ABSTRACT

Two strains of Kluyveromyces fragilis (145 and 276) and one of Kluyveromyces lactis were tested for their abilities to produce beta-D-galactosidase in cheese whey. Kluyveromyces fragilis 145 was selected for its higher beta-D-galactosidase activity per cell at the end of the exponential growth phase.

Addition of ammonium sulfate (.3%) and yeast extract (.1%) to the deproteinized cheese whey increased cell mass and enzyme yield.

Addition of 3% lactose did not affect beta-D-galactosidase activity per cell, which responded positively to a reduction in aeration from 1 to .25 air volume/medium volume/min.

The harvested yeast cells were ethanol- and acetone-permeabilized to enhance lactose hydrolysis by beta-D-galactosidase.

INTRODUCTION

Cheesey whey constitutes a major disposal problem in the dairy industry of Minas Gerais, Brazil. Its lactose content reaches 4.8%, and it includes relatively high levels of other nutrients (8, 22, 33) that make it suitable as a microbial culture medium (3, 7, 31). Microorganisms capable of using lactose as the sole carbon and energy source are producers of beta-D-galactosidase, an enzyme that breaks down lactose to glucose and galactose (1, 2, 4, 6, 11, 14, 18).

Lactose is the major component of nonfat milk solids. In products such as ice cream, condensed milk, "dulce de leche," etc., this sugar may crystalize during long storage, resulting in a defect known as "sandiness," that makes the product nonmarketable (5, 13, 20). Furthermore, the presence of lactose in some dairy products discourages consumption of milk and some milk products by lactose-intolerant adults (12, 19).

There is widespread interest, both from nutritionists and commercial producers, in reducing the lactose content of some dairy products. One of the most promising methods utilizes hydrolysis of lactose to glucose and galactose by beta-D-galactosidase.

Although beta-D-galactosidase is found in microorganisms, plants, and animals, only the enzyme of microbial origin is of industrial interest (19). Beta-D-galactosidase from different microorganisms exhibits different properties (10, 15, 16, 23, 25, 26, 29). In screening for microorganisms as a source of beta-D-galactosidase for lactose hydrolysis, some properties of this isoenzyme must be considered, particularly the optimum pH for hydrolytic activity.

According to Van Dam et al. (30) and Wendoff and Amundson (32), beta-D-galactosidase produced by the yeast Kluyveromyces fragilis is most active at pH 6.0 to 8.0. Thus, beta-D-galactosidase produced by this yeast should be able to hydrolyze lactose in milk, which has a pH of 6.6 (30).

Our study was to establish conditions for using cheese whey as a medium for the production of beta-D-galactosidase. A yeast strain was selected with high enzyme activity per cell throughout the exponential growth phase. Use of permeabilized yeast cells were also investigated.
MATERIALS AND METHODS

Reagents and Organisms

Two yeast strains of Kluyveromyces fragilis (145 and 276) and one of K. lactis were obtained from the Food Science Department, University of California, Davis. Cultures were refrigerated (4°C) in a medium described by Wickerham (1951) and cited by Sheetz and Dickson (27). Acid cheese whey was obtained from the Dairy Plant of the Food Technology Department, Universidade Federal de Viçosa, Viçosa, Minas Gerais, Brazil, and stored at -18°C until used. Analytical grade ortho-nitrophenyl-beta-D-galactopiranoside (ONPG) was obtained from Sigma Chemical Co., St. Louis, MO.

Selection of the Microorganism

The number of yeast cells was determined by the microdrop method (9, 24). The same method was used for growth in deproteinized cheese whey. Hydrolysis of ONPG was used to measure beta-D-galactosidase activity. Each strain of Kluyveromyces was incubated in 60 ml of medium that has been previously sterilized at 121°C for 10 min in 250-ml Erlenmeyer flasks, and maintained at 28°C with continuous agitation in a water bath. For the selection of K. fragilis strains, incubation was at 35°C.

Selection of the Culture Medium

Kluyveromyces fragilis 145 culture was inoculated in cheese whey and deproteinized cheese whey treated with activated carbon (DWTAC) supplemented with ammonium sulfate (.3%), yeast extract (.1%), and lactose (3%). Whey was deproteinized by pH adjustment to 4.6 with lactic acid, then heated to about 97°C. After cooling, the precipitated proteins were removed by filtration through cheese cloth. Ten milliliters of each culture media in 13 × 18-mm test tubes were sterilized at 121°C for 10 min, cooled before inoculation with K. fragilis 145, and then incubated at 35°C with gentle agitation in a water bath. Determination of beta-D-galactosidase activity and number of yeast cells (by microdrop) was after a 10 h incubation. Deproteinized cheese whey was supplemented with 2% activated charcoal, agitated, and centrifuged at 2800 × g for 10 min. Charcoal was removed by filtration of the treated whey through qualitative filter paper.

Fermentor Assays

A 15-liter fermentor (New Brunswick Scientific Co., New Brunswick, NJ) with agitation, aeration, and temperature controls was utilized. To each 4.4 liters of DWTAC medium, yeast extract (.1%) and ammonium sulfate (.3%) were added. Cheese whey was sterilized at 121°C for 50 min.

Determination of the cell number was in a Neubauer chamber. Beta-D-galactosidase activity, pH, and lactose content were monitored at 2-h intervals during fermentation.

Lactose at 4.2 and 7.2% was tested under constant agitation (700 rpm) and aeration (1 VVM = air volume/medium volume/minute).

The effect of two levels of aeration (.25 and 1 VVM) was assessed in cheese whey without supplementary lactose, with agitation (700 rpm), and during incubation at 35°C.

