Lipid-Enhanced Ethanol Production by *Kluyveromyces fragilis*

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The fermentation ability of a strain of *Kluyveromyces fragilis*, already selected for rapid lactose-fermenting capability, was improved dramatically by the addition of unsaturated fatty acids and ergosterol to the medium. The fermentation time of a 20% whey-lactose medium was decreased from over 90 h to less than 60 h. The lipids were shown to be taken up by the organism, and the effects on specific growth rate and biomass production were determined.

In 1981, $41.8 \times 10^9$ lbs (ca. $19 \times 10^3$ kg) of fluid whey were generated in the United States of America. Approximately 50% of this whey was utilized within the dairy industry, generally as a substitute in products in which nonfat dry milk is utilized. The other half was discarded, causing a loss of a potential energy source and creating a serious environmental problem (S. W. Baker, 11th Annual Meeting, Whey Products Institute, Chicago, Ill., 21–23 April 1982). As a result, the dairy industry has utilized components of the whey such as the nutritious protein (12) and the lactose portion as a fermentation substrate for several products: single cell protein, vinegar, wine, and ethanol production, mainly (2, 18; G. Palmer, Fermented alcohol beverage from whey, Proceedings, Whey Products Conference, Minneapolis, Minn. 9–10 October 1978).

Ethanol production is a particularly attractive alternative. Unfortunately, very few yeasts are able to ferment lactose, the major carbohydrate of whey (4). Since whey contains only 4.5% fermentable carbohydrates, it will yield only about 2.5% ethanol. To distill ethanol from a solution containing such a low concentration is expensive. This has caused some workers to attempt to concentrate the whey before fermentation (5). From whey permeate concentrates containing 24% lactose, Kosikowski and Wzorek (15) obtained up to 12% (vol/vol) ethanol, but only after 2 weeks of fermentation. Izaguirre and Castillo (11) obtained similar results with different yeast strains, decreasing the fermentation time to 8 days. The best results were obtained by Moulin et al. (17), who produced 11% (vol/vol) ethanol from 20% lactose in 90 h. These protracted fermentation times are mainly attributed to end-product inhibition; that is to say, the ethanol adversely affects cellular physiology, resulting in reduced sugar consumption rates and an overall decrease in metabolic rate.

Recent advances in the study of ethanol tolerance by yeasts of the genus *Saccharomyces* indicate that the cellular membrane composition is an important aspect of end-product tolerance (7, 8, 22). These studies have shown that the incorporation of unsaturated fatty acids, sterols, or both into the cellular membrane helps to alleviate ethanol inhibition. In addition, a 58.4% increase in fermenter productivity has been reported for *Saccharomyces cerevisiae* by the addition of a protein-phospholipid complex (13). The addition of these components to the membrane results in an overall increase in membrane fluidity (10), which theoretically overcomes the decrease in membrane fluidity attributed to ethyl alcohol.

In this study, we evaluated the effect of sterol and unsaturated fatty acid additions on the production of ethanol from whey lactose by *Kluyveromyces fragilis*.

**MATERIALS AND METHODS**

*Organism.* *K. fragilis* CBS 397 was obtained from Centraalbureau Voor Schimmelcultures (Baarn, the Netherlands) and maintained on YPL slants (1% yeast extract, 2% peptone, 2% lactose) at 4°C. Active cultures for inoculation were prepared by growing the organism in YPL broth twice for 24 h with agitation at 30°C. Other strains tested in the initial screening were: NRRL 2415, NRRL 665, NRRL 610, NRRL 1109, NRRL 2415, NRRL 1140, and NRRL 1114.

*Substrate.* Spray-dried, deproteinized whey powder was obtained from the Mississippi Valley Milk Producers (Davenport, Iowa) and used in suitable concentrations. The percentage of substrate concentration in the text refers to the lactose content. The reconstituted whey medium was supplemented with 0.5% Bactopeptone (Difco Laboratories) and buffered to pH 5.8 with 0.05 M McIlvaine buffer (citric acid-Na3HPO4). All substrates were sterilized at 120°C for 20 min.

The stock solution of lipids and sterols was prepared by dissolving 0.6 g of ergosterol in ethanol and then

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adding 100 ml of Tween 80 and 1 ml of linoleic acid. This mixture was flushed with nitrogen and used at the rate of 5 ml/liter.

