APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Sept. 1984, p. 577–580
0099-2240/84/090577-04$02.00/0

Vol. 48, No. 3

Ethanol from Whey: Continuous Fermentation with a Catabolite Repression-Resistant *Saccharomyces cerevisiae* Mutant

SCOTT L. TERRELL,* ALAIN BERNARD,† AND RICHARD B. BAILEY‡
Biotechnology Branch, Solar Energy Research Institute, Golden, Colorado 80401

Received 12 March 1984/Accepted 11 June 1984

An alternative method for the conversion of cheese whey lactose into ethanol has been demonstrated. With the help of continuous-culture technology, a catabolite repression-resistant mutant of *Saccharomyces cerevisiae* completely fermented equimolar mixtures of glucose and galactose into ethanol. The first step in this process was a computer-controlled fed-batch operation based on the carbon dioxide evolution rate of the culture. In the absence of inhibitory ethanol concentrations, this step allowed us to obtain high biomass concentrations before continuous fermentation. The continuous anaerobic process successfully incorporated a cell-recycle system to optimize the fermentor productivity. Under conditions permitting a low residual sugar concentration (≤1%), maximum productivity (13.6 g liter^{-1} h^{-1}) was gained from 15% substrate in the continuous feed at a dilution rate of 0.2 h^{-1}. Complete fermentation of highly concentrated feed solutions (20%) was also demonstrated, but only with greatly diminished fermentor productivity (5.5 g liter^{-1} h^{-1}).

In the search for a satisfactory solution to the problem of cheese whey disposal, technologies have become available to recover the nutritious protein fraction. However, the lactose portion remains unused and is often subjected to costly waste treatment processes (R. L. Goldsmith, Annu. Meet. Whey Products Inst., 10th, 1981). As an alternative to waste treatment, fermentation of the lactose into ethanol for use as a fuel or chemical feedstock would be advantageous (6, 7, 13, 14). Such operations normally use the lactose-fermenting yeast *Kluyveromyces fragilis* or *Candida pseudotropicalis*. Since cheese whey lactose is an inexpensive substrate, its fermentation into ethanol is a product-recovery intensive process. Therefore, concentration of the lactose (ca. 20%) is a prerequisite for an economical process design. Although the best strains of *K. fragilis* will ferment 20% lactose, they do so very slowly, often requiring up to 1 week in batch culture (7, 14). Another difficulty is that their growth and rate of fermentation are greatly attenuated when they are cultured in a medium containing more than 4% ethanol (M. B. Mumford, M.S. thesis, University of Oklahoma, Norman, 1981). Clearly, improvements are needed in the conventional fermentation processes and in the yeast strains themselves before a widely accepted method for whey lactose fermentation can be realized.

To eliminate the need for waste treatment and disposal, the process should be improved so that it does not result in effluent sugar. In addition, fermentor productivity and ethanol concentration should be maximized to reduce reactor size and distillation costs, respectively. The maximum volumetric productivity reported to date was 14.7 g liter^{-1} h^{-1} with unconcentrated cheese whey and immobilized *K. fragilis* cells (Mumford, M.S. thesis). However, these figures represented a conversion efficiency of only 40% of the theoretical maximum yield. When this system was operated with zero residual sugar, the maximum productivity was only 5.3 g liter^{-1} h^{-1}, and the ethanol concentration was less than 2.5%. Several continuous processes have been described which use cell-recycle systems (3, 4, 8). These studies report enhanced conversion efficiencies and increased volumetric productivities. Recently, our laboratories have achieved a productivity of 7.1 g liter^{-1} h^{-1} with a conversion efficiency of 88% while maintaining a residual sugar concentration of less than 1%. This process utilized a selected strain of *K. fragilis* and a single-stage continuous culture fermentation with 100% cell recycle (10). However, difficulties in obtaining high biomass concentrations, i.e., >25 g (dry cell weight [DCW]) liter^{-1} for steady-state operation were encountered, indicating that a refinement in the aerobic fed-batch step was needed. Such refinement should manifest increased biomass concentrations before the onset of continuous fermentation, thereby reducing the time required to reach maximum ethanol productivity.

Since β-D-galactosidase is commercially available (12), O'Leary et al. (15) suggested that prehydrolysis of whey lactose into mixtures of glucose and galactose would allow fermentation by highly fermentative strains of *Sacchromyces cerevisiae*. However, these investigators were unable to ferment the galactose in such a hydrolysate with their strains. Several *S. cerevisiae* mutants resistant to catabolite repression have been isolated in our laboratory. Of these mutants, a strain designated SR has demonstrated an exceptional ability to ferment equimolar mixtures of glucose and galactose into ethanol (1).

