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Changes in steady state on introduction of a *Lactobacillus* contaminant to a continuous culture ethanol fermentation

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Lactobacillus paracasei **was introduced as a contaminant into a multistage continuous culture ethanol fermentation system at ratios of 1:100, 1:1, and 70:1 with *Saccharomyces cerevisiae*, but failed to overtake the yeast. None of the inoculation ratios allowed *L. paracasei* to affect *S. cerevisiae* in the first fermentor in the multistage system. *S. cerevisiae* remained constant at $\sim 3\times10^7$ CFU/ml regardless of the bacterial inoculation level, and even at the 70:1 inoculation ratio, glucose, ethanol, and lactic acid concentrations did not change from the steady-state concentrations seen before bacterial inoculation. However, *L. paracasei* decreased steadily from its initial inoculation level of $\sim 2.2\times10^9$ CFU/ml and stabilized at 3.7×10^5 CFU/ml after 10 days of steady-state operation. Both organisms then persisted in the multistage system at an approximate *L. paracasei*/*S. cerevisiae* ratio of 1:100 which confirms that, in continuous fuel ethanol production, it would be difficult to eliminate this bacterium. Only when the pH was controlled at 6.0 in fermentor 1 (F1) were changes seen which would affect the multistage system. Ethanol concentration then decreased by 44% after 4 days of pH-controlled operation. This coincided with an increase in *L. paracasei* to $>10^{10}$ CFU/ml, and a 4× increase in lactic acid concentration to 20 g/l. When the clarified contents from other fermentors (F2–F5) in the multistage system were used as growth media, *L. paracasei* was not able to grow in batch culture. This indicated that the first fermentor in the multistage system was the only fermentor capable of supporting the growth of *L. paracasei* under the described conditions.** *Journal of Industrial Microbiology & Biotechnology* (2001) **27,** 39–45.

Keywords: contamination; *Lactobacillus*; *Saccharomyces cerevisiae*; very high gravity (VHG); fuel alcohol; multistage continuous culture

Introduction

Virtually all fuel alcohol plants use a *Saccharomyces cerevisiae*-based fermentation to convert substrate to ethanol by batch, continuous, or multistage continuous fermentations. Many advantages are said to accrue in the use of multistage continuous culture including higher yields of ethanol, more efficient use of substrate, and higher ethanol productivities [6]. The primary disadvantage of continuous culture fermentations is the ongoing risk of bacterial or wild yeast contaminations which lead to unplanned shutdowns and losses of yield.

In the fuel alcohol industry, *Lactobacillus* is the most commonly found bacterial contaminant [11] with serious consequences for production. Faster-growing (alcohol and pH tolerant) bacteria or wild yeast can quickly outnumber culture yeast and rapidly produce undesirable end products. The inhibitory effects on yeast growth of a number of fermentation products have been assessed in batch and continuous culture fermentations. In one study, ethanol, acetic acid, and lactic acid were added to continuous culture fermentations where *S. cerevisiae* had reached steady state [9]. An 80% reduction in yeast density was seen with concentrations of ethanol, lactic acid, and acetic acid of 70, 38, and 7.5 g/l, respectively [9]. Yeast cell viability was also decreased when backset contaminated with *L. fermentum* or *L. delbrueckii* was recycled in sequential 14° Plato (140 g dissolved solids per liter) wheat mash batch fermentations [5]. A 60% reduction of yeast viability was observed when a lactic acid concentration of 14 g/l was attained in the fifth sequential fermentation [5]. A lactic acid concentration of 8 g/l in a beet molasses batch fermentation reduced yeast viability by 95% and alcohol production rate by 80% [16]. Sequential 18° Brix (18 g per 100 g mash) fed-batch fermentations (co-inoculated in the first fed-batch fermentation with yeast and *L. fermentum*) inhibited yeast bud formation once lactic acid (measured as total acidity) surpassed 4.8 g/l [18]. The threshold level of lactobacilli required to influence the specific growth rate of yeast was 10^4 lactobacilli/ml when an initial yeast concentration of 3×10^6 cells/ml in a 14% (w/v) beet molasses batch fermentation (adjusted to pH 5.0) was used [16].

Ethanol yields are also affected by contamination. Losses in ethanol yield as high as 11% have been reported when batch fermentations were contaminated by lactobacilli [7]. Ethanol productivity in a cell-recycle continuous fermentation of a 20° Plato (200 g dissolved solids per liter) glucose medium fell by 30% when the system was also inoculated with *L. fermentum* [4]. Production losses of 7% ethanol were observed when 10^9 CFU/ml of *L. fermentum* were introduced with 10^6 CFU/ml yeast at the beginning of a 22–24° Plato (220–240 g dissolved solids per liter) batch fermentation of wheat mash [13]. The same authors found that more than 2% of total ethanol was lost with the introduction of only 10^5 CFU/ml of either *L. paracasei* or *Lactobacillus* #3 at the beginning of batch fermentations. Any reduction in ethanol yield has significant economic consequences to the fuel alcohol industry where profit margins can be very low [12].

