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Improved Ethanol Production by Mixed Immobilized Cells of *Kluyveromyces marxianus* and *Saccharomyces cerevisiae* from Cheese Whey Powder Solution Fermentation

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Abstract Ethanol productions from cheese whey powder (CWP) solution were investigated by using free or immobilized cells of *Kluyveromyces marxianus* in monocultures or mixed cultures with free or immobilized cells of *K. marxianus* and *Saccharomyces cerevisiae*. *K. marxianus* free cells produced 3.8% *v*/*v* ethanol in monocultures, while *S. cerevisiae* immobilized cells produced 5.3% *v*/*v* ethanol in mixed cultures. The percentage of theoretical yield was found to be higher in mixed cultures than that in monocultures. The maximum ethanol fermentation efficiency was achieved (79.9% of the theoretical value) using mixed cultures of immobilized cells of *K. marxianus* and *S. cerevisiae*. The beads were relatively stable without significant reduction in activity for about eight batches of fermentation.

Keywords Ethanol · Mixed cultures · Immobilization · Cheese whey powder

Introduction

Cheese whey powder (CWP) is a by-product of cheese industry, and its disposal entails increasing pollution problems. Since CWP has very high content of carbohydrate and abundant availability, many studies have been done to investigate the possibilities of using cheese whey as a medium in single-cell protein production and ethanol fermentation [1, 2].

CWP is a dried and concentrated form of cheese whey. It contains lactose and other essential nutrients. Previous studies showed that CWP was better than cheese whey as a substrate for ethanol fermentation as CWP is a concentrated form of cheese whey with considerable advantages as substrate including reduced volume, concentrated lactose content, long-term stability, easy storage, and transportation [3, 4].

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Not many yeast strains are capable of fermenting lactose to ethanol. The most commonly used distiller yeast *Saccharomyces cerevisiae* cannot ferment lactose since it lacks both β-galactosidase and a lactose permease system. This inability to ferment lactose prevents *S. cerevisiae* from using cheese whey as fermentation substrate. Alternative methods have been explored for utilization of CWP by *S. cerevisiae*. Champagne used one β-galactosidase-positive microorganism to hydrolyze lactose first to provide suitable substrate for subsequent fermentation by *S. cerevisiae* [5].

Most of the *Kluyveromyces* species are capable of using lactose in cheese whey for ethanol fermentation. Despite their close phylogenetic relationship, there are still certain technological aspects which *Kluyveromyces* cannot industrially compete with *Saccharomyces*. However, fermentation strategies of mixed culture employed to overcome substrate limitations were considerably successful, which has been widely used in producing ethanol fermentation and single-cell protein, vitamin production, and disposing of waste water [6–9]. Although *Zymomonas* cannot utilize lactose, a coculture of *Zymomonas mobilis* with *Kluyveromyces fragilis* did produce high concentration of ethanol. And an improvement of the ethanol production from lactose has been achieved by using coimmobilized cells of *Z. mobilis* and *K. fragilis* [10].

So far, there are only a few reports available on fermentation of CWP [3, 4, 11]. In the present study, we describe the high ethanol production from lactose fermentation using mixed cultures of immobilized cells of *K. marxianus* and *S. cerevisiae* in alginate gel.

Materials and Methods

Media

The growth medium used for cultivation of inoculums cultures consist of yeast extract (5 g/l), peptone (5 g/l), NH_4Cl (2 g/l), KH_2PO_4 (1 g/l), $MgSO_4 \cdot 7H_2O$ (0.3 g/l). The pH of the medium was adjusted to 5.0. Media used for the fed-batch experiments contained CWP with different concentrations. The CWP was obtained from Tianjin Aokai Company in Tianjin, China, and was dried at 80 °C before use. The initial total sugar concentration in CWP solution was 100 g/l, and the solution was used directly without any additional nutrients supplements.

Microorganisms

K. marxianus TY-3 and *S. cerevisiae* AY-5 strains were preserved in our laboratory.

Immobilization and Coimmobilization

The methods of immobilization or coimmobilization of *K. marxianus* and *S. cerevisiae* were reported previously [12], and they were described briefly as follows: *K. marxianus* and *S. cerevisiae* cells were immobilized with alginate. Precultured cells (of different dry weight) were each mixed with 50 ml 2.5% Na-alginate solution. For coimmobilization, precultured *K. marxianus* and *S. cerevisiae* cells were mixed together in different ratio of dry weight in 50 ml 2.5% Na-alginate solution. The resulting mixture was added dropwise to 150-ml 0.1 M $CaCl_2$ solution to make cell-embodied beads. $CaCl_2$ solution was gently stirred at room temperature during the process. The mean diameter of the resulting Ca-alginate gel beads was about 2.8 mm.