In this study, we describe a two-step, single-stage continuous process that uses strain SR to simultaneously ferment mixtures of glucose and galactose. The first step, an aerobic fed-batch operation, was used to accumulate biomass in the absence of inhibitory ethanol concentrations. This was accomplished by using a computer-controlled substrate pulse which is initiated upon a decrease in the carbon dioxide evolution rate (CER) of the culture.

MATERIALS AND METHODS

Organism. Catabolite repression-resistant mutant SR of an industrial *S. cerevisiae* strain was used throughout these studies. The mutation and selection of the strain as well as the batch fermentation kinetics have been described (1). Cultures were maintained on slants at 4°C.

Media. Two basic media were used in these experiments. The first, YP (1% yeast extract, 2% peptone) supplemented

* Corresponding author.
† Present address: CEN de Cadarache, Laboratoire de Bioenergie Solaire, BPN1, 13115 Saint Paul lez Durance, France.
‡ Present address: Engenics Inc., Menlo Park, CA 95024.

with 0.15% 2-deoxyglucose (Sigma Chemical Co.) and 2% galactose (YPGal + 2DOG), was used for maintenance slants and to grow inocula. The sugar analog 2-deoxyglucose was prepared as previously described (1) and added during inoculum preparation to prevent reversion in the mutant. YP was also supplemented with 5 or 25% (wt/vol) substrate for use in the aerobic batch and fed-batch operations, respectively. For clarity, the percentage of substrate in the text will always refer to an equimolar mixture of glucose and galactose (both Sigma).

A second medium was employed as the nutrient-substrate feed during anaerobic operation. The formulation was similar to that used by Inloes et al. (9) and contained the following (grams liter of water^{-1}): yeast extract (8.5), $(NH_4)_2SO_4$ (1.3), $CaCl_2$ (0.06), and $MgSO_4 \cdot 7H_2O$ (0.1). Lipid addition to the feed was as previously described (11), with final concentrations of 5, 30, and 45 mg of ergosterol, oleic acid, and linoleic acid, respectively, per liter. The substrate was sterilized separately (120°C, 20 min) and added to compose 10, 15, or 20% (wt/vol) of the feed.

Process equipment. All fermentations were carried out in a Braun 6-liter Biostat S fermentor (B. Braun Inst., San Francisco, Calif.) equipped with continuous feed addition, pH, temperature, and foam control. Process data were continuously logged in by an Apple II computer system. Interfacing with the computer was made possible by an Isaac unit (Cyborg Corp., Newton, Mass.) with 16 analog input and output channels. All analog inputs (pH, temperature, agitation, airflow rate, CO_2) were direct current signals of 0 to 5 V which were converted to a 12-bit digital value by the analog-digital converter. One output was used during the fed-batch operation to control the substrate feed pump (K-07534-30; Cole Parmer Inst., Co., Chicago, Ill.), which was initiated by a drop in the CER of the culture. The fermentor control software was written in our laboratories and combined two versions of BASIC language: Applesoft (Apple Computer Inc., Cupertino, Calif.) and Labsoft (Cyborg Corp.).

The continuous cell-recycle system (Fig. 1) was facilitated by a Millipore Pellicon ultrafiltration unit (Millipore Corp., Bedford, Mass.) housing two PTHK coarse-screen cassettes (100,000 nominal molecular weight, 10.0 ft^2 [ca. 9,290 cm^2]). Ultrafiltrate removal was controlled by a Cole Parmer peristaltic pump fitted with a 10:1 reduction head (C-7561-00). A

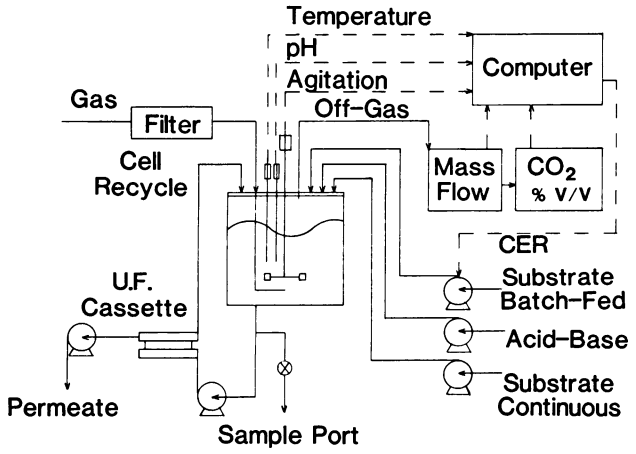

FIG. 1. Continuous fermentation process with cell recycle, computer-controlled substrate addition (during fed-batch operation), and data logging.

