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Fed-batch and continuous fermentation of *Selenomonas ruminantium* for natural propionic, acetic and succinic acids

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An anaerobic fermentation process was developed for production of natural propionic, acetic and succinic acids from L-lactic acid using *Selenomonas ruminantium*. The L-lactic acid was quickly converted to a racemic mixture and there was no enantiomeric preference for further metabolism. The lactic acid was metabolized to propionic, acetic and succinic acids typically in a molar ratio of about 5 : 3 : 1. However, the ratio of propionate : succinate started high (as much as 22 : 1), before declining to as low as 5 : 1 after the first 48 h. Nutrients in corn steep liquor and yeast extract were necessary for optimal production of propionic acid. The corn steep liquor and yeast extract were heat stable at neutral pH, but some nutritional qualities were lost when heated at pH 2.4. In fed-batch fermentation on lactic acid 2.0% propionic acid was produced in 48 h and 2.3% in 68 h. A continuous culture operated at a dilution rate of 0.055 h^{-1} and a lactic acid feed concentration of 30 g L^{-1} had a propionic acid producitivity of 0.59 g L^{-1} h^{-1}. The steady state results were: lactic acid 0.6%, propionic acid 1.1%, acetic acid 0.50%, and succinic acid 0.33%.

Keywords: corn steep liquor; yeast extract; lactic acid; sodium sulfide; cysteine

Introduction

Natural flavors are currently recognized as materials being: a) plant or animal products or extracts; b) made by biological fermentation of natural starting materials; c) made by enzymatic alteration of natural starting materials; and/or d) made by gentle physical processes similar to those that might be used by the consumer in food preparation. Natural flavors are perceived to offer enhanced value to the consumer and command higher prices than artificial flavors. Kosher certification is important to many consumers, both for religious reasons and as a symbol of quality. Kosher certification is available from numerous rabbinical supervision services. Since dairy- and animal-derived ingredients cannot be mixed in kosher foods, it follows that flavors for kosher applications are similarly restricted. A flavor that can be certified as both kosher and natural has the most general application and thus offers the greatest price and market advantage.

Although propionic acid has some flavor uses, its various esters are much more important; these find use in such flavors as apple, banana, raspberry, rum and strawberry. *Propionibacterium shermanii* has long been known to produce propionic acid from dairy whey [10], and has been used commercially to make natural calcium propionate, but the fermentation is slow. Using pH control and a medium with 12% whey solids and 1% yeast extract, 1.9% propionic acid was obtained after 70 h [1]. A co-culture of *Lactobacillus casei* speeds the production of lactic acid and generally improves the yield. When the medium was changed to 18% whey solids and with a co-culture of *L. casei*, as much as 6.5% propionic acid was produced in 100 h of fermentation.

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Another whey-based fermentation process for propionic acid uses a bacterial co-culture of *Veillonella parvula* and *Lactobacillus casei* [6]. This co-culture process is somewhat faster, but yields only 2% propionic acid after 48 h. Elimination of whey would be necessary for a kosher parve product, but unfortunately these processes do not work well without whey. Also, maintaining the proper balance of microorganisms in these co-culture processes is often difficult.

We wished to find a rapid, high yielding single bacterial fermentation that would use glucose or lactic acid as the substrate, and not require whey. *Selenomonas ruminantium* is a nonpathogenic anaerobic bacterium that appeared to be a likely candidate. Carbohydrates are first degraded to lactic acid whereupon the metabolic pathway branches to produce a mixture of propionic, acetic and succinic acids. CO_2 is a requirement for growth on lactate (to make oxaloacetate); since it is also a product of the fermentation made in a 1 : 1 molar ratio with acetate, an external source of CO_2 is required only during early growth [5]. Unlike many other anaerobic fermentations, H_2 production is not a safety issue because only traces are produced [8].

This paper describes the application of *S. ruminantium* for commercial production of propionic, acetic and succinic acids [2]. The process uses an inexpensive production medium, does not require a co-culture and achieves a propionic acid concentration much higher than the relatively low values reported in the literature for *S. ruminantium* (about 0.2–0.3%) [3,11]. In addition, the process offers a rapid fermentation along with kosher and natural status.

