APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Mar. 1989, p. 573–578
0099-2240/89/030573-06$02.00/0

Vol. 55, No. 3

Enhanced Conversion of Lactose to Glycerol by *Kluyveromyces fragilis* Utilizing Whey Permeate as a Substrate

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Received 6 June 1988/Accepted 12 December 1988

***Kluyveromyces fragilis* (CBS 397) is a nonhalophilic yeast which is capable of lactose utilization from whey permeate and high glycerol production under anaerobic growth conditions. However, the optimum yields of glycerol (11.6 mg/ml of whey permeate medium) obtained in this study occurred only in the presence of 1% Na_2SO_3 as a steering agent. The use of other concentrations of Na_2SO_3, as well as 5% NaCl and 1% ascorbic acid, had no or detrimental effects on cell growth, lactose utilization, and glycerol production. Glycerol yields were greater in cultures grown from a light inoculum of *K. fragilis* than in cultures in which a resuspended mass of cells was introduced into the medium. The results of this study suggest that this strain of *K. fragilis* may be useful commercially in the utilization of cheese whey lactose and the concomitant production of glycerol.**

Cheese whey represents a commercial by-product generated in such massive quantities that its safe disposal is a major problem for many municipal sewage treatment plants. In 1986, Paluch (14) surveyed 49 cheese whey processing plants in southern Wisconsin and northern Illinois and calculated the potential dollar value of reclaimed whey as well as the cost of dumping the whey into municipal sewage systems. For a medium-size plant generating 3.0×10^7 lb (ca. 1.36×10^7 kg) of whey per year, she determined that the maximum return on processed whey could be $13.9 million and that the corresponding annual biological oxygen demand costs would be $2.07 million. Whey permeate can be readily produced from cheese whey by membrane filtration and contains approximately 5% lactose. It would be of considerable value if an environmentally safe microorganism could utilize the lactose of whey permeate and generate potentially profitable quantities of a commercially useful by-product. However, most microorganisms have limited or no β-galactosidase activity, which prevents them from making effective use of lactose as a carbon source.

Recently, we have found that the yeast *Kluyveromyces fragilis* (strain CBS 397) is a promising nonhalophilic lactose-utilizing yeast that is capable of considerable glycerol production. In fact, this strain can be compared with a *Saccharomyces cerevisiae* alcohol dehydrogenase I mutant grown in batch fermentation using YEPD (6% glucose) medium, which has a reported maximum yield of 11 mg/ml (10). With the addition of up to 4% sodium sulfite, immobilized *S. cerevisiae* cells in a 10% glucose-containing broth yielded glycerol up to 26 mg/ml (3). Therefore, if glycerol production could be enhanced in *K. fragilis* through use of a steering agent, that organism could be of considerable economic significance while, at the same time, being an effective degrader of the lactose in cheese whey.

During World War I, it was discovered in Germany that glycerol could be made for the manufacture of explosives. The process involved adding small amounts of sodium bisulfite to a batch-type alcoholic fermentation of *S. cerevisiae*. Up to 1,000 metric tons of glycerol per month was produced in Germany during that time by this procedure (5). Today, glycerol is typically produced chemically from petroleum derivatives, which is less expensive than processing by sugar fermentation.

Biological production of glycerol may be carried out by using various osmophilic yeasts, algae, and cyanobacteria in which glucose is the typical carbon source. In most cases, laboratory production of glycerol uses osmophilic microorganisms (grown in media containing up to 20% NaCl) which presumably produce the glycerol as an osmotic regulator, thereby protecting the cell against dehydration (1, 4, 9, 11). Glycerol formation by some osmotolerant yeasts such as *Zygosaccharomyces bailii* may also occur under strictly aerobic conditions (13, 16). Two of the most effective osmophilic yeasts used for fermentative glycerol production are *Pichia farinosa* (ATCC 20210), which can yield 18 mg of glycerol per ml on a synthetic medium with 0.1% yeast extract and 10% glucose incorporated (18), and *Debaryomyces hansenii* (Zopf) van Rij strain 26, which has yielded 11 mg of glycerol per ml on a synthetic complete medium containing vitamins, salts, and 5% glucose (2).

