

APPLICATION OF MULTISTAGE CONTINUOUS CULTURE TO VHG BASED  
ETHANOL FERMENTATIONS: PERFORMANCE AND CONTROL OF BACTERIA  
BY PH AND PULSED ADDITION OF ANTIBIOTIC

A Thesis

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by

A black rectangular redaction box covering the author's name.

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Fall, 2002

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## ABSTRACT

The application of very high gravity (VHG) technology to multistage continuous culture technology proved to be possible. Multistage fermentations were conducted with concentrations of glucose from 152 to 312 g/L in the medium. Steady state concentrations of *Saccharomyces cerevisiae*, glucose, and ethanol were established in each fermentor at each condition tested. A maximum ethanol concentration of 17% v/v was achieved in the last fermentor in the multistage system when 312 g/L glucose was fermented at a dilution rate of  $0.05 \text{ h}^{-1}$  at  $28^\circ\text{C}$ . The productivity of ethanol in the multistage system proved to be higher (12.7 g/L/h) than in identical fermentations conducted in batch (2.4 g/L/h) or in a single stage fermentor (1.25 g/L/h). *Lactobacillus paracasei* was introduced into a multistage fermentation that had been previously brought to steady state with *S. cerevisiae*. The effects of the contamination were then documented and the fermentation data were compared to the non-contaminated control. Control of *L. paracasei* in the multistage continuous culture fermentation (MCCF) system was then investigated by both pH and pulsed additions of penicillin G. Both pH and penicillin G addition were effective at reducing (but not eliminating) *L. paracasei*. The application of penicillin G every six hours (pulsed addition) to give an overall concentration of 2475 U/L in the multistage system was 3.5 times more effective in reducing the viable numbers of *L. paracasei* than penicillin G maintained continuously at 2475 U/L. Other frequencies of addition and overall average concentrations of penicillin G were tested and documented. Lastly, various unstructured mathematical models were run and assessed for their ability to accurately predict experimental steady state concentrations of biomass (yeast), substrate (glucose), and product (ethanol) concentrations in each fermentor at each condition in the multistage system. The Aiba model (which showed the best fit to experimental data) was then used to predict the course of the fermentation under multistage conditions.

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## NOMENCLATURE

D	Dilution rate ( $\text{h}^{-1}$ ) = $F/V$
V	Working volume of culture (L)
F	Flow rate of medium (L/h)
X or $X_t$	Total biomass concentration (g/L)
$X_v$	Viable biomass concentration (g/L)
$X_m$	Biomass concentration above which cells do not grow (g/L)
B	Bleed ratio (dimensionless)
P	Product concentration (g/L)
C	Concentration of substance of interest (g/L, mol/L)
$C_o$	Initial concentration of substance of interest (g/L, mol/L)
$P_m$	Product concentration (ethanol ) above which cells do not grow (g/L)
$P'_m$	Product concentration (ethanol ) above which cells do not produce ethanol (g/L)
S	Substrate concentration (g/L)
$S_o$	Initial substrate concentration (g/L)
$S_r$	Reservoir concentration of substrate (g/L)
$S_c$	Substrate concentration critical in reference to another parameter (g/L)
$K_s$	Saturation constant for growth (g/L)
$K_{si}$	Substrate inhibition constant for growth (g/L)
$K'_{si}$	Substrate inhibition constant for ethanol production (g/L)
$K_{pi}$	Product inhibition constant for growth (g/L)
$K'_{pi}$	Product inhibition constant for ethanol production (g/L)
$\mu$	Specific growth rate ( $\text{h}^{-1}$ )
$\mu_m$	Maximum specific growth rate ( $\text{h}^{-1}$ )

$Y_{x/s}$	Yield of biomass on consumed substrate (dimensionless)
$Y_{p/s}$	Yield of product on consumed substrate (dimensionless)
$v_p$	Specific ethanol production rate ( $h^{-1}$ )
$v_{pm}$	Maximum specific ethanol production rate ( $h^{-1}$ )
$v_s$	Specific substrate consumption rate ( $h^{-1}$ )
$v_{sm}$	Maximum specific substrate consumption rate ( $h^{-1}$ )
$\alpha$	Stephens and Lyberatos (1987) Adaptability of microbe to new culture conditions (dimensionless).
	Luong (1985) Dimensionless modeling constant
$n, m, \beta, b$	Dimensionless modeling constants
$m_s$	Cellular maintenance coefficient (g/g/h)
$dX/dt$	Cell growth rate (g/L/h)
$dP/dt$	Product formation rate (g/L/h)
$dS/dt$	Substrate consumption rate (g/L/h)

## 1 INTRODUCTION

The most economical way to produce ethanol is by fermentation of sugar substrates using *Saccharomyces cerevisiae*. In fact, worldwide production of ethanol in 1999 was  $24 \times 10^9$  L / year (Dixon, 1999). In North America, fuel and industrial grade ethanol is produced at a rate of  $7.0 \times 10^9$  L/year by both continuous and batch fermentation (Ingledeew, 1999a). Although the production of fuel ethanol in industry by batch fermentation is fairly well established and understood, the production and fermentation characteristics of ethanol produced by continuous methods is not. Knowledge of how a multistage continuous system would perform under different operating conditions, and the sensitivity of a multistage system to changes in media, operating conditions, and microbial contamination is lacking. Very High Gravity (VHG) fermentation technology has been successfully applied and proven in industry to increase ethanol productivity in batch, but has yet to be applied and studied in continuous production of ethanol. Furthermore, the production characteristics of ethanol in a VHG multistage system have yet to be studied before this merged system can be used confidently in industry.

A number of objectives existed for this study. One objective was to study the fermentation performance characteristics of a multistage continuous culture system with *S. cerevisiae* when VHG technology was applied. Comparisons of production performance to batch and other fermentation methods were made. Another objective was to deliberately introduce a microbial contaminant (*Lactobacillus paracasei*) into the VHG multistage continuous system and monitor the changes in fermentation characteristics. This appears to be the first time that the population and production dynamics of a contaminated multistage continuous system have been studied. Another objective of this study was to reduce the level of microbial contamination in a multistage fermentation in order to restore ethanol productivity. Two methods were studied and evaluated as to their effectiveness in reducing the level of microbial contamination in the

multistage system used in this study. One method of control involved standard pH control while the other method involved unique methods of addition of penicillin G. These novel methods of addition are possible only in fermentations that are continuous and have not been explored previously. Lastly, a number of unstructured mathematical models were evaluated for their ability to accurately predict experimental steady state values and the course of fermentation in the multistage system under a broad range of operating conditions.

## 2 LITERATURE REVIEW

### 2.1 Methods to produce ethanol by fermentation.

#### 2.1.1 Fermentation methods.

##### 2.1.1.1 Batch culture.

Currently, ethanol production in industry is done predominantly by batch fermentation. This technology has been employed traditionally to make ethanol since batch technology is well understood, mature, and easy to implement and operate. In batch fermentation, all of the medium for the fermentation is present in the fermentor at the start of fermentation. The inoculum is added to the fermentor and the cells grow at specific growth rates ( $\mu$ ) determined by the conditions present in the fermentor. The specific growth rate is not a constant value as is seen in continuous culture fermentations, but rather, changes in relation to the conditions present in the fermentor as time progresses. This produces a concentration of biomass ( $X$ ) which increases with time until conditions in the fermentor no longer permit further growth. Unlike continuous fermentations where steady state (*i.e.* unchanging) concentrations of biomass ( $X$ ), substrate ( $S$ ), and product ( $P$ ) are eventually reached, the concentrations of these parameters in batch fermentations are continuously changing. An advantage of batch fermentation over other fermentation methods (except total cell recycle) is that none of the cells leave the fermentor during operation. This permits all the cells created in the fermentor to produce  $P$  from  $S$ . Another advantage of batch systems is that discrete batch runs are made. If one particular batch shows difficulties in production, the problems giving rise to the lower production can be determined and fixed before the next run is started.

Most of the fuel alcohol by batch methods are made in concentrations of 8-13% v/v. In the laboratory, ethanol concentrations as high as 23.8% v/v have been reached.

with *S. cerevisiae* in very high gravity (VHG) wheat mash fermentations using more than 300 g/L dissolved solids (Thomas *et al.*, 1993). This is currently the highest reported batch concentration of ethanol achieved with all substrate present at zero time and without the use of conditioned or genetically modified *Saccharomyces* yeasts. VHG technology is gradually being applied to the fuel alcohol industry where goals of 15-16% or more alcohol in some locations are being set in order to lower costs (Ingledew, 1999b). In brewing, VHG technology has been successfully tested in pilot plants to produce beers with higher alcohol content which, according to sensory panels, were of acceptable quality when diluted to the alcohol concentration of commerce (D'Amore *et al.*, 1991; McCaig *et al.*, 1992). Every major brewery in the world is producing or experimenting with production of beers from higher gravity worts than were previously considered possible.

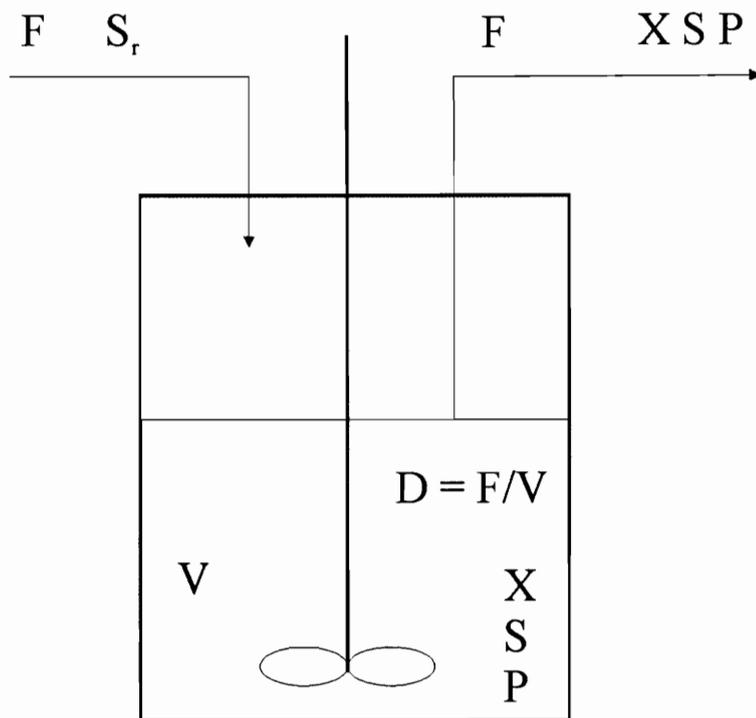
#### **2.1.1.2 Continuous culture.**

Continuous methods of fermentation are also used in industry to produce fuel alcohol. Continuous fermentations provide advantages over batch fermentations which include: optimized process conditions for maximal product productivity, long-term continuous productivity, higher volumetric productivity, reduced labor costs once steady state is reached, reduced vessel down time for cleaning, filling, and sanitizing, and easier process control and operation than batch during steady state operation (Cysewski and Wilkie, 1978; Sinclair and Cantero, 1990; Kelsall and Lyons, 1999a). One major disadvantage is that continuous fermentations are more susceptible to long term bacteriological problems. Another disadvantage is that a higher degree of knowledge is required to properly optimize process conditions to yield desired productivities. These disadvantages are important concerns when plants are designed for continuous operation.

Within continuous culture technology, two major technological variations exist - single stage and multistage stirred tank reactor (CSTR) systems.

### 2.1.1.2.1 Single stage.

A single stage system is the most simple form of continuous culture and is depicted in Figure 2.1. In continuous culturing, four key factors are important. One factor is that the reactor volume remains unchanged during the course of the experiment. This, in turn, means that the flow ( $F$ ) of medium into the reactor and the flow of fermentor contents leaving the reactor are matched. Another factor is that the dilution rate ( $D$ ), which is defined as the ratio of medium flow into the reactor to the reactor working volume, is constant. To have a constant dilution rate, the flows in and out of the reactor must be accurately known. Another important factor is that there is rapid and complete mixing of the reactor contents. Theoretically, instantaneous mixing must occur but in practice the mixing may not be instantaneous. Dunlop and Ye (1990) have shown mathematically and in experimentation that even in highly agitated continuous culture fermentations, the cell metabolism of *S. cerevisiae* in a fermentor could vary. Their calculation initially showed that the smallest eddy of turbulence was typically 50-300  $\mu\text{m}$  diameter in highly agitated fermentors yet the diameter of most microbes is between 1-5  $\mu\text{m}$ . The authors reasoned that the microbes may be spending much of their time in stagnant pools of turbulence where nutrients may be potentially depleted. Their calculations have further shown that in order to eliminate half of the eddy size, approximately 16 times as more energy would be needed to power the impeller (assuming the energy could be evenly distributed in the fermentor). To test their theory, a continuous culture fermentor was set up with five different medium injection ports. Each port was positioned and welded at places in the fermentor where extremes of mixing could be anticipated - below the stirrer, beside the stirrer, at the liquid surface, before a baffle, and between the baffles. Each port was characterized as to the size of the eddy each would create under identical operating conditions. The optical density of the yeast fermentation oscillated from 4 to 5.5 when the glucose feed was alternatively cycled at the end of each new steady state through the 50 and 160  $\mu\text{m}$  characterized ports. In addition, the dry weight of the yeast changed from 4.6 to 2.4 g/L,  $Y_{\text{xs}}$  decreased from



**Figure 2.1** Schematic diagram of a single stage continuous culture system.

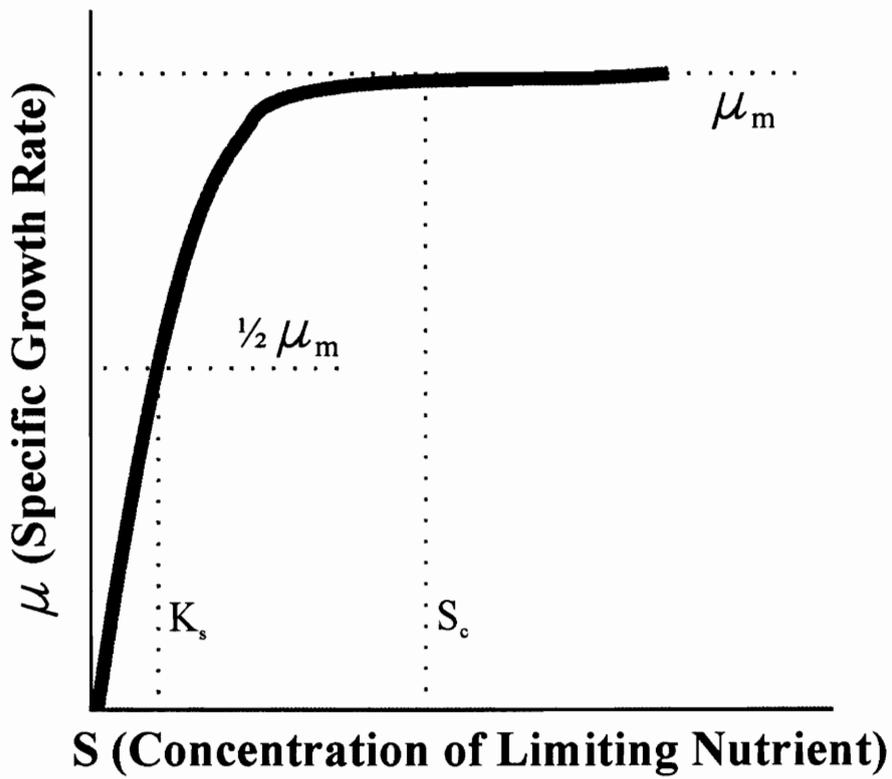
0.42 to 0.24 g cells/g glucose consumed, and the protein synthesis rate decreased from 2.94 to 1.07 mg protein/mg glucose/min when the medium feed was changed from the 50 to the 160  $\mu\text{m}$  characterized port. Another factor considered in continuous culture is that the cell culture does not change in any of its characteristics - *i.e.* a “common” cell type is seen across different dilution rates. Work by Hill and Robinson (1988) have shown this assumption to be incorrect. The volume and morphology of *S. cerevisiae* dramatically changed with changes in continuous culture dilution rate while few changes were seen in yeasts grown in batch. Their work also showed that the growth rates of *S. cerevisiae* and the continuous culture flowrates were not directly responsible for the morphological changes. The authors concluded that some unknown environmental condition arising in the medium during continuous culture was the cause. Changes in cellular morphology were also reported by Veldkamp (1976) where the surface to volume ratio of a *Spirillum* sp. and a *Pseudomonas* sp. (both unspiciated organisms isolated from a natural water source) decreased as the dilution rate in the chemostat was increased.

In theory, the operation of a single stage continuous culture fermentor is straightforward when using a complete (non-limiting) medium. In this situation, the cells in the fermentor are growing at the maximum specific growth rate ( $\mu_m$ ) permitted under those experimental conditions. In Figure 2.1, a constant flow (F) (and thus a constant D) of substrate from the reservoir ( $S_r$ ) is fed into the fermentor. Cells multiplying in the fermentor use a portion of the supplied substrate to produce biomass (X) and product (P). Since the culture volume in the fermentor is fixed and a flow is continuously leaving the fermentor, X, S, and P are also exiting the vessel. The difference between the production of cells in the fermentor (based on  $\mu_m$ ) and the rate of cells leaving the fermentor (based on D) provides a condition where a constant biomass (X) is maintained in the fermentor. This is termed steady state. With this constant X, a constant S and P are also reached in the fermentor. In conditions where  $D < \mu_m$ , X and P increase while S decreases until new steady state conditions of each are reached. Where  $D > \mu_m$  the opposite occurs and cells are eliminated from the fermentor (washout). Thus, in production one needs to balance the desire to have a higher X to provide a faster

conversion rate (*i.e.* a higher resulting P with a lower D) with the wish for higher volumetric productivity (*i.e.* a lower resulting P with a higher D). Care must also be taken to ensure that the flow of medium entering the fermentor does not lead to washout of the cells.

Where the medium used is limiting in one or more nutrients, the specific growth rate ( $\mu$ ) of the organism in the fermentor cannot reach  $\mu_m$ . The biomass concentration in continuous culture under these conditions will be less than that where all nutrients are in excess. Consequently the ethanol production rate will decrease, reducing profit for the producer. In such a case the specific growth rate of the organism is governed by the concentration of the limiting nutrient. A typical graph of  $\mu$  vs. concentration of a limiting nutrient under batch conditions appears as in Figure 2.2. There,  $\mu$  increases as the concentration of the limiting nutrient increases to  $S_c$ . Once  $S_c$  is reached, further increases in the limiting nutrient do not result in increases in  $\mu$ , and  $\mu_m$  is reached. In a continuous culture utilizing a nutrient limiting medium, the biomass concentration in the fermentor is now governed by D and by S. Another parameter of interest in Figure 2.2 is  $K_s$  (traditionally called the Monod constant), which is defined as the concentration of limiting nutrient which provides half maximum specific growth rate, a value used in modeling equations.

In fuel alcohol production, many different sources of carbohydrates have been used in fermentation. Media based on grains, molasses, whey, waste candies, spoiled fruit, potato waste, offspec glucose and fructose syrups, and waste soft drink syrups have been used as feedstocks for fuel alcohol production (Monceaux and Madson, 1999). It is hoped that cellulose will also be used in the near future. Care must be taken in each medium to provide the yeast with all its necessary nutrients in order to strive for the highest  $\mu_m$  possible for the conditions used in continuous culture. Unlike batch fermentation with these feedstocks where, in some cases, longer fermentation times can be used to compensate for the decreased concentration of required nutrients, continuous fermentation is extremely sensitive to  $\mu$ . Washout can easily occur if a value for D is used where it is assumed that the medium is nutritionally complete. This potential danger



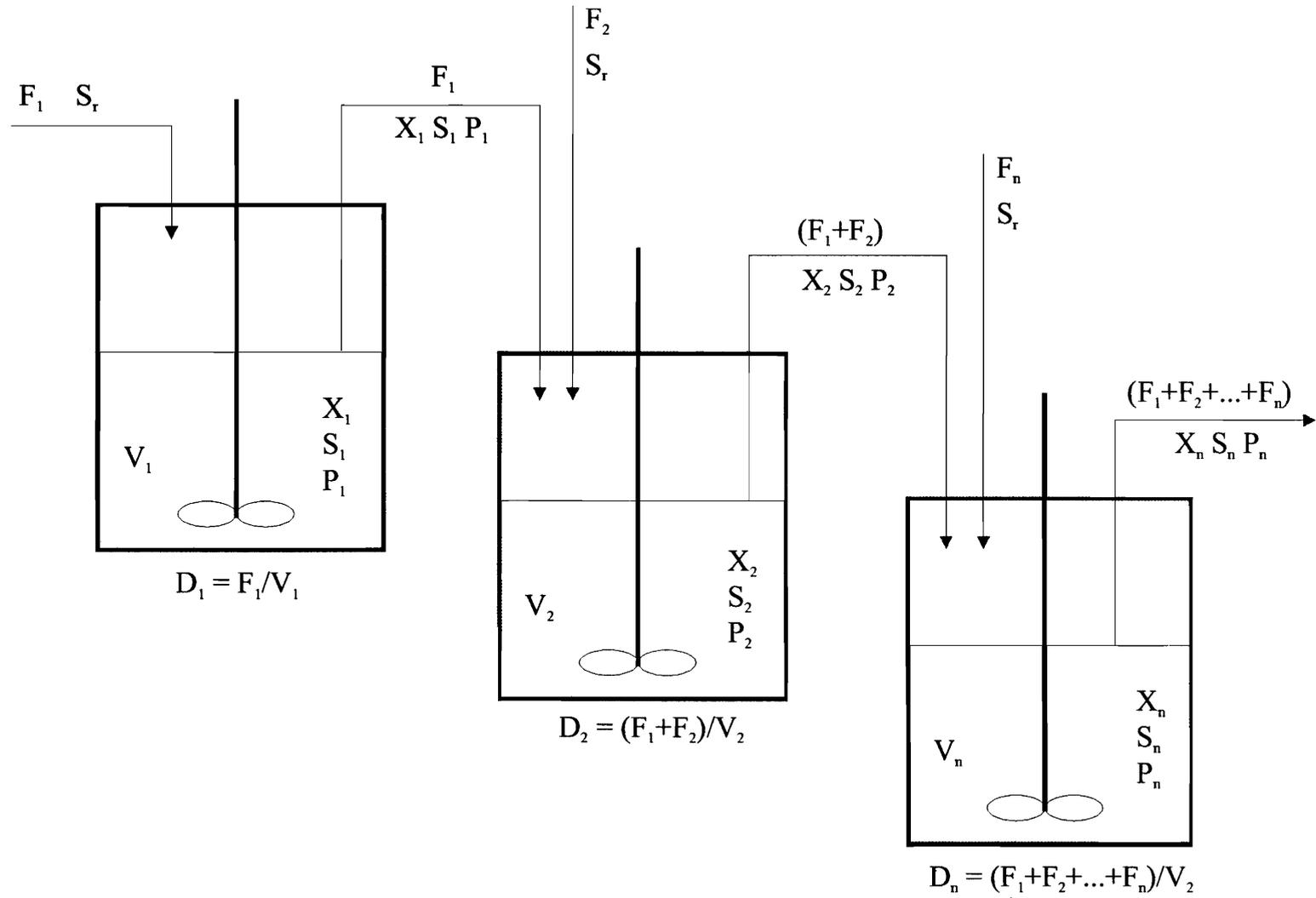
**Figure 2.2** Specific growth rate of an organism under increasing concentrations of a limiting nutrient under batch conditions.

requires the fuel alcohol producer to ideally know both the exact composition of the medium used, and what is nutritionally required by the yeast used in the fermentation. In addition, many of the feedstocks proposed for use in fuel alcohol production may be deficient in more than one required nutrient. The effects of multiple nutrient limitation on  $\mu$  in continuous culture have been identified (Bailey and Ollis, 1986), but research is lacking on the behaviour and productivity of organisms in continuous culture under these conditions.

Examples exist in industry for the use of single stage continuous culture for the production of ethanol. The successful fermentation of beet juice to ethanol in a distillery under continuous culture (with cell recycle) yielded a maximum ethanol productivity of 15 g/L/h (Ramirez and Boudarel, 1983). A review by Pilkington *et al.*, (1998) documents the industrial use of an immobilized continuous culture. In the conversion and startup of a commercial fuel alcohol plant from batch to continuous operation, Brodl (1992) concluded that a steady state operation of continuous fermentation system allowed better design and utilization of equipment. A two stage continuous fermentation system utilizing an ethanol resistant strain (IFGB 0762) was designed and planned to be adapted in a large scale batch pilot plant where the ethanol productivity was expected to increase to 3 g/L/h from the 0.8 g/L/h seen in batch operations (Dellweg and Luca, 1988). Commercial fuel alcohol plants utilizing a proprietary Biostil® process that relies on single stage continuous fermentation have been installed and operated successfully in more than 30 plants (Danielsson, 1992). The advantages offered by the Biostil® process include utilization of concentrated feedstock, higher ethanol yield, and a reduced amount of effluent from the plant.

#### **2.1.1.2.2 Multistage.**

In multistage continuous culture fermentations, a number of fermentors are connected serially in a train as depicted in Figure 2.3. In its simplest form, the multistage system has only one medium feed input ( $F_1$ ) and the fermentor volumes are identical.



**Figure 2.3** Schematic diagram of a multistage continuous culture system.

Here, medium (limiting or non-limiting) enters the first fermentor at rate  $F$  and the overflow from each fermentor enters the subsequent fermentor at rate  $F$  - thus the dilution rate for each fermentor is identical. Consumption of substrate and the concentrations of biomass and product ( $S$ ,  $X$ ,  $P$  respectively) in the first fermentor follow the principles outlined for a single stage continuous culture system (Section 2.1.1.2.1). In subsequent fermentors, the resulting concentrations of  $S$ ,  $X$ , and  $P$  become more complicated, as the concentrations of  $S$ ,  $X$ , and  $P$  in a given fermentor rely not only on the amount of biomass produced (or lost), but also on the incoming concentrations of  $X$ ,  $P$ , and  $S$  provided from all previous fermentors.

One advantage of multistage continuous culture systems is that the production of biomass and the production of product(s) can be separated into different physical stages - allowing the conditions in each corresponding stage to be set for the maximal production of either biomass or product(s). This advantage has been explored by Callow and Pirt (1961) who indicated that although a single stage chemostat permitted maximum production of bacterial cells, it would be inadequate for the production of extracellular product. The same authors have devised and tested a two stage serial chemostat system where the first and second stage volumes were from 2-3 and from 6-14 L respectively. Optimal conditions for cell growth could now be provided in the first chemostat while optimal conditions for product formation could be provided in the second.

A single feed multistage continuous culture system in some cases is required to allow complex media to be completely utilized. Harte and Webb (1967) found that *Klebsiella aerogenes*, when grown on a mixture of glucose and maltose in a single feed single stage continuous system at high growth rates, preferentially exhausted glucose while maltose utilization was repressed. This resulted in a wastage of substrate (maltose) leaving the single stage fermentor. By adding a second stage to their system, *K. aerogenes* was now able to completely utilize maltose (without glucose repression) from the medium in the second stage. With the two stage system, both glucose and maltose were completely utilized. These results have important implications for the fuel alcohol producer - particularly those who use a blend of substrates from a variety of sources. A

one-stage continuous culture fuel alcohol plant may not utilize a portion of their blended medium - lowering expected ethanol yields and profitability. More research is needed on multistage continuous systems utilizing complex media.

Ethanol production by immobilized *Zymomonas mobilis* in a three stage single feed continuous culture system was accomplished by Klein and Kressdorf (1986). A maximum stable ethanol productivity of 108 g/L/h was achieved with a very high dilution rate of 4 h<sup>-1</sup>. The system was run for four weeks under nonsterile conditions since the high dilution rate used did not allow bacterial contaminants to establish in the fermentors. An equally impressive continuous fermentation run of 442 days was achieved in the production of beer with immobilized *S. cerevisiae* in a two stage reactor (Virkajarvi, 1998). No problems with contamination or yeast viability were reported with the flows used. The authors state that the only reason for terminating the run was because of a mechanical break in the column lid.

In a more complicated multistage continuous culture system, the flow of medium into the system occurs in the first fermentor only (F<sub>1</sub>), but the volumes of the fermentors are changed. Thus, the resulting dilution rate for each fermentor in the system can be varied to meet the needs of the organism in any fermentor. In the production of fuel alcohol by multistage continuous systems, the first fermentor is typically larger to provide a lower D (*i.e.* higher retention time) for the first fermentor only. This provides an opportunity for a larger concentration of biomass to form in the first fermentor which subsequently provides a higher conversion rate of glucose to ethanol (due to the higher X) in all fermentors in the system. Similarly, a single feed, serial three stage continuous system utilizing *S. cerevisiae* to produce ethanol was utilized by Wall and Hill (1992) which had decreasing fermentor working volumes from the first to the third fermentor. Each reactor volume was adjusted so that the overall total conversion achieved was 99.9 and 99.99% of the theoretical value. Klein and Kressdorf (1983) achieved a stable ethanol productivity of 74 g/L/h with immobilized *Z. mobilis* in a three stage continuous culture system with different working volumes in each fermentor. The system was operated continuously for 65 days at a dilution rate > 2 h<sup>-1</sup> that prevented any

contaminants from growing in the system.

In a distributed feed continuous culture system, the flow of medium into the system occurs across two or more fermentors in the system ( $F_1$ - $F_n$ ). The value of  $D$  in each fermentor depends on the sum of the medium flows from previous fermentors. In the case where all fermentor working volumes are equal, the dilution rate generally increases downstream from the first fermentor and reaches a maximum value in the last fermentor. By manipulating the fermentor working volumes, any desired dilution rate can be achieved in any fermentor in the distributed feed system. In a distributed feed system, fresh medium is available to microbes in later fermentors in the multistage system whereas nutrient depletion may occur in a single feed system. In particular, the distributed feed continuous culture system is an advantage for production where the medium is inhibitory to growth or product formation (Pirt, 1975). The inhibitory action in the medium may stem from high substrate concentration (conditions present in VHG fermentations) or from a toxic substance present in the original medium. In a distributed feed system, the concentration of the inhibitory substrate can be adjusted in each fermentor to desired values. A six stage distributed feed continuous culture system with identical working volumes utilizing *S. cerevisiae* to make ethanol was researched by Dourado *et al.* (1987). Experimental data with distributed feeding was compared to data obtained from models derived for a distributed feed continuous culture system. The authors found that, out of the large number of possible base equations to use for their model, the equations from the work by Ghose and Tyagi (1979a) provided data that most closely matched the actual experimental data. Using static optimization techniques, Dourado and Calvert (1987) mathematically analysed the results of Dourado *et al.* (1987) and determined the relation between the strength of the substrate inhibition (as  $K_{si}$  which is defined the concentration of substrate (greater than that providing  $\mu_m$ ) which reduces an organism's  $\mu_m$  by 50%) and the number of fermentors participating in distributed feeding in a six stage serial continuous culture system. With a set ethanol concentration of 80 g/L leaving the system, the authors found that the ethanol productivity (g/L/h) increased when  $K_{si}$  was equal to 50 g/L (high substrate inhibition) as the number of

fermentors which participated in distributed feeding increased. With  $K_{si} = 150$  g/L (moderate inhibition) and 250 g/L (low inhibition), little or no increase in ethanol productivity was seen. At a set ethanol concentration of 30 g/L, large increases in ethanol productivity were seen with all  $K_s$  values as the number of fermentors involved in distributed feeding increased.

An example of an industrial fuel ethanol plant utilizing a distributed feed continuous culture system is outlined by Pascal *et al.* (1995). Eight fermentors in the plant were connected serially and were operated in continuous mode. Only beet juice was fed to the first fermentor while beet molasses (65% sucrose) was distributed to the first four fermentors. The last four fermentors in the continuous system were used to ensure complete consumption of sugars. Models constructed by the authors to simulate the distributed feed system were validated by on-site measurements from the production plant.

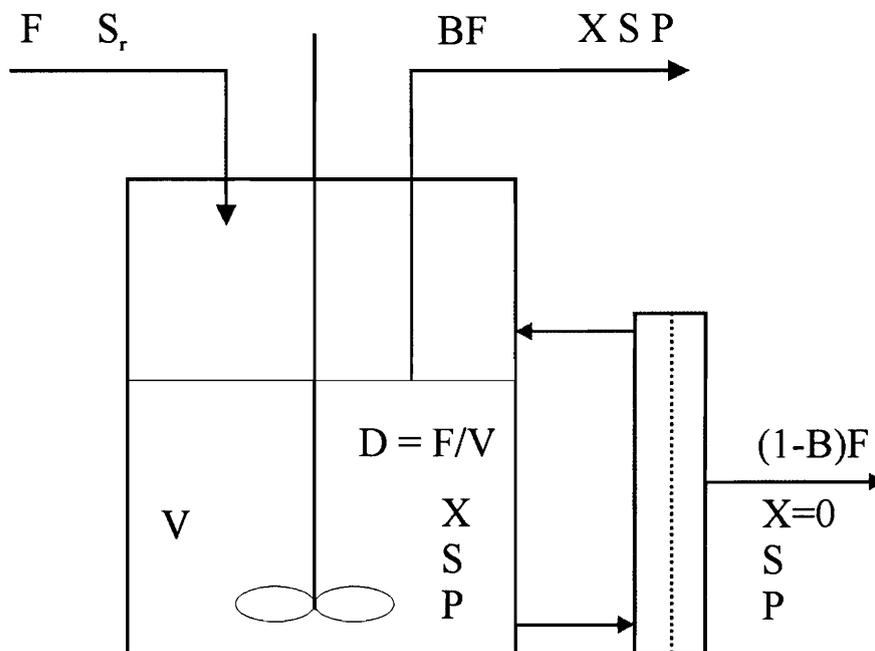
Many papers have dealt with the advantages of multistage as compared to single stage continuous culture systems. Herbert (1962) has shown that at identical dilution rates, a higher product yield was achieved and a more efficient use of substrate was seen in a multistage system than in a single stage continuous system. In a mathematical analysis of single stage and multistage backmix systems, Bischoff (1966) showed that two equal volume, continuous backmix reactors had a total residence time that was less than one reactor with the combined volumes of the two smaller reactors. In like manner, Ghose and Tyagi (1979b) showed mathematically (based on actual experimental data) that the required total residence time (42.4 h) of a single stage CSTR producing ethanol with *S. cerevisiae* was dramatically reduced (to 18.0 h) by utilizing two equal sized fermentors in series that had a combined volume equal to the single stage CSTR. In other work, Tyagi and Ghose (1980) have been able to increase the dilution rate from  $0.1 \text{ hr}^{-1}$  with three stages to  $0.14 \text{ h}^{-1}$  with four stages - producing the same amount of ethanol (86.5 g/L) in both systems. Dourado and Calvert (1987) derived a complex mathematic model to determine the optimal number of fermentor stages required in a multistage system to maximize ethanol productivity when ethanol inhibition kinetics were included.

The experimental results of Wall and Hill (1992) showed that a properly designed serial multistage continuous culture system achieved higher substrate conversions (with a higher throughput of materials) than a single stage continuous system. In addition, the authors found that a larger amount of glucose exited a single stage system at a  $D$  of  $0.14 \text{ h}^{-1}$  as compared to their three stage serial system running at a  $D$  of  $0.23 \text{ h}^{-1}$  (*i.e.* operating at a higher volumetric flowrate). Nishiwaki and Dunn (1999) have calculated that a two stage cell recycle continuous culture chemostat always had a total fermentor volume less than a single chemostat with cell recycle.

It has been documented that in a commercial continuous fuel ethanol plant, the ethanol levels in a five fermentor multistage fermentation system may have the following profile: 6-7% (v/v) in the first two fermentors (first two both receive mash), 7-8% (v/v) in the third fermentor, and 10-11% (v/v) in the last two fermentors (Kelsall, 1995). Such a multistage system is in use in some fuel alcohol plants.

#### **2.1.1.2.3 Cell recycle.**

A cell recycle continuous fermentation system is depicted in Figure 2.3. Here medium enters the fermentor from the reservoir ( $S_r$ ) at a flow rate  $F$  as shown in continuous culture systems (Figs. 2.1, 2.3). The fermentor contents are recirculated through a device which separates the cells from the fermentor contents when a flow is removed from the system. A portion of the flow exiting the fermentor ( $BF$ ) leaves the fermentor with concentrations of  $X$ ,  $S$ , and  $P$  as found in the fermentor. The remaining portion of the flow exiting the fermentor ( $(1-B)F$ ) is taken from the device which concentrates the cells returning to the fermentor and leaves no cells exiting with this flow. By decreasing  $B$  (the bleed ratio), the biomass concentration ( $X$ ) in the fermentor can be increased to levels which cannot be achieved in any other continuous culture system. This higher biomass concentration results in a faster conversion of substrate to product, a higher concentration of product, and less substrate leaving the system. Under conditions where  $B = 0$  the system operates under conditions termed total cell recycle, when



**Figure 2.3** Schematic diagram of a single stage cell recycle continuous culture system.

$0 < B < 1$ , the system operates under conditions termed partial cell cycle, and when  $B = 1$  the system operates as a classical CSTR. In experiments involving partial cell recycle, the models generated by Lee *et al.*, (1983) from experimental data predicted a 400 to 500% increase in ethanol productivity in partial cell recycle fermentations as compared to fermentations where cell recycle was not used. The biomass concentration achieved in a cell recycle continuous culture system is governed by the specific growth rate  $\mu$  of the culture (which is governed by the medium composition and the environmental conditions), the biomass concentration ( $X$ ) in the fermentor, and the fraction ( $B$ ) of the medium inlet flow ( $F$ ) that leaves the fermentor natively and through the cell concentrating device.

An apparatus has been designed and used as a cell concentrating device. The most typical system is the use of a membrane filter. Ethanol productivities as high as 70 g/L/h in a single stage cell-recycle continuous fermentations with cross-flow membrane filtration have been reported whereas only 8.6 g/L/h was obtained in similar conditions without cell-recycle (Warren *et al.*, 1994). Lee and Chang (1987) used a hollow-fiber membrane filter with tangential flow in their single stage cell recycle continuous system to produce an ethanol concentration of 65 g/L and an ethanol productivity of 85 g/L/h when 140 g/L glucose was used as the feed. Interestingly, their results also showed that specific ethanol production decreased continuously as  $X$  increased, while the yeast growth rate decreased only after  $X$  was greater than 50 g/L. The authors extrapolated their findings and determined that at an  $X$  of 255 g/L, cell growth would stop while at an  $X$  of 640 g/L, ethanol production would stop. No explanations were given by the authors for the decrease in both specific ethanol production and yeast growth rates as  $X$  increased with the complex medium they used. One possibility may be that the concentration of some critical nutrients may have become limiting once a certain biomass concentration was met. A variation of the membrane filter technique was employed by Chang *et al.*, (1993) where a rigid internal stainless steel filter module was used in their single stage cell recycle system. The porous filter module had a total surface area of 440 cm<sup>2</sup> with a uniform pore size of 0.2  $\mu$ m. With a high agitation rate inside the fermentor, the authors

operated the cell recycle system continuously for 10 days and produced 50 g/L ethanol in their system at 92.7% of theoretical ethanol yield. The authors found no loss in separation performance for the filter module at the end of their experiment and suggested that longer operation times with particulate media would be possible in their system. One disadvantage of a cell-recycle system based on filtration is that the medium should not contain any particulates since the particulates would quickly foul membrane filters. Also, filters typically need changing and purging periodically which results in the system becoming contaminated. Membrane filter systems based on tangential flow are less prone to fouling since cell deposition on the membrane surface is minimized (Lee and Chang, 1987). In the fuel alcohol industry, media containing particulate materials are almost always used. This challenges the potential application of cell recycle based on membrane filtration to be used in this industry. Published data are lacking on industrial examples of cell recycle systems based on filtration.

Centrifugation can replace membrane filtration to concentrate cells from the fermentor. A fuel alcohol plant utilizing centrifugation is described by Pascal *et al.*, (1995). The plant uses a serial multistage distributed feed continuous culture system where the effluent from the last fermentor is centrifuged. A portion of the cells from the centrifugation are acid washed to kill bacteria, and returned to the “seed” fermentor (whose volume is twice as that of any other fermentor) to increase the biomass concentration in the system. One concern regarding the use of centrifugation in cell recycle systems is the potential risk of bacterial contamination since aseptic conditions are difficult to achieve during centrifugation.

Sedimentation tanks have been used in place of membrane filters in cell recycle continuous systems. The work of Davis and Parnham (1989) illustrated that an incline settling tank was capable of separating and concentrating a faster growing nonflocculent yeast from a slower growing flocculent yeast. With no cell recycle, the faster growing yeast dominated the fermentation. With partial recycle, their experiments showed that the slower growing yeast could be maintained as the dominant species in the fermentor. Unfortunately no ethanol productivity or concentration data was shown as their work

focussed on the population dynamics of both yeasts. Two major disadvantages of sedimentation methods are that the culture must be flocculent and the dilution rate in a system employing sedimentation is limited to the settling velocity of the yeast (Lee and Chang, 1987). Another disadvantage is that longer residence times may be required in sedimentation tanks to concentrate the cell culture which removes the cells for an extended period of time from the conditions provided in the fermentor.

### **2.1.2 Microbial engineering.**

Another approach for the ethanol industry is the genetic modification of an organism to enhance/create ethanol production capabilities. Modified organisms have not been used in industry to date because of their instability and concerns regarding regulation, safety, and potential release of a genetically modified organism to the environment.

*S. cerevisiae* had been genetically modified by Murai *et al.* (1997) to produce glucoamylase that is expressed and anchored to the cell wall. The constructed plasmid consisted of a glucoamylase from *Rhizopus oryzae* fused to the C-terminal half of the  $\alpha$ -agglutinin gene. The resulting *S. cerevisiae* can utilize starch directly. The batch fermentation of 10 g/L starch yielded 2 g/L ethanol by 70 h. These yields are rather low.

It has been estimated that if the cellulose fraction of grain could be utilized in fermentation, then an additional 0.3 gallons of ethanol could be produced per bushel of grain (Leathers, 1998). To this end, LaGrange *et al.* (1996) utilized a *S. cerevisiae* in which  $\beta$  xylanase activity was engineered. Their work focussed on the stability of the secreted  $\beta$  xylanase and on the continuous expression of the enzyme. The recombinant enzyme maintained more than 90% activity when incubated at 50°C for 50 minutes. Potential exists for utilizing this organism in producing ethanol; although, test fermentations were not done. Cellulosic materials contain a number of five carbon sugars (primarily xylose and arabinose) which cannot normally be utilized by *S. cerevisiae*. Attempts have also been made to genetically engineer *S. cerevisiae* to enable it to ferment

xylose to ethanol (Chen and Ho, 1993; Moniruzzaman *et al.*, 1997a; Toon *et al.*, 1997) although none of the engineered organisms are considered ready for industrial use.

*Escherichia coli* is another organism that has been genetically modified to produce ethanol from both arabinose and xylose. Moniruzzaman *et al.* (1998) constructed and tested a recombinant *E. coli* to which the genes for alcohol dehydrogenase and pyruvate dehydrogenase from *Zymomonas mobilis* were inserted into the *E. coli* genome. The resulting strain (*E. coli* KO11) fermented in batch a 91 g/L unsterilized hemicellulose hydrolysate mixture (per L: 14 g glucose, 72 g xylose, 4 g arabinose, 1 g mannose) to 42.6 g/L ethanol (93% of theoretical yield) in 96 h. Antibiotics were not needed to maintain the maximum catalytic activity of *E. coli* KO11 during fermentations - even when the fermentation were deliberately contaminated with 10% unsterile soil. Moniruzzaman *et al.* (1997b) further genetically engineered *E. coli* KO11 by inserting cellobiase and phospho- $\beta$ -glucosidase activities from *Klebsiella oxytoca*. This new construct was able to ferment 100 g/L of waste office paper to 34.5 g/L ethanol (76% theoretical yield - based on cellulose). Work by Dumsday *et al.* (1989) on *E. coli* KO11 in batch and continuous culture fermentations have shown major differences in ethanol productivity in the two modes of fermentation. Batch fermentations with *E. coli* KO11 on glucose, mannose, xylose, and galactose provided stable ethanol production through 3 repeated serial batch runs while in continuous culture, *E. coli* KO11 produced stable ethanol production only with glucose. Fermentations with any other sugar resulted in a progressive loss in ethanol production capacity and, in the case of xylose, the loss in ethanol productivity was irreversible.

## **2.2 Microbial contamination.**

In the fuel alcohol industry, *Lactobacillus* spp. are the most commonly found bacterial contaminants and they are a continual and persistent problem (Makanjuola and Springham, 1984). One negative aspect of such contamination is that faster growing (alcohol and pH tolerant) bacteria or wild yeast will quickly outnumber culture yeast and

aberrant end products will appear. Lactic and acetic acid producing bacteria are known to inhibit yeast growth and metabolism through production of their toxic metabolic endproducts (Thomas *et al.*, 2002, Narendranath *et al.*, 2001a). Another concern is that these microbes will compete with culture yeast for nutrients. As a result, nutrients are diverted to growth and end product production by the contaminant(s).

### **2.2.1 Binary populations - types of symbiosis.**

The potential for multiple interactions between two organisms raises questions as to what types of interactions are possible in a mixed culture system and how many of these interactions are at present in fuel alcohol fermentations. Some interactions in binary populations like predation (where one organism consumes another for nutrients which lowers the population of the consumed organism), parasitism (where one organism utilizes another organism for a portion of their own metabolism and survival but keeps the host alive for a period of time) and mutualism (where the interaction of both organisms are necessary for the survival of each) are not considered since these interactions do not have relevance for most *S. cerevisiae*-based fuel alcohol fermentations that are contaminated.

#### **2.2.1.1 Competition for nutrients.**

Two populations of microorganisms with similar nutrient requirements will compete for nutrients when grown together. Competition will have definite effects on the population dynamics of both microbes when at least one of the nutrients competed for in the medium is at a concentration that is rate-limiting for growth for one and/or both microbes (Fredrickson, 1977). The analysis of binary competition in a chemostat is simplified when the following assumptions are taken into account: a single nutrient influences the specific growth rate of each organism; none of the nutrients in the medium pose inhibitory effects for either organism; mixing in the chemostat is perfect; dilution

rate and temperature are constant; and no wall growth occurs. In this ideal situation, an analysis of a plot of specific growth rate ( $\mu$ ) against concentration of rate limiting nutrient (S) shows that if microbe A has a higher specific growth rate than microbe B for all concentrations of the rate-limiting nutrient, then microbe B will be eventually excluded from the chemostat no matter what dilution rate is chosen. However, if the specific growth rate of each microbe crosses at  $\mu_c$  at a rate-limiting nutrient concentration of  $S_c$ , then the outcome of competition will depend on D. If  $D > \mu_c$  then the microbe that grows more slowly at concentrations of rate-limiting nutrient greater than  $S_c$  will be excluded while at  $D < \mu_c$ , the other microbe will be excluded. Thus, the microbe that eventually establishes itself in the chemostat is based entirely on D. The validity of this analysis has support from many studies where a change in dilution rate determined the microbe that dominated in the chemostat (Jannash, 1967; Meers, 1971; Harder and Veldkamp, 1971).

In one study, competition for thiamine in grape must fermentation by a wild-type yeast led to a sluggish fermentation by *S. cerevisiae* (Bataillon *et al.*, 1996). These authors observed that a wild yeast in the fermentation depleted the thiamine in the medium in a few hours, which immediately affected the growth and ethanol production of *S. cerevisiae*. Although *S. cerevisiae* is able to produce thiamine, the presence of thiamine in the grape must stimulated further the growth and ethanol production of *S. cerevisiae*.

To date, research involving nutritional competition in fuel alcohol fermentations involving *S. cerevisiae* and *Lactobacillus* spp. is lacking. A thorough analysis of the requirements of both organisms may provide key differences which may be exploited in the production of media that could be used to limit contamination by *Lactobacillus* spp. Examples exist where the reverse is true. The review article by Vandevoorde *et al.* (1992) concluded that since lactic acid bacteria do not need iron for their metabolism, environments where iron is limiting or absent will favour their dominance. Another avenue of research that may lead to the control of contamination by nutrition is the examination of the specific growth rates of each organism for a number of nutrients at increasing concentrations. Specific nutrient concentrations may favour a higher specific

growth rate for *S. cerevisiae* than for the contaminant. Media formulation could thus be altered to provide a competitive advantage for *S. cerevisiae*.

### 2.2.1.2 Commensalism.

The definition of a commensalistic interaction is where one organism benefits by the interaction with a second organism but the second organism is not inhibited by the first (Fredrickson, 1977). One example of this is the production of ethanol by a mixed culture of *Kluyveromyces marxianus* IMB3 and *Talaromyces emersonii* CBS 814.70 on starch as shown by Ward *et al.* (1995). *K. marxianus* grew and produced ethanol from glucose but not from starch. *T. emersonii* grew on starch and also produced a high amount of extracellular amylase. When both organisms were co-cultured in batch on a 4% (w/v) starch medium, 12 g/L ethanol was produced by *K. marxianus* in 65 h. This compared to an ethanol concentration of 15 g/L when the amylase enzyme from *T. emersonii* was added to the fermentation instead of adding the *T. emersonii* as a co-inoculant. The 3 g/L drop in ethanol concentration was attributed to the necessary diversion of some glucose for the production of *T. emersonii* biomass. The work of Daeschel *et al.* (1988) showed that benefits may arise when using mixed cultures. In batch fermentations with cucumber juice at 30°C for six days, *S. cerevisiae* produced 86 mM (4.0 g/L) ethanol, while in a mixed culture where *Lactobacillus plantarum* was added 24 h after inoculation with *S. cerevisiae*, 92 mM ethanol was produced. No ethanol was produced when only *L. plantarum* was used.

Although most mixed culture fermentations involving *S. cerevisiae* and *Lactobacillus* sp. for fuel alcohol production result in some level of competition for nutrients and inhibition by toxic byproducts, there may be unexplored benefits in having both organisms together in the fermentor. For example *S. cerevisiae* yeasts are unable to use protein from the medium as a source of utilizable nitrogen since they lack extracellular protease activity. Most lactobacilli are able to hydrolyse protein extracellularly and thus may provide a source of utilizable nitrogen that previously was

unavailable to the yeast. This would be classified as a commensal relationship if the amount of lactic acid produced by the contaminant would be below the threshold amount required to inhibit *S. cerevisiae* and if the amount of growth of the contaminant does not deplete critical nutrients required by *S. cerevisiae*. Since competition for nutrients does occur with both organisms the overall relationship would be classified as commensal and competitive.

### **2.2.1.3 Protocooperation.**

In protocooperation, both organisms benefit by their interaction in mixed culture as compared to individual cultures of each. Unlike mutualism, the survival of each organism does not depend on the other. One example of protocooperation is with yogurt production using *Lactobacillus bulgaris* and *Streptococcus thermophilus* (Veldkamp, 1976). The growth of *S. thermophilus* in milk is limited since the amino acid content in milk is low and the streptococcus is unable to hydrolyse milk proteins. *L. bulgaris* can however hydrolyse milk protein and thus increases the growth of *S. thermophilus* when the two are present. *L. bulgaris* requires formate for optimal growth which is provided by *S. thermophilus* under anaerobic fermentation of lactose. Both organisms grow better in milk together (as compared to individually) which results in more acid for yogurt production.

A good possibility exists that a fuel ethanol fermentation contaminated with *Lactobacillus* may provide additional available nitrogen to the yeast through the hydrolysis of protein. What is not known is whether the yeasts provide nutrients to the lactobacilli. As most lactobacilli are fastidious organisms, the possibility exists that the yeast may produce a complex nutrient that the lactobacilli would need that may be limiting in the medium used.

## 2.2.2 Inhibition by metabolic endproducts.

The majority of the studies involved with the inhibition of *S. cerevisiae* in fuel alcohol production center around the production of lactic and acetic acids by one or more contaminants.

### 2.2.2.1 Lactic acid.

The inhibitory effects on yeast of a number of fermentation end products in continuous culture conditions have been assessed. Ethanol, formic acid, lactic acid, 1-propanol, 2-methyl-1-butanol, 2,3-butanediol, acetaldehyde, and glycerol were added to yeast that had achieved steady state conditions. The concentration of each by-product that resulted in an 80% decrease in yeast cell density was then recorded. In particular, the concentration of lactic acid that resulted in an 80% reduction in yeast density was 38 g/L (Maiorella et al., 1983). No effects on yeast cell morphology were seen with lactic acid at its inhibitory concentrations. The work of Ngang *et al.* (1990) demonstrated a difference in the specific ethanol production rate and yeast growth in batch when lactic acid was either added directly to the fermentor or produced by inoculated *Lactobacillus casei* var *pseudopiantarum* (a homofermentative lactic acid bacterium). The medium for all conditions was adjusted to pH 5.0. At a lactic acid concentration of 8 g/L, approximately 60% of the yeast growth rate and 90% of the specific ethanol production rate remained when lactic acid was added directly to the fermentor. With *L. casei* var *pseudopiantarum*, the corresponding values were 5% and 30%. The authors subsequently showed that the depletion of nutrients could not have been the reason for the difference in inhibition.

In other work, yeast cell viability also decreased when backset recovered from fermented mash and contaminated with *L. fermentum* or *L. delbrueckii* was recycled in sequential 14<sup>o</sup>P wheat mash batch fermentations. The lactic acid concentration attained in the fifth sequential fermentation was 14 g/L which reduced the viability of the yeast by

60% (Chin and Ingledew, 1994). Other authors reported that a lactic acid concentration of 8 g/L in beet molasses batch fermentation reduced yeast viability by 95% and alcohol production rate by 80% (Ngang *et al.*, 1990). Additional work showed effects on yeast by lactic acid at 0.9% (w/v) and by acetic acid at 0.04% (w/v) (Narendranath *et al.*, 2001a). It was concluded in the latter study that *Lactobacillus* inhibition of alcohol production by yeast was not linked to competition for sugar since a high concentration of glucose (125 g/L) remained unused. Lactic acid accumulation in sequential 18°Brix 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 (Oliva-Neto and Yokoya, 1994). Morphological changes in the yeast cells were also visible at this concentration of lactic acid - yeast cells became elongated and yeast buds were irregular under conditions with lactic acid present.

Reduction in ethanol production by yeast due to the synthesis of lactic acid by various lactobacilli was studied by Hynes *et al.* (1997). In wheat mash fermentations to which  $1 \times 10^5$  lactobacilli and  $1 \times 10^6$  yeast per g mash were added at the start of fermentation, the amount of ethanol loss at the end of batch fermentation was strain specific. *Lactobacillus paracasei* reduced the ethanol production by 6.5% (of the total) with the production of 11.76 mg/ml lactic acid. Other lactobacilli tested were *Lactobacillus plantarum* with a loss of 6.3% ethanol, *Lactobacillus rhamnosus* with a loss of 3.2%, and *Lactobacillus delbrueckii* with a loss of 0.41%. Reductions in ethanol concentration between 3.8 and 7.6% (of the total) were determined with different strains of lactobacilli in normal gravity wheat mash fermentations when  $10^9$  CFU bacteria /ml was introduced to  $10^7$  CFU yeast/ml (Narendranath *et al.*, 1997).

#### **2.2.2.2 Acetic acid.**

Acetic acid was added to yeast that had achieved steady state conditions in continuous fermentation. The concentration of acetic acid that resulted in 80% reduction in yeast biomass was 7.5 g/L as compared to lactic acid at 38 g/L - reaffirming that acetic

acid is more inhibitory than lactic acid under similar conditions (Maiorella *et al.*, 1983). Furthermore, acetic acid at its inhibitory concentration changed the yeast cell morphology. Cells became irregular and elongated. The growth of *Pectinatus cerevisiophilus* with *S. cerevisiae* in brewers wort medium with a cell concentration of each at  $2.5 \times 10^5$  cells/ml also inhibited the growth of *S. cerevisiae* (Chowdhury *et al.*, 1997). The authors concluded that acetic acid produced by *P. cerevisiophilus* completely inhibited the growth rate of *S. cerevisiae* at a concentration of 1.6 g/L when the wort medium was controlled at a pH of 5.2. Pampulha and Loureiro-Diaz (1989) reported a good correlation between intracellular pH and fermentation rate of *S. cerevisiae*. The addition of acetic acid to the fermentation resulted in a decrease in the fermentation rate and in the intracellular pH of *S. cerevisiae*. The authors concluded that the intracellular pH did not depend on the total concentration of acetic acid but rather on the amount of undissociated acid that could diffuse across the cell wall from the medium and cause an acidification of the cytoplasm. The amount of undissociated acetic acid that is present in the medium is dependant on the total amount of acetic acid and the fermentation pH. With acetic acid, no fermentation by *S. cerevisiae* was observed when the resulting intracellular pH decreased below approximately 4.5 with any combination of medium pH and total concentration of acetic acid. The inhibition of *S. cerevisiae* primarily by the undissociated forms of lactic, acetic, and other short chain carboxylic and fatty acids is also supported by other publications (Alexandre and Charpentier, 1998; Cásio *et al.*, 1987; Capucho *et al.*, 1994; Eklund, 1983; Kashket, 1985; Narendranath *et al.*, 1997; Narendranath *et al.*, 2001b; Verduyn *et al.*, 1990a; Verduyn *et al.*, 1990b; Verduyn *et al.*, 1992). An acetic acid concentration as little as 0.04% (w/v) severely limited yeast growth and fermentation in a complex batch medium (Narendranath *et al.*, 2001a), although in other work, no reduction in the specific growth rate ( $\mu$ ) or growth yield ( $Y_{xs}$ ) of *S. cerevisiae* by acetic acid (0 to 10 g/L) was observed at pH 5.5 in defined medium (Palmqvist *et al.*, 1999). Inhibition of *S. cerevisiae* by the production of acetic acid by *Lactobacillus kunkeei* was demonstrated by Edwards *et al.* (1999). Their work demonstrated that the minimal inhibitory concentration (MIC) of acetic acid varied from

4.5 to 7.5 g/L in Chardonnay grape juice (pH adjusted to 3.8) depending on the strain of *S. cerevisiae* used. The authors also demonstrated that the MIC values obtained in their fermentations could not have been due solely to the presence of acetic acid generated by *L. kunkeei*. The diffusion of the undissociated form of acetic acid to the cytoplasm of glucose-grown *S. cerevisiae* at pH 3.0 increased 4 fold when ethanol in the medium was increased from 1.6 to 6.3 g/L (Casal *et al.*, 1998). This synergism of ethanol and acetic acid was postulated by the authors to explain the low tolerance of *S. cerevisiae* to conditions where these two fermentation products were present.

### **2.2.3 Control of contamination.**

#### **2.2.3.1 Antibiotics.**

Most of the literature that deals with the use of antibiotics refer to batch fermentations - little attention has been paid to studying the effects of antibiotics on continuous fermentations for fuel alcohol production. The sensitivity of an organism to antibiotics has been shown to vary in a generic continuous culture depending on the dilution rate chosen and the limiting nutrient. *Pseudomonas aeruginosa* when cultured at a dilution rate of 0.05 in a base medium limiting in either Mg, C, or P showed altered survivor profiles when exposed to 50 units/ml of polymixin (Dean *et al.*, 1969). The survivor profile (expressed as % of nutritionally sufficient controls) for Mg, C, and P, was 104%, 62%, and 1.1% respectively. As the dilution rate was increased, the % survivors decreased in each, until at a dilution rate of 0.50, the % survivors for each condition was 92%, 0.8%, and 0.15% respectively. Questions regarding bacterial resistance to antibiotics in long-term operation of continuous fuel ethanol systems have not been addressed.

In an attempt to control growth of contaminating *Lactobacillus* during fuel alcohol production, antibiotics such as penicillin have been frequently used during fermentation. In batch fuel alcohol production using blackstrap molasses, penicillin at

concentrations as high as 500,000 U/L does not affect yeast performance and doses as low as 500 U/L effectively control contamination (Aquarone, 1960). Unfortunately penicillin, although inexpensive, is degraded relatively quickly at the pH (~4.0) of many fuel alcohol fermentations (Kelsall, 1995) although it is still adequately effective at pH 4.5 (Narendranath, 2000). The stability of penicillin G has also been determined in a complex medium containing corn steep powder and lactose (Kheirrolomoom *et al.*, 1999). In that work, the authors incubated the penicillin G in buffer solutions at discrete temperatures ranging from 0°C to 52°C and at pH values ranging from 1.8 to 10. The resulting experimental data was mathematically analysed to give an overall equation (with excellent agreement over a large volume of experimental data) describing penicillin decomposition rate as a function of pH and temperature:

$$C = C_o \times e^{-\left(10^{(-64.29 - 1.825 \text{ pH} + 0.4144T + 0.1280 \text{ pH}^2 - 0.0006187T^2)}\right) \times t} \quad (2.1)$$

where C is the concentration of penicillin G (mol/L) at time t, C<sub>o</sub> is the initial concentration of penicillin G (mol/L), T is the absolute temperature of the solution (K), pH is the pH of the solution, and t is the elapsed time (h). Maximum stability of penicillin G occurred at a pH of 6.0 at 0°C. Decreasing stability was seen as the temperature was increased and as the pH was increased or decreased from a pH of 6.0. Thus, in fuel alcohol production at 28°C with fermentors controlled to a pH of 4.0, equation 2.1 predicts that only 49.8% of the original penicillin G would remain after 3.2 hours of incubation and only ~ 1% would remain after 21.1 hours. In other work, an intriguing proposed schedule of penicillin addition was mathematically determined for a situation where unsterilized sugar cane molasses was fermented in repeated sequential batches (Borzani, 1986). Unfortunately, the author did not conduct experiments to verify the derived equations.