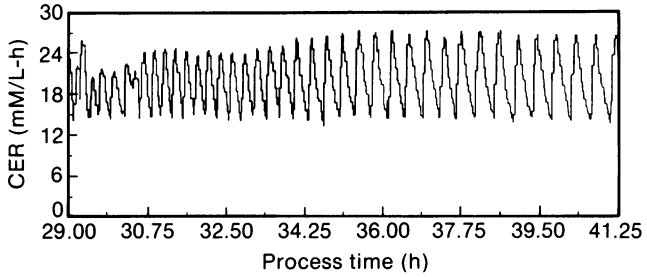

FIG. 2. Plot of the CER of the fed-batch culture versus time. This figure is a redrawing of the direct output from the computer control system. The pulse feed solution contained 25% substrate. At 29 h, the biomass concentration was 16 g (DCW) liter^{-1} and increased to 30 g (DCW) liter^{-1} at 41 h. A 2.7-ml substrate pulse was delivered each time the CER declined to 15 mmol liter^{-1} h^{-1}.

sanitary finish, pneumatic bellows pump (K-7152-10; Cole Parmer) removed culture fluid from the fermentor and supplied the operating pressure for the ultrafiltration unit (40 lb in^{-2}).

Fermentations. Inocula were generated by aseptically transferring strain SR cells from maintenance slants into a 125-ml Erlenmeyer flask containing 50 ml of YPGal + 2DOG. This culture was allowed to incubate overnight at 30°C with agitation. The entire contents were then added to a 1-liter flask containing 250 ml of the same medium and incubated under similar conditions for 24 h. The resultant culture was examined microscopically for purity and used to inoculate the fermentor (3.2 liters of YP plus 5% substrate). Overnight operation of the fermentor was then carried out with the following parameters: airflow rate, 1 volume of air per volume of medium per min (vvm); agitator speed, 500 rpm; and pH maintained at 4.5. CER was monitored continuously. After reaching a maximum of ca. 90 mmol of CO_2 liter^{-1} h^{-1}, the CER would begin to fall, indicating the beginning of substrate limitation. When the CER declined to 15 mM liter^{-1} h^{-1}, automatic substrate addition (YP + 25% substrate) would ensue. Here, the biomass concentration was generally 16 to 18 g (DCW) liter^{-1}. Each pulse lasted 15 s, delivered 2.7 ml of substrate solution (0.2 g of substrate liter of culture^{-1}), and allowed the CER to rise. A new pulse was then delivered each time the CER declined to 15 mM liter^{-1} h^{-1} and was repeated until the biomass had accumulated to 30 g (DCW) liter^{-1}.

Upon attaining a satisfactory biomass concentration, the process was shifted to continuous anaerobic fermentation. This was accomplished by turning off the air flow and starting the pumps for cell recycle, ultrafiltrate removal, and nutrient feed. The nutrient and ultrafiltrate pumps were adjusted to equal flow rates as dictated by the desired dilution rate (D). Nitrogen was sparged through the fermentor (1 vvm) to remove CO_2 from the culture. Both the dilution rate and the substrate concentration were varied during a given experiment, and each variation was maintained for at least 3 fermentor volumes (10.5 liters).

Analytical procedures. Glucose and galactose were both measured with a Yellow Springs Instrument model 27 sugar analyzer. Galactose measurements were accomplished by using a lactose membrane standardized with galactose.

Ethanol analysis was made on a Hewlett-Packard 5880A gas-liquid chromatograph with a flame-ionization detector. Ethanol was quantified by using isopropanol as an internal standard. A Porapak Q steel column (183 by 0.3 cm) was operated isothermally at 175°C with the detector at 250°C and the injection port at 225°C.

DCWs were estimated by filtering portions of the sample through tared 0.45-μm filters. The filters were then rinsed with 100 ml of deionized water, dried at 105°C for 2 h, and reweighed.