In addition to the use of salt as an osmotic agent, two techniques employing steering agents are commonly used to generate enhanced glycerol production. In one case, bisulfite or sulfite can be added to the culture medium, in which it combines with acetaldehyde, interfering with the reduction of acetaldehyde to ethanol and thus diverting fermentative reduction to dihydroxyacetone phosphate which, through glycerol phosphate, yields glycerol (6). In the other case, sodium carbonate added to culture media reportedly promotes the dismutation of acetaldehyde, which then cannot be reduced by NADH (19). The NADH can preferentially reduce dihydroxyacetone phosphate which, upon dephosphorylation, produces glycerol. Although glycerol can be produced in reasonable amounts without sulfites or alkaline steering agents, such alternative procedures have generally not been cost effective.

This communication seeks to demonstrate that glycerol production can be achieved from the fermentation of whey permeate lactose and that such production can be enhanced through the use of a specific steering agent.

MATERIALS AND METHODS

K. fragilis (Jörgensen) van der Walt (1971) was obtained from Centraalbureau voor Schimmelcultures, Delft, The

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Netherlands, as strain CBS 397 and maintained on a medium of 1.0% yeast extract, 2% Bacto-Peptone (Difco Laboratories, Detroit, Mich.), and 2% dextrose at 4°C as laboratory stock cultures.

The growth medium for experimental purposes was whey permeate (WP). It was obtained in powder form from Express Foods Co., Louisville, Ky., and contained 87% lactose. To prepare a liquid medium containing 5% lactose (50.0 ± 0.5 mg/liter), 57.5 g was suspended in 1 liter of distilled water. To remove precipitated protein, the medium was vacuum filtered through sterile polycarbonate filters (average pore size, 0.45 μm) after autoclaving. The pH of the medium was 6.2. For purposes of experimental growth, 75 ml of WP was dispensed into each 250-ml screw-cap or rubber-stoppered Erlenmeyer flask used.

Inoculations were cell suspensions (optical density at 650 nm [OD_{650}] of 0.05) from overnight starter cultures grown at 30°C (120 rpm) and inoculated either as a 1.0% volume or as a resuspended cell mass representing established growth at OD_{650} 1.5 from 3-day nonshaken cultures at 30°C. In anaerobic cultures, the CO_2 generated from fermentation was expelled through a saturated aqueous KOH solution. Air was originally present in the flasks at the time of inoculation but quickly became displaced by CO_2 generated from fermentation. Sampling at each time period (5 ml each) was performed anaerobically through long hypodermic needles suspended in the medium through the rubber stopper closures. All growth was at 30°C. Anaerobic growth continued for up to 240 h.

At each sampling time, cell density was checked after proper dilution by reading the OD_{650} with a Spectronic 20 instrument (Bausch & Lomb, Inc., Rochester, N.Y.). Supernatant fluid samples were collected, frozen in Eppendorf microcentrifuge tubes, and assayed within 3 days of the time of collection. Lactose assays were performed by the UV absorption method of Boehringer Mannheim Biochemica GmbH (catalog no. 176303) in which the NADH produced stoichiometrically with lactose hydrolysis was measured by A_{340} with a Lambda 3A spectrophotometer (The Perkin-Elmer Corp., Norwalk, Conn.) and evaluated against a standard curve. Assays for glycerol content in supernatant fluids were performed by using reagents obtained from Boehringer Mannheim Biochemica GmbH (catalog no. 148-270) and the spectrophotometer described above, operating at A_{340}; readings were compared against a glycerol standard curve. Assays for glucose (generated from the lactose along with galactose) were done by the hexokinase method (procedure 115; Sigma Chemical Co., St. Louis, Mo.), in which the reduced product was measured colorimetrically at 520 nm. Absorbance was directly proportional to glucose concentration (over the range used) in the medium samples.

For cell growth, WP was supplemented with either 5 or 15% NaCl as an osmotic agent, 0.5, 1.0, or 2.0% Na_2SO_3 as a steering agent, or 1.0% ascorbic acid as a reducing agent (alone and in conjunction with 1.0% Na_2SO_3). Glycerol production is expressed as milligrams of glycerol generated per milliliter of WP.