Materials and methods

Cultures and monitoring of growth

S. ruminantium strain ATCC 27209, obtained from the American Type Culture Collection (Rockville, MD, USA),

was first adapted on 2% lactic acid (in Corn Steep Liquor Medium). Culture purity was checked by streaking on pre-reduced Brucella Blood Agar plates (Anaerobe Systems, San Jose, CA, USA) and incubating in a Difco (Detroit, MI, USA) anaerobe jar with a CO_2-H_2 generator pack and resazurin indicator. Serum vials (6.0 ml containing 5.0 ml medium) sealed with butyl rubber stoppers were used for small scale experiments; inoculation was 0.1 ml by syringe. Incubation was at 39° C under anaerobic conditions, and growth was monitored by increase in optical density at 600 nm. The entire 6-ml serum bottle was placed in a Spec 21 spectrophotometer (Milton Roy, Rochester, NY, USA) for a measurement of optical density; the method was accurate enough for determining growth differences.

Media

The Corn Steep Liquor (CSL) Medium contained 34.3 g L^{-1} 88% L-lactic acid (PURAC® USP Heat Stable, Purac America, Lincolnshire, IL, USA; 87.5–88.5% lactic acid with >95% of this acid in L form), 5.0 g corn steep liquor (50% solids, National Starch and Chemical Corp, Bridgewater, NJ, USA), 2.0 g yeast extract (Tastone® 154, Universal Foods Corp, Milwaukee, WI, USA), 0.25 g $(NH_4)_2SO_4$, 0.07 g $CaCl_2 \cdot 2H_2O$, 0.04 g $MgSO_4 \cdot 7H_2O$, 0.1 mg biotin (Pfizer Chemical Division, New York, NY, USA), 0.01 mg PABA (*p*-aminobenzoic acid, Sigma Chemical Co, St Louis, MO, USA), 1.0 ml 1% (aqueous) resazurin (redox indicator) and 11.0 ml trace minerals in 1.0 L water. The pH was adjusted to 7.0 with NaOH (11.6 g L^{-1}; medium $[Na^+]$ = 0.3 M), 4.0 g L^{-1} Na_2CO_3 were added, the solution was sparged with CO_2 until the pH reached 6.8 then 0.5 g L^{-1} of reducing agent (cysteine-HCl or $Na_2S \cdot 9H_2O$) was added. The trace minerals contained 5.5 g citric acid, 0.95 g $FeSO_4 \cdot H_2O$, 0.39 g $ZnSO_4 \cdot 7H_2O$, 0.09 g H_3BO_3, 0.08 g $MnSO_4 \cdot H_2O$, 0.05 g $CuSO_4 \cdot 5H_2O$, 0.025 g $Na_2MoO_4 \cdot 2H_2O$, 0.005 g $NaCrO_4 \cdot 4H_2O$, and 0.001 g $NaSeO_3$ in 1.0 L water.

For all fermenter experiments, growth was monitored by diluting a sample to the range of 0.0–0.5 A_{600} (linear range), then determining optical density at 600 nm. A microscopic count (Petroff Hausser Counting Chamber, Hausser Scientific, Horsham, PA, USA) indicated that there were 2.8×10^9 cells ml^{-1} per absorbance unit (standard deviation = 0.5×10^9). *S. ruminantium* 27209 is not pigmented nor are extracellular pigments produced during growth. Typically the cells were motile, curved-rods with a length of 5 μm, but about 5% were longer (5–25 μm).

Fermentation conditions

The experiments reported in Figures 1 and 2 were done using a 7-L fermenter (Chemap® model 7.14.20, B. Braun Biotech International, Melsungen, Germany) containing 4.0 L of medium. The experiment reported in Figure 3 used a 14-L fermenter (model MF-214, New Brunswick Scientific, Edison, NJ, USA) which was initially filled with 2.5 L of CSL Fermenter Medium. Starting at 12 h, 10 L of fresh medium were then fed over a period of 5 h, bringing the fermenter volume to 12.5 L. Autoclaved solutions of sodium lactate (pH 7) were added to the fermenter at 29 h (284 g 88% L-lactic) and at 56 h (60 g 88% L-lactic acid). The experiment reported in Figure 4 used the 14-L fermenter that was initially filled with 2.5 L of dilute filter-sterilized lactic acid (34.1 g L^{-1}, adjusted to pH 7.0 with NaOH, with 4.0 g L^{-1} $NaCO_3$ added). A heat-sterilized slurry concentrate (pH 7.1) containing 12.5 g CSL, 5.0 g yeast extract, 0.62 g $(NH_4)_2SO_4$, 0.18 g $CaCl_2 \cdot 2H_2O$, 0.10 g $MgSO_4 \cdot 7H_2O$, and 32.6 ml water was autoclaved for 30 min. Then the slurry, a filter-sterilized vitamin concentrate, and filter-sterilized Na_2S were added to the fermenter before inoculation with *S. ruminantium*. Twelve hours after inoculation, fresh medium was added over a 4-h period to bring the fermenter volume to 11.5 L. At 29 h 268.1 g 88% L-lactic acid (as a 44% filter-sterilized solution of sodium lactate) was added, and at 54 h a feed of 10.1 g h^{-1} of 38% sodium lactate was begun and continued for 27 h. The continuous culture experiment reported in Figure 5 used a 2.4-L fermenter (Model KLF2000, Bioengineering AG, Switzerland). One liter of medium was heat-sterilized, Na_2S

