and aeration were stopped to allow flocs to settle. After the sedimentation by flocculation, the supernatant was pumped out carefully so as not to disturb the flocs cells. About 80% (*i.e.*, 0.8 l) of culture broth was removed, leaving about 0.2 l culture broth containing the flocculated cells in the bioreactor. Then, 0.8 l of fresh medium was added to resuspend the flocculated cells, and subsequently the next batch started under the above same conditions. On the assumption that all media components were almost consumed at the end of each batch, the feeding of 0.8-l fresh media contained 125 g/l xylose, 12.5 g/l yeast extract, and 12.5 g/l bacto-peptone, the same composition as the first batch.

Cell growth was monitored by A_{600} using a spectrophotometer (UV-1201, Shimadzu, Japan). Dry cell weight (DCW) was estimated using a calibration curve of the relationship between A_{600} and DCW. Xylitol and D-xylose were determined by high-performance liquid chromatography (Waters, U.S.A.) using a carbohydrate analysis column (Waters, U.S.A.) with a refractive index detector (Waters, U.S.A.). The column was eluted with 80% acetonitrile at a constant flow rate of 2 ml/min at room temperature [15].

Results and Discussion

Flocculation Characteristics of *C. tropicalis* HY200

The yeast strain *C. tropicalis* HY200, which was recently isolated from rice paddy, produced xylitol from xylose with the novel capability of flocculation at the end of culture [15]. The flocculation occurred so fast that cells were sedimented within 15 min in the 2.5-l bioreactor with 1-l working volume (Figs. 1A and 1B) at the end of cultivation in the formulated xylitol-producing medium. Compared with that for non-flocculated cells from the early exponential phase, the flocculated cells from the stationary phases showed large cell aggregates or flocs composed of tens of cells under microscopy (Figs. 1C and 1D), consistent with the flocs morphology of *S. cerevisiae* [22]. No matter what kinds of carbon sources were used in the growth media, such as xylose, glucose, galactose, fructose, maltose, and sucrose, flocculation was always observed at the end of cultivation [15].

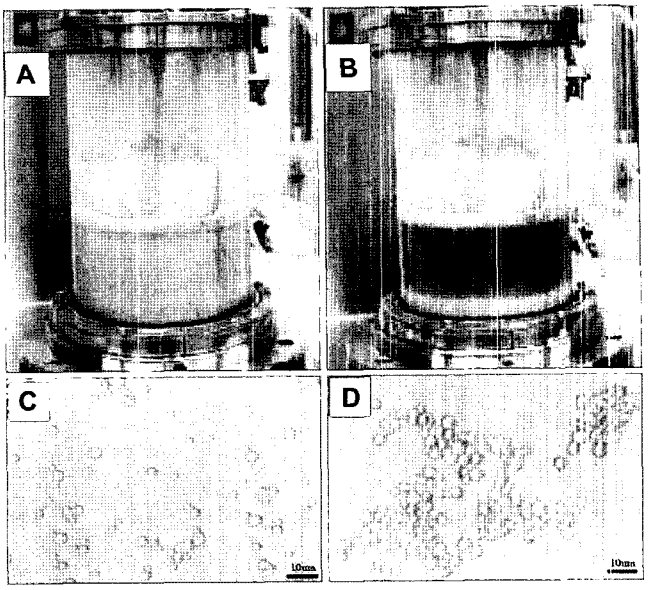

Fig. 1. Flocculation of *C. tropicalis* HY200.
The flocculation of HY200 in a 2.5-l jar bioreactor containing 1-l working volume, after stopping agitation immediately (**A**) and for 15 min (**B**). Cells were grown in the medium composed of 100 g/l xylose, 10 g/l yeast extract, and 10 g/l bacto-peptone at 30°C, 360 rpm, and 1.0 vvm for 30 h. Light microscopic images of non-flocculent cells from the early exponential phase (**C**) and flocculated cells from the stationary phase (**D**). Scale bars represent a scale of 10 mm.