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In a recent work, very high gravity (VHG) fermentation and multistage continuous culture technologies were merged successfully for the first time to produce fuel ethanol concentrations as high as 17% (v/v) without the use of modified *S. cerevisiae* and without major changes to existing production equipment [1]. This merger allows the fuel ethanol industry an opportunity to produce higher ethanol concentrations in a continuous manner.

This work was designed to examine the long-term effects of a deliberate contamination of *L. paracasei* in a merged VHG–multistage continuous culture system and to investigate how this contamination might affect ethanol yield and productivity.

Materials and methods

Multistage continuous fermentation system

Five Bioflo III fermentors (New Brunswick Scientific, Edison, NJ) were connected in series to produce a multistage continuous culture fermentation (MCCF) system as described previously [1] and depicted in Figure 1. All materials and procedures regarding construction of the medium reservoir, MCCF medium formulation and preparation, MCCF preparation, and operation and sampling of the MCCF were followed as previously described [1] with one addition. A steam sterilizable pH probe was inserted into the fermentor headplates before autoclaving them to provide optional pH control. Where pH control was needed, 6N KOH was used along with the pH control system present on the Bioflo III fermentors. Fermentations were conducted at 28°C with 100 rpm agitation. Sterile air was supplied to each fermentor at 2 SLPM. Medium and sterile air entered the fermentor through a common port, but the medium entered through a flow breaker before mixing with the air flow (to prevent back contamination of the medium reservoir). In addition, yeast growth benefitted from the presence of oxygen in air as oxygen is essential for synthesis of required membrane unsaturated fatty acids [17].

Yeast master culture and storage

A slant of *S. cerevisiae* ("Allyeast Superstart") was provided by Alltech Inc. (Nicholasville, KY). This yeast was inoculated into 100 ml YEPD medium in a 250-ml screw-capped side-arm flask and was grown overnight with shaking (150 rpm) at 30°C. Sterile

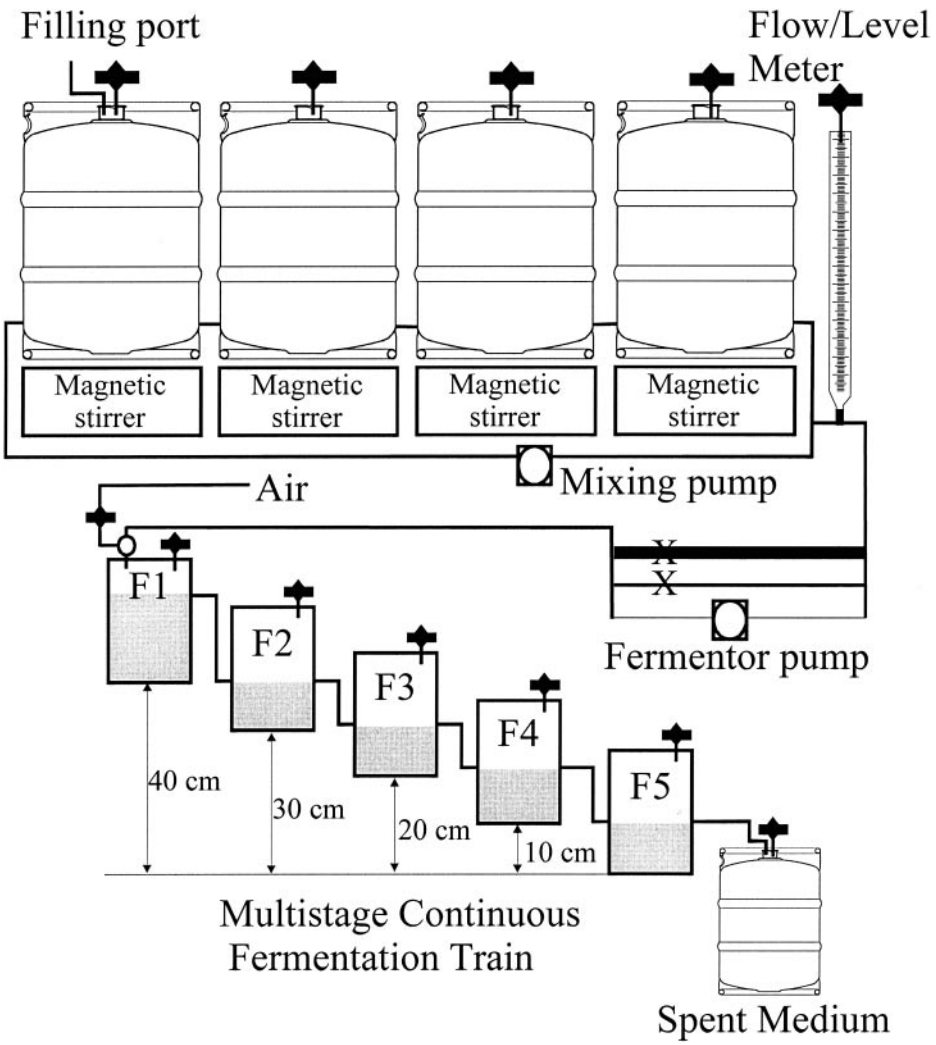

Figure 1 Schematic diagram of the constructed MCCF system.