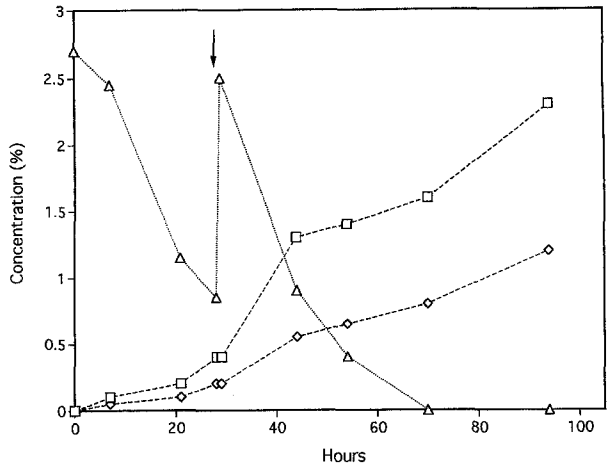

Figure 1 Batch fermentation with CSL and cysteine. A 7-L fermenter with 4.0 L of CSL medium with cysteine was heat-sterilized, and was then inoculated with *S. ruminantium*. At 29 h an additional 91.2 g lactic acid (as sodium lactate) was added (arrow). Concentrations of propionic acid (□), acetic acid (◇) and lactic acid (Δ) are shown

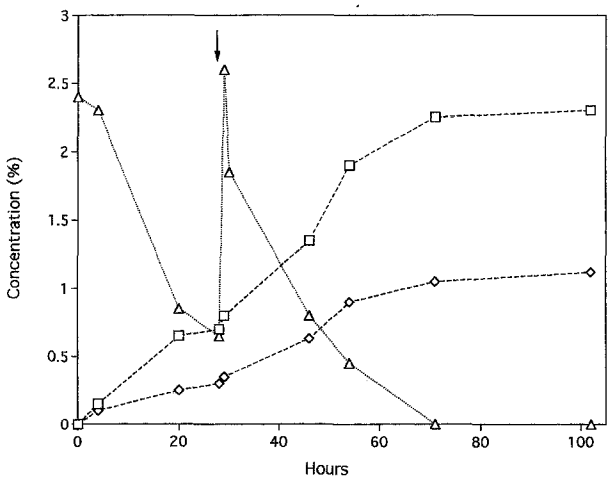

Figure 2 Batch fermentation with CSL and sodium sulfide. A fermenter with CSL medium with Na_2S was heat-sterilized in place, and inoculated with *S. ruminantium*. At 29 h an additional 91.2 g lactic acid (as sodium lactate) was added (arrow). Concentrations of propionic acid (□), acetic acid (◇) and lactic acid (Δ) are shown

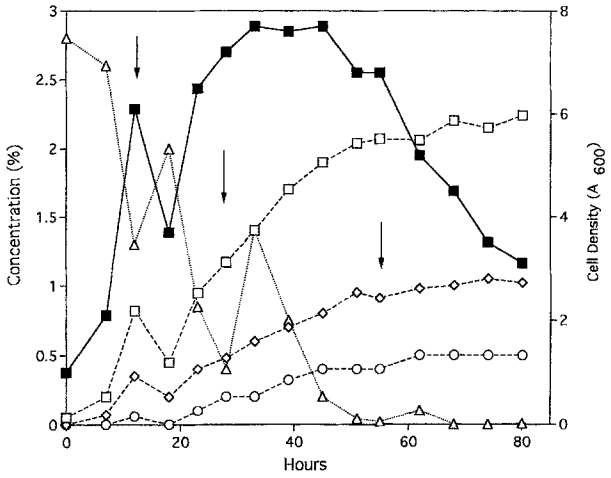

Figure 3 Fed-batch fermentation with heat-sterilized medium. A 14-L fermenter with 2.5 L of the CSL medium was heat-sterilized. Filter-sterilized vitamins and Na_2S were added, and the fermenter was inoculated with *S. ruminantium*. Addition of 10 L of fresh heat-sterilized medium was begun at 12 h at a rate of about 1.8 L h^{-1} (arrow). Arrows indicate when autoclaved solutions of sodium lactate (pH 7) were added to the fermenter. Propionic acid (□), acetic acid (◇), succinic (○), lactic acid (Δ), and cell density (■) are shown