RESULTS

K. fragilis grown aerobically from starter inoculation (OD_{650} of 0.05) in unsupplemented WP reached stationary phase after 28 h of growth. Concurrently, lactose was depleted while the glycerol yield reached 1.9 mg/ml. When 5% NaCl was added to WP, the stationary phase was reached at 34 h and the maximum OD_{650} of *K. fragilis* was slightly depressed. Lactose utilization was nearly complete at the end of 28 h, and the glycerol yield was only slightly increased. No growth at all was obtained in WP supplemented with 15% NaCl. *K. fragilis* growth in WP plus 0.5% Na_2SO_3 showed almost no change in OD or lactose utilization during growth in WP alone (control). The glycerol yield increased to 3.2 mg/ml over the course of growth. However, when the concentration of Na_2SO_3 was increased to 1.0%, lactose utilization was slowed and glycerol production reached 3.0 mg/ml at 70 h (Fig. 1).

More significant differences were found when *K. fragilis* was grown in the same medium with the same supplements but in which the cell masses were harvested and resuspended in fresh WP followed by anaerobic growth. In the WP control, cells reached maximum OD only after 30 h, which also corresponded to the greatest depletion of lactose. Maximum glycerol yield (3.4 mg/ml) occurred by 88 h after resuspension. Near-stationary OD (with possibly a reduction in viable cell counts) occurred in the resuspended cell masses in WP with each of the supplements, whereas curves for lactose utilization were all similar in that near-complete degradation occurred by 64 h. Whereas supplemented NaCl showed only a marginal increase in glycerol yield over the control value 120 h after resuspension, addition of Na_2SO_3 (particularly 1.0%) produced very significant increases, to 8.8 mg/ml at 88 h in WP plus 1.0% Na_2SO_3 (Fig. 2). Since the glycerol yield was lower with addition of 0.5% Na_2SO_3 than with addition of 1.0% Na_2SO_3, no further studies were performed with 0.5% Na_2SO_3.

When low inoculum (OD_{650} of 0.05) was introduced into WP followed by anaerobic growth in which WP was supplemented with 5% NaCl or 1.0% Na_2SO_3, a reduction (by nearly 50%) in OD_{650} at the stationary phase was recorded, and near-complete lactose utilization was delayed to 48 h, compared with delay of the control to 24 h. Whereas there was almost no difference in glycerol yield between growth of *K. fragilis* in control WP and in WP plus 5% NaCl, a substantial and highly significant increase in glycerol yield occurred in WP supplemented with 1.0% Na_2SO_3. The glycerol yield reached 11.6 mg/ml by 192 h (Fig. 3).

When 2% Na_2SO_3 was added to WP, overall growth was slowed, OD_{650} was reduced, the rate of lactose degradation was slowed, and the yield of glycerol was slightly reduced. Consequently, it was determined that 1.0% Na_2SO_3 as a steering agent represented the optimum concentration for glycerol yield and a concentration which did not appreciably inhibit cell growth.

The addition of 1.0% ascorbic acid as a reducing agent alone and in conjunction with 1.0% Na_2SO_3 had an overall negative effect on glycerol production (Fig. 4 and 5). The addition of only ascorbic acid to WP did not affect the overall growth of *K. fragilis* but slowed both the rate of growth and the utilization of lactose (Fig. 4a and d). At 96 h (upon the depletion of lactose), the residual sugars, especially glucose, were absent in the controls, with 0.24% remaining in WP plus 1.0% Na_2SO_3 and 0.46% remaining in WP plus 1.0% Na_2SO_3 plus 1.0% ascorbic acid. The highest yields of glycerol were consistently found in WP plus 1.0% Na_2SO_3 without ascorbic acid at all time periods sampled over the course of 240 h (Fig. 5). However, when WP was again supplemented with 2.0% Na_2SO_3, the glycerol yield (while reaching 10.5 mg/ml at 192 h) consistently remained below the levels recorded with supplementation by only 1.0% Na_2SO_3 (Fig. 5).

