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Ethanol production from deproteinized whey by β-galactosidase coimmobilized cells of *Saccharomyces cerevisiae*

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SUMMARY

The performance of β-galactosidase coimmobilized cells of *Saccharomyces cerevisiae* was evaluated during shake flask fermentation of deproteinized cheese whey lactose to ethanol. The performance of the coimmobilized enzyme treatment was compared to that of a treatment using acid prehydrolyzed whey lactose (a readily available substrate). Enzyme coimmobilization resulted in a slower rate and a lower extent of substrate utilization, thus giving a lower maximum ethanol concentration (13.5 versus 16.7 g/l). It did result, however, in a better ethanol yield (95% vs. 89% theoretical). It appears that, compared to acid prehydrolysis of whey lactose, through β-galactosidase coimmobilization we could succeed in obtaining substantial process simplifications, thus saving in equipment and operating cost, while gaining in ethanol yield at the cost of some reasonable loss in the rate and the extent of lactose utilization.

INTRODUCTION

As demand for the limited global supply of non-renewable energy resources increases, the prices of oil and natural gas keep increasing. As a result, production of ethanol from renewable carbohydrate raw materials for use as an alternative liquid fuel has been attracting a worldwide interest.

Ethanol fermentation has been studied by many workers; a review of the literature is to be found in Margaritis and Merchant [8].

Among the many carbohydrate materials used for the production of ethanol, cheese whey lactose reserves special consideration. Cheese whey is a clean, wholesome, abundant food-grade material and a potential environmental pollutant. A large number of researchers have investigated different aspects of ethanol production from cheese whey permeate. Most work in this area has been done on free cell fermentation with two lactose fermenting yeasts; *Kluyveromyces marxianus* [13, 14] and *Kluyveromyces fragilis* [1,4,7,12].

Recently, efforts to optimize whey permeate lactose fermentation has been concentrated on the use of immobilized cell reactors [3,6,9]. A major advantage of Immobilized Cell Reactors (ICR) is that they allow high cell densities with little cell washout, even at very short residence times, thus eliminating costly cell removal before distillation.

Hahn-Hägerdal [6] reported that *Saccharomyces cerevisiae* coimmobilized with β-galactosidase is a more efficient way to continuously ferment whey permeate than using *K. fragilis*, even if this organism can ferment lactose directly.

The main objective of this study was to evaluate the performance of β-galactosidase coimmobilized of *S. cerevisiae* during batch fermentation of deproteinized cheese whey to ethanol. The performance of the co-immobilized lactase set-up was compared to that of a treatment using acid prehydrolyzed whey as production medium. The method of batch fermentation employed in this study was selected between shake flask and static fermentation through a preliminary comparative experiment with acid prehydrolyzed whey (a readily available substrate).

MATERIALS AND METHODS

Organism and culture conditions. Saccharomyces cerevisiae SU No Y6 was used throughout this investigation. The strain was maintained at 4 °C on MYGP medium (Malt extract 0.3%, yeast extract 0.3%, glucose 1.0%, peptone 0.5% and agar 1.5%). Cells of *S. cerevisiae* for immobilization were obtained from cultures grown in 200 ml MYGP broth (MYGP medium without agar) at 30 °C for 24 h. The cells were harvested from the fermen-

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tation broth (3.6×10^8 cells/ml) by centrifugation at $10000 \times g$ for 20 min, and were resuspended in 15 ml distilled water.

Immobilization of cells. 15 ml of a cell suspension of *S. cerevisiae* were mixed with 10 ml of a 10% sterile sodium alginate solution (BDH, 30105). The mixture was then extruded drop by drop with a peristaltic pump into a sterile 2% $CaCl_2$ solution at room temperature while stirring it continuously. The beads (2–3 mm diameter) were hardened in this solution for two hours. The particles were then washed with sterile physiological salt solution prior to use to remove excess calcium ions and untrapped cells.

The coimmobilization of *S. cerevisiae* cells with β-galactosidase (Sigma, G-6512) was carried out as described by Hahn-Hägerdal [6] and Büyükgüngor [3].

Treatment of whey. Cheese whey was obtained from a local feta cheese plant. The pH of the whey was adjusted to 5.2 with 1 N NaOH. Protein precipitation was induced by heating the whey at 90 °C for 20 min. Precipitated proteins were removed by centrifugation at $4000 \times g$ for 15 min. In the treatments using enzyme coimmobilization, immediately after centrifugation the pH was adjusted to 4.5. In the case of acid prehydrolyzed whey, the pH of the supernatant was adjusted to 1.2 with concentrated HCl and the medium was heated at 121 °C for 30 min. After cooling, the pH of the medium was adjusted to 4.5 with 20 N NaOH.

Production medium. The production medium consisted of 100 ml deproteinized or deproteinized, acid hydrolyzed whey (pH 4.5) supplemented with 0.3 g malt extract, 0.3 g yeast extract and 0.5 g peptone.