It is well known that penicillin (functional against Gram positive microbes) reduces the levels of a number of microbes that pose a risk to fuel ethanol production

(Stroppa, 2000). In batch media containing 4% w/v glucose and up to 4.0 mg/L penicillin, *Lactobacillus fermentum*, *Lactobacillus plantarum*, *Lactobacillus buchneri*, *Bacillus coagulans*, *Bacillus megaterium*, and *Bacillus subtilis* were inoculated at  $10^4$  cells/ml in separate 120 ml batch runs and incubated at 32°C for 48 hours at 150 RPM. At a dosage of 4.0 mg/L penicillin, *L. fermentum* and *L. buchneri* levels decreased extensively - less than 10 CFU/ml remained for each organism after 48 hours. Under the same conditions, *L. plantarum*, *B. coagulans*, *B. megaterium*, and *B. subtilis*, decreased by 1.05, 1.69, 1.61, and 1.67 log units from the original inoculation level. The authors concluded that *L. plantarum* and *L. fermentum* were the most and least resistant organisms tested with penicillin. It should be noted that *Bacillus* species are rarely found in fuel alcohol fermentations.

Tetracycline (functional against Gram positive and negative microbes) has been examined for use in fuel alcohol fermentations. Doses up to 20 mg/L in batch fuel alcohol production using blackstrap molasses (with contaminants normally found in molasses) were evaluated (Aquarone, 1960). In that study, ethanol concentration increased from 9.3% v/v to 10.4% v/v when 1.2 mg/L tetracycline was used. No further increases in ethanol content were observed with higher amounts of tetracycline. The same authors concluded that at a dosage of 1.5 mg/L to 30 mg/L, no discernable effects on yeast growth or ethanol production were seen with tetracycline. The effectiveness of chlortetracycline and oxytetracycline have been evaluated in grain based alcohol batch fermentations where  $1 \times 10^5$  bacteria (from contaminated commercial fuel alcohol fermentations) were present at the start of fermentation (Day *et al.*, 1954). Compared to penicillin which was effective in controlling contamination between 0.75 and 2.0 units/ml mash, the oxytetracycline required between 20 and 40 units /ml mash and chlortetracycline required between two to 10 units/ml mash to accomplish the same control of contamination that penicillin offered.

Virginiamycin has also attracted interest as a replacement for penicillin. Virginiamycin is composed of two factors, M and S. Factor M ( $C_{28}H_{35}N_3O_7$  - a polyunsaturated cyclic peptolide) and factor S ( $C_{43}H_{19}N_7O_{10}$  - a cyclic hexadepsipeptide)

both diffuse into Gram positive microbes and attach to the 50S ribosomal subunit to inhibit protein synthesis (Cocito, 1979; Allignet *et al.*, 1992). For Gram negative microbes, factor M generally cannot enter the cell. Both factors together inhibit protein synthesis 10-100 times better than either factor alone. Virginiamycin is produced in bulk, is active at low fermentation pH, and has been shown to be effective at reducing *Lactobacillus rhamnosus*, *Lactobacillus paracasei*, *Lactobacillus plantarum*, and *Lactobacillus delbrueckii* in doses as small as 0.5 mg/kg (Hynes *et al.*, 1997). In that work, the difference in ethanol (g/L) produced by the yeast co-inoculated with each bacterium ( $1 \times 10^6$  cells / g mash) in the presence and absence of virginiamycin was determined. After 48 hours of fermentation, the difference in ethanol produced (mg/ml) by the yeast (with and without virginiamycin) with *L. rhamnosus* present was 5.92, with *L. paracasei*, 9.16; with *L. plantarum*, 3.36; and with *L. delbrueckii*, -2. The authors concluded that a virginiamycin dosage of 0.5 mg /kg mash did not affect yeast performance or viability.

Other antibiotics have been tested for controlling contamination in fuel alcohol fermentations. Monensin, for example, caused greater reductions in bacterial contamination by the end of fermentation than did penicillin - even when different doses were used for each (Stroppa *et al.*, 2000). Chloramphenicol, streptomycin, tyrothricin, and bacitracin were tested in grain-based alcohol batch fermentations where  $1 \times 10^5$  bacteria were present at the start of fermentation (Day *et al.*, 1954). In that work, none of the tested antibiotics performed as well as penicillin in reducing the bacteria inoculated into the mash.

### **2.2.3.2 Chemicals.**

Another approach to the control of *Lactobacillus* contamination showed that *Lactobacillus casei* and *Lactobacillus fermentum* were sensitive to sulfite treatment while yeasts were not (Chang *et al.*, 1997). When *L. fermentum* was introduced into an equilibrated, aerated, continuous cell-recycle fermentation without sulfite, the yeast

population decreased from  $2.5 \times 10^8$  (control with no *L. fermentum*) to  $5.3 \times 10^7$  CFU/ml and *L. fermentum* levels equilibrated to  $3.1 \times 10^8$  CFU/ml. The yield of ethanol decreased from 0.5 to 0.32 g ethanol/g glucose. When sulfite was then introduced at 400 ppm, the yeast increased to  $1.2 \times 10^8$  CFU/ml, while *L. fermentum* decreased to  $1.9 \times 10^7$  CFU/ml, and the yield of ethanol increased from 0.32 to 0.43 g ethanol/g glucose. The antimicrobial affect of sulfite however was limited to conditions where oxygen is present. In a typical fuel alcohol plant, oxygen is not deliberately added to the fermentation (except in small amounts at the beginning of fermentations as required for yeast membrane synthesis in anaerobic growth), and the evolution of large amounts of CO<sub>2</sub> would drive off any residual oxygen. Thus, the benefit of using sulfite to control bacterial contamination is not likely to work well in an industrial setting.

Hydrogen peroxide was also successful in reducing the number of *L. fermentum* in a mixed culture containing yeast (Chang *et al.*, 1997). Viable *S. cerevisiae* and *L. fermentum* were combined in PYM broth to give an initial viable cell count of  $1.4 \times 10^8$  CFU/ml and  $1.4 \times 10^9$  CFU/ml respectively. Hydrogen peroxide was added to aliquots of the mixed culture to give final hydrogen peroxide concentrations from 0 to 200 mM. Each treated sample was then incubated at 30°C for two hours and then re-assessed for viable numbers of *S. cerevisiae* and *L. fermentum*. At a dosage of 200 mM hydrogen peroxide, only  $9.7 \times 10^5$  CFU/ml *L. fermentum* remained (~1500 fold decrease) while  $8.4 \times 10^7$  CFU/ml *S. cerevisiae* remained (~2 fold decrease). The authors also concluded that the magnitude of the effect of hydrogen peroxide on *L. fermentum* in a mixed culture with yeast was less than in a pure culture. This was likely due to the decomposition of hydrogen peroxide by yeast catalase/peroxidase.

Urea hydrogen peroxide (UHP) has been assessed for its ability to reduce the numbers of a variety of different lactobacilli in wheat mash fermentations with yeast (Narendranath *et al.*, 2000). Unlike hydrogen peroxide, whose stability decreases with increasing pH (Luck, 1956) and rapidly decomposes to water and oxygen when in contact with organic compounds, the stability of the UHP adduct is greater and it is a solid at room temperature (Banerjee, 1947). Viable *L. plantarum*, *L. paracasei*, *L.*

*rhamnosus*, *L. fermentum*, and *Lactobacillus sp.* strain #3 were inoculated separately to 500 ml aliquots of unclarified wheat mash (21 g dissolved solids / 100 ml mash) to give initial viable cell counts of  $1 \times 10^7$  CFU/ml in each mash (Narendranath *et al.*, 2000). UHP was then added to give an initial concentration of 32 mM in the mash, and the system incubated at 30°C for four hours. Samples were withdrawn at 0, 2, and 4 hours and assayed for viable counts. All of the lactobacilli were reduced in viable numbers by 4.0 to 4.5 logs in the particulate wheat mash. The authors also determined that no inhibitory effects on yeast (at  $\sim 1 \times 10^6$  CFU/ml) occurred with 30 mM UHP and that in fermentations conducted with yeast ( $\sim 1 \times 10^6$  CFU/ml), 30 mM UHP, and *L. paracasei* ( $\sim 1 \times 10^7$  CFU/ml), the fermentation profile was virtually identical to fermentations conducted with yeast alone. In addition to the antimicrobial properties that UHP provides, there are additional benefits in using UHP. Unlike using antibiotics where the antibiotic or its residues may remain in the final product, UHP degrades to water, oxygen, and urea. UHP provides the yeast with these two essential nutritional factors (oxygen and assimilable nitrogen from urea) that are deficient in many fuel alcohol fermentations. Oxygen is required by the yeast to synthesize essential unsaturated fatty acids and sterols for membranes (Andreasen and Stier, 1953; O'Connor-Cox and Ingledew, 1989; Ingledew and Kunkee, 1985) which are required for optimal fermentative performance by the yeast (Munoz and Ingledew, 1989). Urea provides additional assimilable nitrogen to the yeast to increase yeast growth and fermentation rate. The benefits of supplementing the base fermentation medium with additional assimilable nitrogen (especially for higher gravity fermentations) has been well documented in the literature (Thomas *et al.*, 1993; Jones *et al.*, 1995; Jones and Ingledew, 1994a; Jones and Ingledew, 1994b; Jones and Ingledew, 1994c; Thomas and Ingledew, 1990; O'Connor-Cox *et al.*, 1991). At 30 mM, UHP provides both these nutrients at near optimal concentrations. Patent protection is being sought for this application.

The antimicrobial properties of liberated hydrogen peroxide is generally attributed to the lack of catalase or peroxidase in *Lactobacillus* bacteria (Chang *et al.*, 1997;

Condon, 1987; Connolly, 2000; Narendranath *et al.*, 2000). However, under certain circumstances, *L. plantarum* and some pediococci have been shown to produce catalase (Johnston and Delwiche, 1965). The toxicity of hydrogen peroxide is attributable primarily to free radical formation - especially the OH<sup>·</sup> radical formed by its decomposition. The hydroxyl radical had been known to damage multiple sites within the cell - including DNA where single strand breaks (Ananthaswamy and Eisenstark, 1977), and altered bases (Demple and Lin, 1982) are reported. Other work has shown that hydrogen peroxide has the ability to generate more reactive and cytotoxic hydroxyl radicals (Gregory and Fridovich, 1974).

### **2.2.3.3 Heat.**

Some fuel alcohol manufacturers utilize a separate holding tank to fully saccharify liquefied and dextrinized starch to glucose before proceeding with fermentation. These “saccharification tanks” can become badly contaminated with lactic acid bacteria because these tanks are typically held at 60°C in order to optimize glucoamylase activity. The residence time of the mash in the saccharification tank is typically between 45 and 90 min. Although most bacteria do not survive in the saccharification tank at 60°C, many *Lactobacillus* can survive. The conditions in gelatinization and liquefaction provide some pasteurization to the mash (which has some benefit), but sterility is not accomplished. The saccharification tank provides conditions favourable for the multiplication of lactobacilli without competition from *S. cerevisiae*. This can be the source of major contamination in fuel alcohol plants which use such tanks (Kelsall and Lyons, 1999b). If a higher temperature glucoamylase was available for the saccharification tank, it would allow an increase in the temperature of saccharification and reduce the risk of bacterial multiplication. The result of the above is that simultaneous saccharification and fermentation (SSF) is now extensively practised where liquefied starch is saccharified to glucose in the fermentor during fermentation (Kelsall, 1995). This greatly reduces the possibility for contamination since mash can be delivered to the fermentor in better

bacteriological condition and because glucose in SSF systems is consumed by yeast at a rate similar to its liberation by added enzyme and thus little excess glucose is therefore present during fermentation.

To combat the increased risk of contamination in the fermentors, one commercial plant continuously separates a portion of the yeast from the mash in the fermentor and passes the yeast through a pasteurization column to limit contaminants (Danielsson, 1992). Although no parameters for pasteurization was given, the plant claims that the pasteurization significantly reduces the risk of contamination. From a microbiological standpoint, many bacterial species are more resistant to heat treatment than yeasts. A pasteurization treatment of the fermentor contents would thus kill off a higher proportion of yeast cells than bacteria which, in the long run, concentrates the bacteria in the fermentor. Without further data, it is not possible to conclude if such a heat treatment would be effective.

#### **2.2.3.4 Very High Gravity (VHG) fermentations.**

Most fuel alcohol plants producing ethanol use "normal" gravity substrate concentrations (for the fuel alcohol industry) of approximately 22-23°Plato (P) (~22-23 g glucose/100 ml mash). In the absence of contamination and/or incomplete starch saccharification, the concentration of ethanol reported is typically between 11-12% (v/v). For the fuel alcohol producer, very high gravity (VHG) technology is a promising and exciting innovation. Instead of fermenting a 22°P mash, the gravity is increased to 28-30°P (or as high as 34-38°P) by adding adjuncts, by double mashing in the preparation of the substrate, or, most likely, by reducing the water content during mashing (Thomas *et al.*, 1996). Special precautions must be taken in the preparation of VHG mashes. In particular, increased concerns regarding nutrients must be addressed – the assimilable nitrogen content must be adequate for yeast growth, and adequate oxygen must be provided during fermentation (Ingledeew, 1995). In laboratory batch cultures, VHG technology leads to ethanol levels as high as 23.8% (v/v) from a substrate delivering 38°P

dissolved solids (Thomas *et al.*, 1993). Numerous practical advantages of VHG technology include reduced water consumption, increased ethanol concentrations, increased ethanol yield, and reduced labor cost per unit ethanol produced (Ingledeu, 1995).

VHG fermentations are also somewhat “self regulating” with respect to bacterial contamination. Bacteria were followed in 12°P and 28°P wort fermentations in a study published by Magnus *et al.* (1986). The species of bacteria studied were common to the brewing industry and included beer spoilage bacteria representative of the coliform group (*Citrobacter freundii* ATCC 8090, *Enterobacter agglomerans* #127, *Flavobacterium proteus* ATCC 12841, *Hafnia sp.* BSO 105, *Klebsiella oxytoca* #52), the acetic acid group (*Acetobacter sp.* BSO 5, *Gluconobacter oxydans* NCIB 9013), the anaerobic group (*Zymomonas anaerobia* BSO 57), and the lactic acid group (*Lactobacillus brevis* BSO 31, *Lactobacillus frigidus* NCIB 8518, *Pediococcus acidilactici* NCIB 6990, *Pediococcus sp.* BSO 77). All the bacteria grew in 12°P and 28°P wort medium (with no yeast added) – inoculations of 10<sup>3</sup> /ml were able to multiply to at least 10<sup>6</sup> bacteria/ml. In the 28°P worts, however, none of the coliform bacteria survived past three days (6-7 days is the normal fermentation time for lager wort) of fermentation while all of the acetic acid, anaerobic, and lactic acid bacterial groups (except for *L. frigidus*) survived in these fermentations (Magnus *et al.*, 1986). *L. frigidus* however, did survive in 12°P fermentations. The authors concluded that death of the organisms studied was due to the higher alcohol content produced in the 28°P fermentations (11% v/v) since all the contaminants grew in 12°P and 28°P worts without yeast inoculation. It was also concluded that acetic acid bacteria, lactic acid bacteria, and *Zymomonas* posed the greatest threat in high-gravity fermentations (Magnus *et al.*, 1986). The authors indicated that higher ethanol contents would not eliminate all contamination caused by lactic acid bacteria since there are species of lactobacilli which contaminate fermentations (saki for example) with ethanol contents as high as 20% (v/v).

#### 2.2.3.4.1 Process conditions.

An intriguing proposal which may possibly control contamination has been outlined by Stephens and Lyberatos (1987). The authors showed theoretically that in a mixed continuous culture system containing two bacterial species with different specific growth rates, it is possible to choose which bacterial species will dominate in numbers in the system. A general unstructured competitive model in a chemostat is defined by the following equations:

$$x_1 = \mu_1(s)x_1 - Dx_1 \quad (2.2)$$

$$x_2 = \mu_2(s)x_2 - Dx_2 \quad (2.3)$$

$$s = -\frac{\mu_1(s)}{Y_1}x_1 - \frac{\mu_2(s)}{Y_2}x_2 + D(s^0 - s) \quad (2.4)$$

Unstructured models predict a faster adaptation of a microbe to step changes in D or S than is experimentally observed. The authors reasoned that bacteria require time to adjust to changing environmental and/or nutritional conditions and that the time required is different for different bacteria. This difference in adaptation time can be exploited to alter the bacterial population in the chemostat. Equations 2.2 and 2.3 were modified to account for the adaptation of the two bacteria to give the following equations:

$$x_1 = \mu_1(z_1)x_1 - Dx_1 \quad (2.5)$$

$$x_2 = \mu_2(z_2)x_2 - Dx_2 \quad (2.6)$$

$$z_1 = \alpha_1(s - z_1) \quad (2.7)$$

$$z_2 = \alpha_2(s - z_2) \quad (2.8)$$

where  $\alpha_i$  represents the adaptability of bacteria  $i$  - larger  $\alpha$  values indicate shorter response times. To test their constructed model (in dimensionless form), two hypothetical bacteria, 1 ( $\mu_{1m} = 0.6$ ) and 2 ( $\mu_{2m} = 0.55$ ) were subjected in a chemostat to feed substrate concentrations varied periodically in a square wave fashion. During half of the period,  $S^0$

had a maximal value and during the other half, it was 0. At a cycling frequency of 0.5, the model showed that bacteria 1 (faster growing microbe) declined (sawtooth pattern) to extinction while bacteria 2 (slower growing microbe) increased (sawtooth pattern) and remained at a high concentration in the chemostat. At a cycling frequency of 0.25, the reverse was found. The authors also found that varying D provided the same results as varying S although much higher reservoir substrate concentrations would be needed when varying D. The authors concluded that their method could be used to control contamination *in situ* when a contaminant was detected. From a microbiological standpoint, it would be much more difficult (if not impossible) for a contaminant to increase its specific growth rate past its maximal experimentally determined value to counteract its elimination by the cycling of S or D than for a contaminant to acquire resistance to an antibiotic. Thus, if cycling of S or D can be put into practical use, the generation of a resistant bacteria (to the cycling) is unlikely to occur. Unfortunately, the authors did not validate their models experimentally with a cycling of S or D and so the applicability of their findings is not known. The one disadvantage of cycling S or D in a continuous production plant is that steady state is not reached (as S or D is constantly changing) and thus the production and productivity of ethanol may be different (and more unpredictable) than what is seen in a system where steady state is achieved.

### **2.3 Factors affecting ethanol production by *Saccharomyces cerevisiae*.**

#### **2.3.1 Osmotic effects.**

Physiological changes have been observed for yeasts in fermentations with higher sugar or other solute concentrations. For example, osmotic effects have been noted in high gravity fermentations which led to decreased yeast growth rate and fermentative performance (Jakobsen and Piper, 1989). In their study, the growth rate of two lager yeast strains decreased by 70% when the glucose concentration in the medium was increased from 10 g/100 ml to 30 g/100 ml. Decreases in growth rates were also

observed for these two lager yeast strains when sucrose was used, although the decrease in the growth rate was only 30%. Osmotic stresses are, in part, prevented by generating dextrin rather than glucose to increase dissolved solids and relying on SSF for metered production of glucose in the fermentor (Thomas *et al.*, 1994).

In other work, *S. cerevisiae* was exposed to hypertonic levels of NaCl, methanol, and glycerol. The results showed that there was a direct relationship between loss of viability and the extent of water loss from within the cell (Morris *et al.*, 1983). In addition, rhodamine dye staining of yeast cell mitochondria before and after hypertonic stress indicated morphological changes in the mitochondrial structure. No reasons for these changes were given or for the role these changes had on viability.

In VHG fuel alcohol production, noticeable osmotic effects were found to be reduced by the addition of osmoprotectants. Combinations of glycine, betaine, and proline were added to 35% (w/v) glucose batch fermentations with the result that total glucose utilization increased from 15 g to 30.5 g. The viability of the yeast under these conditions increased from 12% to 80% (Thomas *et al.*, 1994). Although there is documentation on the effects of osmotic stress on yeast viability and fermentative performance during fermentation, there is little information on the mechanisms behind the loss of viability or fermentative performance with osmotic stress. *S. cerevisiae* is able to gradually adapt to increased osmotic stress or decreased water activity by synthesizing the compatible solute glycerol inside the cell. This allows the cell to cope to some degree with increased stress.

In other work, *S. cerevisiae* was shown to remain viable at very low water activities ( $a_w \sim 0.40$ ) (Gervais *et al.*, 1992). In their work, exponential, early stationary, stationary, and late stationary phase cultures of *S. cerevisiae* were exposed to osmotic stress (maximum of 126.5 MPa ( $a_w \sim 0.40$ )) with four different glycerol addition rates which corresponded to osmotic pressure changes of 0.298 MPa/s, 0.063 MPa/s, 0.039 MPa/s, and a “step” addition where the total amount of glycerol required to reach the maximum osmotic pressure was immediately added. Early stationary phase *S. cerevisiae* showed the highest viability at all rates of osmotic pressure increase. Furthermore, early

stationary *S. cerevisiae* cells showed the highest viability (80% as compared to control) when the increase in osmotic pressure was increased from 0.039 MPa/s to a maximum of 126.5 MPa ( $a_w \sim 0.40$ ). The authors concluded that the high resistance to osmotic stress at the lower rates of increase could be due to mechanical preservation of the membrane by the reduction of the water flow induced by osmotic stress, and/or the adaptation of the cell to higher osmotic stress by osmoregulation. The work of Maiorella *et al.* (1984) showed inhibition of *S. cerevisiae* by various inorganic salts that could be a product of normal medium preparation. In particular, continuous culture fermentations with these salts have shown that water activity alone does not lead to accurate prediction of the amount of inhibition by the salts used. The specific ethanol production rate increased and cell production decreased with increasing concentrations of salts.

### 2.3.2 Dilution rate.

In continuous culture, the dilution rate has been shown to affect the percentage of glucose partitioned between the glycolytic and pentose phosphate pathways. Radiorespirometric studies with *S. cerevisiae* in complex media were made under continuous culture at 30°C. Glucose was radiolabelled at C-1 and C-6 with  $^{14}\text{C}$  and the amount of  $^{14}\text{CO}_2$  evolved was determined in a Warburg respirometer for each dilution rate tested (Mian *et al.*, 1974). The authors observed that as  $D$  increased from 0.1 to 0.5  $\text{h}^{-1}$ , the percentage of glucose that was channelled through the glycolytic and pentose phosphate pathways changed. The percentage of glucose entering the glycolytic pathway decreased from 91% at 0.1  $\text{h}^{-1}$  to 66% at 0.5  $\text{h}^{-1}$  while the percentage of glucose entering the pentose phosphate pathway increased from 9 to 34% with the same dilution rates. Presumably, the results from these authors would indicate that amount of ethanol produced by *S. cerevisiae* would decrease as the dilution rate increased in continuous cultures.

The work of van Hoek *et al.* (1998) showed that a critical dilution rate ( $D_{\text{crit}}$ ) exists for *S. cerevisiae*. The authors showed for an industrial baker's yeast under aerobic

(60% of air saturation as determined by an oxygen electrode) continuous culture conditions in a glucose limited, defined medium that if  $D < D_{crit}$ , then the yeasts respired glucose and if  $D > D_{crit}$ , aerobic fermentation was observed. For their yeast,  $D_{crit}$  was  $0.28 \text{ h}^{-1}$ . The specific ethanol productivity ( $\text{mmol/g dry yeast/h}$ ) of their yeast at  $D < 0.28 \text{ h}^{-1}$  was nonexistent while it increased from  $0.11$  at  $D = 0.28 \text{ h}^{-1}$  to  $13.9 \text{ mmol/g dry yeast/h}$  at  $D = 0.4 \text{ h}^{-1}$ . The value of  $D_{crit}$  is dependant on the strain of *S. cerevisiae* and on experimental conditions. Values of  $D_{crit}$  from  $0.16 \text{ h}^{-1}$  (Petrik *et al.*, 1983) to  $0.38 \text{ h}^{-1}$  (Postma *et al.*, 1989) have been reported in the literature.

### 2.3.3 Ethanol.

Ethanol tolerance of yeast is a factor to consider in VHG alcohol production. Kalmokoff and Ingledew (1985), measured ethanol tolerance using methods which assessed effects of ethanol on yeast fermentative ability, yeast growth, yeast viability, and ethanol production capacity. Four yeast strains were tested – *Saccharomyces sake* IFO 2347, *S. cerevisiae* NCYC 366, *S. uvarum* NCYC 1324, and a baker's yeast. When each yeast was grown under exactly the same conditions and then incubated in various amounts of ethanol (4, 7, 10, 13, 15, and 18% v/v), the fermentation rate (measured as  $\text{CO}_2$  evolution) decreased as the ethanol content increased. A plot of the fermentation rate against ethanol content resulted in a strong negative linear correlation. This linear inhibition of growth by ethanol was also determined by Luong (1985). More importantly, the authors determined that all *Saccharomyces* strains continued to ferment until the ethanol content reached 25% (v/v). This finding is important for the fuel alcohol industry, as it shows that *S. cerevisiae* can produce high levels of ethanol under VHG conditions. This is supported by other work where 23.8% (v/v) ethanol has been produced from 38°P VHG wheat mash using a commercial active dry yeast in batch fermentation (Thomas *et al.*, 1993).

The growth of *S. uvarum* (brewer's yeast) was also studied in various amounts of ethanol (2, 4, 6, 8, 10, 12, and 14% v/v) added after an initial amount of growth had

occurred. A negative linear correlation between specific growth rate and ethanol levels was seen: growth ceased when ethanol levels reached 11.8% (v/v). Surprisingly, growth inhibition at this ethanol level was found to be similar in the other strains of yeast tested – *Saccharomyces sake* IFO 2347 (13% v/v), *S. cerevisiae* NCYC 366 (12% v/v), and the baker's yeast (12% v/v). The authors also assessed viability of these yeasts at different concentrations of ethanol (10, 13, 15, 18, and 21% v/v). What was found was that ethanol had a threshold effect on viability - most of the strains did not decrease in viability until the ethanol content reached 15-18% (v/v). In all probability, the rate of death is influenced by alcohol in the presence of a number of other stress-causing factors.

The authors concluded that there were no significant differences in ethanol tolerance with the limited number of *Saccharomyces* strains tested. At higher ethanol contents, growth may cease, but the yeasts are still metabolically active and capable of producing ethanol. If the above holds true in continuous culture (single stage) this would pose some difficulty to the fuel alcohol producer since washout of the yeast cells would occur at higher ethanol contents (> 15% v/v) since the yeast are no longer capable of significant reproductive growth.

Inhibition of yeast growth by ethanol depends on the strain of yeast used and on experimental conditions. In one study, ethanol was added to yeast that had achieved steady state conditions in continuous culture. The concentration of ethanol that resulted in an 80% decrease in yeast cell density was recorded to be 70 g/L (Maiorella *et al.*, 1983). Differences in the specific growth rate of five thermotolerant yeasts isolated from industrial sources decreased in the presence of ethanol in batch fermentations (Peres and Lalue, 1998). Decreases as high as 71% in specific growth rate were observed when ethanol at 7% v/v was initially added to batch fermentations. In other work, the inhibition by ethanol on the growth of a number of different yeast strains was evaluated in complex media (Antoce *et al.*, 1997). These authors demonstrated that, out of the different yeasts evaluated, *S. cerevisiae* proved the most resistant showing only a 50% inhibition in growth at an ethanol concentration of 6.09% v/v, and 100% inhibition in growth at 12% v/v. Hallsworth (1998) calculated the available water ( $a_w$ ) values that would be present

with the levels of ethanol reported in a number of published works. His argument was that, aside from the other known mechanisms by which ethanol inhibits yeast growth, the concentration of ethanol alone in the fermentation could inhibit yeast growth by lowering  $a_w$ . As most non-osmotolerant yeasts cannot grow below an  $a_w$  of 0.92, this level of water activity corresponds to an ethanol concentration of 15.9% w/v. The authors concluded that the reduced  $a_w$  caused by ethanol concentrations as small as 5% w/v may directly impact the specific growth rate of most yeasts. Differences in response times of *Zymomonas mobilis* and *S. cerevisiae* upon inoculation into similar complex media with 30 g/L ethanol was observed by Hobley and Pamment (1994). In particular, the lag time of *S. cerevisiae* was considerably longer (10 h) compared to *Z. mobilis* (4 h) when equivalent biovolumes of each ( $2 \times 10^{-6}$  ml cells /ml) were used. The authors concluded that the high metabolic activity of *Z. mobilis* may, in part, be the reason for the difference in adaptation times seen.

Ethanol has been shown to produce physical and chemical changes in yeast cells. Irreversible physical damage was demonstrated by Dombek and Ingram (1987). In their work, *S. cerevisiae* was grown in batch, centrifuged, and cells resuspended in fresh medium containing various amounts of ethanol. In addition, an aliquot of the same *S. cerevisiae* cells was pre-exposed to various amounts of ethanol and then resuspended into fresh medium with various amounts of ethanol. The inhibition of fermentation caused by ethanol at concentrations above 5% v/v was only partially reversed when the same cells were resuspended in fresh medium. The authors eliminated the possibilities that pH, viability, or inhibition of glycolysis were the reason for the reduced fermentative activity since the intracellular pH was constant, viability was > 90%, and the measured activities of glycolytic enzymes remained high for all conditions. Thus, they concluded that physical damage by ethanol is through another mechanism. In other work, the membrane phospholipid composition of *S. cerevisiae* changed as ethanol concentrations were increased in the course of batch fermentations (Chi *et al.*, 1999). The concentration of saturated fatty acids ( $C_{10:0}$ ,  $C_{12:0}$ ,  $C_{14:0}$ ,  $C_{16:0}$ , and  $C_{18:0}$ ) increased and the concentration of unsaturated fatty acids ( $C_{16:1}$  and  $C_{18:1}$ ) decreased when *S. cerevisiae* was fermented on

glucose at 30°C. However, when grown under identical conditions but with added phosphatidylinositol (PI), *S. cerevisiae* maintained a higher amount of saturated fatty acids and a lower amount of unsaturated fatty acids than cells grown without PI. The cells grown with added PI produced ethanol faster and tolerated higher concentrations of ethanol. In addition, the viability of *S. cerevisiae* under 18% v/v ethanol shock remained higher for PI grown cells. Cells grown with PI retained 74% viability after one hour as compared to cells grown without PI at 47%. The authors concluded that the inhibition of yeast by ethanol could be related to cell membrane composition and that this inhibition was partially reversed by the addition of PI. Other authors (Alexandre *et al.*, 1994; Hanson and Lester, 1980) would support the work of Chi *et al.*, (1999) as they showed that a decrease in the unsaturation index in cell membranes of *S. cerevisiae* led to increased ethanol tolerance.

Enzymatic activity is affected by ethanol through both inhibition and denaturation. Glycolytic enzymes of *S. cerevisiae* have been isolated and purified and then subjected to various ethanol concentrations for 30 min at pH 6.0 to determine the extent of denaturation. In addition, optimized enzymatic assays with purified enzymes were subjected to increasing amounts of ethanol to determine inhibition. Denaturation of the glycolytic enzymes did not occur below an ethanol concentration of 12% v/v and no significant inhibition of the enzymes occurred below an ethanol concentration of 5% v/v (Millar *et al.*, 1982). At a minimum ethanol concentration of 19% v/v, a 90% reduction in the activity (by denaturation) of pyruvate decarboxylase was observed. At this same ethanol concentration, most of the glycolytic enzymes were non-competitively inhibited in activity by 50%. The authors concluded that at an ethanol concentration above 10-12% v/v, inhibition of glycolytic enzymes by both denaturation and competitive inhibition can limit ethanol production by yeast.

## 2.3.4 Nutritional deficiencies.

### 2.3.4.1 Nitrogen.

In 1973, Patel and Ingledew reported that in normal 12°P brewing fermentations not all the nitrogen present in wort could be used by yeast. In particular, the nitrogen content provided by the protein fraction of the wort was not utilized while the non-protein fraction was rapidly utilized. The authors reasoned that nitrogen may actually be nutritionally limiting in all worts despite the fact that ample total nitrogen was present. Casey *et al.*, (1985) later showed that stuck or sluggish fermentations of very high gravity worts could be eliminated by addition of “usable” nitrogen to these fermentations. Beers with increased alcohol of at least 16% v/v were then possible. The commercial significance of this work including the advantages of the technology were shown by McCaig *et al.*(1992).

In other work, the rate and extent of fermentation of commercial grape juices were both increased as the free amino nitrogen (FAN) level of the juices was increased by the addition of 1% yeast extract (Ingledew and Kunkee, 1985). The fermentation of the selected juices in industry resulted in sluggish or stuck fermentations if supplements were not added. More recently, an increase in ethanol productivity in batch fermentation by 50% to 3.3 g/L/h was achieved when a combination of 12 g/L yeast extract, 0.3 g/L yeast cells walls, 0.3 g/L glycine, and 20 g/L soya flour was added to provide additional FAN to a medium containing 300 g/L glucose (Bafrcová *et al.*, 1999). It has become clear that supplementation of available nitrogen is necessary to provide complete utilization of sugar especially when the initial concentration of sugar in the medium is high. D’Amore *et al.*, (1991) observed that as the concentration of sugar in brewing wort was increased, a progressive decrease in the rate and extent of fermentation were seen. At worts above 24°P, complete utilization was not achieved and the amount of ethanol produced was less. This realization led other authors to determine the amount of FAN required for complete utilization of sugar at higher sugar concentrations. In batch fermentations where

the concentration of sugar is between 10 to 12°P (normal gravity), a 140-150 mg FAN/L addition was recommended by Ingledew *et al.* (1986) to ensure complete utilization of sugar. This value increases to 250 mg FAN/L when the concentration of sugar is > 18°P (very high gravity for brewing). Levels of FAN in the media beyond the recommended amounts stimulate the rate of fermentation so that less time is needed to complete the fermentation. In other work, the amount of available nitrogen that a yeast needs in fermentation was shown to vary according to the yeast strain. The nitrogen demand of 13 commercial *S. cerevisiae* cultures was assessed in batch fermentations with both synthetic simulated grape must (containing 200 g/L of glucose and fructose and a total nitrogen content from added amino acids of 125 mg/L) and with three different natural grape musts (Manginot *et al.*, 1998). The evolution rate of CO<sub>2</sub> at stationary phase was determined by the progressive loss of weight of the fermentor. Computer controlled pulses of concentrated solutions of either ammonium phosphate, glycine, methionine, or a mixture of amino acids (with a total nitrogen content of g g/L) was added at stationary phase to maintain a constant evolution rate of CO<sub>2</sub>. The dilution caused by the addition of the concentrated stock solutions of nitrogen was less than 5%. For each yeast, a range of constant CO<sub>2</sub> evolution rates were studied and the amount of nitrogen needed to maintain a CO<sub>2</sub> evolution rate between 10 and 50 g/L was calculated for each. The nitrogen demands of the 13 *S. cerevisiae* strains were different and a range of nitrogen demand between ~1 to ~2.5 mg N / g CO<sub>2</sub> was found. The nitrogen demand for each yeast was reproducible and did not depend on the type of nitrogen source used in the pulses or on the type of medium used.

The results of one continuous culture study appear to be different from those obtained from batch. Liden *et al.* (1995) fermented a complex medium (the yeast nitrogen base component used did not have amino acids or ammonium sulphate) in either carbon limiting (20 g/L glucose, 5.0 g/L (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>) or nitrogen limiting (20 g/L glucose, 0.36 g/L (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>) conditions at various dilution rates. At all dilution rates the specific yield of ethanol ( $Y_{ps}$ ) was higher in the nitrogen limited as compared to the carbon limited conditions. In addition,  $Y_{ps}$  did not depend on D (statistically evaluated) as an average

value of  $Y_{ps}$  under carbon limited conditions was 0.357 g ethanol / g glucose consumed as compared to 0.381 g ethanol / g glucose consumed for nitrogen limited conditions. No explanation by the authors was given for the increased  $Y_{ps}$ . A likely possibility was that with nitrogen limitation, the cells would not be able to multiply to the same extent as when nitrogen was in excess and so more of the consumed glucose would be available for the production of ethanol.

Other approaches to increasing the free available nitrogen in fermentations include adding fresh yeast autolysate (Jones and Ingledew, 1994c; Bafrcová *et al.*, 1999), urea (Jones and Ingledew, 1994b; Jones and Ingledew. 1994d), ammonium salts (Jones and Ingledew, 1994b), glutamic acid (Thomas and Ingledew, 1990), protein hydrolysates (Thomas *et al.*, 1994; Ingledew and Kunkee, 1985), amino acids (Ingledew, 1994), and various commercial “yeast-food” products (Jones and Ingledew, 1994b). In all cases, increased usable nitrogen led to higher viable counts and thereby an increased catalytic power. It is also known that actively growing yeasts make alcohol considerably faster (> 30 times) than yeasts in stationary phase (Kirsop, 1982). Therefore, an alternative approach to eliminating the problems in fermentation caused by nitrogen limitation is to increase the inoculation rate. By increasing the inoculation rate from  $1.6 \times 10^7$  to  $8 \times 10^7$  CFU/ml (5 fold increase), O’Connor-Cox and Ingledew (1991) found that the fermentation completed in 24 h (as compared to 72 h), higher ethanol levels were observed (an increase between 0.5 and 0.8% w/v), and a greater degree of attenuation was achieved. These authors observed that no increases in viable numbers of yeast (due to multiplication during the fermentation) were seen when the higher inoculation rates were employed.

Many mashes used in alcohol production contain an appreciable amount of protein which are not used by yeast. An investigation into protease addition to wheat mash fermentations designed to liberate available nitrogen from proteins was also carried out by Thomas and Ingledew (1990) and by Jones and Ingledew (1994a). Commercial proteases were added to the fermentors 30 min after inoculation of wheat mash containing 350 g dissolved solids /L. At a 0.01% w/v concentration, all the proteases

reduced the fermentation time as compared to the control - with some proteases reducing the time by as much as 55% (Jones and Ingledew, 1994a). The authors concluded that protease addition to the fermentation could reduce or eliminate the need for supplementation of VHG fermentations with additional FAN.

#### **2.3.4.2 Minerals.**

A deficiency of certain minerals in media used for fuel alcohol fermentation could lead to stuck and sluggish fermentations. Minerals such as magnesium are vital in yeast for the proper functioning of critical enzymes. A review of the role of magnesium in cellular functions was compiled by Walker (1994). Investigations into magnesium supplementation in fermentation were also performed by Walker (1999), Walker and Maynard (1997), and Rees and Stewart (1999). In the work by Rees and Stewart (1999), the rates of normal and high gravity batch fermentations and the final ethanol concentrations increased for both lager and ale strains of *S. cerevisiae* on supplementing the media with 500 ppm  $Mg^{2+}$ . Increases in ethanol of 0.19 and 0.31% v/v were seen for normal and high gravity worts respectively. In addition, yeast cell numbers increased for both yeasts in both worts. Similar increases in final ethanol concentration and rates of fermentation were seen with a variety of brewing yeasts (Rees and Stewart, 1997; Walker and Maynard, 1997; Walker, 1999). In other work, final ethanol concentrations and consumption of glucose by *S. cerevisiae* increased as the concentration of  $Mg^{2+}$  was increased in the medium and reached maximal values at 500  $\mu M$  (Walker and Maynard, 1997). Further increases in  $Mg^{2+}$  did not promote the formation of additional ethanol. The authors also found a high correlation between the time of maximal accumulation of  $Mg^{2+}$  and the time where the concentration of ethanol, consumption of glucose, and cell density reached maximal values. In addition to its requirement as a nutrient,  $Mg^{2+}$  has been shown to protect yeast against ethanol shock (Walker, 1998). In fermentations where 10% v/v ethanol was present at the start of fermentation, the viabilities of *S. cerevisiae* progressively decreased from the onset of fermentation in the presence of

Mg<sup>2+</sup> supplemented at either 2 or 20 mM. The decrease in viability was slower with 20 mM Mg<sup>2+</sup> and after 24 h, no viable cells were seen with 2 mM Mg<sup>2+</sup> while 53% of the cells were viable with 20 mM Mg<sup>2+</sup>. At an initial concentration of ethanol at 20% v/v in the medium, the viability of *S. cerevisiae* was 85% after 24 hours when 50 mM Mg<sup>2+</sup> was supplemented to the medium.

Minerals such as zinc have also been shown to be important for optimal fermentation by yeast. In normal and high gravity brewing worts, the rate of batch fermentation and the final ethanol concentration increased for six commercial strains of *S. cerevisiae* when media were supplemented with Zn<sup>2+</sup> up to a concentration of 327.5 ppm (Rees and Stewart, 1998). Above a concentration of 327.5 ppm, Zn<sup>2+</sup> greatly reduced both the final ethanol concentration and the rate of fermentation.

#### 2.3.4.3 Oxygen.

Unless the media is supplemented with a mixture of ergosterol and unsaturated fatty acids or yeast hulls, oxygen is required for yeast growth under anaerobic conditions. The effect of a lack of oxygen on yeast performance was shown in commercial grape juice preparations. Ingledew and Kunkee (1985) inoculated *S. cerevisiae* into fermentations containing a commercial grape juice preparation where the head space was either slowly but continuously flushed with N<sub>2</sub> gas (reducing oxygen access) or air. The fermentations became stuck in fermentations where the headspace was flushed with N<sub>2</sub> - the concentration of soluble solids decreased from 260 g/L to ~180 g/L in 18 days and did not decrease further. In contrast, in fermentations where the headspace was slowly flushed with air, the concentration of soluble solids decreased from 260 g/L to ~30 g/L in 13 days. The same authors showed that oxygen was not required by yeast when ergosterol and unsaturated fatty acids were provided. Fermentations conducted where the headspace was flushed with N<sub>2</sub> gas, and where a usable nitrogenous source as well as ergosterol (40 mg/L) and a source of unsaturated fatty acids (0.4% v/v Tween 80) were provided, completed in ~7 days with the final concentration of soluble solids at ~30 g/L.

Under aerobic conditions, *S. cerevisiae* is able to synthesize sterols and unsaturated fatty acids for its membranes. It is unable to do so under strict anaerobic conditions. Providing the yeast with ergosterol (a sterol normally found in yeast) and unsaturated fatty acids (such as oleic acid) removes the anaerobic inadequacy in the synthesis of these compounds (Andreasen and Stier, 1953; Andreasen and Stier, 1954).

This accepted dogma that ergosterol and unsaturated fatty acid supplementation can allow for the growth of *S. cerevisiae* under anaerobic conditions was, in part, challenged by Thomas *et al.* (1998). In experiments involving 10 industrial strains of *S. cerevisiae*, no growth was observed in any strain in minimal medium under anaerobic conditions and only one strain grew anaerobically with additions of ergosterol and Tween 80. Growth was restored to all yeasts anaerobically with a mixture of added amino acids. The authors determined that even trace amounts of oxygen (< 5 ppm) in a typical N<sub>2</sub> gas cylinder was sufficient to promote yeast growth. Upon removal of the trace oxygen, none of the yeast strains grew in minimal medium under anaerobic conditions even with additions of ergosterol and Tween 80.

The amount of oxygen and the timing of oxygen addition to VHG fermentations was previously determined by O'Connor-Cox and Ingledew (1989). In fermentations with 280 g/L dissolved solids, the rate of anaerobic fermentation increased as the dissolved oxygen concentration in the medium was increased from 0 to 10 ppm. A maximum rate of fermentation was found at 7.5 ppm beyond which no further increases in fermentation rate was observed. When media were oxygenated in the fermentor at various times after inoculation, the optimal time of oxygenation to provide the fastest rate of fermentation for VHG fermentations was 14 h after inoculation. This coincides with the second division of the growing yeast. Other authors are in agreement with the dose of oxygen added (Lodolo *et al.*, 1999) but differences exist on the optimal timing for the dose depending on the medium composition (Lodolo *et al.*, 1999).

## **2.4 Mathematical modeling.**

Many mathematical models have been generated from experiments that use yeast to produce ethanol. Two types of mathematical models have been developed which attempt to predict the course of actual fermentations.

### **2.4.1 Unstructured models.**

Unstructured mathematical models predict the course of a fermentation based on a limiting substrate concentration (S), and both product concentration (P), and biomass concentration (X) (or derivatives of the three parameters) as well as an assortment of other parameters. More than 50 such models have been proposed or used to simulate the course of a fermentation. In a review of the more prevalent mathematical models proposed to predict ethanol fermentation, Marin (1999) concluded that not one of the models could predict fermentations across conditions different from the experimental conditions that yielded the model. In effect, each model that was reviewed could only be applied to the particular conditions under which the model was generated. A “universal” unstructured model predicting ethanol fermentation is therefore not available.

From a microbiological standpoint, the ideal model would not have ethanol production linked exclusively to growth. Ethanol production can occur from both multiplying and non-multiplying cells. Growth would be linked to the concentration of viable cells. Inhibition of growth and ethanol production would occur due to the concentrations of ethanol and substrate. Also, substrate utilization would be linked both to growth and to ethanol production. As cells die, a death constant for yeast must be included. These parameters would be the most obvious first choices for a fermentation microbiologist to include in a new model. None of the models discussed in the following subsections fulfill these requirements although aspects of such requirements are included in some. In yeast-mediated fuel alcohol production, ethanol at increasing concentrations becomes toxic to yeast. However, it has been shown that fermentation by a variety of

industrial yeasts can continue well past 12% (v/v) ethanol – the level where yeast growth is inhibited (Kalmokoff and Ingledew, 1985). Thus, the production of ethanol is not coupled exclusively to growth in many fermentations. Another complication is that the substrate used in fuel alcohol fermentations is typically at higher concentrations. VHG fermentations have been run up to 38°P. This raises concerns for osmotic effects or reduced water activity which would translate into a need to include a new term in a model for the inhibition of growth (and possibly ethanol production) by substrate. Yet another complication is that all models based on Monod's theory require that growth is limited by a single substrate during fermentation (defined as S). In VHG and other fermentations, the culture medium is designed to be a complete medium with high levels of substrate. The importance of this is shown in the work by Liu (1998) who demonstrated (based on data from other authors) that the yield of biomass in substrate-sufficient chemostat cultures is lower than in substrate-limiting chemostats. The authors concluded the reason for the lower yield was that at high substrate concentrations, catabolism is seriously dissociated from anabolism in continuous cultures. Most models utilize the total biomass concentration (X) as a parameter. Moreover, although only viable cells can multiply, it is well known that both viable and metabolically active (but nondividing) cells can produce ethanol although the latter make alcohol at a reduced rate (Kirsop, 1982). Jarzebski *et al.* (1989) included terms in their model that take into account the proportion of viable and dead (or possible nonviable but metabolically active?) cells in the total cell population. When the authors modeled data (provided by others) from cell recycle experiments at high cell concentrations, they noted a much closer agreement in X, S and P with all experimental data when their model was used as compared to models which do not take cell viability into account.

A review of the most commonly used and reported models in light of these desired requirement and facts is provided in the following subsections.

### 2.4.1.1 Monod model.

The classical approach to model a fermentation is to use an model based on kinetics derived by Monod (1949). The equations for the classical model are:

$$\frac{dX}{dt} = \mu X \quad (2.9)$$

$$\frac{dS}{dt} = - \frac{1}{Y_{x/s}} \frac{dX}{dt} \quad (2.10)$$

$$\frac{dP}{dt} = - Y_{p/s} \frac{dS}{dt} \quad (2.11)$$

where  $\mu$ , the specific growth rate of the organism ( $\text{h}^{-1}$ ), is equal to

$$\mu = \mu_m \frac{S}{K_s + S} \quad (2.12)$$

This model works well in instances where neither the substrate (S) nor the product (P) is inhibitory, where all cells are viable, and where product formation is tightly coupled to cell growth (X). In this model, substrate is only consumed and product only formed when cell growth occurs. The application of this model to VHG fermentations would not produce satisfactory results since substrate is in excess, and ethanol production occurs with viable and nonviable (but metabolically active) cells. In addition, inhibition by ethanol is not taken into account in these equations.

To model a multistage system the differential equations need to be modified to include the concentrations of X, S, and P entering and leaving a particular fermentor. Thus, in its simplest form, the differential Monod style equations for a multistage system consist of the following equations:

$$\frac{dX_i}{dt} = DX_{i-1} + \mu X_i - DX_i \quad (2.13)$$

$$\frac{dS_i}{dt} = DS_{i-1} - \frac{1}{Y_{x/s}} \frac{dX}{dt} - DS_i \quad (2.14)$$

$$\frac{dP_i}{dt} = DP_{i-1} - Y_{p/s} \frac{dS}{dt} - DP_i \quad (2.15)$$

where  $i$  is the fermentor number. This system of equations along with parameters and initial starting conditions can be input to software and the model can be solved over time. Other models have different equations governing  $\mu$ ,  $v_s$ , and  $v_p$  which can be substituted in place of the Monod equations.

#### 2.4.1.2 Ghose and Tyagi model.

The model proposed and utilized by Ghose and Tyagi (1979a) for ethanolic fermentations of hydrolysed bagasse may be better suited for modelling VHG fuel alcohol fermentations. Their model is based on the Monod equations and takes into account that inhibition of the yeast can occur from both glucose and ethanol, that ethanol production is not coupled exclusively to growth, and that the inhibition of ethanol on growth is linear (Dourado *et al.*, 1987). Other models have alcohol production coupled with growth so that alcohol cannot be produced without growth. The following equations make up the Ghose and Tyagi (1979b) model:

$$\mu = \mu_m \left( \frac{S}{K_s + S + \frac{S^2}{K_{si}}} \right) \left( 1 - \frac{P}{P_m} \right) \quad (2.16)$$

$$v_s = - \frac{v_p}{Y_{P/S}} \quad (2.17)$$

$$v_p = v_{pm} \left( \frac{S}{K'_s + S + \frac{S^2}{K'_{si}}} \right) \left( 1 - \frac{P}{P'_m} \right) \quad (2.18)$$

In this model, substrate inhibition and growth limitation by substrate are taken into account. However, substrate consumption is linked to product formation only and consumption of substrate for growth is not taken into consideration. Ethanol inhibition in this model is linear, and a non-linear relationship exists between  $\mu$  and  $v$ . It is also one of the few models that has been used to model an ethanol fermentation where the substrate concentration is higher (157 g/L) than what is normally used in most models.

#### 2.4.1.3 Sevely model.

Another model based on the Monod equations was constructed by Sevely *et al.*, (1980). The equations which govern this model are:

$$\mu = \mu_m \left( \frac{S}{K_s + S} \right) \left( \frac{K_p}{P + K_p} \right) \left( 1 - \frac{P}{P_m} \right) \quad (2.19)$$

$$v_s = - \frac{v_p}{Y_{P/S}} - \frac{\mu}{Y_{X/S}} \quad (2.20)$$

$$v_p = \alpha\mu + \beta \quad (2.21)$$

This model is one of the few that has incorporated both the substrate consumed for growth and substrate used for product formation. No inhibition by substrate is taken

into account in this model and the inhibition by ethanol is nonlinear.

#### 2.4.1.4 Luong / Levenspiel model.

Luong (1985) proposed a model where the specific growth rate and specific ethanol production rate are governed by the following three equations:

$$\mu = \mu_m \left( 1 - \left( \frac{P}{P_m} \right)^\alpha \right) \left( \frac{S}{K_s + S} \right) \quad (2.22)$$

$$v_p = v_{pm} \left( 1 - \left( \frac{P}{P_m'} \right)^\beta \right) \left( \frac{S}{K_s' + S} \right) \quad (2.23)$$

$$v_s = - \frac{\mu}{Y_{X/S}} \quad (2.24)$$

where  $\mu_m$  and  $v_m$  are based on Monod type equations and  $\alpha$  and  $\beta$  are unitless constants which determine the type of relationship between the specific growth rate, the ethanol production rate and the ethanol concentration. Setting these constants to 1 forms a linear relationship between the ethanol concentration and both  $\mu$  and  $v$  while values  $< 1$  form nonlinear (concavity upward) relationships and values  $> 1$  form nonlinear (concavity downward) relationships. The author performed fermentations with *S. cerevisiae* and the hard data obtained for  $\mu$  and  $v$  in the experiments were predicted accurately by the models. In addition, the model accurately predicted  $\mu$  and  $v$  values from experimental data taken from four other authors utilizing *S. cerevisiae* under different experimental conditions. Unfortunately, none of the data tested had substrate concentrations at higher gravities. A similar equation governing specific growth rate (Equation 2.23) was used by Levenspiel (1980) to theoretically model the growth of *S. cerevisiae* with increasing ethanol concentrations.

#### 2.4.1.5 Aiba model.

Aiba *et al.* (1968) proposed a model where the specific growth and ethanol production rates were inhibited by ethanol. The equations for this model are as follows:

$$\mu = \mu_m \left( \frac{S}{K_s + S} \right) \left( \frac{K_p}{K_p + P} \right) \quad (2.25)$$

$$v_p = v_{pm} \left( \frac{S}{K'_s + S} \right) \left( \frac{K'_p}{K'_p + P} \right) \quad (2.26)$$

$$v_s = - \frac{\mu}{Y_{X/S}} \quad (2.27)$$

Unfortunately, the equations were constructed with a respiratory-deficient mutant of *S. cerevisiae* and it has not been determined if they are applicable to fermentations with respiratory-sufficient *S. cerevisiae*.

#### 2.4.1.6 Lee, Poulard, and Coulman model.

Lee *et al.* (1983) proposed a model based on theoretical calculations in a cell recycle system.

$$\mu = \mu_m \left( \frac{S}{K_s + S} \right) \left( 1 - \frac{X}{X_m} \right)^n \left( 1 - \frac{P}{P_m} \right)^m \quad (2.28)$$

$$v_s = - \frac{\mu}{Y_{X/S}} \quad (2.29)$$

$$v_p = \frac{\mu}{Y_{X/P}} \quad (2.30)$$

The most unique feature of this model is that the authors included a term which inhibited the specific growth rate based on the concentration of biomass. For the inhibition of growth by ethanol and biomass, the type of inhibition could be set to be linear or nonlinear (parabolic). As well, a linear relationship between  $v$  and  $\mu$  is evident in the model. The model unfortunately links ethanol production and substrate utilization exclusively to growth such that neither parameter is affected unless growth occurs. Substrate utilization is also linked only to conditions where growth occurs and not also to situations where ethanol is produced where growth does not occur. The equations in this model were not tested under experimental conditions and so the validity of the model is not proven.

#### 2.4.1.7 Andrews model.

Andrews (1968) proposed the following equation to link the specific growth rate of an organism to the concentration of an inhibitory substrate during a fermentation:

$$\mu = \mu_m \left( \frac{1}{1 + \frac{K_i}{S} + \frac{S}{K_{P_i}}} \right) \quad (2.31)$$

The equation bears close resemblance to enzymatic functions to describe the inhibition of an enzyme due to high substrate concentrations. Calculations made by the author using this model showed various curves relating  $\mu$  to  $P$  but no hard data was used to verify the accuracy of the equation.

#### 2.4.1.8 Jarzebski model.

The importance of viable cells in the production of biomass and ethanol production in a cell recycle system was recognized by Jarzebski *et al.*, (1989). The following equations were proposed by the authors.

$$\mu = \mu_m \left( \frac{S}{K_s + S} \right) \left( 1 - \frac{X_t}{X_m} \right)^n \left( 1 - \frac{P}{P_m} \right)^m \quad (2.32)$$

$$v_s = - \frac{\mu}{Y_{X/S}} - m_s \quad (2.33)$$

$$v_p = e^{-bX_v} \quad (2.34)$$

Instead of using the total biomass concentration in all the generated differential equations (as is typically done in most modeling situations), the authors instead used the concentration of viable biomass. In addition, substrate is considered to be consumed for cellular maintenance purposes but the consumption by nonviable (but metabolically active) cells was not included. Likewise, the production of ethanol includes viable but not nonviable (but metabolically active) cells. In spite of these shortcomings, the model has experimental validity. The authors modeled cell recycle experimental data from other authors which resulted in a closer agreement in all modeled parameters than for models which did not take cell viability into account.

#### 2.4.1.9 Dourado model.

A more complicated model successfully applied to a multistage fermentation using *Saccharomyces bayanus* was constructed by Dourado *et al.*, (1987). The equations for this model are as follows:

$$\mu = \mu_m \left( \frac{S}{K_s + S + \frac{S^2}{K_{si}}} \right) \left( \frac{P}{K_p + P + \frac{P^2}{K_{pi}}} \right) \left( 1 - \frac{P}{P_m} \right)^R \quad (2.35)$$

$$v_s = - \frac{v_p}{Y_{P/S}} \quad (2.36)$$

$$v_p = v_{pm} \left( \frac{S}{K'_s + S + \frac{S^2}{K'_{si}}} \right) \left( \frac{P}{K'_p + P + \frac{P^2}{K'_{pi}}} \right) \left( 1 - \frac{P}{P'_m} \right)^R \quad (2.37)$$

Data generated from these equations accurately predicted the actual data obtained from multiple multistage fermentations with *S. cerevisiae*. In this model, ethanol inhibition on growth and product formation is exponential with a unitless modifier (R) which is a value dependant on whether the product concentration is greater or less than  $P_m$ . Unfortunately, the consumption of substrate is linked only to the formation of product and not at all on the formation of biomass.

#### 2.4.2 Structured models.

An alternative approach to model the course of a fermentation mathematically is to consider the metabolic reactions that occur in a cell. In structured models, a full or partial metabolic map of the organism is typically constructed and the known equations and reactions of the organism are mathematically modeled. A major advantage of structured models is that if correctly constructed and applied to actual experimental data, information on other areas of metabolism can be obtained that could/would not be obtained in the experiment - including concentrations of cellular intermediates, rates of reactions, and potential bottlenecks in growth and product formation. A disadvantage of structured models is that if an observed fermentation condition or enzymatic reaction

does not have an equation to describe it, then either the condition and/or the reaction is not included in the model and/or assumptions are made in the model. An example of a observed fermentation condition is the effect of osmotic pressure on the viability of yeast. It is well known that increased osmotic pressure affects both viability and product formation of yeast. Yet no mathematical equations that can be used in structured models have been generated that describe this relationship. In either case the accuracy of the generated model will be reduced. Another disadvantage is that, in contrast to unstructured models, there are a large number of equations and parameters that need to be balanced mathematically which greatly increase the complexity of the model.

A number of examples of structured models exist concerning ethanol fermentation with *S. cerevisiae*. Grosz and Stephanopoulos (1990b) modeled ATP and NADH metabolism of *S. cerevisiae* under anaerobic fermentation. In particular, the model was simplified by having only four rate equations: glucose uptake, ethanol production rate, glycerol production rate (consumes NADH and forms NAD) and transport rate out of the cell. Unfortunately, the formation of NADH in glycolysis was not considered in the model although this limitation was balanced with an “additional products” reaction where NADH is produced. In spite of this error, the model, for the most part, accurately predicted the specific ethanol production rate, specific glycerol production rate, specific glucose consumption rate, and specific growth rate as a function of intracellular ATP concentration in *S. cerevisiae* from the authors experimental data in continuous culture. The authors stated that their model was only applicable in micro-aerobic fermentations where glucose was in excess.

In a more elaborate model, Cortassa and Aon (1994) modelled all the enzymatic reactions in the glycolytic pathway and a single reaction that described the TCA cycle. The 11 metabolic reactions and their associated parameters that comprised this model were used to theoretically model the behaviour of *S. cerevisiae* under aerobic conditions in defined medium with different dilution rates and under either carbon, nitrogen, or phosphate limitation. According to the data generated from their model (based on experimental data from other authors), the uptake of glucose in continuous culture was

the single most important “reaction” that governed both the control of the glycolytic flux and also the control of flux ratio at the metabolic branch where glucose could either go towards ethanol production or to the TCA cycle. Regardless of the type of nutrient limitation, the ethanol production rate increased as D was increased with nearly 80% of the carbon consumed by the cells channelled towards ethanol production. Unfortunately, the explicit concentrations of ethanol and glucose were not provided to facilitate a comparison between actual and predicted values. Although more complete and more informative than the model proposed by Grosz and Stephanopoulos (1990b), this model does not take into account the formation of biomass, the reactions occurring in the TCA cycle, or the placement of enzymatic reactions within various membrane-bound organelles found within yeast. The TCA cycle in bacteria occurs in the cytoplasm while in yeast and other eukaryotes it resides in mitochondria. Transport of TCA intermediates across the mitochondrial membrane will influence enzymatic reactions on both sides of that membrane.