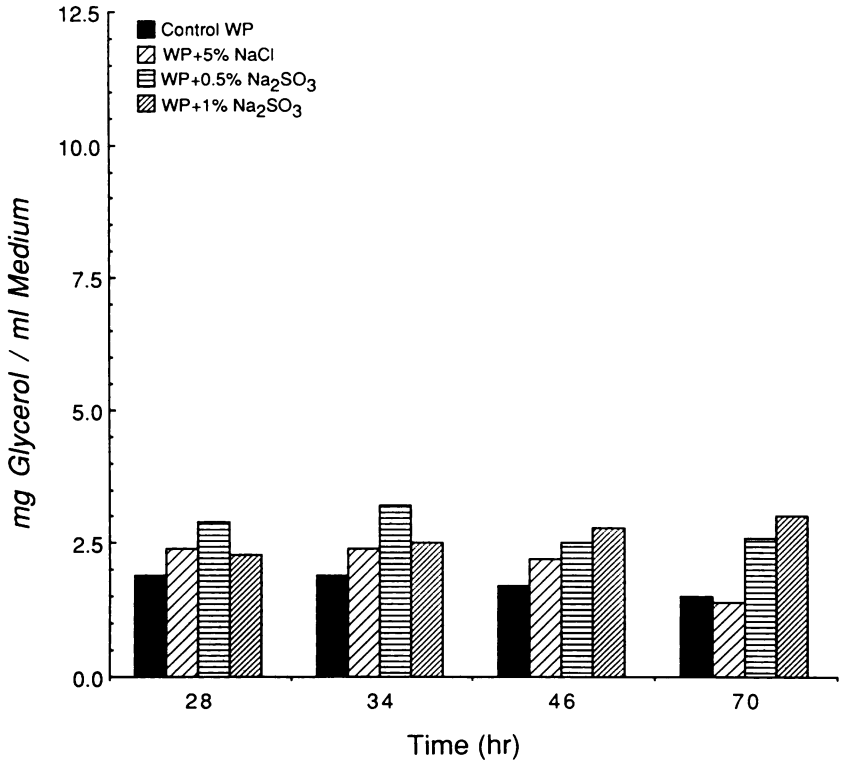

FIG. 1. Glycerol yield over 70 h of *K. fragilis* grown aerobically from low inoculum in WP alone (control) and supplemented with NaCl or various concentrations of Na_2SO_3.

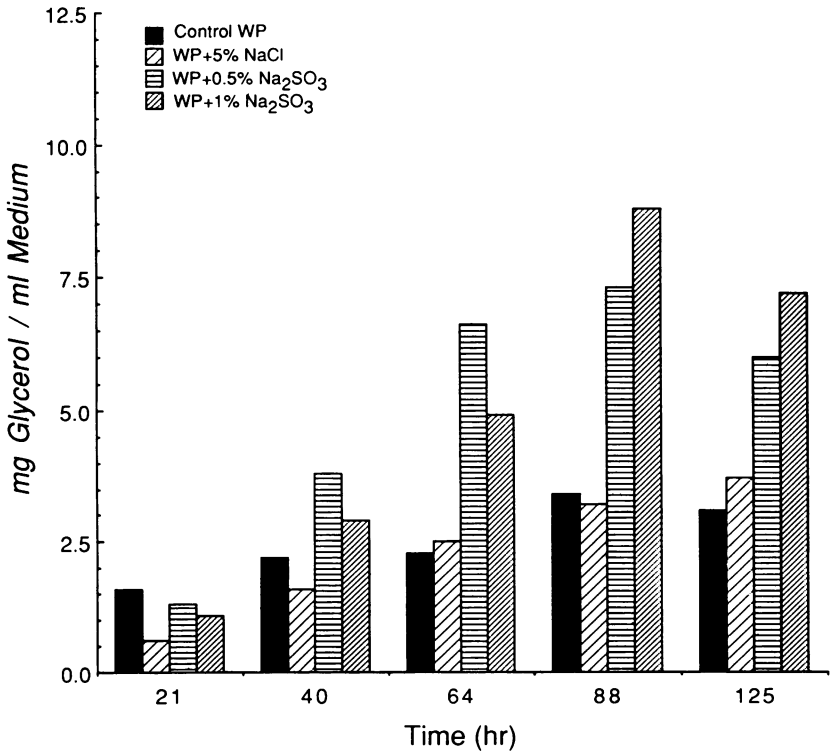

FIG. 2. Glycerol yield over 125 h of *K. fragilis* grown anaerobically from resuspended cell mass in WP alone (control) and supplemented with NaCl or various concentrations of Na_2SO_3.