glycerol was added to the culture to bring the final concentration of glycerol in the culture to 20%. The culture was then aseptically dispensed into sterile 1.8-ml cryogenic vials and stored at $-70^{\circ}C$.

L. paracasei culture and storage: *L. paracasei* isolated from a fuel alcohol plant [7] was subcultured on screw-capped MRS (Unipath, Nepean, Ontario, Canada) agar slants. The headspaces in the slants were flushed with filter-sterilized CO_2 on inoculation to provide an oxygen-free environment suitable for growth. Inoculated slants were incubated overnight at $30^{\circ}C$ in a CO_2 incubator (National Appliance Co., Portland, OR), removed, and stored at $4^{\circ}C$. The slants in storage were subcultured monthly.

Inoculum preparation for the MCCF

One cryogenic vial containing *S. cerevisiae* was thawed and aseptically added to a 1-l screw-capped flask containing a 500-ml portion of the medium currently used in the fermentor. This inoculum was grown overnight with shaking (100 rpm) at $30^{\circ}C$.

L. paracasei for the MCCF was prepared by transferring cells from a slant to a 250-ml screw-capped Erlenmeyer flask containing 100 ml MRS broth. The pre-inoculum flask was then flushed with filter-sterilized CO_2 and incubated in a rotary shaker at 100 rpm at $30^{\circ}C$ for 24 h. To obtain 1:100, 1:1, and 70:1 inoculation ratios of *L. paracasei*/*S. cerevisiae*, the viable numbers per milliliter of medium of each microbe were determined. From published work, the steady-state viable count of *S. cerevisiae* in the MCCF operating at $28^{\circ}C$, 100 rpm, and at a flowrate of 0.336 l/h was 3.8×10^{7} cells/ml [1]. As well, work in this laboratory determined the relationship between CFU/ml of *L. paracasei* grown in MRS broth and absorbance in a Klett-Summerson colorimeter (Klett, New York, NY) equipped with a #66 red filter (640–700 nm). For this bacterium, 100 Klett units are equivalent to 8.54×10^{8} CFU/ml, and mid-exponential *L. paracasei* were removed from MRS at approximately 352 Klett units [15]. Then, to prepare a 70:1 inoculation ratio in the MCCF immediately after inoculation, $70\times3.8\times10^{7}$ CFU/ml (the steady-state yeast population) or 2.7×10^{9} CFU/ml of *L. paracasei* was required in the MCCF. With a working volume of 5060 ml in fermentor F1, the number of cells of *L. paracasei* required was 1.4×10^{13} CFU. With a target Klett value of 352 (3.0×10^{9} CFU/ml), the volume of inoculum required was 4.7 l. Similar calculations were performed for other desired ratios. The calculated volume of MRS broth was prepared, inoculated with a 10-ml pre-inoculum, flushed with filter-sterilized CO_2, and incubated in a rotary shaker at 100 rpm at $30^{\circ}C$. Once a Klett value of 352 was reached, cells were aseptically harvested by centrifugation at $10{,}200\times g$ for 15 min at $4^{\circ}C$. To eliminate the problem of diluting the steady-state *S. cerevisiae* with a large volume of the resuspended bacterial pellet, a 500-ml volume from F1 was removed and used as the final resuspension fluid for the *L. paracasei* pellet. Once resuspended, the *L. paracasei* inoculum was immediately inoculated into F1.

MCCF inoculation and system equilibration

Medium in the medium reservoir was pumped into all the fermentors to fill each fermentor to its respective working volume. Agitation and cooling lines on each fermentor were turned on and set to 100 rpm and $28^{\circ}C$. As well, the medium flowrate was set at 0.336 l/h (D for F1 = 0.066 h^{-1}; D for F2 and other fermentors = 0.12 h^{-1}). Dilution rates differ because F1 holds twice the medium volume as in F2–F5, a practice often seen in industrial MCCF designs. The yeast inoculum was added to F1 and allowed to inoculate (by overflow) all fermentors in the system. The system was allowed to run for 7 days to allow the yeast to reach steady state as confirmed when the glucose concentrations in successive measurements over 3 days varied by less than 5% in each fermentor. Once steady state was achieved, the *L. paracasei* inoculum was introduced into the system.