HY200 cells from the various growth phases were submitted to a flocculation assay with the same amount of cells (5×10^7 cells/ml) after washing with double-distilled water and resuspension in 50 mM sodium acetate buffer (pH 5.0). Cells from the late exponential (61.3%) and stationary (85.9%) phases were efficiently flocculated after 5 min of standing in the buffer (Fig. 2A). However, the cells from the early and middle exponential phases did not flocculate, and only showed slow sedimentation by gravity. The cells of stationary phase efficiently flocculated over the wide range of cell density forming the plateau at over 5×10^7 cells/ml (Fig. 2B). In contrast, no significant flocculation was observed for the cells from the early exponential phase up to 9×10^8 cells/ml (Fig. 2B). Thus, the growth phase of cells was a critical factor to induce flocculation of HY200, rather than cell density, suggesting that some factors responsible for the flocculation were only present and/or active in the stationary phase. This observation was consistent with the flocculation for the widely characterized flocculent *Saccharomyces* sp., the flocculation of which dominantly occurred at the

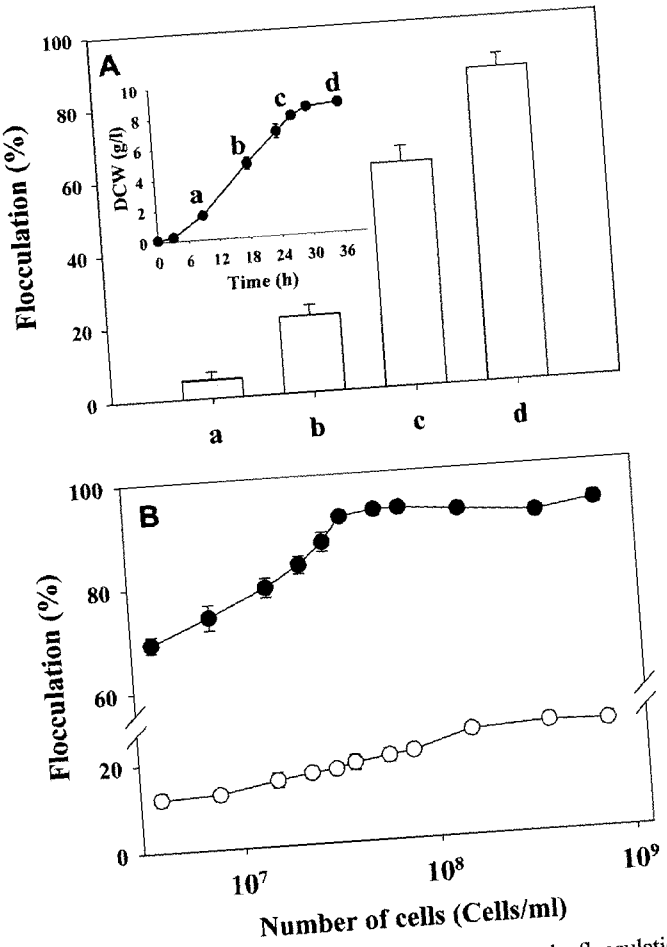

Fig. 2. Effects of growth phases and cell density on the flocculation of HY200.
A. Flocculation of cells from various growth stages of early exponential (a), middle exponential (b), late exponential (c), and stationary (d) phase. *Inset:* the growth profile of HY200 in the shake flask, where cells were taken from the denoted points. The same amount of cells (5×10^7 cells/ml) was used for the assay. **B**. Flocculation assay with various cell densities taken from the early exponential phase (○) and the stationary phase (●). Error bars represent the standard deviation from triplicate experiments.

stationary phase under nutrient starvation conditions [29]. To elucidate which mechanism is involved in the flocculation of HY200, further studies were carried out as discussed below.