In other work, Nissen *et al.*, (1997) generated a structured model which contained 37 metabolic reactions involving 43 compounds. The authors based the concentrations of 13 of those compounds on their own experimental data from the anaerobic fermentation by *S. cerevisiae* in continuous culture using defined medium with glucose as the limiting nutrient. In addition to compiling a large number of enzymatic reactions, the authors also included the full TCA cycle compartmentalized in a mitochondrion and included equations for the transport of compounds across the mitochondrial membrane. The data generated from the model using metabolic flux analysis (MFA) fit perfectly all experimental data and also the rates of glucose consumption, ethanol production, and glycerol production. The power of this structured model is realized when, once the model parameters were determined and fitted to experimental data, vast amounts of information were obtained. The model showed at different dilution rates information about all specific enzymatic activities, yields of CO<sub>2</sub>, ethanol, biomass, glycerol, succinic acid, acetic acid, and pyruvic acid, cellular maintenance coefficients, concentrations of protein, glycogen, trehalose, RNA and DNA, and fluxes through

reactions at important branch points such as the flux of carbon through the pentose phosphate and glycolytic pathways. The difference in activity between cytosolic and mitochondrial alcohol dehydrogenases was also predicted by the model. The authors conducted a sensitivity analysis of the model and concluded that in order for the model to be accurate, only the concentrations of glucose, CO<sub>2</sub>, ethanol, glycerol, and protein need to be determined during the fermentation. The authors constructed the model using data from glucose limited chemostats and so it would be of great interest to see if their model could be used to predict the course of fermentations under other conditions. The validity of this type of model and the analysis behind the model was shown by Lin *et al.* (2001, 2002). Lin used experimental data from the five stage multistage continuous fermentation system (described later in this thesis) under “aerobic fermentation” conditions employing *S. cerevisiae* in a complete optimized medium at 15% w/v glucose. Using the model of Nissen *et al.*, (1997) with small modifications, and the experimental data from the chemostats, Lin successfully modeled the course of fermentation in all the fermentors in the multistage system. The authors concluded that MFA has the potential to provide information about the health of the yeast as well as potential medium formulation limitations. In addition, MFA could also serve as a predictive tool to assess the performance of yeasts in fermentations at higher gravity and under altered process conditions.

Although more complex to construct and to implement than unstructured models, structured models have the ability to model data obtained from experimentation and also predict data that were not obtained during the fermentation.

### 3 METHODS AND MATERIALS

#### 3.1 Master cultures.

##### 3.1.1 *S. cerevisiae*.

###### 3.1.1.1 Source and purity tests.

An industrial strain of *Saccharomyces cerevisiae* (“Allyeast Superstart”) was provided on a slant by Alltech Inc. (Nicholasville, KY). The following procedure was used to determine purity of the master yeast.

The slant was streaked onto two screw-capped YEPD slants to maintain a source of master yeast during the check for contaminants. YEPD slants (and plates) had the following composition (per L): 10 g Bacto yeast extract (Difco, Detroit, MI), 10 g Bacto peptone (Difco), 20 g D-glucose (BDH, Toronto, Ontario), and 15 g/L Bacto Bacteriological Technical agar (Difco). Two additional YEPD slants adjusted to a pH of 5.0 that contained 0.005% w/v gentamycin and 0.01% w/v oxytetracycline were inoculated. The low pH and the addition of gentamycin and oxytetracycline would permit growth of all yeasts present in the original slant but would inhibit growth of any bacteria. All YEPD slants were incubated at 27°C for 24 hours, and stored at 4°C.

Dilutions from the YEPD/gentamycin/ oxytetracycline agar slants were made by adding 2 ml of 0.1% w/v sterile peptone water to the slant and suspending the cells. Dilutions of  $10^{-2}$  to  $10^{-5}$  were then prepared in sterile 0.1% w/v peptone water.

To determine the presence of non-*Saccharomyces* wild yeasts (if present in the preparation), all dilutions were membrane filtered (see Section 3.2.1 for procedure) onto lysine agar plates with the following composition (all chemicals from Sigma, St. Louis, MO) (per L): 44.5 g glucose, 1.78 g  $\text{KH}_2\text{PO}_4$ , 0.89 g  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.178 g  $\text{CaCl}_2$ , 0.089 g NaCl, 1.78 mg adenine, 0.891 mg DL-methionine, 0.891 mg L- histidine, 0.891 mg DL-tryptophan, 0.0089 mg boric acid, 0.0356 mg  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.178 mg

$(\text{NH}_4)_5\text{Mo}_7\text{O}_{24}$ , 0.0356 mg  $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ , 0.2225 mg  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 1.0 g lysine, 0.02 g inositol, 0.002 g calcium pantothenate, 0.4 mg aneurine, 0.4 mg pyridoxine, 0.2 mg p-aminobenzoic acid, 0.4 mg nicotinic acid, 0.2 mg riboflavin, 0.002 mg biotin, 0.001 mg folic acid, and 17.8 g agar. Inoculated plates were incubated at 27°C for one week. Plate counts were performed daily.

To determine the presence of *Saccharomyces* wild yeasts, all dilutions were membrane filtered (see Section 3.2.1 for procedure) and the membranes then put onto Lin's Wild Yeast Medium (LWYM) with the following composition (all chemicals from Sigma, St. Louis, MS) (per L): 4 g yeast extract, 2 g malt extract, 2 g peptone, 10 g glucose, 1.0 g  $\text{K}_2\text{HPO}_4$ , 0.5 g  $\text{NH}_4\text{Cl}$ , 0.4 mg crystal violet, 0.1 g fuchsin-sulfite mixture, and 20 g agar. Inoculated plates were incubated at 27°C for one week. Plate counts were performed daily.

To determine the presence of bacterial contaminants, all dilutions were membrane filtered (see Section 3.2.1 for procedure) and membranes put onto YEPD and MRS plates both with 20 mg/L cycloheximide to inhibit yeast growth. The inoculated YEPD plates were incubated at 27°C for one week. The inoculated MRS plates were incubated for a week in a  $\text{CO}_2$  incubator (National Appliance Co, Portland, OR). Plate counts were performed daily.

None of the test plates showed any colonies after incubation for one week. This indicated that the provided yeast culture was free from bacteria, and from any *Saccharomyces* and non-*Saccharomyces* wild yeasts detectable by the media used. The selected culture was further characterized on a API 20C strip (API Laboratory Products Ltd., St. Laurent, Quebec) which verified at 96.8% confidence that the culture was *Saccharomyces cerevisiae*. Microscopic evaluations of the selected yeast culture confirmed the absence of bacteria, and the presence of individual yeast cells of similar size and shape.

### 3.1.1.2 Master culture preparation and storage.

Once the purity of the Alltech yeast was confirmed, it was inoculated into 100 ml of YEPD broth in a 250 ml screw-capped sidearm flask and grown overnight with shaking (150 RPM) at 30°C. Sterile glycerol was then added to the culture and mixed to bring the final concentration of glycerol in the culture to 20% v/v. Aliquots of the culture were then aseptically dispensed into 150 sterile 1.8 ml cryogenic vials and stored at -70°C (Parton and Willis, 1990; Gherna, 1981). These master vials were used when required over the course of the project.

### 3.1.2 *Lactobacillus paracasei*.

#### 3.1.2.1 Source.

An industrial strain of *L. paracasei* was isolated from a fuel alcohol plant by Dr. Jaime Finguerut at the Centro de Tecnologia Copersucar, Bairro Santo Antonio, Piracicaba, Brazil. Identification was confirmed as *L. paracasei* using an API 50 CHL kit for *Lactobacillus*, and subcultured onto MRS slants to provide a laboratory master stock (Hynes *et al.*, 1997). This industrial isolate is homofermentative and produces a higher amount of lactic acid than heterofermentative lactic acid strains. Homofermentative lactic acid bacteria produce lactic acid as the major metabolic by-product; this acid is inhibitory to *S. cerevisiae* at ~0.8% in batch fermentation (Narendranath *et al.*, 2000b). In addition, this strain grows quickly. Colonies of food or brewing lactobacilli require up to a week to develop on MRS plates in sharp contrast to only 24 h required for this *Lactobacillus*. Lactic acid bacteria that show this characteristic have been dubbed “ferocious” lactobacilli (Alexandre and Charpentier, 1998). Thus, this “worst case”, industrially relevant *Lactobacillus* was chosen as the contaminant for this project.

### **3.1.2.2 Master culture preparation and storage.**

A loopful of the laboratory master stock was inoculated into screw-capped MRS (Unipath, Nepean, Ontario, Canada) agar slants. The headspaces in the slants were flushed with filter-sterilized CO<sub>2</sub> on inoculation to provide an oxygen-free environment suitable for growth. Inoculated slants were incubated overnight at 30°C in a CO<sub>2</sub> incubator (National Appliance Co, Portland, OR), removed, and stored at 4°C. The slants in storage were subcultured monthly.

## **3.2 Analyses.**

### **3.2.1 Viable count by membrane filtration.**

The membrane filtration procedure for viable cell counting was used (Ingledeew and Burton, 1980). For each fermentation sample processed, three membrane filtration units were pre-washed in distilled water, pre-dried in an oven at 105°C for five minutes (to ensure absence of moisture in the sintered glass base), and then sterilized for 15 min in a Millipore XX63 700 00 sterilizer cabinet equipped with four Sylvania G8T5 UV lamps (Millipore Corporation, Bedford, MA). Triplicate aliquots of the appropriately diluted fermentation sample were vacuum-filtered through sterile 0.45 µm 47 mm grided GN-6 Gelman membrane filters (Gelman Sciences Inc., Ann Arbor, MI) aseptically placed on the sterile filter base. The funnel and filter were then rinsed with 5 ml of 0.1% sterile peptone water and the filter was removed and “rolled” onto the surface of an appropriate prepoured agar plate.

#### **3.2.1.1 Viability of *S. cerevisiae*.**

For the enumeration of *S. cerevisiae* in the absence lactobacilli, the medium on which membranes were placed was YEPD agar. Inoculated plates were incubated

aerobically at 27°C for 2-3 days.

### **3.2.1.2 Viability of *S. cerevisiae* in a mixed culture containing *L. paracasei*.**

For the enumeration of *S. cerevisiae* in the presence of lactobacilli, final concentrations of 0.005% w/v filter sterilized gentamicin and 0.01% w/v filter sterilized oxytetracycline were aseptically added to molten YEPD agar (after autoclaving and cooling to ~50°C). Poured plates were cooled, filtered membranes transferred, and incubated aerobically at 27°C for 2-3 days.

### **3.2.1.3 Viability of *L. paracasei* in a mixed culture containing *S. cerevisiae*.**

For the enumeration of *L. paracasei* when yeasts were present, deMan, Rogosa, Sharpe (MRS) agar (Unipath, Nepean, ON) was used with 0.001% w/v filter sterilized cycloheximide. Plates to which membranes were added were incubated at 27°C in a CO<sub>2</sub> incubator for 3-4 days.

### **3.2.2 Viable, dead, and budding yeast cells by methylene blue staining.**

The methylene blue procedure as outlined by Thomas *et al.*, 1990 was used. A one ml aliquot of sample was diluted with an appropriate amount of Ringer's solution (with methylene blue) to give a total cell count of 400-500 (in five of 25 squares in an improved Neubauer hemacytometer) when viewed under the microscope. After an incubation time of five min, total, viable, and dead yeast cells were all enumerated. The composition of Ringer's solution was as follows (per L): 8.6 g NaCl, 0.3 g KCl, 0.33 g CaCl<sub>2</sub> (anhydrous), 10 g sucrose, and 0.5 g Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub>•5H<sub>2</sub>O. Methylene blue was added at a concentration of 250 mg/L of Ringer's solution.

### **3.2.3 Yeast dry mass.**

The medium formulation used in the experiments complicated dry weight determinations since all media used in this study contained particulate matter (from the corn steep powder (CSP) fraction). To overcome this, a medium reservoir sample was processed in addition to the fermentor samples. A 10 ml aliquot from each sample was filtered (in triplicate) through a preweighed 0.45  $\mu\text{m}$  47 mm grided Gelman GN-6 membrane filter, which was then placed into pre-weighed aluminum foil pans, dried at 105°C for two hours to constant weight, cooled in a desiccator, and reweighed to four decimal places (Bayrock and Ingledew, 1997). The yeast dry mass was determined by subtracting the mass of the medium reservoir sample (particulate matter) from the fermentor samples (yeast and particulate matter).

### **3.2.4 HPLC analysis.**

Lactic acid, glycerol, ethanol, and glucose concentrations were determined by HPLC analysis. The samples were thawed and diluted to the required extent with Milli-Q water. Aliquots of the diluted samples (5  $\mu\text{l}$ ) were each mixed with an equal volume of 2% w/v boric acid (internal standard), and injected into a Biorad HPX-87H Aminex column equilibrated at 40°C. The eluant was 5 mM sulfuric acid flowing at a rate of 0.7 ml/min. The components were detected by a differential refractometer (Model 410, Waters Chromatographic Division, Milford, MA) and the subsequent data processed by the supplied Maxima 810 software (Waters Chromatographic Division). During the project, the Maxima 810 software was replaced by Waters Millennium<sup>32</sup> software. Analyses of identical chromatograms by both pieces of software yielded no differences in the concentrations of components. No difference in sample component concentrations were found when randomly-chosen identical samples were re-injected following storage for 3 weeks.

The concentration of undissociated lactic acid at the pH of each fermentation medium was determined by calculation using the Henderson-Hasselbach equation (Narendranath *et al.*, 2000). Unless otherwise stated, all lactic acid concentrations recorded in this work refer to undissociated lactic acid concentrations.

### **3.2.5 Mathematical analysis of continuous culture experiments.**

All differential equations used in each model to simulate MCCF fermentations were entered into an Excel 97 spreadsheet along with constants determined from batch experiments in Section 3.4.2. Finite time analysis procedures (with 0.005  $\Delta t$  values) were used in the determination of  $dX/dt$ ,  $dS/dt$ , and  $dP/dt$  at each time interval and from these values, the X (biomass concentration), S (glucose concentration), and P (ethanol concentration) were determined by "walking" X, S, and P concentrations using corresponding  $dX/dt$ ,  $dS/dt$ , and  $dP/dt$  values. The constants used in the models were held constant across all equations in all models. In each model, the number of rows used in Excel to calculate X, S, and P was increased until the calculated values of X, S, and P reached steady state. Most of the models required 10,000 rows x 7 columns or 70,000 cells to reach steady state in X, S, or P - which meant that each model required a minimum of 210,000 cells (210,000 calculations) to clearly show steady state. Some models required more than 1,000,000 calculations to show steady state in X, S, and P.

Ideally, one would determine X, S, and P in each model from their differential equations by performing numerical integration over each  $\Delta t$  interval to provide true values of X, S, and P. Some of the more common numerical integration types which are routinely used are: rectangular area integration, Simpson integration, and fourth order Runge-Kutta integration. Of these, it is generally agreed upon that fourth order Runge-Kutta integration consistently provides integrated values which closely match actual values. Unfortunately, Excel does not have any native numerical integration routines. In addition, published algebraic formulae do exist that calculate fourth order Runge-Kutta values that could be modified to work in Excel, but these formulae require that only one

variable be present in all equations. All of the models used in this work have 3 variables (X, S, and P) and thus deriving fourth order Runge-Kutta formulae that would work in Excel would be difficult. Other excellent software packages such as Matlab ([www.mathworks.com](http://www.mathworks.com)) and Octave ([www.octave.org](http://www.octave.org), an open source, free clone of Matlab which runs under Windows, Macintosh, and Linux operating systems) have the capability of integrating a system of algebraic-differential equations containing multiple variables, but need to be provided the values of all constants. This limits the usefulness of these other software packages since many models have constants that are not known in advance. By contrast, in Excel the "Solver" function can determine the value of one or multiple unknown constants in each model and the model therefore provides desired values. The values from the "walking" method ( $0.005 \Delta t$ ) in Excel were compared to fourth order Runge-Kutta integration in Octave ( $0.05 \Delta t$ ) when the Aiba model was simulated in each with identical conditions. The steady state values returned by each were nearly identical and differed by only 2% (maximum). Thus, the "walking" method used in this work, though not ideal, provides results that compare favourably to fourth order Runge-Kutta integration when  $\Delta t$  in the "walking" method was reduced by a factor of 10.

The value for each constant which was determined experimentally in this work was uniformly applied (where applicable) to all models containing the same constants. In models where all the constants were known, the "walking" method was used to predict the X, S, and P concentrations in all fermentors in the MCCF. In models where not all of the constants were known, the "Solver" function was used to determine the value of the unknown constants that resulted in the model providing values in steady state that matched experimental steady state values in F1 under conditions where the medium reservoir contained 152 g/L glucose. The time taken for "Solver" to determine the unknown values for a model ranged from one to 3 hours for one iteration with 10 iterations usually required. To determine in each model the steady state values of X, S, and P at other medium reservoir sugar concentrations, only the dilution rate and the medium reservoir glucose concentration were changed in the models. Ideally, one would determine the value of unknown constants in a model by attempting to fit the values

generated from the model to as many experimental data points as possible to increase the accuracy of the model over the entire simulation time. However, in the present work, samples were taken from the MCCF to confirm steady state and did not include samples over the course of the non-steady state fermentation. Also, at best, only one sample per day per fermentor could be processed from the MCCF (8-10 hours) - a very low degree of resolution. In this work, the objective of applying mathematical models to the MCCF data was to see which model(s) best predicted the steady state values of X, S, and P in the MCCF and not the course of each leading up to steady state.

### **3.2.6 Mathematical analysis of batch growth curves.**

All batch growth data for *S. cerevisiae* were analyzed mathematically according to the procedure described by Zwietering *et al.*, (1990). The curve-fitting mathematical routines calculated the maximum slope (and therefore maximum growth rate) for exponential growth of yeast in each flask. Statistical analysis (t test) of each original and curve-fitted sigmoidal curve pair indicated no significant differences in any curve pair (95% confidence interval).

### **3.3 Medium optimization.**

The base medium used for all continuous culture and batch experiments was composed of crude glucose (cerelose Casco #020010, Canada Starch Company, Etobicoke, ON), corn steep powder (CSP, Marcor Development Corporation, Hackensack, NJ), and diammonium phosphate (DAP) (Monsanto, Trenton, MI) abbreviated as G, C, and P below. In order to determine the proper amounts of each component to provide *Saccharomyces cerevisiae* with sufficient nutrients that it would need to completely ferment very high gravity (VHG) media, a 1x4x4 factorial experiment was designed with levels of each component as shown in Table 3.1. Flasks of G<sub>1</sub>C<sub>1</sub>P<sub>1</sub> and G<sub>1</sub>C<sub>4</sub>P<sub>4</sub> were prepared and the pH, turbidity (Klett units), and a<sub>w</sub> were determined to see

**Table 3.1** Factors and factor levels used in medium optimization experiments:

Factor	Factor level in factorial experiment			
	1	2	3	4
Glucose <b>G</b>	34 g/100 ml	34 g/100 ml	34 g/100 ml	34 g/100 ml
Corn Steep Powder <b>C</b>	0.5 g/100 ml	1 g/100 ml	2 g/100 ml	4 g/100 ml
Diammonium Phosphate <b>P</b>	100 mg P/L <sup>1</sup> (91 mg N/L)	200 mg P/L (180 mg N/L)	400 mg P/L (362 mg N/L)	800 mg P/L (724 mg N/L)

<sup>1</sup> Shown are the mg P/L and mg N/L determined from added  $(\text{NH}_4)_2\text{HPO}_4$ .

if any differences existed between the lowest and highest factorial condition. The difference found in pH ( $G_1C_1P_1 = 4.93$ ,  $G_1C_4P_4 = 4.80$ ) between the two flasks was 0.13 which would not seriously impact yeast performance. However, the difference of 28.5 found in turbidity (Klett units) of the media before inoculation ( $G_1C_1P_1 = 19$ ,  $G_1C_4P_4 = 47.5$ ) indicated that each factorial condition required its own uninoculated control. Also, the difference of 0.08 found in  $a_w$  between the two flasks (which translates to 4.02 g /100 ml dissolved solids) indicated that differences existed in osmotic pressure that the media would exert. Differences in osmotic pressure may affect the fermentative performance of *S. cerevisiae* and make a comparison between factorial conditions difficult. This difference in osmotic pressure was eliminated from all conditions by calculating the difference in total dissolved solids for each condition as compared to  $G_1C_4P_4$  and adding sorbitol (non-metabolizable by *S. cerevisiae*) to each condition to make up the difference. The 16 combinations listed in Table 3.1 were each run with an uninoculated control to give 32 flasks to process in one factorial experiment. Two sets of experiments were run to provide enough data for statistical evaluation.

Concentrated stock solutions of crude glucose (42.5 g /100 ml), CSP (25 g/100 ml), and sorbitol (25 g/100 ml) were prepared in two litre volumetric flasks and autoclaved for 20 min at 121°C. After cooling to room temperature in a laminar flow hood, the volume lost during autoclaving in each volumetric flask was made up by aseptically adding sterile distilled water to each flask. A concentrated sterile stock solution of DAP (17.0544 g /100 ml) was prepared by filter sterilizing a two litre volume of the solution through a sterile filtration unit which contained a 0.45  $\mu\text{m}$  47 mm grided GN-6 Gelman membrane filter. Each factorial condition listed in Table 3.1 was aseptically formulated from the sterile stock solutions in a laminar flow hood, and the difference in calculated dissolved solid concentration in each flask (as compared to  $G_1C_4P_4$ ) was made up by adding sterile concentrated sorbitol. Sterile distilled water was added to bring up the volume of each flask to 250 ml while preserving the desired factor levels in each flask. One ml of a pre-inoculum of *S. cerevisiae* was added to each flask and they were incubated at 28°C at 100 RPM in an rotary shaker incubator. The growth of *S. cerevisiae*

was monitored by measuring the absorbance using a Klett-Summerson colorimeter (Klett Mfg Co, New York, NY, USA) equipped with a #66 red filter (640-700 nm). Samples (1 ml) were withdrawn from each flask at four hour intervals, filtered through 0.45  $\mu$ m 47 mm grided Gelman GN-6 membrane filters and frozen for future HPLC analysis.

The results from factorial experiments were analysed on the SAS statistical analysis program with the following statistical tests: Analysis of Variance, Duncan's Multiple Range Test, Ryan-Einot-Gabriel-Welsch Multiple Range Test, and Tukey's Studentized Range Test (HSD).

### **3.4 Batch culture experiments.**

#### **3.4.1 Growth of *S. cerevisiae* with lactic acid.**

Batch growth of yeast was followed using a medium which contained the same glucose (26% w/v), CSP (2% w/v), and DAP (20 mM) concentrations as media formulated for continuous culture experiments (Section 3.5.1.2). A clarified and concentrated solution of CSP with DAP was prepared and autoclaved at 121°C for 15 min, simulating normal medium preparation for continuous culture experiments. It was then clarified by centrifugation at 10,200 x g for 15 min and filter sterilized through a 0.45  $\mu$ m 47 mm grided Gelman GN-6 membrane filter. A concentrated sterile solution of lactic acid was also prepared by first autoclaving the solution at 121°C for 30 min to hydrolyze all lactic anhydride to lactic acid, and then filter sterilizing. A concentrated solution of glucose was sterilized similarly by membrane filtration. Media for batch growth were combined aseptically from concentrated stocks to provide (in duplicate) a 0-7% w/v range of final lactic acid concentrations in flasks with identical glucose and CSP concentrations as in the medium used for continuous culture experiments. The pH of each medium was measured in a 10 ml sample. Another 10 ml aliquot from each flask was frozen and stored for subsequent HPLC analysis.

A 100 ml aliquot of each medium was dispensed into sterile 250 ml screw-capped, side-armed Erlenmeyer flasks. One ml of a pre-inoculum of *S. cerevisiae* was added and each flask was incubated at 28°C at 100 RPM in a rotary shaker incubator. The growth of *S. cerevisiae* was monitored at hourly intervals by measuring absorbance using a Klett-Summerson colorimeter. When the yeast entered stationary phase, a 10 ml sample from each flask was removed, filtered through a 0.45 µm 47 mm grided Gelman GN-6 membrane filter, and the filtrate was frozen for subsequent HPLC analyses.

#### **3.4.2 Growth of *L. paracasei* with media from MCCF fermentors.**

*L. paracasei* was grown in batch conditions using media withdrawn from each of the five fermentors in the MCCF experiments. Each medium was clarified by centrifuging at 10,200 x g for 15 min at 4°C), and then sterilized by membrane filtration through a 0.45 µm 47 mm grided Gelman GN-6 membrane filter into sterile Erlenmeyer flasks.

A 100 ml aliquot of each clarified and sterilized medium was then dispensed into sterile 250 ml screw-capped side-armed Erlenmeyer flasks and inoculated with *L. paracasei* from a MRS agar slant. Each flask was flushed with filter sterilized CO<sub>2</sub>, capped tightly, and incubated at 28°C at 100 RPM in a rotary shaker incubator. Growth of *L. paracasei* was monitored by measuring absorbance using a Klett-Summerson colorimeter.

#### **3.4.2 Determination of the kinetic growth parameters ( $K_s$ , $K_{si}$ , and $\mu_m$ ).**

Batch growth of yeast was followed using a medium which contained the same CSP (2% w/v) and DAP (20 mM) concentrations as in media formulated for continuous culture experiments. A clarified and concentrated solution of CSP with DAP was prepared and autoclaved at 121°C for 15 min, simulating normal medium preparation for continuous culture experiments. It was then clarified by centrifugation at 10,200 x g for 15 min and filter sterilized through a 0.45 µm 47 mm grided Gelman GN-6 membrane

filter. A concentrated solution of glucose was sterilized similarly by membrane filtration. Media for batch growth were combined aseptically from concentrated stocks. For the determination of  $K_s$  and  $\mu_m$ , a range of glucose concentrations (in triplicate) between 0 and 8.0 g/L in 0.2 g/L increments were formulated while for  $K_{si}$ , a range of glucose concentration (in triplicate) between 0 and 400 g/L in 20 g/L increments was formulated. All flasks contained identical CSP and DAP concentrations as used in the medium for continuous culture experiments. Sorbitol was not added to equalize the osmotic pressure across the flasks for two reasons. First, the classical constants used in modeling (such as  $K_{si}$  - the inhibitory substrate concentration) do not distinguish between the primary biochemical effects the substrate has on the yeast and the secondary osmotic pressure effects on the yeast due to the concentration of the substrate. Both effects are “included” in each constant. Second, the experiments conducted in the MCCF did not include sorbitol in the medium formulation to balance osmotic strength when the glucose concentrations were raised to VHG levels.

A 100 ml aliquot of each medium was dispensed into sterile 250 ml screw-capped, side-armed Erlenmeyer flasks. One ml of a pre-inoculum of *S. cerevisiae* was added and each flask was incubated at 28°C at 100 RPM in a rotary shaker incubator. The growth of *S. cerevisiae* was monitored hourly by measuring the absorbance using a Klett-Summerson colorimeter. When the yeast entered stationary phase, a 10 ml sample from each flask was removed, filtered through a 0.45  $\mu\text{m}$  47 mm grided Gelman GN-6 membrane filter, and frozen for subsequent HPLC analyses.

### **3.5 Continuous culture experiments.**

#### **3.5.1 Medium reservoir.**

##### **3.5.1.1 Construction.**

Four 59 L stainless steel kegs (Sabco Industries, Toledo, OH) were connected in

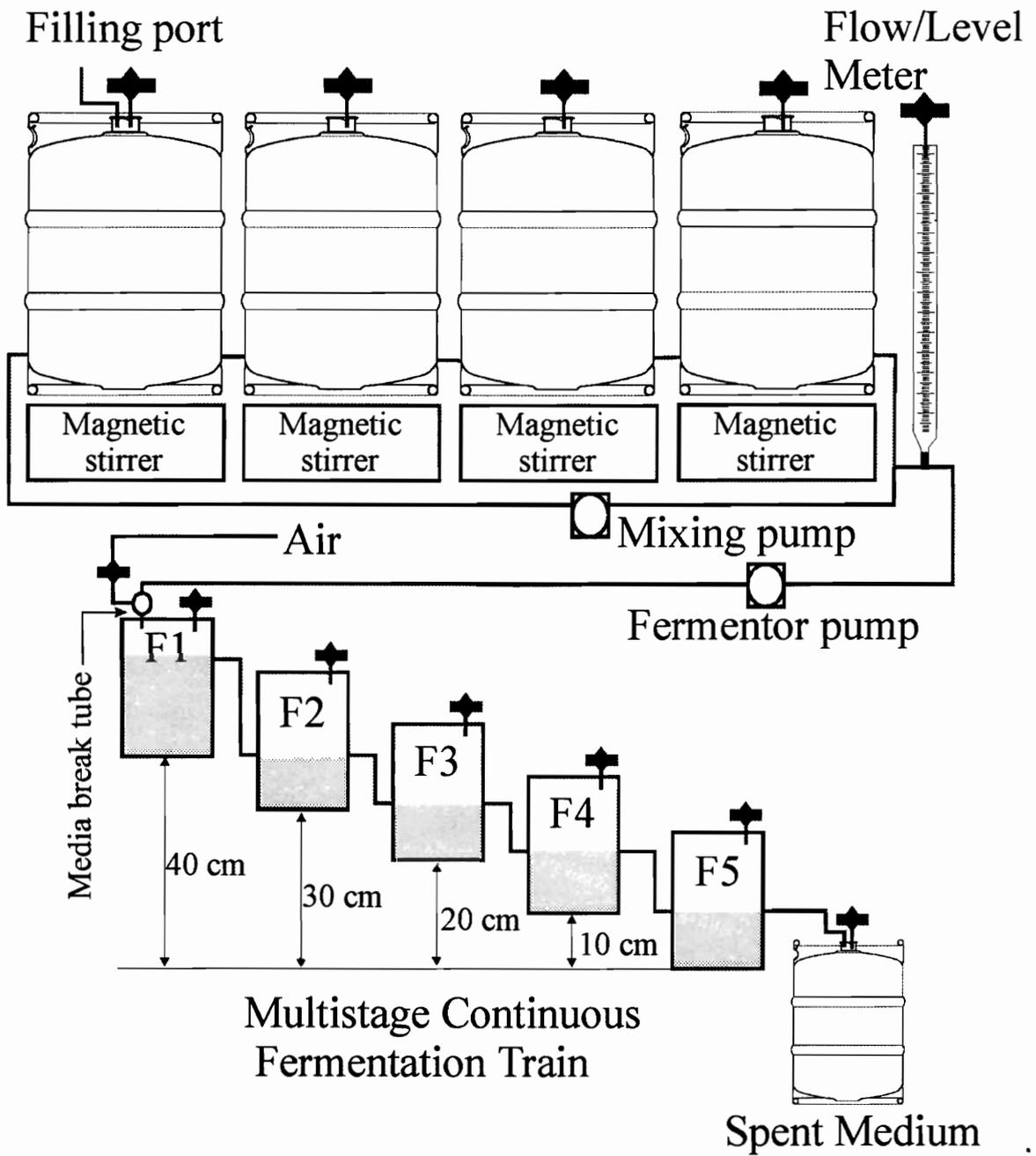
series to provide a medium reservoir capacity of > 200 L as depicted in Figure 3.1. Each keg was custom modified with the addition of two stainless steel threaded ports placed 180° to each other and welded (stainless steel welds) to the keg on the external side wall at the lowest point where the vertical wall begins to taper inward to the base of the keg. Stainless steel hose-barb or blunt end fittings were screwed into the threaded ports with teflon tape to form a mechanical seal.

In preparation for autoclaving, each keg was stoppered with a #9 butyl rubber stopper through which a short stainless steel tube was inserted. An Acrodisk 50 filter disk (Gelman Sciences Inc., Ann Arbor, MI ) was attached to the stainless steel tubing to provide sterile venting. The entire stopper was tightened to the keg by a collection of clamps, and the stopper was sealed to the keg with silicone sealant and allowed to set overnight. On one of the kegs, an additional port was added through the stopper to allow fresh medium to be added to the multi-keg reservoir.

To seal the ports at the bottom of each keg, a short piece of silicone tubing with a tubing clamp was attached to each port and one of the tubing-ports was wrapped to provide an aseptic connector after autoclaving. Each assembled keg was then autoclaved empty for 30 min at 121°C, allowed to cool overnight, and positioned on a Thermolyne Maxi-Stirrer magnetic stirrer (VWR-Canlab, Edmonton, AB, Canada). The kegs were aseptically connected to each other by cutting the tubing off the non-wrapped tubing port, flushing the port with ethanol, and connecting the tubing end in the wrapped port to the exposed port. Each keg contained a large octagonal, teflon magnetic stirbar (8 mm diameter, 51 mm length) to continuously mix the contents of each keg during all experiments.

### **3.5.1.2 Media formulation, preparation, and transfer.**

The medium composition for all continuous culture experiments conducted in the BioFlo III fermentors was composed of: 14-31.4% w/v solutions of starch-derived glucose (cerelose - Casco #020010, Canada Starch Company, Etobicoke, ON), 2% w/v



**Figure 3.1** Schematic diagram of the constructed multistage continuous culture fermentation (MCCF) system.

CSP (determined in Section 3.3), and 20 mM DAP (determined in Section 3.3). Large volumes of media were required on a regular basis. For example, at a working volume of 3 L in the fermentors and a dilution rate of 0.2, the multistage continuous culture system required 14 L of media every 24 h. The glucose and CSP/DAP medium components were autoclaved separately and combined after cooling. The amounts of glucose and water to use in the 59 L keg formulations depended on the desired glucose concentration. For example, to prepare 40 L of 14% w/v glucose media, 6087 g cerelose (92% glucose content), and 26 L of distilled water were prepared in the 59 L stainless steel keg while 1000 g CSP, 105.7 g DAP, and 9.4 L distilled water were prepared in the 12 L stainless steel keg.

The CSP/DAP medium component was autoclaved for 30 min in a 12 L stainless steel keg (Spartanburg Steel Products, Spartanburg, SC) equipped with an Acrodisk 50 filter disk and a wrapped aseptic transfer line. The glucose solution was prepared by dissolving the calculated amount of glucose with the remainder of the calculated amount of deionized water. The temperature of the glucose solution was gradually increased to ~50-60°C in a sanitized pilot plant steam kettle with constant stirring to aid dissolution. The hot, mixed glucose solution was then poured into a 59 L stainless steel keg, ports sealed with an assortment of clamps, autoclaved for 50 minutes, and removed from the autoclave to cool (see Section 6.2). The following day, the sterile transfer lines on the glucose and CSP/DAP kegs were aseptically connected and the contents of the CSP/DAP keg were transferred to the glucose keg to make one 50 L batch of complete medium.

Prepared medium was added to the medium reservoir by aseptically connecting a keg containing the sterile medium to the multi-keg medium reservoir at the medium addition port, and pressurizing the medium keg with sterile air at 3 psi to transfer the contents. The multi-keg medium reservoir was then pumped from the first to the fourth keg for nine hours using an external peristaltic mixing pump. After nine hours, the pump on the external mixing loop was shut off. The nine hour mixing time was determined to be sufficient to ensure complete mixing in the reservoir when fresh medium was added to the reservoir (see Section 6.1).

### **3.5.2 Multistage continuous culture fermentation (MCCF).**

#### **3.5.2.1 Construction and preparation.**

Five Bioflo III fermentors (New Brunswick Scientific, Edison, NJ) were connected in series to produce a multistage continuous culture system simulating, in miniature, typical industrial designs (Fig. 3.1). In order to minimize the equipment necessary to move the fermentation liquid from one fermentor to another in the multistage system, each fermentor was raised 10 cm from the previous fermentor to allow gravity to move (via overflow ports) the fermentor contents. Custom stainless steel port connectors with a large internal bore to prevent clogging were constructed and installed on each fermentor. A 50 ml burette with an Acrodisk 50 filter disk connected on top was positioned between the medium reservoir and the first fermentor to act both as a medium flowmeter, and as a medium level indicator for the medium reservoir. Fermentor 1 (F1) had a working volume of 5.04 L while fermentors two to five (F2-F5) each had a working volume of 2.8 L. Fermentations were conducted at 28°C with 100 RPM agitation. The medium and the sterile air at two standard litres per minute (SLPM) entered F1 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 benefited from the presence of oxygen in air as oxygen is essential to yeast even in anaerobic fermentations for synthesis of required membrane unsaturated fatty acids and sterols (Andreasen and Stier, 1953; O'Connor-Cox and Ingledew, 1989).

#### **3.5.2.2 Fermentor autoclaving.**

All fermentor ports and tubing ends were clamped with tubing clamps and carefully wrapped. All Acrodisk 50 filter disks used on the fermentors, medium kegs, flowmeter, and the reservoir were tested for water integrity as prescribed by Gelman. The

50 ml burette, along with a length of silicone tubing that was used in the peristaltic pump, was attached to the first fermentor and autoclaved. After autoclaving the empty fermentors at 121°C for 30 min, the five fermentors were aseptically connected (serially), the first fermentor connected to the medium reservoir, and the last fermentor connected to the effluent bottle.

### **3.5.2.3 Inoculum preparation for MCCF.**

One cryogenic vial containing the yeast (See Section 3.1.1.2) was thawed and aseptically added to a one litre screw-capped flask containing a 500 ml portion of the medium under current use in the fermentor. This inoculum was grown overnight with shaking (150 RPM) at 30°C.

### **3.5.2.4 Fermentor inoculation and equilibration for MCCF.**

The medium in the medium reservoir was pumped overnight into all the fermentors to fill each fermentor to its respective working volume. The following day the agitation and cooling lines on each fermentor were turned on and set to the desired values. As well, the medium flowrate into F1 was adjusted to the desired value and rechecked to ensure stability during the course of the experiment. The yeast inoculum was then poured into F1 and allowed to simultaneously inoculate (by overflow) all the fermentors in the system. The system was allowed to run for a week to allow the yeast to reach steady state in each of the five fermentors before withdrawing any samples. Steady state was confirmed when the glucose concentration in each fermentor did not vary by more than 5% over a three day sampling period. If any glucose was detected in F5 at steady state, the medium flowrate to F1 was reduced and the system was allowed to re-equilibrate for a week. This was repeated until the glucose concentration in F5 reached a minimum value (as close to 0 as possible).

### **3.5.2.5 Measuring medium flowrate in MCCF.**

To determine the medium flow rate into the MCCF, the burette flowmeter was first flushed twice with medium from the reservoir bottle and then medium was drawn up to the top of the 50 ml burette. The line connecting the medium reservoir to the flowmeter was then clamped and the time taken for the flowmeter to empty was determined. The medium flow rate was measured daily three times to ensure consistent flow.

### **3.5.2.6 Fermentor sampling.**

Each fermentor was sampled using the sampling port present on the fermentor headplate. The sampling line was first purged by removing and discarding the contents of the sampling line (~10 ml). A sterile sampling flask was then aseptically connected to the sampling port and a 100 ml test sample from the fermentor was removed and placed on ice. A sterilized 50 ml vial was then placed on the sampling port to prevent contamination until the next sampling.

A portion of each sample was used immediately for viable plate counts and microscopic evaluations and the remaining amount was filtered through a 0.45  $\mu\text{m}$  25 mm Gelman membrane filter (Gelman Sciences Inc., Ann Arbor, MI) and frozen for future chemical analysis.

## **3.5.3 Individual continuous stirred tank reactor (CSTR) fermentations with lactic acid addition.**

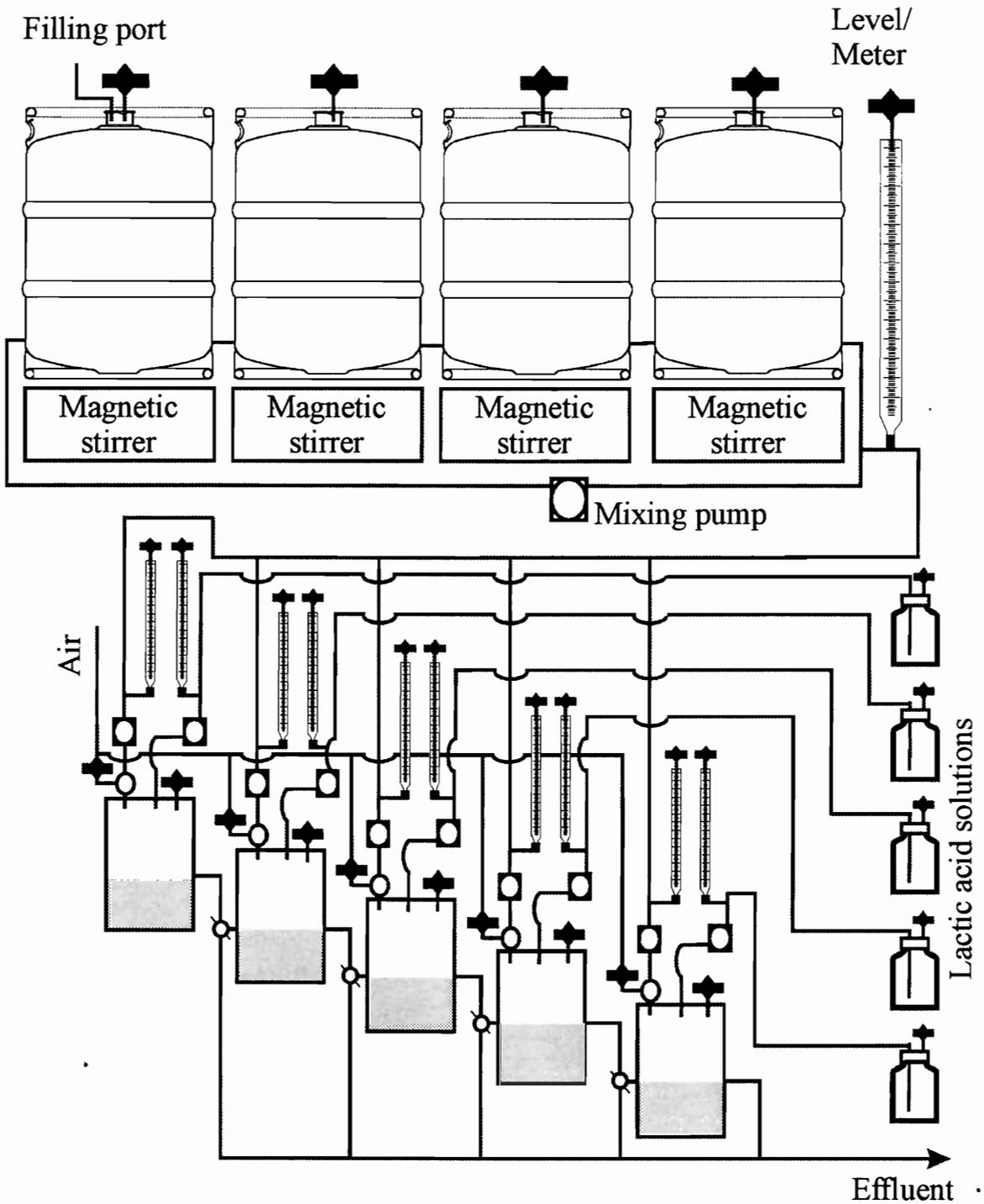
### **3.5.3.1 Construction and preparation.**

The MCCF system as described in Section 3.5.2.1 was used as a base system with the following modifications. On each fermentor effluent port line, a "T" connection was

made so that a choice could be made either to allow the fermentor contents to proceed to the next fermentor in the MCCF, or to drain the overflow into a common effluent line. This arrangement allowed the constructed fermentation system to be used either in MCCF or in individual CSTR modes, and also allowed switching from one mode to another while the system was operating. Each fermentor had an independent supply of sterile medium, air, and lactic acid. For each fermentor, the medium feed was supplied by an external peristaltic pump and the lactic acid feed was supplied by the on-board Bioflo pump. The medium and lactic acid feeds on each fermentor were calibrated and monitored during operation as previously described (Section 3.5.2.5) using inline sterile ten and one ml burettes with an Acrodisk 50 filter disk attached to the top of each. Lastly, the working volume for the first fermentor in the MCCF was changed to 2.8 L to match volumes of other MCCF fermentors. The modified fermentation system is shown in Figure 3.2.

### **3.5.3.2 Inoculation and equilibration of CSTR fermentors.**

Individual CSTR mode fermentations were performed by preparing (Sections 3.5.2.2, 3.5.2.3 ) and inoculating (Section 3.5.2.4) the system initially in MCCF mode. After 24 h, the system was converted to create five individual and identical CSTR fermentations by redirecting the effluent flows in each fermentor, and by providing sterile air and medium to each fermentor to match conditions provided in the first fermentor (F1) of an MCCF system. Fermentations were conducted at 28°C with 100 RPM agitation. Sterile air was supplied to each fermentor at two standard litres per minute (SLPM). The medium and the 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). The fermentors were run for at least seven days (11 working volume displacements) at a chosen dilution rate to allow the system to reach steady state. A steady state was reached when the glucose concentration in each fermentor varied by less than 5% over three consecutive days of sampling. On



**Figure 3.2** Schematic diagram of the constructed multistage continuous culture fermentation (MCCF) system with lactic acid addition.

reaching steady state, lactic acid was introduced into each fermentor in the system.

### **3.5.3.3 Lactic acid addition to CSTR fermentors.**

Sterile medium and diluted lactic acid (Purac, Lincolnshire, IL) were pumped separately into the fermentors at the rates of 3.1 ml/min and 0.5 ml/min respectively. With these rates of pumping, the effective dilution rate was  $0.077 \text{ h}^{-1}$ . The lactic acid concentration in each fermentor was varied by adjusting the concentration of stock solution while maintaining the rate of addition (0.5 ml/min) and dilution rate ( $D = 0.077 \text{ h}^{-1}$ ) constant. A control fermentor with only the endogenous level of medium lactic acid was run at 0.5 ml/min with sterile distilled water which served as a comparison to treated fermentors. Following the introduction of acid, the individual CSTR fermentors were then run for seven days (13 working volume displacements) to allow equilibration to new steady states which were confirmed when the glucose concentrations in each fermentor each varied again by less than 5% over three subsequent days of sampling. Sampling of the fermentors was performed as in Section 3.5.2.6. All lactic acid concentrations reported for continuous culture fermentations in this work correspond to the lactic acid concentrations achieved in the continuous fermentors once steady state has been achieved.

### **3.5.4 MCCF with introduction of *L. paracasei*.**

#### **3.5.4.1 Construction and preparation.**

Five Bioflo III fermentors (New Brunswick Scientific, Edison, NJ) were connected in series to produce an MCCF system as outlined in Section 3.5.2.1. All materials and procedures regarding MCCF medium formulation and preparation, MCCF preparation, and operation and sampling of the MCCF were followed as outlined in Section 3.5.2. A steam sterilizable pH probe was inserted into each fermentor headplate

prior to autoclaving to provide optional pH control. Where pH control was needed, 6M 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 two SLPM.

### **3.5.4.2 Inoculum preparation.**

#### **3.5.4.2.1 *S. cerevisiae*.**

The *S. cerevisiae* inoculum was prepared as in Section 3.5.2.3.

#### **3.5.4.2.2 *L. paracasei*.**

*L. paracasei* for the MCCF was prepared by transferring cells from a stock MRS 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<sub>2</sub> and incubated in a rotary shaker at 100 RPM at 30°C for 24 h. In order to obtain 1:100, 1:1, and 70:1 inoculation ratios of *L. paracasei* : *S. cerevisiae*, the viable numbers/ml of medium of each microbe was determined. The steady state viable count of *S. cerevisiae* in the MCCF operating at 28°C, 100 RPM, and at a flowrate of 0.336 L/h was  $3.8 \times 10^7$  cells/ml. As well, work done in this laboratory had verified the relationship between CFU/ml of *L. paracasei* grown in MRS broth and absorbance in a Klett-Summerson colorimeter. For this bacterium, 100 Klett units are known to be equivalent to  $\sim 8.54 \times 10^8$  CFU/ml, and mid-exponential *L. paracasei* were removed from MRS at approximately 352 Klett units (Narendranath and Ingledew, 2000). To prepare a 70:1 inoculation ratio in the MCCF containing  $70 \times 3.8 \times 10^7$  CFU/ml yeast (the steady state yeast population),  $2.7 \times 10^9$  CFU/ml of *L. paracasei* were required in the MCCF. With a working volume of 5060 ml in fermentor F1, the number of cells of *L. paracasei* required was  $1.4 \times 10^{13}$  CFU. With a target Klett value of 352 ( $3.00 \times 10^9$  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<sub>2</sub>, and incubated in a rotary shaker at 100 RPM at 30°C. Once a Klett value of 352 was reached on the Klett-Summerson colorimeter, cells were aseptically harvested by centrifugation at 10,200 x g for 15 min at 4°C. To eliminate the problem of diluting the steady state *S. cerevisiae* with a large volume of the resuspended bacterial pellet, a volume (500 ml) from F1 was removed and used as the final resuspension fluid for the pellet of *L. paracasei*. Once resuspended, the *L. paracasei* was immediately inoculated into F1.

#### **3.5.4.3 Fermentor inoculation and equilibration of the MCCF.**

Medium from the 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°C. As well, the medium flowrate was set at 0.336 L/h (D for F1 = 0.066 h<sup>-1</sup>; D for F2 and other fermentors = 0.12 h<sup>-1</sup>). Dilution rates differ because F1 holds twice the medium volume as in F2-F5, a practice often seen in industrial MCCF designs. The yeast was added to F1 and allowed to inoculate (by overflow) all the fermentors in the system. The system was allowed to run for seven 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.

#### **3.5.5 MCCF with penicillin G addition.**

##### **3.5.5.1 Construction and preparation.**

The MCCF system as described in Section 3.5.3.1 was used as a base system with

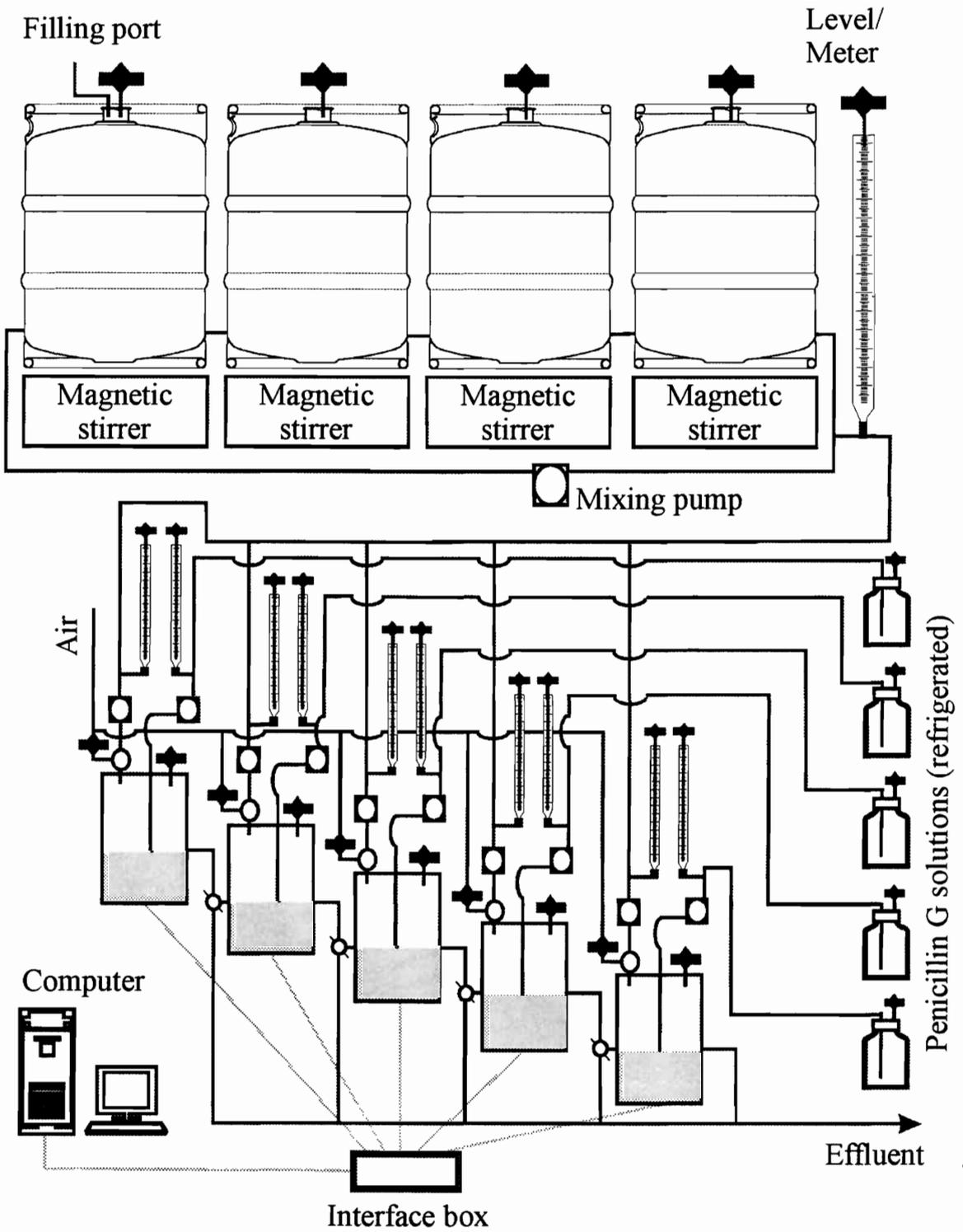
the following modifications. The modified fermentation system is shown in Figure 3.3.

#### **3.5.5.1.1 pH probes.**

Custom sleeves for pH probes were constructed. Each pH probe was a gel-filled standard combination pH electrode (#CA33221-034, VWR-Canlab, Edmonton, AB, Canada), inserted into stainless steel piping, secured with a ferrule and an O-ring. The stainless steel pipe was 15 cm long and had an outer diameter that fitted snugly through the medium addition port on the Bioflo III headplate, and an inner diameter that allowed the top portion of the pH probe to fit inside the pipe. The electronic connector on the pH probe was fed through the stainless steel pipe and the pH probe was inserted into the pipe until the bottom of the pH probe sheath was positioned approximately 0.5 cm inside the pipe. Silicone sealant was then injected into this void to form a water-tight seal when cured (24 hours). The O-ring was positioned near the top of the pipe where the electrical connector exited the pipe and the ferrule was placed on top of the O-ring (taper up). The custom pH probes were mated to the Bioflo III fermentors by inserting the pH probe unit into the medium addition port, allowing the pH probe to rest on the headplate via the O-ring. The O-ring was then tightened to form an air-tight seal with the existing cap from the medium addition port. This design allowed the positioning of the tip of the pH probe at any depth in the fermentation medium by simply adjusting the position of the O-ring on the stainless steel pipe before tightening.

#### **3.5.5.1.2 Penicillin G reservoir and supply.**

For each fermentor the penicillin G feed was supplied by the on-board Bioflo nutrient pump using a custom penicillin stock storage and transfer line. Penicillin G decomposition rate is governed by temperature and pH (Kheirilomoom *et al.*, 1999). To minimize the effect of temperature on long term operation and to be able to accurately use a constant temperature decomposition rate in calculations, all penicillin G reservoirs



**Figure 3.3** Schematic diagram of the constructed multistage continuous culture fermentation (MCCF) system with penicillin G addition.

were refrigerated at 5°C during fermentation. The reservoirs used for penicillin G were constructed in duplicate jacketed two litre Celstir bioreactors (Wheaton Scientific, Millville, NJ, USA) which were connected to a common circulating water bath set to 5°C. An Acrodisk 50 filter disk was silicone sealed to one of the sampling ports on the Celstir fermentors to allow the passage of sterile air into the reservoir. To minimize the effect of diluting the medium components in the fermentor with each addition of penicillin G, the reservoir solutions of penicillin G were made in a clarified, filter-sterilized medium aseptically obtained from the medium reservoir. This preparation would eliminate the dilution of medium components in the fermentor but would not minimize the dilution of *S. cerevisiae* from the fermentor via washout. The maximum possible rate of penicillin G addition in any experiment was 0.3 ml /min (100% setpoint on Bioflo III fermentor). With a medium addition rate of 5.6 ml/min ( $D = 0.12 \text{ h}^{-1}$ ), the addition of penicillin G at 0.3 ml /min increased the total flow of liquid into the fermentor by 5.35% to give a new dilution rate of  $0.126 \text{ h}^{-1}$ .

The transfer line from the penicillin G reservoir to the fermentor was constructed from 1.58 mm OD x 0.254 mm ID Teflon tubing (VWR-Canlab, Edmonton, AB, Canada) which was inserted to the bottom of the penicillin G reservoir through one of the sampling ports, and sealed in place at the sampling port with silicone sealant. This tubing was chosen so as to minimize the volume of penicillin G between the refrigerated reservoir and the fermentor. This “dead” volume of penicillin G would have a different decomposition kinetic constant than either the reservoir or the fermentor - thus this volume had to be minimized. The teflon tubing was connected and sealed to a “T” connection where an inline 0.2 ml pipette-Acrodisk flowmeter was attached. A length of tubing from the flowmeter was cut so that the end of the teflon tubing reached the penicillin pump on the Bioflo III fermentor. To this end of tubing, a length of 0.78 mm ID x 4.1 mm OD silicone tubing (VWR-Canlab, Edmonton, AB, Canada) was attached which provided the length of tubing required in the penicillin pump head. More teflon tubing was attached to the other end of the length of silicone tubing and both connections on the silicone tubing length were sealed with silicone sealant. The length of teflon tubing

from the penicillin pump head was chosen so that the resulting teflon tubing end would descend 5 cm into the fermentor working volume when inserted through one of the smaller accessory ports on the Bioflo III headplate. This end of teflon tubing was inserted into a stainless steel pipe that fit into the accessory port and descended from the headplate to 5 cm below the fermentor working volume, and sealed to the pipe with silicone sealant to provide a water-tight seal. An assortment of nuts, ferrules, and o-rings required to seal each pipe to the fermentor headplate was attached to each pipe. Each penicillin transfer line was made with the same lengths of tubing and piping.

The penicillin transfer line was calibrated by adding water to the reservoir and determining the length of time required to dispense 0.2 ml when the penicillin pump was set from 5 to 100% in increments of 5%. From this, a mathematical relationship between the penicillin pump and the volume dispensed per min was constructed.

Each reservoir and transfer line was autoclaved as a single unit at 121°C for 20 min. Prior to autoclaving, the stainless steel section of the unit was carefully wrapped.

#### **3.5.5.1.3 Computer interface.**

Each RS232C DB25 connection on the Bioflo III fermentors was connected to a custom constructed DB25 interface hub by straight through (no crossover) DB25 connectors. The hub was constructed from a salvaged serial DB25 switch box with seven DB25 interfaces. The switch inside the box was removed and the pins on each DB25 interface were soldered to each corresponding pin on all the other DB25 interfaces. This allowed any signal that entered the hub to “broadcast” the signal to all equipment connected to the hub. One DB25 interface on the hub was connected to the IBM computer.

The communications protocol on each Bioflo III fermentor was changed to accommodate the communications protocol from the Basic program running on the computer. Specifically, the version of Basic used on the computer did not allow even parity serial communications which, by default, were set on the Bioflo III fermentors. The

back from each Bioflo III fermentor was removed and two labelled banks of DIP switches used for configuring the fermentors were found. On bank 1, switch 3 was set to “Off” to disable all parity. The switches on bank two were not changed and from the switches on bank two the unique unit address for each fermentor was recorded. The resulting serial communications protocol which was set for each fermentor was 9600 baud, no parity, 8 data bits, and 1 stop bit. This protocol along with the unique unit address for each fermentor was used in the Basic program to send commands and receive data from individual fermentors.

Once all communication between the computer and the Bioflo III fermentors was set to the same protocol, the proprietary command syntax required to control the penicillin pump and receive data to/from fermentors required deciphering. The manual which was provided with the Bioflo fermentors contained errors in the command syntax - the commands sent from the Basic program on the computer to any fermentor did not work as outlined in the manual. To overcome this, an EasyCom (Avance Systems, <http://www.avance-systems.com>) serial hardware port sniffer cable was constructed and the corresponding software was downloaded and run on a second computer with the cable connecting the main computer, the second computer, and the Bioflo III fermentor. From the data captured by the EasyCom software, the proper ASCII command syntax for changing and receiving data from any of the Bioflow III data loops such as pH, temperature, agitation, dissolved oxygen, and nutrient (penicillin) addition was deciphered.

The deciphered command syntax, communication protocol, and unique unit addresses were incorporated into a custom programmed 475 line Basic program which incorporated all the necessary drivers for each loop, algorithms for programming all setpoints and functions to set each setpoint to values along a constructed mathematical function. Once the program began operation, all setpoints were set automatically by the program at the proper time with the proper value based on initial user input with initial conditions and desired functions.

### 3.5.5.2 Penicillin G addition.

The half life of penicillin G is dependant on pH and temperature (Kheirulomoom *et al.*, 1999). In the case of a stable compound, the concentration of the compound in continuous culture depends solely on the rate of addition of the compound, and the washout of the compound from the fermentor due to the dilution rate. With the addition of an unstable compound such as penicillin G, the picture becomes more complicated. During a continuous culture experiment, the concentration of penicillin in a continuous culture is determined by the decomposition kinetic constant of penicillin in the reservoir based on the reservoir pH and temperature, the decomposition kinetic constant of penicillin in the fermentor based on the fermentor pH and temperature, the rate of penicillin added to the fermentor, and the flow rate out of the fermentor (dilution rate). These four factors must be balanced in order to achieve the desired concentration. The calculation to achieve this is outlined below. The process is automated by using the "Solver" function in Excel 97 which also provides the resulting equations governing penicillin setpoints. These equations are incorporated into the Basic program to control the fermentors.

