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# Production of propionic acid by mixed cultures of *Propionibacterium shermanii* and *Lactobacillus casei* in autoclave-sterilized whey

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## SUMMARY

Pure cultures of *Propionibacterium freudenreichii* ss. *shermanii* did not grow in autoclave-sterilized cheese whey (121°C, 15 psi, 20 min) at whey concentrations greater than 2% (w/v) spray-dried sweet dairy whey. Propionic acid was produced from autoclave-sterilized whey by growing *P. shermanii* in mixed culture with *Lactobacillus casei*. In medium containing 5–12% autoclaved whey solids and 1% yeast extract, the mixed culture produced 1.3–3.0% propionic acid, 0.5–1.0% acetic acid, and 0.05–0.80% lactic acid. All the lactose was consumed. Using pH-controlled fermentors (pH = 7.0), mixed cultures produced at least 30% more propionic acid than cultures in which pH was not controlled.

## INTRODUCTION

Cheese whey is a fluid medium containing about 6% solids, of which 12% is milk protein and 70% lactose. As it is highly nutritious and expensive to dispose of, it is advantageous to convert it to a usable product. A great deal of effort has gone into using whey as a substrate for microorganisms. For example, whey has been used as a substrate for producing single cell protein (SCP) [1,21], for the biosynthesis of vitamin $B_{12}$ [3,9] and, more recently, for the production of xanthan gum [16].

Lactobacilli and propionibacteria are often grown together. In fermented dairy products, such as Swiss cheese, the action of both microorganisms is necessary to obtain the desirable characteristics of the final product [5]. In silage fermentations, lactic acid bacteria are needed for rapid pH reduction, and propionic acid inhibits the growth of undesirable yeast [12]. In Sherman's early propionic acid fermentations, lactic acid bacteria were used as 'accelerator' organisms [17,18,22]. The lactic acid bacteria were used in 'minute amounts' and apparently increased the rate of the fermentation.

Several studies examining the 'synergistic' interaction of species of *Lactobacillus* and *Propionibac-*

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*terium* have been done [10,13,14]. *L. plantarum* and *P. shermanii* exhibit features typical of mutualism when growing together anaerobically in a glucose-minimal medium [8]. Initially, competition for glucose exists until *L. plantarum* produces a small quantity of lactic acid. As *P. shermanii* metabolizes lactic acid in preference to glucose, it will switch to using this substrate [7]. Propionic acid, acetic acid and $CO_2$ are produced as by-products. The benefit that this relationship offers to *L. casei* is the constant removal of lactic acid [11,13]. This mutualism is now shown to exist when lactose in whey is the substrate.

*P. shermanii* grows slowly compared with many other bacteria and prefers neutral pH and mesophilic temperatures. Hence, fermentations using non-sterile whey media are susceptible to contamination and unacceptable variability in product quality [2]. We previously demonstrated that propionic acid production decreased with increasing whey concentration in medium sterilized by autoclave, and that increasing the yeast extract concentration reduced the inhibitory effect of autoclaving [2]. As yeast extract is expensive, it is desirable to find other means of reducing the inhibition while fermenting high-solids whey medium. High solids are desired to maximize product concentration. In this paper we demonstrate that a mixed culture fermentation may be used to overcome the inhibition, allowing the use of high concentrations of autoclave-sterilized whey.

## MATERIALS AND METHODS

*Microorganisms. Lactobacillus casei* strain TA-101 was isolated from Kraft Swiss cheese. Twenty grams of Kraft natural Swiss cheese were homogenized in 180 ml cold sterile saline for 3 min in a Waring blender. The homogenate was plated on sodium lactate agar (10 g trypticase, 10 g yeast extract, 10 g sodium lactate, 0.25 g $K_2HPO_4$, 20 g agar, 1 l deionized water) at various dilutions, and incubated for 5 days, anaerobically, at 30°C. A clonal isolate was subsequently identified as *Lactobacillus casei* and its authenticity was confirmed by the American Type Culture Collection (Rockville, MD).

*Propionibacterium freudenreichii* ss. *shermanii* strain PS-109 was a single colony isolate obtained from a PS-1 Dry-Vac specialty culture (Chris Hansen's Laboratory, Milwaukee, WI) [2].