Batch growth of L. paracasei

L. paracasei was grown in batch conditions using media withdrawn from each fermentor in the MCCF. Each medium was clarified by centrifuging it at $10{,}200\times g$ for 15 min at $4^{\circ}C$, and then sterilized by membrane filtration through GN-6 membrane filters (Gelman Sciences Inc., Ann Arbor, MI) into sterile Erlenmeyer flasks.

A 100-ml aliquot of each clarified and sterilized medium was then dispensed into sterile 250-ml screw-capped side-arm Erlenmeyer flasks and inoculated with *L. paracasei* from an MRS agar slant. Each flask was flushed with filter-sterilized CO_2, capped tightly, and incubated at $28^{\circ}C$ at 100 rpm in a rotary shaker incubator. Growth of *L. paracasei* was monitored by measuring absorbance using a Klett-Summerson colorimeter.

HPLC analysis

Lactic acid, glycerol, ethanol, and glucose concentrations were determined by HPLC analysis. Each sample was thawed and diluted to the required extent with Milli-Q water. Aliquots of the diluted sample were mixed with an equal volume of 2% (w/v) boric acid (internal standard), and 5-μl aliquots were injected onto an HPX-87H column (Biorad, Richmond, CA) equilibrated at $40^{\circ}C$. The eluent was 5 mM sulfuric acid flowing at a rate of 0.7 ml/min. Separated components were detected by a differential refractometer (Model 410; Waters Chromatographic Division, Milford, MA) and identified and quantitated using Waters Maxima 810 software.

Viability by membrane filtration

The membrane filtration procedure for viable cell counting [8] was used. Triplicate aliquots of an appropriately diluted fermentation sample were vacuum-filtered through sterile 0.45-μm (pore size), 47-mm grided GN-6 membrane filters (Gelman Sciences Inc.), rinsed with 5 ml of 0.1% sterile peptone water and placed onto YEPD plates (per liter: yeast extract [Difco], 10 g; Bacto peptone [Difco], 10 g; AnalaR D-glucose [BDH], 20 g; Bacto bacteriological technical agar [Difco], 15 g) with 0.005% (w/v) gentamicin and 0.01% (w/v) oxytetracycline (Sigma Chemical Co.). The plates were incubated at $27^{\circ}C$ for 2 days. For the enumeration of *L. paracasei*, filtered samples were placed on MRS plates containing 0.001% (w/v) cycloheximide (Sigma) to inhibit the growth of yeast. Plates were incubated in a CO_2 incubator (National Appliance Co.) at $30^{\circ}C$ for 2 days after two cycles of evacuating and refilling the chamber with commercial-grade (>99.5%) CO_2. Triplicate plating was performed.

Results and discussion

L. paracasei was chosen as the contaminant in this project for a number of reasons. This particular *Lactobacillus* is an isolate from a fuel alcohol plant. It grows extremely rapidly. MRS plates with "normal" brewing and distillery lactobacilli require up to a week or

more to develop in contrast to only 24 h for this *Lactobacillus*. Due to this extremely rapid growth, some *Lactobacillus* strains in grape juice have been termed "ferocious" lactobacilli [2]. *L. paracasei* is also homofermentative which leads to more efficient production of metabolic energy than with a heterofermentative bacterium. Lactic acid produced is inhibitory to *S. cerevisiae* when over 0.8% w/v is present in batch fermentations [14]. Thus, this "worst case," industrially relevant organism was chosen as the contaminant for this work. The growth of *L. paracasei* and increases in its metabolic end products were followed only in the first fermentor in the MCCF system. The first fermentor in this system provides the best opportunity for *L. paracasei* to compete with the yeast because all nutrients are abundant and ethanol concentrations are at their lowest levels in F1. *L. paracasei* produced in F1 would then continue to produce lactic acid and scavenge trace nutrients if viability was maintained as they passed into later fermentors in the MCCF.

In particular, it was of interest to see which microorganism would prevail and what population dynamics would result from competition and inhibition. It was expected that the introduction of a very fast-growing *Lactobacillus* into a nutrient-rich environment in competition with an established, but slower-growing yeast, would result in the eventual washout of the yeast from the MCCF. In Figure 2, the MCCF system was run for 8 days to allow *S. cerevisiae* to reach its steady state. *L. paracasei* was then inoculated into F1 at a 1:100 ratio of *L. paracasei*/*S. cerevisiae* and tracked over time.