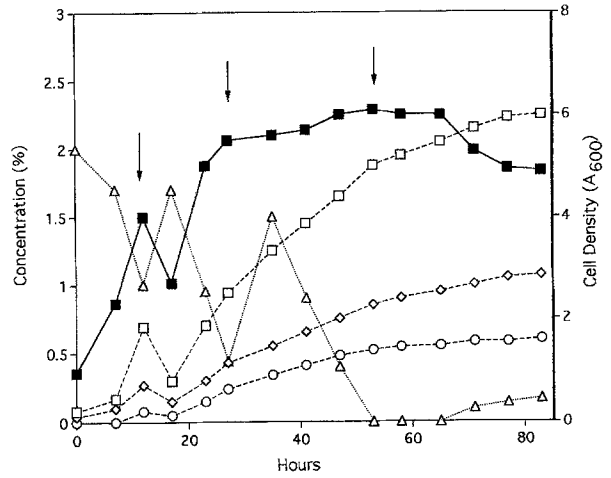

Figure 4 Fed-batch fermentation with filter-sterilized medium. A heat-sterilized slurry concentrate containing CSL, yeast extract and salts was added to a 14-L sterile fermenter. Then 2.5 L of sodium lactate, vitamin concentrate, and a Na_2S solution were filter-sterilized and added to the fermenter prior to inoculation with *S. ruminantium*. At 12 h, addition of 9 L of fresh medium was begun at a rate of about 2.25 L h^{-1}. At 29 h sodium lactate was added. At 54 h a feed of 10.1 g h^{-1} of sodium lactate was begun and continued for 27 h. Propionic acid (□), acetic acid (◇), succinic (○), lactic acid (Δ), and cell density (■) are shown

was added, and the fermenter was inoculated with *S. ruminantium*. After completion of batch growth, 400 ml was removed to allow a transition period of fed-batch mode. The medium was fed at 75 ml h^{-1} for 11 h. Then, the volume was reduced from 1.4 L to 1.0 L where it was controlled using feed and effluent peristaltic pumps (Masterflex®, Barnant Co, Barrington, IL, USA).

Heat sterilization (121° C) was a minimum of 20 min. Upon cool-down and throughout the fermentation, the medium was slowly sparged with CO_2 (15–45 cc L^{-1} min^{-1}). Fermenter parameters were 39° C, 100–

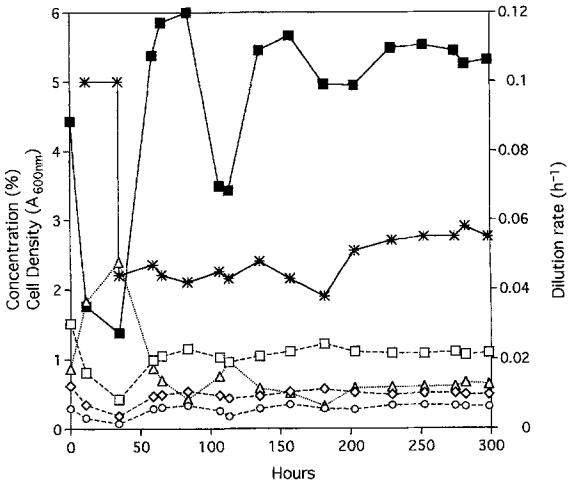

Figure 5 Continuous culture fermentation. After batch growth of *S. ruminantium*, medium feed was initiated at 0 h. Propionic acid (□), acetic acid (◇), succinic (○), lactic acid (Δ), optical density (■), and dilution rate (*) are shown

200 rpm and atmospheric pressure. Foam was controlled with 0.5 ml L^{-1} chemical antifoam (Mazu DF245-59A, PPG Specialty Chemicals, Gurnee, IL, USA). Lactic acid was always fed as sodium lactate, pH 7.0.

Determination of metabolites

Succinic and total lactic acids were determined by HPLC, propionic and acetic acids by GC, and L-lactic acid by the Model 27 Analyzer (Yellow Springs Instrument Co, Yellow Springs, OH, USA). The GC was a Varian 3300 (Palo Alto, CA, USA) equipped with flame ionization detector and a 1.2-m glass column (2 mm ID) packed with Chromosorb 102 80/100 mesh (Celite Corp, Lompoc, CA, USA). The HPLC was a Perkin-Elmer Series 3B (Cupertino, CA, USA) equipped with a refractive index detector and an Aminex HPX87H 300 mm × 7.8 mm column (Bio-Rad Laboratories, Hercules, CA, USA). The mobile phase was 0.012 N sulfuric acid (0.6 ml min^{-1}, 1500 psi, 65° C).