On-line measurement of the CO_2 concentration in the fermentor off-gas was accomplished with an infrared CO_2 analyzer (Mine Safety Appliances Co., Pittsburgh, Pa.) and a mass flow meter (Sierra Instruments, Inc., Carmel Valley, Calif.). These instruments allowed continuous monitoring of the CO_2 (percent [vol/vol]) and gas flow rate (liters minute^{-1}), respectively. With these data, the computer determined the CER as follows: CER (millimoles liter^{-1} hour^{-1}) = $F \times CO_2 \times 1/V_m \times 1/V$, where F is the gas flow rate (liters hour^{-1}); CO_2 is the concentration of CO_2 in the off-gas (percent [vol/vol]), V_m is the molar volume (liters) and V is the fermentor volume (liters).

RESULTS

Figure 2 represents a plot of the CER data obtained during a typical aerobic cycle. The average period between substrate pulses lasted 20 min for a pulse size of 0.2 g of substrate liter of culture^{-1} pulse^{-1}. Under these conditions, the biomass increased from 18 to 30 g (DCW) liter^{-1} within 13 h after the onset of substrate addition. In this example, the area of the CER peaks increased after 35 h. This expansion was attributed to a slight variation in the substrate pump speed and not to a decrease in the viability of *S. cerevisiae* SR during the fed-batch cycle. The overall growth yield coefficient ($Y_{x/s}$, where x is grams of cells liter^{-1} and s is grams of sugar liter^{-1}) was equal to 0.51.

The ability of strain SR to ferment equimolar mixtures of glucose and galactose was evaluated by using the continuous process with cell recycle. Table 1 summarizes the results of several contiuous runs with variations in the initial substrate concentration and dilution rate. When the restraint of <1% residual sugar was maintained, the highest productivity was 13.6 g liter^{-1} h^{-1} with a dilution rate of 0.2 h^{-1} and 15% initial substrate. The maximum conversion efficiency was also observed under these conditions and was 94% of the theoretical product yield. When a 10% substrate feed was used, both the productivity and efficiency continued to rise with increases in the dilution rate. An increase in the residual sugar concentration was not observed at any of the dilution rates tested. To achieve a satisfactory ethanol productivity from a 10% feed solution, the dilution rate would have had to be at least 0.25 h^{-1}. However, a gradual increase in the filtration cassette backpressure made it difficult to equalize flow rates between the feed and permeate pumps at dilution rates exceeding 0.25 h^{-1}. Therefore, we increased the initial substrate concentration to 15% to avoid pushing the dilution rate to a point of residual sugar accumulation. Strain SR did ferment a 20% feed to completion but only with low productivity and efficiency. Attempts to raise the dilution rate only resulted in a significant increase in the residual sugar concentration at dilution rates greater than 0.08 h^{-1}.

TABLE 1. Results of continuous fermentations with various dilution rates and initial substrate concentrations

Substrate (%)[a]	Dilution rate (h^{-1})	Ethanol (g liter^{-1})	Productivity (g liter^{-1})	Residual sugar (%)	Efficiency (%)[b]
10	0.05	22	1.1	0.2	43
	0.08	29	2.3	0.2	58
	0.10	36	3.6	0.2	72
	0.15	42	6.3	0.2	84
15	0.05	48	2.4	0.4	64
	0.08	51	4.1	0.4	68
	0.10	64	6.4	0.4	86
	0.13	68	8.8	0.5	92
	0.20	70	13.6	0.5	94
	0.25	61	15.3	1.9	91
	0.30	56	16.8	2.0	84
20	0.08	69	5.5	0.4	69
	0.10	73	7.3	1.5	77
	0.15	73	10.9	3.3	85

[a] Total substrate consisted of 50% glucose and 50% galactose.
[b] % Efficiency is calculated as the amount of ethanol produced × 100 divided by the theoretical maximum of the sugar consumed.

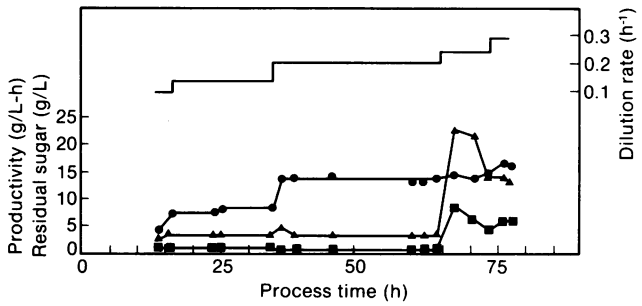

FIG. 3. Graphical representation of a typical continuous culture with 15% substrate in the feed solution. Symbols: ●, fermentor ethanol productivity; ▲, residual galactose; and ■, residual glucose. The solid line illustrates a stepwise increase in the dilution rate.