Effect of Sodium Chloride, Triton X-100, Enzyme Treatments, and Sugars on Flocculation

To investigate whether the flocculation of HY200 is mediated by nonspecific interactions, flocculation was assayed after adding NaCl (0.25 to 1 M) and 1% Triton X-100 to the cell suspension in 50 mM sodium acetate buffer (pH 5.0). Even though the flocculation efficiency gradually decreased with increase of NaCl up to 1 M, the flocculation occurred substantially in the presence of NaCl up to 1 M and 1% Triton X-100, excluding the possibility of flocculation through nonspecific interactions.

Next, in order to test whether the flocculation of HY200 is mediated by specific interactions between zymolectins and carbohydrates, like the lectin-like model, HY200 from the stationary phase was tested for flocculation after treatments

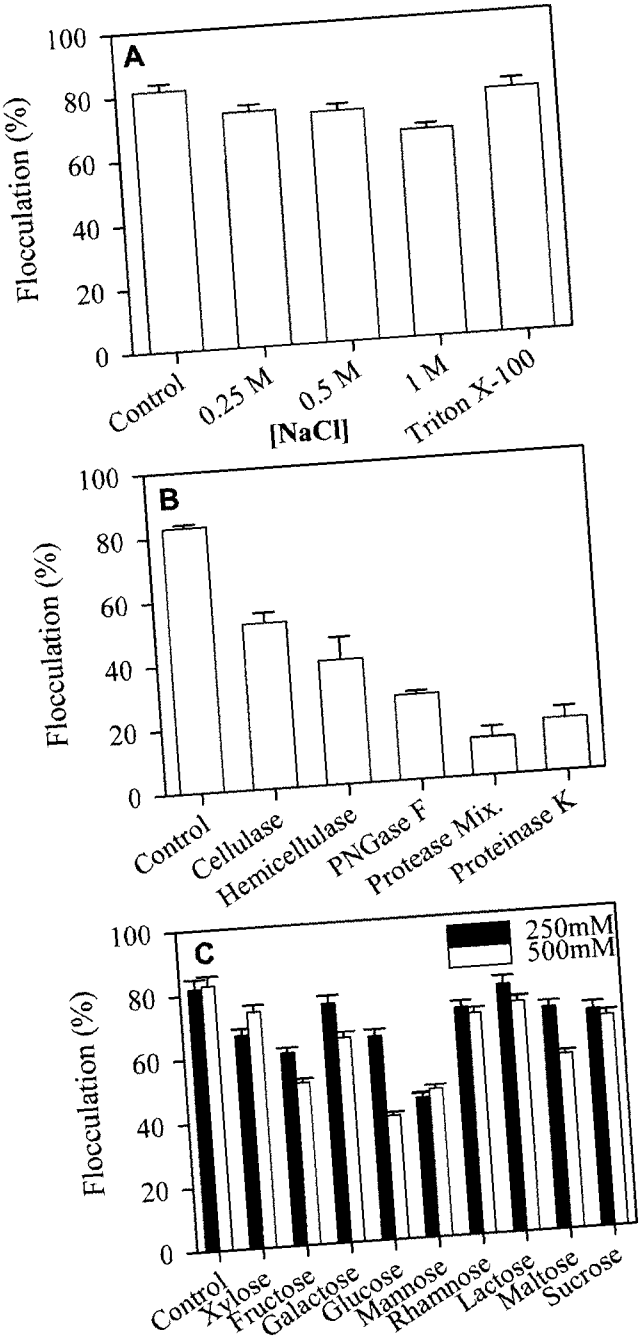

Fig. 3. Effects of NaCl, Triton X-100, enzymes, and sugars on the flocculation of HY200.
A. Flocculation of cells from the stationary phases in the presence of various concentrations of NaCl and 1% Triton X-100. **B**. Flocculation of cells from the stationary phases after treatments of cells with various enzymes at 37°C for 1 h. **C**. Flocculation of cells from the stationary phases in the presence of free sugars (0.25 and 0.5 M). Error bars represent the standard deviation from triplicate experiments.