Heat treatment

In an experiment to determine the effect of heat treatment (Table 1) duplicate 100-ml serum bottles containing 60 ml medium were heated at 121° C in an autoclave. The experimental conditions were: A) heat 30 min, then cool to room temperature; B) heat 30 min, cool, heat again for 30 min, then cool; and C) adjust pH to 2.4, heat 30 min, cool, adjust to pH 7.0, heat again for 30 min, then cool. There were three additional conditions, D, E and F, that were identical to A, B and C respectively, except that filter-sterilized vitamins (0.6 ml of a 100-fold concentrate of biotin and PABA) were added after the heat treatments. After inoculation, growth was monitored by measuring optical density of a diluted sample at 600 nm.

Results

Corn steep liquor medium

Media described in the literature for *S. ruminantium* typically contain glucose, vitamins, minerals and yeast extract,

Table 1 Nutritional effects of heat treating medium components[a]

Condition	Δ Optical density[b]		% Propionate	
	19 h	43 h	19 h	43 h
A	3.3 ± 0.9	3.0 ± 0.4	0.84	1.23
B	3.7 ± 0.1	2.9 ± 0.1	1.09	1.25
C	1.0 ± 0.5	3.9 ± 0.7	0.29	1.26
D	3.2 ± 0.0	3.3 ± 0.1	0.80	1.00
E	3.4 ± 0.6	3.3 ± 0.0	0.70	1.29
F	0.6 ± 0.6	4.0 ± 0.1	0.32	1.29

A = Autoclave 30 min pH 7.0
B = Autoclave 30 min pH 7.0; cool; autoclave for an additional 30 min
C = Autoclave 30 min pH 2.4; cool; adjust pH, add cysteine and Na_2CO_3, sparge with CO_2; autoclave an additional 30 min (pH 7.0)
D = Condition A + filter sterilized PABA and biotin
E = Condition B + filter sterilized PABA and biotin
F = Condition C + filter sterilized PABA and biotin

[a]Corn steep liquor was from Corn Products
[b]Samples were taken from duplicate 100-ml serum bottles prior to measurement. Standard deviation is shown as ±

but result in low propionate yield. The Corn Steep Liquor Medium in this study consisted of corn steep liquor, lactic acid, yeast extract, mineral salts, and vitamins. CSL greatly stimulated growth and was always added to the medium. Three percent lactic acid was used, since higher concentrations inhibited growth. We chose not to use glucose in our fermentation medium because: a) lactic acid is a possible end product and might not always be fermented to propionic acid [9,11]; and b) unlike lactate medium, pH would have to be controlled. The cost difference between natural lactic acid and glucose is insignificant in this application.

Yeast extract could differ from supplier to supplier, but when several high quality yeast extracts from different suppliers were tested there was little difference in growth rate or final propionate concentration. CSL could vary even more than yeast extract. CSL samples were obtained from three different manufacturers and there were obvious differences in color and texture. Normally CSL is sold as a thick liquid (50% solids), but a spray-dried CSL was also obtained for comparison. The results showed that the growth with the spray-dried CSL at 24 and even at 32 h was not as good as with either of the liquid CSLs, but there were no differences in propionate concentration (Table 2). The pH did not change appreciably during the fermentation. Slow growth may have been caused by heat destruction of a nutrient during spray drying or simply by the poor quality of the CSL; liquid CSL was used in all further experiments. The effect of addition of trace salts was also tested, and for two of the three CSLs there was some stimulation of growth (Table 2). For this reason trace salts were used in all further experiments.

Heat sterilization of medium components

An experiment was designed to determine if excess heat sterilization (121° C) could result in loss of nutritional content. We were suspicious because growth was poor when spray dried corn steep liquor was used in the medium. Growth and propionate production were compared after 30- and 60-min heat treatments. Heat treatment at pH 2.4 was tested because stability of vitamins is sometimes better under acidic conditions. Filter-sterilized vitamins were also tested. The results are shown in Table 1. The 60-min autoclave time did not slow growth over that of the control. However, heating at acidic pH resulted in much lower rates of growth and propionate production, although eventually all cultures reached the same maximum. The effect of the acidic pH heat treatment was not caused by destruction of biotin or PABA since there was no benefit from the use of filter-sterilized vitamins.