Anomalous conversion efficiency data were observed at the lower dilution rates for each initial substrate concentration (Table 1). Under these conditions the efficiencies should have been higher since the substrate was almost completely metabolized.

Figure 3 is a plot of the data obtained during a typical continuous operation with a 15% initial substrate concentration in the feed solution. As in all experiments, the residual galactose concentration was only slightly higher than that of the residual glucose. The sharpest increase in productivity was observed as the dilution rate was changed from 0.13 to 0.2 h^{-1}, and low residual substrate could not be steadily maintained at a dilution of ≥0.2 h^{-1}.

Throughout the continuous process, the biomass concentration gradually increased due to the 100% cell recycle. With the more concentrated feed solutions (15 and 20%), the biomass continued to accumulate until the ethanol concentration reached 74 g liter^{-1}. The highest biomass concentration attained during any one experiment was 80 g (DCW) liter^{-1}. However, at concentrations greater than 55 g (DCW) liter^{-1}, the filtration cassette required daily cleaning, which resulted in contamination of the culture. As a result, a periodic cell bleed was instituted in remaining experiments to maintain a biomass concentration of 50 g (DCW) liter^{-1}, the maximum workable concentration with this system.

DISCUSSION

Our results have demonstrated that strain SR is able to simultaneously ferment equimolar mixtures of glucose and galactose in a continuous process. A cell-recycle system was successfully applied to this process with the advantage of an increase in volumetric productivity. Also, by incorporating a computer-controlled fed-batch step, we rapidly accumulated high biomass concentrations before the continuous feeding of substrate. This method allowed us to maximize the growth yield coefficient ($Y_{x/s}$) and to achieve a higher ethanol productivity at the onset of continuous fermentation.

It must be pointed out that our efficiency results should have shown the opposite relationship, i.e., increased efficiency with decreased feed rate (Table 1). One likely reason for these results could be ethanol loss via the fermentor offgas. To obtain the CER data, it was necessary to continuously sparge the culture with nitrogen. During more recent ethanol fermentations, we have experienced a stripping of the ethanol by nitrogen gassing and a resultant loss of ethanol at low rates of product formation. Since the rate of product formation was faster with an increase in dilution rate, this effect might be accountable for the discrepancy in our calculations. A second explanation might lie in the reported growth of the biomass during continuous fermentation. The dissolved-oxygen concentration was not measured in these experiments because of the low sensitivity of the instruments in a range characteristic of anaerobic fermentation. Oxygen may have entered the system, through incomplete scrubbing of the gassing mixture or by partial saturation of the continuous feed, and allowed the yeast to respire. All experiments were initiated at a low dilution rate, which was slowly increased to the point of residual sugar accumulation. As the dilution rate was increased, the concentration of intracellular sugar may have increased and effectively repressed the mitochondrial function in respiring cells. This situation would have caused an increase in the fermentation efficiency of the biomass.

Although whey lactose is an inexpensive fermentable substrate, it is more importantly considered an environmental pollutant. Therefore, our primary concern was to design an efficient continuous process in which the effluent would not contain residual sugar. Under conditions adhering to this restraint, our most promising results were obtained with a 15% substrate feed at a dilution rate of 0.2 h^{-1}. Under those conditions, the ethanol productivity and conversion efficiency showed a dramatic improvement over operations employing *K. fragilis* to ferment lactose under the same restraints (10; Mumford, M.S. thesis). In addition, the resultant ethanol concentration (ca. 68 g $liter^{-1}$) lies near the maximum concentration allowable without causing significant inhibition of cell growth or ethanol production in *S. cerevisiae* (2). In the mutant strain SR, inhibition of cell growth was observed at ethanol concentrations exceeding 74 g $liter^{-1}$. Because of this, a balance in high ethanol concentration and metabolic activity appeared to be optimized when the continuous feed contained 15% substrate. This indicates that strain SR may be suited for fermentation of concentrated cheese whey lactose after hydrolysis to the component sugars. Whether or not the reduction in capital and distillation costs would compete with the added cost for hydrolysis is, however, beyond the scope of this study. Still, this process represents a significant improvement over the use of *K. fragilis* in the conversion of whey lactose into ethanol.

