Efficient Production of Ethanol from Saccharified Crops Mixed with Cheese Whey by the Flex Yeast *Kluyveromyces marxianus* KD-15

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Ethanol production from a mixture of wheat flour or potato tubers and cheese whey was examined using the flex yeast *Kluyveromyces marxianus* KD-15, a 2-deoxyglucose-resistant mutant of strain NBRC 1963 that can produce ethanol from sugar beet thick juice diluted with whey. Strain KD-15 simultaneously converted glucose and lactose to ethanol within 48 h, in media containing 10.0% to 15.0% (w/v) total sugars from saccharified filtrate and whey. For efficient production of ethanol from 15.0% (w/v) total sugars, KD-15 cells were collected after fermentation and inoculated into fresh media. Batch fermentation was successfully repeated at least ten times in medium composed of saccharified potato tubers mixed with whey. Yeast cells began to convert all the sugars to ethanol within 24 h, accompanied by cell propagation after the third batch.

Keywords: ethanol production, flex yeast, *Kluyveromyces marxianus*, wheat flour, potato tubers, cheese whey

Introduction

Renewable biofuels have received attention due to worldwide concern for the depletion of fossil fuels, thereby potentially reducing the reliance on petroleum and decreasing greenhouse gas emissions (Antoni *et al.*, 2007). Ethanol produced from sugarcane, corn, and other crops is used as a gasoline substitute or supplement for automobiles in many countries (Walter *et al.*, 2008). Lignocellulosic biomass is a potential source for the growing demand for fuel ethanol, as the excess utilization of crops for ethanol production impacts the global food supply; however, a number of challenges impede its industrial application (Balat *et al.*, 2008; Chen and Qiu, 2010).

Hokkaido, the northernmost island of Japan, is a major agricultural region of crop rotation farming with sugar beets, beans, potatoes, and wheat. Crops deemed unsuitable as food or food ingredients are used as material for fuel ethanol production. Maximum amounts of domestic sugar beet, which are subject to government subsidies, are annually set to balance with sugar imports. Excess amounts of sugar beet are diverted from the market for ethanol manufacturing in the form of raw juice or juice concentrate. An alternative crop source for ethanol is low-grade wheat, evaluated using the standards for test weight, imperfect grains, moisture content, damaged kernels, and the presence of foreign material. Most substandard wheat is utilized as animal feed and occasionally for ethanol fermentation after enzymatic hydrolysis.

Another form of biomass, other than locally available crops, accessible for ethanol production is whey, excreted in cheese-making processes with 90% of the volume of milk used (Siso, 1996). The yeasts employed are typically *Kluyveromyces marxianus* strains capable of fermenting lactose, the principal sugar in whey, and not the conventional yeast *Saccharomyces cerevisiae* (Guimarães *et al.*, 2010). However, fermentation of crude whey containing about 5% lactose generates only 2.5% ethanol, which is far from economically feasible due to the high cost of distillation. Even when a lactose-fermenting strain is used, supplementation with glucose to elevate the initial sugar concentration triggers catabolite repression, preventing the yeast cells from fermenting lactose (Wang *et al.*, 1987).

We previously isolated several mutants resistant to 2-deoxyglucose from *K. marxianus* NBRC 1963 and selected a

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catabolite-repression insensitive mutant, KD-15, capable of synthesizing ethanol from sucrose, lactose, and a mixture of sucrose and lactose (Oda and Nakamura, 2009). Strain KD-15, named flex yeast after flex-fuel vehicles, was shown to produce ethanol from a medium composed of sugar beet thick juice diluted with crude whey (Oda *et al.*, 2010). In the present study, experiments assessed the applicability of this strain to ethanol production from local starchy materials mixed with crude whey.

Materials and Methods

Raw materials Flour comprised of 75.0% (w/w) starch was prepared by milling wheat (*Hokushin* variety) harvested in Tokachi District of Hokkaido in August 2010 and evaluated as substandard. Potato (*Hokkaikogane* variety) used for processed foods, with a tuber starch content of 17.9% (w/w), was produced in the experimental field of Memuro Research Station, National Agricultural Research Center for Hokkaido Region (Memuro, Hokkaido) in September 2010. Crude whey (6.0% (w/v) lactose) was a by-product of Camembert cheese production using fresh milk at the Tokachino Fromages Co. (Nakasatsunai, Hokkaido) in March 2010.