**Fermentations.** The rate of alcohol production was determined in reconstituted whey of various lactose concentrations, supplemented with 0.5% of Bactopeptone. Inoculation was at an initial cell concentration of \(30 \times 10^7\) to \(60 \times 10^9\) cells per ml (10\%, vol/vol).

The fermentations were carried out in 1-liter Bellco stirbar flasks fitted with water traps to allow escape of carbon dioxide formed during fermentation while maintaining anaerobic conditions. All fermentations were performed isothermally at 30°C.

**Analytical methods.** (i) **Determination of lactose concentration.** Lactose concentration was determined with a model 27 industrial sugar analyzer (Yellow Springs Instrument Co., Yellow Springs, Ohio).

(ii) **Yeast cell counts.** Enumeration of cell populations during growth and fermentation was carried out with a Petroff-Hausser counting chamber.

(iii) **Biomass concentration and maximum growth rate.** Biomass concentration \((X_m)\) was followed by measuring the optical density at 590 nm and was expressed in grams (dry weight) of cells per liter by referring to a calibration curve showing a linear correlation between optical density and cell weight. One optical density unit corresponded to approximately 0.25 g of cells per liter. The maximum value of the specific growth rate \((\mu_m)\) was determined by the exponential growth law (slope of ln x against time).

(iv) **Ethanol analysis.** Ethanol was measured by gas chromatography, using a Hewlett-Packard 5880A gas chromatograph with automatic sampler 7671A. A column (1.8 m by 3.2 mm) packed with Supelco Porapak Q was used with a flame ionization detector. The injector and detector temperatures were 225 and 250°C, respectively, and the column oven operated isothermally at 175°C.

(v) **Fatty acid and sterol analyses.** Freeze-dried yeast samples were weighed and placed in acid-washed glass centrifuge tubes. Each sample was extracted by the method of Taylor and Parks (206).

Fatty acids were analyzed as their methyl ester derivatives. A known portion of each extract sample was blown to dryness under a stream of nitrogen and then placed in a vacuum over CaSO₄ desiccant for a few minutes. Methyl esters were formed from the dry total lipids by refluxing for 1 or 2 h with anhydrous methanol containing 2.5% HCl by weight. The esters and non-saponifiable lipids were then extracted with petroleum ether (boiling point, 20 to 40°C). The solvent was removed under a stream of nitrogen, and the methylated samples were dissolved in a known amount of chloroform containing octanol as an internal standard. Quantitative gas chromatography was performed utilizing a model HP5840 gas chromatograph (Hewlett Packard) containing a 6-foot (1.8-m) column with a 10% SP2330 liquid phase supported on Chromasorb WAW. The oven temperature was programmed to go from 130 to 225°C at a rate of 4°C per min with a nitrogen carrier gas flow of 30 ml min⁻¹.

The modified Lieberman-Burchard assay (20) for sterols was performed on a portion of each hydrolyzed lipid sample. One milliliter of cold Lieberman-Burchard reagent (acetic anhydride-sulfuric acid, 19:1) was added to a disposable sample tube containing a diluted 3-ml lipid sample in chloroform. Readings of optical density at 680 nm were recorded after 10 and 15 min at room temperature. Sterol concentrations were calculated from a curve prepared with ergosterol standards run concurrently with each assay. The presence of ergosterol was confirmed by UV spectroscopy of the non-saponifiable lipids giving characteristic absorption maxima at 262, 271, 282, and 293 nm.

**Chemicals.** Ergosterol, Tween 80, and fatty acids used to supplement the fermentations were all purchased from the Sigma Chemical Co. Fatty acid methyl ester standards were purchased from Supelco.

**RESULTS**

**Effect of lipid supplementation on fermentation kinetics.** A preliminary screening of several strains of *K. fragilis* or *Kluyveromyces lactis* from different culture collections indicated that CBS 397, a strain of *K. fragilis*, was superior in both rate of ethanol production and the maximum final ethanol concentration obtained. The data for this yeast strain are summarized in Table 1, in which the average time for total lactose consumption, maximum final ethanol concentration, and fermentation efficiencies are listed as a function of the initial lactose concentration. These data show a proportionate increase in both fermentation time and ethanol production in the range of 5 to 15% initial lactose concentration, with only a slight, but significant, decrease in fermentation efficiency. However, the data for 20% lactose fermentations show a disproportionate increase in fermentation time and a relatively small incremental increase in ethanol production (1.6%), owing to the inability of the organisms to completely ferment all of the lactose. The decrease in fermentation efficiency to 84% is relatively minor, considering the drastic increase in fermentation time. None of the other strains tested (data not shown) were capable of completely fermenting even a 15% lactose-containing medium.