In the Bioflo III fermentor, the fraction of an original amount of penicillin G is governed by decomposition (affected by pH and temp) and by washout from dilution. The equation that predicts the fraction remaining from decomposition is outlined in the work by Kheirulomoom *et al.*(1999):

$$\frac{C_t}{C_o} = e^{-\left(10^{(-64.29 - 1.825 \text{ pH} + 0.4144T + 0.1280 \text{ pH}^2 - 0.0006187T^2)}\right) \times t} \quad (3.1)$$

where  $C_t$  is the concentration of penicillin at time  $t$ ,  $C_o$  is the initial concentration of penicillin G,  $T$  is the absolute temperature of the solution ( $^{\circ}\text{K}$ ),  $\text{pH}$  is the pH of the solution, and  $t$  is the elapsed time (h). The equation that predicts the fraction remaining from washout is :

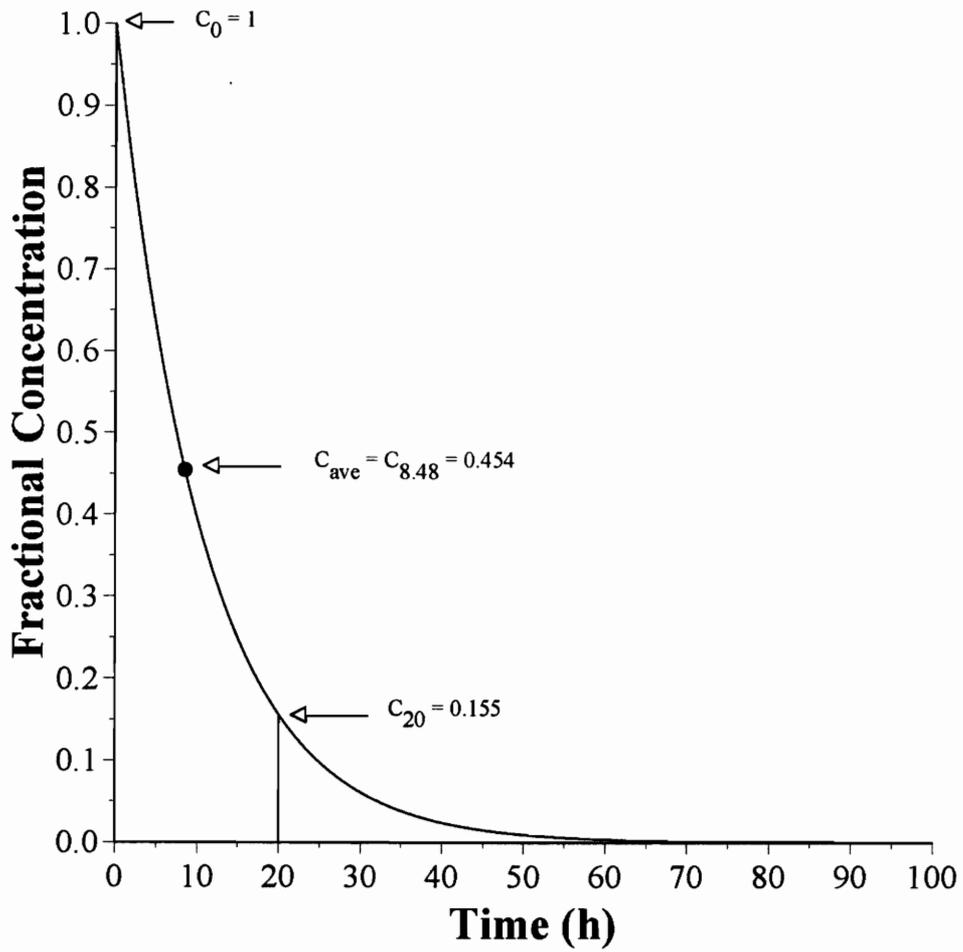
$$\frac{C_t}{C_o} = e^{(-Dt)} \quad (3.2)$$

where D is the dilution rate (per h), and t is the elapsed time (h). The fraction remaining at any time is governed by the product of these two equations:

$$\frac{C_t}{C_o} = e^{-(10^{(-64.29-1.825 pH+0.4144T+0.1280 pH^2-0.0006187T^2)} \times t)} \times e^{(-Dt)} \quad (3.3)$$

Thus, the concentration of penicillin G in the fermentor can be calculated for any time t. By calculating (with very small time intervals) the concentration of penicillin throughout a time interval between  $t_o$  and  $t_n$ , an average overall concentration ( $C_{ave}$ ) of penicillin can be determined (with a corresponding  $t_{ave}$ ). This is depicted schematically in Figure 3.4. To set the average overall concentration of penicillin at a desired concentration ( $C_x$  at  $t_{ave}$ ), one can determine the initial concentration ( $C_o$ ) of penicillin required by substituting  $C_{ave}$  for  $C_t$  and  $t_{ave}$  for t in the previous equation and solving for  $C_o$ . Also, by knowing the final concentration of penicillin at  $t_n$ , one can determine the amount of penicillin to add to the fermentor at  $t_n$  to bring the concentration of penicillin to the level seen initially in the fermentor. From this, one can construct a repeating function of any frequency and magnitude for penicillin in the Bioflo III fermentors.

To determine what penicillin pump setpoint is needed to increase the penicillin G concentration in the fermentor at  $t_n$ , one needs to know the amount of penicillin remaining in the reservoir at  $t_n$ , and the rate of volume addition provided by the penicillin pump. The concentration of penicillin in the reservoir is solely determined by the decomposition kinetic constant of penicillin based on the reservoir pH and temperature and can be calculated. By knowing the amount of penicillin required in the fermentor at  $t_n$ , and the amount remaining in the reservoir at  $t_n$ , one can calculate the volume required (for a particular penicillin reservoir concentration) in a short pulse (1 min) to provide the necessary amount of penicillin. The rate of pumping (ml/min) that the penicillin pump



**Figure 3.4** Depiction of average fractional penicillin G concentration over a time interval from 0 to 20 hours in the Bioflo III fermentor.

provides with the penicillin transfer line at various setpoints on the Bioflo III fermentor is governed by the equation  $y = 0.003 \times S$  [ $r^2 = 0.98$ ] (determined experimentally) where S is the penicillin pump setpoint (0-100%) on the Bioflo III fermentor and y is the resulting flow (ml/min). Once the volume needed from the reservoir is determined, the penicillin pump setpoint on the Bioflo III fermentor can be calculated and implemented.

All the above calculations and parameters concerning the reservoir concentration of penicillin G, frequency of the pulses, pulse duration, and desired overall average concentration of penicillin in the fermentor were determined with multiple Solver function runs in Excel 97. The resulting equations governing penicillin pump setpoints were incorporated into the Basic program. Once the program was started, no manual setting of setpoints was needed for the duration of the experiment.

#### **3.5.5.2.1 Constant fermentor concentration.**

A fuel alcohol plant typically adds 454 g of penicillin G to 81250 gallons of fermentation volume every 24 hours while adding fresh medium in continuous mode at rate of 290 gpm (dilution rate of  $0.0036 \text{ h}^{-1}$ ). This translates to a peak concentration of the potassium salt of penicillin G of 2475 U/L.

To provide a constant 2475 U/L of penicillin G in the fermentor, the Excel 97 spreadsheet was used to determine the penicillin G reservoir concentration. The pulse duration (which adds penicillin G to the fermentor) and the pulse frequency (time between pulsed additions) were set to ten minutes and entered into the spreadsheet. The solver function was used to determine the required penicillin G concentration in the reservoir that would give an overall average concentration of 2475 U/L over ten minutes. The resulting penicillin pump equation was directly programmed into the Basic program to control one fermentor with this equation.

### **3.5.5.2.2 Variable fermentor concentration.**

In variable penicillin G concentration experiments, the magnitude (overall average concentration) and/or the pulse frequency of penicillin G addition were set while keeping the pulse duration constant at two minutes. Experiments were run where the pulse frequency was kept constant at six hours with the overall average penicillin G concentration set to 2475, 1237.5, and 618.75 U/L. Likewise, the overall average penicillin G concentration was kept constant at 2475 U/L while the pulse frequency was set to 12, 18, and 24 hours. In each condition, the required penicillin G reservoir concentrations and the resulting penicillin pump equations were calculated by the Excel spreadsheet and entered into the Basic program.

### **3.5.5.3 Inoculum preparation.**

#### **3.5.5.3.1 *S. cerevisiae*.**

The *S. cerevisiae* inoculum was prepared as in Section 3.5.2.3.

#### **3.5.5.3.2 *L. paracasei*.**

The *L. paracasei* inoculum was prepared as outlined for the pre-inoculum in Section 3.5.4.2.2.

### **3.5.5.4 Fermentor preparation, inoculation and equilibration.**

The MCCF system was autoclaved and assembled as described in Section 3.5.2.2. Once assembled, medium from the medium reservoir was pumped into the MCCF to fill each fermentor to its respective working volume. Each pH probe unit was calibrated using the Bioflo III fermentor electronic controls with pH 4.0 and 7.0 buffers, and then

sterilized by immersion in 70% ethanol for 20 min. Each pH probe unit was then aseptically inserted into each fermentor through the medium addition ports.

The system was inoculated in MCCF mode with a 500 ml inoculum of *S. cerevisiae* and a 300 ml inoculum of *L. paracasei*, and the system was operated for one day in MCCF mode with 26% w/v glucose in the medium with the fermentors set to 28°C and a dilution rate of 0.066 h<sup>-1</sup> (0.186 L/h medium flow). The fermentors were then set to individual CSTR mode with the following parameters: pH adjusted to 5.5 with 6N KOH, temperature set to 28°C, agitation at 100 RPM, and D set to 0.066 h<sup>-1</sup>. During the experiments, the pH of each fermentor (as displayed by the fermentor) was confirmed daily by manually determining the pH of each fermentor sample externally. If any differences existed between the two pH readings, the manual pH determination was assumed correct and the pH setpoint on the fermentor was adjusted to correct the difference. The fermentors in CSTR mode were run for an entire week to allow both cultures to reach their respective steady states. All medium flows were monitored (and adjusted if needed) daily to maintain a constant dilution rate as outlined previously in Section 3.5.2.5.

The penicillin transfer lines were aseptically inserted into each fermentor and the penicillin reservoirs were refrigerated to 4°C. The reservoir concentrations of penicillin G were formulated in medium taken from the medium reservoir. The calculated amount of the potassium salt of penicillin G (determined from the Excel 97 spreadsheet) was dissolved in one litre of clarified medium and the pH determined. The solutions were then filter sterilized into pre-sterilized containers and aseptically transferred to their respective penicillin reservoirs. From pH and temperature of both the penicillin in reservoirs and the fermentors, the desired pulse frequency, the dilution rate of the experiment, and the desired overall penicillin concentration in the fermentors, corresponding penicillin pump equations were generated and incorporated into the Basic program. The penicillin transfer lines were primed to the point where penicillin was seen at the entrance of each fermentor headplate.

The beginning of the penicillin experiment was initiated once the Basic computer program was activated. Samples were withdrawn from each fermentor as described previously in Section 3.5.2.6.

### **3.5.6 Single stage with 16.2 L working volume.**

The combined working volume of the five stage MCCF system totaled 16.2 L. A single continuous stirred tank reactor (CSTR) was constructed using a 20 L glass carboy containing the same working volume. Mixing was accomplished using a large magnetic stirbar and a Thermolyne 25500 Maxistirrer (VWR-Canlab, Edmonton, AB, Canada). The carboy was temperature controlled using an immersion heater with continuous cold water circulation. The custom CSTR was constructed with similar ports and controls as found on the Bioflo III fermentors used for MCCF experiments.

Prior to starting a fermentation run, the CSTR was autoclaved empty at 121°C for 20 min and allowed to cool. Sterile medium was pumped aseptically into the CSTR reactor to fill the reactor to its working volume of 16.2 L. The medium was provided to the CSTR by an external peristaltic pump with an upstream inline 50 ml burette (with an Acrodisk 50 filter disk on positioned on top) to calibrate medium flow. Calibration and maintenance of a consistent medium flow into the CSTR was followed as in Section 3.5.2.5. The medium and the sterile air at two SLPM entered the CSTR 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). Once this was accomplished, a 200 ml aliquot of steady state fermentation broth (containing only *S. cerevisiae*) from F1 in the MCCF was introduced to inoculate the CSTR. Fermentations were conducted at 28°C, at 100 RPM, and with a medium flow rate of 0.34 L/h providing a dilution rate ( $D$ ) of  $0.02 \text{ h}^{-1}$  in the CSTR. The system was allowed to run for at least seven days (8.4 displacements) to allow the system to reach steady state. A steady state was reached when the glucose concentration in the CSTR varied by less than 5% over three consecutive days of sampling. Sampling was performed as in Section 3.5.2.6.

## 4 RESULTS AND DISCUSSION

### 4.1 Medium optimization.

#### 4.1.1 Examination of nutrients required by yeast and provided in medium formulations.

The medium formulation for all experiments in the multistage continuous culture (MCCF) system for this project consisted of glucose (cerelose), with corn steep powder (CSP) and diammonium phosphate (DAP) as nitrogen sources to stimulate fermentation. In order to determine if the medium formulation met the nutritional requirements of the yeast, calculations were made to determine the amounts of each component that yeasts require. In addition, it is known that the addition of excess nutrients stimulates fermentation - at least to an upper addition limit where further levels may not provide any benefit to the yeast. Thus, the strategy for medium optimization experiments was to:

1. Examine the nutrients provided by the proposed medium formulation.
2. Examine the nutritional requirements of the yeast.
3. Compare what is provided to what the yeasts require for growth.
4. Conduct experiments to determine the optimal medium formulation providing maximal yeast performance.

Table 4.1 gives a typical composition of the CSP used in medium formulation. The CSP provides the yeast with amino acids, vitamins, and minerals required for growth. Corn steep powder does not contain glucose, DAP, or starch.

The cellular composition of *S. cerevisiae* grown aerobically and anaerobically in continuous culture with complex media is presented in Table 4.2. In particular, the composition of anaerobically-grown cells was used in the optimization of the media formulation in this work since the yeast in the MCCF metabolizes anaerobically.

**Table 4.1** Composition of corn steep powder<sup>1</sup>.

Component	g/100g dry weight		Component	g/100g dry weight	
	Traders <sup>2</sup>	Marcor <sup>2</sup>		Traders	Marcor
Dry matter	95		Thiamine	0.000001	
Nitrogen <sup>3</sup>	7.68		Methionine <sup>4</sup>	1.9	1.1
Protein	48		Phenylalanine <sup>4</sup>	4.4	1.7
Fat	0.4		Threonine <sup>4</sup>	4	1.9
Carbohydrate	0		Tryptophan <sup>4</sup>	0	0.1
Fiber	0		Tyrosine <sup>4</sup>	3.4	1.1
Ash	17		Valine <sup>4</sup>	5.8	2.6
Calcium	0.06	0.3	Alanine <sup>4</sup>		3.7
Magnesium	1.5	1.5	Aspartic acid <sup>4</sup>		2.9
Phosphorus	3.3	3.9	Glutamic acid <sup>4</sup>		7.3
Available Phosphorus	1.1		Proline <sup>4</sup>		4.2
Potassium	4.5	5.2	Serine <sup>4</sup>		2.2
Sulfur	0.58		Leucine <sup>4</sup>	11.3	4.2
Biotin	0		Isoleucine <sup>4</sup>	3.6	1.6
Choline	0.00056		Lysine <sup>4</sup>	2.5	1.7
Niacin	0.000016		Arginine <sup>4</sup>	3.3	2.3
Pantothenate	0.000003		Cystine <sup>4</sup>	1.9	1.7
Pyridoxine	0.000002		Glycine <sup>4</sup>	5.1	2.3
Riboflavin	0.000001		Histidine <sup>4</sup>	2.8	1.6

<sup>1</sup> Data from Trader's Guide to Fermentation Media Formulation (Trader's Protein, P.O Box 80367, Memphis, TN 38108 USA), and Marcor Development Corporation (108 John Street, Hackensack, N.J. 07601-4130 USA.)

<sup>2</sup> Compositional analysis of corn steep powder provided by Trader's and Marcor. The reproducibility of the data and the methods used for analysis are unknown.

<sup>3</sup> Calculated on basis of total crude protein x 0.16 (a typical protein contains 16% N).

<sup>4</sup> Amino acids are expressed as % of total unhydrolyzed protein not as free amino acids.

**Table 4.2** Compositional analysis of *S. cerevisiae*.

Component	Aerobic growth <sup>1</sup>	Anaerobic growth <sup>2</sup>	Component	Aerobic growth
Carbon	47	45.0 (D = 0.10 h <sup>-1</sup> )	Lead	0.0007
		45.4 (D = 0.20 h <sup>-1</sup> )	Cobalt	0.0005
		45.3 (D = 0.27 h <sup>-1</sup> )		
Oxygen	32		Iodine	0.0004
Nitrogen (~54% protein)	9	8.8 (D = 0.10 h <sup>-1</sup> )	Arsenic	0.00001
		9.9 (D = 0.20 h <sup>-1</sup> )	Molybdenum	0.000009
		10.9 (D = 0.27 h <sup>-1</sup> )		
Hydrogen	6.5			
Potassium	3.5		Choline Chloride	0.55
Phosphorus	2		Inositol	0.5
Calcium	0.9		Niacin	0.0585
Sulfur	0.5		Thiamin Hydrochloride	0.0165
Magnesium	0.5		Para Aminobenzoic acid	0.016
Sodium	0.2		Calcium Pantothenate	0.012
Zinc	0.12		Riboflavin	0.01
Iron	0.1		Pyridoxine Hydrochloride	0.005
Chloride	0.1		Folicin	0.0049
Copper	0.012		Biotin	0.0002
Manganese	0.0035			

<sup>1</sup> g/100g Dry weight. Aerobically grown yeast. Data from Harrison 1971.

<sup>2</sup> g/100g Dry weight. Anaerobic continuous culture studies (D = dilution rate). Data from Verduyn *et. al.* 1990a.

Composition of cells provides a good estimation of nutrient requirements. Although many of the components needed for growth are not listed, calculations based on Table 4.2 still give a reasonable estimation of those specific nutritional requirements of the yeast.

To be able to compare the nutrients supplied by the CSP fraction in Table 4.1 and the requirements suggested by Table 4.2, an amount of yeast produced in a quantity of medium must be known. To determine this amount, data from preliminary MCCF experiments were used. In those continuous experiments, the condition that gave the highest biomass density used the following formulation:

Glucose	140	g/L	
Corn steep powder	20	g/L	
Ammonium sulphate	2.64	g/L	( 20 mM)

Continuous fermentations using this medium led to yields of 12.23 g yeast (dry weight) per L of medium in the fifth fermentor in the MCCF. With this value, it was then possible to calculate nutrient concentrations (Table 4.1) in one litre of the above medium, and also the amount of each nutrient required (Table 4.2) for the production of 12.23 g yeast dry weight /L. Both of these calculations are summarized in Table 4.3. A direct comparison between nutrients provided by the medium and nutrients needed by the yeast could then be made. Some of the nutrients that the yeast required for growth were not provided in sufficient quantities by the medium. In particular two major nutrients, nitrogen and phosphorus, were deficient. A deficiency of these major nutrients would likely seriously impact on the growth and performance of yeast. To alleviate the deficiency in phosphorus, DAP was used in place of ammonium sulphate for all subsequent formulations in this work since sulfur was not limiting in the experiments (Table 4.3). Clearly the amounts of CSP and available nitrogen needed to be increased in the formulation to address nutrient limitations in continuous culture experiments. A shift in the type of nutrient that limits growth may occur as additional N and P are provided.

**Table 4.3** Comparison between the amount of nutrients provided in one litre of medium to the nutrients required to produce 12.23 g yeast (dry weight) in one litre under experimental conditions with 14 g glucose/100ml.

Component	Amount required (mg/L) to produce 12.23 g dry weight yeast/L		Amount provided by 1 L medium (mg) <sup>1</sup>	Difference (mg/L) <sup>2</sup>	
	Aerobic	Anaerobic		Aerobic	Anaerobic
Available Nitrogen	1101	1076 (D=0.10 h <sup>-1</sup> ) 1211 (D=0.20 h <sup>-1</sup> ) 1333 (D=0.27 h <sup>-1</sup> )	560	541	516 (D=0.10 h <sup>-1</sup> ) 651 (D=0.20 h <sup>-1</sup> ) 773 (D=0.27 h <sup>-1</sup> )
Potassium	428		855	427	
Free Phosphorus	245		209	36	
Calcium	110		11	99	
Choline	67		0.11	66.9	
Magnesium	61		285	224	
Sulphur	61		Corn Steep (Marcor) 110	49	
			Corn Steep+(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> 750	689	
Niacin	7.2		0.00304	7.2	
Thiamine	2.02		0.00019	2.02	
Pantothenate	1.5		0.00057	1.49	
Riboflavin	1.2		0.00019	1.2	
Pyridoxine	0.61		0.00038	0.61	
Biotin	0.024		0	0.024	

<sup>1</sup> Nutrient values calculated do not take into account losses of nutrients during autoclaving.

<sup>2</sup> Shaded values indicate deficiencies.

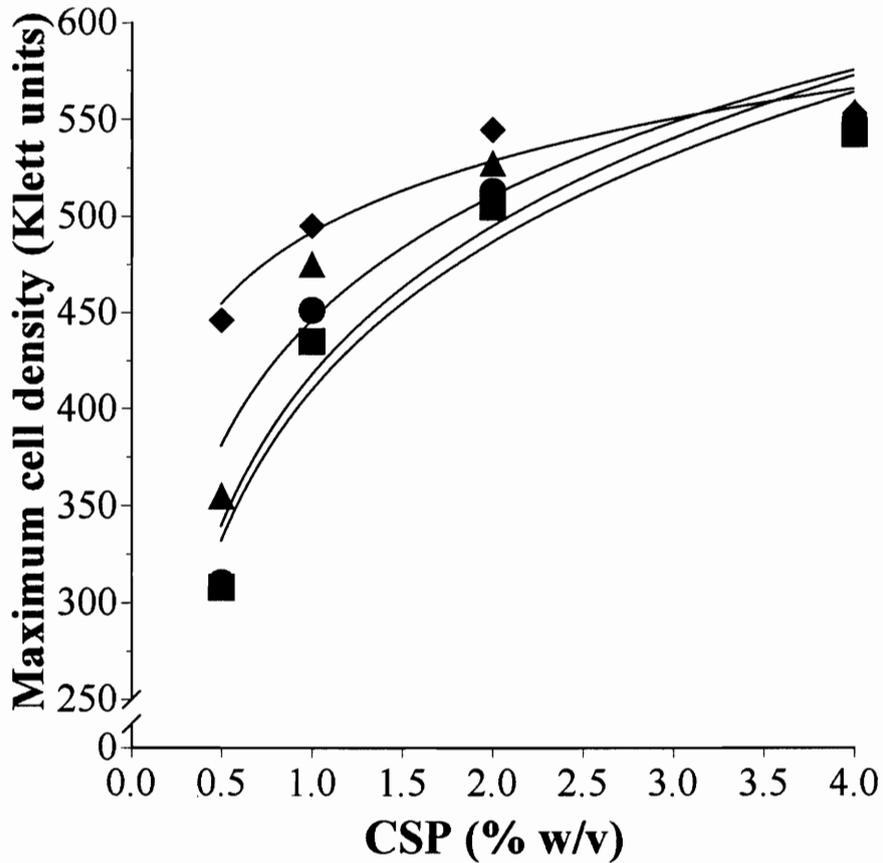
#### **4.1.2 Determination of optimal levels of medium components.**

A 1x4x4 factorial experiment was performed to determine the optimal concentrations of CSP (C) and DAP (P) needed for VHG fermentations with a fixed concentration of glucose (G). Batch fermentations were conducted with levels of factors as outlined in Table 3.1. The results for each parameter are discussed briefly in the following subsections and the conclusions from a rigorous statistical evaluation of data are presented in the next section.

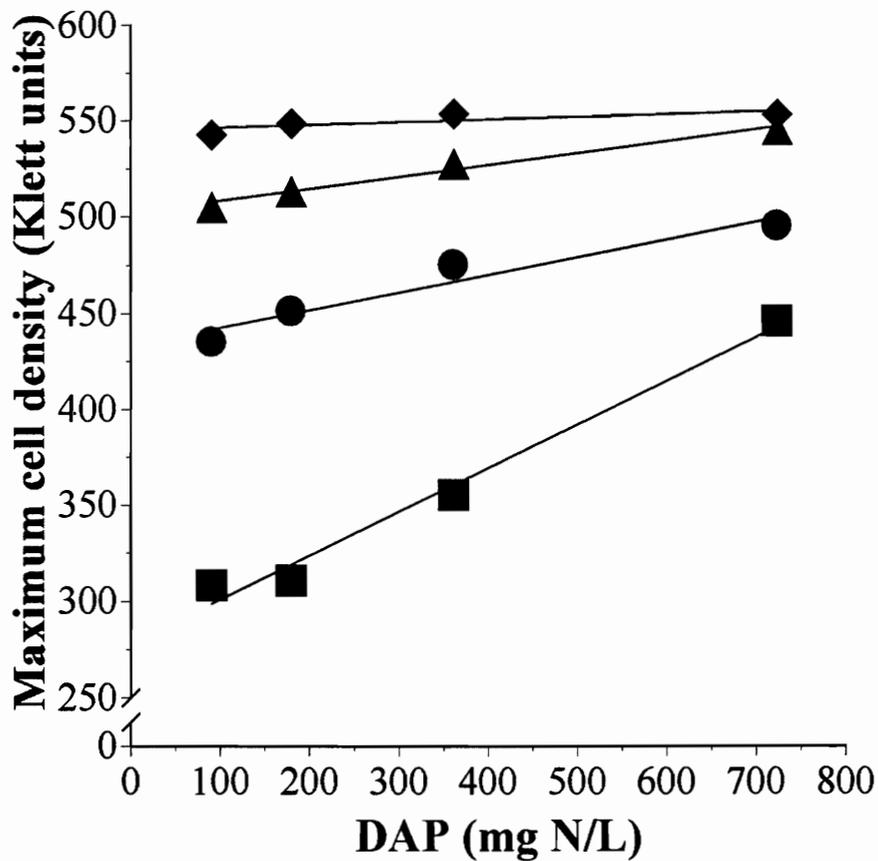
##### **4.1.2.1 Maximum biomass concentration (MaxX) of *S. cerevisiae*.**

It is important to evaluate the maximum yeast biomass concentration achieved in an alcohol fermentation. In fermentations, increased production of biomass concentration results in a faster rate of ethanol production. Studies on biomass concentrations that can be obtained from test media under batch conditions give an indication as to the maximum yeast biomass concentration that might be possible under continuous fermentations with a given medium and the combination of factors which might provide this biomass.

Figure 4.1 and 4.2 show the maximum biomass concentration (MaxX) as measured by optical density after the culture entered stationary phase as CSP (Fig. 4.1) and DAP (Fig. 4.2) concentrations were increased. Biomass concentration continuously increased with increases in CSP and DAP levels from C1 to C4 and P1 to P4 (see Table 3.1 for concentrations of each factor). As expected, the highest biomass produced was with the C4 and P4 test conditions. In Figure 4.1, all DAP conditions allowed a maximum Klett value of ~550 to be reached when CSP was added at the C4 level. This was not the case with increasing DAP concentrations (Fig. 4.2) as each CSP condition reached different maximal biomass concentrations when P4 additions of DAP were made. An interesting observation in Figure 4.2 is that, at the C4 CSP addition level, the addition of DAP had no effect on the maximum biomass concentration. The biomass concentration remained at ~550 Klett units regardless of the amount of DAP added. Thus, it appears



**Figure 4.1** Maximum growth of *Saccharomyces cerevisiae* at stationary phase with increasing corn steep powder (CSP) levels at each diammonium phosphate (DAP) level (■, P1 (91 mg N/L, 100 mg P/L); ●, P2 (180 mg N/L, 200 mg P/L); ▲, P3 (362 mg N/L, 400 mg P/L); ◆, P4 (724 mg N/L, 800 mg P/L)).



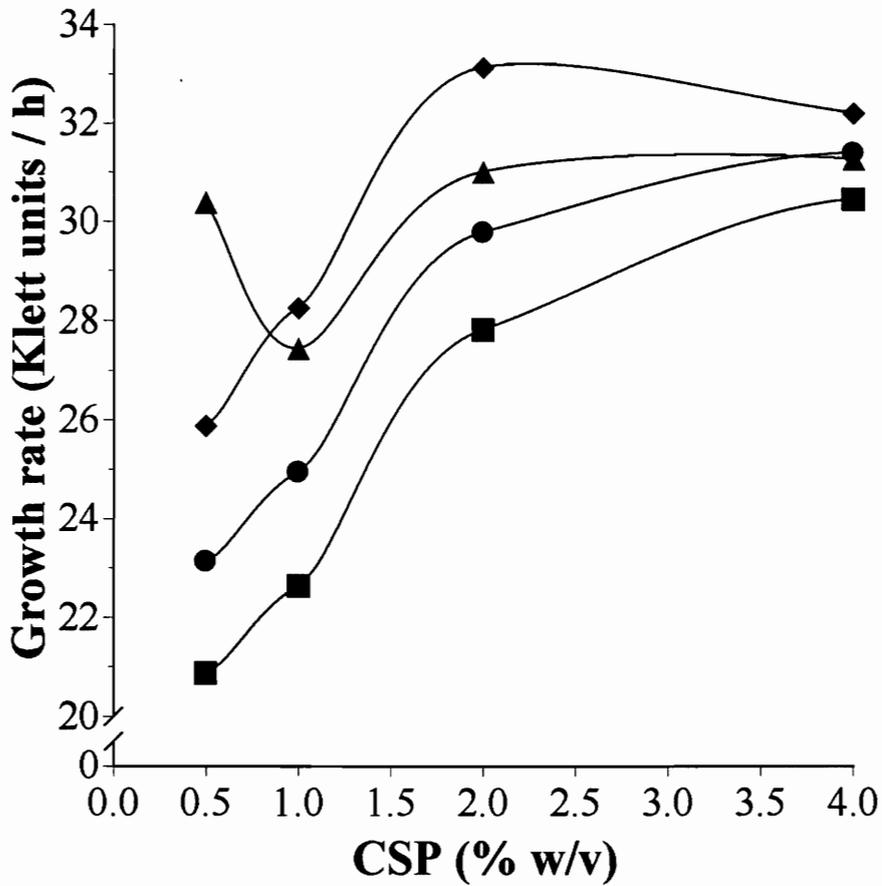
**Figure 4.2** Maximum growth of *S. cerevisiae* at stationary phase with increasing DAP at each CSP level (■, C1 (0.5% w/v CSP); ●, C2 (1% w/v CSP); ▲, C3 (2% w/v CSP); ◆, C4 (4% w/v CSP)).

that increased phosphate is not needed when a CSP concentration of C4 is used. At lower levels of CSP, the addition of DAP clearly effected the maximal biomass concentration. The most dramatic effect on biomass concentration was with the addition of CSP (Fig. 4.1) where larger increases in biomass were seen than in DAP additions (Fig. 4.2). Thus, at first glance, a medium containing the C4 CSP concentration would seem to be the best level to use in the medium formulation as it provided maximal biomass concentration without the addition of DAP.

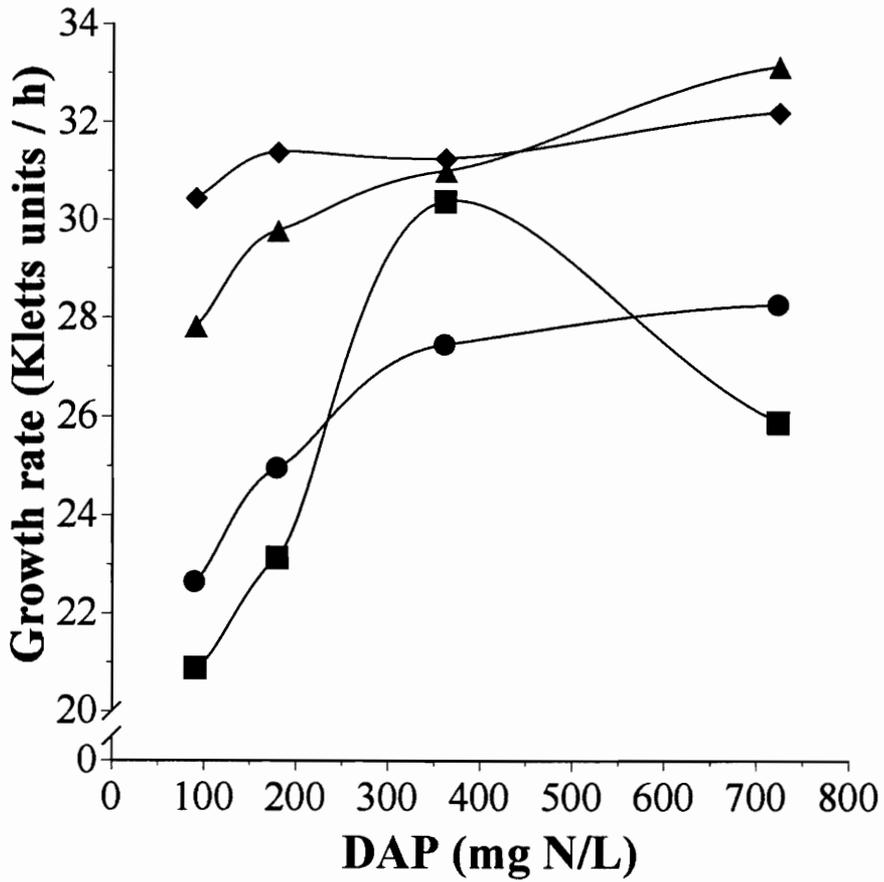
#### **4.1.2.2 Maximum exponential growth rate (MaxU) of *S. cerevisiae*.**

The maximum exponential growth rate of yeast under experimental conditions is another important parameter to consider in the production of ethanol. A higher specific growth rate as well as a higher growth rate would lead to a higher density of yeast cells present in the fermentor under continuous operation which would lead to a faster rate of ethanol production. In addition, medium conditions which promote a higher exponential growth rate would allow the continuous culture system to operate at higher dilution rates, increasing the volumetric productivity.

Figures 4.3 and 4.4 show the maximum exponential growth rate (MaxU) of yeast under batch conditions with increasing CSP (Fig. 4.3) and DAP (Fig. 4.4) concentrations. In general with CSP addition (Fig. 4.3), the maximum exponential growth rate increased rapidly with CSP from C1 to C3 at all levels of DAP. Beyond the C3 level, the maximum exponential growth rate tapered off regardless of DAP additions. This suggests that there is no apparent benefit for yeast exponential growth rate in adding CSP to the medium at levels beyond C3. Unlike the results for CSP additions, additions of DAP (Fig. 4.4) from P1 to P4 in general resulted in the continued increase in maximum exponential growth rates - no "plateau" was observed. Also, as the level of CSP in the medium was increased (Fig. 4.4), a reduction in the maximum exponential growth rate occurred with increases in DAP at each CSP level. At a CSP level of C4, increases in DAP from P1 to P4 had little effect on the maximum exponential growth rate. This indicates that DAP is not



**Figure 4.3** Maximum exponential growth rate of *S. cerevisiae* with increasing CSP levels at each DAP level. (■, P1 (91 mg N/L, 100 mg P/L); ●, P2 (180 mg N/L, 200 mg P/L); ▲, P3 (362 mg N/L, 400 mg P/L); ◆, P4 (724 mg N/L, 800 mg P/L)).



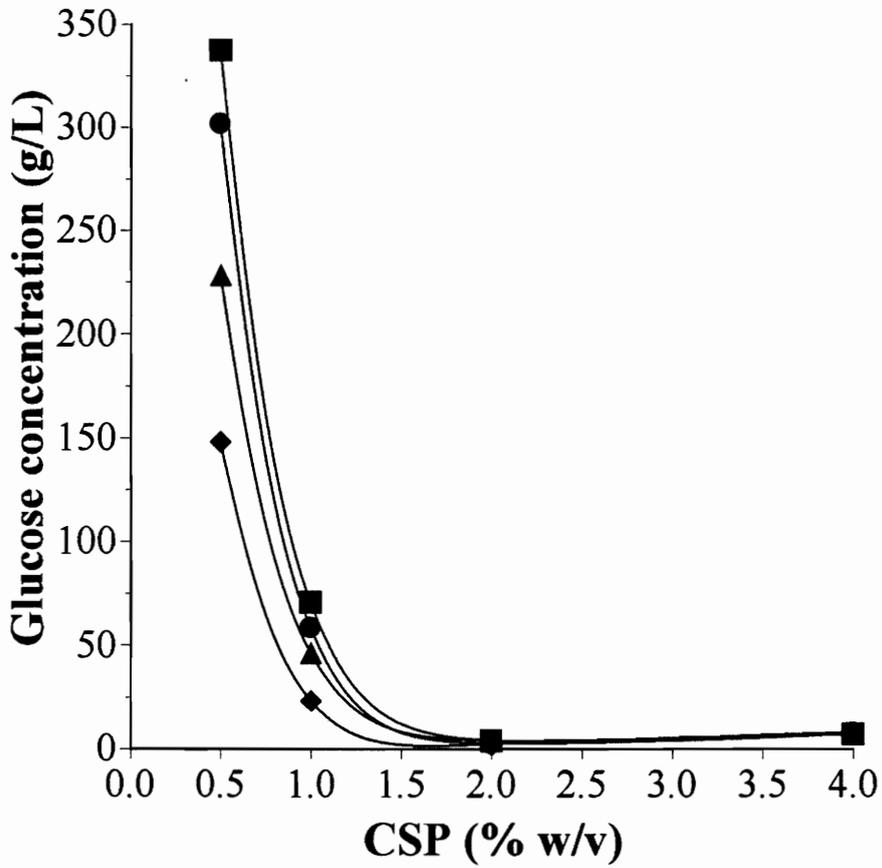
**Figure 4.4** Maximum exponential growth rate of *S. cerevisiae* with increasing DAP levels at each CSP level (■, C1 (0.5% w/v CSP); ●, C2 (1% w/v CSP); ▲, C3 (2% w/v CSP); ◆, C4 (4% w/v CSP)).

needed when C4 levels of CSP are used.

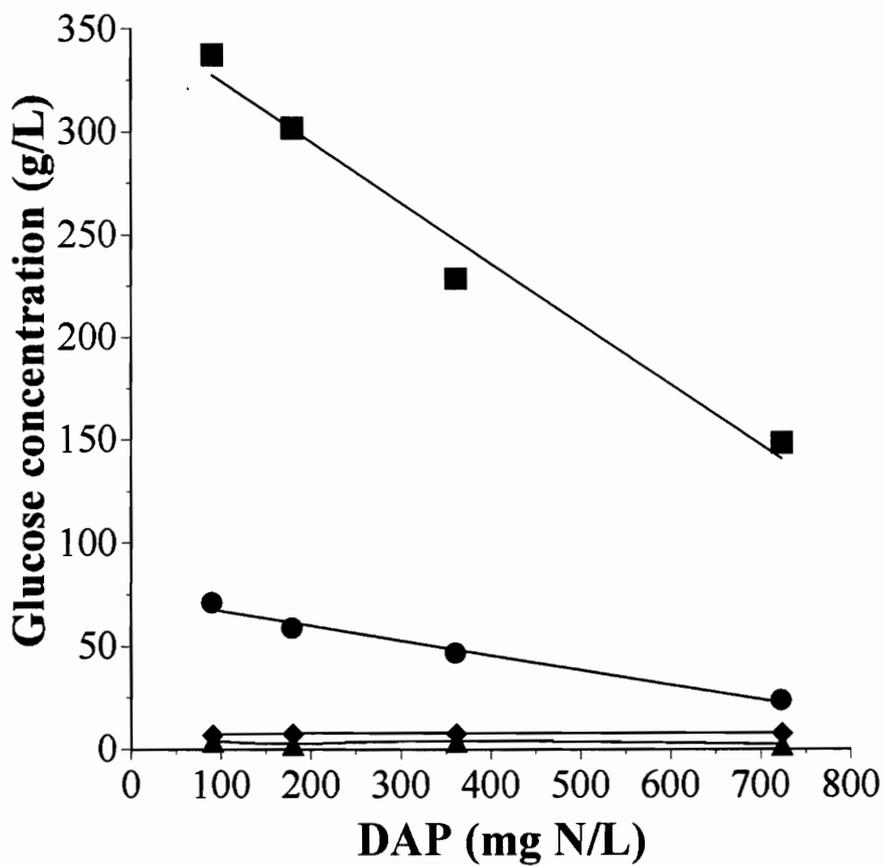
#### **4.1.2.3 Glucose (GLU) concentration at the end of fermentation.**

A primary concern for fuel alcohol producers is to produce as much ethanol as possible from the sugar present in the fermentor. Any sugar leaving a fermentor in the effluent is a waste of substrate and a loss in ethanol yield (which reduces profitability). As the cost of raw materials for media in fuel alcohol fermentations at times has reached as high as 60% of the operating cost, complete utilization of sugar in mash during fermentation is desired. In continuous culture, complete utilization of sugar can usually be achieved by decreasing the dilution rate of the system to provide a longer residence time in the fermentor. However, by decreasing the dilution rate, the volumetric productivity of the system also decreases which adversely affects profitability. Another method to achieve complete utilization of sugar is to optimize the composition of the medium to accommodate the nutritional requirements of the yeast. This method requires that the fuel alcohol producer knows the nutritional status of the medium used in the fermentations, and that he has an intimate knowledge of the nutritional requirements of the yeast.

Figures 4.5 and 4.6 illustrate the residual glucose (GLU) concentration seen at the end of batch fermentations as CSP (Fig. 4.5) and DAP (Fig. 4.6) concentrations were increased. The GLU in batch fermentation decreased sharply for all levels of DAP as the CSP concentration increased (Fig. 4.5) and reached a minimum value when the C3 level of CSP was used. Increases in CSP did not decrease the residual of glucose further. Using the C3 condition (2 g/100ml), less than 1% glucose was seen at the end of fermentation at all P levels. Even the C2 level of addition (1 g/100ml) had significant effects on the residual glucose concentration as a 88% decrease was observed as compared to C1. These results suggest that the optimal level of addition of CSP should be set at C3 (2 g /100ml) to minimize effluent glucose concentration in continuous fermentations. The addition of DAP at four established CSP levels had a less dramatic



**Figure 4.5** Glucose concentration at the end of batch fermentation with *S. cerevisiae* with increasing CSP levels at each DAP level (■, P1 (91 mg N/L, 100 mg P/L); ●, P2 (180 mg N/L, 200 mg P/L); ▲, P3 (362 mg N/L, 400 mg P/L); ◆, P4 (724 mg N/L, 800 mg P/L)).



**Figure 4.6** Glucose concentration at the end of batch fermentation with *S. cerevisiae* with increasing DAP levels at each CSP level (■, C1 (0.5% w/v CSP); ●, C2 (1% w/v CSP); ▲, C3 (2% w/v CSP); ◆, C4 (4% w/v CSP)).

impact on residual glucose concentration (Fig. 4.6). Over the C2 to C4 CSP supplementation range, an increase in DAP did not significantly change the residual glucose concentrations. The C3 and C4 levels of CSP enhanced glucose utilization - no residual glucose remained at end fermentation. Increases in DAP were not needed under these conditions to ensure glucose would be totally used. The C2 and C1 levels of CSP allowed a decrease in GLU to be seen as DAP was increased but the magnitude of glucose utilization decreased from the C1 to the C2 levels of CSP. In addition, ~ 150 g/L glucose remained under the C1 CSP condition when DAP was increased to P4. The work indicates that large amounts of DAP (> 800 mg N/L) would be needed to reduce the GLU to levels below 10 g/L. In comparison, at a CSP level of C2 and at a DAP level of P1, the residual glucose concentration was ~75 g/L - less than 1/2 the value with C1 and P4 of ~150 g/l was obtained. These results clearly show the importance of CSP addition to reduce the residual glucose concentration in the present medium formulation and that DAP addition is not sufficient to overcome the need for additional nutrients provided by CSP.

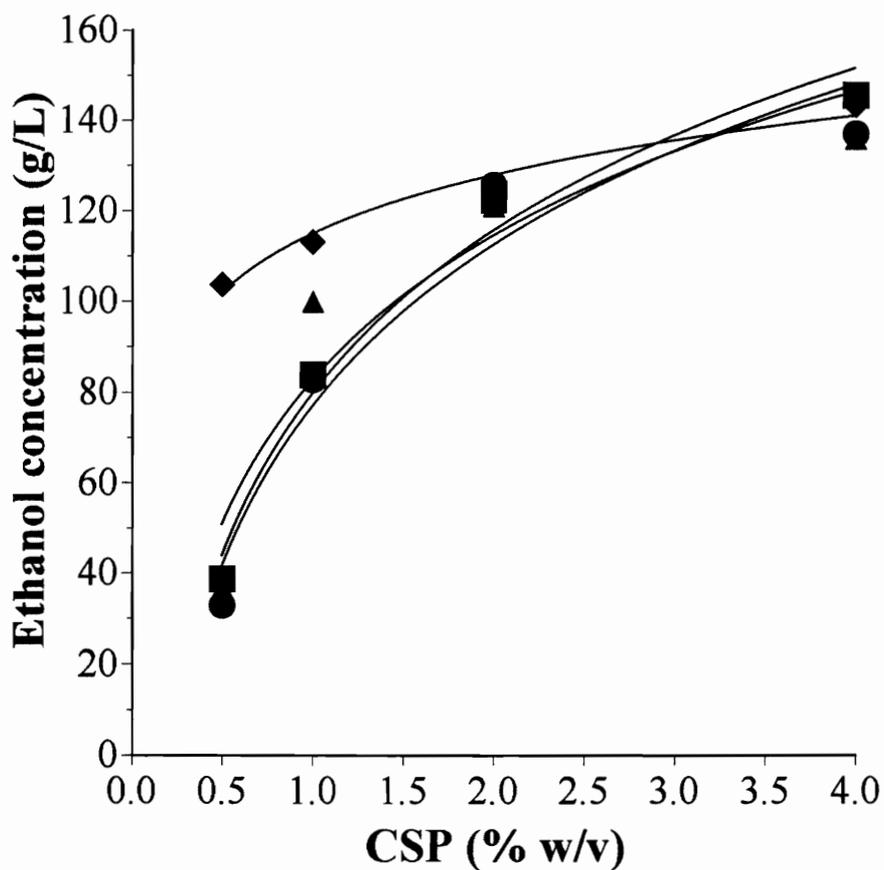
#### **4.1.2.4 Final ethanol concentration (MaxE).**

Another goal for fuel alcohol producers is to produce as much ethanol as possible in a fermentation. Yeast cells are able to produce ethanol both when actively multiplying and when under stationary phase or non-multiplying conditions. However, the rate of ethanol production is ~30x faster with actively growing yeasts than with yeasts in stationary phase (Kirsop, 1982). In batch systems, multiplying and non-multiplying cells produce ethanol. However, the time over which the cells remain in active growth and in stationary phase will vary. In VHG wheat mash batch fermentations, Thomas *et al.*, (1993) demonstrated that yeast cell numbers increased only in the first 50 h of fermentation. No further increases in cell numbers were observed beyond 50 h of fermentation although ethanol was still produced by stationary phase cells. Ethanol production continued for an additional 150 h until all sugars were consumed. In a

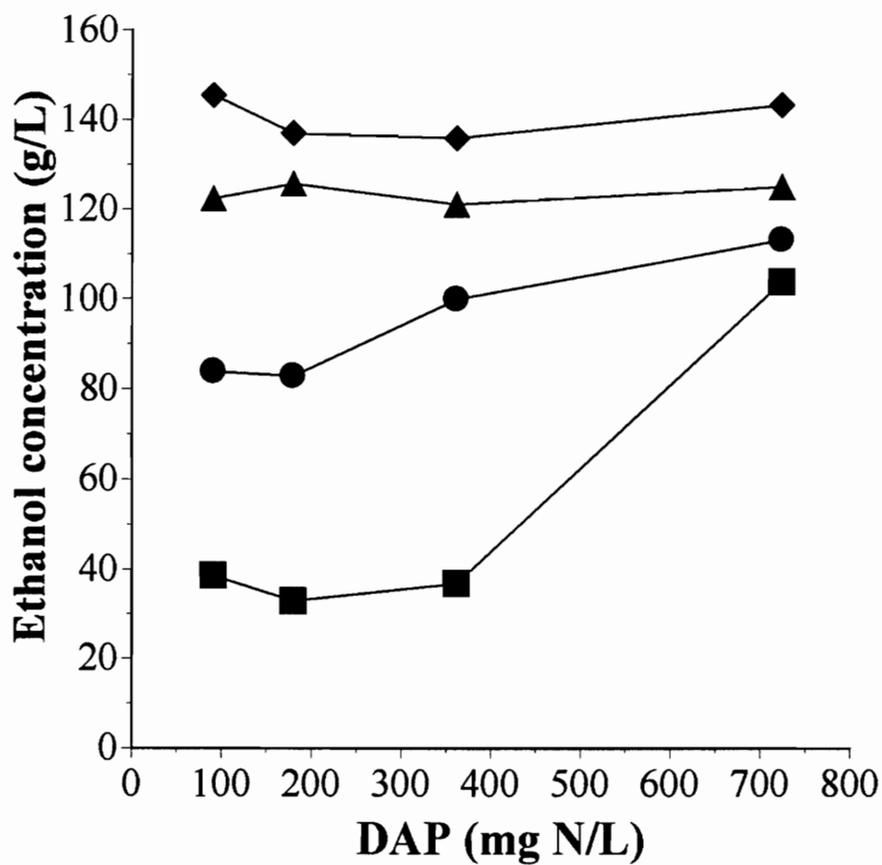
multistage continuous culture, ethanol production by both multiplying and non-multiplying cells is distributed across multiple fermentors. In the first fermentor(s) where conditions are most favorable for growth and the ethanol content is low, the yeast population would be in exponential phase producing yeast biomass continuously. In later fermentors in the multistage system, the yeast cell population would not multiply as well due to a higher ethanol content and depleted nutrients and so the cell would enter stationary phase. Thus, an examination of ethanol production in continuous culture conditions by both multiplying (exponential phase) and non multiplying (stationary phase) yeast cells is important.

Figures 4.7 and 4.8 illustrate the maximal ethanol concentration achieved (MaxE) at the end of batch fermentations as CSP (Fig. 4.7) or DAP (Fig. 4.8) are increased. Although the experiments were conducted in batch where the ethanol concentration achieved is a result of both multiplying and non-multiplying yeast cells in a single fermentor over time, the batch experiments would approximate the ethanol concentrations leaving a multistage continuous system (where ethanol production by multiplying and non-multiplying yeast cells is distributed over multiple fermentors at steady state). The maximum ethanol concentrations achieved by batch in the present experiments is also useful in showing which medium formulations lead to the highest ethanol concentrations in the fermentation.

MaxE increased for each level of DAP as CSP was increased (Fig. 4.7) and reached a maximum of 140 g/L for each DAP level where a C4 addition of CSP was present. The increases in MaxE for the P1 to P3 levels of DAP additions were nearly identical as the level of CSP was increased - which indicated that no differences in maximal ethanol production is apparent with CSP addition at DAP levels of P1 to P3. Only when a P4 level of DAP was used was there a change in MaxE production as compared from P1 to P3 when CSP was increased. Here an optimal concentration of CSP cannot be easily recommended as one must balance the cost of additional CSP with the ethanol concentrations reached with CSP additions. DAP additions had no effect on maximal ethanol concentration when C3 and C4 levels of CSP were used (Fig. 4.8).



**Figure 4.7** Maximum ethanol concentrations of *S. cerevisiae* with increasing CSP levels at each DAP level. (■, P1 (91 mg N/L, 100 mg P/L); ●, P2 (180 mg N/L, 200 mg P/L); ▲, P3 (362 mg N/L, 400 mg P/L); ◆, P4 (724 mg N/L, 800 mg P/L)).

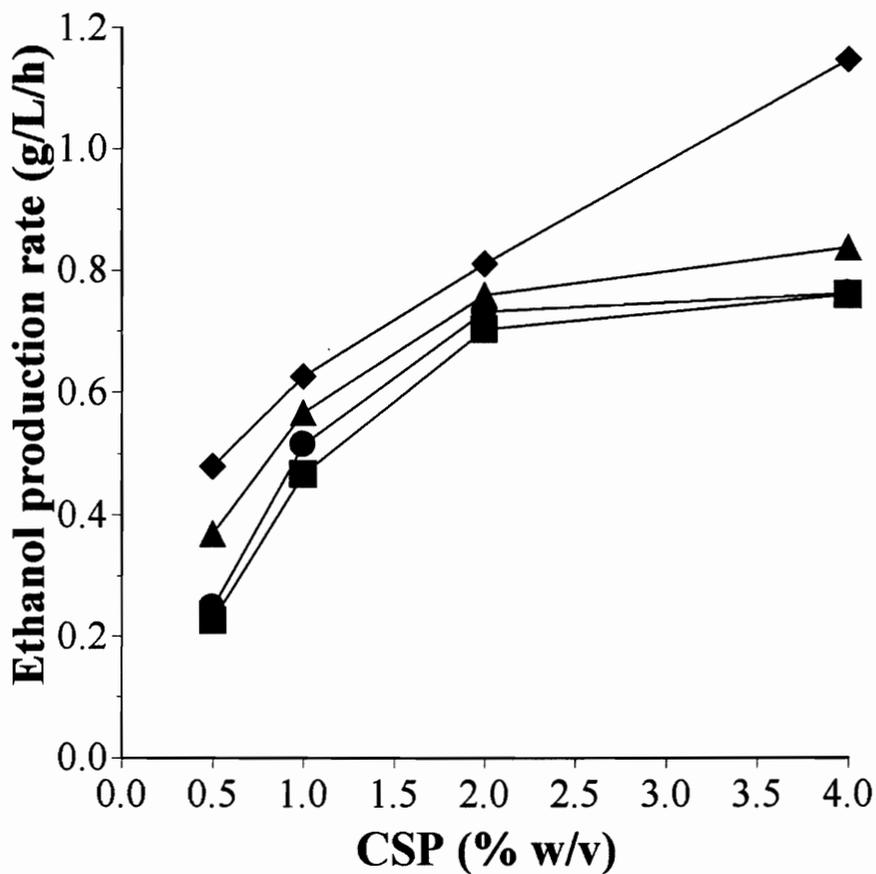


**Figure 4.8** Maximum ethanol concentrations of *S. cerevisiae* with increasing DAP levels at each CSP level. (■, C1 (0.5% w/v CSP); ●, C2 (1% w/v CSP); ▲, C3 (2% w/v CSP); ◆, C4 (4% w/v CSP))

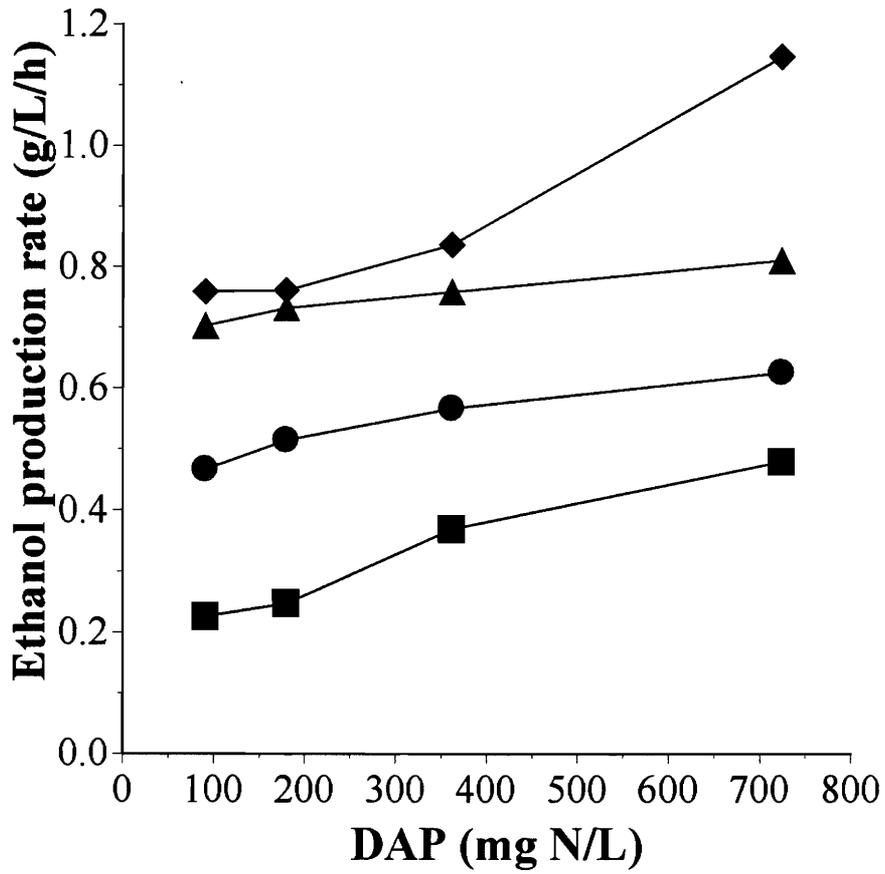
However, the concentration of ethanol achieved was ~140 g/L at a C4 level of CSP and ~120 g/L with a C3 level of CSP regardless of the DAP level used. This indicated that the nitrogen and phosphorus concentrations provided at these two levels of CSP are sufficient to promote maximal ethanol concentrations in each without the need for DAP. No additions of DAP would be needed in the medium if either of these CSP levels were used. The importance of CSP addition to the medium formulation is apparent in the C3 CSP level as no addition of DAP resulted in an increase in MaxE to the level achieved in the C4 condition. Thus, DAP addition alone is not sufficient to achieve maximal ethanol concentrations. In general the maximum ethanol concentration increased when DAP was increased for the C2 and C1 levels of CSP.

#### **4.1.2.5 Ethanol production rate (EPR) of exponentially growing *S. cerevisiae*.**

In continuous culture systems, the ethanol production rate in the first few fermentors in the system would be influenced greatly by exponentially growing yeast cells. Figures 4.9 and 4.10 illustrate the ethanol production rate (EPR) at the highest determined yeast growth rate for each condition with increasing CSP (Fig. 4.9) and DAP (Fig. 4.10) concentrations. Ethanol production rates were calculated at the time when the slope of the exponential growth curve for each condition was at its highest value. The effect of CSP addition on ethanol production rate at each level of DAP is shown in Figure 4.9. For all levels of DAP, CSP addition resulted in an increase in the ethanol production rate. For the P1 to P3 levels of DAP, the ethanol production rate increased to ~ 0.7 g/L/h when CSP addition was between C1 to C3. Further increases in EPR did not occur with a CSP addition of C4. For these levels of DAP, a C3 level of CSP appears to be optimal for the production of ethanol with exponentially growing yeast cells regardless of the DAP concentration - increases beyond C3 would appear to be a waste of nutrients. Also, the results illustrate that the ethanol production rate does not appear to be as dependant on DAP as it is on CSP. Three levels of DAP showed similar values with increases in CSP.



**Figure 4.9** Maximum exponential ethanol production rate of *S. cerevisiae* with increasing CSP levels at each DAP level. (■, P1 (91 mg N/L, 100 mg P/L); ●, P2 (180 mg N/L, 200 mg P/L); ▲, P3 (362 mg N/L, 400 mg P/L); ◆, P4 (724 mg N/L, 800 mg P/L)).



**Figure 4.10** Maximum exponential ethanol production rate of *S. cerevisiae* with increasing CSP levels at each DAP level (■, C1 (0.5% w/v CSP); ●, C2 (1% w/v CSP); ▲, C3 (2% w/v CSP); ◆, C4 (4% w/v CSP)).

In the P4 condition, the EPR matched the trend of the other levels of DAP but showed a large increase when a C4 level of CSP was added. It is unclear why this increase in EPR was only seen with C4 and not in any of the other levels of CSP. The effect of DAP on the ethanol production rate is depicted in Figure 4.10. All of the levels of CSP except C4 showed a similar slope (but poised at different values) when DAP was increased from P1 to P4. No clear conclusions can be made concerning the optimal level of DAP to add as all the levels of CSP led to stepwise increases in ethanol production rate.