Enzymes The commercial enzyme preparations used were Liquozyme SC (120 Kilo Novo α-amylase Unit [KNU]/g) as α-amylase, Spirizyme Fuel (750 amyloglucosidase unit [AGU]/mL) as glucoamylase, Viscozyme L (100 fungal β-glucanase unit [FBG]/g) as β-glucanase, and Viscozyme Wheat FG as cellulase and endo-1,4-xylanase, obtained from Novozymes A/S (Bagsvaerd, Denmark).

Saccharification For preparation of 100 mL of saccharified slurry from wheat flour, 20 μL of Liquozyme SC was dissolved with stirring in 86 mL of whey (pH 5.7-6.0) at room temperature. With continued stirring, 20 g of flour was gradually added to the whey-enzyme mixture and subjected to stepwise heating for 30 min each at 75°C, 85°C, and 98°C. After cooling to 65°C, 20 μL of Spirizyme Fuel and 2 μL of Viscozyme Wheat FG were added and the mixture was incubated for 16 h.

About 500 g of mashed raw potato tubers was mixed with 50 mL of whey, 500 μL of Liquozyme SC, and 50 μL of Viscozyme L and heated for 30 min each at 65°C, 75°C, and 85°C. The slurry was mixed by hand with a spatula every 15 min in 1 h and every 30 min thereafter. After cooling to 65°C, 500 μL of Spirizyme Fuel was added and the mixture was further incubated for 16 h.

The saccharified slurries were centrifuged and filtrated with cheese cloth to obtain a fluid composed of glucose and lactose that was completely hydrolyzed from starch. Glucose and lactose concentrations were 20.2% and 6.0% (w/v) in the fluid of wheat flour and 17.0% and 0.8% (w/v) in

that of potato tubers, respectively. Galactose was not detected in the fluids, indicating that the enzymes used here lack β-galactosidase, which hydrolyzes lactose into glucose and galactose. Recovery of glucose from starch was calculated to be 89.0% (w/w) in wheat flour and 76.6% (w/w) in potato tubers.

Fermentation tests *K. marxianus* KD-15 and its parental strain NBRC 1963 were grown in 60 mL of YPD medium (1.0% yeast extract, 2.0% polypeptone, and 2.0% glucose) at 30°C for 24 h with shaking (150 rpm). An aliquot (50 mL) of the medium was centrifuged, and harvested cells were inoculated into 100 mL of fermentation medium (initial concentration, 2×10^8 cells/mL) in a 200 mL Erlenmeyer flask. The fermentation media were prepared by mixing the saccharified fluid and whey filtrate, previously heated to remove the precipitate and sterilized at 121°C for 20 min. Total sugars were adjusted to 15.0%, 12.5%, and 10.0% (w/v) by increasing the volume of whey filtrate, resulting in a higher ratio of lactose. Each flask was stopped with a Silicosen culture plug (Shin-Etsu Polymer Co., Ltd., Tokyo) and further covered with Saran Wrap (Asahi Kasei Chemicals Corp., Tokyo), punctured with a pin to allow the emission of CO_2 gas, and incubated at 30°C with shaking (90 rpm). When necessary, cells were collected from the entire volume of fermented broth by centrifugation and inoculated into fresh medium for the repeated batch-fermentations.

Analytical procedures Ethanol and total sugars were determined using a high-performance liquid chromatograph (LaChrom Elite, Hitachi High-Technologies Corp., Tokyo) equipped with a packed column (Shodex KS-801, Showa Denko Co., Tokyo) and an RI monitor.

For the β-galactosidase assay, the crude enzyme (0.05 mL), prepared from a cell suspension (Oda and Nakamura, 2009), was added to 1.0 mL of 50 mM phosphate buffer (pH 6.8) containing 5 mM *o*-nitrophenyl β-D-galactopyranoside and incubated at 30°C for 30 min. The reaction was stopped by the addition of 1.0 mL of 3.0% (w/v) Na_2CO_3, and the release of *o*-nitrophenol was determined by absorbance at 410 nm. One unit of activity was defined as the amount of enzyme that released 1 μmol of *o*-nitrophenol per min.