Figure 2 illustrates the viability of *S. cerevisiae* and *L. paracasei* (CFU/ml) in fermentor 1 (F1) of the MCCF operated at a dilution rate of 0.066 h^{-1}, a 26% medium reservoir glucose concentration, and at 28°C and 100 rpm agitation. Surprisingly, viable numbers of *L. paracasei* decreased after the time of inoculation. This decrease could not be due to nonpermissive growth conditions in the MCCF. The theoretical washout line plotted in Figure 2 was calculated

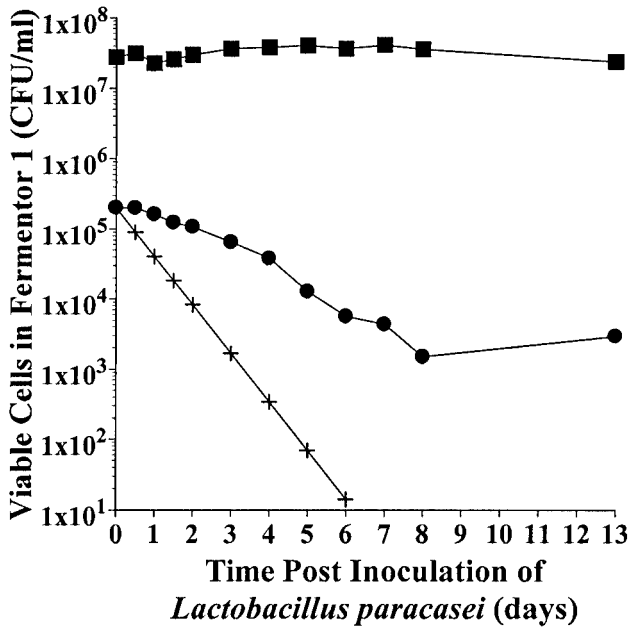

Figure 2 Viabilities (CFU/ml) of *S. cerevisiae* and *L. paracasei* inoculated into F1 in the MCCF system with the medium reservoir containing 26% (w/v) glucose. *L. paracasei* was inoculated at a 1:100 ratio (*L. paracasei*/*S. cerevisiae*) after steady-state levels of yeast were attained (■, *S. cerevisiae*; ●, *L. paracasei*; +, theoretical washout).

based on the initial inoculation level of *L. paracasei*, the dilution rate of the MCCF, and on the assumption that *L. paracasei* behaved as nonviable particles. It was theorized that if the conditions in the MCCF did not allow *L. paracasei* to multiply, then the trendline for *L. paracasei* should match the theoretical washout trendline. This did not happen. The theoretical and actual trendlines continued to diverge from the time of inoculation which indicated that *L. paracasei* in the MCCF were multiplying to a limited extent. It was expected that viable *L. paracasei* would increase and not decrease in the MCCF — especially because (in optimal conditions) lactobacilli grow faster than yeasts. Clearly, either the inoculation level of *L. paracasei* was not sufficient to compete on an equal (or superior) basis with *S. cerevisiae*, or environmental factors (such as ethanol, yeast, temperature, pH, and/or dilution rate) did not permit a rapid enough growth of *L. paracasei*, or the medium was nutritionally deficient.

Although viable *L. paracasei* decreased in Figure 2, the level of viable *S. cerevisiae* remained at its steady-state value throughout the experiment. Thus, neither the growth of *L. paracasei*, nor the metabolic by-products of its growth (lactic acid), influenced *S. cerevisiae* in this experiment. It is likely that the rather small numbers of viable *L. paracasei* were not sufficient to compete with the yeast for trace nutrients or they could not produce enough metabolic by-products to inhibit the yeast.

Another surprising finding in Figure 2 was that *L. paracasei* appeared to stabilize at a steady-state value of approximately 3×10^3 CFU/ml. *L. paracasei* and *S. cerevisiae* coexisted in the MCCF at different steady-state cell populations (at an approximately 1:10,000 ratio of *L. paracasei*/*S. cerevisiae*). These results suggest that the MCCF system is capable of supporting growth of two microorganisms at different steady-state values.

In another experiment, the inoculation ratio of *L. paracasei* was increased to 1:1 (Figure 3). The results in Figure 3 again showed that viable *L. paracasei* decreased from the time of inoculation, whereas the yeast maintained steady-state numbers. The unchanging steady-state values of *S. cerevisiae* in Figures 2 and 3 strongly suggest that the inoculation level of *L. paracasei* does not play a role in inhibiting *S. cerevisiae* (at least up to a 1:1 ratio) and that regardless of the inoculation level, *L. paracasei* will eventually achieve its own steady state. The expected rapid dominance of the MCCF by a contaminating *Lactobacillus* (which plagues continuous fuel alcohol production) was not achieved. As well, the fact that viable *L. paracasei* in both Figures 2 and 3 decreased with time indicates that the MCCF is only capable of supporting *L. paracasei* at a lower (noninhibitory for yeast) steady-state level compared to *S. cerevisiae*. From a production perspective, the data show that, under optimal operating conditions for fuel alcohol production with a glucose concentration of 26% w/v, the MCCF was capable of reducing the load of a contaminant (*L. paracasei*) while maintaining the unwavering dominance of the yeast culture — all without any antimicrobial chemicals! It appeared that the system was "self regulating." Similar results were reported from continuous fuel alcohol plants [3], in VHG brewing [10], and in laboratory experiments [19].