#### **4.1.3 CSP and DAP interactions on fermentation parameters.**

The preceding subsections illustrated quantitative data from factorial batch fermentations conducted to elucidate the effects of CSP and DAP on various fermentation parameters (MaxU, GLU, EPR, MaxX, and MaxE) needed to optimize the base medium for VHG fermentations in continuous culture. Although optimal addition levels of DAP and CSP can be concluded in some of the figures, it is difficult to determine from the graphs the optimal level of DAP and CSP that would optimize as many of the fermentation parameters as possible. Also, the figures do not provide a means to determine if CSP and DAP have any combination effects (synergistic or antagonistic) on each of the fermentation parameters if provided simultaneously. To this end, the data were statistically evaluated using the ANOVA (F test) and the Ryan-Einot-Gabriel-Welsch (REGWQ) test for similarity. This REGWQ statistical test is considered in statistical circles to be more rigorous than Duncan's or Tukey's statistical tests in determining similarities and differences in data trends and also determining interaction effects.

Table 4.4 displays the significance of each listed effect on the outcome of each parameter (MaxU, EPR, GLU, MaxX, and MaxE) obtained from the statistical evaluation (F test) of data gathered for each fermentation parameter for all factor and factor levels. It is now possible to conclude from this table which effect (or combination of effects) exerted an influence on the fermentation parameters tested to optimize the medium for

**Table 4.4** Main and interaction effects of CSP and DAP at four levels during batch fermentation by *S. cerevisiae*.

Effect	Outcome parameter				
	MaxU <sup>1</sup>	EPR <sup>2</sup>	GLU <sup>3</sup>	MaxX <sup>4</sup>	MaxE <sup>5</sup>
CSP <sup>6</sup>	27.158 <sup>8</sup>	22.19	287.43	1953.55	59.34
DAP <sup>7</sup>	13.03	3.99	15.09	202.46	6.17
DAP*CSP	2.11	0.42	11.7	46.01	2.63
MaxX			0.2		0.56
MaxU			0.79		0.56

<sup>1</sup> MaxU = Maximum exponential growth rate

<sup>2</sup> EPR = Maximum exponential ethanol production rate

<sup>3</sup> GLU = Stationary phase glucose concentration

<sup>4</sup> MaxX = Maximum stationary phase yeast cell density

<sup>5</sup> MaxE = Final ethanol concentration

<sup>6</sup> Corn steep powder

<sup>7</sup> Diammonium Phosphate

<sup>8</sup> F value at 95% confidence limits

Shaded values indicate significant effects at 95% confidence limits.

future continuous culture experimentation. The data in Table 4.4 that was obtained from the F test must be interpreted in the proper way. Due to the nature of the F test, the magnitude of values (and comparisons of values) in Table 4.4 cannot be compared. The values are only used to differentiate whether or not an effect has an influence on the fermentation parameters (*i.e.* “yes” or “no”).

Based on the CSP, DAP, and glucose concentration levels used in the batch factorial experiments, it is clear that both CSP and DAP influence (at the 95% confidence level) all fermentation parameters. The data in Table 4.4 also yield additional data about the influence of CSP and DAP that is not evident in the previous subsections where only the trends for each fermentation parameter were plotted. The combined effect of CSP\*DAP does not significantly effect MaxU or EPR, but does effect GLU, MaxX, and MaxE. This combination of CSP and DAP in the medium obviously influences (synergistically) some fermentation parameters but not others. In addition, the maximum biomass concentration (MaxX) and maximum exponential growth rate (MaxU) do not affect either the final glucose concentration (GLU) or the maximum ethanol concentration (MaxE). These last few conclusions could not have been reached without using the F test on the factorial data. If additional potential factors were to be tested in the medium formulation, this type of analysis would quickly show not only if the factor had an influence on the fermentation parameters (or could be removed from the medium formulation if no influences were seen), but also to show if the factor has synergistic or antagonistic influences on the fermentation parameters in combination with other factors.

Table 4.5 displays a breakdown of all CSP and DAP levels and their significance to various fermentation parameters as reported by the REGWQ statistical test. This table allows one to compare the effects of each factor level on each fermentation parameter, and the effectiveness of different factor levels within each fermentation parameter. The bar groupings in Table 4.5 indicate which factor levels show the same magnitude of effect for each fermentation parameter (at 95% confidence level). For exponential growth rate (MaxU), there are no statistical differences between C1 and C2, C2 and C4, P1 and P2, nor between P3 and P4. There are, however, statistical differences between [C1 and C2]

**Table 4.5** Interactions between CSP and DAP on various fermentation parameters for *Saccharomyces cerevisiae* grown in shake flask cultures.

Fermentation parameter	g CSP <sup>1</sup> /100 ml (Factor level)				mg N/L ,mg P/L from DAP <sup>2</sup> (Factor level)			
	0.5	1	2	4	91	180	362	724
	(C1)	(C2)	(C3)	(C4)	(P1)	(P2)	(P3)	(P4)
MaxU <sup>3</sup>	██████████		██████████		██████████		██████████	
EPR <sup>4</sup>	██████████		██████████		██████████			
GLU <sup>5</sup>	████	████	██████████		██████████			
MaxX <sup>6</sup>	████	████	████	████	████	████	████	████
MaxE <sup>7</sup>	████	████	██████████		██████████			

<sup>1</sup> Corn steep powder

<sup>2</sup> Diammonium phosphate

<sup>3</sup> MaxU = Maximum exponential growth rate

<sup>4</sup> EPR = Maximum exponential ethanol production rate

<sup>5</sup> GLU = Stationary phase glucose concentration

<sup>6</sup> MaxX = Maximum stationary phase yeast cell density

<sup>7</sup> MaxE = Final ethanol concentration

Bar lines indicate no significant effects at 95% confidence limits.

Bar line groupings using Ryan-Einot-Gabriel-Welsch (REGWQ) Multiple Range Test.

and [C3 and C4], and between [P1 and P2] and [P3 and P4]. Thus, examining these results in a practical light, adding more CSP than C3 or more DAP than P3 would not improve MaxU and would thus be a waste of materials.

For exponential ethanol production rate (EPR), similar conclusions can be drawn about the CSP levels but the DAP levels require more interpretation. Here, there are no significant differences between P1, P2, and P3, nor between P3 and P4, but there are differences between [P1, P2, and P3] and [P3 and P4] groups. The P3 level overlaps into both groups which stems from the fact that statistically P3 can be included in both groups. To optimize the exponential ethanol production rate, CSP and DAP levels of C3 and P3, respectively, would offer the best combination.

For stationary phase glucose concentration (GLU), three groups are evident for CSP- C1, C2, and [C3 and C4], while two groups are evident for DAP- [P1, P2, and P3] and P4. Practically, C3 would give the optimal CSP level while any of P1, P2, or P3 would be adequate to optimize stationary phase glucose concentration. Increasing to P4 would give a lower glucose concentration, but at a significant cost as far as DAP addition is concerned. Similar conclusions can be made for the maximum ethanol concentration at stationary phase (MaxE).

For maximum stationary phase yeast cell density (MaxX), each CSP and DAP level is significantly different from any other. Adding C4 and P4 would give the highest cell density, but at a significantly higher cost. In addition, C4 and P4 would not increase the performance of most other fermentation parameters. Thus, addition at C4 and P4 levels is not cost-effective.

Based on the above factorial experiments with statistical analysis by the F and REGWQ tests, the levels of CSP and DAP that would optimize most of the fermentation parameters would be C3 (2 g of CSP/100ml) and P3 at 1.706 g/L (400 mg phosphorus/L with 362 mg available nitrogen/L). Higher levels of either factor would not improve most of the fermentation parameters and could be a waste of materials. Therefore, concentrations of CSP (C3) and DAP (P3) levels were used in all medium formulations for the remainder of this work. All of the fermentation parameters in Table 4.5 are

important in considering the performance of yeast in the production of ethanol in batch systems. However, in continuous culture, more emphasis needs to be placed on the exponential growth rate of the yeast. The reason for this is that in batch systems a longer fermentation time can be used (in general) to overcome a yeast that is growing more slowly. In contrast, a yeast that is growing at a slower rate in a continuous system would consequently form a lower steady state biomass concentration (and consequently a lower ethanol conversion rate) or be washed out of the system.

Ideally, the factorial experiments should have been performed in continuous culture since not only is the optimized medium to be used in continuous work, but the interaction effects between factors and the fermentation parameters (Table 4.4) and the effects of each factor level on the fermentation parameters (Table 4.5) may be different in batch and continuous systems. For example, one interaction in Table 4.4 that would probably change if the factorial experiments were conducted in continuous culture is the maximum exponential growth rate (MaxU). Since the growth rate of yeast in a continuous system directly impacts on the steady state glucose and ethanol concentrations, there would probably be significant effects on the residual (GLU) and ethanol (MaxE) concentrations with maximum exponential growth rate (MaxU). Practically, the sixteen combinations in the factorial experiments in duplicate would have been extremely difficult to conduct in continuous mode and the time involved to gather the data from the experiments would have been substantially greater. Thus, although not ideal, the factorial experiments conducted in batch still led to a better understanding and optimization of medium components than if the experiments had not been conducted at all.

#### **4.2 Multistage continuous culture fermentation (MCCF).**

MCCF experiments were conducted using the optimized medium formulation described in the previous section. The aim of the MCCF experiments was to provide data on ethanol production using VHG media and also to provide a “baseline” to which other

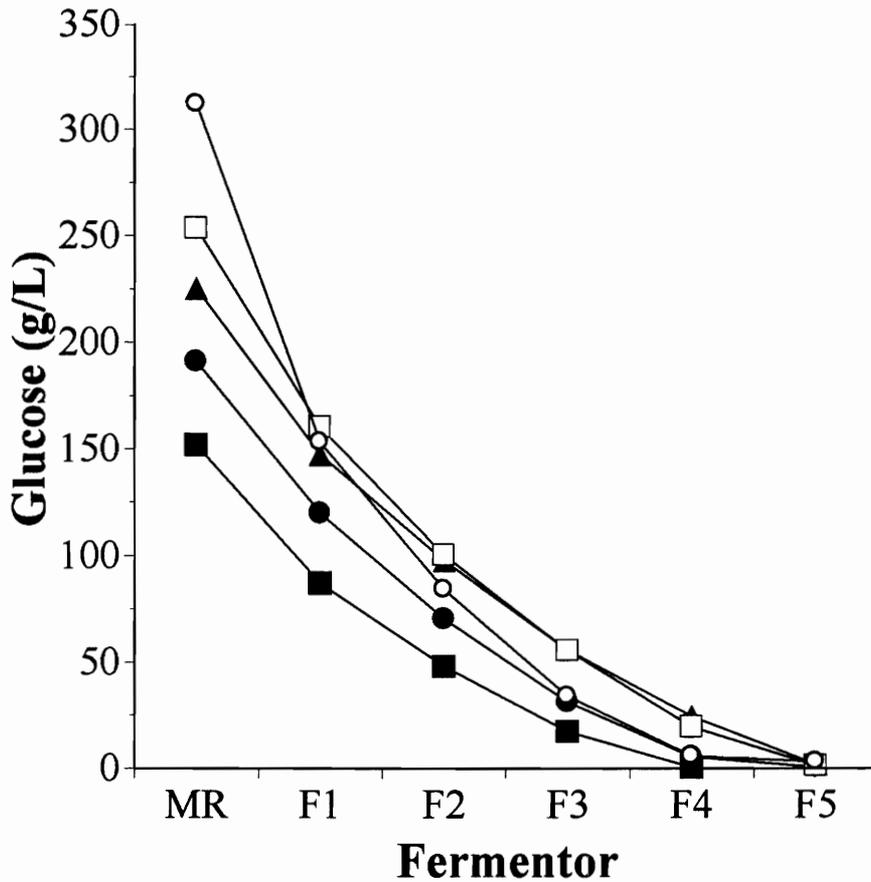
experiments based on the MCCF can be compared. To this end the results of the MCCF fermentations are detailed in the following subsections. Where possible, the results from the MCCF will be compared to values from batch, cell-recycle, and single and multistage continuous systems in other published work.

#### **4.2.1 Glucose concentration.**

Figure 4.11 illustrates glucose concentrations in the medium reservoir (MR) and steady state glucose levels in each of the five fermentors in the multistage continuous culture fermentation (MCCF) system over a range of glucose concentrations from 152 to 312 g/L. One of the primary objectives for these experiments was to determine what dilution rate was needed to ensure complete ( $< 5$  g/L) glucose utilization in the effluent from the fifth fermentor for each concentration of glucose used (including glucose concentrations meeting VHG level requirements). The dilution rate required at each glucose concentration to ensure complete utilization by the last fermentor was determined in each condition as outlined in Section 3.5.2.4. The maximum residual glucose concentration in F5 at any medium reservoir (MR) glucose concentration was three g/L. With an initial MR glucose concentration of 312 g/L, a dilution rate (D) of  $0.05 \text{ hr}^{-1}$  was required to achieve this low residual glucose concentration. These values translate into a consumption of 99.03% glucose in all of the conditions tested.

The total residence time in the MCCF when operated at 312 g/L concentration of glucose was 116 h. In batch experiments, wheat mash fermentations starting with 370 g/L dissolved solids were fermented in 96 h when high yeast pitching rates ( $150 \times 10^6$  cells/g mash) were used and alcohol concentrations were over 21% v/v (Thomas and Ingledew, 1992).

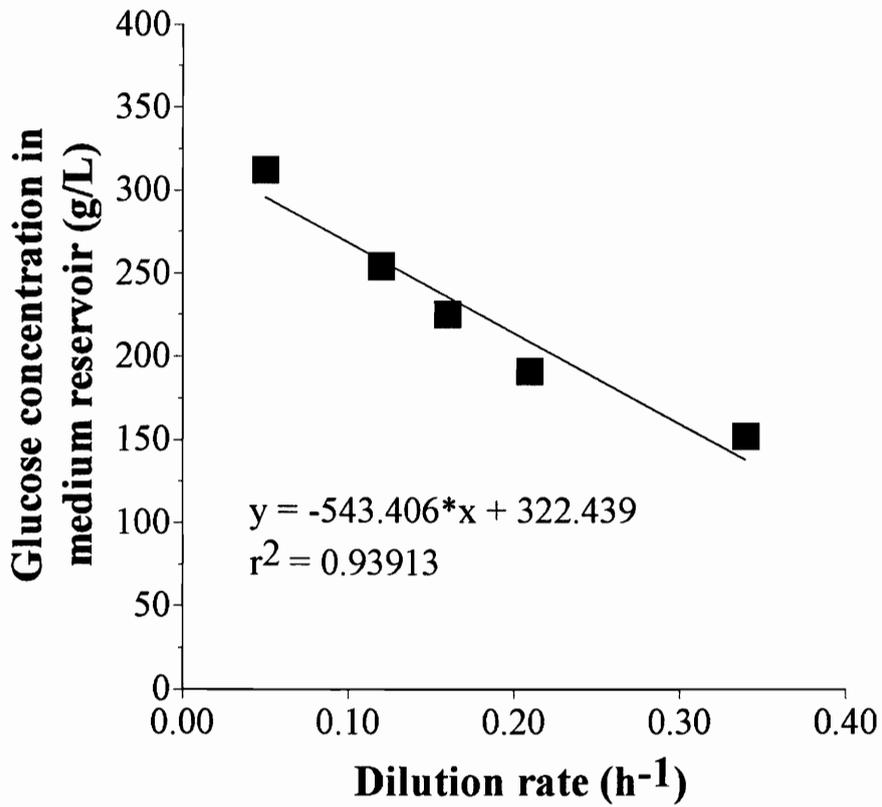
It is clear from Figure 4.11 that the yeasts are capable of consuming all of the glucose provided from the medium reservoir in the MCCF system and that VHG level medium formulations (greater than 27 g dissolved solids /100g grain mash) can be successfully fermented in a MCCF system under the conditions used. As in VHG



**Figure 4.11** Glucose concentrations in each fermentor at steady state in the MCCF with the medium reservoir containing from 152 to 312 g/L glucose (■, 152 g/L glucose ( $D = 0.34$ , Flow = 15.87 ml/min); ●, 191 g/L glucose ( $D = 0.21$ , Flow = 9.80 ml/min); ▲, 225 g/L glucose ( $D = 0.16$ , Flow = 7.47 ml/min); □, 254 g/L glucose ( $D = 0.12$ , Flow = 5.60 ml/min); ○, 312 g/L glucose ( $D = 0.05$ , Flow = 2.33 ml/min); MR = medium reservoir; F1-F5 = fermentors 1-5 in the MCCF system).

fermentations performed in batch mode, the yeast in the MCCF are inoculated into the fermentor initially containing medium at VHG concentrations of substrate. In both batch and continuous fermentations, the concentration of substrate decreases in the fermentor as the yeast multiply and produce ethanol. However, unlike in batch VHG fermentations where the substrate concentration (ideally) decreases to zero, the substrate concentration in a continuous system is maintained at a steady state concentration in the fermentor. This consequently reduces the concentration of ethanol that could have been achieved in that fermentor. Therefore, if the definition of a VHG based fermentation were to be based on the concentration of ethanol produced rather than the concentration of glucose used, the MCCF system would not qualify as a VHG fermentation.

For each glucose concentration used in the MR, the dilution rate of the MCCF was adjusted to provide near quantitative consumption of glucose by the time effluent exits the last fermentor (F5). Figure 4.12 shows the relationship between the MR glucose concentration and the dilution rate used to achieve complete glucose consumption by F5 for each condition. A linear relationship with a high correlation ( $r^2 = 0.94$ ) exists between the two parameters. In general, as the MR glucose concentration increased, the dilution rate required for the MCCF decreased. From a production point of view, the dilution rate for the MCCF can be adjusted (based on the equation generated in Figure 4.12) to achieve total glucose consumption at any MR glucose concentration. This provides a company with the opportunity to adjust the operation of an MCCF to fluctuations in incoming glucose concentrations. Another observation from Figure 4.12 is that the maximum glucose concentration that can be used in an MCCF as described in this work is a value less than 320 g/L, since at 320 g/L,  $D$  approaches 0 and thus at that point, the MCCF is virtually in batch mode. Thus, although the MCCF system is capable of operating under VHG conditions with this medium, there appears to be an upper limit of substrate (320 g/L) beyond which continuous fermentation cannot be performed. In contrast, VHG wheat mashes as high as 380 g/L were successfully fermented in the laboratory in batch mode (Thomas *et al.*, 1993). Further research into the MCCF system and into optimization of medium components may allow increased initial substrate



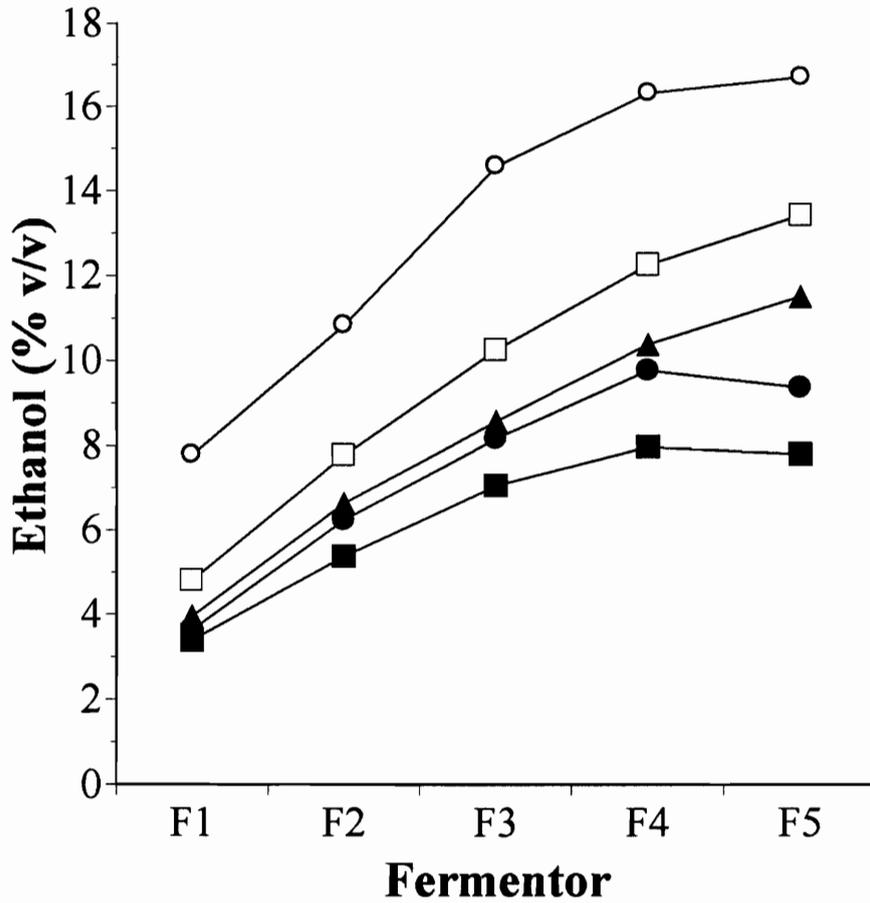
**Figure 4.12** Dilution rates required in the multistage continuous culture system to ensure complete glucose utilization by F5 as medium reservoir glucose concentrations are increased.

concentration closer to that reported for batch fermentation.

Tyagi and Ghose (1980) successfully fermented a bagasse hydrolysate where the reducing sugar concentration in the feed was 314 g/L (of which 220 g/L was glucose). In that work they utilized a four-stage, serial continuous system operating at a dilution rate of 0.05 h<sup>-1</sup>. In comparison, the five-stage MCCF system in this work was able to totally end ferment a glucose concentration of 312 g/L at a dilution rate of 0.05 h<sup>-1</sup>. Unfortunately, it is not clear if any residual sugar remained in the four-stage system used by Tyagi and Ghose (1980) as measurements of sugar were not made in the third and fourth fermentors at their chosen dilution rate. Assuming that sugar was totally used in their system, it is intriguing but unclear why a four-stage serial continuous system would out-perform the five-stage MCCF used in this work when the fermentable sugar concentration in their work and the glucose concentration used in the present work were nearly identical. Possible explanations include differences in the yeast used for the experiments, the level of yeast growth supported by the bagasse hydrolysate, and/or other differences in medium formulation. In other work, Dourado and Calvert (1987) required a six-stage serial continuous system in order to completely exhaust glucose by the sixth fermentor when only 160 g/L glucose was fermented by *S. cerevisiae* at a dilution rate of 0.054 h<sup>-1</sup>. Thus, in their serial continuous culture system, they were only able to ferment approximately half of the sugar concentration as used in the present MCCF, and only when six stages were used rather than the five used here. Clearly this reduction in ethanol productivity would be less profitable for the fuel alcohol producer.

#### 4.2.2 Ethanol.

Figure 4.13 illustrates the individual steady state ethanol concentrations in the five fermentors when the glucose concentrations in the medium reservoirs were set at a range of 152 to 312 g/L. In general, the steady state ethanol concentration increased from F1 to F5 for all sugar concentrations. The maximum ethanol concentration reached in F5 was 132.0 g/L (16.73% v/v) when the 312 g/L MR glucose condition was used. At 152 and



**Figure 4.13** Steady state ethanol concentrations in the five fermentors in the multistage continuous culture system with the medium reservoir containing 152 to 312 g/L glucose (■, 152 g/L glucose ( $D = 0.34$ , Flow = 15.87 ml/min); ●, 191 g/L glucose ( $D = 0.21$ , Flow = 9.80 ml/min); ▲, 225 g/L glucose ( $D = 0.16$ , Flow = 7.47 ml/min); □, 254 g/L glucose ( $D = 0.12$ , Flow = 5.60 ml/min); ○, 312 g/L glucose ( $D = 0.05$ , Flow = 2.33 ml/min); F1-F5 = fermentors 1-5 in the MCCF system).

191 g/L MR glucose conditions, a slight decrease in ethanol concentration from F4 to F5 was seen which suggested that ethanol was consumed in F5. This indicates that the MCCF dilution rate could have been increased for these conditions. Further support for this is found in Figure 4.11 where the glucose in F4 for these conditions was at very low levels. An increase in the dilution rate would have increased the flow through the MCCF (increased productivity) and increased the glucose level in F4. The ethanol concentration continued to increase in Figure 4.13 for all medium glucose levels from F1 to F5, however the rates of ethanol production decreased from F1 to F5. This was no doubt due to inhibition of yeast growth by ethanol, to inhibition of yeast ethanol production rate, and/or to the shift of biomass from actively growing to non-growing cells where the rate of ethanol production becomes up to 30 times lower than seen in actively growing cells (Kirsop, 1982).

In the work of Tyagi and Ghose (1980), a maximum of 10.32% v/v ethanol in a single feed ( $D = 0.05 \text{ h}^{-1}$ ), four-stage, continuous, cascade, stirred tank fermentor system was produced utilizing 220 g/L glucose (314 g/L reducing sugar concentration) generated enzymatically from cellulosic bagasse. In the present work, however, by increasing the number of fermentors to five in the MCCF system, the yeasts were able to produce 16.7% v/v ethanol from 312 g/L glucose concentration at the same dilution rate. This increase was achieved by raising the concentration of glucose used in the feed (reducing the amount of water present). For fuel alcohol plants producing ethanol by continuous culture, ethanol concentrations normally achieved are often less than 11% v/v. The present work demonstrates that ethanol concentrations as high as 16.7% v/v can be achieved utilizing VHG level substrate concentrations and multistage continuous culture technologies.

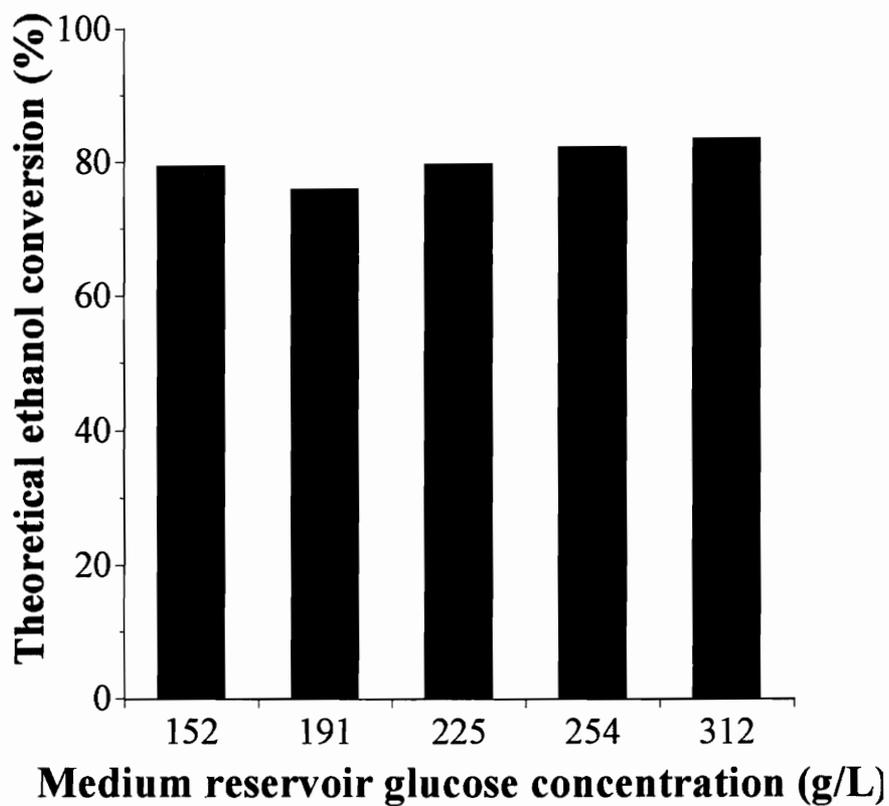
#### **4.2.3 Theoretical ethanol conversion.**

The stoichiometric amount of ethanol that *S. cerevisiae* can produce from glucose is 51.1% (weight basis). Thus, the fermentation of one gram of glucose will yield

theoretically 0.511g ethanol if all the glucose could be converted to ethanol and none was diverted to biomass or metabolic endproducts other than CO<sub>2</sub>. In the MCCF, the amount of glucose consumed (at steady state) in the system can be determined and from this, the theoretical amount of ethanol that could be produced can be calculated. Since the amount of ethanol actually produced in the system is known (at steady state), deviation from theoretical yield can be calculated.

The resulting theoretical ethanol conversion rates for each medium reservoir concentration of glucose used in the MCCF is shown in Figure 4.14. In general at all concentrations of glucose, similar ethanol conversion rates were observed and they ranged from 76% at 191 g/L glucose to 83% at 312 g/L glucose (which meant that ~20% of the glucose was used for purposes other than producing ethanol or that ethanol was lost in the MCCF). The fact that the theoretical ethanol yield was nearly constant across MR glucose concentrations was unexpected. The increasing MR glucose concentrations was anticipated to impose additional stresses on the yeast - *i.e.* osmotic stress. Thus, it was expected that the yeast would divert more glucose for maintenance purposes as the concentration of glucose in the medium reservoir was increased. From the data in Figure 4.14, one could conclude, in general, that *S. cerevisiae* converts a similar proportion of glucose to ethanol whether cultured in normal, high, or very high gravity continuous fermentations and that the fraction of consumed glucose going towards ethanol production could not be increased by increasing the glucose concentration in the medium reservoir.

This comparatively low theoretical ethanol conversion rate was surprising for a number of reasons. This value is low compared to the typical 90%+ yields seen in batch fuel alcohol fermentations. Fermentations of a VHG wheat mash in batch mode in the lab have produced ethanol concentrations at 95% of the theoretical yield (Jones and Ingledew, 1994d). Other VHG fermentations in batch with high maltose brewing syrups and lager worts resulted in theoretical ethanol yields of 98-99% (O'Connor-Cox *et al.*, 1991). In continuous culture, a theoretical ethanol conversion rate of 92.7% was found with cell recycle (Chang *et al.*, 1993) although VHG levels of substrate were not used.



**Figure 4.14** Theoretical ethanol conversions in the MCCF with the medium reservoir containing from 152 to 312 g/L glucose.

Calculations from the data of Tyagi and Ghose (1980) indicate that a theoretical ethanol conversion of 92% was attained in their four-stage serial multistage continuous system ( $D = 0.14 \text{ h}^{-1}$ , MR glucose concentration 220 g/L) although not all of the glucose was consumed by the last fermentor in the series. It is unclear what factors gave rise to the lower ethanol conversion rate in the MCCF study reported here for all sugar concentrations particularly since higher values can be obtained not only in batch and in continuous operations, but also where the medium used was not investigated for optimal nutrition. Possible reasons for the lower ethanol conversion rates obtained in the MCCF include an increased diversion of consumed glucose for cell growth and production of other endproducts (but none were seen), and/or the increased diversion of consumed glucose for cellular maintenance (due to the increased stress posed on the yeast from medium and/or fermentation conditions and/or an inherent higher demand for cellular maintenance for this particular strain of yeast). Lastly, the production of  $\text{CO}_2$  and introduction of air into the fermentors may have led to losses of ethanol due to gaseous "stripping".

#### **4.2.4 MCCF ethanol productivity.**

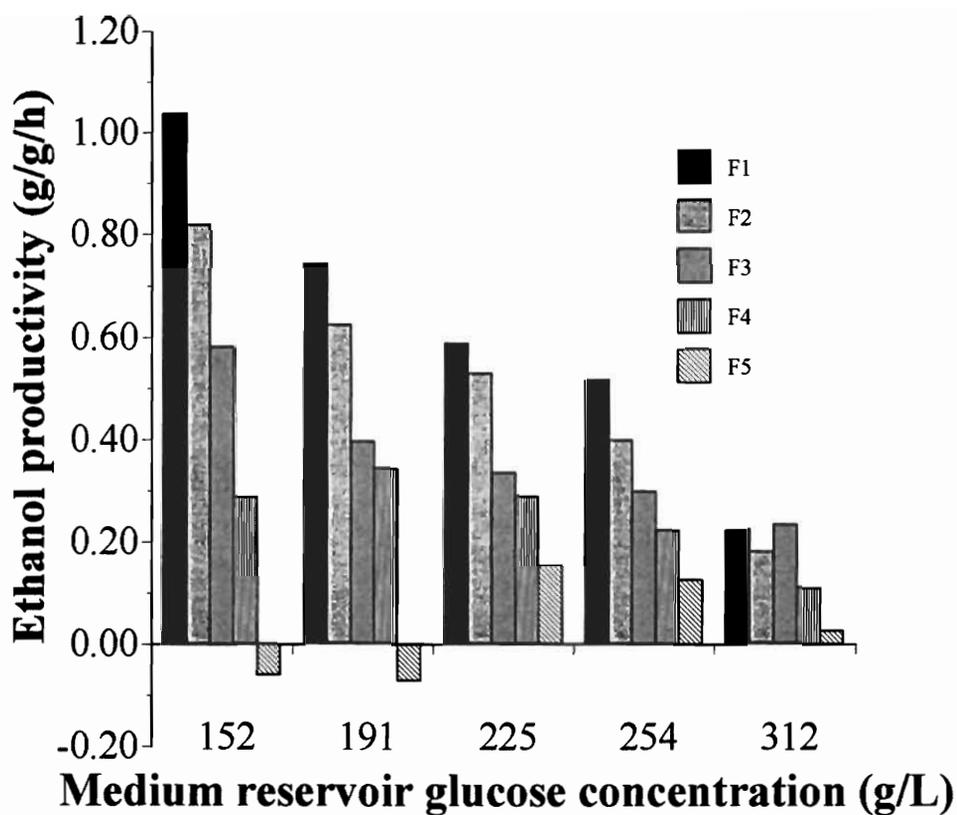
##### **4.2.4.1 Volumetric productivity.**

Figure 4.15 shows the volumetric productivity (g/L/h) in the MCCF when the glucose concentration in the MR was varied between 152 and 312 g/L. A linear relationship with a high correlation ( $r^2 = 0.96$ ) exists between the medium reservoir glucose concentration and outflow productivity. As expected, the ethanol productivity decreased as the glucose concentration in the MR increased. The decrease was due to the fact that the dilution rate was also decreased as the glucose concentration was increased in the MR to achieve minimal loss of glucose from the MCCF system. At first glance, Figure 4.15 would lead to the conclusion that it would be better to run the MCCF system with 152 g/L glucose in the medium reservoir since a higher ethanol productivity (~21

g/L/h) is achieved. Upon further examination, even though the 312 g/L condition gave a lower ethanol productivity (~6 g/L/h) as compared to the 152 g/L condition (~21 g/L/h), the concentration of ethanol produced at the 312 g/L condition (132 g/L, ~17% v/v) was much higher than the 152 g/L condition (62 g/L, ~8% v/v). Thus, a fuel alcohol producer should not only be concerned about ethanol productivity achieved in a fermentation system, but also what concentrations of ethanol were achieved and what were the glucose losses experienced in effluents. Many reports of high ethanol productivities exist in the literature but at these productivities, the role of ethanol concentration is downplayed. An ethanol productivity of 32 g/L/h was achieved from bagasse hydrolysate in the single-stage cell recycle continuous system of Ghose and Tyagi (1979a) with a steady state ethanol concentration of only 60.5 g/L. In other single-stage experiments with cell recycle, an ethanol productivity of only 15.2 g/L/h was achieved from beet juice supplemented with sucrose (Ramirez and Boudarel, 1983) while only 57.2 g/L ethanol was produced from the medium containing 117 g/L sucrose. An ethanol productivity of 70 g/L/h was achieved in a single-stage cell recycle system at a steady state ethanol concentration of 70 g/L (Warren *et al.*, 1994). A higher ethanol productivity of 85 g/L/h was achieved in a single-stage cell recycle system but an ethanol concentration of only 90 g/L was produced (Lee and Chang, 1987). Thus, although ethanol productivity is a useful parameter to compare the performance of one fermentation system to another and the changing conditions within a system, it must be tempered with other fermentation parameters that give information to the fuel alcohol producer which help to optimize a total fermentation system.

#### **4.2.4.2 Specific ethanol productivity.**

Figure 4.16 shows the specific ethanol productivities for the individual fermentors within the MCCF as the glucose concentration in the medium reservoir was changed from 152 to 312 g/L. Specific ethanol productivity is defined as ethanol produced per unit biomass per hour (g/g/h). This figure is a useful indication of how well the cells are



**Figure 4.16** MCCF individual fermentor specific ethanol productivities with the medium reservoir containing 152 to 312 g/L glucose.

producing ethanol. In Figure 4.16, the negative specific ethanol productivity values for F5 for the 152 and 191 g/L conditions reflect the fact that ethanol concentration decreased from F4 to F5 under both conditions. These results indicate that fermentations were over in F4 and both of these runs could have been made at a higher dilution rate to prevent this decline in ethanol concentration. Figure 4.16 shows at a glance which fermentors in the MCCF were optimally producing ethanol. As expected, the specific ethanol productivity decreased from F1 to F5 in each condition as the MR glucose concentration increased. The data in Figure 4.16 clearly show that on a cellular basis, the ethanol production capability of the yeast is highest in the first fermentor in each condition and that it decreases as one progresses to the last (F5) fermentor. The fact that the specific ethanol productivity decreased from F1 to F5 is not surprising since the nutritional quality of the medium is decreasing, yeast growth slows down or stops (which has an impact on yeast performance), and ethanol incrementally increases to inhibitory levels (which also has an impact on yeast performance).

The production of ethanol by yeast occurs in multiplying and in non-multiplying cells (although not at the same rate). To this end, another way to depict specific ethanol productivity is to determine the net ethanol productivity from cells that were newly produced in each fermentor rather than as a function of total cells. An attempt has been made to differentiate the specific ethanol production by both types of cells to see if there is any difference in ethanol productivity by multiplying and non-multiplying cells. Upon closer examination, the specific ethanol productivity in F1 for each condition in Figure 4.16 is in reality the net specific ethanol productivity for yeast cells that multiplied since none of the cells in F1 have come from earlier fermentors. For later fermentors in the MCCF, the yeast cell concentration is a result of cells that have multiplied in the fermentor and also cells that have passed through from earlier fermentors. Likewise, the ethanol concentration in fermentors other than F1 is a result of production from both cells that have multiplied in the fermentor and from cells that have carried over from previous fermentors. Thus, it is impossible to determine the net specific ethanol productivity in all fermentors in the MCCF based on the data obtained from the experiments.

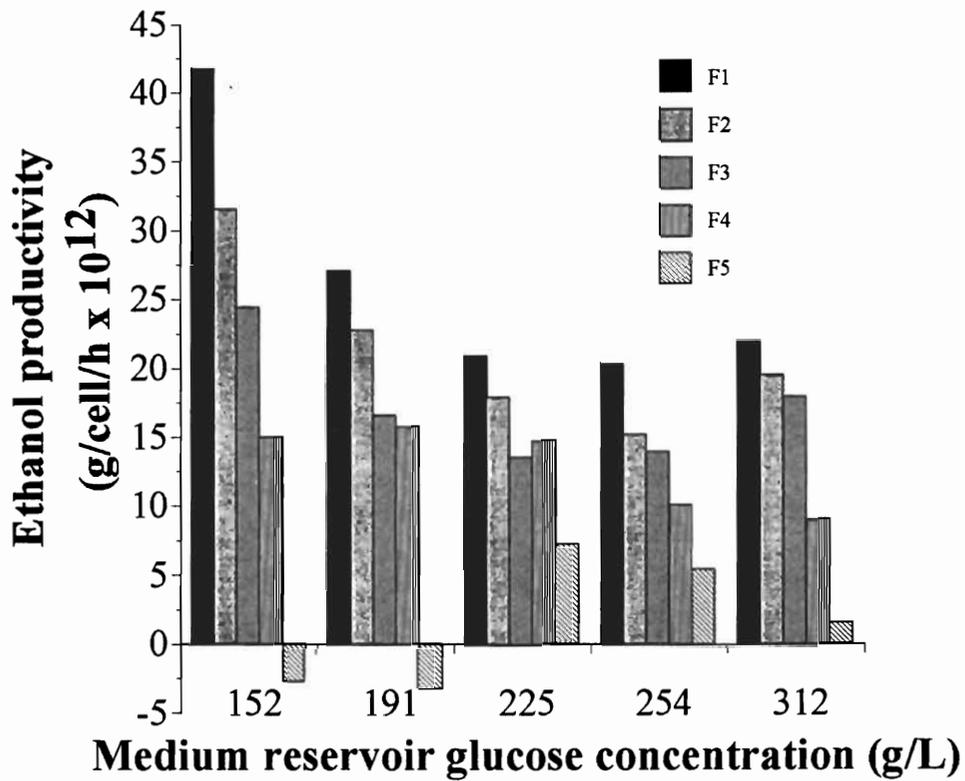
One difficulty in reporting specific ethanol productivity from cells on a weight basis is that there may be changes in yeast size and/or yeast cell density as the cells travel from F1 to F5 in the MCCF. Support for this hypothesis is found in work which reported that the volume and morphology of *S. cerevisiae* dramatically changed with changes in continuous culture dilution rate while few changes were seen in yeasts grown in batch (Hill and Robinson, 1988). In addition, if the medium used contains particulates, then corrections need to be performed on each yeast measurement (which increases the error in calculations). To overcome this, specific ethanol productivity can be determined on a per cell basis. These results are depicted in Figure 4.17. The observations and conclusions reached for Figure 4.16 also apply to Figure 4.17. This method of reporting specific ethanol productivity would be more accurate than reporting specific ethanol productivity from weight and also would immediately tie ethanol productivity to individual cells which is an attractive scenario to microbiologists. However, this method of reporting specific ethanol productivity is not common in the literature.

#### **4.2.5 Yeast cell counts.**

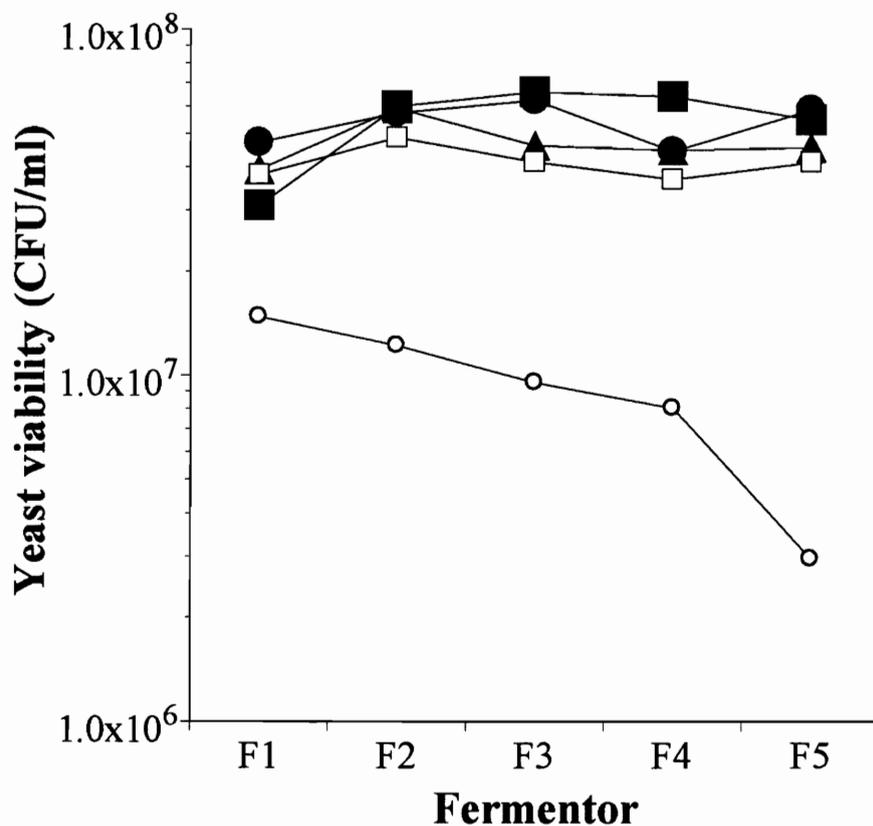
##### **4.2.5.1 Plate counts.**

Figure 4.18 illustrates the individual fermentor steady state viable yeast concentrations in CFU/ml when the medium reservoir glucose concentrations ranged from 152 to 312 g/L. The level of magnitude of standard deviation in viable numbers obtained by the membrane filtration technique is small compared to other plating methods, provided that the plating conditions and medium are as close to the experimental conditions in the fermentor as possible. Plating methods remain the most preferred and reproducible methods used by microbiologists to assess viability and they are the benchmark to which other methods are compared.

In Figure 4.18, the data for the five fermentation trains containing glucose in the MR ranging from 152 to 254 g/L showed a net initial increase in viable cells (F1-F2)



**Figure 4.17** MCCF individual fermentor specific ethanol productivities (g net ethanol produced in each fermentor per cell per h) with the medium reservoir containing 152 to 312 g/L glucose.

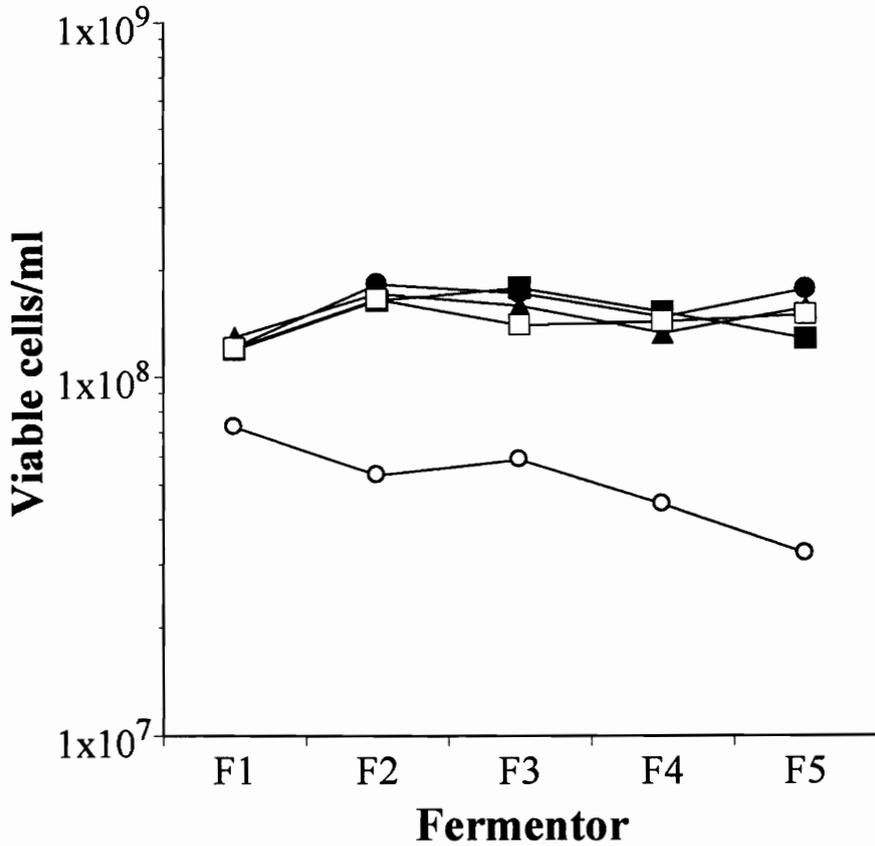


**Figure 4.18** Viabilities (CFU/ml) of *S. cerevisiae* at steady state in each fermentor in multistage continuous culture with the medium reservoir containing 152 to 312 g/L glucose (■, 152 g/L glucose (D = 0.34, Flow = 15.87 ml/min); ●, 191 g/L glucose (D = 0.21, Flow = 9.80 ml/min); ▲, 225 g/L glucose (D = 0.16, Flow = 7.47 ml/min); □, 254 g/L glucose (D = 0.12, Flow = 5.60 ml/min); ○, 312 g/L glucose (D = 0.05, Flow = 2.33 ml/min)).

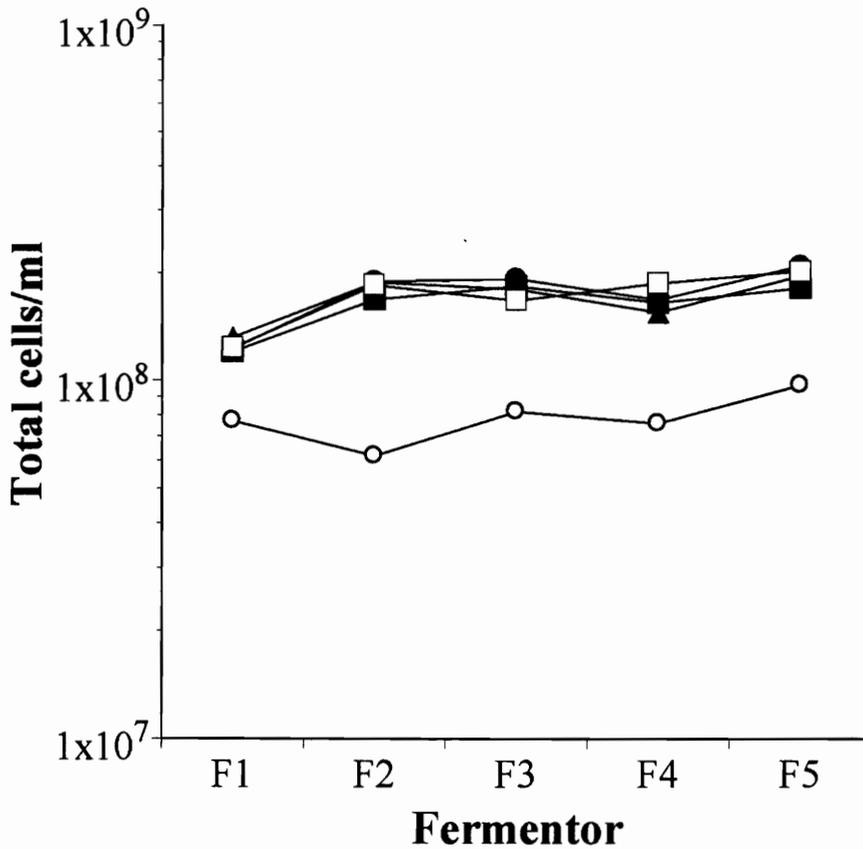
followed by no further increases through to F5. Thus, any biomass increase in the MCCF system was the result of growth in F1 and perhaps in F2. The steady state overall average concentration of viable cells from F2 to F5 was  $\sim 5 \times 10^7$  CFU/ml for all conditions (except at 312 g/L substrate). In contrast, the 312 g/L condition did not reach a plateau and continually decreased from  $\sim 1.5 \times 10^7$  CFU/ml in F1 to  $\sim 3 \times 10^6$  CFU/ml in F5. There are possible reasons for the yeast decrease at 312 g/L. One possibility was that the glucose concentration in the MR (and all subsequent glucose concentrations in the fermentors) was more inhibitory towards yeast specific growth rate. Another possibility was that the higher ethanol concentrations in the fermentors for the 312 g/L condition inhibited yeast growth. Another possibility is the increased death rate of yeast in successive fermentors. Lastly, the dilution rate of  $0.05 \text{ h}^{-1}$  for this condition was the slowest which meant that the yeast culture had a longer residence time in each fermentor to consume trace nutrients. Thus, the media nutritional status may have deteriorated faster from F1 to F5 at 312 g/L than in other conditions.

#### **4.2.5.2 Microscopic counts with vital stain (methylene blue).**

The methylene blue test distinguishes viable yeast from non-viable yeast cells because viable yeast can exclude the methylene blue dye. Viable cells are clear while non-viable cells are blue in the test. Figures 4.19 and 4.20 illustrate the viable and total yeast cell concentrations at steady state in each fermentor (as assessed by methylene blue) with incoming glucose concentrations ranging from 152 to 312 g/L. The analysis carried out at the 152 to 254 g/L glucose concentrations in both figures showed an initial increase in viability in the F1 and F2 stages followed by cessation of growth between F2 and F5 (see also Figure 4.18). In Figure 4.19 for the 152 to 254 g/L conditions, the viable number of cells in F1 averaged  $1.23 \times 10^8$  cells/ml and increased to a combined average in F2-F5 of  $1.57 \times 10^8$  viable cells/ml. In Figure 4.20 for the 152 to 254 g/L conditions, the total number of cells in F1 averaged  $1.25 \times 10^8$  cells/ml and increased to a combined average in F2-F5 of  $1.83 \times 10^8$  cells/ml. At 312 g/L of substrate, the number of viable cells (Fig. 4.19)



**Figure 4.19** Viable cell concentrations (as assessed by microscopic count with methylene blue as the vital stain) of *S. cerevisiae* at steady state in each fermentor in multistage continuous culture with the medium reservoir containing 152 to 312 g/L glucose (■, 152 g/L glucose (D = 0.34, Flow = 15.87 ml/min); ●, 191 g/L glucose (D = 0.21, Flow = 9.80 ml/min); ▲, 225 g/L glucose (D = 0.16, Flow = 7.47 ml/min); □, 254 g/L glucose (D = 0.12, Flow = 5.60 ml/min); ○, 312 g/L glucose (D = 0.05, Flow = 2.33 ml/min)).

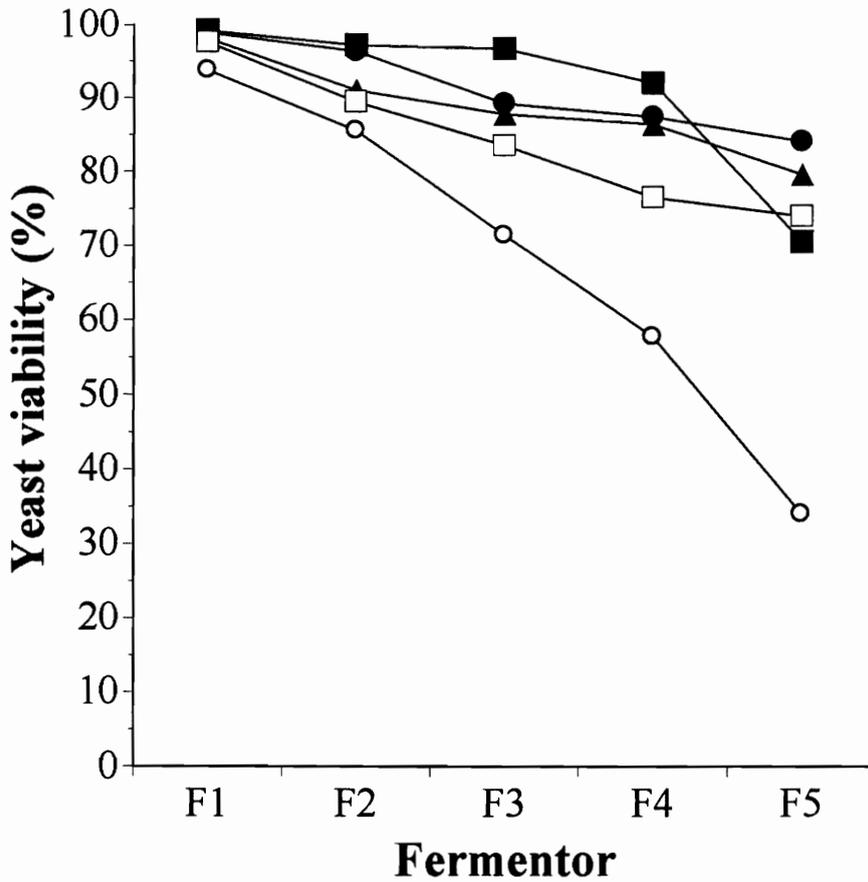


**Figure 4.20** Total cell concentrations (as assessed by microscopic count with methylene blue as the vital stain) of *S. cerevisiae* at steady state in each fermentor in multistage continuous culture with the medium reservoir containing 152 to 312 g/L glucose (■, 152 g/L glucose (D = 0.34, Flow = 15.87 ml/min); ●, 191 g/L glucose (D = 0.21, Flow = 9.80 ml/min); ▲, 225 g/L glucose (D = 0.16, Flow = 7.47 ml/min); □, 254 g/L glucose (D = 0.12, Flow = 5.60 ml/min); ○, 312 g/L glucose (D = 0.05, Flow = 2.33 ml/min)).

continually decreased while the total number of cells (Fig. 4.20) decreased from  $7.73 \times 10^7$  cells/ml (F1) to  $6.18 \times 10^7$  cells/ml (F2) and then showed a gradual increase to  $7.93 \times 10^7$  cells/ml in F5.

From the data in Figures 4.19 and 4.20, the percent viable cells can be calculated and is presented in Figure 4.21. Here, the general trend was that % viability decreased from F1 to F5 for all conditions. In general, the losses in viability increased across the train as the MR glucose concentration was increased. More than 70% of the yeast were still viable by F5 in the MCCF for all MR glucose concentrations up to 254 g/L. This contrasts with the 34% viability seen in F5 in the MCCF with a glucose concentration of 312 g/L (a 59.8% decrease). Presumably, the yeasts were experiencing more stressful conditions with the higher glucose concentrations used (higher glucose and ethanol concentrations than in other MR conditions). The low viability in the 312 g/L MR condition is alarming since only 34% of the cells were able to produce ethanol in the latter stages in the MCCF. If the viability could be increased in F5 (or the number of yeasts increased), the ethanol production rate could potentially be increased.

The viable cells/ml in Figure 4.19 parallel the results seen for viable cells assessed by plating in CFU/ml (Fig. 4.18). The most important difference is that results obtained by plating are on average nearly 0.6 logs lower than the corresponding viable cells/ml measured by methylene blue. Ideally, both values should be close. Upon further examination, an interesting observation is seen when the data in Figure 4.21 are compared with the data in Figures 4.18 and 4.20. Using methylene blue, the viability of yeast decreased in each fermentor in the MCCF at each sugar concentration used (Fig. 4.21) and yet using the viable cell plating technique in Figures 4.18 and in the methylene blue method in Figure 4.20, the yeast viability and total yeast cells increased slightly in most conditions from F1 to F2, and then remained constant along the train (this observation holds true even when all figures were compared on a logarithmic basis). Taken together, these observations suggest that the methylene blue procedure may overestimate the fraction of yeast cells that are non-viable. Support for this hypothesis comes from work in which viabilities have been compared using plate counts, citrate



**Figure 4.21** Percent yeast viability (as assessed by microscopic count with methylene blue as the vital stain) of *S. cerevisiae* at steady state in each fermentor in multistage continuous culture with the medium reservoir containing 152 to 312 g/L glucose (■, 152 g/L glucose ( $D = 0.34$ , Flow = 15.87 ml/min); ●, 191 g/L glucose ( $D = 0.21$ , Flow = 9.80 ml/min); ▲, 225 g/L glucose ( $D = 0.16$ , Flow = 7.47 ml/min); □, 254 g/L glucose ( $D = 0.12$ , Flow = 5.60 ml/min); ○, 312 g/L glucose ( $D = 0.05$ , Flow = 2.33 ml/min)).

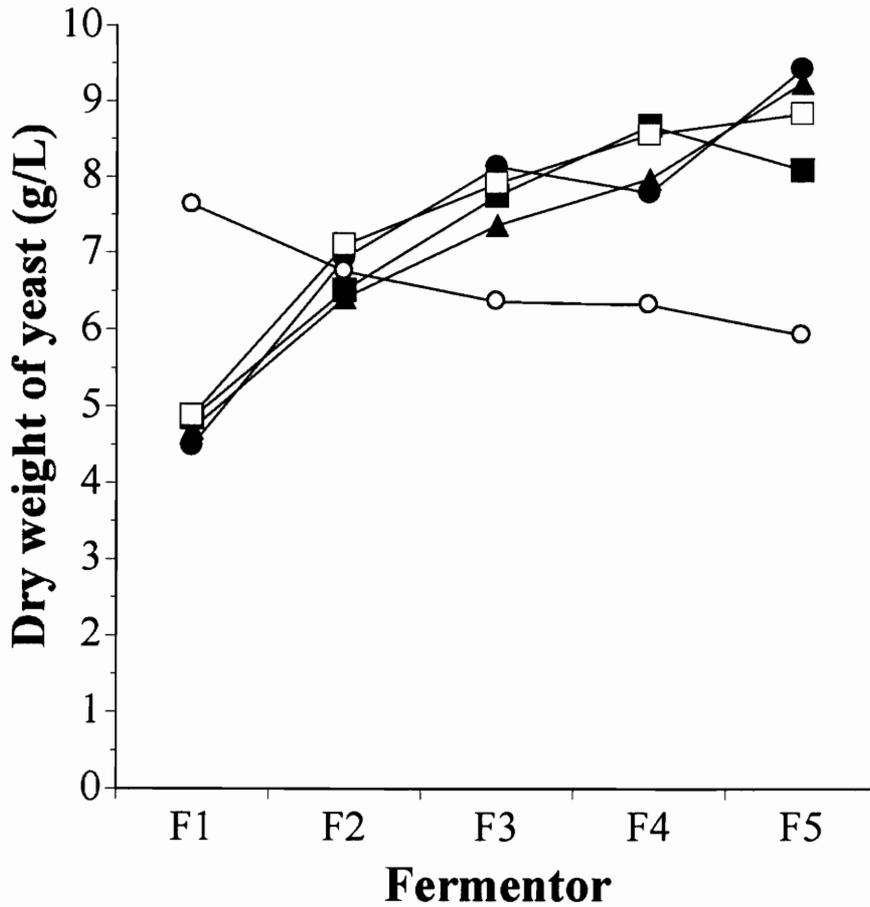
methylene blue, and citrate methylene violet procedures for three yeast strains which were assessed in “healthy” (exponential and stationary phase cells), “stressed” (exponential cells were incubated in water for 0-72 hours) and “non-viable” (exponential and stationary phase cells heat treated at 70°C for 2-4 hours in water) states (Smart *et al.*, 1999). The authors concluded that the plate count method is the most reproducible method of determining the numbers of viable and dead cells.