Whole and viable numbers of yeast cells were enumerated using a hemocytometer without and with alkaline methylene blue staining (Sami *et al.*, 1994), respectively.

Results

Fermentation of saccharified flour mixed with whey *K. marxianus* KD-15 and NBRC 1963 were cultured in media composed of saccharified flour mixed with whey (containing about 10.0%, 12.5%, and 15.0% total sugars) (Fig. 1). The ratio of lactose decreased in proportion to the increase in total sugars (Table 1). The two strains consumed 10.0% of all
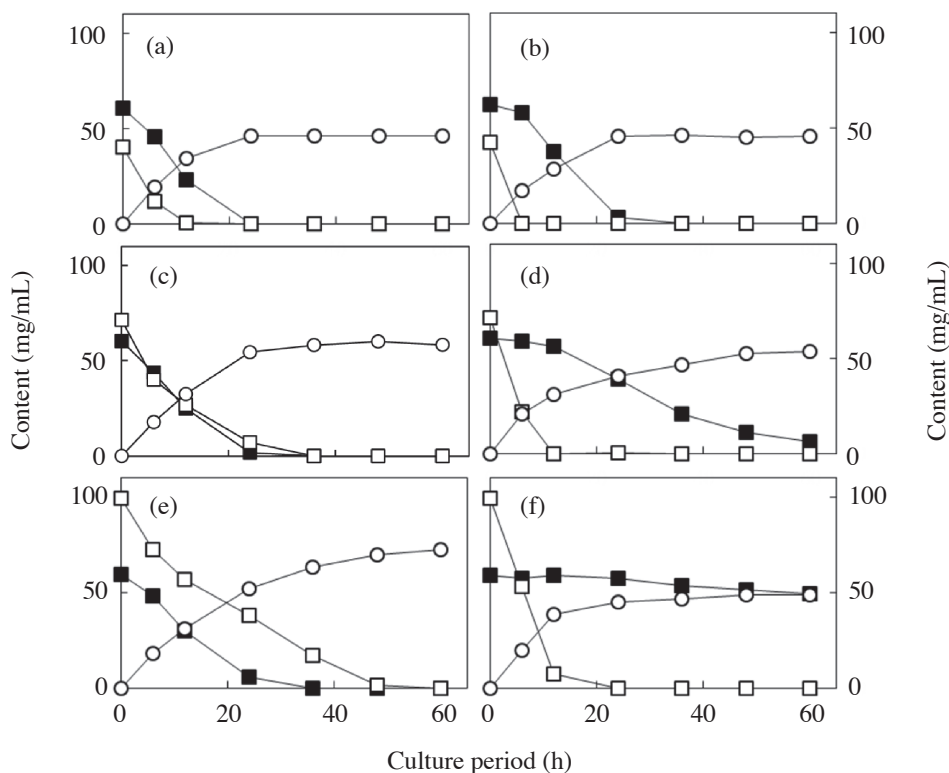
Fig. 1. Ethanol production in media composed of saccharified flour mixed with whey. Strains KD-15 (a, c, e) and NBRC 1963 (b, d, f) were grown in media containing about 10.0% (a, b), 12.5% (c, d), and 15.0% (e, f) total sugars, derived from saccharified flour mixed with whey. Data are shown as the average values and standard deviations from four independent experiments. Symbols: ○, ethanol; □, glucose; ■, lactose.

Table 1. Parameters of fermentation in media composed of saccharified flour mixed with whey.

| Strain | Total sugar concentration (% [w/v]) | Initial content (mg/mL) | | | E_{max} (mg/mL)[a] | $Y_{E/S}$ (%)[b] |
		Glucose	Lactose	Total		
KD-15	10.0	40.4 ± 4.8	60.9 ± 7.4	101.3 ± 3.0	46.3 ± 1.0	45.8 ± 2.2
	12.5	71.4 ± 8.7	60.2 ± 5.2	131.6 ± 6.1	59.7 ± 3.3	45.4 ± 3.5
	15.0	99.3 ± 8.5	59.4 ± 6.4	158.7 ± 10.0	71.4 ± 2.6	45.1 ± 2.7
NBRC 1963	10.0	42.3 ± 6.0	62.4 ± 8.5	104.8 ± 4.5	46.9 ± 0.8	44.8 ± 2.4
	12.5	71.6 ± 7.4	60.5 ± 5.9	132.1 ± 4.2	54.1 ± 5.3	41.1 ± 4.8
	15.0	99.4 ± 7.9	59.3 ± 6.1	158.7 ± 8.7	49.1 ± 4.7	31.0 ± 3.8

[a] Maximum ethanol concentration reached.