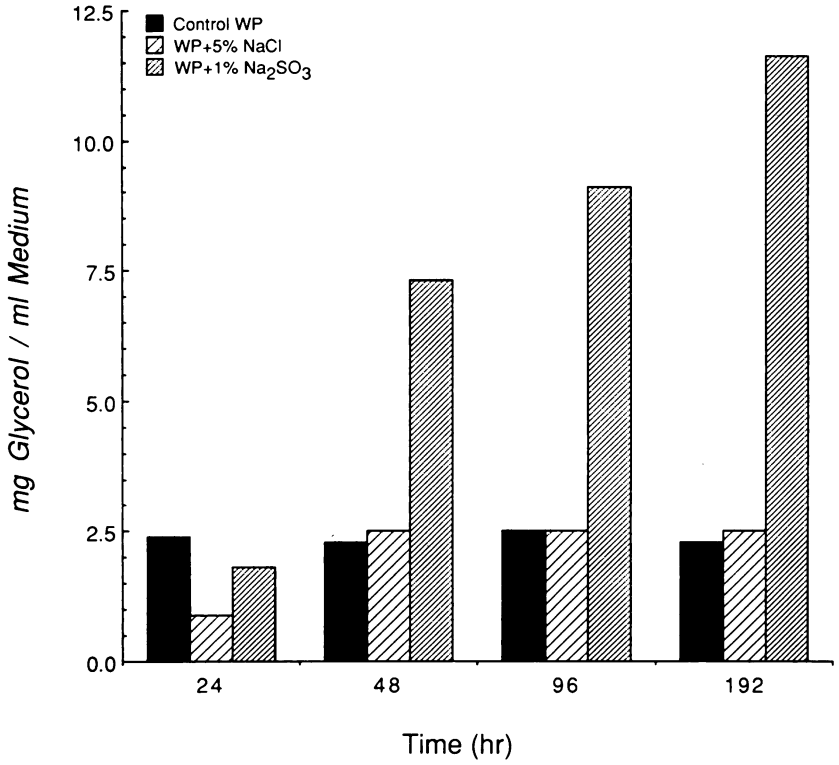

FIG. 3. Glycerol yield over 192 h of *K. fragilis* grown anaerobically from low inoculum in WP alone (control) and supplemented with either 5% NaCl or 1.0% Na_2SO_3.

DISCUSSION

Although *K. fragilis* grew rapidly to a high OD under aerobic conditions, the generation of glycerol, particularly in the presence of sodium sulfite, was very low compared with growth in anaerobic conditions. The strain of *K. fragilis* used in this study (CBS 397) is essentially Crabtree negative, since it is capable of only very weak aerobic fermentation. It has been shown in batch culture that only 4% of glucose provided to *K. fragilis* can be metabolized to ethanol (20). Thus, acetaldehyde, a precursor of ethanol, is not believed to exist in substantial quantity. The mechanism of action of sodium sulfite is believed to involve its binding to acetaldehyde, which then results in a shift of fermentation to glycerol production (10). Under these conditions, sodium sulfite is likely to be effective only if alcoholic fermentation is occurring, a situation which in *K. fragilis* requires anaerobic growth. Furthermore, Na_2SO_3 in aerobic solution may rapidly become oxidized to Na_2SO_4, which is not an effective steering agent. At concentrations of greater than 1.0%, Na_2SO_3 inhibited growth, presumably through its propensity for toxicity.

Whereas Na_2SO_3 supplementation significantly increased glycerol production, addition of ascorbic acid had no such effect. In fact, it had a negative effect on glycerol production by itself or in conjunction with Na_2SO_3. Ascorbic acid may function by chemically reducing acetaldehyde without the involvement of NADH. If so, this could alter the ratio of NAD to NADH. Since cells need NAD to degrade glucose to ethanol or glycerol, that process is likely to be interrupted by the presence of ascorbic acid. Therefore, high levels of NADH alone do not ensure elevated glycerol levels. It is also possible that in this study, much of the ascorbic acid was in the salt form, since the pH of the medium was maintained at 7.76. Ascorbic acid lowered the pH of WP to 3.2, and NaOH was used to readjust the pH.

The presence of 5% NaCl depressed the overall growth of *K. fragilis* under both aerobic and anaerobic conditions, although it showed no effect on the production of glycerol in either case. Since *K. fragilis* did not grow at all in WP supplemented with 15% NaCl, it cannot be considered salt tolerant, as has been demonstrated with *D. hansenii* (2, 9). Thus, *K. fragilis* has been shown in this study not to be a halophilic yeast, and the presence of 5% NaCl in WP does not appear to significantly elicit the response of intracellular glycerol production for osmotic stabilization, as has been found in known halophilic yeasts such as *Zygomyces rouxii*, *D. hansenii*, and selected strains of *S. cerevisiae* (2, 15, 18). No previous reports defining the halophilic nature of *K. fragilis* strains have been published; however, Gawel and Kosikowski (8) have shown that several strains of *K. fragilis* (but not CBS 397) can be osmotolerant in media containing up to 22% lactose.