Fermentation with CSL and cysteine

Although the use of CSL medium resulted in fast growth and high (1.3%) propionate yield, this experiment was conduted using serum bottles. Feeding of lactic acid might have produced more propionic acid, but this could not be done in the 6-ml serum bottles. Consequently, the process was scaled up to a fermenter with 4.0 L of CSL medium containing 0.5 g L^{-1} cysteine as a reducing agent. Initially the production of propionate was slightly slower than with the serum bottles. Only about 0.5% propionic acid was produced in 22 h and 1.2% in 48 h (Figure 1). After 29 h of fermentation we added another 17.1 g L^{-1} lactic acid to determine if more propionate might be produced. The propionate concentration increased markedly after the sodium lactate was fed. A total of 2.3% propionate and 1.2% acetate were produced in 4 days.

Fermentation with Na_2S and CSL

Although the use of Na_2S as reducing agent in serum bottles resulted in a similar yield of propionate as when cysteine was used, the medium was reduced much faster with Na_2S as indicated by disappearance of the resazurin red color. The use of Na_2S as the reducing agent was examined at the fermenter scale; the previous fermentation using CSL was repeated, but with Na_2S replacing cysteine in the medium (Figure 2). When lactic acid declined to 0.6% on day 2 of the fermentation, an additional 2% lactic acid was added (as sodium lactate). A total of 2.3% propionate and 1.0% acetate was produced, but this time in only 3 days of fermentation (vs 4 days with cysteine). For this reason Na_2S was used in all further experiments.

Fermentation with medium feed

In some production facilities the inoculum size that can be produced for the full scale fermenter may be much less than the desirable range of 1–10%. This problem can be alleviated in practice by initially filling the full size fermenter just enough to cover the lower mixing impeller, inoculating, then slowly filling the fermenter with medium as the culture grows. This procedure was tested with *S. ruminantium*.

The medium was then inoculated with *S. ruminantium*, and after 12 h the optical density was 6.1, lactic acid was 1.3% (L-lactic acid was 0.7%), and propionic acid was 0.8% (Figure 3). Fresh medium was then fed slowly over a period of 5 h. By 21 h the lactic acid had declined to 0.4% (L-lactic acid was 0.2%) and propionic acid had increased to 1.17%, so 568 g of a sodium lactate concentrate was rapidly pumped into the fermenter. After 55 h the

Table 2 Comparison of corn steep liquors

CSL[a]	Type[b]	Trace Min.[c]	21 h Δ Optical density[d]	32 h Δ Optical density[d]	48 h % Propionate
CP	wet	no	1.00 ± 0.01	0.99 ± 0.01	1.30
R	dry	no	0.58 ± 0.04	0.63 ± 0.02	1.27
NS	wet	no	0.96 ± 0.04	0.97 ± 0.00	1.30
CP	wet	yes	1.09 ± 0.01	0.98 ± 0.00	1.28
R	dry	yes	0.66 ± 0.02	0.69 ± 0.01	1.19
NS	wet	yes	0.95 ± 0.03	0.90 ± 0.02	1.30

[a]Sources of corn steep liquor were: CP = Corn Products (Englewood Cliffs, NJ, USA); R = Roquette Corp (Gurnee, IL, USA); and NS = National Starch (Bridgewater, NJ, USA). Corn Steep Liquor Medium containing CSL (2.5 g solids L^{-1}) was added to triplicate 5-ml serum bottles. Reducing agent was $Na_2S{\cdot}9H_2O$
[b]The CSL was either wet (50% solids) or spray dried
[c]Trace minerals were added where indicated
[d]The 48-h time points are not shown. Standard deviation is shown as ±

lactic acid concentration had declined to 0.2% (L-lactic acid was 0.06%), and the propionic acid had risen to 2.07%. One hour later 120 g of a sodium lactate concentrate was transferred to the fermenter. After 80 h lactic acid was not detected by HPLC (L-lactic acid was 0.06%) and propionic acid reached a maximum of 2.24%. A propionic acid concentration of 2% was obtained in only 48 h, 10 h less than in the batch fermentation (Figure 2). The experiment was repeated with similar results: propionic acid reached 2.1% in 51 h and 2.25% in 73 h (data not shown).

It should be noted that *S. ruminantium* quickly converted the L-lactic acid used in the medium to a racemic mixture and there was little or no enantiomeric preference for further metabolism. L-lactic acid does not racemize under the incubation and heat sterilization conditions used in this experiment [4] so the racemization resulted from microbial activity. Further evidence of this was found in the next experiment where similar results were obtained with filter sterilization.