[b] Yield of ethanol per substrate in g/g.

Each parameter was calculated from the data obtained in Fig. 1. Data are shown as the average values and standard deviations from four independent experiments.

the sugars in 24 h and produced 50 mg/mL of ethanol. Strain KD-15 took up lactose more quickly than strain NBRC 1963. In the medium containing 12.5% total sugars, strain KD-15 converted equal amounts of glucose and lactose to 62 mg/mL of ethanol in 36 h. Lactose was consumed by strain NBRC 1963 when glucose disappeared from the medium. Differences in the two strains were obvious when the sugar concentration of the medium increased to 15.0%. Strain KD-15 utilized glucose and lactose simultaneously and accumulated

the highest level of ethanol (72.3 mg/mL) in 48 h. Even after consumption of glucose for 24 h, strain NBRC 1963 hardly took up any lactose within 72 h, resulting in a lower yield of ethanol per substrate (g/g).

The two strains showed basal β-galactosidase activity immediately after inoculation (Fig. 2). Strain KD-15 actively synthesized β-galactosidase, which increased three-fold in the three media containing 10.0% to 15.0% total sugars. In strain NBRC 1963, the activity was elevated with 10.0% to-
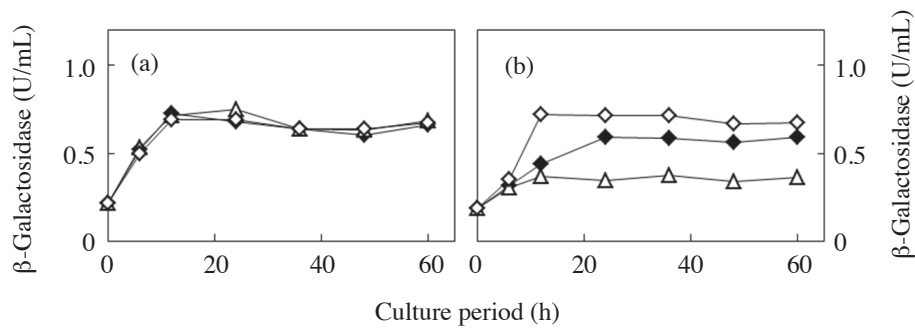
Fig. 2. β-Galactosidase activity in media composed of saccharified flour mixed with whey. Cells of strains KD-15 (a) and NBRC 1963 (b) in Fig. 1 were used for the assay of β-galactosidase activity. Symbols: ◇, 10.0%; ◆, 12.5%; △, 15.0%.
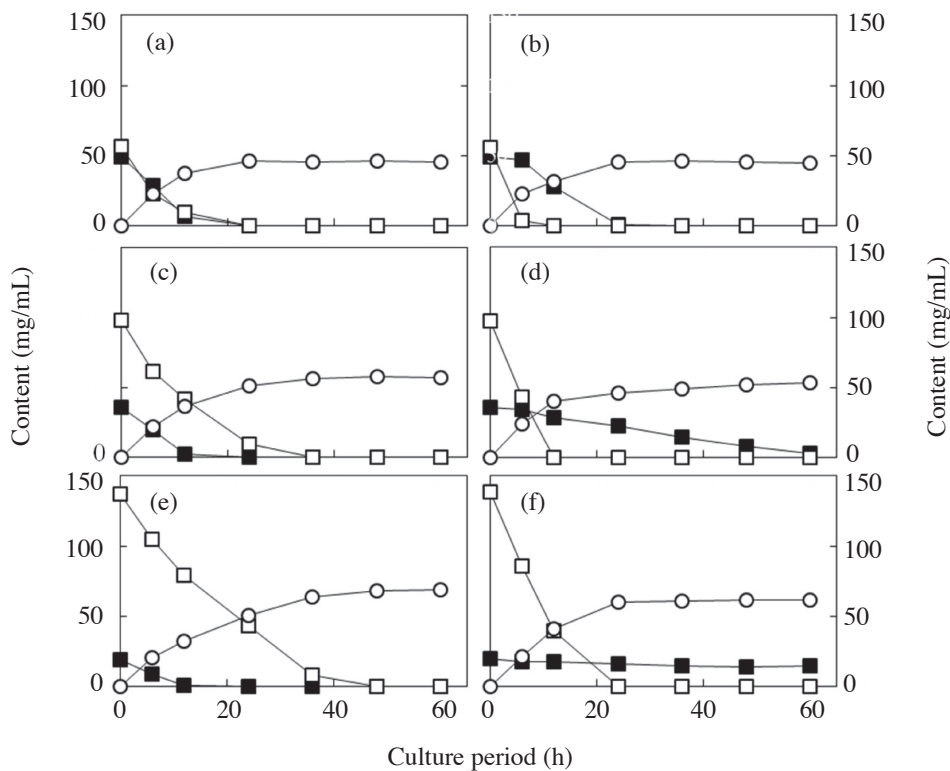