Fermentation conditions. The fermentation was carried out in 500 ml conical flasks containing 100 ml production medium and 20 g of Ca-alginate beads with entrapped cells of the microorganism or Ca-alginate entrapped cells coimmobilized with β-galactosidase. The flasks were incubated at 30 °C in a rotary shaker/incubator (Lab-Line Orbit-Environ Shaker, Lab-Line Instr., Inc.) at 200 rpm or static in an incubator.

Analytical techniques. Concentration of living cells entrapped in Ca-alginate beads was determined by dissolving 6 beads in 10 ml of 0.3 M sodium citrate solution for 30 min with continuous shaking. The number of living cells liberated from the gel was determined by plate counting.

Ethanol concentration was determined enzymatically as described by Bernt and Gutmann [2]. Reducing sugars from acid hydrolyzed lactose were determined by the 3,5-dinitrosalicylic acid (DNS) method [10] and expressed as equivalent lactose.

The reported data are average values of two separate experiments.

RESULTS AND DISCUSSION

Shake flask versus static fermentation

As shown in Fig. 1, shake flask gave higher ethanol concentrations compared to static fermentation. Maximum ethanol concentrations were 16.7 and 12.7 g/l for shake flask and static fermentation respectively. These concentrations were reached 12 h from start of fermentation.

The higher ethanol concentration in the case of shake flask fermentation is probably due to better growth of the yeast. As it appears in Table I, within the first 12 h of shake flask fermentation biomass concentration increased by a factor ca. 3.5, while it remained practically constant during the same period of static fermentation.

Shaking could be beneficial to the growth and performance of entrapped yeast cells by improving the mass transfer characteristics with respect to substrate, products/byproducts and oxygen. Shaking results in mixing of the growth medium outside the beads, thus helping maintain a concentration gradient between the interior and the exterior of the beads. This concentration gradient works in both directions; through better diffusion it helps maintain a satisfactory supply of sugars and other nutrients to the entrapped cells, while it facilitates the removal of ethanol, CO_2 and other byproducts of catabolism from the microenvironment of the cells. Besides, shaking keeps the beads floating around. As a result, the entire surface of the bead is available for mass

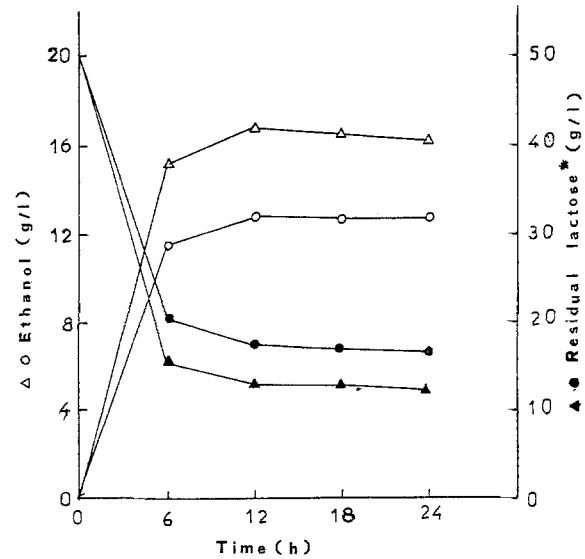

Fig. 1. Ethanol (△, ○) and residual lactose* (▲, ●) concentration during shake flask (△, ▲) and static (○, ●) fermentation of acid hydrolyzed whey by *S. cerevisiae* cells immobilized in Ca-alginate. * Residual lactose concentration represents concentration of reducing sugars expressed as equivalent lactose concentration.

TABLE 1

Biomass concentration during shake flask and static fermentation of cheese whey lactose to ethanol by immobilized cells of *S. cerevisiae*

Fermentation time (h)	Acid hydrolysis		Hydrolysis by coimmobilized β-galactosidase/shake flask (cfu/g beads)
	Shake flask fermentation (cfu/g beads)	Static fermentation (cfu/g beads)	
0	5.2×10^8	5.2×10^8	5.2×10^8
6	12.0×10^8	6.8×10^8	6.0×10^8
12	18.0×10^8	7.1×10^8	6.7×10^8
18	16.0×10^8	5.8×10^8	6.2×10^8
24	13.0×10^8	4.6×10^8	6.2×10^8

transfer. Finally, moderate shaking favors oxygen supply to the yeast; and this is especially important for high biomass concentrations [5].

Based on the results of this experiment, shake flask was selected over static fermentation for the coimmobilized enzyme experiments.

Performance of β-galactosidase coimmobilized cells

Results from shake flask fermentation of lactose hydrolyzed by coimmobilized lactase or acid are presented in Fig. 2 and Table 2.