Fermentation with filter-sterilized medium

Heat-sterilization was not detrimental to the medium (Table 1); however only filter-sterilization equipment is available in some production scale facilities. Therefore, to have the widest possible application, this process was adapted for filter-sterilization. Heat treatment was used only for the salts, yeast extract and CSL.

The initial concentration of lactic acid in the fermenter assayed at 2% (Figure 4) rather than the expected 3%. This was because 88% lactic acid contains 34% lactate esters (concentrated lactic acid self esterifies) [4]. Upon dilution to less than 5% in water the lactate esters hydrolyze, but while this takes several days at room temperature, complete hydrolysis can be obtained by boiling for 5–10 h. The hydrolysis time at 121° C is even shorter; our results suggest that most esters were hydrolyzed by the heat sterilization in the previous experiment.

Twelve hours after inoculation with *S. ruminantium*, lactic acid had declined to 1.0% (L-lactic acid was 0.48%) and propionic acid was 0.69%, and fresh medium was added over a 4-h period. By 27 h propionic acid had risen to 0.94% and lactic acid had declined to 0.44% (L-lactic acid was 0.2%); more sodium lactate was then added. By 53 h total lactic acid was 0.04% (L-lactic acid was not detected), so a feed of sodium lactate (10–13 g h^{-1}) was started and maintained for the rest of the fermentation. The final concentration of lactic acid at 83 h was only 0.17% (L-lactic acid was 0.063%), and the final product concentrations were 2.25% propionic acid, 1.07% acetic acid, and 0.60% succinic acid. The experiment was repeated two more times with essentially similar results (final propionate was 2.29% and 2.33% at 90 and 85 h, respectively).

Greater growth and acid production rates were consistently obtained in heat-sterilized fed-batch fermentation as compared to filter-sterilized fed-batch fermentation, but the reason for this is unclear. One might suspect poor hydrolysis of the lactate esters in the filter-sterilized medium. In this scenario the inferior performance would result from the lower concentration of free lactic acid and/or from the presence of the esters. As discussed above a large portion of the lactic acid is present as esters following filter-sterilization. However, *Selenomonas* is able to eventually metabolize these esters, since the same final acid concentration was obtained with filter-sterilization as with heat-sterilization. In one fermentation we tried to prove that the slow growth and acid formation were due to lactate esters. To hydrolyze the esters the lactic acid was diluted and then boiled for 8 h prior to use; contrary to our expectations this did not result in a significant improvement in growth or acid production rate. That is, the fermentation with the boiled acid had a yield of 2.3% propionic acid in 90 h whereas the control produced 2.2% in 87 h. Thus, heating the fermentation medium produced another beneficial effect.

Continuous culture fermentation

To increase the productivity of the process, the fermentation was adapted to continuous culture using heat-sterilized medium (Table 3). Initially *S. ruminantium* was grown in batch mode for 12 h until lactate was nearly depleted, then the medium feed was started at a dilution rate of 0.1 h^{-1} (Figure 5). After 50 h it became clear that this was too high a dilution rate, because the culture performance was deteriorating. The dilution rate was slowed to about 0.045 h^{-1}. The small variations in dilution rate shown in

Table 3 Steady state[a] parameters for continuous fermentation

	Concentration	SD[b]
Lactic acid (100% basis)	6.2 g L^{-1}	0.03
Propionic acid	10.8 g L^{-1}	0.16
Acetic acid	5.0 g L^{-1}	0.01
Succinic acid	3.3 g L^{-1}	0.01
Absorbance (600 nm)	5.40	0.12
Dilution rate	0.055 h^{-1}	0.0015
Lactic acid feed (100% basis)	30.0 g L^{-1}	
Propionic acid productivity	0.59 g L^{-1} h^{-1}	
Lactic acid utilization	80%	
Propionate : succinate molar ratio	5.2 : 1	
Reduced : oxidized molar ratio	2.1 : 1	
Propionate : succinate : acetate ratio	5.2 : 3.0 : 1	

[a]Steady state was considered to be last 69 h (5 time points) of the continuous fermentation. Values are averages of these data
[b]Standard deviation

Figure 5 are due to equipment limitations. At about 120 h there was an unexplained upset and the absorbance decreased, but the culture recovered quickly. The decrease in the dilution rate at 170–190 h was due to an equipment difficulty. At 242 h the dilution rate was increased to 0.055 h^{-1}, and the lactic acid, propionic acid and optical density were stable. The continuous culture was run for a total of 310 h with steady state conditions maintained for 69 h. As expected the productivity (0.59 g L^{-1} h^{-1}) was substantially higher than that of the fed-batch fermentations (0.42 g L^{-1} h^{-1}). Productivity does not consider fermenter clean-out and turn around times—if these are taken into account the advantage of the continuous culture is even more pronounced. The continuous fermentation $Y_{P/S}$ values were 0.45, 0.21 and 0.14 g g^{-1} for propionic, acetic and succinic acids respectively.