Vijaikishore and Karanth (18) reported reduced fermentation times and reduced glycerol production in yeast cultures from high-inoculum sources. Those authors suggested that the greater initial cell growth favored the anaerobic production of ethanol over glycerol, but they did not use sodium sulfite as a steering agent. Indeed, in this study it has been shown that the highest glycerol production occurred only when a small inoculum (OD_{650} of 0.05) was used along with the steering agent. This result may have been due to the greater ability of Na_2SO_3-tolerant or physiologically adapted cells to survive from young growth and to produce glycerol. These cells may more readily become adapted to the steering agent. Similar adaptation of *K. fragilis* to high lactose levels

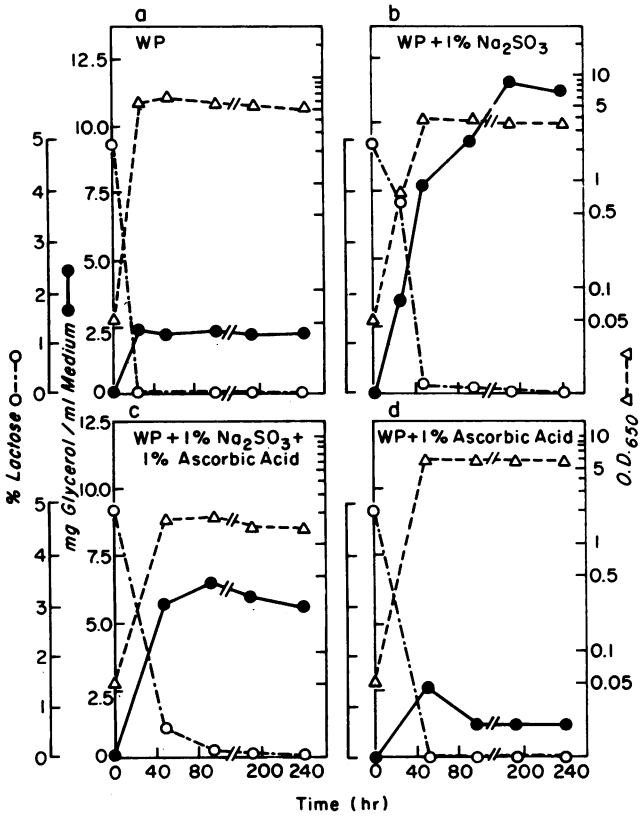

FIG. 4. Growth curves, lactose utilization, and glycerol yield of *K. fragilis* grown under anaerobic conditions from low inoculum in WP alone (a) and with 1.0% Na_2SO_3 (b), 1.0% Na_2SO_3 plus 1.0% ascorbic acid (c), and 1.0% ascorbic acid (d).

resulting in enhanced ethanol production has been reported (8).

Theoretically, 100 g of yeast biomass should be capable of producing 124 g of glycerol (5 g/liter) when grown in a glucose medium (25 to 30 liters) under anaerobic conditions, although with considerable energy utilization (17). Through the enzyme β-galactosidase, lactose is broken down into equimolar concentrations of glucose and galactose. However, both monosaccharides can be further metabolized in the formation of glycerol, as is the case with *K. fragilis*. Although this process can occur under aerobic conditions, it is far more efficient in anaerobic growth because many Crabtree-negative yeasts, including *K. fragilis*, form no or small amounts of ethanol and glycerol under aerobic conditions. The NADH generated during catabolism is reoxidized with oxygen (7). As a carbon source, 5% lactose can be considered as roughly equivalent to 5% glucose. If lactose, or glucose, is reduced to 0%, then the biological oxygen demand is likewise reduced to 0%. We have shown that when 1.0% Na_2SO_3 is added to whey permeate, and after glycerol fermentation, there is 0.23% glucose remaining (Table 1). Therefore, a small residual biological oxygen demand remains.