Analytical Methods

Measuring Dry Weight of the Biomass Yeast cells were harvested by centrifugation for 10 min at 10,000 rpm. The pellets were washed twice with distilled water and weighed after 24 h of drying at 100 °C.

Determination of Suspended Cell Concentrations Suspended dry cells in fermentation media measured the culture absorbance at 600 nm and converted the absorbance to the suspended cells concentrations as described previously [12].

Measuring the Dry Weights of Immobilized Cells in the Carriers The dry weight of cells immobilized in the carriers was obtained as described [13]. After cultivation, about 0.5-ml carrier beads bearing immobilized cells were placed on a glass filter to remove the fluid and then transferred to a 5-ml graduated cylinder containing 3.5-ml sterilized waters. The height of the liquid was recorded to measure the volume increase of the carrier. Immobilized cells were then squeezed out from carrier in sterilized water using a glass stick, and the process was repeated three times. The dry weight of these previously immobilized cells was determined as the dry cell weight obtained by measuring the absorbance of the cells contained in the sterilized water.

Analysis of Residual Sugar in Culture The residual sugar concentration was determined by the phenol-sulfuric method [14].

Ethanol Production Assay Ethanol was estimated by the dichromate colorimetric method, which is based on the complete oxidation of ethanol by dichromate in the presence of sulfuric acid to form acetic acid [15].

Fermentation

Free-cell fermentation was done statically in incubator at 30 °C. Batch immobilized cell fermentation was performed at 30 °C in 500-ml Erlenmeyer flasks in an anaerobic shaker at an agitation of 120 rpm. Ten percent (*v*/*v*; 20-mg dry wt) free cells or 20% (*w*/*v*) beads carrying immobilized cells were added to 100 ml of CWP solution as the inoculums. Samples were collected at an interval of 12 h. After measuring absorbance at 600 nm, the remaining sample was centrifuged at 4,800 rpm for 15 min. The supernatant was removed and stored at 4 °C for sugar and ethanol determination.

Repeated-batch operations were carried out as follows: after immobilization culturing, the medium was removed by using sterilized gauze and washed twice with sterilized distilled water; the immobilized cells and fermentation medium were mixed in a flask, and ethanol fermentation was carried out on a rotary shaker at 120 rpm at 30 °C.

Results and Discussions

Mixed Culture

Analysis of ethanol production were carried out in free-cell fermentations of monoculture of *K. marxianus* or mixed cultures of *K. marxianus* and *S. cerevisiae*, respectively. The experiments were performed under static condition at 30 °C. The results obtained from

these experiments were shown in Fig. 1. The ethanol concentration was 4.6% *v*/*v* of mixed culture, which was higher compared with that of monoculture (only 3.8% *v*/*v*); moreover, the residual sugar concentration and the fermentation time were lower and shorter from the mixed culture than those of monoculture. So mixed culture was found to be more efficient in CWP fermentation than monoculture regarding final ethanol yield and residual sugar concentration. Similar observations were reported with mixed culture of *K. fragilis* and *Z. mobilis* [10].

Optimization of the Cells Proportion of *K. marxianus* and *S. cerevisiae* of Mixed Culture

The proportion of *K. marxianus* and *S. cerevisiae* cells for mixed culture would be very important for ethanol yield. The differences of ethanol productions were investigated among the cultures with different proportions of *K. marxianus* and *S. cerevisiae* cells (Fig. 2). Ethanol concentration increased from 4% *v*/*v* to 4.6% *v*/*v* as the *S. cerevisiae* cell proportion rose from one fourth to one half, whereas there was a decrease in ethanol concentration as the *S. cerevisiae* cells proportion improved from one half to three fourths. The results suggested that the suitable proportion of *K. marxianus* and *S. cerevisiae* cells is very important for getting higher ethanol yield. In this study, equal volume of *K. marxianus* and *S. cerevisiae* was chosen as the optimum proportion.

Comparison of Ethanol Fermentations by Immobilized and Free Cells

In order to find out the best condition for ethanol production, fermentations were performed with different microorganism groups: (1) monoculture of free or immobilized *K. marxianus* cells; (2) mixed culture of free cells of *K. marxianus* and *S. cerevisiae*; (3) both strain cells immobilized within the same bead; (4) mixed cultures with one type of yeast cells embodied in one bead. The results showed that immobilized cell fermentation was faster than that of free cells (Fig. 3). The average fermentation time is 48 h by immobilized cells, but, for free cells of *K. marxianus* and mixed culture of free cells of *K. marxianus* and *S. cerevisiae*, fermentation time is 96 and 72 h, respectively.