Fig. 2 gives ethanol and residual lactose concentration during shake flask fermentation of cheese whey lactose by immobilized and β-galactosidase coimmobilized cells of *S. cerevisiae*. These results indicate a slower rate and a lower extent of lactose utilization, a slower rate of ethanol production and a lower maximum ethanol concentration for the coimmobilized enzyme treatment compared to the acid prehydrolysis treatment. The different rate and extent of lactose utilization reflect differences in microbial growth between treatments. As it appears in Table 1, biomass concentration remained practically constant throughout fermentation for the coimmobilized enzyme treatment, while it increased substantially (by more than triple) in the acid prehydrolysis treatment. In turn this difference in microbial growth is mainly due to differences in substrate availability and salt concentration. Acid prehydrolysis of whey lactose secures maximum availability of fermentable substrate to the yeast, which is not true in the case of coimmobilized lactase, where substrate availability depends on the rate of enzymatic hydrolysis of whey lactose. Besides, microbial growth could be stimulated by the presence of a low salt concentration [11]. The salt (NaCl) is formed during the pH adjustment in the acid prehydrolysis treatment. For reasons of comparison it is useful to mention that the total amount of lactose utilized by the coimmobilized lactase treatment was ca. 80% of the total amount utilized by the acid prehydrolysis treatment.

Important kinetic parameters describing the performance of the two treatments are presented in Table 2. Acid hydrolyzed lactose supported a higher ethanol productivity compared to enzyme hydrolyzed lactose (2.53 versus 1.62 g/l per h). Both treatments gave maximum ethanol productivities within the first 6-h period. On the other hand, the enzyme treatment gave higher values of specific ethanol productivity and specific sugar uptake rate, indicating a higher efficiency of the lactase coimmobilized cells. Finally, the lactase treatment resulted in a slightly better ethanol yield compared to the acid prehydrolysis treatment (0.47 vs. 0.45 g ethanol/g sugars utilized or 95% vs. 89% of theoretical). Working with immobilized cells of a lactose fermenting yeast

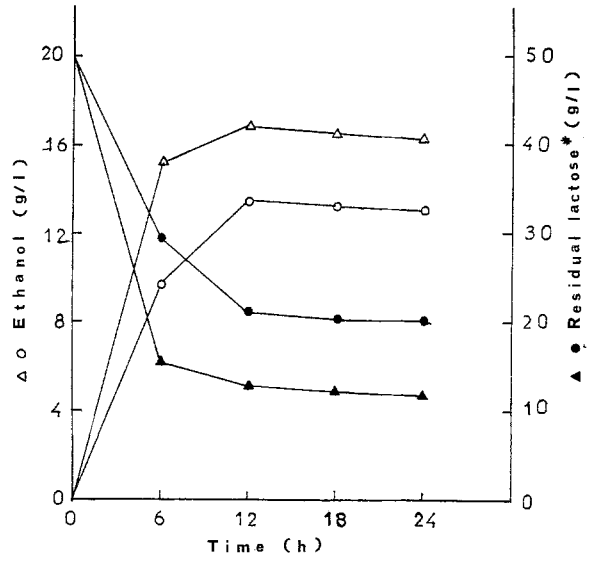

Fig. 2. Ethanol (△, ○) and residual lactose* (▲, ●) concentration during shake flask fermentation of deproteinized whey by Ca-alginate immobilized cells of *S. cerevisiae* using two methods of lactose hydrolysis: β-galactosidase coimmobilization (△, ▲) and acid prehydrolysis (○, ●). * Residual lactose concentration represents concentration of reducing sugars expressed as equivalent lactose concentration.

TABLE 2

Kinetic parameters describing shake flask fermentation of acid and coimmobilized enzyme hydrolyzed lactose in deproteinized cheese whey using immobilized *Saccharomyces cerevisiae* cells

Kinetic parameter	Acid hydrolysis				Coimmobilized enzyme hydrolysis			
	Fermentation time, h				Fermentation time, h			
	6	12	18	24	6	12	18	24
Biomass concentration $\times 10^{-8}$ (cfu/g beads)	12.0	18.0	16.0	13.0	6.0	6.7	6.2	6.2
Specific biomass productivity $\times 10^{-8}$ (cfu/g sugars utilized/h)	6.57	5.71	3.19	1.73	1.30	0.87	0.38	0.28
Ethanol concentration (g/l)	15.20	16.70	16.50	16.20	9.71	13.50	13.20	13.00
Ethanol productivity (g/l per h)	2.53	1.39	0.92	0.68	1.62	1.13	0.73	0.54
Specific ethanol productivity $\times 10^{11}$ (g ethanol/cfu/h)	1.05	0.39	0.29	0.26	1.35	0.84	0.59	0.44
Specific sugar uptake rate $\times 10^{11}$ (g sugar/cfu/h)	2.40	0.87	0.65	0.60	2.85	1.79	1.31	0.99
Ethanol yield (g ethanol/g sugar used)	0.44	0.45	0.44	0.43	0.47	0.47	0.45	0.44
Percent sugars utilized	69.0	74.8	75.2	75.3	41.0	57.7	58.3	59.0

(*K. marxianus*) on the same fermentation medium, Marwaha and Kennedy [9] reported substantially lower ethanol yields (up to 0.44 g/g or 83.7% theoretical).

Overall, through enzyme coimmobilization we could gain a great deal in terms of process simplification (i.e. avoid pH adjustments, heating, cooling), we could save in equipment and operating cost, and we could benefit from a higher ethanol yield while we would suffer some loss in the rate and extent of lactose utilization.

In conclusion, β-galactosidase coimmobilization seems to be a promising alternative for industrial batch fermentation of cheese whey lactose to ethanol.

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