The drastic increase in fermentation time when initial lactose concentrations over 15% were fermented indicated that the problem was due to either alcohol intolerance or osmotic sensitivity. We therefore tried various lipid sup-

<table>
<thead>
<tr>
<th>Initial lactose concn (%, wt/vol)</th>
<th>Fermentation time (h)</th>
<th>Maximum ethanol concn (%, vol/vol)</th>
<th>Fermentation efficiencya</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>15</td>
<td>3.2</td>
<td>92</td>
</tr>
<tr>
<td>10</td>
<td>28</td>
<td>6.0</td>
<td>91</td>
</tr>
<tr>
<td>15</td>
<td>36</td>
<td>9.0</td>
<td>87</td>
</tr>
<tr>
<td>20</td>
<td>&gt;90</td>
<td>10.6</td>
<td>84</td>
</tr>
</tbody>
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a Time required for consumption of all of the lactose utilized with a 10% (vol/vol) inoculum.

b (Actual alcohol yield/theoretical yield) × 100, based on lactose consumed.
plemations to the medium as a remedial measure. The various lipid mixtures tried were the following: phosphatidylcholine-albumin, 5:10 g/liter; phosphatidylcholine-albumin-ergosterol, 5:10:0.03 g/liter; ergosterol-Tween 80 (Tween 80 was prepared as by Hayashida et al. [6] and was assumed to contain 0.6% oleic acid [19]), 0.03:5 g/liter; Tween 80, 5 g/liter; Tween 80-linoleic acid, 5:0.05 g/liter; Tween 80-ergosterol-linoleic acid, 5:0.03:0.045 g/liter. As long as ergosterol (other sterols were not tried) and an unsaturated fatty acid were present in the fermentation medium, a marked improvement was seen. Figure 1 presents the kinetics of ethanol production in a 20% whey-lactose medium with or without lipid supplementation. The lipid mixture for this experiment, ergosterol and linoleic acid in Tween 80, resulted in a decrease in fermentation time to under 60 h and an increase in ethanol production to 10.9%. It should also be pointed out that in a similar experiment, except with only 10% initial lactose concentration, no differences in the kinetics of ethanol production were seen whether or not lipid supplementation was used.

**Fatty acid and sterol composition of K. fragilis.**

As a result of the above observations, qualitative and quantitative fatty acid profiles were made from K. fragilis cultures harvested after aerobic growth, anaerobic growth, and lipid-supplemented anaerobic growth. The anaerobically grown cells were routinely poisoned with both chloramphenicol (20 mg/liter) and cycloheximide (20 mg/liter) before harvesting. To prevent contamination of the pellets by the unsaturated fatty acids and sterols added to the medium, the cells were washed four times before analysis. These data are listed in Tables 2, 3, and 4. As expected, based on the molecular oxygen requirements for squalene cyclization and fatty acid desaturation (3, 21), aerobically grown cells contained predominately unsaturated fatty acids (83.4%), primarily linoleic and oleic acids, whereas the anaerobically grown cells contained negligible levels of the unsaturated fatty acids. The decrease in the quantitative fatty acid composition expressed as a percentage of cellular biomass was equally dramatic (Table 3). Fatty acids constituted 7.9% of the dry weight of aerobically grown cells and only 0.4% of that of anaerobically cultured cells. In lipid-supplemented cultures containing exogenous linoleic and oleic acids and ergosterol, fatty acids constituted 1.7% of the dry weight. Linoleic and oleic acid represented 38% and 14%, respectively, a very significant increase over the anaerobic unsupplemented cultures. It is implied that K. fragilis under anaerobic conditions does not synthesize more unsaturated fatty acid in response to unsaturated lipid supplements, but does incorporate a portion of the supplement.

The sterol content of anaerobically grown cells was also much reduced over the levels found in aerobically grown cells, as expected. The major sterol in both cases was found to be ergosterol by its characteristic UV absorption maxima (data not shown). However, when the medium was supplemented with ergosterol, the sterol content of the cells increased from 0.06% (micrograms of sterol per milligram of dry weight) to 0.16%, which represents an increase from 15 to 40% of the sterol content of aerobically grown cells. These data are summarized in Table 4.