The methylene blue test for viability is routinely used in the ethanol industry and in laboratories to determine the viability of yeast in many different types of fermentations. However, its accuracy at reporting low viabilities has been called into question. In an experimental survey of different staining techniques for the determination of yeasts at different physiological states and pH, Smart and coworkers (1999) concluded that methylene violet was not only more accurate than methylene blue in determining the viability of yeast cells at low viabilities, but it also allowed better differentiation of living, stressed, and dead cells. Similar concerns about using methylene blue at low viabilities were voiced by O’Connor-Cox *et al.*, (1997).

In other work, the number of viable cells (CFU/ml) inoculated into normal gravity (22-24°P) batch fermentations at  $\sim 1 \times 10^6$  CFU/ml increased to  $\sim 3 \times 10^8$  CFU/ml, and then decreased to  $\sim 1 \times 10^8$  CFU/ml by the end of fermentation (Narendranath *et al.*, 1997). As well, 28°P VHG wort batch fermentations were inoculated at  $3 \times 10^7$  CFU/ml and reached  $\sim 2.5 \times 10^8$  CFU/ml at stationary phase (O’Connor-Cox and Ingledew, 1989). The inoculation level of  $3 \times 10^7$  CFU/ml in the latter work required approximately 166 h to completely ferment medium at 28°P (and in this time the viable number of cells was increasing). In the MCCF reported here, the steady state viability for the 312 g/L condition started at  $\sim 1.5 \times 10^7$  CFU/ml in F1 and continually decreased to  $\sim 2.5 \times 10^6$  CFU/ml by F5. The total residence time in the MCCF to completely ferment glucose at the 312 g/L concentration was 120 h. Thus, the MCCF required (in comparison to batch) less time and fewer viable yeast to ferment medium containing more substrate than the work done in batch. Clearly the productivity in the MCCF is higher than what is observed in batch.

#### 4.2.6 Biomass by dry weight.

Figure 4.22 illustrates the individual yeast dry weight data at steady state in fermentors with the initial glucose concentration set from 152 to 312 g/L. The biomass concentration for all conditions, except at 312 g/L, continually increased from fermentor to fermentor down the train from an average of 4.73 g/L (F1) to an average of 8.89 g/L (F5) (a 4.17 g/L increase in biomass). These dry weight results would initially suggest that the yeast numbers increased across the MCCF. However, the viable yeast cell numbers by plating on YEPD (Fig. 4.18) and the total yeast cell numbers (Fig. 4.20) remained constant over the same conditions where dry weights increased in Figure 4.22 (even when all charts were compared on a logarithmic basis). The only explanation to account for a constant yeast cell number (viable or total) but an increasing yeast dry mass in the MCCF would be that either the density of the yeast cell is increasing or that the yeast cell volume (and thus dry weight per cell) is increasing by a factor of  $\sim 1.87$  ( $8.89/4.73$ ). Support for the latter explanation is found in work which reported that the volume and morphology of *S. cerevisiae* dramatically changed with changes in continuous culture dilution rate, whereas few changes were seen in yeasts grown in batch (Hill and Robinson, 1988). Their work also showed that the growth rates of *S. cerevisiae* and the continuous culture flowrates were not directly responsible for the morphological changes. The authors concluded that some unknown environmental condition arising in the medium during continuous culture was the cause of morphological changes. In Figure 4.22, the biomass in the fermentors with 312 g/L glucose in the MR continually decreased from 7.63 g/L in F1 to 5.92 g/L in F5. Possible reasons for this biomass decrease include an increase in cell death or lysis rates from F1 to F5 (supported by the CFU/ml and total cell count decreases seen in Figures 4.18 and 4.20 respectively), an inhibition of growth rate due to osmotic inhibition and/or ethanol concentration (each either exerting influence individually or acting synergistically), and/or a decrease in cell volume because of the higher osmotic strength in F1-F5. Support for the latter reason is found in work showing that cell volumes of *S. cerevisiae* decreased as



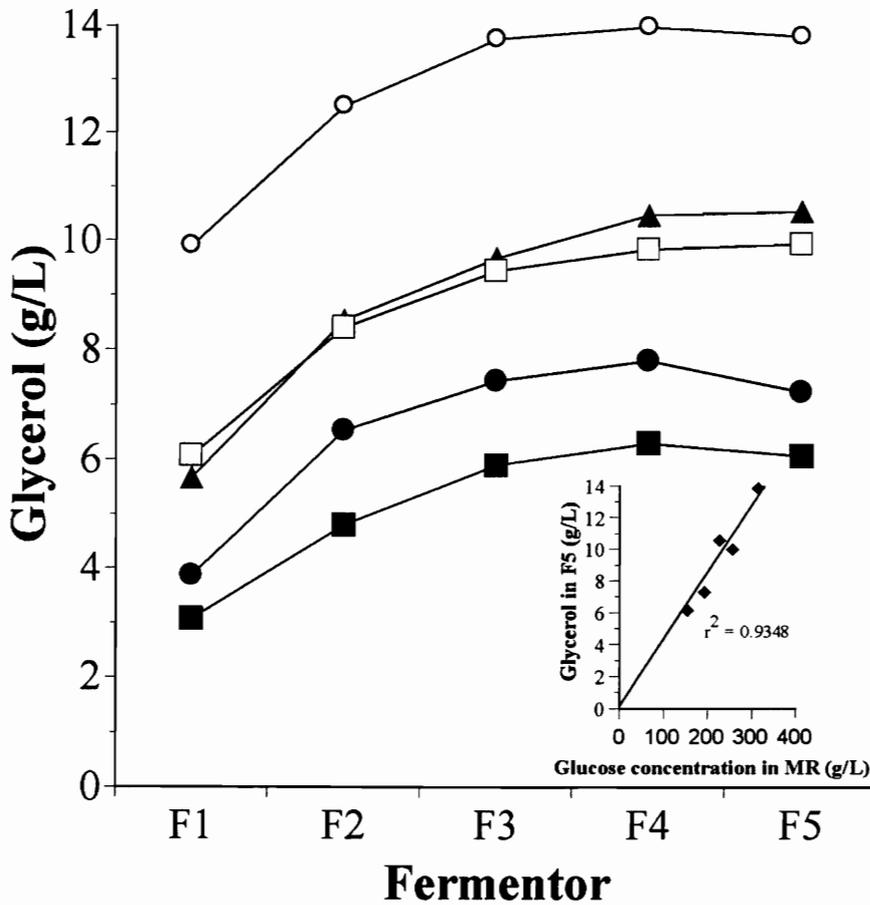
**Figure 4.22** Dry weights of *S. cerevisiae* at steady state in each fermentor in multistage continuous culture with the medium reservoir containing 152 to 312 g/L glucose (■, 152 g/L glucose ( $D = 0.34$ , Flow = 15.87 ml/min); ●, 191 g/L glucose ( $D = 0.21$ , Flow = 9.80 ml/min); ▲, 225 g/L glucose ( $D = 0.16$ , Flow = 7.47 ml/min); □, 254 g/L glucose ( $D = 0.12$ , Flow = 5.60 ml/min); ○, 312 g/L glucose ( $D = 0.05$ , Flow = 2.33 ml/min)).

the osmolality of NaCl in the medium increased (Morris *et al.*, 1983).

In one study reported by Chang *et al.* (1993), the concentration of biomass (dry weight) with a cell recycle system reached a steady state of ~55 g/L using medium containing 100 g/L glucose (with no glucose exiting the single fermentor), and with a dilution rate of 0.41 h<sup>-1</sup> and a bleed ratio of 0.15. With total retention (bleed ratio = 0) the authors managed to obtain a maximum cell concentration of 160 g/L above which it was difficult to mix the contents of the fermentor. The higher concentration of cell mass achievable in a cell recycle system is one of the main advantages of this type of fermentation system. In single-stage continuous culture, Liden *et al.* (1995) obtained a cell concentration of ~1.5 g/L at a dilution rate of 0.1 h<sup>-1</sup> but they used a concentration of only 20 g/L glucose in the medium. Tyagi and Ghose (1980) managed to obtain a maximum cell concentration of 13.4 g/L in the first stage of a four-stage serial continuous system operating at a dilution rate of 0.05 h<sup>-1</sup> with 220 g/L glucose (314 g/L reducing sugar) in the incoming medium. All of the glucose was consumed by the third fermentor. Another set of operating conditions performed by the authors ( $D = 0.12 \text{ h}^{-1}$ , 314 g/L reducing sugar (220 g/L glucose)) yielded a dry weight cell concentration profile of 3.85 g/L in F1; 11.7 g/L in F2; 12.0 g/L in F3; 11.95 g/L in F4; and 11.1 g/L glucose leaving the system. Comparing these values to the dry weight values obtained for the 254 g/L condition (Fig. 4.22) in this study, most of the dry weight values published by Tyagi and Ghose (1980) exceed the dry weights obtained for F1 to F4 in this MCCF. These higher dry weight values are puzzling since the base medium is a cellulose hydrolysate which could contain compounds inhibitory for yeast growth. The authors did add a complex nitrogen source, but do not specify any information other than it was obtained from an “agro-based waste material” and had 7.08% total nitrogen. The type of yeast used may also have contributed to higher dry weight values.

#### **4.2.7 Production of glycerol.**

Figure 4.23 illustrates the individual steady state glycerol concentrations in MCCF

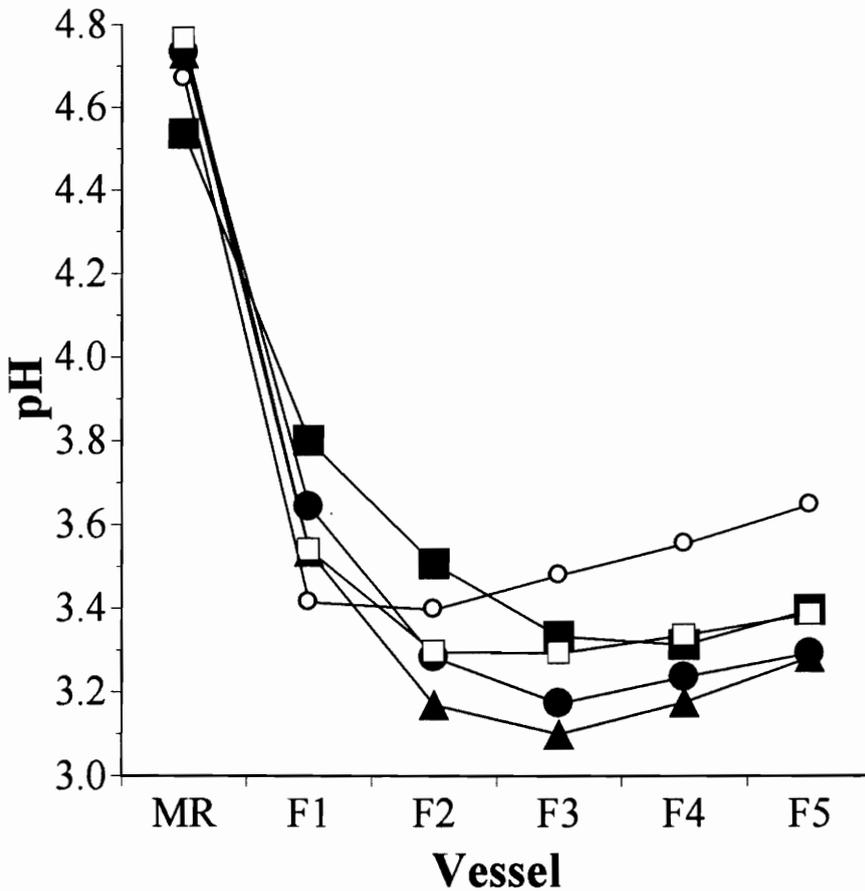


**Figure 4.23** Steady state glycerol concentrations in each fermentor in the multistage continuous culture system with the medium reservoir containing 152 to 312 g/L glucose (■, 152 g/L glucose ( $D = 0.34$ , Flow = 15.87 ml/min); ●, 191 g/L glucose ( $D = 0.21$ , Flow = 9.80 ml/min); ▲, 225 g/L glucose ( $D = 0.16$ , Flow = 7.47 ml/min); □, 254 g/L glucose ( $D = 0.12$ , Flow = 5.60 ml/min); ○, 312 g/L glucose ( $D = 0.05$ , Flow = 2.33 ml/min); ◆, glycerol concentration in F5 with increasing glucose concentration in the MR; F1-F5 = fermentors 1-5 in the MCCF system).

fermentations set with glucose concentrations from 152 to 312 g/L. In these pure culture MCCF experiments, glycerol was the only other yeast metabolic product detected by HPLC. The glycerol concentrations reached a maximum value of 13.98 g/L in F4 in the 312 g/L condition. There is a clear correlation ( $r^2 = 0.9348$ ) between the amount of glycerol produced and the glucose concentration in each medium reservoir. The increased glycerol concentrations (between fermentors and between different glucose conditions) appear to indicate that the yeast was under increased levels of stress in the MCCF. The production of glycerol is undesirable (to an alcohol plant) since potentially more ethanol could have been made with the glucose that was diverted to the formation of glycerol. Diversion of glucose for the production of 14 g/L glycerol corresponds to a loss of  $\sim 7$  g/L ethanol. If this loss did not occur, the theoretical ethanol conversion would have increased to 89.8% from 83.5%, a 6.3% gain (Fig. 4.14). While this gain does help to bring the theoretical ethanol conversion rate to values seen in other fuel alcohol fermentations, it does not fully explain the reduction in theoretical ethanol conversion in the MCCF as compared to the  $> 90\%$  theoretical ethanol conversion rates seen in other fuel ethanol fermentations.

#### **4.2.8 Changes in pH during fermentation.**

Figure 4.24 illustrates the steady state pH values in individual fermentors set with medium reservoir glucose concentrations from 152 to 312 g/L. As expected, the pH decreased extensively in F1 and F2 in the MCCF. The initial MR showed an average pH of 4.69. The pH dropped by at least one pH unit by F1 in all conditions (where an average pH of 3.59 was seen). The pH continued to decrease in each fermentor until F3 reaching an average minimum pH of 3.28, and then increased gradually to an average of 3.32 and 3.40 in F4 and F5. The pH profiles for most of the conditions in the MCCF were similar in spite of the fact that the media used were not extensively buffered. In general, as the concentration of glucose in the MR was increased (which necessitated the dilution rate to be decreased), the steady state pH decreased in each fermentor for each



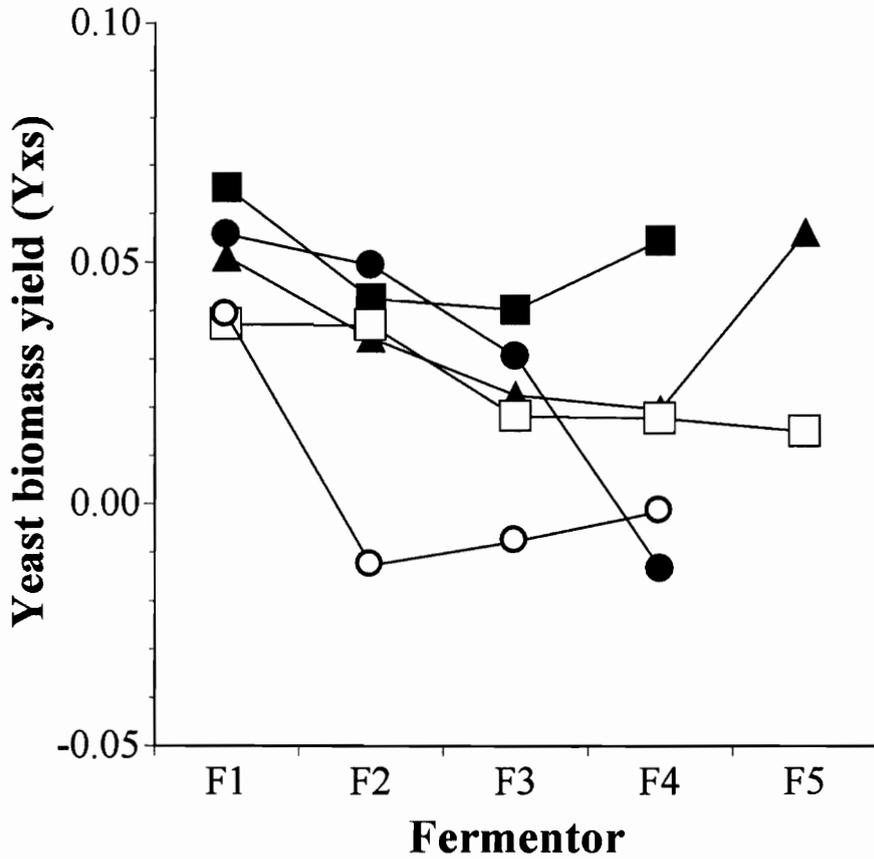
**Figure 4.24** Steady state pH in each fermentor in the multistage continuous culture system with the medium reservoir containing 152 to 312 g/L glucose. (■, 152 g/L glucose ( $D = 0.34$ , Flow = 15.87 ml/min); ●, 191 g/L glucose ( $D = 0.21$ , Flow = 9.80 ml/min); ▲, 225 g/L glucose ( $D = 0.16$ , Flow = 7.47 ml/min); □, 254 g/L glucose ( $D = 0.12$ , Flow = 5.60 ml/min); ○, 312 g/L glucose ( $D = 0.05$ , Flow = 2.33 ml/min); MR = Medium Reservoir; F1-F5 = fermentors 1-5 in the MCCF system).

condition. The low pH ( $< 4.0$ ) in the MCCF is an advantage in that the yeast are able to multiply and ferment at this low pH where most other organisms would be inhibited. Thus, the MCCF as it is operated is “naturally” antimicrobial due to the low pH (Magnus *et al.*, 1986).

#### **4.2.9 Yields.**

##### **4.2.9.1 Biomass yield ( $Y_{xs}$ ).**

Figure 4.25 illustrates the individual fermentor steady state  $Y_{xs}$  (yield of biomass, defined as the g biomass produced per g substrate consumed) with MR glucose concentrations set from 152 to 312 g/L.  $Y_{xs}$  is a measure of how much substrate is diverted to biomass formation. Typical fermentations with glucose show a  $Y_{xs}$  value of approximately 0.05 g/g ( $\sim 5\%$  of the weight of glucose is used for biomass formation). This is a typical figure for anaerobic yeast fermentations. It is desirable to have  $Y_{xs}$  as low as possible in fuel alcohol fermentations to reduce the amount diverted towards biomass. However, yeast growth and carbohydrate flux through the glycolytic pathway to ethanol are coupled. The yeast makes ATP through glycolysis and growth of yeast is proportional to and limited by ATP generated. The  $Y_{xs}$  in the MCCF in general decreased from an average in F1 of 0.0497 g/g to an average in F3 of 0.0279 g/g with the exception when the medium contained glucose at 312 g/L.  $Y_{xs}$  may decrease due to a decrease in yeast multiplication rate and/or the consumption of glucose by non-multiplying cells. From F3, some of the  $Y_{xs}$  values continued to decrease while some increased to F5. The  $Y_{xs}$  values for the 312 g/L condition from F2-F4, and the  $Y_{xs}$  values in F4 for the 191 g/L condition, were negative values. The reason for this was that biomass in these conditions decreased in the MCCF and thus the loss in biomass concentration resulted in negative values. Overall, the range of positive  $Y_{xs}$  values agrees with the 0.05 g/g value seen in the literature with other glucose fermentations performed at higher substrate concentrations. However, the  $Y_{xs}$  values in Figure 4.25 did



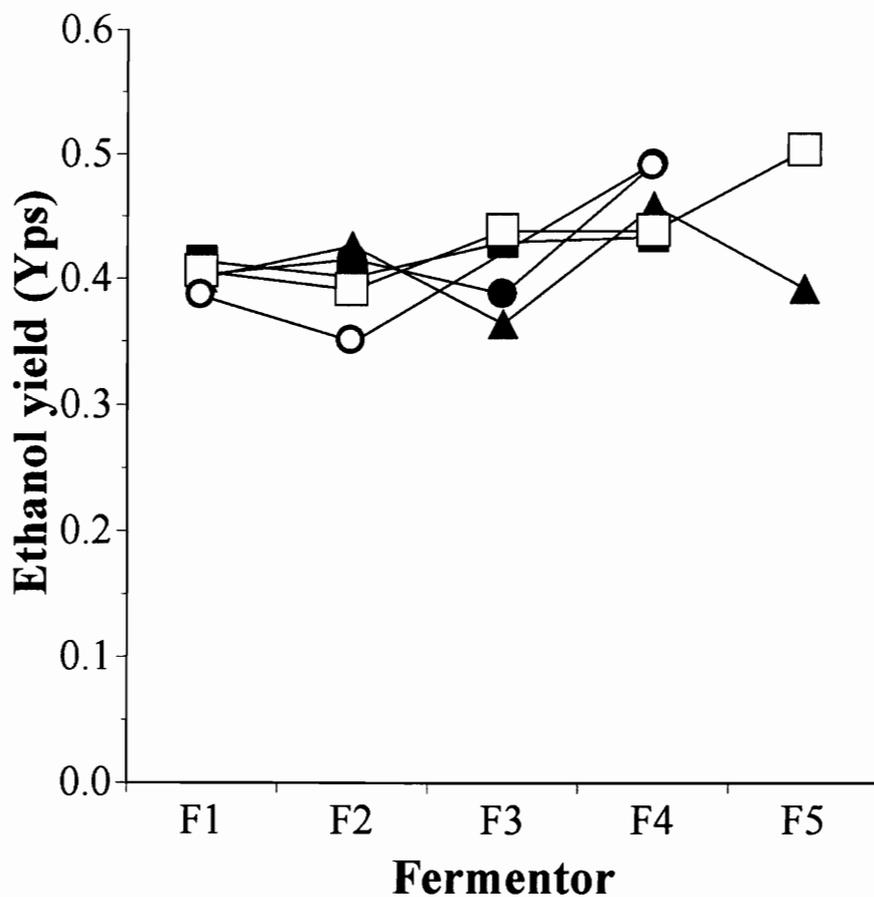
**Figure 4.25** Steady state  $Y_{xs}$  (g biomass formed per g glucose consumed) in each fermentor in the multistage continuous culture system with the medium reservoir (MR) containing 152 to 312 g/L glucose (■, 152 g/L glucose ( $D = 0.34$ , Flow = 15.87 ml/min); ●, 191 g/L glucose ( $D = 0.21$ , Flow = 9.80 ml/min); ▲, 225 g/L glucose ( $D = 0.16$ , Flow = 7.47 ml/min); □, 254 g/L glucose ( $D = 0.12$ , Flow = 5.60 ml/min); ○, 312 g/L glucose ( $D = 0.05$ , Flow = 2.33 ml/min); F1-F5 = fermentors 1-5 in the MCCF system).

change for all conditions which indicates that a single value for  $Y_{xs}$  cannot be used in mathematical modelling as is assumed by most modeling engineers when using unstructured models.

A changing  $Y_{xs}$  has been reported previously in the literature. Thatipamala *et al.* (1992) observed that  $Y_{xs}$  decreased from  $\sim 0.144$  to  $0.056$  g/g when the initial substrate (glucose) concentration in the medium reservoir was increased from  $\sim 10$  g/L to 280 g/L in batch fermentations. In other work,  $Y_{xs}$  decreased from 0.45 to 0.2 g/g in a single-stage continuous system when the dilution rate was increased from 0.025 to  $0.4$  h<sup>-1</sup> during the course of the experiment (van Hoek *et al.*, 1998). In a three-stage, single feed, serial continuous system with different working volumes in each fermentor,  $Y_{xs}$  decreased from 0.06 g/g in the first stage to 0.03 g/g in the second stage and increased to 0.11 g/g in the third stage when a flowrate of 58 ml/h was used with a 26 g/L concentration of glucose in the medium reservoir (Wall and Hill, 1992). These data, together with the data from the present MCCF experiments, clearly demonstrate the need to experimentally determine  $Y_{xs}$  on an experiment by experiment basis in order to accurately determine correct values.

#### 4.2.9.2 Product yield ( $Y_{ps}$ ).

Figure 4.26 illustrates the individual fermentor steady state  $Y_{ps}$  (yield of product, defined as the g product produced per g substrate consumed) with medium reservoir glucose concentrations set from 152 to 312 g/L.  $Y_{ps}$  is a measure of how much substrate is converted to product (ethanol). A high value is desirable. Typical fermentations with glucose show a  $Y_{ps}$  of approximately 0.3-0.5 (30-50% of the weight of glucose used for product formation). Some of the  $Y_{ps}$  values calculated from the MCCF data where little metabolism was in progress gave negative results (presumably due to ethanol consumption in some fermentors) and, as a result, such values were not included in Figure 4.26. With the exception of the 312 g/L condition, the  $Y_{ps}$  in the MCCF remained constant at an average of 0.406 g/g from F1 to F3, and then increased



**Figure 4.26** Steady state  $Y_{ps}$  (g ethanol formed per g glucose consumed) in each fermentor in the multistage continuous culture system with the medium reservoir containing 152 to 312 g/L glucose (■, 152 g/L glucose ( $D = 0.34$ , Flow = 15.87 ml/min); ●, 191 g/L glucose ( $D = 0.21$ , Flow = 9.80 ml/min); ▲, 225 g/L glucose ( $D = 0.16$ , Flow = 7.47 ml/min); □, 254 g/L glucose ( $D = 0.12$ , Flow = 5.60 ml/min); ○, 312 g/L glucose ( $D = 0.05$ , Flow = 2.33 ml/min); F1-F5 = fermentors 1-5 in the MCCF system).

to an average of 0.451 g/g in F4 to F5. Thus, the yield of ethanol increased slightly in the later fermentors in the MCCF. Roughly 40% of the weight of glucose was accounted for as ethanol in F1-F3, while 45% was converted in F4-F5. Some of the glucose not converted to ethanol and CO<sub>2</sub> is used by the yeast for maintenance purposes and much is diverted to biomass production and alternate by-product formation (especially glycerol). The increase in Y<sub>ps</sub> in the MCCF results from the fact that the yeasts are producing ethanol in all the fermentors in the MCCF but most growth occurs in F1; no net increase in yeast numbers occurs in F2 to F5 (see Figures 4.18 and 4.20). Thus, one would expect that the ethanol yield will increase in the latter fermentors since glucose would not be needed for an increase in cell multiplication. This finding had two important consequences. First, ethanol is more effectively produced from glucose in the later stages (F2-F5) in the MCCF where cell growth is minimized (cell growth occurring at a rate balancing cell death, maintaining overall yeast numbers) even though the rate of ethanol production is higher with actively growing cells which are there in smaller numbers. And second, in chemical engineering circles, the notion that ethanol productivity (Y<sub>ps</sub>) is a constant in fermentation experiments (and/or mathematical modelling) is not correct. As with Y<sub>xs</sub>, Y<sub>ps</sub> must be experimentally derived on an experiment by experiment basis. Changes in Y<sub>ps</sub> have been documented in the literature; many of the references cited in the previous section present evidence for changes in Y<sub>xs</sub>.

#### **4.3 Introduction of *L. paracasei* into an MCCF equilibrated with *S. cerevisiae*.**

The fermentation characteristics of an MCCF fermentation were clearly established in the previous section. In this series of experiments, *L. paracasei* was introduced into an MCCF that had first been allowed to reach steady state with yeast (after 5-7 days of operation). The growth of both organisms as well as other fermentation parameters were then tracked for up to 25 additional days.

The great length of time involved in a typical MCCF contamination experiment (as well as the amount of time for preparation) meant that not all glucose concentrations

that were used in the previous section could be run in the contamination studies. In the MCCF experiments, as the glucose concentration in the MR was increased, the length of time required for the yeast to reach steady state increased greatly. The decreased dilution rate at these higher glucose concentrations also increased the residency time in the fermentors. A common practice in continuous culture is to allow ten volume displacements during an experiment before the culture is considered to be in steady state (with confirmation by sampling at this point). If the 312 g/L condition had been chosen for future experiments, ten volume displacements would require 200 h or 8.3 days of further operation under these conditions before the yeast would be considered to be in steady state (confirmed by sampling). All the above considerations pointed towards using a lower MR glucose concentration to try to reduce the amount of time in each experiment. At the same time however, a high glucose concentration in the MR was desired for all contamination conditions as the work has relevance to VHG technology. In addition, a higher concentration of glucose would lead to lower medium use since the dilution rate is lower under conditions of higher concentrations of glucose. A compromise for all these considerations was to run the experiments at a MR glucose concentration of 260 g/L where only four days operation would be needed to accomplish ten volume displacements and this concentration is close to VHG levels.

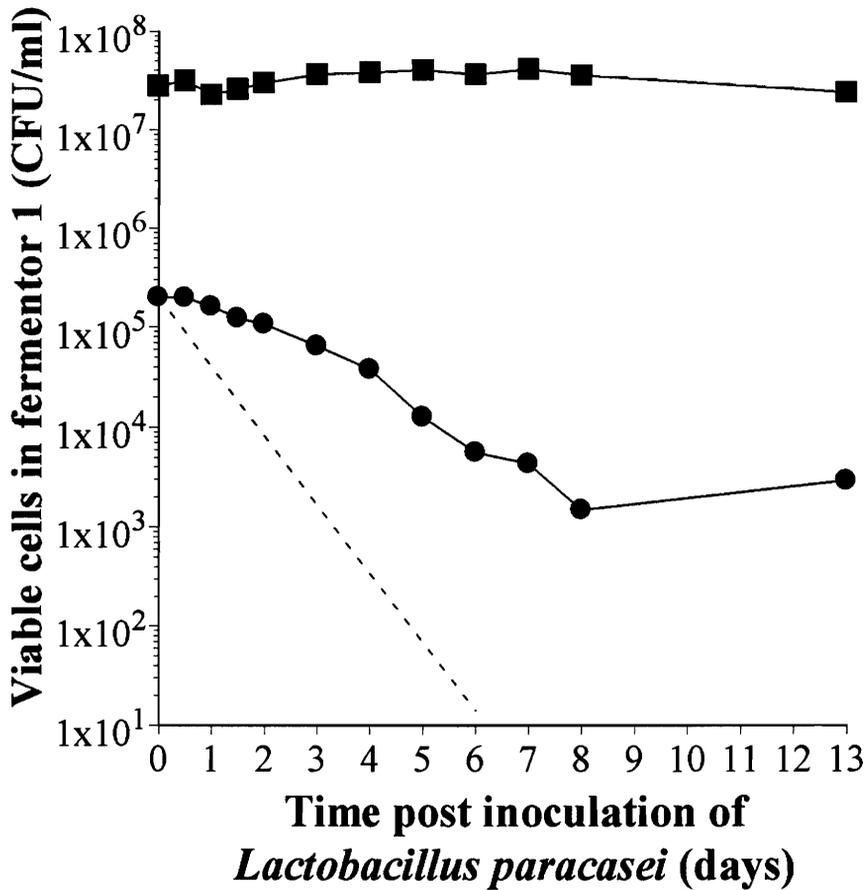
*L. paracasei* was chosen as the contaminant in this project for a number of reasons. First, in the fuel alcohol industry, *Lactobacillus* spp. are the most commonly found bacterial contaminants and they are a continual and persistent problem (Makanjuola and Springham, 1984). The *Lactobacillus* strain used in these experiments was isolated from a fuel alcohol plant and it grows extremely rapidly. MRS plates with “normal” brewing and distillery lactobacilli require up to a week or more to produce colonies large enough to count. This is in contrast to only 24 hours required by this *Lactobacillus*. Some extremely rapid growing *Lactobacillus* strains in grape juice have been termed “ferocious” lactobacilli (Alexandre and Charpentier, 1998). *L. paracasei* is also homofermentative which leads to more efficient production of metabolic energy (and more lactic acid) than produced by a heterofermentative *Lactobacillus*. Thus, this “worst

case”, industrially relevant organism was chosen as the contaminant for this work. The growth of *L. paracasei* and increases in metabolic endproducts were followed only in the first fermentor (F1) of a multistage continuous culture fermentation (MCCF) system. The first fermentor in this system provides the best opportunity for *L. paracasei* to compete with yeast since nutrients are more abundant, ethanol concentrations are at their lowest levels, and pH is highest. *L. paracasei* produced in F1 would continue to produce lactic acid and scavenge trace nutrients if viability was maintained in later fermentors in the MCCF.

#### 4.3.1 *L. paracasei* : *S. cerevisiae* at 1:100 and 1:1 inoculation ratios.

Figure 4.27 illustrates the viability of *S. cerevisiae* and *L. paracasei* (CFU/ml) in Fermentor 1 (F1) of the MCCF operated at a dilution rate of  $0.066 \text{ hr}^{-1}$  (corresponding to  $D = 0.12 \text{ h}^{-1}$  in F2), at a 260 g/L MR glucose concentration, and at 28°C and 100 RPM agitation. In particular, it was of interest to see which microorganism would prevail and what population dynamics would result from competition and inhibition. According to classical continuous culture theory, when many different organisms are present in a continuous culture, only one organism will prevail as the dominant organism because of a combination of advantages afforded to it by cultural conditions, medium composition, growth rate, and resistance to growth inhibitors. 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.

To create the data in Figure 4.27, the MCCF system was run for eight days to allow *S. cerevisiae* to reach steady state. *L. paracasei* was then inoculated into F1 at a 1:100 ratio of *L. paracasei* : *S. cerevisiae* and tracked over time. Surprisingly, viable numbers of *L. paracasei* decreased after the time of inoculation. This decrease could not be due to non-permissive growth conditions in the MCCF. The theoretical washout line plotted in Figure 4.27 was calculated based on the initial *L. paracasei* inoculation level,



**Figure 4.27** Viabilities (CFU/ml) of *S. cerevisiae* and *L. paracasei* inoculated into F1 in the multistage continuous culture fermentation (MCCF) system with a medium reservoir containing 260 g/L 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 if no growth occurs).

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 levels of *L. paracasei* would increase and not decrease in the MCCF, especially since (under optimal conditions) lactobacilli have faster specific growth rates than yeasts. Clearly either the inoculum of *L. paracasei* was not sufficient to compete on an equal (or superior) basis with *S. cerevisiae*, the environmental factors (e.g. ethanol from yeast, temp, pH, and/or dilution rate) did not permit rapid growth of *L. paracasei*, or the medium was nutritionally deficient for *L. paracasei*. It was not possible from this experiment to deduce which combination of these factors or which single factor was responsible for the reduction of *L. paracasei*.

Although viable *L. paracasei* decreased in Figure 4.27, the level of viable *S. cerevisiae* remained at its steady state value throughout the experiment (the same steady state CFU/ml value obtained in Figure 4.18). Thus, neither the growth of *L. paracasei* to the cell number indicated, nor any metabolic by-products of its growth, influenced *S. cerevisiae* in this experiment. The rather small numbers of viable *L. paracasei* were not sufficient to compete with the yeast for trace nutrients and they were unable to produce enough metabolic by-products to inhibit the yeast.

Another finding in Figure 4.27 was that *L. paracasei* appeared to stabilize at a steady-state value of approximately  $3 \times 10^3$  CFU/ml. *L. paracasei* and *S. cerevisiae* coexisted in the MCCF at different steady-state cell populations (at an approximate 1:10,000 ratio of *L. paracasei* : *S. cerevisiae*). These results suggest that the MCCF system is capable of supporting the coexistence of both microorganisms at different steady state values.

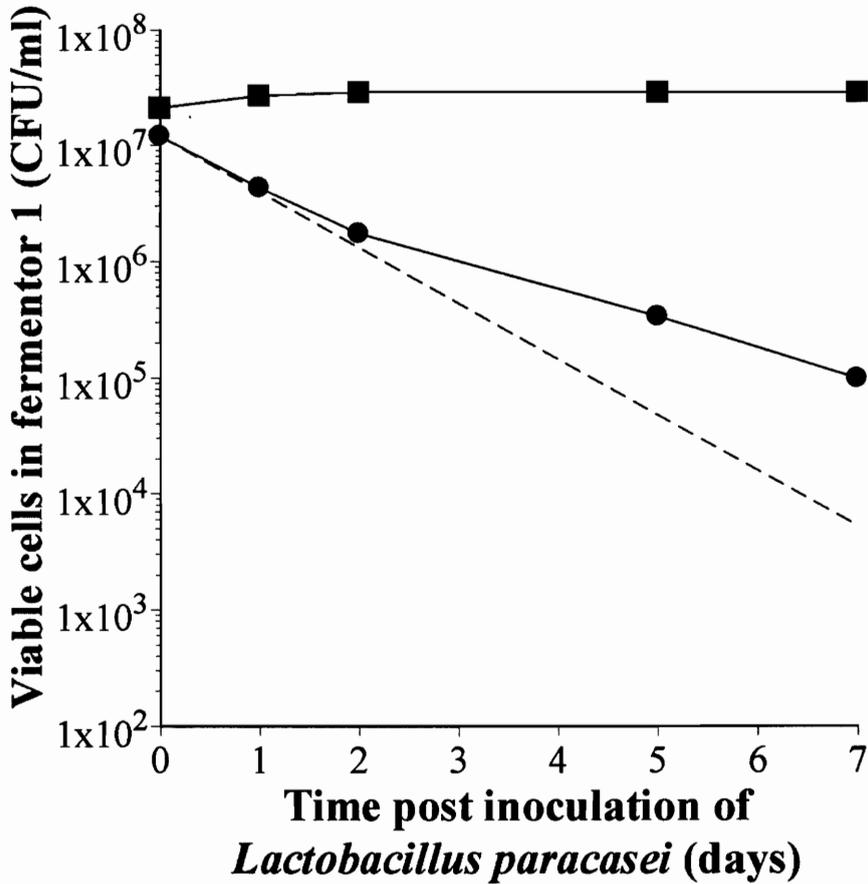
In order to determine if the inoculation level of a contaminant can influence the steady state production parameters, the inoculation ratio of *L. paracasei* was increased to

1:1. The results in Figure 4.28 showed that viable *L. paracasei* again decreased from the time of inoculation while the yeast maintained steady state numbers. The unchanging steady state values of *S. cerevisiae* in Figures 4.27 and 4.28 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. A rapid dominance of the MCCF by a contaminating *Lactobacillus* (which plagues continuous fuel alcohol production) was not observed in these experiments. As well, the fact that the *L. paracasei* in both Figures 4.27 and 4.28 decreased with time and reached a steady state cell number indicates that the MCCF is only capable of supporting *L. paracasei* at a lower (non yeast inhibitory) steady state level as compared to *S. cerevisiae*.

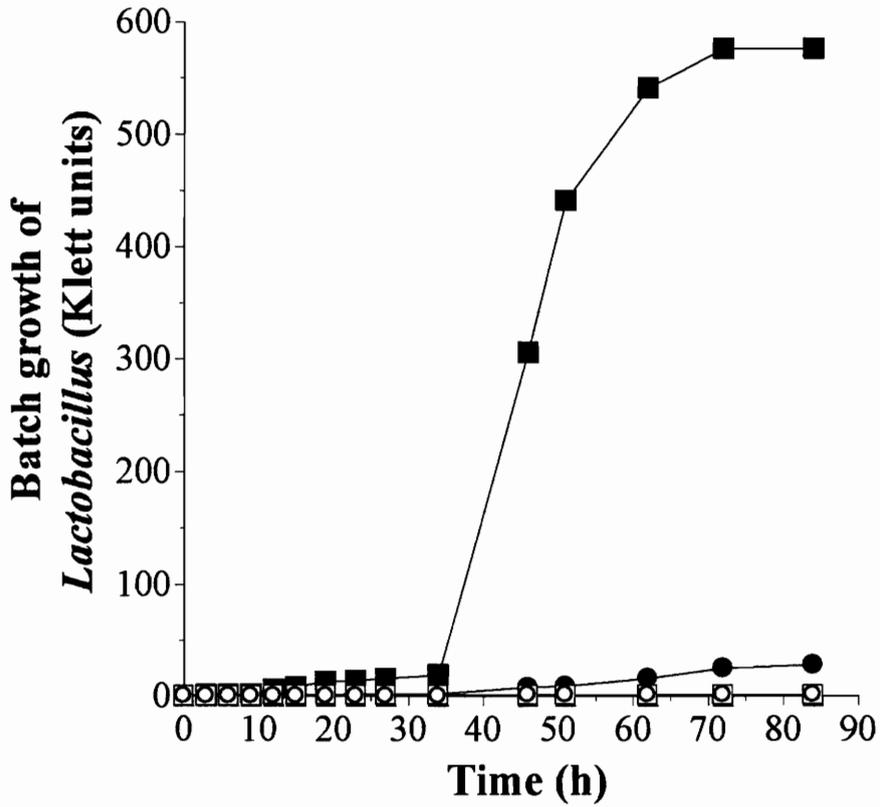
From a production perspective, the data showed that, under optimal operating conditions for fuel alcohol production with an incoming glucose feed concentration of 260 g/L and pH of 3.3 in the fermentor, the MCCF was capable of reducing the load of a contaminant (*L. paracasei*) while maintaining the 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 (Brodl, 1992), in VHG brewing (Magnus *et al.*, 1986), and in laboratory experiments (Oliva-Neto and Yokoya, 1994).

#### **4.3.2 Batch growth of *L. paracasei* in clarified MCCF media.**

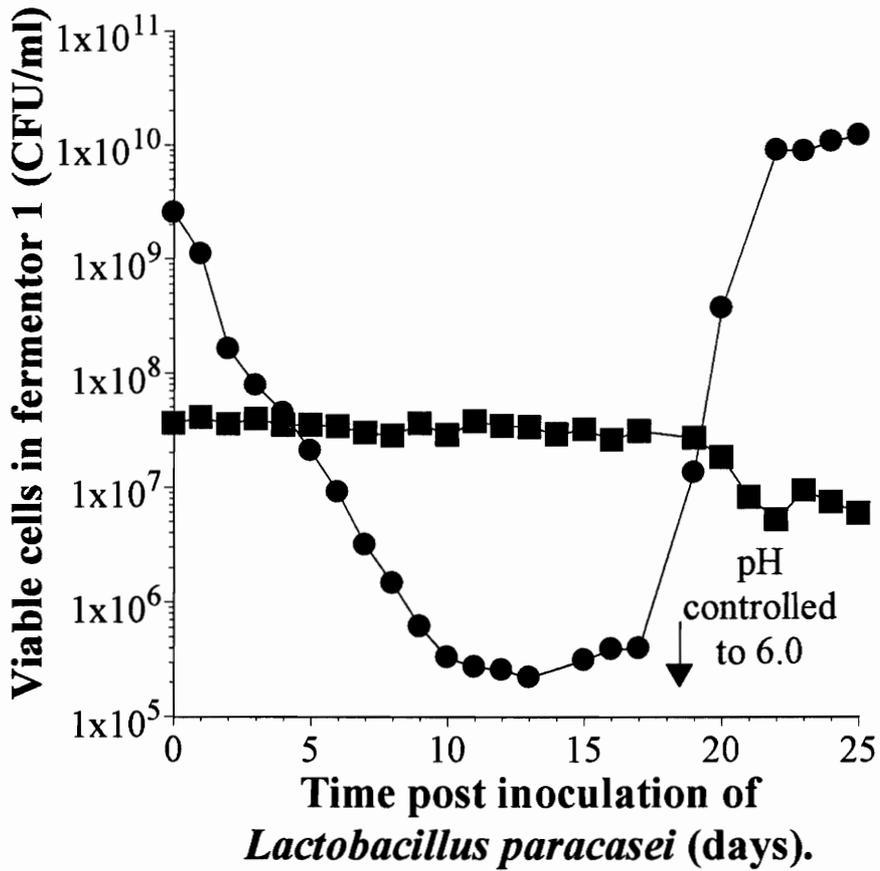
As mentioned previously, one possible reason why *L. paracasei* did not grow well in the MCCF was that the medium used was in some way nutritionally deficient for *L. paracasei*. To test this possibility, the medium and fermentor samples from an earlier MCCF run (without introduction of *L. paracasei*) were filtered to remove *S. cerevisiae* and any particulate materials present in the MCCF medium. *L. paracasei* was then inoculated into each clarified fermentor “medium”, flushed with CO<sub>2</sub>, and its growth under batch conditions was followed. The results of the batch growth tests are presented



**Figure 4.28** Viabilities (CFU/ml) of *S. cerevisiae* and *L. paracasei* inoculated into F1 in the MCCF system with the medium reservoir containing 260 g/L 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 if no growth occurs).



**Figure 4.29** Batch growth of *L. paracasei* in clarified media obtained from a *S. cerevisiae*-equilibrated MCCF system with the medium reservoir containing 260 g/L glucose. Fermentor contents (F1-F5) in the MCCF were collected and filtered to produce the corresponding clarified medium prior to bacterial inoculation (■, Clarified medium reservoir; ●, Clarified F1; ▲, Clarified F2; ◆, Clarified F3; □, Clarified F4; ○, Clarified F5).



**Figure 4.30** Viabilities (CFU/ml) of *S. cerevisiae* and *L. paracasei* inoculated into F1 in the MCCF system with medium containing 260 g/L 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*)

*paracasei* entered a steady state which was previously suggested by the data in Figure 4.27. This steady state was confirmed in Figure 4.30 by tracking the viable number of *L. paracasei* (average  $3.7 \times 10^5$  CFU/ml) for an additional seven days. At steady state for both organisms, the ratio of *S. cerevisiae* to *L. paracasei* was 100:1.

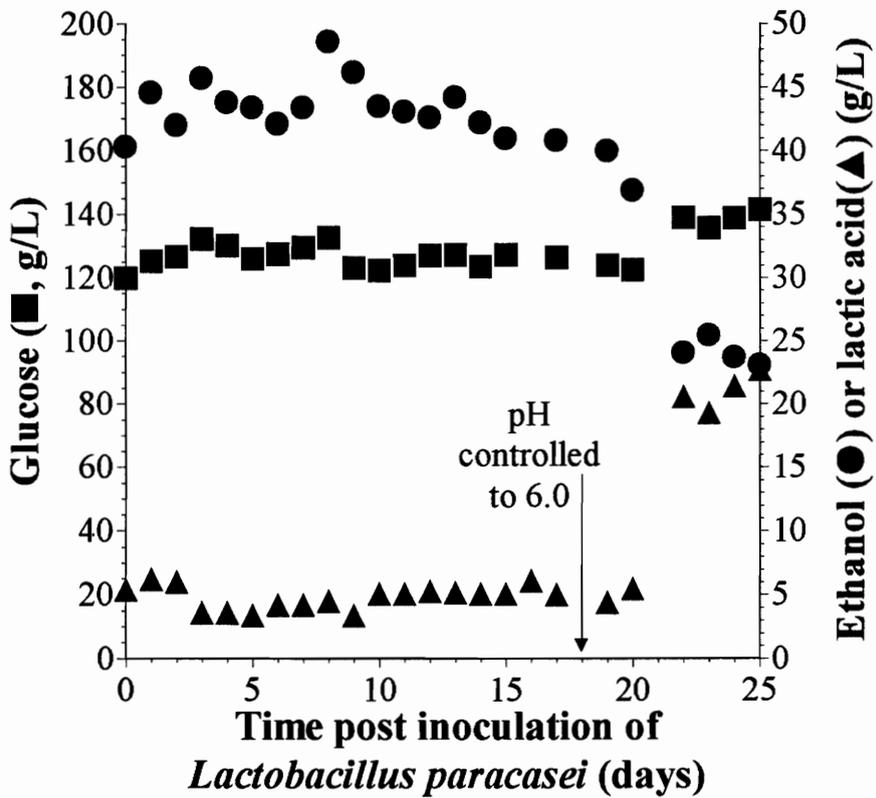
From a production point of view, the fact that the two organisms can exist in two steady states means that one might never be able to eliminate a contaminant from a continuous culture. A contaminant may exist in an MCCF even if production parameters (e.g. growth of *S. cerevisiae*, ethanol production rate) show “normal” values. The lower and stable steady state bacterial contaminant level would be a “ticking time bomb” for fuel alcohol producers. If aberrations in pH, temperature, or other process parameters were to change even intermittently, the contaminant might suddenly increase in number and cause problems.

In Figure 4.30, pH control in the MCCF was activated and set to pH 6.0 at the 7<sup>th</sup> day of bacterial steady state (day 18) in order to determine if the normal pH in F1 of 3.2 contributed to the low steady state viable numbers of *L. paracasei*. Within three days after starting pH control, *L. paracasei* increased from its steady state value of  $3.7 \times 10^5$  to approximately  $1 \times 10^{10}$  CFU/ml - a 4.4 log increase! Competition by *S. cerevisiae* and inhibition by ethanol did not appear to inhibit the growth of *L. paracasei* from its 100 fold lower steady state value. One can therefore conclude from Figure 4.30 that the most likely factor preventing *L. paracasei* from achieving a high steady state value of viable cells in the MCCF was the pH. *L. paracasei* entered steady state at approximately  $1 \times 10^{10}$  CFU/ml after three days of pH control at 6.0. *S. cerevisiae* remained in steady state throughout the experiment ( $\sim 3 \times 10^7$  CFU/ml) up until the time when pH control was activated. To that point, its growth had not been affected by the introduction of a high initial level of *L. paracasei*. Only when *L. paracasei* increased to its new steady state cell value (pH control activated) did the viability of yeast begin to 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.

The results in Figure 4.30 also show that high numbers of a contaminant in continuous culture does not necessarily indicate that the contaminant will cause problems during the fermentation. The viable numbers of *L. paracasei* added at the time of inoculation and viability at steady state after pH control was activated were nearly the same (at most ~2x more at the new steady state) yet, at the time of inoculation, the viable numbers of yeast (Fig. 4.30) and the glucose and ethanol concentrations (discussed in the next section) maintained steady states. The advantage of continuous culture over batch methods is that if the conditions present in continuous culture are inhibitory for the contaminant the contaminant will wash out or be reduced in numbers. In contrast in batch culture, all *L. paracasei* that are introduced into the system remain. Theoretically, even if the growth of *L. paracasei* is inhibited in both systems, the inhibition of yeast growth and ethanol production by *L. paracasei* is potentially higher in batch since a greater number of *L. paracasei* can produce (albeit at a low level of production) lactic acid and consume more trace nutrients than in the case with continuous culture.

#### **4.3.3.2 Glucose, ethanol, lactic acid.**

Figure 4.31 illustrates the corresponding glucose concentrations in F1 over the course of the 70:1 (bacterium:yeast) mixed culture experiment. Glucose remained relatively constant at 125 g/L from the time when *L. paracasei* was inoculated to the time when pH control was activated. However, after pH control was activated, the glucose concentration increased from its steady state value of approximately 125 g/L to 140 g/L as *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. It is not known at this time why the glucose consumption decreased at a time when the combined biomass of both organisms in F1 was at a peak. Most likely, since yeast are approximately 50x larger than bacteria, the rate of



**Figure 4.31** Glucose, ethanol, and lactic acid concentrations in a mixed culture of *S. cerevisiae* and *L. paracasei* in the first fermentor in the MCCF system with the medium reservoir containing 260 g/L glucose. *S. cerevisiae* was equilibrated for five days before inoculation of *L. paracasei* at a 70:1 ratio of *L. paracasei* to *S. cerevisiae* (■, Glucose; ●, Ethanol; ▲, Lactic acid).

consumption of glucose by this number of yeast is much higher than the rate of conversion of glucose to lactic acid catalyzed by the smaller 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 the lowered consumption by growth-inhibited *S. cerevisiae*.

Ethanol concentration over the course of the mixed culture experiment in F1 in the MCCF is also depicted in Figure 4.31. 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 their steady state, the ethanol concentration fell in F1 to 25 g/L - a 44% decrease. This represents a 50% decrease in ethanol concentration due to the presence of high numbers of *L. paracasei*.

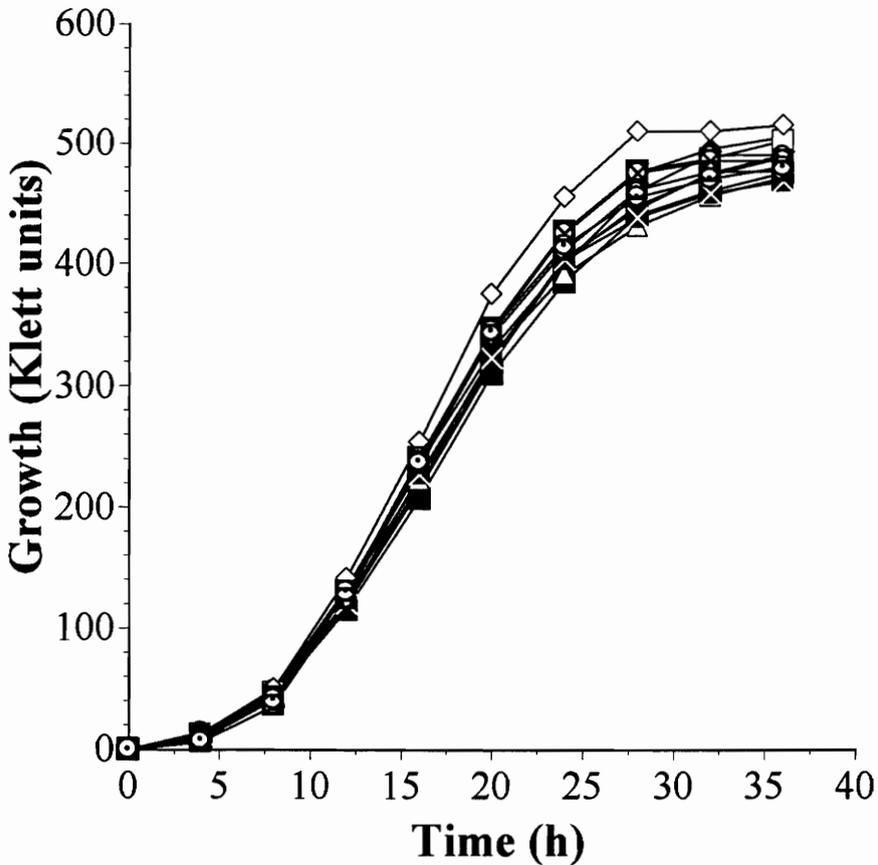
Lactic acid levels in the mixed culture experiment are depicted in Figure 4.31. As seen for glucose and ethanol, the lactic acid concentrations generally remained between 3 and 6 g/L when *S. cerevisiae* was in steady state. The corn steep powder used in the medium formulation contributed some lactic acid (~4.1 g/L) as shown in Figure 4.31. 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 to at least 20 g/L once the pH was controlled at 6.0. This 4 to 5 fold increase is attributable entirely to the corresponding increase in *L. paracasei* to greater than  $1 \times 10^{10}$  cells/ml when the pH was changed to 6.0. Of concern at this point was the reason for the decrease in *S. cerevisiae* when *L. paracasei* reached its new steady state value after pH control was activated. The three likely reasons for the decrease in *S. cerevisiae* are the high amount of lactic acid formed (20 g/L) by *L. paracasei* at the new steady state, the possibility that *L. paracasei* is now able to compete effectively with *S. cerevisiae* for critical nutrients in F1, and/or the effect of pH at 6.0 on the growth of *S. cerevisiae*. Recent work by Narendranath *et al.* (2001b) explains the negative effects of lactic acid and why it affects yeast growth.

#### 4.3.4 Batch growth in pH-controlled clarified F1 medium.

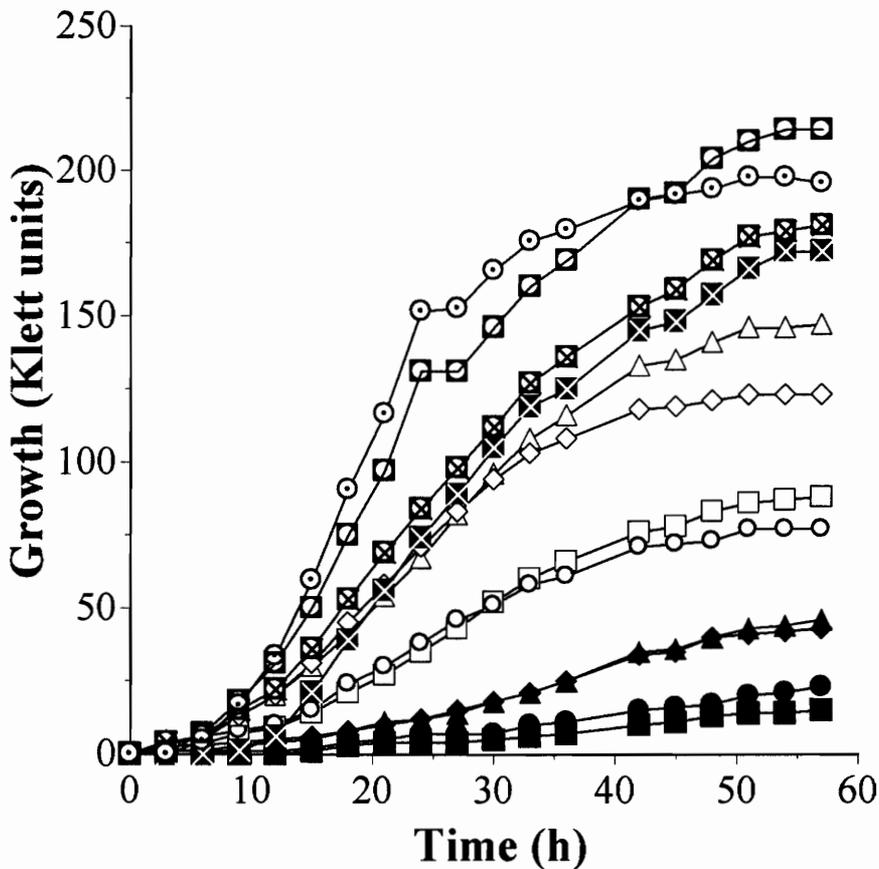
##### 4.3.4.1 Batch growth curves.

The results in Figure 4.30 indicated that the pH in F1 in the MCCF was the cause for the low steady state viable numbers of *L. paracasei* when introduced into the system. To further examine the effect of pH on *S. cerevisiae* and *L. paracasei* in F1, the pH of clarified media from F1 from an earlier MCCF run (partially spent in nutrients due to previous fermentation) was adjusted in the range of 3.5 to 6.0 with  $\text{NH}_4\text{OH}$  or  $\text{KOH}$ . *S. cerevisiae* or *L. paracasei* were introduced and the growth was followed in batch.

*S. cerevisiae* (Fig. 4.32) and *L. paracasei* (Fig. 4.33) were inoculated separately into identical flasks spanning the pH range of 3.5 to 6.0 (adjusted with  $\text{NH}_4\text{OH}$  or  $\text{KOH}$ ) and their batch growth followed over time. For *S. cerevisiae*, all the growth profiles were nearly identical in shape and placement. This indicated that *S. cerevisiae* was capable of growing at almost the same rate and to the same extent over a range of pH from 3.5 to 6.0. Thus, pH does not affect the growth of *S. cerevisiae* in batch and rules out the possibility that the pH of 6.0 in Figure 4.30 was responsible for the decrease in *S. cerevisiae*. The results also show that in the MCCF, changes in the medium pH due to batch to batch variation would not affect growth and ethanol production by *S. cerevisiae*. The fact that *S. cerevisiae* was able to grow under each condition in Figure 4.32 in the clarified, partially spent medium (where significant growth of *S. cerevisiae* had already occurred) indicated that the medium formulation used for the MCCF system was not growth limiting. With *L. paracasei*, the outcome was different (Fig. 4.33). The growth curves differed with changes in pH. Very little growth was seen at pH 3.5, a value slightly higher than pH values seen in most fermentors in a normal or contaminated MCCF system where typical pH values are: Medium Reservoir, 4.75; F1, 3.54; F2, 3.30; F3, 3.29; F4, 3.34; F5, 3.39. Rapid growth of *L. paracasei* however was seen over the pH range of 5.0 to 6.0. Clearly, the growth of *L. paracasei* in F1 in the MCCF was extensively affected by low pH ( $< 3.4$ ) maintained by the yeast in the chosen medium



**Figure 4.32** Batch growth of *S. cerevisiae* in clarified F1 media obtained from a *S. cerevisiae*-equilibrated MCCF system with the medium reservoir containing 260 g/L glucose. The pH of clarified F1 media was subsequently adjusted with either KOH or NH<sub>4</sub>OH prior to re-inoculation with *S. cerevisiae* (■, pH 3.5 (KOH); ●, pH 3.5 (NH<sub>4</sub>OH); ▲, pH 4.0 (KOH); ◆, pH 4.0 (NH<sub>4</sub>OH); □, pH 4.5 (KOH); ○, pH 4.5 (NH<sub>4</sub>OH); △, pH 5.0 (KOH); ◇, pH 5.0 (NH<sub>4</sub>OH); ⊠, pH 5.5 (KOH); ⊞, pH 5.5 (NH<sub>4</sub>OH); ⊙, pH 6.0 (KOH); ⊚, pH 6.0 (NH<sub>4</sub>OH)).