Fig. 3. Ethanol production in media composed of saccharified potato tubers mixed with whey. Strains KD-15 (a, c, e) and NBRC 1963 (b, d, f) were grown in media containing about 10.0% (a, b), 12.5% (c, d), and 15.0% (e, f) total sugars, derived from saccharified potato tubers mixed with whey. Data are shown as the average values and standard deviations from three independent experiments. Symbols are the same as those in Fig. 1.

tal sugars, similarly to strain KD-15, and was stimulated, in a limited manner, with 12.5% and 15.0% total sugars. The pattern of enzyme activity in the two strains corresponded with their utilization of lactose.

Fermentation of saccharified potato tubers with whey
Fermentation was assessed in the media composed of saccharified potato tubers mixed with whey (Fig. 3). These media included smaller amounts of lactose than those prepared from flour, because the volumes of whey added to moist tubers are limited for the saccharification process (Table 2). Strain KD-15 fermented all the sugars in the media, while

consumption of lactose by strain NBRC 1963 was prolonged in the media containing 10.0% and 12.5% total sugars and repressed in that containing 15.0% total sugars, due to the high initial content of glucose. The β-galactosidase activities were about twice those in the media composed of saccharified flour mixed with whey (Fig. 4). Strain KD-15 showed varied activities in the media containing 10.0%, 12.5%, and 15.0% total sugars. Changes in β-galactosidase activity were not the same as those in the media composed of saccharified flour mixed with whey. However, the activity of strain KD-15 was apparently higher than that of NBRC 1963, indicat-

Table 2. Parameters of fermentation in media composed of saccharified potato tubers mixed with whey.

| Strain | Total sugar concentration (% [w/v]) | Initial content (mg/mL) | | | E_{max} (mg/mL)[a] | $Y_{E/S}$ (%)[b] |
		Glucose	Lactose	Total		
KD-15	10.0	56.1 ±4.0	48.9 ± 2.3	105.1 ± 5.4	46.1 ± 2.4	43.8 ± 0.3
	12.5	98.0 ± 1.5	36.0 ± 0.6	134.0 ± 1.7	57.6 ± 3.0	43.0 ± 1.7
	15.0	137.0 ± 10.8	19.1 ± 4.0	156.1 ± 7.0	69.1 ± 3.9	44.2 ± 0.5
NBRC 1963	10.0	55.9 ± 3.4	48.8 ± 0.3	104.7 ± 3.2	46.2 ± 2.7	44.2 ± 1.9
	12.5	97.6 ± 4.8	35.9 ± 1.3	133.5 ± 5.9	53.9 ± 3.4	40.4 ± 3.6
	15.0	138.8 ± 9.4	19.6 ± 4.4	158.4 ± 5.0	62.4 ± 3.2	39.6 ± 1.2

[a] Maximum ethanol concentration reached.

[b] Yield of ethanol per substrate in g/g.