with various enzymes, such as cellulase, hemicellulase, endoglycosidase (PNGase F), and proteases. Treatments of cells with the protease mixture (papain, pepsin, pronase, and trypsin) and proteinase K at 37°C for 1 h caused a complete irreversible loss of flocculation ability of HY200 (Fig. 3B), indicative of a critical role of cell wall proteins in the flocculation. Flocculation mediated by zymolectins has been known to be irreversibly abolished by protease treatments in other yeasts, such as *S. cerevisiae* [23, 29, 30] and *K. apiculata* [11]. Treatments of cells with carbohydrate-hydrolyzing cellulase and hemicellulase significantly reduced flocculation ability below 50% (Fig. 3B). In particular, PNGaseF, which cleaves all types of oligosaccharides from N-linked glycoproteins, almost got rid of cells flocculation ability (Fig. 3B). To examine further influences of sugars on the flocculation, various free sugars (0.25 and 0.5 M) were added to the cell suspension for the flocculation assay. Some sugars significantly inhibited the flocculation in the order of mannose, glucose, fructose, and maltose (Fig. 3C). However, xylose, sucrose, lactose, rhamnose, and galactose only slightly reduced the flocculation (Fig. 3C). Thus, it is most likely that sugars on the cell surface, mainly mannose and glucose, are involved in the flocculation of HY200. With *S. cerevisiae*, flocculation is inhibited by only mannose for Flo1 phenotype and by mannose, glucose, sucrose, and maltose for NewFlo phenotype [8, 29, 30, 33]. On the other hand, galactose specifically inhibited the flocculation mediated by galactose-specific zymolectins of *Kluveromyces* sp. [1, 9] and *K. apiculata* [11]. The inhibition of flocculation by free sugars has been explained by specific binding of sugars to the zymolectins so that they can no longer bind the sugar residues on the cell wall of adjacent cells [8, 29, 30, 33].

Taken together, it is most likely that the flocculation of HY200 is mediated by the specific interactions between zymolectins and sugar residues of mannose and glucose in the cell wall of adjacent cells. The flocculation ability of HY200 in the stationary phase only suggest that the unknown zymolectin might be induced under nutrients starved conditions, like the zymolectins responsible for flocculation in other yeasts [22, 29, 30, 33].

Effect of pH, Calcium Ion, and EDTA on Flocculation

Flocculation of other yeasts has been observed over a wide pH range of 2–9 [21, 33], with optimal efficiency between pH 3.0–5.5 for *S. cerevisiae* [13, 29] and *K. apiculata* [11]. The influence of pH on the flocculation of HY200 from the stationary phase was examined over the various pHs in the range of 2–10. Flocculation occurred optimally across a narrow pH range of 3.0–5.0, with significant inhibition at lower (pH<3) and higher (pH>6.0) ranges (Fig. 4). The optimal flocculation within the narrow pH ranges suggests that zymolectins might be inactive at extreme acidic and basic pH owing to conformational changes that occur when the

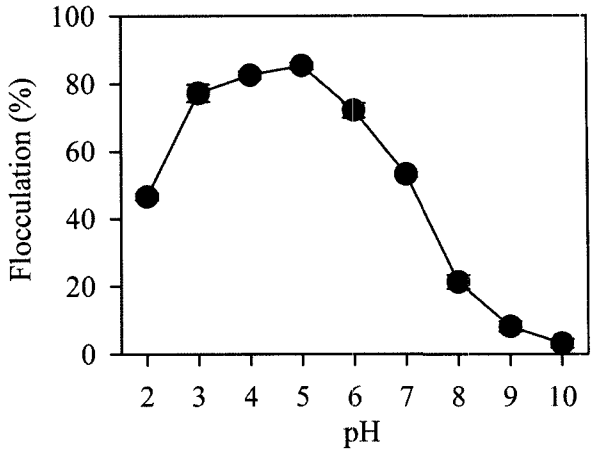

Fig. 4. Effects of pH on the flocculation of HY200.
Error bars represent the standard deviation from triplicate experiments.

electrostatic charge of surface proteins changes, as proposed for other yeasts [13, 29]. The optimal flocculation around pH 4.0 was ideal to induce flocculation at the end of culture, because the medium pH fell from 6.4 before culture to around 4.0 by the end of culture in the xylitol-producing medium [15].