**Figure 4.33** Batch growth of *L. paracasei* in clarified F1 media obtained from a *S. cerevisiae*-equilibrated MCCF system with the medium reservoir containing 260 g/L glucose. The pH of clarified F1 media was subsequently adjusted with either KOH or NH<sub>4</sub>OH prior to inoculation with *L. paracasei* (■, pH 3.5 (KOH); ●, pH 3.5 (NH<sub>4</sub>OH); ▲, pH 4.0 (KOH); ◆, pH 4.0 (NH<sub>4</sub>OH); □, pH 4.5 (KOH); ○, pH 4.5 (NH<sub>4</sub>OH); △, pH 5.0 (KOH); ◇, pH 5.0 (NH<sub>4</sub>OH); ⊠, pH 5.5 (KOH); ⊠, pH 5.5 (NH<sub>4</sub>OH); ○, pH 6.0 (KOH); ■, pH 6.0 (NH<sub>4</sub>OH)).

under steady state conditions. The medium formulation was not nutritionally deficient or limiting for *L. paracasei* as shown by good growth at pH values greater than 4.5 despite the fact that growth of *S. cerevisiae* had already occurred with the medium in the MCCF, and that ethanol produced by the yeast in the MCCF was part of the clarified medium.

Another interesting observation in Figures 4.32 and 4.33 was that, in most cases, adjusting the pH with  $\text{NH}_4\text{OH}$  or  $\text{KOH}$  did not make a difference in the growth of either organism at any pH. If pH adjustment would be needed in future MCCF experiments, then the choice of base to use and the method of control would become important. Bases are most often used to adjust pH to higher values in industry are  $\text{NH}_4\text{OH}$ ,  $\text{KOH}$ , and  $\text{NaOH}$ . Buffers are not used.  $\text{NaOH}$  is the least attractive chemical of the three to use in the control of pH since yeast cells may be sensitive to high amounts of  $\text{Na}^+$  ions and salts accumulate in the effluent from the still. The advantages of using  $\text{NH}_4\text{OH}$  to control pH is that it supplements yeast with utilizable nitrogen. As ammonium ion is taken up by the yeast, less residual salts is left to inhibit yeast or create effluent problems. A primary disadvantage is the volatile nature of  $\text{NH}_4\text{OH}$  which creates safety concerns for workers. Potassium hydroxide is preferred over  $\text{NaOH}$  for toxicity reasons ( $\text{K}^+$  are not as toxic as  $\text{Na}^+$  ions to yeast) and it has no volatility. However, the addition of  $\text{KOH}$  does lead to the accumulation of  $\text{K}^+$  ions in the fermentation. The methods of adding these chemicals to fermentations to control pH includes direct addition of the chemical to the medium reservoir, or dosing of chemical into the fermentor (in-line control) as needed. In-line control has the advantages that tight control of a desired pH value can be accomplished during the fermentation and the desired pH value can be changed during the fermentation. However, additional equipment is necessary to control the pH. Direct addition of chemical in or before cooking, or prior to glucoamylase addition is a very attractive method for industry since a one time addition of chemical could be performed. However, it would be difficult to determine the amount of chemical to be added to a tank to provide a desired pH value during fermentation at steady state operation. In addition, medium is almost always made continuously, and it would be difficult to change the pH in the fermentors during the course of fermentation. Any additions of chemicals to a holding

tank would require time for thorough mixing. Lastly, as the composition of the medium used may change during the course of the fermentation, the amount of chemical required to maintain a set pH will change - the amount of change being dependent on yeast metabolism and on variations in medium components. Any change in the consistency of the pH obtained during steady state will impact (particularly in continuous culture) the production characteristics of the system. As a result of the numerous disadvantages in adding the pH control chemical to a medium reservoir or holding tank, the in-line approach to controlling pH in individual vessels was used exclusively in this work.

The results in Figures 4.32 and 4.33 clearly show that there is no advantage (other than from a pollution standpoint) in using  $\text{NH}_4\text{OH}$  to control the pH in the MCCF. If the medium formulation had been deficient in nitrogen or if the concentration of  $\text{K}^+$  ions had been inhibitory, there might have been better growth for both organisms when  $\text{NH}_4\text{OH}$  was used to adjust pH. This was not the case here.

#### **4.4 Lactic acid addition into F1 in an MCCF equilibrated with *S. cerevisiae*.**

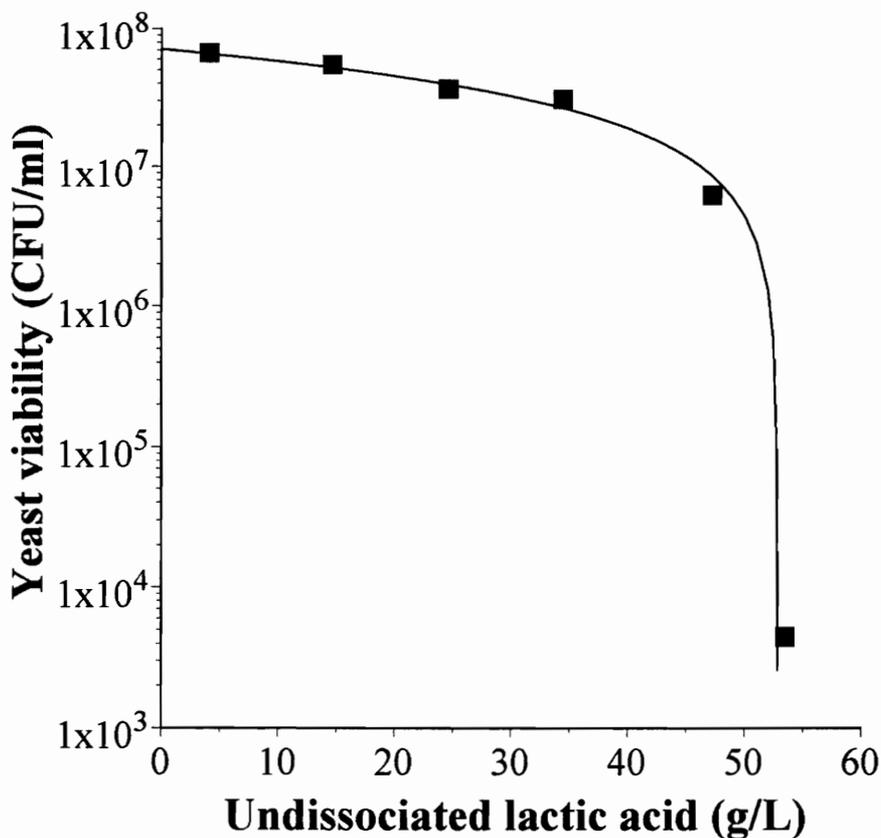
As shown in Figure 4.30 viable *S. cerevisiae* decreased as viable *L. paracasei* increased when pH control was activated. The only possible reason for the decrease in the cell number of *S. cerevisiae* was that the production of lactic acid (20 g/L) by *L. paracasei* was inhibitory to yeast growth or that *L. paracasei* was competitively scavenging trace nutrients away from *S. cerevisiae*.

The prevailing understanding of inhibition of *S. cerevisiae* by organic acids is that inhibition is caused by undissociated acid readily diffusing into cells and subsequently dissociating inside the cells (at its higher pH) generating  $\text{H}^+$  ions that lowers internal pH (Cásio *et al.*, 1987; Capucho and San Romao, 1994; Eklund, 1983; Kashket, 1985; Verduyn *et al.*, 1992; Narendranath *et al.*, 2001a, 2001b). To determine if lactic acid production is the cause for the decrease in viable yeast, each fermentor in the MCCF was provided with its own separate medium feed from the medium reservoir and the effluent from each fermentor was disconnected from the inlet of the following fermentor - setting

up five independent CSTR fermentors each with characteristics of the original F1 fermentor. The medium flowrate was adjusted in each fermentor to give a dilution rate identical to the dilution rate in F1 (F1 in the MCCF has ~2x the volume of any later fermentor). *S. cerevisiae* was inoculated into each F1 fermentor and was allowed a five day equilibration to achieve steady state. The flow of any lactic acid solution to any fermentor was monitored and held constant at 0.5 ml/min which represented an increase in the overall dilution rate of 8.9% ( $D = 0.077 \text{ h}^{-1}$ ) as compared to MCCF experiments without the addition of any non-medium volume ( $D = 0.066 \text{ h}^{-1}$ ). To achieve different steady state lactic acid concentrations in each fermentor, the concentration of lactic acid in each of the sterile lactic acid feed solutions was adjusted - taking into account the combined flows of medium and lactic acid and the level of lactic acid desired in each fermentor. A control fermentor was run with sterile distilled water. All fermentors were then tracked over time to determine in each when steady state was reached with the different concentration of lactic acid used. No lactic acid bacteria were present.

#### 4.4.1 Growth of *S. cerevisiae*.

Figure 4.34 portrays steady state levels of viable *S. cerevisiae* when a range of 14 to 70 g/L of undissociated lactic acid (calculated on the basis of pH) was fed to the individual CSTR fermentors. The viability of *S. cerevisiae* decreased by 53% as compared to the control when the undissociated lactic acid increased to 34.4 g/L in the fermentor. This was remarkable since in many published reports, *S. cerevisiae* has been reported in batch experiments to be completely inhibited by 20 g/L undissociated lactic acid. In this study, *S. cerevisiae* was inhibited by more than one log only when the undissociated lactic acid in the fermentor reached 53.5 g/L. At an undissociated lactic acid concentration of 70 g/L, no viable *S. cerevisiae* were recovered. Thus, with lactic acid at 70 g/L, *S. cerevisiae* washed out. The inhibition of *S. cerevisiae* presented in Figure 4.34 was probably not due to any pH effects (see Section 4.4.2). Based on the shape of the curve in Figure 4.34, total inhibition of *S. cerevisiae* in the MCCF would



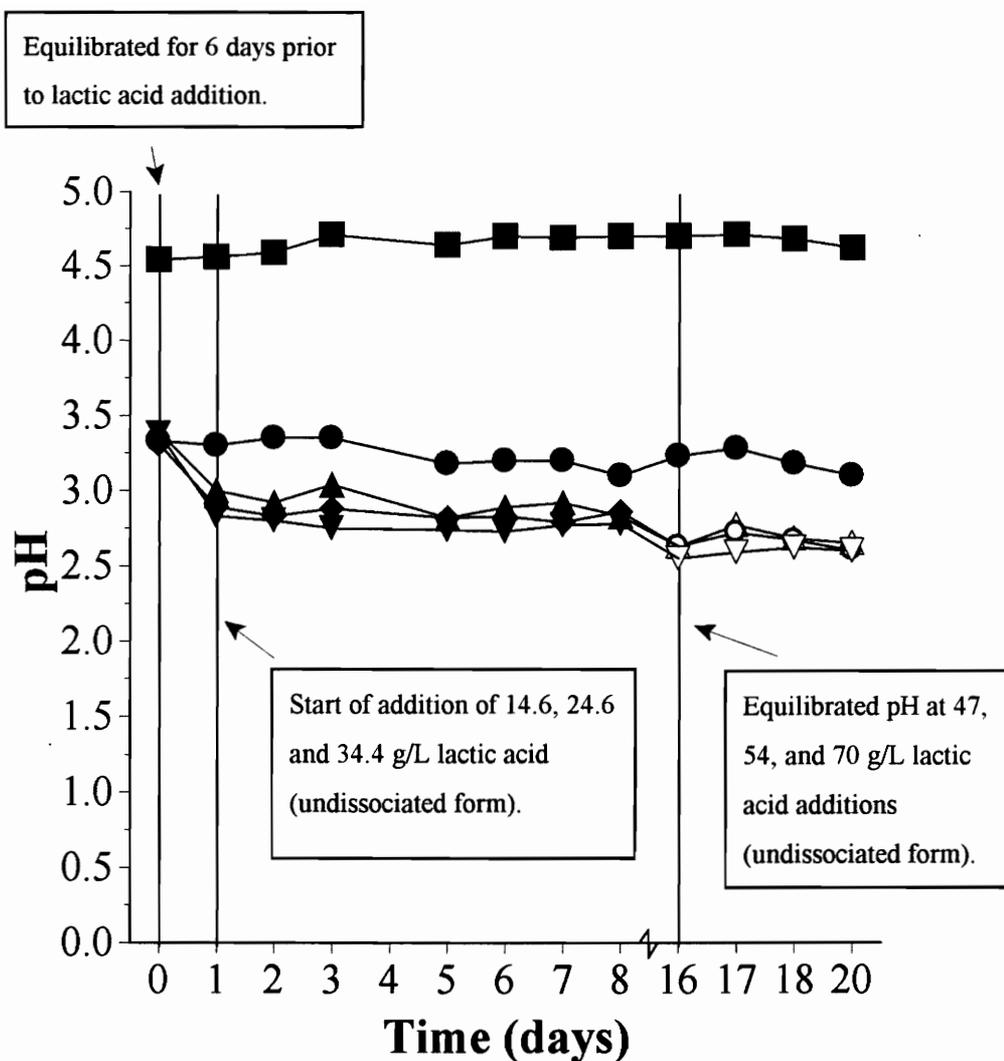
**Figure 4.34** Survival of *S. cerevisiae* in increasing amounts of undissociated lactic acid in a medium containing 260 g/L glucose. The control value of 4.1 g/L is contributed by the corn steep powder used in the medium. Prior to, and at the end of each lactic acid addition, the steady state viabilities of *S. cerevisiae* were confirmed. The total (and undissociated lactic acid) concentrations (% w/v) at the corresponding pH values were determined: 5.2 (4.1) at pH 3.16; 16.7 (14.6) at pH 2.88; 27.7 (24.6) at pH 2.82; 38.1 (34.5) at pH 2.76; 51.5 (47.3) at pH 2.68; and 58.1 (53.6) at pH 2.66.

appear to occur between a concentration of 52 and 57 g/L undissociated lactic acid.

#### 4.4.2 pH.

The pH profiles for all fermentors with added lactic acid are shown in Figure 4.35. Before any lactic acid was added to the individual fermentors, each fermentor was run at a dilution rate of  $0.066 \text{ h}^{-1}$  with *S. cerevisiae* for five days to allow it to reach steady state. This is represented by the first vertical line in Figure 4.35. Following equilibration, the lactic acid solutions (or sterile distilled water in the case of the control) were started at day 1. The vertical line at day 1 represents the start of the addition of lactic acid. The pH was monitored for an additional seven days of operation to confirm the steady state medium pH in each of these fermentors. After the steady state pH was confirmed, the concentration of lactic acid solutions that was pumped to each fermentor was changed so that the next three concentrations of lactic acid could be tested. After an additional seven days (note time break in x axis), the pH in these fermentors were tentatively in new steady states. The vertical line in Figure 4.35 at day 16 represents this point in time. The pH was followed for an additional three days to confirm the steady state pH in these fermentors.

At 14.6, 24.6, and 34.5 g/L undissociated lactic acid conditions, all three equilibrated to a steady state pH of 2.8 after six days. At 47.2, 53.5, and 70.0 g/L undissociated lactic acid conditions, all three equilibrated to a pH of 2.6. It is unlikely that a 0.2 pH difference at steady state (2.8 - 2.6) between the 34.5 and 47.2 g/L undissociated lactic acid conditions would account for the large change (an approximately  $\sim 0.77$  log decrease) for the corresponding viable numbers of *S. cerevisiae* in Figure 4.34. Furthermore, the  $\sim 4.1$  log decrease in viability occurring over the 47.3 and 53.6 g/L change in lactic acid (Fig. 4.34) resulted over a time when the overall pH of the medium remained constant at 2.6 (Fig. 4.35). The only parameter which changed was the concentration of lactic acid added. This strongly indicates that the concentration of undissociated lactic acid was responsible for the decrease in viable numbers of *S.*



**Figure 4.35** Fermentor pH with increased amounts of undissociated lactic acid at steady state utilizing medium containing 260 g/L glucose (■, Medium reservoir; ●, Control (4.1 g/L lactic acid (undissociated)); ▲, 14.6 g/L lactic acid (undissociated); ◆, 24.6 g/L lactic acid (undissociated); ▼, 34.5 g/L lactic acid (undissociated); △, 47.2 g/L lactic acid (undissociated); ○, 53.5 g/L lactic acid (undissociated); ▽, 70.0 g/L lactic acid (undissociated)).

*cerevisiae* in Figure 4.34. Even if pH played a role in determining the viable numbers of *S. cerevisiae*, differences can be seen in viable counts if one compares the 14.6 to 34.5 g/L and the 47.2 to 70.0 g/L undissociated lactic acid concentrations where the pH in each group remained constant at 2.8 and 2.6 respectively. At a constant pH of 2.8 (Fig. 4.35), the viable counts of *S. cerevisiae* decreased from  $5.4 \times 10^7$  to  $3.0 \times 10^7$  CFU/ml (Fig. 4.34) - a 44% decrease. At a constant pH of 2.6 (Fig. 4.35), *S. cerevisiae* decreased from  $6.7 \times 10^6$  to  $4.4 \times 10^3$  CFU/ml (Fig. 4.34) - a decrease of > 99.9%! Thus, even if the maximum pH change of 0.2 had influenced the viable numbers of *S. cerevisiae*, pH did not influence the viability of *S. cerevisiae* as undissociated lactic acid ranged from 14.7 to 34.5 or as it ranged from 47.2 to 70.0 g/L.

#### 4.4.3 Comparison of added lactic acid to produced lactic acid.

At the highest level of bacterial viability in the experiment reported in Figure 4.30, *L. paracasei* produced a total of 20 g/L of lactic acid. At a pH of 6.0 (the controlled pH), the concentration of lactic acid that remains undissociated in the fermentor would only be 0.1 g/L. As the experiments in Sections 4.3.3 and 4.4 were performed under similar operating conditions, it is now possible to determine the inhibition of *S. cerevisiae* by lactic acid in Figure 4.30. By extrapolating 0.1 g/L undissociated lactic acid data in Figure 4.35, it becomes very clear that undissociated lactic acid could not have been responsible for the 83% reduction seen for *S. cerevisiae* at pH 6.0 (Fig. 4.34). Competition for nutrients has been suggested in other work to be one possible reason why there was reduced growth of lactobacilli when yeast were present at equal numbers (Thomas *et al.*, 2001). These data raise the question as to how high a lactic acid concentration produced by *L. paracasei* would have to be to affect the yeast. From Figure 4.34, in order to have a 50% reduction in yeast numbers in continuous operation, ~34 g/L of undissociated lactic acid needs to be present in the fermentors. This translates to a total lactic acid concentration at corresponding pH values of: 98.4 g/L at pH 4.0; 237 g/L at pH 4.5; 675 g/L at pH 5.0; 2060 g/L at pH 5.5; and 6440 g/L at pH 6.0. It is

obvious that the latter values are not possible and that inhibition by lactic acid at those pH values does not exist. This also raises the question at higher lactic acid concentrations as to how (or if) dissociated lactic acid could inhibit the yeast. Evidence exists that the total as well as the undissociated forms of an organic acid may affect yeast growth. Thomas *et al.*, (2002) achieved steady state biomass concentrations in continuous cultures where the undissociated acetic acid concentration was set at 102 mM and the pH was controlled either at 4.5 or 3.0. The steady state biomass concentrations achieved were higher at pH 4.5 (340 Klett units) than at pH 3.0 (250 Klett units) with identical undissociated concentrations of acetic acid in both conditions but where the total concentration of acetic acid was higher at pH 4.5 (167 mM) than at pH 3.0 (104 mM). The authors suggested that inhibition of yeast can occur from the dissociated as well as the undissociated fraction of acetic acid because the two species are in equilibrium. In their experiments, both pH and the total amount of lactic acid were changed (2 parameters changed per experiment). As well, the steady state biomass concentration was different for both pH values where no acetic acid was added. In addition, identical concentrations of undissociated acetic acid at both pH values show an opposite trend in steady state biomass concentration. This intriguing suggestion by the authors can be definitively proven only if the range of pH values and total and undissociated acetic acid concentrations (as well as other organic acids such as lactic acid) are expanded and conducted in a factorial manner with statistical analysis.

With the data gathered in this work, it is unfortunately not possible to clearly state the reason as to why viable numbers of *S. cerevisiae* decreased in Figure 4.30. In hindsight, a continuous addition of fresh nutrients to the mixed culture at pH 6.0 once *S. cerevisiae* had reached its new steady state value would have clearly shown if competition for nutrients by *L. paracasei* was the reason. As it stands, the total amount (and not the undissociated fraction) of lactic acid produced by *L. paracasei* may ultimately be the reason for the decrease in viable *S. cerevisiae*. The present work suggests that, in addition to the well documented chemical stresses that a yeast may encounter during fermentation due to a bacterial contaminant (Connolly, 2000; Ingledew,

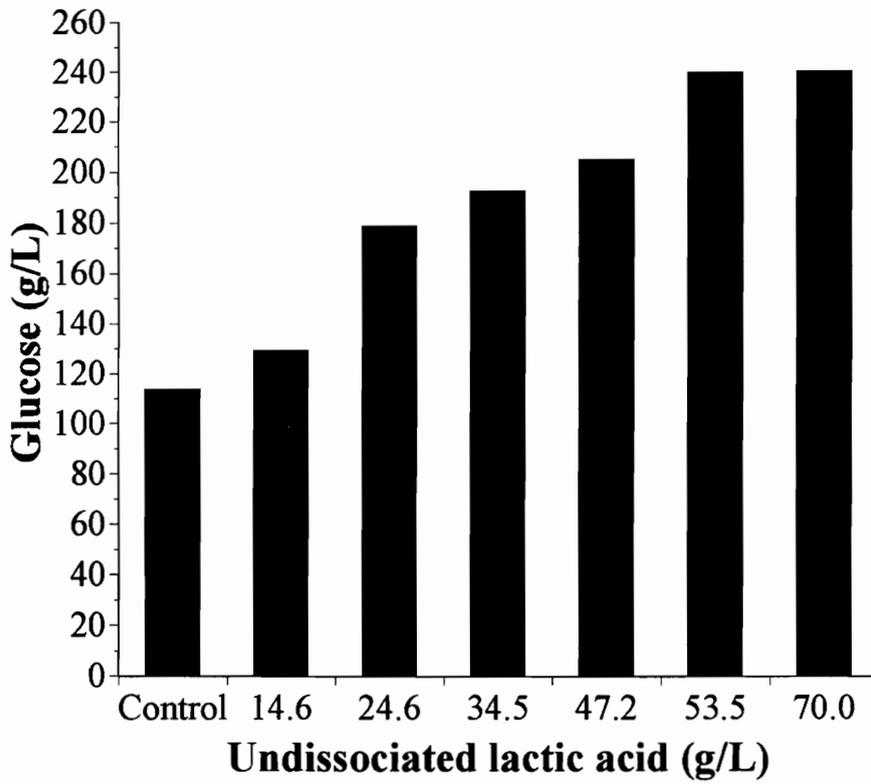
1999), nutritional stress due to competition for trace nutrients by the contaminating bacteria may also affect yeast growth and performance.

#### **4.4.4 Glucose.**

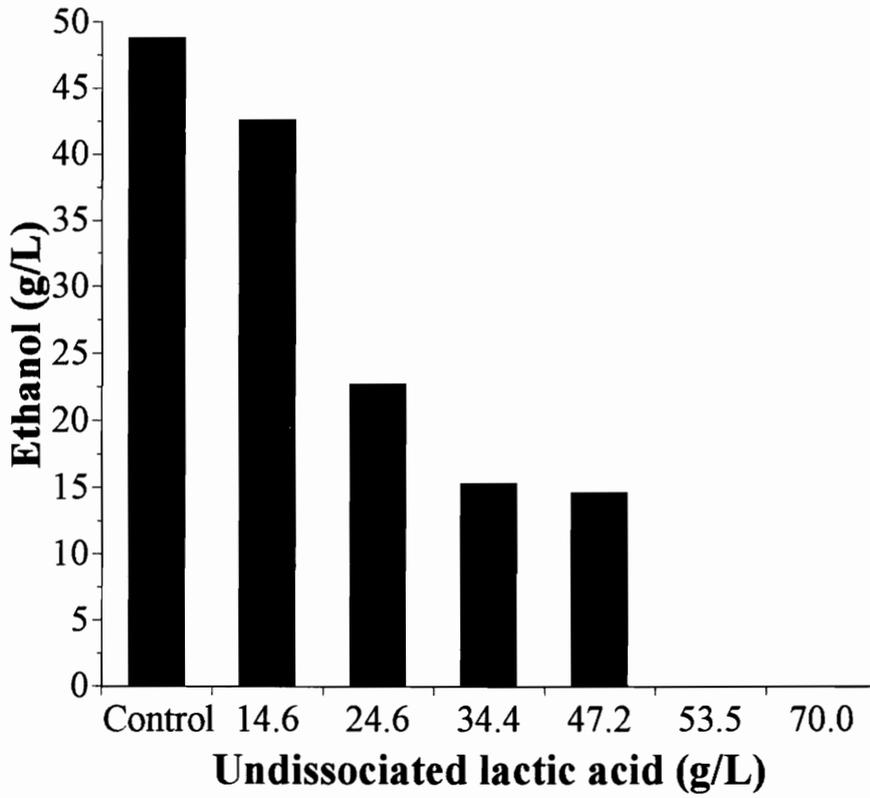
Changes in glucose concentrations with different undissociated lactic acid concentrations are shown in Figure 4.36. Here the amount of glucose left by *S. cerevisiae* in F1 is displayed for each undissociated lactic acid condition. As expected, less glucose was consumed when the undissociated lactic acid concentrations were increased. There was a corresponding decrease in the number of viable *S. cerevisiae* cells. In the control (which contained 4.1 g/L undissociated lactic acid), nearly 50% of the glucose supplied was not used and would have passed to F2 in the MCCF. Glucose consumption decreased a further 50% when 24.6 g/L lactic acid was present. As the undissociated lactic acid level increased to 70.0 g/L, more glucose remained in each fermentor, until at 53.5 and 70.0 g/L undissociated lactic acid, very little glucose was used. F1 fermentation at these concentrations effectively ceased.

#### **4.4.5 Ethanol.**

The steady state ethanol concentrations attained with different concentrations of undissociated lactic acid in the medium are shown in Figure 4.37. Ethanol concentrations decreased with increased undissociated lactic acid concentrations. Nearly 50% of the theoretical yield (calculated on the basis of glucose consumed) was still achieved with 24.6 g/L undissociated lactic acid present. This decreased to 30% with both 34.5 and 47.2 g/L undissociated lactic acid. No ethanol was detected when the medium contained 53.5 and 70.0 g/L undissociated lactic acid.



**Figure 4.36** Residual glucose (corrected for metered lactic acid additions) at steady state in the presence of increasing undissociated lactic acid utilizing medium containing 260 g/L glucose.

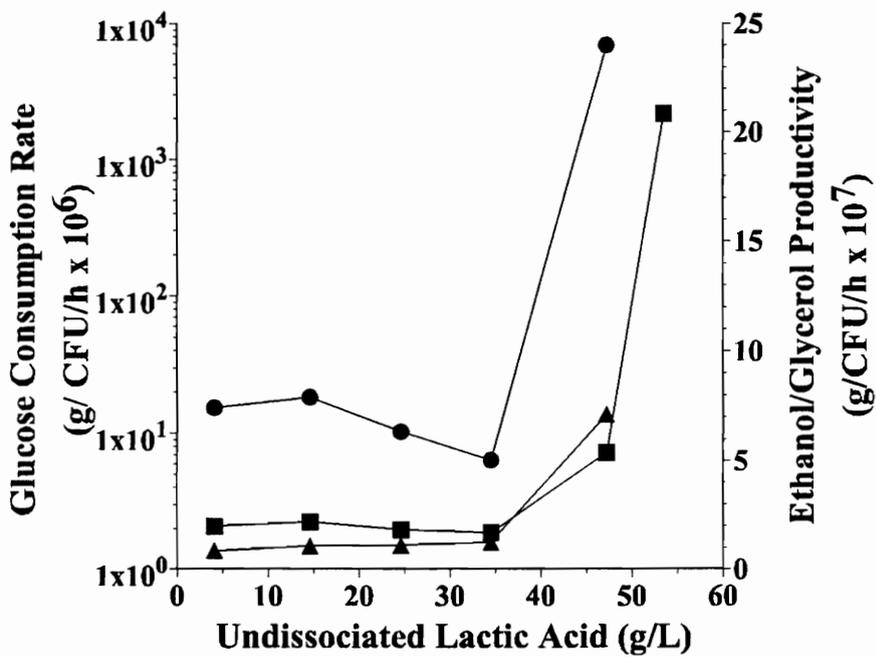


**Figure 4.37** Ethanol production at steady state in the presence of increasing undissociated lactic acid in a medium containing 260 g/L glucose.

#### 4.4.6 Specific consumption and productivities.

The absolute values of viable cell counts along with glucose and ethanol concentrations (Figs. 4.34, 4.36, and 4.37) depict what is happening in each CSTR fermentor as undissociated lactic acid was increased. To determine how cells of *S. cerevisiae* were responding to added lactic acid, specific productivities and consumption rates were required. Specific productivity (or consumption) rates were calculated as g ethanol or glycerol produced (or g glucose consumed) per hour per CFU. All data used in the calculations were taken from verified steady state values achieved at each undissociated lactic acid concentration.

The specific glucose consumption rate and specific ethanol and glycerol productivities for each lactic acid condition are shown in Figure 4.38. The specific consumption remained constant at  $2 \times 10^{-6}$  (g glucose/CFU/h) up to a concentration of 34.5 g/L undissociated lactic acid in the medium. It then increased 4 fold at 47.2 g/L undissociated lactic acid and 4400 fold at 53.5 g/L undissociated lactic acid. In other words, as the undissociated lactic acid concentration increased beyond 34.5 g/L, the yeasts which survived dramatically increased consumption of glucose on a per viable cell basis. One hypothesis to account for this increase is that *S. cerevisiae* needs the energy (ATP) produced from the increased fermentation of glucose to expel the increased hydrogen ion concentration resulting from the influx of undissociated lactic acid (which dissociates inside the yeast cell at the increased pH). As the lactic acid increases, *S. cerevisiae* needs correspondingly more ATP energy to expel the larger amount of  $H^+$  (formed from the lactic acid diffusing into the cell) and this “drives” the yeast to ferment more glucose in order to survive. This hypothesis was put forward by Verduyn *et al.* (1992) in their work using benzoic acid (nonmetabolizable by yeast) where they documented that the specific glucose flux (uptake) increased when benzoic acid levels were increased in continuous culture, and by Narendranath *et al.* (2001b) using acetic and lactic acids.

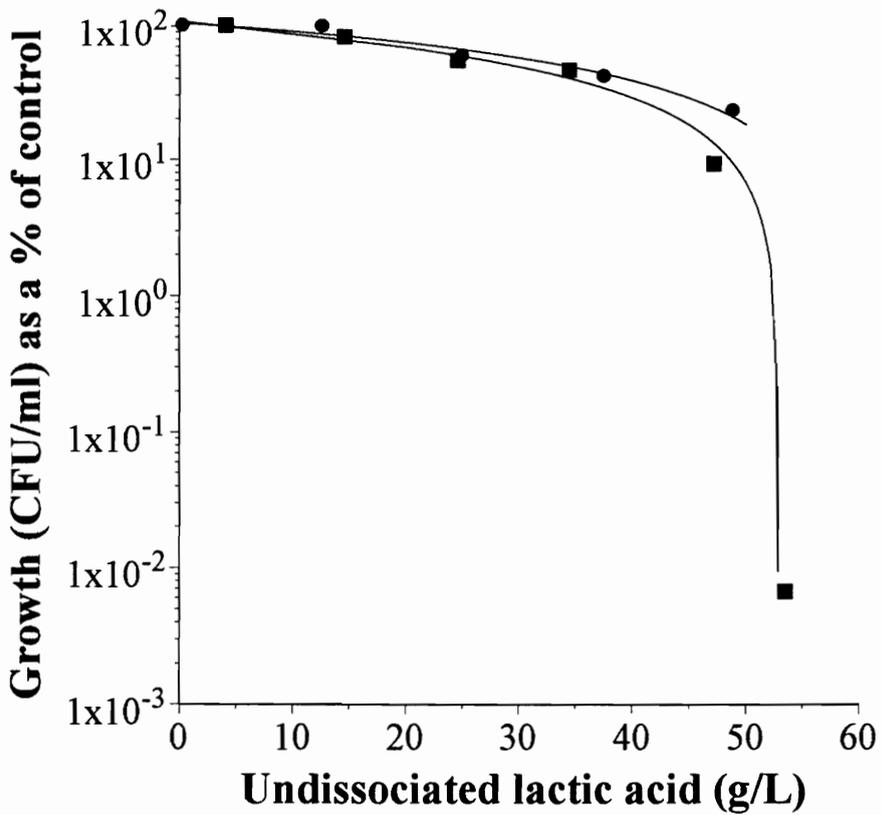


**Figure 4.38** Specific glucose consumption and specific ethanol and glycerol productivities in the presence of increasing undissociated lactic acid in a medium containing 260 g/L glucose (■, specific glucose consumption; ●, specific ethanol productivity; ▲, specific glycerol productivity).

Since the specific glucose consumption by *S. cerevisiae* increased with increasing lactic acid concentrations, and since *S. cerevisiae* in the CSTR fermentors cannot metabolize aerobically under the conditions of high sugar in these experiments (DeDenken, 1966; Petrik *et al.*, 1983), it stands to reason that the specific ethanol and glycerol production rates would increase as well. These are also shown in Figure 4.38. As expected, both the specific ethanol and glycerol production rates increased in parallel with the specific glucose consumption rate. The specific ethanol production rate increased by ~3.2 fold to a maximum of  $2.4 \times 10^{-6}$  g ethanol per h per CFU as compared to the control ( $7.4 \times 10^{-7}$  g ethanol per h per CFU). One conclusion from this data is that although lactic acid does cause a decrease in the number of viable cells of *S. cerevisiae* in the MCCF (Fig. 4.34), the stress imposed by the lactic acid forces surviving cells of *S. cerevisiae* to produce more ethanol per cell (Fig. 4.38) (Thus, depending on one's point of view, not all stress is bad). Unfortunately, this boost in specific productivity comes at the price of reduced cell numbers. If cell recycle were added to the MCCF to raise viable *S. cerevisiae* (CFU/ml) at the highest specific ethanol production rate (47.2 g/L undissociated lactic acid) to the same CFU/ml where no lactic acid was added (control), ethanol would be produced much faster which would translate into a higher ethanol productivity for the fuel alcohol plant.

#### **4.4.7 Batch vs continuous culture.**

The apparent resistance of *S. cerevisiae* to the high levels of undissociated lactic acid shown in Figure 4.34 may be due to the natural resistance of this strain of *S. cerevisiae* to lactic acid, to medium composition, or to the fact that the experiment was operating in continuous mode. To address whether the culturing mode affects the apparent resistance of yeast, similar experiments were performed in batch growth with media formulated using identical concentrations of glucose and corn steep powder in the CSTR experiments, but with varying lactic acid concentrations. The results are shown in Figure 4.39. Here a direct comparison can be made between the steady state viable



**Figure 4.39** Comparison of inhibition (% of maximum growth) of viable cell numbers from MCCF fermentations and growth rate from batch fermentations with increasing undissociated lactic acid present in the 260 g/L glucose medium. No growth was observed in batch when the undissociated lactic acid concentration was above 50 g/L (■, % of maximum CFU/ml in MCCF fermentations as compared to MCCF control; ●, % of maximum growth rate in batch fermentations as compared to batch control).

counts from CSTR experiments, and the maximum slope (OD/h) calculated from batch experiments with lactic acid, when both sets of data were plotted as a percent of their respective (maximum) control values. The comparison in batch and continuous culture modes are based on the fact that lactic acid inhibits the specific growth rate of *S. cerevisiae*. However, each culturing method reacts differently at reduced specific growth rates. In continuous culture, a reduction in the specific growth rate would result in a new lower steady state viable numbers of *S. cerevisiae*. In batch, a decrease in the specific growth rate would result in a decreased exponential growth rate as the lactic acid concentration is increased. By calculating each set of data as a percentage of their respective (maximum) control values, both culturing modes can be compared on an equal basis.

In Figure 4.39, both plots showed similar inhibition profiles when the undissociated lactic acid concentrations in the media were increased to 40 g/L. A 50% reduction (inhibition) of growth in both plots occurred when the lactic acid concentration was ~35 g/L. Only when undissociated lactic acid was greater than 40 g/L did the inhibition of yeast in the two culturing methods begin to differ. At a concentration of ~50 g/L lactic acid, batch culturing resulted in a ~77% reduction while continuous culturing showed ~91% reduction in comparison to the appropriate control. No detectable growth occurred in batch over 50 g/L lactic acid while growth was still present in the CSTR experiments as evidenced by a steady state yeast population.

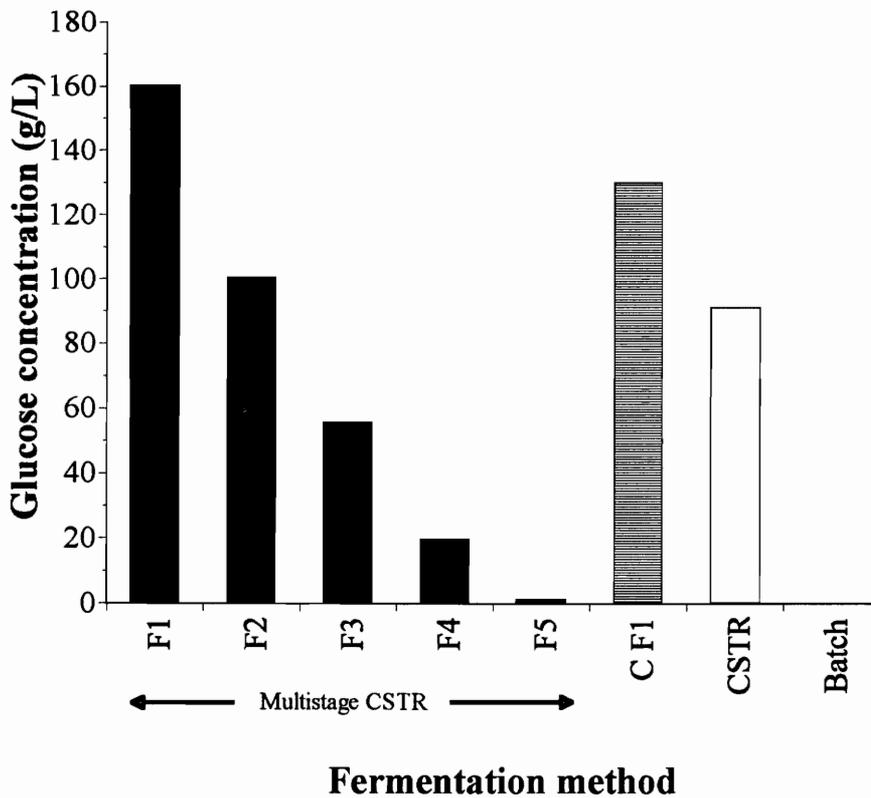
One can conclude from this data that there appears to be no discernable difference between the methods of cultivation in inhibition of *S. cerevisiae* by undissociated lactic acid up to 40 g/L. Thus, the apparent lack of inhibition at high levels of lactic acid must be due to either the natural resistance to lactic acid of this strain of *S. cerevisiae*, or due to unidentified protective components in the medium used for this work.

## 4.5 Comparison of fermentations by batch, single-stage CSTR, and MCCF methods.

In Section 4.2, the fermentation characteristics and ethanol productivity of the MCCF at steady state with *S. cerevisiae* were studied in detail. It is of interest, however, to compare the fermentation of *S. cerevisiae* in batch, single-stage CSTR, and in an MCCF with regards to glucose utilization and ethanol production when all three modes of fermentation used identical media and identical fermentation conditions. In addition, the work performed with the deliberate introduction of *L. paracasei* to F1 in the MCCF permits a comparison of the ethanol production capabilities between a contaminated and non-contaminated MCCF. The following subsections show the results from each fermentation mode.

### 4.5.1 Glucose concentration.

Figure 4.40 illustrates the steady state glucose concentrations in fermentations conducted in the MCCF (which is equal to multistage CSTR), single-stage CSTR, in the CF1 fermentor (F1 of the MCCF system) contaminated with *Lactobacillus paracasei*, and in batch mode. The single-stage fermentor had the equivalent combined volume of the MCCF as well as a flowrate identical to the MCCF (0.34 L/h). These conditions provided an overall dilution rate ( $D$ ) of  $0.12 \text{ hr}^{-1}$  for F2-F5 and  $0.061 \text{ h}^{-1}$  for F1 in the MCCF, and  $0.02 \text{ hr}^{-1}$  for the single-stage CSTR. The glucose concentration exiting the MCCF was 1.2 g/L while in the single-stage CSTR, the glucose concentration that exited the system was 91 g/L. Thus, for the same concentration of glucose and flowrate to each system containing the same total volume, the MCCF consumed  $> 99.5\%$  of the glucose while only 64% was consumed in the single-stage CSTR. One can conclude from this data that the MCCF is superior to the single-stage continuous system in utilizing glucose (1.76 fold better) because the substrate was almost completely utilized. As the cost of raw materials can comprise up to 60% of the production cost of a typical fuel alcohol



**Figure 4.40** Glucose concentrations at steady state in continuous (multistage CSTR, CF1, CSTR), and at the end of batch fermentations using media containing 260 g/L glucose. All continuous fermentations utilized a flow of 5.60 ml/min ( $D$  for F1 and CF1 =  $0.066 \text{ hr}^{-1}$ ;  $D$  for F2-F5 =  $0.12 \text{ hr}^{-1}$ ;  $D$  for CSTR =  $0.021 \text{ hr}^{-1}$ ) (F1-F5 = fermentors 1-5 in the MCCF system; CF1 = F1 contaminated with *L. paracasei*; CSTR = one fermentor with a working volume equivalent to the sum of all working volumes in the multistage CSTR).

plant, wastage of raw materials has to be avoided. The glucose utilization in the MCCF was essentially the same amount that was seen in batch and thus is a better fermentation method than a single-stage CSTR for ethanol production. This has potentially some practical implications. The MCCF system could permit the fuel alcohol producer to invest in less costly smaller fermentors instead of purchasing one large equivalent fermentor (in this case 5x the volume of any of the individual MCCF fermentors) to maximize ethanol production. Desired increases in volumetric productivity could be more easily made in a multistage system than a single-stage system since the multistage system is more easily scalable for higher ethanol productivities as additional fermentors can be easily added to the fermentation train.

In Section 4.3.3, the first fermentor (F1) of a multistage CSTR system was deliberately contaminated with *Lactobacillus paracasei* and allowed to reach a steady state with *S. cerevisiae*. The glucose concentration at steady state from this contaminated fermentor (CF1) is included in Figure 4.40. As expected, the glucose concentration in CF1 was lower than in F1 (by 30.5 g/L). If not metabolized to ethanol, this reduction (extra consumption of glucose) of 30.5 g/L represents a maximum theoretical loss of 15.6 g/L ethanol for the alcohol producer. Although the presence of *L. paracasei* did not significantly change the steady state of number of viable yeast in CF1 as compared to F1 (Figs. 4.18 and 4.30), the presence of  $3.7 \times 10^5$  CFU/ml viable *L. paracasei* at steady state in CF1 must have caused the decrease in glucose concentration. This decrease may have been due to either the additional consumption of glucose in CF1 for growth and lactic acid production by *L. paracasei*, and/or a possible increase in the specific ethanol production rate of *S. cerevisiae* due to interaction with *L. paracasei* and its end product of metabolism.

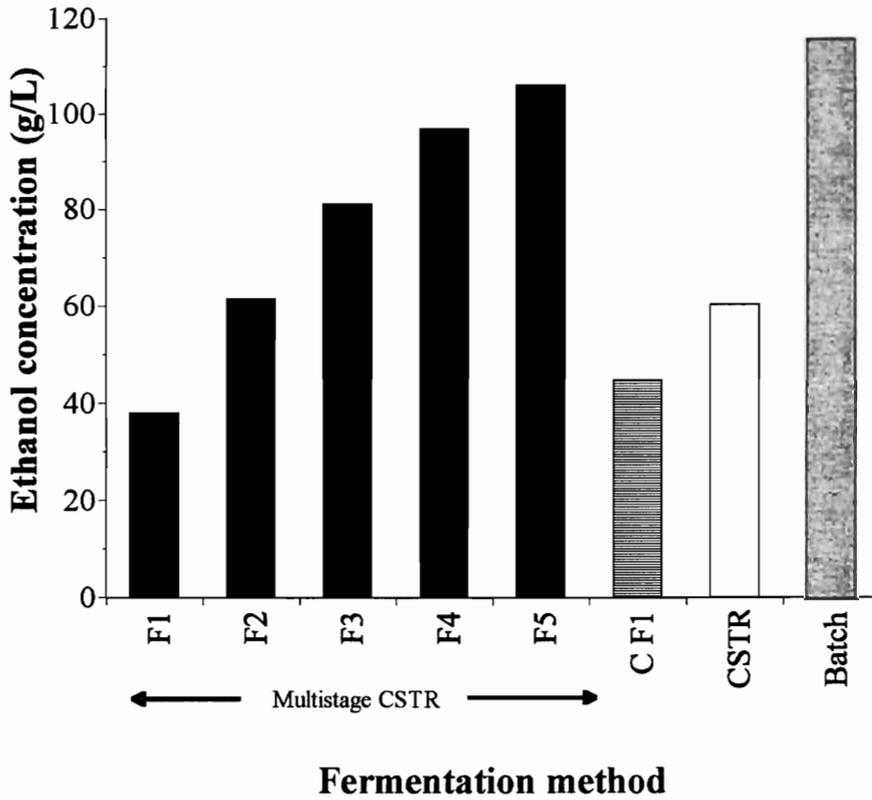
In Figure 4.40, the fermentation methods which provide the best glucose utilization were the MCCF at the flow rates chosen and batch fermentations. In the batch fermentation, no detectable amount of glucose remained once fermentation was complete. Although some glucose was detected in the effluent leaving the MCCF (1.2 g/L), it represented a loss of only 0.5% of the total glucose entering the system.

#### 4.5.2 Ethanol concentration and productivity.

Figure 4.41 depicts the cumulative ethanol concentrations in MCCF and the ethanol concentration in fermentations conducted in single-stage CSTR, CF1 fermentor (F1 of the MCCF system deliberately contaminated with *Lactobacillus paracasei*), and batch methods. As expected, the ethanol concentration in the MCCF increased from 38 g/L in F1 to a final concentration of 106 g/L in F5. In comparison, the ethanol concentration in the single-stage CSTR reached a maximum of 60 g/L - only 57% of the ethanol concentration reached in F5 in the MCCF. Thus, by simply dividing the working volume of a large CSTR into smaller, multiple, serially arranged CSTR's, higher ethanol concentrations and lower concentrations of glucose are clearly achieved in the effluent. Clearly the MCCF provides economic and practical advantages over an equivalent single-stage CSTR system operated at the same flow rate.

The highest concentration of ethanol was reached in the batch fermentor (Fig. 4.41). It was 9.4% greater than the ethanol concentration produced in the MCCF. The approximate 10 g/L increase in ethanol concentration in batch fermentation came from the complete utilization of glucose as opposed to the 1.2 g/L glucose remaining in the multistage CSTR system. The utilization of the remaining glucose in the MCCF accounts for only 0.61 g/L ethanol which means that ~ 9.3 g/L is not accounted for. The higher ethanol concentration produced in batch was possibly achieved from a combination of nutrients provided by dead yeast cells (since, unlike continuous fermentations, all cells are retained) and from the possible degradation of proteins in the medium from the release of proteases from lysed cells. If this is true, then experiments in cell recycle fermentations should also yield a similar (but possibly smaller) increase in ethanol since a portion of the dead cells are retained in the fermentor as a result of the process.

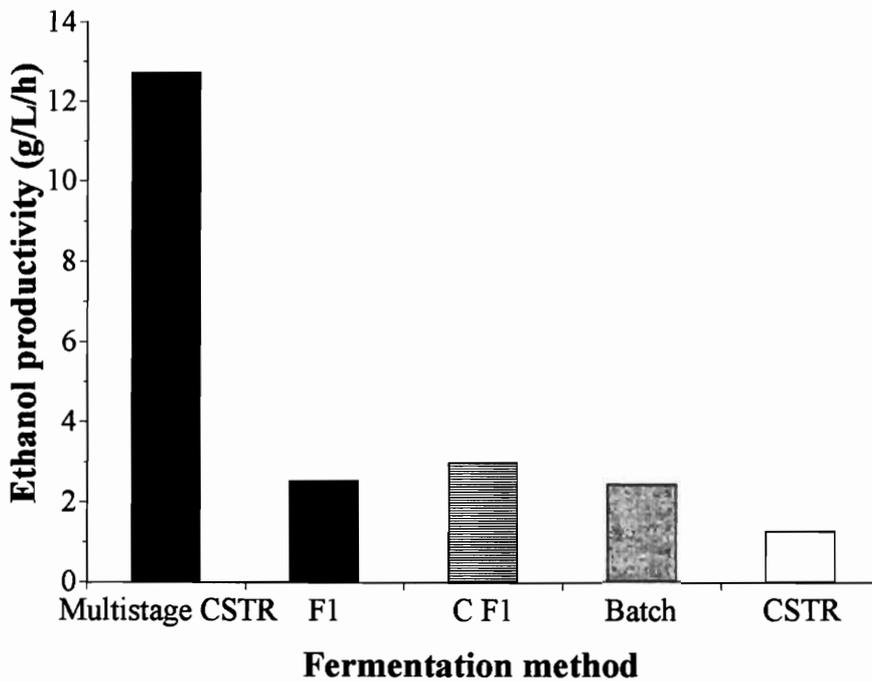
An interesting observation in Figure 4.41 is that the ethanol concentration of 44.7 g/L in CF1 was higher than the 37.9 g/L seen for F1 - an 18% increase in ethanol concentration! This increase is more remarkable when considering the facts that the viable number of *S. cerevisiae* decreased from  $3.8 \times 10^7$  CFU/ml in F1 (Fig. 4.18) to



**Figure 4.41** Ethanol concentrations at steady state in continuous (multistage CSTR, CF1, CSTR), and at the end of batch fermentations using media containing 260 g/L glucose. All continuous fermentations utilized a flow of 5.60 ml/min ( $D$  for F1 and CF1 =  $0.066 \text{ hr}^{-1}$ ;  $D$  for F2-F5 =  $0.12 \text{ hr}^{-1}$ ;  $D$  for CSTR =  $0.021 \text{ hr}^{-1}$ ) (F1-F5 = fermentors 1-5 in the MCCF system; CF1 = F1 contaminated with *L. paracasei*; CSTR = one fermentor with a working volume equivalent to the sum of all working volumes in the multistage CSTR).

$3.1 \times 10^7$  CFU/ml in CF1 (Fig. 4.30) and also that in CF1, competition for nutrients by both organisms must be occurring. One possible reason may be that the production of lactic acid (5.2 g/L in CF1 compared to 3.3 g/L in F1) by *L. paracasei* may “drive” the yeast to produce more ethanol. This effect of “driving” the yeast to produce ethanol was clearly seen in Figure 4.38 when lactic acid was added to steady state CSTR fermentations with *S. cerevisiae*. This hypothesis has support from the work by Verduyn *et al.* (1992) where they documented that the specific glucose flux (uptake) increased and ethanol concentration increased when benzoic acid levels were increased in continuous culture. Another possible reason for the increased concentration of ethanol in CF1 may be that the low steady state numbers of *L. paracasei* ( $3.7 \times 10^5$  CFU/ml as compared to  $3.1 \times 10^7$  CFU/ml for *S. cerevisiae*) may be providing *S. cerevisiae* with additional nutrients from the catabolism of medium components which are not normally metabolizable by yeast. One example may be the protein in the CSTR medium which exists at 10 g/L. Proteins cannot be utilized by yeasts but *L. paracasei* may be able to hydrolyze the protein into amino acids and peptides which can in part be utilized by yeast. If this interaction is confirmed, then the two organisms are not only competitors, but are also commensal in their symbiosis. Further research would be needed to confirm this interaction.

The ethanol productivities (g/L/h) in fermentations conducted in the MCCF, single-stage CSTR, CF1 fermentor and batch are shown in Figure 4.42. The MCCF showed the highest ethanol productivity of 12.7 g/L/h followed by CF1 at 3.0 g/L/h, batch at 2.4 g/L/h, and single-stage CSTR at 1.25 g/L/h. Clearly, even though the highest ethanol concentration was seen in batch (Fig. 4.41), the MCCF out-performed batch fermentations in ethanol productivity at steady state by a factor of 5.3. This dramatic increase in multistage productivity can be attributed to a number of reasons. A multistage CSTR system allows one to “extend” the productivity of the culture in that the population of cells generated in the first few fermentors continue to metabolize and produce product in later stages even though their growth may be totally inhibited (Pirt, 1975). As well, the yeast cells in the first few fermentors in the multistage system are



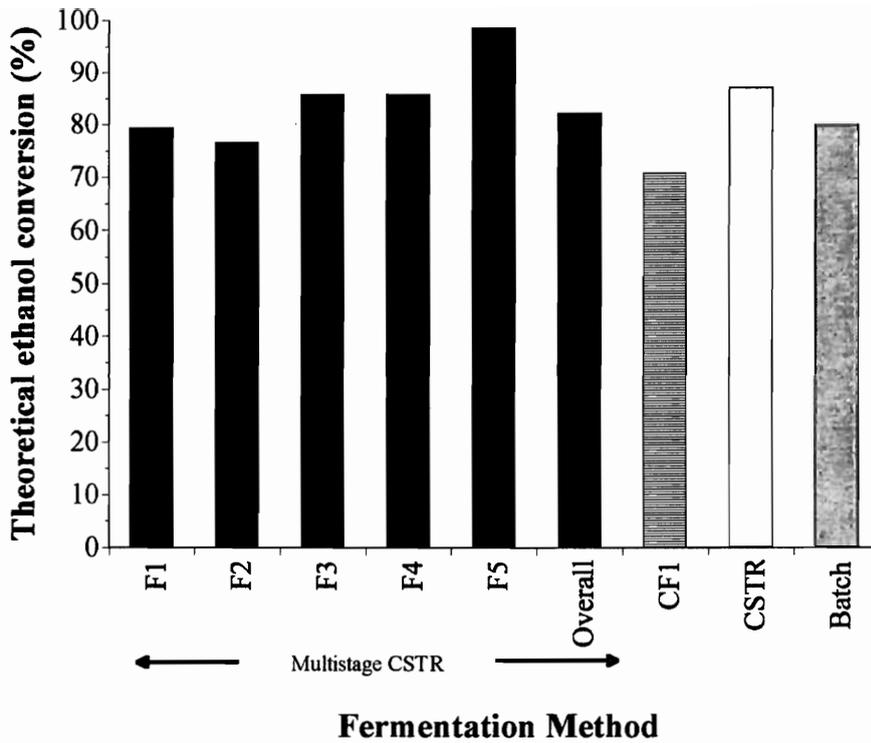
**Figure 4.42** Ethanol productivity at steady state in continuous (multistage CSTR, CF1, CSTR), and at the end of batch fermentations using media containing 260 g/L glucose. All continuous fermentations utilized a flow of 5.60 ml/min ( $D$  for F1 and CF1 =  $0.066 \text{ hr}^{-1}$ ;  $D$  for CSTR =  $0.021 \text{ hr}^{-1}$ ) (F1 = fermentor F1 in the MCCF system; CF1 = F1 contaminated with *L. paracasei*; CSTR = one fermentor with a working volume equivalent to the sum of all working volumes in the multistage CSTR).

exposed to the best conditions of growth possible (high amounts of nutrients, low amounts of inhibitory products) - unlike a single-stage CSTR where higher amounts of inhibitory products and lower amounts of nutrients would be present. Lastly the ethanol concentration in the multistage CSTR system rises gradually from fermentor to fermentor which gradually inhibits ethanol productivity as compared to a single-stage CSTR where inhibition is greater because a higher amount of ethanol would be present right at the beginning of the fermentation. For ethanol producers, this would mean that the highest volumetric throughput can be achieved with a MCCF.

Not surprisingly, the ethanol productivity in Figure 4.42 was higher in CF1 than in F1. This was due to the fact that the ethanol concentration achieved in CF1 was higher than F1 (Fig. 4.41). Indeed the increase in ethanol productivity in Figure 4.42 indicates that the introduction of *L. paracasei* actually improved the production rate of ethanol. This further implies that, as far as ethanol production is concerned, the introduction and growth of *L. paracasei* to the MCCF was actually beneficial to the yeast as the two organisms may not only be competitors but also commensalistic in their interaction. This finding is opposite to what one would expect when contamination occurs in fuel alcohol fermentations. A reduction in ethanol productivity was seen for *S. cerevisiae* cultured in a cell recycle continuous system that was deliberately contaminated with *Lactobacillus fermentum* #7-1 (36% reduction) or with an inoculum from a contaminated industrial fermentor using tapioca (33% reduction) (Chang *et al.*, 1995). The ethanol productivity for CF1 was slightly higher than the batch fermentation which is remarkable since this showed that even the first fermentor (out of five) in a MCCF (contaminated or not) produced alcohol at a higher rate than a batch fermentation.

#### **4.5.3 Ethanol conversion efficiency.**

The ethanol conversion efficiency of each fermentation system expressed as a percentage of the theoretical amount of ethanol that can be converted from the amount of glucose consumed in each system is shown in Figure 4.43. In general, the ethanol

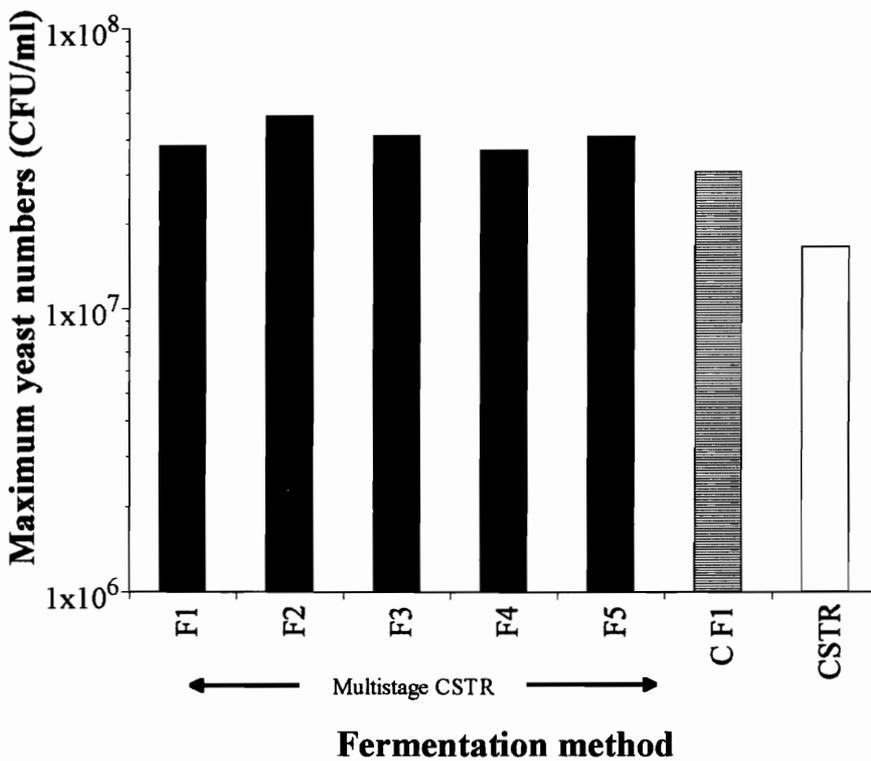


**Figure 4.43** Conversion of glucose to ethanol as a percent of ethanol that can be theoretically converted from glucose consumed. Fermentations were conducted in continuous (multistage CSTR, CF1, CSTR), and in batch fermentations using media containing 260 g/L glucose. All continuous fermentations utilized a flow of 5.60 ml/min ( $D$  for F1 and CF1 = 0.066  $\text{hr}^{-1}$ ;  $D$  for F2-F5 = 0.12  $\text{hr}^{-1}$ ;  $D$  for CSTR = 0.021  $\text{hr}^{-1}$ ) (F1-F5 = fermentors 1-5 in the MCCF system; CF1 = F1 contaminated with *L. paracasei*; CSTR = one fermentor with a working volume equivalent to the sum of all working volumes in the multistage CSTR).

conversion efficiency in the MCCF increased from 79.3% in F1 to a maximum of 98.5% in F5 with an overall ethanol conversion efficiency of 82.1% for the entire MCCF. The ethanol conversion efficiency for batch and MCCF (overall value) showed similar values at 80.0 and 82.1% respectively. This indicates that both systems “chanell” glucose to ethanol production with about the same efficiency. The ethanol conversion efficiency of 70.1% for CF1 was the lowest out of all the fermentation methods. This was not unexpected as a portion of the glucose in the medium was utilized by *L. paracasei* for growth and lactic acid production and for yeast growth. Thus, a portion of the glucose consumed would not be available for ethanol production. An unexpected observation in Figure 4.43 was that the single-stage CSTR system showed a 87% ethanol conversion rate - the highest ethanol conversion rate of all systems studied here. This indicated that *S. cerevisiae* in the single-stage CSTR was diverting the highest amount of glucose towards ethanol production - which would mean that less amount of glucose was diverted for growth. Unlike the MCCF where the tasks of growth and ethanol production by *S. cerevisiae* could be, to some extent, separated in distinct fermentors in the system, *S. cerevisiae* in the single-stage CSTR must accomplish both tasks in the same fermentor. This is borne out by comparing the individual fermentors in the MCCF to the single-stage CSTR. The last fermentor (F5) in the multistage CSTR system would be expected to have the highest ethanol conversion efficiency (albeit at a slower rate of production) since nearly all of the biomass formed in the system occurred in F1 and F2 - and indeed a 98.5% ethanol conversion efficiency was seen.