Each parameter was calculated from the data obtained in Fig. 3. Data are shown as the average values and standard deviations from three independent experiments.
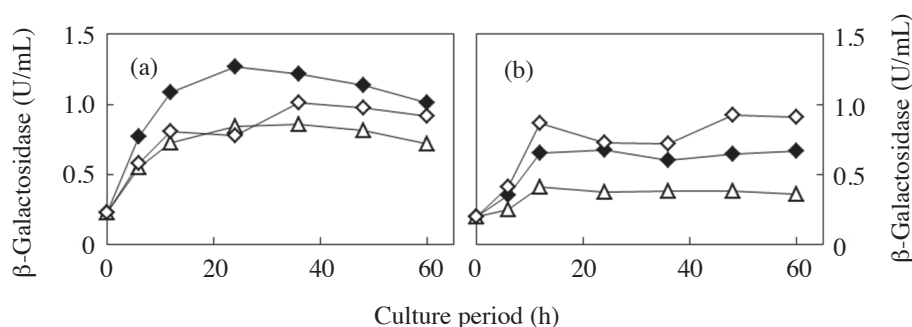

Fig. 4. β-Galactosidase activity in media composed of saccharified potato tubers mixed with whey. Cells of strains KD-15 (a) and NBRC 1963 (b) in Fig. 3 were used for the assay of β-galactosidase activity. Symbols are the same as those in Fig. 2.
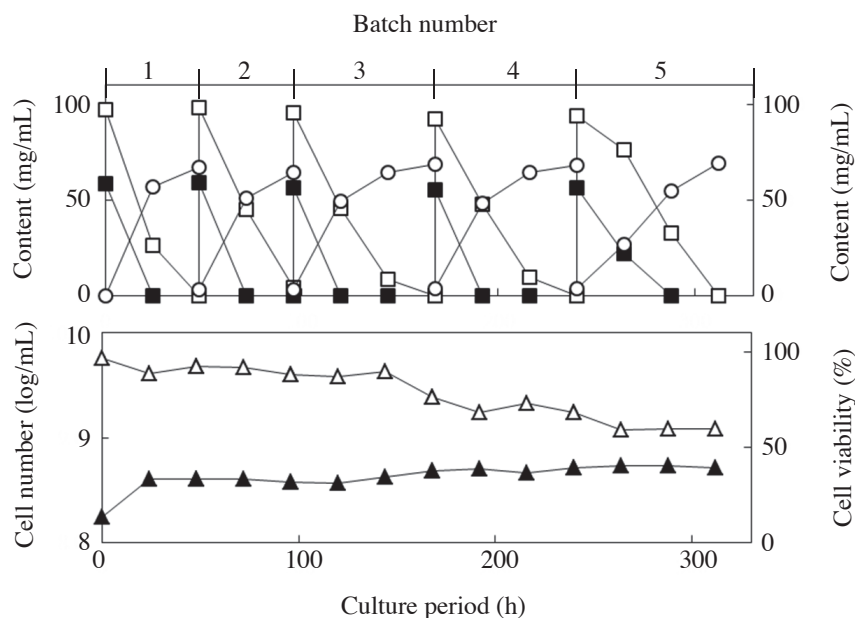