Figure 4 shows a long-term mixed fermentation of *S. cerevisiae* and *L. paracasei* in F1 in the MCCF. An inoculation ratio of 70:1 (*L. paracasei*/*S. cerevisiae*) was used with the bacteria added after 5 days of equilibration to steady state by *S. cerevisiae*. As in the previous experiment, the viable numbers of *L. paracasei* decreased steadily from the time of inoculation. This decrease strongly suggests that, even in the "friendly" environmental conditions of

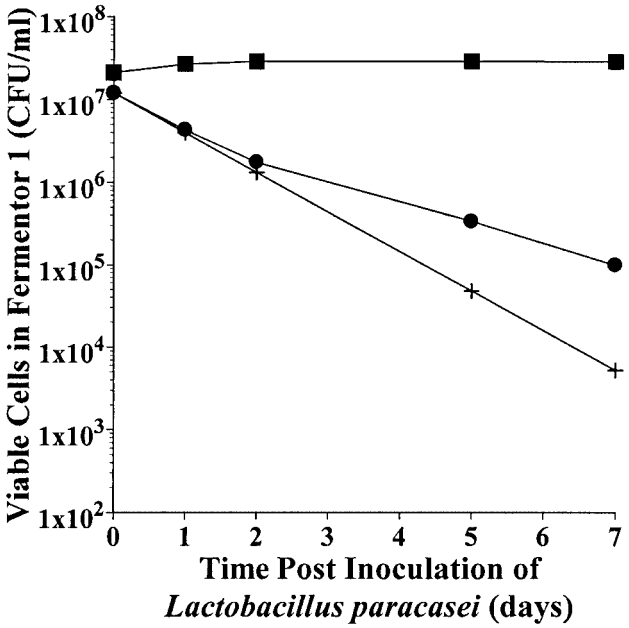

Figure 3 Viabilities (CFU/ml) of *S. cerevisiae* and *L. paracasei* inoculated into F1 in the MCCF system with the medium reservoir containing 26% (w/v) glucose. *L. paracasei* was inoculated at a 1:1 ratio (*L. paracasei*/*S. cerevisiae*) after steady-state levels of yeast were attained (■, *S. cerevisiae*; ●, *L. paracasei*; +, theoretical washout).

F1, *L. paracasei* could not overtake *S. cerevisiae* in the equilibrated MCCF system — at least up to a 100:1 ratio of bacteria to yeast. In fact, *L. paracasei* decreased in viability from the time of inoculation. Thus, the inoculation level of *L. paracasei* would not appear to be a major factor in determining the final cell numbers of *L. paracasei* and resulting MCCF kinetics. After 10 days of operation, *L. paracasei* entered a steady state as described above and in Figure 2. This was confirmed by tracking the steady-state value of *L. paracasei* (average 3.7×10^5 CFU/ml) for an additional 7 days. From a production point of view, the fact that two steady states can exist means that one might never be able to eliminate a contaminant from a continuous culture. A contaminant may exist in an MCCF even if production parameters (e.g. growth of *S. cerevisiae*, ethanol production rate) show "normal" values. The lower and stable steady-state bacterial contaminant level would be a "ticking time bomb" for fuel alcohol producers. If conditions such as pH, temperature, or other process parameters were to change, the contaminant might suddenly increase in number and cause problems. In this work, pH control in the MCCF was activated and set to pH 6.0 at the seventh day of bacterial steady state. Within 3 days, *L. paracasei* increased from its steady-state value of 3.7×10^5 to approximately 1×10^{10} CFU/ml — a 4.4 log increase! Competition by *S. cerevisiae* and possible inhibition by ethanol, or high glucose concentrations did not appear to affect the growth of *L. paracasei* from its 100-fold lower steady-state value. One can conclude from Figure 4 that the most likely significant factor preventing *L. paracasei* from achieving a high steady-state value of viable cells in the MCCF was the pH. *L. paracasei* entered a steady-state value of approximately 1×10^{10} CFU/ml after 3 days of pH control at 6.0. *S. cerevisiae* remained in steady state throughout the experiment ($\sim3\times10^7$ CFU/ml) up until pH control was activated, and its growth was not affected by the introduction of, nor by the high initial inoculation level of *L. paracasei*. Only when *L. paracasei* increased to its new steady-state cell value (pH control activated) did the yeast cell viability decrease due to the competition for nutrients by large numbers of *L. paracasei* and/or because of the lactic acid produced. At the new steady state (at pH 6.0), *L. paracasei* now outnumbered *S. cerevisiae* by 3.2 logs.