*Whey media preparation, shake flask, and fermentor operation.* The whey media contained 2–12% (w/v) Teklac and 1% (w/v) yeast extract. (Teklac is a spray-dried sweet dairy whey obtained from Foremost Food Co., San Francisco, CA.) The medium was adjusted to pH 7.0 with 50% NaOH before autoclaving. For shake flask experiments, 500 ml screw-capped flasks (Bellco, Vineland, NJ) containing 250 ml of whey medium and 2% (w/v) $CaCO_3$ were used. Sterilization was for 20 min at 121°C, under 15 psi steam. The shake flasks were incubated at 30°C, at 160 rpm on a NBS G-25 shaker (New Brunswick Scientific Co., Edison, NJ). For fermentors, 3 l whey medium in a 6-l Erlenmeyer flask, and a New Brunswick 7.5-l Magnaferm Fermentor containing 1 l water, were sterilized for 60 min at 121°C, under 15 psi steam. After sterilization, the water was pumped out of the fermentor and the sterile whey medium was added aseptically. Fermentors were operated at 30°C and 120–160 rpm; no gas was sparged. The pH was controlled at 7.0 with 25% NaOH using New Brunswick pH 40 controllers.

*Culture storage.* Storage and recovery of *P. shermanii* has been described [2]. *L. casei* was stored in MRS stabs (Lactobacilli MRS Broth (Difco) and 2% agar). Stabs were inoculated, incubated at 30°C for 48 h and stored at 4°C for up to 2 months. A stab was revived by overlaying it with 5 ml MRS broth and incubating for 48 h at 30°C.

*Preparation of inocula.* A shake flask containing 2% Teklac medium was inoculated with either 5 ml Hansen glucose broth (HGB) from one revived stab *(P. shermanii)* or 5 ml MRS from one revived stab *(L. casei)* and incubated for 48 h. These cultures were used to inoculate mixed cultures in shake flasks or fermentors at 2.5% (v/v) per culture. Flasks containing pure culture received a 5% (v/v) inoculum and were used as controls.

*Assays.* D- and L-lactic acids, and lactose were

Table 1

Mixed culture fermentation using *P. shermanii* PS-109 and *L. casei* TA-101 growing in shake flasks

Values are given as g/l after incubation for 48 or 72 h.

| Teklac[a] (%, w/v) | Lactose (g/l at $t_0$) | 48 h | | | | | 72 h | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | pH | lactose | lactic acid | HAc[b] | HPr[b] | pH | lactose | lactic acid | HAc[b] | HPr[b] |
| 5 | 38 | 5.30 | 4 | 2.2 | 3.8 | 10.3 | 5.84 | <0.5 | <0.5 | 5.3 | 12.5 |
| 6 | 44 | 5.26 | 17 | 2.8 | 3.7 | 9.1 | 5.60 | <0.5 | 4.4 | 5.2 | 16.0 |
| 7 | 47 | 5.26 | 22 | 3.2 | 3.9 | 9.5 | 5.44 | 2.7 | 7.8 | 5.3 | 15.5 |

[a] The media also contained 1% (w/v) yeast extract and 2% (w/v) $CaCO_3$. Initial pH 6.90. Inocula ratio 1:8 (v/v), strain TA-101 to PS-109.

[b] HAc, acetic acid; HPr, propionic acid.

estimated enzymatically using kits purchased from Boehringer-Mannheim (Indianapolis, IN). Propionic acid (HPr) and acetic acid (HAc) were assayed by gas chromatography [2].

## RESULTS

### *Shake flasks*

Pure cultures of *P. shermanii* in 2% Teklac grew to $1.0 \times 10^{10}$ CFU/ml, produced about 0.6% propionic acid and consumed all the lactose (approx. 14 g/l). At Teklac concentrations higher than 2%, no growth occurred, a negligible amount of propionic acid was produced, and lactose was not metabolized. Pure cultures of *L. casei* grew to about $1.0 \times 10^9$ CFU/ml and converted 80–85% of the lactose to lactic acid at all Teklac concentrations examined. For example, in 7% Teklac (approx. 49 g lactose/l), *L. casei* produced 40–42 g lactic acid/l and consumed all the lactose; i.e., about 85% was converted to lactic acid and about 15% was converted to cell mass, $CO_2$ and other products.

Typical data from mixed cultures in shake flasks at various Teklac concentrations are summarized in Table 1. In 72 h, at Teklac concentrations of 5, 6 or 7%, about 1.3–1.6% propionic acid and 0.5% acetic acid were produced and most of the lactose was consumed. Lactic acid concentrations remained low throughout the fermentation.