**Effect of lipid supplementation on the maximum growth rate (μm) and the final biomass concentration (Xₘ).** It has been shown previously (1) that ethanol is inhibitory to cell growth and fermentation and causes reduced cell viability. To ascertain the effect of lipid supplementation on growth parameters, we measured the variation of the specific growth rate and the maximal biomass production during anaerobic growth as a function of the concentration of ethanol added to the medium. These experiments were per-

![Graph](image-url)

**FIG. 1.** Kinetics of ethanol production in 20% lactose without (■) or with (○) lipid supplementation.

<table>
<thead>
<tr>
<th>Culture</th>
<th>Fatty acid (%)</th>
<th>Total (%)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>14:0</td>
<td>16:0</td>
</tr>
<tr>
<td>Aerobic</td>
<td>14.2</td>
<td>8.4</td>
</tr>
<tr>
<td>Anaerobic</td>
<td>58.9</td>
<td>37.9</td>
</tr>
<tr>
<td>Supplementedb</td>
<td>1.4</td>
<td>28.2</td>
</tr>
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*All calculations were based on averages with inherent rounding errors.*

*Supplemented anaerobic cultures contained exogenous linoleic acid and oleic acids and ergosterol.*

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**TABLE 2.** Relative percent composition for fatty acids detected in total lipid extracts of K. fragilis.
formed in nitrogen-sparged Bellco anaerobe tubes (9) containing 2% lactose. The lipid-supplemented cultures sustained a higher specific growth rate until 6% ethanol was included in the medium, and likewise allowed accumulation of more cellular biomass. The results were most dramatic when the medium contained 2% ethanol; there was a 25% increase in μm and an approximate doubling in biomass produced (Xm) (Table 5). At an external ethanol concentration of 6%, lipid supplementation had no detectable effect.

DISCUSSION

It has been well established by several workers (7, 13) that the addition of lipids to S. cerevisiae fermentation medium results in higher concentrations of ethanol in a relatively shorter time period. In this study, a strain of K. fragilis with a high fermentative activity towards lactose has been selected and likewise shown to have enhanced fermentation rates by the addition of a mixture of oleic and linoleic acids and ergosterol to the fermentation broth. The fermentation time for a 20% lactose solution was reduced to 60 h instead of 90 h or longer. It should be pointed out that the lipid mixture shown in the experiments reported here (ergosterol and linoleic acid in Tween 80) worked no better than the phosphatidylcholine-albumin mixture used by Hayashida et al. (6), but it is cheaper and substantially easier to prepare and use.

There were less total lipids in the anaerobically grown cells compared with those grown aerobically (14), presumably because of less extensive intracellular membrane development (16). The exogeneously provided lipids and sterols are incorporated in the cell membrane. Supplemented cells contain less total lipids than aerobically grown cells and incorporate only a small amount of the sterol provided. The extent of enrichment with the exogeneously supplied fatty acids has to be examined. Our study enforces the hypothesis of interaction between biomembranes and ethanol; the decrease in fluidity caused by the presence of ethanol may inhibit the action of the protein involved in the transport of the lactose into cells, but this inhibition is compensated in cells enriched in unsaturated fatty acids by the greater mobility of these acids (23). The change in fatty acid composition of membrane lipids does not cause any apparent change in the morphology of the organisms. The lipid supplementation has, for low ethanol concentrations, a protective effect on the growth of the strain, allowing faster growth rates and more biomass production. Further studies on the continuous fermentation process with cell recycling employing the lipid-sterol mixture are under investigation.

<table>
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<th>TABLE 3. Dry weight percentages for total fatty acids detected in K. fragilis</th>
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<tbody>
<tr>
<td>Culture</td>
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<tr>
<td>---------</td>
</tr>
<tr>
<td>Aerobic</td>
</tr>
<tr>
<td>Anaerobic</td>
</tr>
<tr>
<td>Supplemented*</td>
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</table>

* Anaerobic supplemented cultures contained exogenous linoleic acid and oleic acids and ergosterol.

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<tr>
<th>TABLE 5. Influence of ethanol concentration on the maximum growth rate (μm) and maximum biomass concentration (Xm) in anaerobic culture</th>
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<tbody>
<tr>
<td>Ethanol concn (% vol/vol)</td>
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<tr>
<td>---------------------------</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>0</td>
</tr>
<tr>
<td>2</td>
</tr>
<tr>
<td>4</td>
</tr>
<tr>
<td>6</td>
</tr>
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LITERATURE CITED

8. Hayashida, S., and K. Ohta. 1980. Effects of phosphatidylcholine or ergosteryl olete on physiological properties of...