Figure 5 illustrates the glucose concentrations in F1 over the course of the mixed culture experiment. Glucose remained

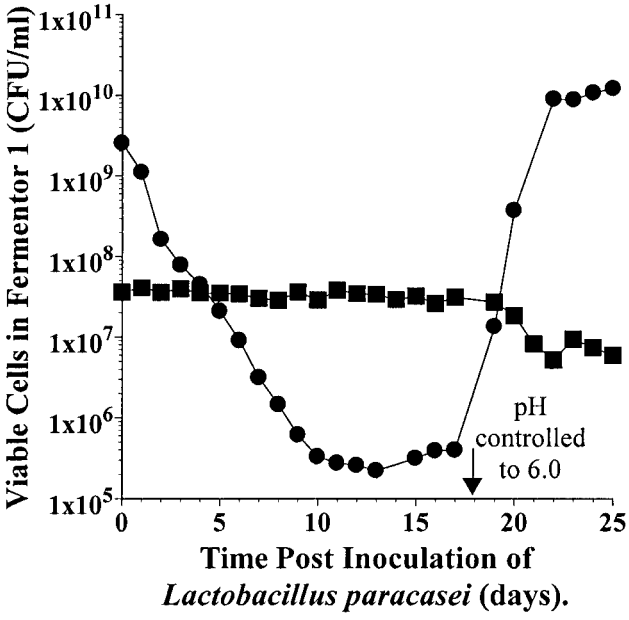

Figure 4 Viabilities (CFU/ml) of *S. cerevisiae* and *L. paracasei* inoculated into F1 in the MCCF system with the medium reservoir containing 26% (w/v) glucose. *L. paracasei* was inoculated at a 70:1 ratio (*L. paracasei*/*S. cerevisiae*) after steady-state levels of yeast were attained (■, *S. cerevisiae*; ●, *L. paracasei*).

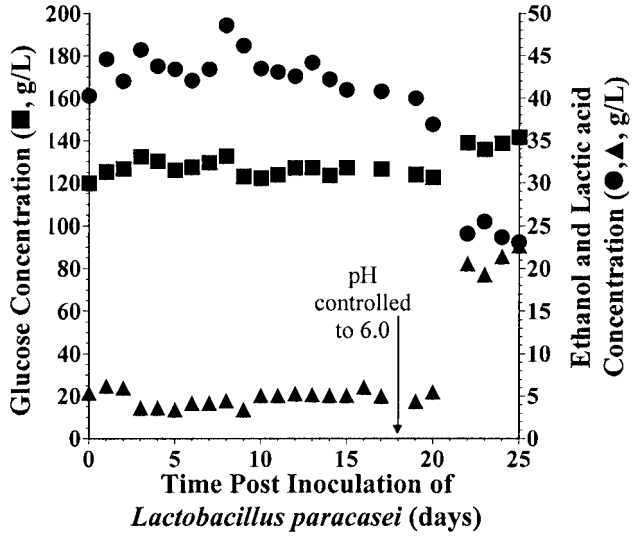

Figure 5 Glucose, ethanol, and lactic acid concentrations in a mixed culture of *S. cerevisiae* and *L. paracasei* in F1 in the MCCF system with the medium reservoir containing 26% (w/v) glucose. *S. cerevisiae* was equilibrated for 5 days before inoculation of *L. paracasei* at a 70:1 ratio (*L. paracasei*/*S. cerevisiae*) (■, glucose; ●, ethanol; ▲, lactic acid).

relatively constant from the time *L. paracasei* was inoculated to the time when pH control was activated. However, the glucose concentration increased from its steady-state value of approximately 125–140 g/l when *L. paracasei* reached its new steady-state level — a 12% increase. The glucose concentration increased at this time despite the fact that there were 100-fold more *L. paracasei* at this time than before pH control was activated. With such a large increase in viable counts of bacteria (2 logs), one would expect that glucose consumption should increase and not decrease. However, the increase in glucose concentration corresponded to the time when cell numbers of *S. cerevisiae* were in decline and not to the time when *L. paracasei* increased. It is not known why the glucose concentration increased at a time when the combined biomass of both organisms in F1 was at a peak. Most likely, since yeast are approximately 50× larger than bacteria, the rate of conversion of glucose to ethanol by this number of yeast is much larger than the rate of conversion of glucose to lactic acid catalyzed by the bacteria present. Thus, with the inhibition of yeast growth and ethanol production by *L. paracasei* (whether by competition for nutrients and/or lactic acid production), glucose concentration in F1 would increase due to lowered consumption by inhibited *S. cerevisiae*.