In other yeasts, zymolectins require calcium ions (Ca^{2+}) to adopt their active conformations for the specific interactions with sugar residues [9, 11, 23, 26, 29]. The influences of Ca^{2+} and EDTA on the flocculation of HY200 were investigated by the addition of Ca^{2+} and EDTA to the initial culture medium and then extents of flocculation were measured in the various growth phases. The addition of 7.5 mM Ca^{2+} to

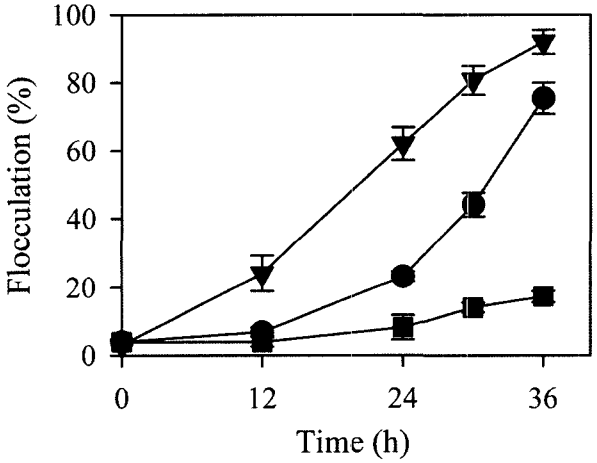

Fig. 5. Effects of calcium ions and EDTA on the flocculation of HY200.
Flocculation of cells from various growth phases grown in medium only (●), in medium containing 7.5 mM $CaCl_2$ (▼), and in medium containing both 7.5 mM $CaCl_2$ and 50 mM EDTA (■). Cells were grown in 250-ml Erlenmeyer flasks containing 50 ml growth medium composed of 20 g/l of xylose, 5 g/l of yeast extract, 3 g/l of malt extract, and 3 g/l of peptone at 30°C and 200 rpm. Error bars represent the standard deviation from triplicate experiments.

the initial growth medium induced flocculation more efficiently, even after the middle exponential phase (Fig. 5), without significant effects on cell growth (data not shown), compared with the control grown in medium only. Interestingly, co-addition of 7.5 mM Ca^{2+} and 50 mM EDTA to the initial medium completely inhibited the flocculation, even at the stationary phase, with slight inhibition of cell growth of about 15%, compared with the control grown in the medium only. The complex nitrogen sources used might contain some Ca^{2+}, which induces efficient flocculation in the medium only. Taken together, the unknown zymolectin most likely requires Ca^{2+} for the flocculation, like the zymolectins of other yeasts. However, other metal ions were not effective to precipitate cells (data not shown).

All these observations were consistent with what has been seen for flocculation through the lectin-like model for other yeasts [9, 11, 13, 21, 22, 29]. Two hypotheses might be proposed for the flocculation of HY200 in the stationary phase only. First, flocculation occurs by specific interactions (molecular recognitions) between cell wall components active in the stationary phase, like the lectin-like model for other yeast genera, such as *Saccharomyces* [21, 22, 29, 30], *Kluyveromyces* [1], *Hansenula* [28], *Schizosaccharomyces* [9, 14], and *Kloeckera* [11]. Secondly, flocculation occurs by nonspecific interactions, such as van der Waals, hydrophobic, and electrostatic interactions, between cells in the stationary phase, though there have been no previous reports of flocculation by nonspecific interactions. Genealogically older cells in the stationary phase tend to be more hydrophobic than those of younger cells, acting as nucleation points for floc formation [4]. However, the exact mechanism of flocculation of HY200 at the molecular and genetic level remains to be determined.