Discussion

We studied the use of *S. ruminantium* for production of propionic, acetic and succinic acids for natural flavors. The fermentation could be run in batch, fed batch, or continuous mode, and is very competitive with other processes to make propionic acid. The key medium component is CSL which allows rapid growth of *S. ruminantium*. The elimination of whey or milk products from the medium results in a product that could be certified as kosher parve. CSL is an excellent stimulant of growth and acid production, and all liquid CSL samples gave good results.

In theory the *S. ruminantium* fermentation should have a product mixture balanced for oxidation-reduction because growth is anaerobic and there is normally no way to get rid of excess reducing power which would otherwise accumulate as reduced nicotinamide adenine dinucleotide (NADH). When grown on lactic acid the production of either propionic or succinic acids results in a deficit of one NADH per mole substrate whereas production of acetic acid results in an excess of two moles of NADH. Thus, to achieve a balanced oxidation-reduction state, *S. ruminantium* must produce twice as many moles of propionic and succinic acids (reduced products) as acetic acid (oxidized product). The reduced : oxidized molar product ratios were calculated, and the results were close to theory. The average molar ratio for all of the data points from Figure 3 was 2.05; for all of the data points from Figure 4 was 2.02; and for all the data points from Figure 5 was 2.06. There is no similar theoretical value for the propionate : succinate ratio. The ratio of propionate to succinate was high initially, but then decreased substantially to about 5–6 (Figure 6). In the continuous fermentation during steady state the propionate to succinate ratio was also fairly low (5.06). High concentrations of propionate can be toxic to *S. ruminantium*, so production of some succinate reduces the amount of propionate and may allow a greater amount of growth before inhibition.

S. ruminantium had the highest propionic acid productivity in a continuous fermentation, but lactate utilization was only 80%. We believe that lactate utilization can be increased with further optimization; on the other hand lactic acid is inexpensive and the economic impact of 20% lactate waste is minimal. We maintained the continuous culture fermentation at steady state for 69 h at a dilution rate of 0.055 h^{-1}, but we believe that it could have been continued indefinitely. Our dilution rate of 0.055 h^{-1} is considerably less than the maximum of 0.2–0.4 h^{-1} reported previously for *S. ruminantium* [9,11,13]. However, this is to be expected because these other fermentations were for growth on glucose (with lactate product), which is faster, and especially because our fermentation had much higher concentrations of propionate and acetate.

The strain of *S. ruminantium* used here quickly racemized lactic acid, and both enantiomers were then metabolized equally. Gilmour [3] reported similar results, but other strains of *S. ruminantium* are apparently not as versatile. Scheifinger *et al* [9] reported that strain HD-4 produced L-lactate and a small amount of D-lactate, but could not ferment the D-lactate, whereas de Vries *et al* [12] found that no D-lactate was produced. The *S. ruminantium* strain used by Wallace [13] produced and fermented D-lactate, but could not interconvert enantiomers.

Although *S. ruminantium* is an anaerobe, it tolerates some oxygen contamination because it contains cytochromes, NADH oxidase, and superoxide dismutase. In fact, intentional introduction of oxygen will result in a more-oxidized product mix with higher relative concen-

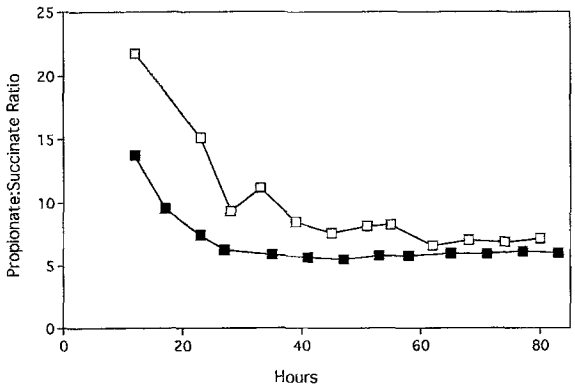

Figure 6 Time course of the ratio of propionate to succinate during fermentation. The molar propionate : succinate ratio was calculated from data in Figures 3 and 4; □ heat-sterilized, ■ filter-sterilized

trations of acetate [7,12]. However, since propionate is more valuable for flavors than acetate, the fermenter operator must be careful to exclude oxygen.

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