The production of glycerol from the fermentation of lactose by a yeast has not previously been reported in the literature. Although the use of sodium sulfite as a steering agent has been known since the time of the first report by Neuberg and Reinfurth (12), it has not previously been reported to be effective in enhancing glycerol production of *Kluyveromyces* sp., a nonhalophilic yeast. This study reports a time of 192 h for maximum glycerol production (Fig. 3). However, under nearly identical anaerobic growth con-

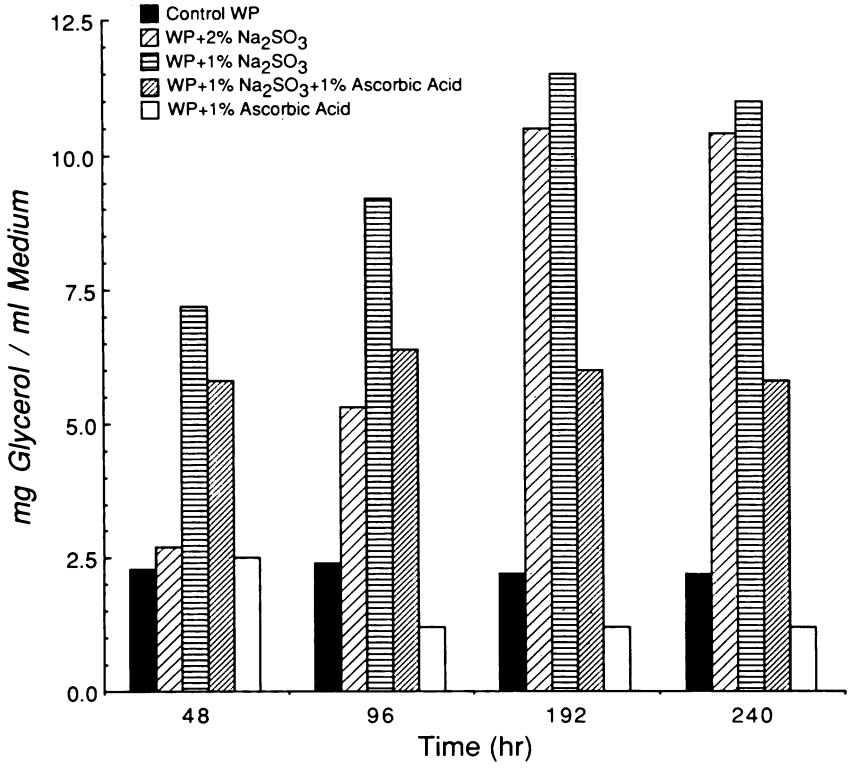

FIG. 5. Glycerol yield over 240 h of *K. fragilis* grown anaerobically from low inoculum in WP alone (control) and supplemented with 2% Na_2SO_3, 1.0% Na_2SO_3, 1.0% Na_2SO_3 plus 1.0% ascorbic acid, and 1.0% ascorbic acid.

TABLE 1. Residual sugars remaining in spent media after growth of *K. fragilis* under conditions indicated

Incubation time (h)	% Residual sugar remaining[a]							
	WP control		WP + 1% Na_2SO_3		WP + 1% Na_2SO_3 ascorbic acid		WP + 1% ascorbic acid	
	Glucose	Galactose	Glucose	Galactose	Glucose	Galactose	Glucose	Galactose
48	0	0	0.21	0.16	0.45	0.12	0.54	<0.01
96	0	0	0.24	0.17	0.46	0.01	0.41	<0.01
192	0	0	0.23	0	0.45	0	0.63	0
240	0	0	0.23	0	0.45	0		

[a] Galactose levels were always lower than the corresponding glucose levels, presumably because of the conversion of galactose to glucose.

ditions but with glucose as the carbon source, Bisping and Rehm (3) reported maximum glycerol production by *S. cerevisiae* within 96 h at 30°C, but in a 10% glucose medium supplemented with Na_2SO_3. The longer time for *K. fragilis* is partially due to the different concentrations of carbon source, the need for lactose to be broken down into simple sugars, and the conversion of galactose to glucose.

This study has, for the first time, demonstrated the ability of *K. fragilis* strain CBS 397 to fully utilize lactose and, in the presence of 1.0% Na_2SO_3, to generate significant yields of glycerol under laboratory conditions. The results suggest that pilot studies or investigations using continuous culture should be conducted to ascertain the commercial practicability of the process.

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