From the data presented in this study, one can envision several approaches to process improvement. (i) Redox potential has been shown to be a better substitute than dissolved oxygen for the control of bacterial leucine fermentations (5). On-line measurement of redox potential would also be helpful in yeast fermentations to monitor needed oxygen at very low concentrations. The optimum oxygen concentration would be one sufficient for intracellular membrane development, which is used in ethanol transport, without causing unnecessary cell growth and a concomitant reduction in fermentation efficiency. (ii) An improved method is needed for cell separation in the recycle process. Cysewski and Wilke (4) report that biomass concentrations of 120 g $liter^{-1}$ are attainable with *S. cerevisiae*. These concentrations were achieved by using a cell settler to facilitate the recycle process. Our results demonstrated that strain SR will also accumulate biomass to high densities (>80 g $liter^{-1}$). If the workable biomass concentration is increased, higher dilution rates and resultant productivities should be possible. (iii) In a single-stage continuous culture system there is only one degree of freedom. Therefore, one cannot optimize both productivity and substrate utilization. In the present case, in which substrate utilization is crucial, a second stage can be added, so that both parameters could be maximized. (iv) This study showed that strain SR will ferment 20% substrate. By incorporating vacuum fermentation technology with our process, it may be feasible to ferment highly concentrated lactose hydrolysate without noticeable end product inhibition (4). Concentration of cheese whey lactose would reduce the volume of water that the fermentation process is required to handle, resulting in decreased capital outlay and distillation costs.

ACKNOWLEDGMENTS

This work was sponsored by the Alcohol Fuels Office of the Department of Energy, Solar Energy Research Institute WPA no. 349.

We thank Karel Grohmann for his many helpful suggestions concerning this research.

LITERATURE CITED

1. **Bailey, R. B., T. Benitez, and A. Woodward.** 1982. *Saccharomyces cerevisiae* mutants resistant to catabolite repression: use in cheese whey hydrolysate fermentation. Appl. Environ. Microbiol. **44**:631–639.
2. **Bazua, C. D., and C. R. Wilke.** 1977. Ethanol effects on the kinetics of a continuous fermentation with *Saccharomyces cerevisiae*. Biotechnol. Bioeng. Symp. **7**:105–118.
3. **Cysewski, G. R., and C. R. Wilke.** 1976. Utilization of cellulosic materials into ethanol. I. Fermentation of hydrolysate to ethanol and single-cell protein. Biotechnol. Bioeng. **18**:1297–1313.
4. **Cysewski, G. R., and C. R. Wilke.** 1977. Rapid ethanol fermentations using vacuum and cell recycle. Biotechnol. Bioeng. **19**:1125–1143.
5. **Dahod, S. K.** 1982. Redox potential as a better substitute for dissolved oxygen in fermentation process control. Biotechnol. Bioeng. **24**:2123–2125.
6. **Friend, B. A., and K. M. Shahani.** 1979. Whey fermentation. N. Z. J. Dairy Sci. Technol. **14**:143–152.
7. **Gawel, J., and F. V. Kosikowski.** 1978. Improving alcohol fermentation in concentrated ultrafiltration permeates of cottage cheese whey. J. Food Sci. **43**:1717–1719.
8. **Hamer, G.** 1982. Bioengineering report: recycle in fermentation processes. Biotechnol. Bioeng. **24**:511–531.
9. **Inloes, D. S., D. P. Taylor, S. N. Cohen, A. S. Michaels, and C. R. Robertson.** 1983. Ethanol production by *Saccharomyces cerevisiae* immobilized in hollow-fiber membrane bioreactors. Appl. Environ. Microbiol. **46**:264–278.
10. **Janssens, J. H., A. Bernard, and B. Bailey.** 1984. Ethanol from whey: continuous fermentation with cell recycle. Biotechnol. Bioeng. **23**:1–7.
11. **Janssens, J. H., N. Burris, A. Woodward, and R. B. Bailey.** 1983. Lipid-enhanced ethanol production by *Kluyveromyces fragilis*. Appl. Environ. Microbiol. **45**:598–602.
12. **Kohlwey, D. E., and M. Cheryan.** 1981. Performance of a β-D-galactosidase hollow fiber reactor. Enzyme Microb. Technol. **3**:64–68.
13. **Lyons, T. P., and J. D. Cunningham.** 1980. Fuel alcohol from whey. Am. Dairy Rev. **November**:42A–42E.
14. **Moulin, G., M. Guillaume, and P. Galzy.** 1980. Alcohol production by yeast in whey ultrafiltrate. Biotechnol. Bioeng. **22**:1277–1281.
15. **O'Leary, V. S., C. Sutton, M. Beacivengo, B. Sullivan, and V. H. Holsinger.** 1977. Influence of lactose hydrolysis and solids concentration on alcohol production by yeasts in acid whey ultrafiltration. Biotechnol. Bioeng. **19**:1689–1702.