Repeated-Batch Cultivations with Cell Recycles by Flocculation for Xylitol Production

The flocculent yeast HY200 produced xylitol from xylose with a decent xylitol yield (~70%) and productivity (~2.6 g/l·h) in the batch cultivations [15]. To take advantage of the efficient flocculation of HY200 at the end of culture, repeated-batch cultivations were carried out by employing cell recycles by flocculation for the xylitol production from xylose. The operating mode of the repeated-batch cultivation was cyclically composed of batch cultivations in the fresh medium, separation of cells from the culture broth by flocculation, withdrawal of the culture broth containing products, and refilling of the fresh medium. Based on the optimized medium composition and operating parameters for the batch cultivation in the 2.5-l bioreactor with 1-l working volume [15], repeated-batch cultivations were performed with 6 rounds of cultivations. It took about 20 min for the flocculation to occur over 85% and about 10 min to withdraw the culture broth and refill the fresh medium, thus requiring only about 30 min for conversion

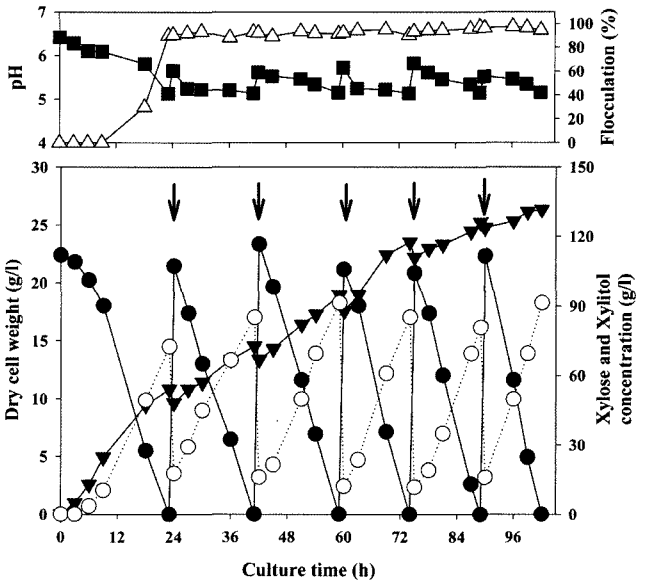

Fig. 6. Representative repeated-batch cultivation of HY200 with cell recycles by flocculation over 6 rounds of cultivation for the production of xylitol from xylose.

Cultivation was performed in a 2.5-l jar bioreactor containing 1-l working volume under the conditions of 420 rpm and 0.5 vvm at 30°C. Arrows indicate the conversion of one batch into the next batch. The symbols represent as follows: DCW, ▼; xylose, ●; xylitol, ○; pH, △; flocculation, ■.

of one batch to the next batch. After the end of the first batch cultivation, HY200 steadily retained a high flocculation ability (over 85%) throughout the repeated batch cultivations (Fig. 6), suggesting that zymolectins responsible for the flocculation remained active after induction in the stationary phase of the first batch.

In the first set of experiments performed with 360 rpm and 1.0 vvm at 30°C, the volumetric productivity and yield of xylitol from xylose was gradually increased with increase of cell recycles, from 2.18 g/l·h and 0.59 g/g in the first batch to 4.01 g/l·h and 0.64 g/g in the final sixth batch, respectively (Table 1). The cell recycles by flocculation significantly increased cell mass, from 9.94 g cell/l in the first batch to 21.28 g cell/l in the final sixth batch, leading to faster xylose consumption rate and consequent shorter culture time from 30 h in the first batch to 15 h in the final sixth batch. The 2-fold shorter culture time resulted in about 2-fold increases in volumetric productivity of xylitol at the final sixth batch, compared with the first batch.

Earlier studies have shown that the volumetric productivity and yield of xylitol from xylose are more effective under micro-aerobic conditions, because the xylose reductase responsible for the conversion of xylose to xylitol requires the reducing cofactor of NADPH [15, 16, 25]. Thus, another set of experiment was performed with slight increase of agitation to 420 rpm and decrease of aeration to 0.5 vvm (Fig. 6). As expected, volumetric productivity and yield of

Table 1. Fermentation kinetic parameters of repeated-batch cultivations with cell recycles by flocculation performed over 6 rounds of batch cultivation for the production of xylitol from xylose.