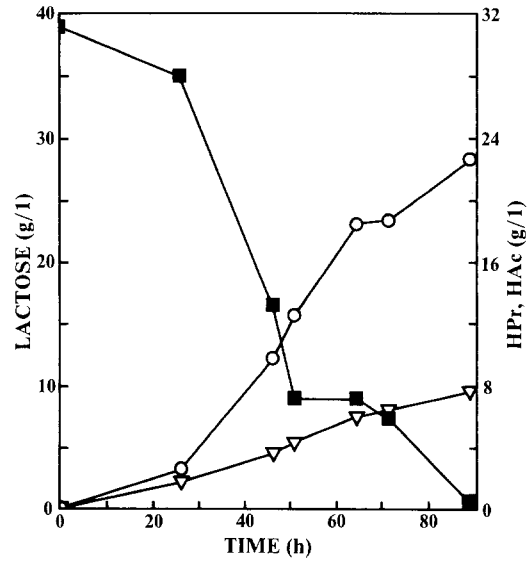


Fig. 1. The time course of lactose utilization and acid production during mixed culture fermentation using *L. casei* and *P. shermanii*. Media contained autoclaved 7% Teklac and 1% KAT yeast extract. NBS fermentors were operated at 30°C, 160 rpm and pH 6.90 ± 0.10. ■, lactose; ○, propionic acid; ▽, acetic acid.

### *Fermentors*

Results from experiments involving pH-controlled mixed-cultures in fermentors, using 7% and 12% autoclaved Teklac, are shown in Figs. 1 and 2, respectively. Within 88 h, in medium containing 7% Teklac, about 2.2% propionic acid and 0.8% acetic acid were produced. In medium containing

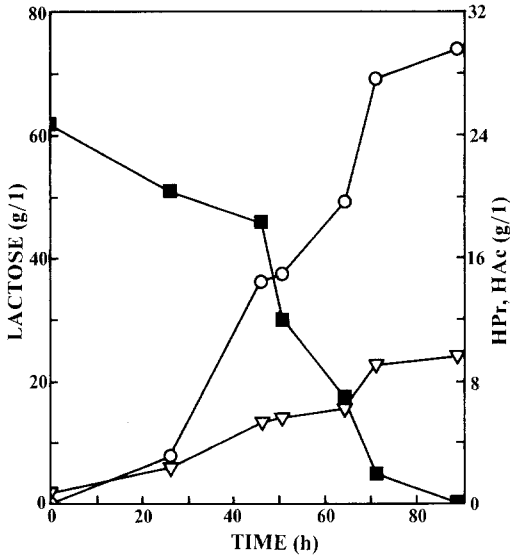


Fig. 2. The time course of lactose utilization and acid production during mixed culture fermentation using *L. casei* and *P. shermanii*. Media contained autoclaved 12% Teklac and 1% Difco yeast extract. NBS fermentors were operated at 30°C, 160 rpm and pH 6.90 ± 0.10. ■, lactose; ○, propionic acid; ▽, acetic acid.

12% Teklac, about 3.0% propionic and 1% acetic acid were produced. In each experiment all the lactose was consumed and lactic acid did not accumulate.

## DISCUSSION

The inhibition of *P. shermanii* at Teklac concentrations greater than 2% was probably due to the method of media sterilization, i.e. autoclaving [2]. Autoclaved Teklac medium was brown in color and obviously caramelized. As the Teklac concentration was increased, the extent of discoloration and darkening also increased. (Interestingly, in mixed culture, the media became an increasingly lighter color as the fermentation proceeded.) In whey, both milk protein (12%) and lactose (70%) are present. Autoclaving whey produces a reaction comparable to the browning reaction of milk. Heating causes free amino groups of the proteins to undergo a Maillard-type reaction with the aldehyde group of lactose. Nutritional value is lost due to the destruction of essential amino acids and vitamins, and loss of biological value and digestibility of protein. Heating may also produce toxic substances and metabolic inhibitors [4]. Another effect of autoclaving on whey is the rearrangement of lactose to lactulose. Many microorganisms cannot use lactulose and/or find it inhibitory [6,20].

Growth in the presence of *L. casei* diminished the inhibitory effects autoclaved whey had on *P. shermanii* and permitted propionic acid to be produced. The mechanisms involved were not determined, though several possibilities exist. For instance, lactic acid bacteria produce proteolytic enzymes [15,19]. Proteases produced by *L. casei* may modify the whey by breaking down the proteins, thus making available amino acids and/or other nutrients required by *P. shermanii*. Toxic and/or inhibitory substances produced during autoclaving may also be destroyed by the proteolytic enzymes. *L. casei* converts lactose to lactic acid. Preferential metabolism of lactic acid by *P. shermanii* compared to lactose may also contribute to the observed results [7].

Thus, mixed culture fermentation allows for the use of significantly higher concentrations of autoclave-sterilized whey, compared to pure culture fermentation, i.e. 12% whey solids vs. 2% whey solids. This results in the production of more propionic acid. Even more propionic acid is produced in pH-controlled fermentors, i.e. 22 g/l or 38% more at 7% whey solids, compared to shaken flasks in which pH was not controlled. At 12% whey solids 30 g/l is produced, representing a 5-fold increase compared to the amount produced by pure culture in shaken flasks in which pH was not controlled.

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