Fig. 5. Repeated-batch fermentation in medium composed of saccharified flour mixed with whey. Strain KD-15 was grown in media containing about 15.0% total sugars, derived from saccharified flour mixed with whey. Data are shown as representative values from three independent experiments. Symbols: ○, ethanol; □, glucose; ■, lactose; ▲, cell number; △, cell viability.
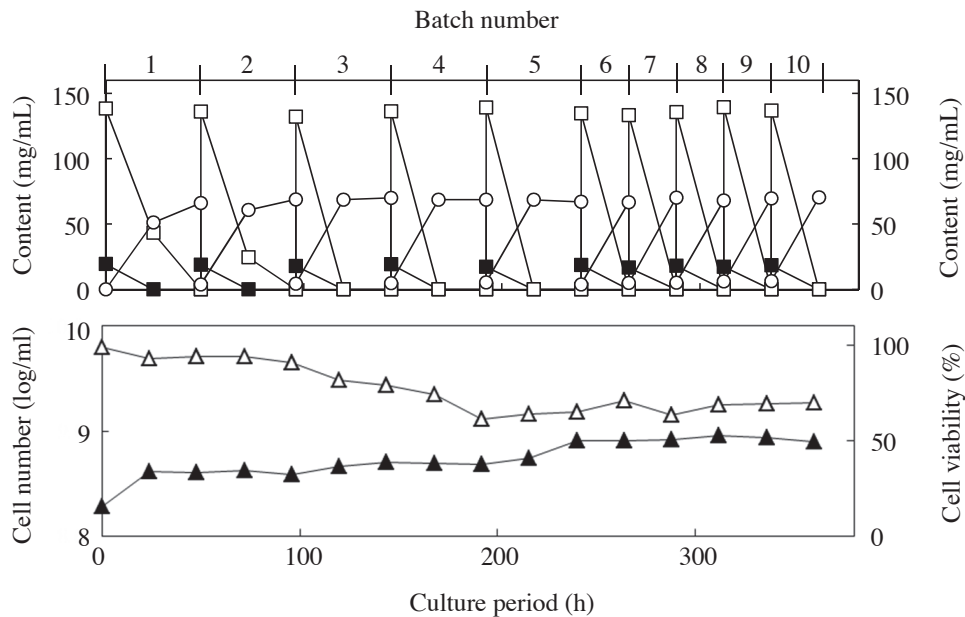
Fig. 6. Repeated-batch fermentation in medium composed of saccharified potato tubers mixed with whey. Strain KD-15 was grown in media containing about 15.0% total sugars, derived from saccharified potato tubers mixed with whey. Data are shown as representative values from three independent experiments. Symbols are the same as those in Fig. 5.

ing its lack of sensitivity to catabolite repression triggered by exogenous glucose.

Repeated-batch fermentation For the efficient production of ethanol from 15.0% total sugars, the cells of strain KD-15 were harvested after fermentation and inoculated into fresh media composed of saccharified crops mixed with whey. These batch fermentations were repeated several times for each medium.

In the third batch fermentation of medium composed of saccharified flour mixed with whey, the cells utilized lactose rapidly, whereas glucose was utilized more slowly than in the first batch (Fig. 5). The experiment was terminated at the fifth batch because glucose was not consumed completely in 48 h after the third batch.

In contrast, batch fermentation was successfully repeated at least ten times in the medium composed of saccharified potato tubers mixed with whey (Fig. 6). The cells took up all the sugars more quickly as the fermentation was repeated, confirming a previous report (Ma *et al.*, 2009). The time to complete fermentation was reduced from 48 h in the first batch to 24 h in the third batch. The average values of the initial content of total sugars, maximum ethanol concentration reached, and yield of ethanol per substrate (g/g) through the 10-batch fermentations were 154.3 ± 2.3 (mg/mL), 64.1 ± 1.4 (mg/mL), and 41.5 ± 1.3 (%), respectively.

The number of vigorous cells in both fermentations seemed to be constant throughout the process, as determined from the findings that the reduction in viable cells was com-

pensated for by an increase in the total number of cells (Figs. 5, 6). The initial pH of the media was 4.9-5.2 and was routinely reduced to 4.4-4.7 after each batch (data not shown).

Discussion

Microbial cells typically utilize certain sugars preferentially, with catabolite repression blocking the synthesis of enzymes catalyzing the metabolism of other carbon sources (Gancedo, 1998). The extent of catabolite repression depends heavily on the species of organism, type of sugar, and culture conditions (Gancedo, 1992). Some microorganisms utilize less-preferred sugars after the concentrations of preferred sugars decrease below a certain level, while others cannot take up less-preferred sugars even after the consumption of preferred sugars (Kim *et al.*, 2010a).

K. marxianus is reportedly free from catabolite repression with respect to the expression of respiratory enzymes, in contrast to *S. cerevisiae* (Fonseca *et al.*, 2008), while preferential utilization of glucose by *K. marxianus* was observed in a medium containing glucose and lactose (Wang *et al.*, 1987).