Operating conditions	Parameters	Batch recycle number					
		1st	2nd	3rd	4th	5th	6th
360 rpm and 1 vvm	Q_P[a] (g /l·h)	2.18	2.43	3.27	3.57	3.99	4.01
	$Y_{P/S}$[b] (g/g)	0.59	0.58	0.60	0.58	0.61	0.64
	Cell mass (g/l)	9.94	12.67	14.68	18.26	20.47	21.28
	q_P[c] (g/g)	5.89	4.60	4.01	2.93	2.92	2.83
420 rpm and 0.5 vvm	Q_P[a] (g /l·h)	3.02	3.73	4.18	4.89	5.75	6.28
	$Y_{P/S}$[b] (g/g)	0.64	0.63	0.65	0.69	0.66	0.67
	Cell mass (g/l)	10.83	14.52	18.95	23.52	25.21	26.32
	q_P[c] (g/g)	6.69	4.62	3.97	3.11	3.42	2.86

[a]Volumetric productivity of xylitol (g xylitol/l·h).
[b]Xylitol yield from xylose (g/g).
[c]Xylitol yield per cell mass (g/g).

xylitol at each batch was significantly higher than those from the first conditions of 360 rpm and 1.0 vvm (Table 1). The maximal volumetric productivity of xylitol was achieved at 6.28 g/l·h at the sixth batch, which was a 2.1-fold higher than that of the first batch. Those results are mainly due to 2-fold shorter culture time at the sixth batch (12 h) than the first batch (24 h). The overall average volumetric productivity and yield of xylitol was 4.6 g/l·h and 0.66 g/g, respectively.

The cell mass in the final sixth batch was about 2-fold higher than that of the first batch in both conditions (Table 1). However, the xylitol yield per cell mass gradually decreased about 2-fold from the first batch (about 5.89 and 6.69 g/g) to the sixth batch (about 2.83 and 2.86 g/g) under both conditions. The decreases in the xylitol yield per cell mass with increase of cell recycles were also observed in other repeated-batch cultivations with cell cycles by centrifugations [6, 18]. Those decreases were most likely attributed to decline of cell viability from shortage of nutrients and intracellular energy [6, 16, 18]. Probably co-substrate feeding, such as glucose, could help maintain cell viability and the cofactor regeneration [6, 24].

Here, it should be noted that, to our knowledge, our study is the first report of repeated-batch cultivations employing cell recycles by flocculation for the production of xylitol. The averaged (4.6 g/l·h) and maximal (6.3 g/l·h) volumetric productivities of xylitol obtained under the condition of 420 rpm and 0.5 vvm were higher or comparable to those previously reported highest in the various operating modes using *C. tropicalis*. The maximal volumetric productivity of xylitol was 3.9 g/l·h in the optimized batch cultivation [6] and 4.9 g/l·h in the optimized fed-batch operations [20], respectively. For the operating modes employing cell recycles by membrane filtration and centrifugations, the maximal volumetric productivity of xylitol was 4.9 g/l·h at the final fed-batch with 3 rounds of cell recycles with cross-flow membrane filtration [6], and 5.4 g/l·h in the final batch with 14 rounds of cell recycle with centrifugation [18]. Kwon *et al.* [20] recently reported that the highest xylitol productivity of 12.0 g/l·h was achieved by cell-recycle fermentation using a submerged membrane bioreactor with suction and air sparging. The xylitol yield from xylose for *C. tropicalis* using cell-recycle fermentations ranged from 0.80–0.85 g/g [6, 18, 20]. However, cell recycles with membrane filtration and centrifugation are usually complicated and expensive processes.

The flocculent HY200 with xylitol-producing capability allowed repeated-batch cultivations with cell recycles by flocculation, providing an efficient xylitol production system with relatively high volumetric productivity. Cell recycle by flocculation is a very fast, convenient, and economically beneficial approach, compared with that by membrane filtration or centrifugation, and thus potentially applicable for the industrial scale of xylitol production.

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