Immobilized cells produced about 0.3% *v*/*v* ethanol, higher than free cells in monoculture (Table 1); however, lower ethanol production was observed in immobilized cell fermentation compared with free cells of *K. marxianus* and *S. cerevisiae* in mixed-culture fermentation. Analyzing ethanol fermentations or the mixed culture of immobilized

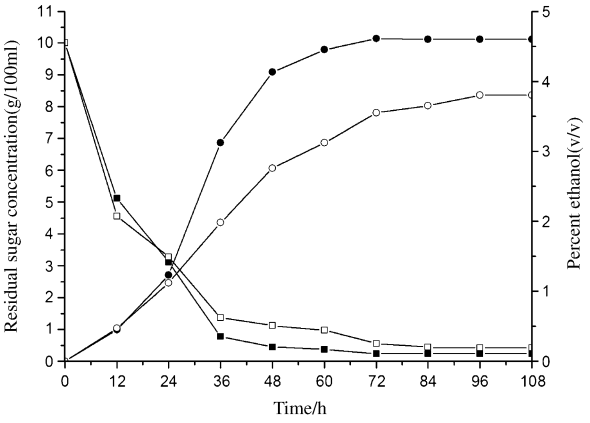

Fig. 1 Comparison of fermentation kinetic parameters of monoculture of *K. marxianus* and mixed culture of *K. marxianus* and *S. cerevisiae*. Ethanol (*v*/*v*): *filled circles*—mixed culture of *K. marxianus* and *S. cerevisiae*; *empty circles*—monoculture of *K. marxianus*; total sugar: *filled squares*—mixed culture of *K. marxianus* and *S. cerevisiae*; *empty squares*—monoculture of *K. marxianus*. The proportion of *K. marxianus* and *S. cerevisiae* in mixed culture was 1:1

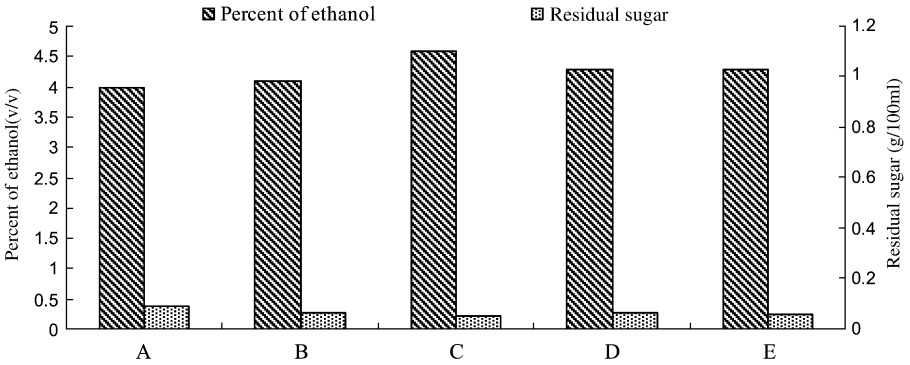

Fig. 2 Comparison of fermentation kinetic parameters of different volume proportion of strains in mixed culture of *K. marxianus* and *S. cerevisiae. A*: *K. marxianus* (15-mg dry wt): *S. cerevisiae* (5-mg dry wt)=3:1; *B*: 2:1; *C*: 1:1; *D*: 1:2; *E*: 1:3;

cells or coimmobilized cells, the highest ethanol concentration was observed from the fermentation of mixed culture of immobilized *K. marxianus* and *S. cerevisiae*. In the fermentation by the mixed culture of both species, *S. cerevisiae* rapidly metabolizes sugar, especially glucose and galactose, thereby resulting in an increased ethanol production.