Cell Permeabilization

Kluyveromyces fragilis 145 cells were grown for 8 h in a shaker G 25 (New Brunswick Scientific Co., New Brunswick, NJ) at 35°C and 150 rpm agitation and were harvested by centrifugation at 2800 × g for 5 min. Cells then were permeabilized either with ethanol or acetone by the method of Van Dam et al. (30). Hydrolysis of ONPG, as a measure of beta-D-galactosidase activity, was determined on a cell suspension in distilled water containing 10 mg of yeast cells/mg.

Analyses

Lactose was determined according to the method described by Nickerson et al. (21). Beta-D-galactosidase activity was determined on a .5-ml sample with 3.5 ml of buffer solution (17) and .1 ml of toluene in 13 × 180-mm test tubes. After incubation at 35°C for 40 min with sporadic agitation, .5 ml of 2.5 mM ONPG was added, followed by further incubation at 35°C. The reaction was stopped after 15 min by adding 1.0 ml 1 M sodium carbonate. Test tube contents were centrifuged at 3000 × g for 5 min, followed by determination of ortho-nitrophenol (ONP) absorbance at 420 nm in a spectrophotometer (Bausch and Lomb Spectronic 20).
RESULTS AND DISCUSSION

Growth curves for the three yeast strains in cheese whey are shown in Figure 1. *Kluyveromyces fragilis* 276, 145, and *K. lactis* produced specific growth rates (28) of .47 h⁻¹, .38 h⁻¹, and .33 h⁻¹.

Increased beta-D-galactosidase activity (Figure 2) was correlated with the fast-growing *K. fragilis* strains.

Beta-D-galactosidase isozymes produced by *K. fragilis* and *K. lactis* (4, 15) might imply...
higher hydrolytic efficiency of *K. fragilis*. Beta-D-galactosidase activity per cell was higher at the beginning of the exponential growth phase in *K. fragilis* 276 and decreased with increased cell number (Figure 3). However, beta-D-galactosidase activity per *K. fragilis* 145 cell increased up to the first half of the exponential phase and from then on decreased.

Despite the higher specific growth rate shown by *K. fragilis* 276 and the higher beta-D-galactosidase activity per milliliter of culture, the high enzymatic activity at the beginning of growth decreased in all experiments with increase in cell number. As for the beta-D-galactosidase activity per cell at the end of the exponential phase, *K. fragilis* 145 revealed the best performance among the strains tested.

Specific growth rates in deproteinized cheese whey of *K. fragilis* 276 and *K. fragilis* 145 were .73 and .58 h⁻¹ (Figure 4). Beta-D-galactosidase activity per milliliter of medium was similar to that obtained in cheese whey medium (Figure 5).

Wasserman (31) observed that the assimilable nitrogen used by *K. fragilis* was available only from noncoagulable nitrogen fractions and that the proteins precipitated by heat and acid were not broken down by the actively growing yeast.

*Kluyveromyces fragilis* 145 grown in deproteinized whey shows either a large number of enzyme molecules per cell at the end of the exponential growth phase or high enzyme activity (Figure 6). Difficulties arise in producing permeabilized yeast cells in cheese whey due to protein precipitation with yeast cells.
These precipitated proteins interfere with the dispersion of permeabilized cells and slow lactose hydrolysis.

Beta-D-galactosidase activity per \( K. \) fragilis 145 cell in deproteinized cheese whey corresponded to 4.27 \( \mu \)mol ONP/10\(^{-11}\) cell/min. In the same medium supplemented with nutrients, this activity was about 4.25 \( \mu \)mol (Figure 7). These data agree with results of Wasserman (31).

\( K. \) fragilis 145 growing at 35°C in a fermentor adjusted 700 rpm agitation and 1 VVM aeration performed (Figure 8). The stationary phase reached its maximum after 8 h incubation when lactose content decreased to 41.33% of its original concentration. Beta-D-galactosidase activity per cell was lowest at the end of the incubation period. Larger cell production, however, makes this last stage the recommended one for cell harvesting.

\( K. \) fragilis 145, when growing in the previous medium, supplemented with 3% lactose and under the same conditions (i.e., 1 VVM, 700 rpm, and 35°C) reached the stationary phase after 8 h incubation (Figure 9). With other conditions constant and aeration reduced to .25 VVM, beta-D-galactosidase activity per cell of \( K. \) fragilis was essentially the same (Figure 10). The report of Mahoney et al. (14) on \( K. \) fragilis UCD 5561 showed that a reduction in aeration resulted in elevated beta-D-galactosidase activity per milligram of yeast. The stationary phase was reached after 8-h incubation when lactose content was reduced to 31.2% of its original value.

\( K. \) fragilis ethanol-permeabilized cells showed activity corresponding to 15 nmol ONP/ml/min, which is similar to that obtained with acetone permeabilization. Milk lactose hydrolysis studies using \( K. \) fragilis permeabilized cells are in progress.

Figure 8. Growth of \( K. \) fragilis 145 on deproteinized cheese whey treated with activated carbon plus supplementary nutrients in fermentor at 35°C, 1 air volume/medium volume/min, and 700 rpm. ONP = Ortho-nitrophenol.

Figure 9. Growth of \( K. \) fragilis 145 on deproteinized cheese whey treated with activated carbon (3% lactose additional) with supplementary nutrients in fermentor at 35°C, 1 air volume/medium volume/min, and 700 rpm. ONP = Ortho-nitrophenol.

Figure 10. Growth of \( K. \) fragilis 145 on deproteinized cheese whey treated with activated carbon with supplementary nutrients in fermentor at 35°C, .25 air volume/medium volume/min, and 700 rpm. ONP = Ortho-nitrophenol.
REFERENCES


BETA-GALACTOSIDASE