Among the 2-deoxyglucose-resistant mutants of *K. marxianus* NBRC 1963, strain KD-15 showed a catabolite-repression insensitive phenotype, as lactose was metabolized in the presence of sucrose with stimulated synthesis of β-galactosidase (Oda and Nakamura, 2009). However, it was not guaranteed that strain KD-15 could actually convert lactose to ethanol in the presence of glucose.

In some *S. cerevisiae* strains, the expression of α-glucosidase, required for maltose utilization, was repressed by glucose and only slightly by sucrose (Zimmermann and Eaton, 1974). Invertase secreted from the cells hydrolyzes sucrose more rapidly than the cellular uptake of resultant monosaccharides (Oda and Ouchi, 1990), leading to the accumulation of extracellular glucose that will not strongly induce catabolite repression of α-glucosidase synthesis. Exogenous sucrose may not necessarily exert catabolite repression through mechanisms identical to those associated with glucose. The expression of invertase, which hydrolyzes sucrose, is repressed in yeast cells by glucose, suggesting that gene regulation by glucose is epistatic to that of sucrose.

In the present study, ethanol production by strains KD-15 and NBRC 1963 was first compared in media composed of saccharified crops mixed with whey. After consumption of a small amount of glucose, strain NBRC 1963 rapidly shifted to adapt itself to lactose fermentation in the medium containing 10.0% total sugars, derived from saccharified flour and whey. An increasing ratio of glucose in the media containing more than 12.5% total sugars induced catabolite repression remarkably upon consumption of lactose. Strain KD-15 completely and simultaneously consumed both lactose and glucose in any concentration.

With respect to potato, the use of harvested tubers evaluated as below food grade might be more economical for ethanol production (Ramesh *et al.*, 2010) than starch powder extracted and purified from tubers. For saccharification, moist potato tubers cannot be supplemented with as high a volume of whey as for flour, as the higher ratio of glucose that may elicit a remarkable degree of catabolite repression. Under these conditions, strain KD-15 converted all lactose and glucose to ethanol. The two sugars included in the medium at any ratio might be fermented completely by strain KD-15, as was observed in ethanol production from sugar beet thick juice diluted with an arbitrary amount of crude whey (Oda *et al.*, 2010).

The cells of strain KD-15 were recycled and applied to repeated-batch fermentation using medium composed of saccharified potato tubers mixed with whey. While the reason for the unsuccessful results with the medium containing flour as a starchy material is still unknown, a number of possible explanations are raised. One is that wheat-derived compounds might affect the cellular activity of fermentation. In addition, the higher ratio of lactose in the medium may gradually diminish the fermentation ability of the cells. Alternatively, the reduced fermentation time after the third batch, in the medium composed of saccharified potato tubers mixed with whey, suggests that specific constituents in the potato tubers stimulate yeast cell fermentation activity. In all,

mixed materials composed of saccharified flour and potato tubers combined with whey might result in a stable and efficient process.

Repeated-batch fermentation is a promising method to increase ethanol production with a high cell density as well as to reduce manufacturing costs (Kim *et al.*, 2010b; Yamakawa *et al.*, 2010). Furthermore, flocculation characteristics might be required for efficient recycling of cells without centrifugation (Silva *et al.*, 2010; Zhao and Bai, 2009).

A conventional *S. cerevisiae* strain exhibited a higher rate of ethanol production from sucrose compared with strain KD-15; however, it cannot consume lactose (Oda and Nakamura, 2009). Enzymatic hydrolysis of lactose by β-galactosidase makes it possible to generate glucose and galactose, which are fermentable by *S. cerevisiae* (Coté *et al.*, 2004). However, preliminary experiments showed that a supplement of commercial β-galactosidase in the saccharification procedure did not completely split lactose derived from whey. Even when lactose is hydrolyzed to glucose and galactose, exogenous glucose represses galactose fermentation in *S. cerevisiae* through catabolite repression (Bailey *et al.*, 1982). Ethanol production from saccharified crops mixed with whey still requires the use of strain KD-15, rather than *S. cerevisiae* strains, for the usual manufacturing process. The crops might be replaced by food-processing by-products, such as starch (Arapoglou *et al.*, 2010; Ebrahimi *et al.*, 2008) and cellulosic material from wheat or rice straw (Binod *et al.*, 2010; Talebnia *et al.*, 2010).

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