Ethanol concentration over the course of the mixed culture experiment in F1 in the MCCF is also depicted in Figure 5. In general, the ethanol concentration remained between 40 and 45 g/l in F1 during the time when *S. cerevisiae* was in steady state. Once pH control was activated and levels of *L. paracasei* increased to steady state, the ethanol concentration fell in F1 to 25 g/l — a 44% decrease. The other fermentors in the MCCF were not tracked in this experiment.

Lactic acid levels in the mixed culture experiment are depicted in Figure 5. As for glucose and ethanol, the lactic acid concentrations generally remained between 3 and 6 g/l when *S. cerevisiae* was in steady state. The amount of lactic acid (~0.41% w/v) in the MCCF medium formulation originated from the corn steep powder. Since the lactic acid concentration remained constant, it follows that *L. paracasei* did not produce inhibitory concentrations of lactic acid at the time of its inoculation or at its eventual steady state. However, the lactic acid concentration rose from 5 to at least 20 g/l once pH was controlled at 6.0. This 4× increase is attributable entirely to the corresponding increase in *L. paracasei* to greater than 1×10^{10} cells/ml.

Why did *S. cerevisiae* decrease when *L. paracasei* reached its new steady-state value after pH control was activated? The most likely reason is the high amount of lactic acid formed during growth of *L. paracasei* (20 g/l). It is also possible that *L. paracasei* was now competing effectively with *S. cerevisiae* for critical nutrients in F1 and that these nutrients were being removed at this stage.

Most of the data presented in this work concerned itself with how yeast and *L. paracasei* interacted in F1 in the MCCF because F1 provided the best opportunity for the growth of *L. paracasei*. Later fermentors in the MCCF provided very poor conditions for growth for this fastidious bacterium. To test the potential for *L. paracasei* to grow in other fermentors in the MCCF, fermentor samples from a noncontaminated MCCF run (i.e., equilibrated with yeast only) were harvested. Each fermentor sample was filtered to remove *S. cerevisiae* and any particulates present in the MCCF medium. *L. paracasei* was then inoculated into each clarified fermentor "medium," flushed with CO_2, and its growth under batch conditions was followed. The results are presented in Figure 6. *L. paracasei* grew in the medium formulated for MCCF

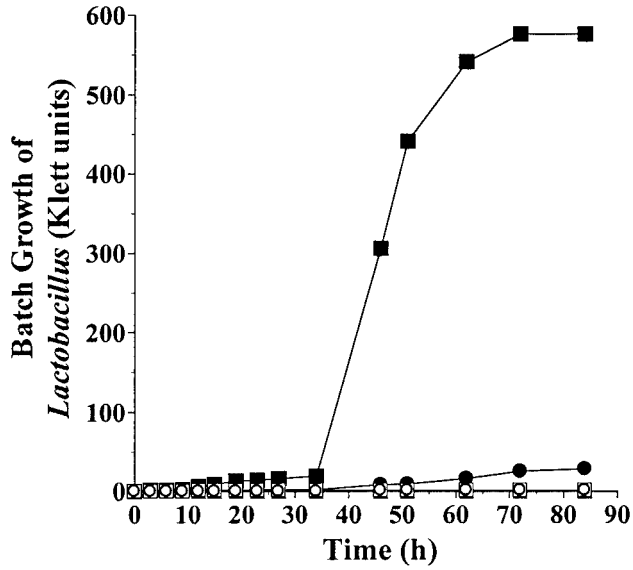

Figure 6 Batch growth of *L. paracasei* in clarified media obtained from an *S. cerevisiae*-equilibrated MCCF system with the medium reservoir containing 26% (w/v) glucose. Medium from each fermentor (F1–F5) in the MCCF was collected and filtered to produce the corresponding clarified medium before bacterial inoculation (■, clarified medium reservoir; ●, clarified F1; ▲, clarified F2; ◆, clarified F3; □, clarified F4; ○, clarified F5).

experiments (the contents of the medium reservoir). This eliminated the possibility that the medium formulation was nutritionally deficient in some manner for *L. paracasei*. In contrast, all five of the partially spent, clarified fermentor "media" severely restricted growth of *L. paracasei*. Only the medium from F1 allowed a small amount of growth of *L. paracasei*. Growth inhibition in these batch fermentations was likely due to the low pH formed in the media by the yeast (medium reservoir, 4.75; F1, 3.54; F2, 3.30; F3, 3.29; F4, 3.34; F5, 3.39), inhibition by yeast-produced ethanol, depletion of trace nutrients by *S. cerevisiae* which may be critical for growth of *L. paracasei*, or a combination of these factors. Thus, other than the low steady-state cell concentration seen in mixed experiments in F1, other fermentors in the MCCF could not support the growth of *L. paracasei*. For fuel alcohol producers, this is good news as the MCCF (under these conditions) permits the production of ethanol without the concern that *L. paracasei* will overtake and dominate *S. cerevisiae*.

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