Repeated-Batch Fermentation

To investigate the fermentation efficiency of immobilized cell recycling, repeated-batch fermentations were carried out. As shown in Table 2, immobilized beads were stable for eight successive batches without any significant changes in ethanol yield. The fermentation time (48 h) was not decreased throughout the batches and the increase of the immobilized cell mass in each batch was not significant. The ethanol production rates in the repeated-batch

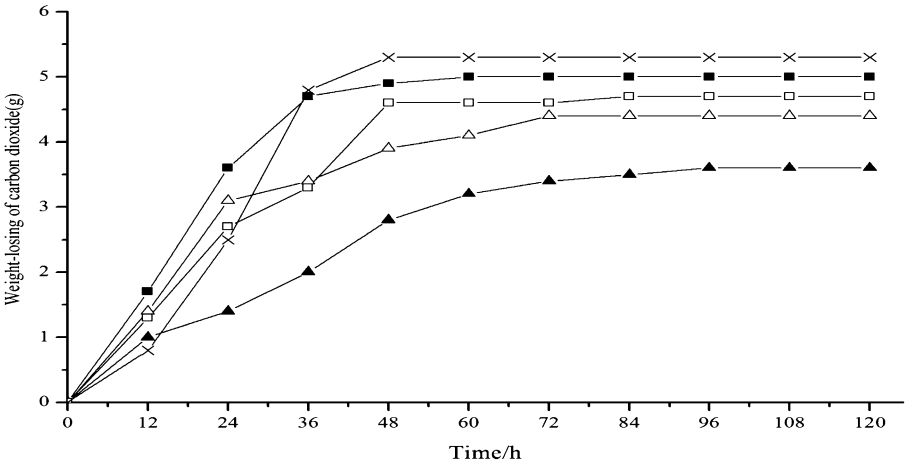

Fig. 3 Comparison of fermentation time about immobilized cells and free cells: *filled triangles*—free cells of *K. marxianus*; *empty triangles*—immobilized *K. marxianus*; *empty squares*—free cells of *K. marxianus* and *S. cerevisiae*; *filled squares*—coimmobilized *K. marxianus* and *S. cerevisiae*; *x marks*—mixed culture of immobilized *K. marxianus* and *S. cerevisiae*

Table 1 Comparison of ethanol productions by free cells and immobilized cells.

Parameters	Free cells of *K. marxianus*	Immobilized *K. marxianus*	Free cells of *K. marxianus* and *S. cerevisiae*	Mixed-culture immobilized *K. marxianus* and *S. cerevisiae*	Coimmobilized *K. marxianus* and *S. cerevisiae*
Final ethanol (*v*/*v*)	3.8	4.1	4.6	5.3	5.1
Residual sugar (g per 100 ml)	0.42	0.38	0.23	0.18	0.19
Rate of ethanol formation (g/l h)	0.31	0.68	0.51	0.88	0.85
Ethanol yield (g/g)	0.32	0.34	0.38	0.43	0.42
Rate of sugar utilization (g/l h)	0.99	2.00	1.35	2.04	2.04
Percentage of theoretical yield	58.76	63.14	69.75	79.95	77.01
Immobilized cell concentration (mg/g bead)	–	18.2	–	22.1	20.4
Free-cell concentration (g/l)	1.1	0.26	1.8	0.18	0.20

fermentations were between 0.80 and 0.88 g/l h by mixed-culture immobilized *K. marxianus* and *S. cerevisiae*.

The immobilized and free-cell concentrations were tested at the end of each batch, respectively. The free-cell concentration in the successive batch fermentation increased from 0.08 to 1.01 g/l at the end of the eighth batch (Table 2). This increase could be due to the growth of free cells leaking from gel beads.

Conclusion

The mixed culture of immobilized cells of *K. marxianus* and *S. cerevisiae* is highly desirable in ethanol fermentation using CWP as medium. This system achieves higher

Table 2 Kinetic parameters for repeated-batch fermentation.

Batch number	Mixed culture of immobilized *K. marxianus* and *S. cerevisiae*					
	P	*S*	*T*	Q_p	X_i	X_f
1	5.3	0.18	79.95	0.88	22.1	0.08
2	5.3	0.18	79.95	0.88	22.8	0.10
3	5.2	0.21	78.68	0.86	25.1	0.22
4	5.1	0.24	77.41	0.85	25.8	0.31
5	5.1	0.26	77.57	0.85	24.6	0.48
6	4.9	0.32	74.99	0.81	22.8	0.62
7	4.9	0.38	75.46	0.81	21.5	0.88
8	4.8	0.41	74.15	0.80	19.2	1.01

P final ethanol concentration (*v*/*v*), *S* residual sugar (g per 100 ml), *T* percentage of theoretical yield, Q_p, rate of ethanol formation (g/l h), X_i final immobilized cell mass concentration (mg/g), X_f free-cell concentration (g/l)

ethanol productivity than that from fermentations by free cells or coimmobilized cells. Our results indicated that the combination of mixed-culture and immobilized technology might be one of the best fermenting strategies employed to overcome substrate limitation and achieve high product yield.

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