#### **4.5.4 Viable number of yeast.**

The steady state viable numbers (CFU/ml) of *S. cerevisiae* in all continuous fermentations are shown in Figure 4.44. The viable numbers of *S. cerevisiae* in the single-stage CSTR system reached a steady state at  $1.7 \times 10^7$  CFU/ml while in the MCCF, the viable numbers were at a minimum at  $3.8 \times 10^7$  CFU/ml for all fermentors. Thus, there was approximately twice the number of cells in the MCCF than the single CSTR system.



**Figure 4.44** Viabilities (CFU/ml) of *S. cerevisiae* at steady state in continuous fermentations using media containing 260 g/L glucose. All continuous fermentations utilized a flow of 5.60 ml/min ( $D$  for F1 and CF1 = 0.066 hr<sup>-1</sup>;  $D$  for F2-F5 = 0.12 hr<sup>-1</sup>;  $D$  for CSTR = 0.021 hr<sup>-1</sup>) (F1-F5 = fermentors 1-5 in the MCCF system; CF1 = F1 contaminated with *L. paracasei*; CSTR = one fermentor with a working volume equivalent to the sum of all working volumes in the multistage CSTR).

The increased number of viable cells in the multistage CSTR is an advantage since more yeast are present to convert glucose to ethanol and thus a faster volumetric productivity is possible. The presence of *L. paracasei* at  $3.7 \times 10^5$  CFU/ml did not seem to adversely affect the viable numbers of *S. cerevisiae* in CF1. Although viable numbers of *S. cerevisiae* in CF1 at  $3.1 \times 10^7$  CFU/ml is not much different than the viable numbers in F1 at  $3.8 \times 10^7$  CFU/ml, 18% more ethanol was produced in CF1 than F1 (Fig. 4.41). In the MCCF, the viable numbers of *S. cerevisiae* ranged between  $3.7 \times 10^7$  (F1) and  $4.9 \times 10^7$  CFU/ml (F4).

#### **4.6 Controlling growth of *L. paracasei* in the MCCF.**

##### **4.6.1 pH control.**

As described earlier (Fig. 4.33), the growth of *L. paracasei* is highly dependant on the pH of the fermentation medium. It stands to reason that one could control *L. paracasei* in the MCCF by controlling the pH. Controlling growth of contaminating bacteria by pH is routine in breweries where an acid wash (decontamination) of the yeast is performed on the yeast in storage after a fermentation run, and a number of fuel alcohol plants lower the pH of fermentors when bacteria become a problem. Although pH is recognized as a primary method to combat and limit contamination and is used in many yeast-based industrial fermentations, there are no published papers on the effects of pH control on a mixed populations of organisms involving yeast in a multistage continuous system.

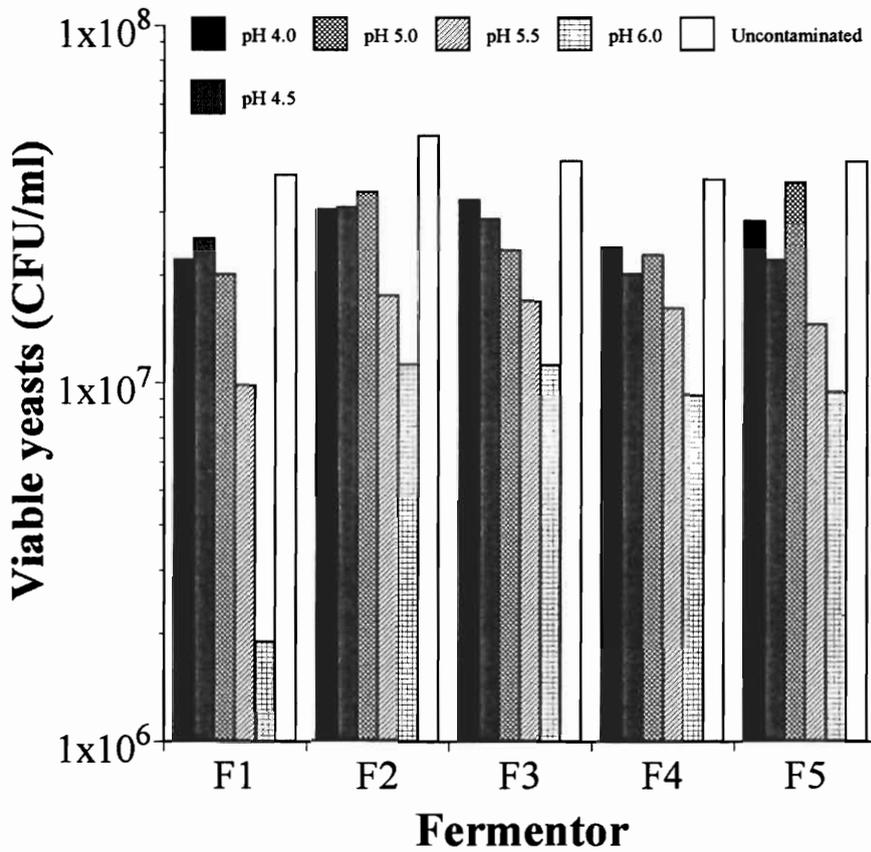
The results from a contaminated MCCF that is pH controlled over a range of pH values can be easily compared to the production of an MCCF that is not contaminated. In addition, the pH of many multistage continuous fermentation systems in industry are known to vary due to media formulation and the recycling of wash waters, caustic and cooling tower or distillation tower streams. Some are operated at different pH values. The following subsections present MCCF fermentations conducted using *S. cerevisiae*

and *L. paracasei* at pH values set from 4.0 to 6.0 in F1. The population dynamics of both organisms and the resulting ethanol production capabilities of the MCCF are presented and compared to uncontaminated MCCF fermentations.

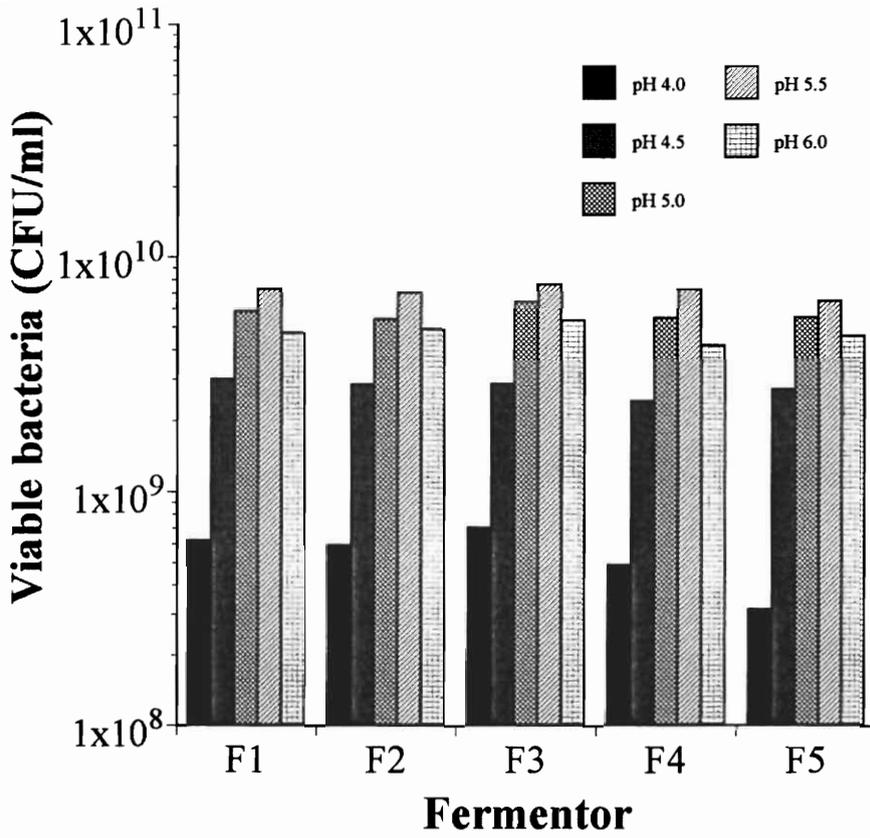
#### 4.6.1.1 *S. cerevisiae* and *L. paracasei*.

Figure 4.45 illustrates the steady-state viable numbers (CFU/ml) of *S. cerevisiae* in all fermentors in the MCCF (D = 0.12, 260 g/L glucose medium reservoir concentration, 28°C, 100 RPM) when the pH was controlled in F1 from pH 4.0 to pH 6.0. In general for all fermentors (with the exception of the condition in F1 at pH 6.0), the highest viable numbers of yeast ( $2\text{-}3 \times 10^7$  CFU/ml) were seen at pH 4.0. This decreased to  $\sim 1 \times 10^7$  CFU/ml as the pH reached 6.0. Another observation is that, for every fermentor, all of the viable numbers of yeast in the pH controlled conditions were lower than in the control where no pH control was utilized. Since the pH within the range of 4.0 to 6.0 does not affect yeast growth (Fig. 4.32), the decrease in viable numbers of yeast from pH 4.0 to 6.0 and in each fermentor (as compared to the control) could not have been due to pH. At each pH value tested, the viable numbers of *S. cerevisiae* across each fermentor in the MCCF were similar. The same number of viable yeast were also found across the fermentors in the MCCF in the control. This indicates that after the initial growth of *S. cerevisiae* in F1, no increase in yeast viable numbers occurred in later fermentors in the MCCF.

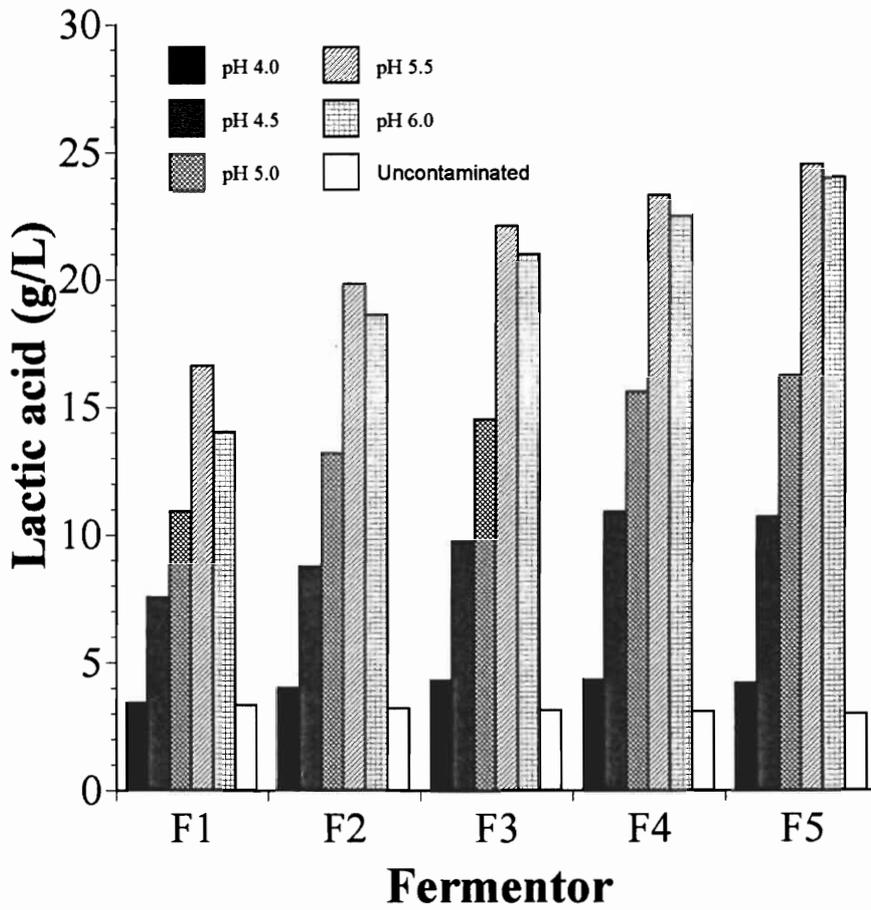
The decrease in *S. cerevisiae* with increasing pH must therefore be due to the actions of *L. paracasei* - by the production of lactic acid and/or by the competition of nutrients. This is borne out by examining the data in Figures 4.46 and 4.47. One observation in Figure 4.46 is that the viable number of *L. paracasei* remained nearly identical across the fermentors in the MCCF at each pH value. From this one can conclude that no net increases in viable numbers of *L. paracasei* occurred in the MCCF after the initial growth seen in F1. However, differences in steady state viable numbers of *L. paracasei* were seen when the pH was changed. In general at each stage, as the pH



**Figure 4.45** Viable cell concentration (in CFU/ml) of *S. cerevisiae* in a pH-controlled mixed culture, multistage continuous culture fermentation (MCCF) system run at 260 g/L glucose.



**Figure 4.46** Viable cell concentrations (in CFU/ml) for *L. paracasei* in a pH-controlled mixed culture, multistage continuous culture fermentation (MCCF) system run at 260 g/L glucose.



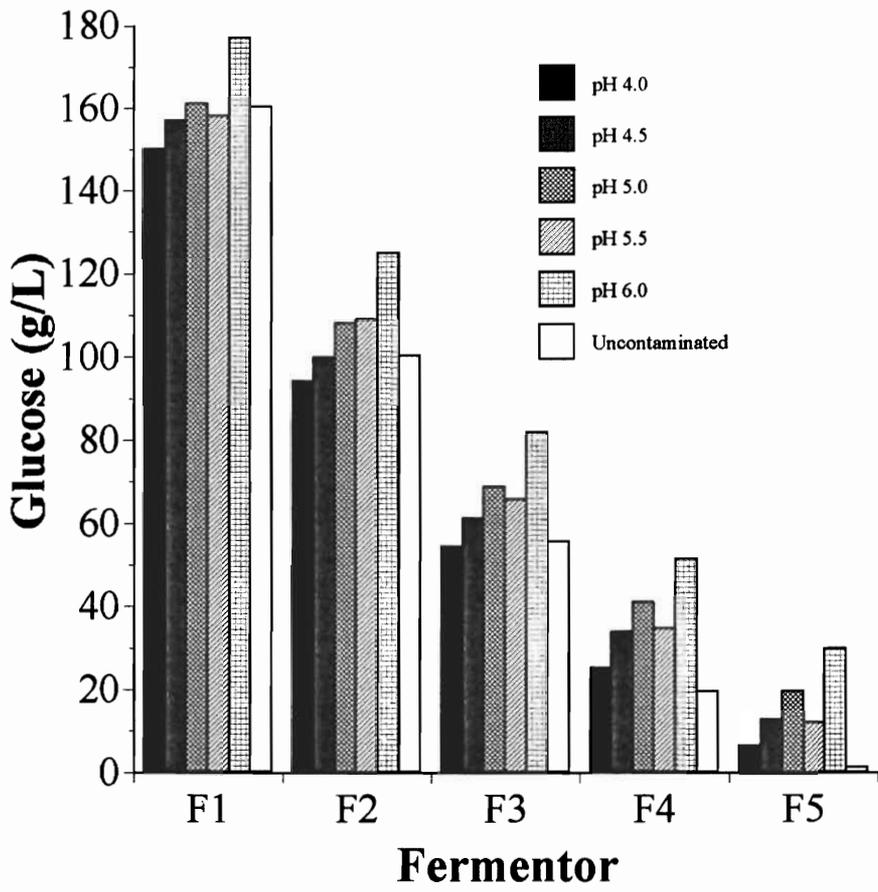
**Figure 4.47** Lactic acid concentrations in a pH-controlled mixed culture, multistage continuous culture fermentation (MCCF) system run at 260 g/L glucose.

increased from 4.0 to 6.0, the viable number of *L. paracasei* also increased. The average viable numbers of *L. paracasei* (CFU/ml) obtained at each pH value were:  $5.41 \times 10^8$  (pH 4.0),  $2.75 \times 10^9$  (pH 4.5),  $5.71 \times 10^9$  (pH 5.0),  $7.12 \times 10^9$  (pH 5.5), and  $4.75 \times 10^9$  (pH 6.0). At a pH of 5.5, more than a 13 fold increase in viable numbers of *L. paracasei* were seen as compared to a pH of 4.0. In Figure 4.47, the lactic acid concentration in each fermentor paralleled the growth of *L. paracasei* in Figure 4.46 - higher concentrations of lactic acid were found when higher numbers of *L. paracasei* were seen. Maximal concentrations of lactic acid in each fermentor were found at pH 5.5 where viable numbers of *L. paracasei* were also maximum. In the MCCF, lactic acid increased from F1 to F5 for all pH values where a maximum of 25 g/L was found in F5 at a pH of 5.5. This amount of lactic acid corresponds to a loss of 25 g/L ethanol since the glucose that *L. paracasei* utilized in producing lactic acid could have been used by *S. cerevisiae* to make ethanol. This increase in lactic acid occurred without the benefit of a net increase in *L. paracasei* (Fig. 4.46) - indicating that (except in F1) lactic acid production in the MCCF occurred by non-growing (but metabolically active) cells. As the pH increased from 4.0 to 6.0 in Figures 4.45, 4.46, and 4.47, the viable numbers of *L. paracasei* and the lactic acid concentration increased while a decrease in viable numbers of *S. cerevisiae* resulted.

Clearly the inhibition of *S. cerevisiae* at higher pH values was the result of the growth of *L. paracasei* and less likely the result of lactic acid production. By lowering the pH of the MCCF, higher viable numbers of *S. cerevisiae* and much lower viable numbers of *L. paracasei* are achieved.

#### **4.6.1.2 Glucose concentration.**

Figure 4.48 illustrates the steady-state glucose concentration in all fermentors in the MCCF using 260 g/L glucose in the incoming medium and when the pH was controlled in F1 from pH 4.0 to pH 6.0. Two trends are evident in the graph. First, as expected, the glucose concentration continually decreased in each MCCF from F1 to F5 with each pH control setpoint. This finding was previously demonstrated and discussed

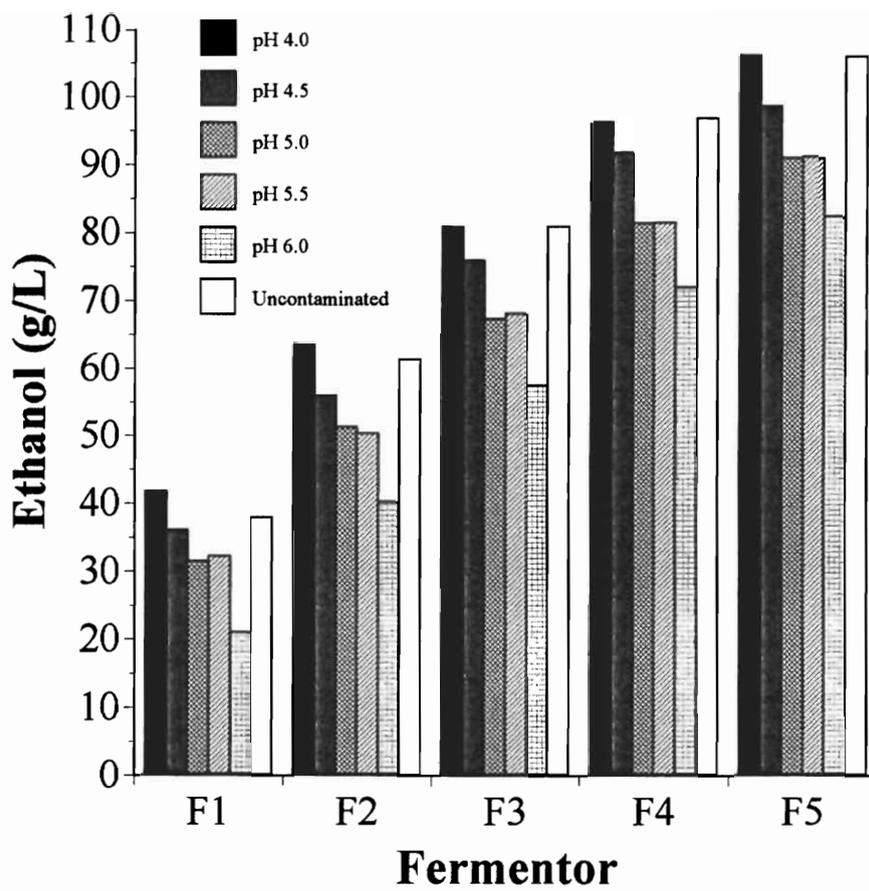


**Figure 4.48** Glucose concentrations in a pH-controlled mixed culture multistage continuous culture fermentation (MCCF) system run at 260 g/L glucose.

for Figure 4.11 where an MCCF was run without *L. paracasei* at different concentrations of glucose in the medium reservoir. Another trend observable in Figure 4.48 is that as the pH was increased from 4.0 to 6.0 in the contaminated runs with *L. paracasei*, the concentration of glucose in each fermentor increased as well. At a controlled pH of 4.0, the glucose concentration in each fermentor most closely matched the glucose concentration in the uncontaminated (control) run. This corresponded to the lowest viable numbers of *L. paracasei* (Fig. 4.48) and the lowest concentration of lactic acid (Fig. 4.47) in the MCCF. Thus, it appears that controlling the pH in the MCCF to a lower value is beneficial as more glucose is consumed by the yeast both because of lower viable numbers of *L. paracasei* and also greater viable numbers of *S. cerevisiae*. In the fifth fermentor, glucose was not totally exhausted from the medium at any pH. However, at a pH of 4.0, the glucose concentration in the effluent leaving F5 was 6.42 g/L, which was 5 fold greater than the concentration of glucose leaving the control without *L. paracasei*. At a pH of 6.0, the glucose concentration leaving the MCCF was 29.8 g/L which is ~ 25x the level leaving in the control. These increases in glucose concentration in F5 correspond to a theoretical loss of ethanol from the MCCF of 3.2 g/L (pH 4.0) and 15.2 g/L (pH 6.0). It is clear that since the pH within the range of 4.0 to 6.0 does not affect yeast growth, it remains that the increase in glucose concentration was a result of decreased numbers of viable *S. cerevisiae* due to the increased concentrations of *L. paracasei* and lactic acid with increasing pH. This provides research data to support the established industrial practice of lowering fermentor pH when bacterial contamination is noted in hopes of maintaining ethanol yields and controlling contamination

#### **4.6.1.3 Production of ethanol.**

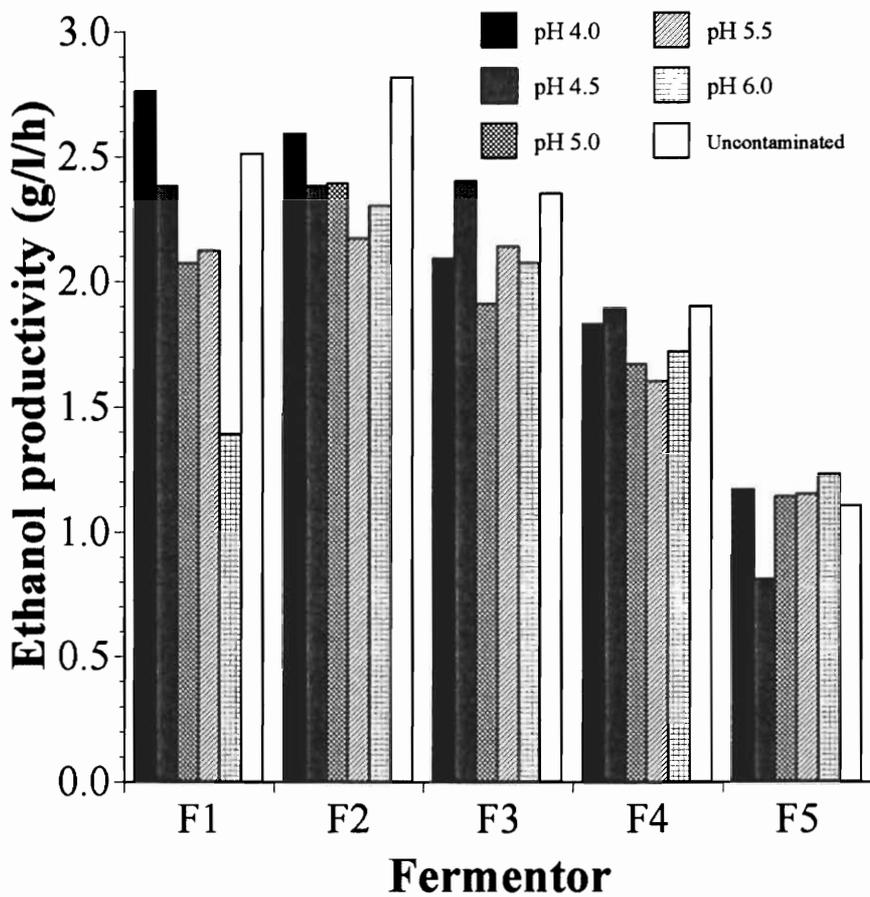
Figure 4.49 illustrates the steady-state ethanol concentration in all fermentors in the MCCF when the pH was controlled in F1 from pH 4.0 to pH 6.0. The controls (open bars, uncontaminated fermentations) show the ethanol concentrations in F1 to F5 at steady state. The shaded bars depict ethanol concentration production with the presence



**Figure 4.49** Ethanol concentrations in a pH-controlled mixed culture multistage continuous culture fermentation (MCCF) system run at 260 g/L glucose.

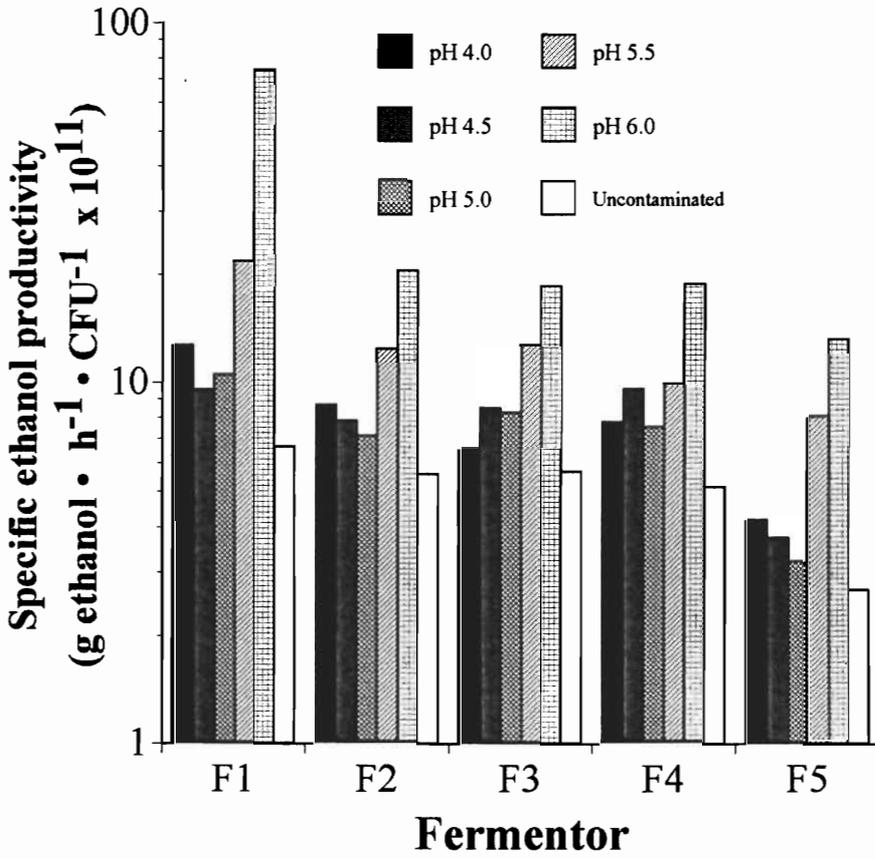
of *L. paracasei* at different pH values. The maximum ethanol concentration achieved was at pH 4.0 in F5 where 105 g/L was produced and is identical to the ethanol concentration achieved in the uncontaminated control. At this pH value, growth of *L. paracasei* is severely limited. This identical ethanol concentration in F5 at pH 4.0 and in the control MCCF experiments occurred even when the glucose concentration at pH 4.0 in F5 was 5.22 g/L higher than in the control. Thus, the yeast in F5 controlled at pH 4.0 must be utilizing nutrient sources other than glucose to produce the same amount of ethanol. One possibility is that the yeast may be scavenging nutrients from dead yeast cells in the later (more stressed) fermentors in the MCCF. The other possibility is that *L. paracasei* may be hydrolysing proteins found in the medium and thus providing the yeast with additional nutrient sources that normally cannot be utilized by the yeast. This may spare the yeast from converting glucose to amino acids, allowing more ethanol (more ATP) to be made via glycolysis. Further studies in the MCCF are needed to confirm these possibilities. In Figure 4.49, as the pH was increased from 4.0, the concentration of ethanol in the effluent of the MCCF as compared to the control was: 93% (pH 4.5), 86% (pH 5.0), 86% (pH 5.5), and 77% (pH 6.0). At a pH of 6.0, 2.4 g/L of ethanol was lost in the MCCF as compared to the control and at this pH value, growth of *L. paracasei* would be more permissive. It is clear that, in a MCCF contaminated with *L. paracasei*, controlling the pH to 4.0 in the first fermentor allowed the MCCF to produce ethanol at the same concentration as in the control and is an effective way to regain ethanol concentration when the MCCF is contamination by *L. paracasei*. The lower concentration of ethanol achieved at higher pH values is most likely due to the increased inhibition of *S. cerevisiae* by the increase in viable numbers of *L. paracasei* and the conversion of glucose (normally fermented to ethanol) to lactic acid by bacterial metabolism. This inhibition of growth is caused by the increased viable numbers of *L. paracasei* and by competitive consumption of nutrients and, to some extent, the production of lactic acid.

Figure 4.50 shows the ethanol productivity (g/L/h) in an MCCF that contained *S. cerevisiae* and *L. paracasei* and was pH controlled in F1 from 4.0 to 6.0. In general, the ethanol productivity of *S. cerevisiae* decreased from F1 to F5 for all pH values. With the



**Figure 4.50** Ethanol productivity (g/L/h) in a pH-controlled mixed culture multistage continuous culture fermentation (MCCF) system run at 260 g/L glucose.

exception of F1, the ethanol productivity in other fermentors had nearly the same value across the pH values tested. The average ethanol productivity of the pH controlled conditions decreased from ~2.4 g/L/h in F2 to ~1.1 g/L/h in F5. This indicates that the conditions are more favourable for ethanol production in the earlier fermentors than in the later fermentors in the MCCF. The fact that ethanol productivity is nearly constant in each fermentor across the pH values tested is remarkable considering that in most fermentors, the viable number of yeast declines by nearly a 1/3 (Fig. 4.45). The only explanation to explain why ethanol productivity can remain constant with decreasing yeast numbers is for each yeast to produce correspondingly more ethanol. This is exactly what is occurring as is evidenced in Figure 4.51 where the specific ethanol productivity is displayed. In Figure 4.51, the highest specific ethanol productivity occurred at pH 6.0 in each fermentor in the MCCF - even though at pH 6.0, the lowest *S. cerevisiae* and ethanol concentrations were found. In fact, the specific productivity of ethanol was better in all fermentors at all pH conditions in the presence of *L. paracasei* than with only *S. cerevisiae* in the MCCF control. This high specific productivity also occurred in an environment where *L. paracasei* and lactic acid concentrations were almost at their maximal values. This phenomenon was also seen in previous experiments where pure lactic acid was added to steady state yeast in continuous culture (Fig. 4.38). The most probable reason why an increase in specific productivity occurred with an increase in lactic acid concentration was discussed in Section 4.4.6. The results in Figures 4.45, 4.46, 4.47, 4.48, 4.49, and 4.50 clearly show that as the pH is controlled to different values in the MCCF, the populations of *S. cerevisiae* and *L. paracasei* change. At pH values approaching 4.0, *L. paracasei* is inhibited which allows for a higher viable population of *S. cerevisiae* to form which, in turn, produces higher amounts of ethanol. More complete utilization of glucose then takes place. In contrast, as the pH is raised to 6.0, the viable numbers of *L. paracasei* increase dramatically which inhibits yeast via the production of lactic acid and by competition for nutrients. The production of lactic acid also diverts a portion of the glucose in the medium away from ethanol production which reduces ethanol yield. For the fuel alcohol producer, the increased cost of pH control in an MCCF



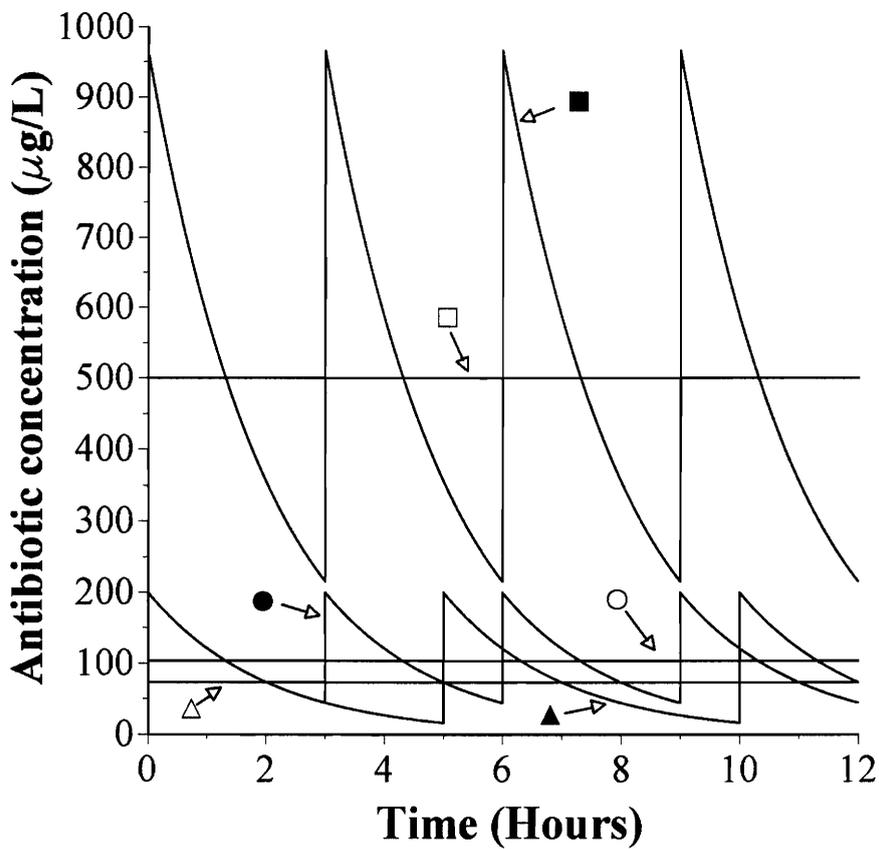
**Figure 4.51** Specific ethanol production (g/CFU/h) in a pH-controlled mixed culture multistage continuous culture fermentation (MCCF) system run at 260 g/L · glucose.

may not be justified if contamination is not present, but may be necessary once a "house flora" establishes itself in the system. If an MCCF system operating at a pH of 6.0 (uncontrolled) is contaminated, then a loss of up to 20 g/L ethanol can be realized. This loss can be potentially eliminated if the pH is controlled to 4.0. Lastly, it is clear from Figure 4.51 that it is not only possible to produce more ethanol by increasing the size of the population of *S. cerevisiae*, and also by either increasing the amount of more efficient ethanol-producing *S. cerevisiae* or by increasing the productivity of the established yeast cells.

#### **4.6.2 Control of *L. paracasei* by penicillin G.**

The strategy of adding an antibiotic to control bacterial contamination during fuel alcohol fermentations in batch mode is well established. Typically a single dose of a chosen antibiotic is added at the beginning of fermentation to combat bacterial contamination. The concentration of antibiotic will remain constant during the fermentation if the antibiotic does not naturally decay in the fermentor and if the population of organisms in the fermentor does not consume and/or degrade the antibiotic. In continuous culture, the situation is very different. If one adds a stable antibiotic at any time to a continuous culture, the concentration of antibiotic will immediately decrease from its concentration at the time of the addition. The rate of reduction of antibiotic under these conditions is based solely on the dilution rate of the fermentor. This may appear to be a disadvantage. However, there are novel methods of addition that arise as a result of the fermentation being in continuous mode. One method of addition involves maintaining the concentration of the antibiotic at a constant level (as is seen in batch). Unlike batch fermentations, the addition of antibiotic in continuous culture requires that the antibiotic be constantly added. An initial pulse of the antibiotic is required to bring the concentration of the antibiotic in the continuous fermentor to the desired concentration. Following this initial pulse, additional antibiotic must be added to match the dilution rate of the fermentor in order to maintain the concentration near the desired level. A second

method of addition involves varying the concentration of antibiotic over time in a pulsed cycle. Instead of maintaining the concentration of antibiotic at a fixed value, the decrease in concentration of the antibiotic due to the dilution rate is exploited. Furthermore, since this addition method is cyclical, the frequency of the pulses as well as the overall average concentration of the antibiotic during each pulse cycle can be varied. To illustrate the practical implications of this addition method, a sample calculation was performed and is shown in Figure 4.52. Here a single-stage CSTR operating at a dilution rate of  $0.5 \text{ h}^{-1}$  is pulsed with antibiotic with either changes to the frequency of the pulses or the magnitude of the pulses. When the simulated fermentor is pulsed to provide  $200 \text{ }\mu\text{g/L}$  antibiotic at 0 h and additional pulses every three hours, the overall average concentration (see Section 3.5.5.2) of the antibiotic between pulses is  $103.5 \text{ }\mu\text{g/L}$ . At a frequency of five hours, the same conditions yields an overall concentration of  $73.3 \text{ }\mu\text{g/L}$ . Thus, with all conditions being equal, a decrease in the pulse frequency results in a lower overall average concentration of antibiotic. If the pulse frequency is increased (theoretically approaching continuous addition), then the overall average concentration of antibiotic approaches  $200 \text{ }\mu\text{g/L}$  which essentially is a continuous addition of antibiotic. One crucial observation is that, with pulsed additions, there exists a time frame between pulses where the concentration of antibiotic is well above the concentration where constant addition of antibiotic is performed - even when the overall average concentrations of both types of addition are identical (*i.e.* the amount of antibiotic added in each mode is identical). This may prove to be an advantage in controlling a contaminant in continuous culture since the contaminant is exposed for a period of time to a higher concentration of antibiotic in pulsed additions as compared to constant additions in spite of the fact that both addition strategies add the same amount of antibiotic. Another observation is that in order for the overall average concentration of antibiotic to be equal for both the three and five h pulse frequency conditions that provide  $200 \text{ }\mu\text{g/L}$  antibiotic, the amount of antibiotic required in each pulse for the five hour frequency condition must be increased. Intuitively, one may reason that as the frequency is decreased, the concentration of the contaminant would also decrease since the concentration of antibiotic at the beginning of each pulse



**Figure 4.52** Simulated pulsed additions (1 minute durations) of an antibiotic with time to a single-stage continuous culture fermentor operating at a dilution rate of  $0.5 \text{ h}^{-1}$ . (■, concentration of antibiotic and □, overall average concentration of antibiotic in the fermentor with pulses that provide  $966 \text{ } \mu\text{g/L}$  every three hours; ●, concentration of antibiotic and ○, overall average concentration of antibiotic in the fermentor with pulses that provide  $200 \text{ } \mu\text{g/L}$  every three hours; ▲, concentration of antibiotic and △, overall average concentration of antibiotic in the fermentor with pulses that provide  $200 \text{ } \mu\text{g/L}$  every five hours).

would be greater than which is seen at higher frequencies. This may be especially effective in continuous culture where hitting the contaminant with a higher dose of antibiotic over the pulse interval would tend to wash out a greater proportion of cells. However, one must take into account that there exists a time frame between pulses where the concentration of antibiotic is lower than that where constant additions are done. This may provide an opportunity for the contaminant to reinitiate growth. Lower frequencies of addition would provide a longer time interval for growth. The effectiveness of pulsed additions of antibiotic may depend on the specific growth rate of the contaminant. The doubling time of the contaminant may be greater, equal to, or lower than the interval between pulses which may have consequences with respect to the biomass of the contaminant. Also, in some cases, the organisms present in the continuous fermentation may consume and/or degrade the antibiotic. In addition to changing the frequency of pulses in continuous culture, the overall average concentration of antibiotic for any frequency may be selected. This is also illustrated in Figure 4.52. The pulse additions providing 200  $\mu\text{g/L}$  every three h gave an overall average concentration of 103  $\mu\text{g/L}$ . Calculations have been performed that show that if a 500  $\mu\text{g/L}$  overall average concentration is desired for the same conditions, then the pulses additions must provide 966  $\mu\text{g/L}$  antibiotic at each pulse. Thus, the overall average concentration of antibiotic may be set to any desired value and the results can be compared to either constant addition of antibiotic in continuous culture or a constant concentration in batch culture. None of the issues discussed above in regards to antibiotic addition to continuous cultures has been reported in published work.

An additional concern arises with the addition of an unstable antibiotic. Many fuel alcohol plants use penicillin in batch fermentations at  $\sim 2475$  U/L. However, penicillin is an unstable antibiotic and decomposes at a rate that is determined by both temperature and pH (Kheirloom *et al.*, 1999). For this reason, any pulsed additions of penicillin to a continuous culture must take into account the decomposition rate of penicillin in order to accurately poise the concentration with each pulse. Furthermore, the concentration of penicillin in the penicillin stock reservoirs decreases due to decomposition. In short, in

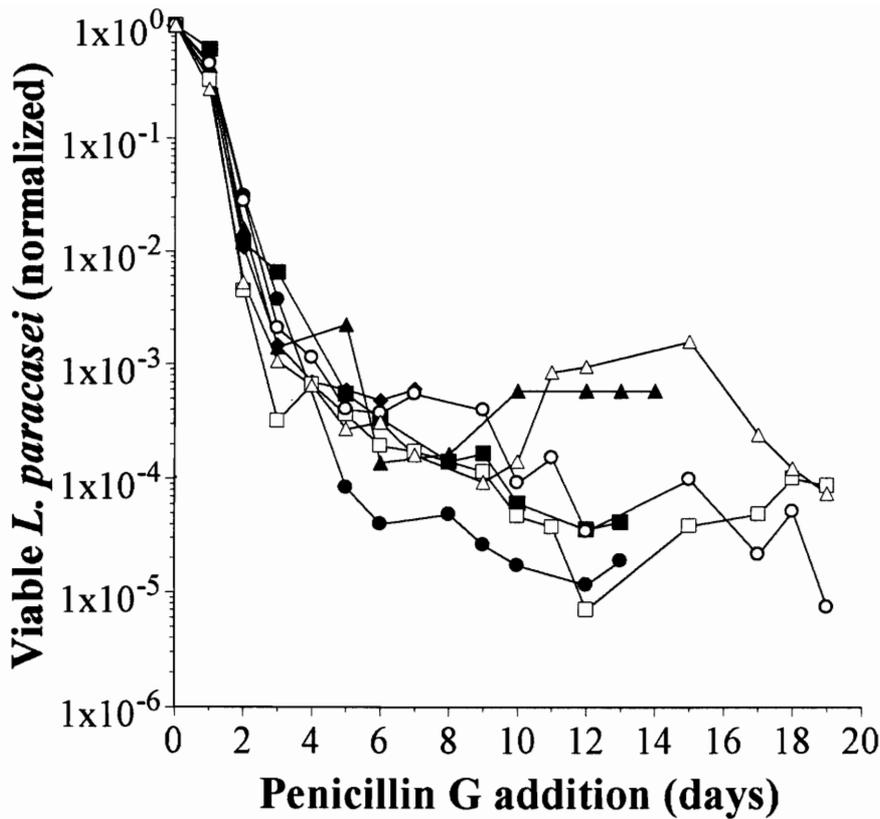
order to accurately pulse a continuous fermentor with penicillin, three decay rates must be known and balanced: the decay of penicillin in the penicillin reservoir at the reservoir pH and temperature, the decay of penicillin in the fermentor at the fermentor pH and temperature, and the "decay" of penicillin concentration in the fermentor due to dilution. The end result when one balances these decay rates is that a steadily increasing volume of stock penicillin solution must be added at each pulsed addition in order that the pulsed concentrations in the continuous fermentor are identical. Consequently, programmable pumps are essential to carry out additions of an unstable antibiotic to a continuous fermentor, and they must be programmed with suitable equations to add the required amounts of antibiotic at each pulse. These calculations have been performed and are introduced in the present work. They represent the first published report that penicillin G has been added to a continuous fuel alcohol fermentation in a pulsed mode to combat microbial contamination. Furthermore, no published data for any type of continuous fermentation were found to indicate frequencies or concentrations of pulsed mode additions to form a basis for the present work. The optimal strategy of addition of an antibiotic to a continuous culture fermentation to combat microbial contaminations has, in fact, not been addressed.

The following subsections highlight the results from experiments where penicillin G was added at different pulse frequencies and concentrations to F1 in the MCCF ( $D = 0.066\text{h}^{-1}$ ,  $28^{\circ}\text{C}$ ,  $260\text{ g/L}$  medium reservoir glucose concentration) where *L. paracasei* and *S. cerevisiae* were previously brought to steady state at a controlled pH of 5.5. A pH of 5.5 was chosen in the experiments since it was previously determined (Fig. 4.46) that maximum viable numbers of *L. paracasei* occurred at this pH value with the present experimental conditions. The experiments performed were very time consuming, complicated in setup, and challenging to maintain. Each condition required seven days to allow *S. cerevisiae* and *L. paracasei* to first reach steady state in the fermentor at pH 5.5, and a minimum of 20 additional days to track the condition once penicillin G addition was initiated. The setup of each F1 continuous fermentor required (in addition to previously described parameters) on-line pH controllers, computerized penicillin G addition, and

refrigerated penicillin G reservoirs (detailed in Section 3.5.5). Lastly, in addition to the normal daily sampling and analysis of each fermentor, the KOH and medium reservoirs needed to be re-filled more frequently since each of the five fermentors in the MCCF was operated in single-stage mode under F1 conditions only.

#### 4.6.2.1 Inhibition of *L. paracasei*.

Figure 4.53 represents the normalized viable numbers of *L. paracasei* in F1 in the MCCF (previously equilibrated with *S. cerevisiae* and *L. paracasei* at a controlled pH of 5.5) with penicillin G added continuously at 2475 U/L or in pulses of differing frequencies and concentrations. All additions of penicillin G decreased the viable number of *L. paracasei* in Figure 4.53 by a minimum of > three log units. It is interesting to note that only three conditions have appeared to reach (6 hour at 1237 U/L) or come close to (constant at 2475 U/L and six hour at 2475 U/L) steady state. The continuous addition at 2475 U/L and the six hour pulsed additions at 2475 U/L approached steady state after 12 days of treatment and resulted in viable numbers of *L. paracasei* of  $2.77 \times 10^5$  CFU/ml and  $1.02 \times 10^5$  CFU/ml. The pulsed addition at six hours at 1237 U/L resulted in a steady state of *L. paracasei* of  $5.00 \times 10^6$  CFU/ml after ten days of treatment. None of the other conditions appear to reach any steady state as large fluctuations of viable numbers were seen in these cases following ten days of treatment. It is clear that the viable number of *L. paracasei* decreased to a greater extent when additions of penicillin G were pulsed at a six hour frequency at 2475 U/L as compared to continuous addition at 2475 U/L. A 3.5 fold difference in viable numbers (day 12) is apparent between the two conditions. All other six hour pulsed treatments at overall average concentrations below 2475 U/L were less effective at reducing viable numbers than the continuous addition at 2475 U/L. Thus, by simply changing the mode of addition of penicillin G from continuous to pulsed (where the overall average concentration is identical in both treatments), a lower level of contamination can be realized. Clearly the mode of addition of an antibiotic holds some promise for more effective control of contaminants in fuel alcohol fermentations. Further



**Figure 4.53** Viable numbers of *L. paracasei* (normalized) in a MCCF ( $D = 0.066 \text{ h}^{-1}$ , 260 g/L glucose in the medium reservoir) plotted against time with various frequencies of addition and concentrations of penicillin G after prior equilibration of the MCCF with both *L. paracasei* and *S. cerevisiae* at a controlled pH of 5.5 (■, constant addition of penicillin G at a concentration of 2475 U/L; ●, six hour pulsed additions of penicillin G at an overall average concentration of 2475 U/L; ▲, six hour pulsed additions of penicillin G at an overall average concentration of 1237 U/L; ◆, six hour pulsed additions of penicillin G at an overall average concentration of 618 U/L; □, 12 hour pulsed additions of penicillin G at an overall average concentration of 2475 U/L; ○, 18 hour pulsed additions of penicillin G at an overall average concentration of 2475 U/L; △, 24 hour pulsed additions of penicillin G at an overall average concentration of 2475 U/L).

research is needed to further explore the effects of frequencies and concentrations of other effective antibiotics which are used in this industry.

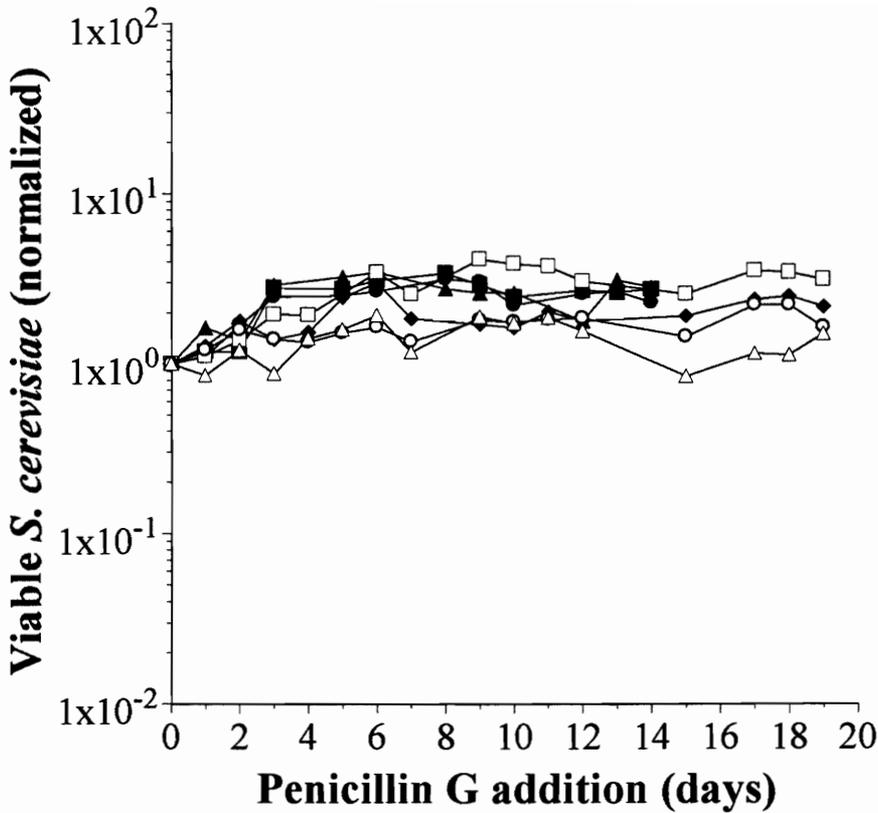
The frequency of antibiotic addition did not appear to effect the viable numbers of *L. paracasei*. A higher initial concentration of penicillin G at the beginning of pulses (where the pulse frequency was decreased) did not reduce the viable number of *L. paracasei* below the viable numbers seen with constant addition at 2475 U/L. At a pulsing interval of 24 hours where the overall average concentration of penicillin G was set to 2475 U/L, a penicillin G concentration of 6175 U/L was added at the beginning of each pulse. For 9.84 h following each pulse, *L. paracasei* would be exposed to a penicillin G concentration that is greater than 2475 U/L and at the end of the pulse period, a concentration of 664 U/L would be reached. In spite of these facts, the viable numbers of *L. paracasei* for this pulse frequency addition at 2475 U/L and other pulse frequencies at 2475 U/L (where the concentration of penicillin G is also high at the beginning of each pulse) did not decrease beyond the viable numbers seen for the constant addition at 2475 U/L. One possible reason for the lack of a further decrease in viable numbers of *L. paracasei* at lower pulse frequencies is that, at the time interval between pulses where the concentration of penicillin G is lower than in continuous addition, the growth of *L. paracasei* is more permissive. For example, for the 24 h pulse frequency, the initial 9.84 h of time has *L. paracasei* "growing" in concentrations greater than 2475 U/L while the remaining 14.16 h is spent at concentrations below 2475 U/L. Even though the initial concentration of 6175 U/L and the decrease in concentration to 2475 U/L may result in a large proportion of viable *L. paracasei* leaving the fermentor, the longer time interval at penicillin G concentrations below 2475 U/L may permit the remaining *L. paracasei* to multiply effectively. This needs to be researched further. As well, longer experimental treatment times are needed to confirm whether changes in pulse frequency at a constant overall average concentration of penicillin G would allow the viable numbers of *L. paracasei* to reach steady states, and if the viable numbers are significantly less than the viable numbers in constant addition.

The decrease in viable numbers of *L. paracasei* due to penicillin G control can be compared to conditions where pH control was utilized to reduce viable numbers of *L. paracasei*. In Figure 4.30, the viable number of *L. paracasei* reached a steady state value of  $3.7 \times 10^5$  CFU/ml when the pH of the MCCF "naturally" dropped to 3.2. This compares to  $1.02 \times 10^5$  CFU/ml with six hour pulsed additions of penicillin G at 2475 U/L (Fig. 4.53). Thus, a 3.2 fold decrease in viable numbers of *L. paracasei* was obtained with pulsed additions of an antibiotic as compared to the "natural" conditions found in MCCF under the described conditions. Although pH values lower than 3.2 in the MCCF would probably result in further reductions of *L. paracasei*, the growth of *S. cerevisiae* would also be affected.

The control of *L. paracasei* in the MCCF appears to be more effective when antibiotic is added rather than when the pH is lowered. However, for the fuel alcohol producer, the costs associated with pH control and penicillin addition may determine the choice in a particular method.

#### 4.6.2.2 Recovery of *S. cerevisiae*.

Figure 4.54 portrays the normalized viable numbers of *S. cerevisiae* in F1 plotted against time in the MCCF (previously equilibrated with *S. cerevisiae* and *L. paracasei* at a controlled pH of 5.5) when penicillin G was added continuously at 2475 U/L or in pulses of differing frequencies and concentrations. The initial viable number in F1 of *S. cerevisiae* in the treatments varied from  $1.5 \times 10^7$  to  $3.73 \times 10^7$  CFU/ml in spite of the care in fermentor setup and operation. For this reason, normalized viable numbers of *S. cerevisiae* were used when comparing the effects between penicillin G treatment conditions. As expected, the addition of penicillin G to the F1 fermentors, regardless of the mode of addition, did not cause a decrease in viability of *S. cerevisiae*. On the contrary, viable numbers of *S. cerevisiae* increased for all treatment conditions and in one case (12 hour pulsed additions at 2475 U/L) increased nearly 4 fold. A steady state viable cell number was not reached for any of the penicillin treatment conditions although



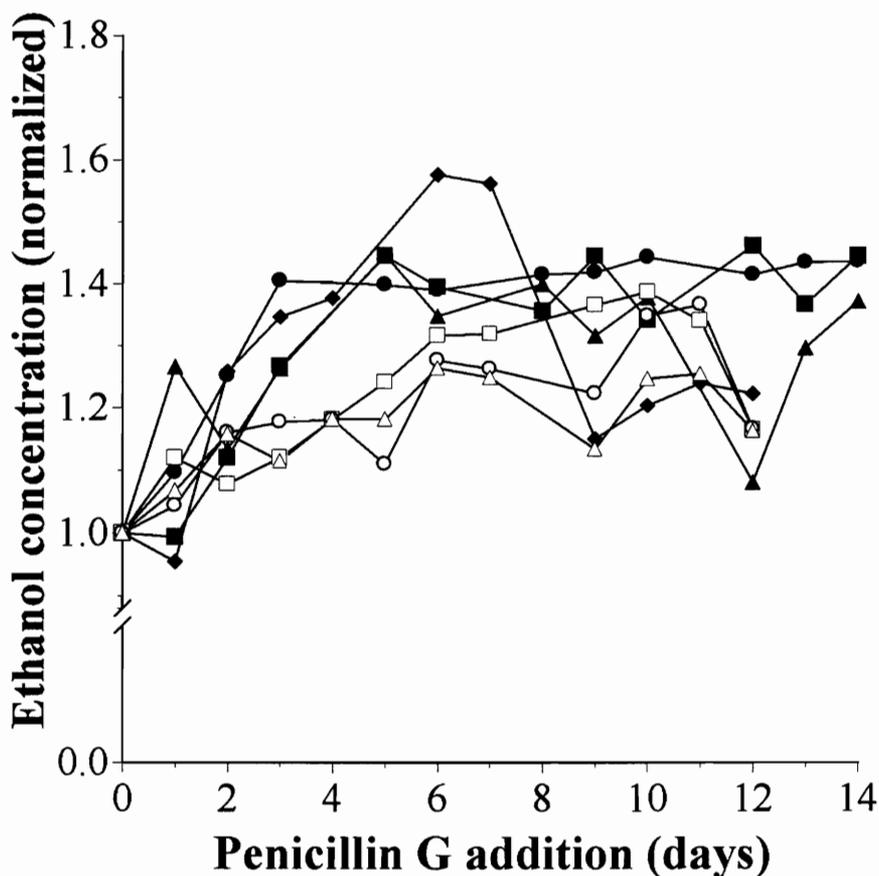
**Figure 4.54** Viable numbers of *S. cerevisiae* (normalized) in a MCCF ( $D = 0.066 \text{ h}^{-1}$ , 260 g/L glucose in the medium reservoir) plotted against time with various frequencies of addition and concentrations of penicillin G after prior equilibration of the MCCF with both *L. paracasei* and *S. cerevisiae* at a controlled pH of 5.5 (■, constant addition of penicillin G at a concentration of 2475 U/L; ●, six hour pulsed additions of penicillin G at an overall average concentration of 2475 U/L; ▲, six hour pulsed additions of penicillin G at an overall average concentration of 1237 U/L; ◆, six hour pulsed additions of penicillin G at an overall average concentration of 618 U/L; □, 12 hour pulsed additions of penicillin G at an overall average concentration of 2475 U/L; ○, 18 hour pulsed additions of penicillin G at an overall average concentration of 2475 U/L; △, 24 hour pulsed additions of penicillin G at an overall average concentration of 2475 U/L).

fluctuations in the number of viable *S. cerevisiae* were considerably smaller than that observed for *L. paracasei*. The inability of *S. cerevisiae* to reach a steady state was most likely due to the changing magnitude in the number of viable cells of *L. paracasei*. The viable numbers of *S. cerevisiae* for all penicillin G treatment conditions exceeded normal steady state viable numbers ( $3 \times 10^7$  CFU/ml) in the MCCF where *S. cerevisiae* and *L. paracasei* were allowed to equilibrate without pH control (Fig. 4.30). The average viable numbers of *S. cerevisiae* under each penicillin G treatment, although not in steady state, were at least 1.07 fold higher (6 hour pulse frequency at 618 U/L) and at most 2.36 fold higher (6 hour pulse frequency at 2475 U/L) than the steady state viable numbers of *S. cerevisiae* reached in Figure 4.30. Clearly, since the pH does not appear to significantly affect yeast growth (Fig. 4.32), the increase in viable numbers of *S. cerevisiae* with the penicillin G treatments over the viable numbers in a "naturally" contaminated MCCF must be due to factors other than pH. Many of the treatment conditions with penicillin G resulted in viable numbers of *S. cerevisiae* that also exceeded the steady state viable number of *S. cerevisiae* ( $4.13 \times 10^7$  CFU/ml) where *L. paracasei* was not added to a MCCF (Fig. 4.18). On average with 18 h pulse treatments at 2475 U/L, the viable numbers of *S. cerevisiae* were 1.45 fold greater than that seen in the MCCF. Other treatments increased the cell numbers 1.38 fold (constant addition at 2475 U/L), 1.72 fold (6 hour pulses at 2475 U/L), 1.47 fold (6 hour pulses at 1237 U/L), and 1.45 fold (18 hour pulses at 2475 U/L) (Fig. 4.18). One possible reason for increased numbers of *S. cerevisiae* in the penicillin treatments over what was observed under pH control may be that this control mechanism (penicillin G) is always in a state of flux in concentration. *L. paracasei* may need to divert a portion of its carbon and energy reserves and/or shift its metabolic pathways to try to maintain homeostasis under conditions where the control mechanism is constantly changing as compared to when the control mechanism is constant with pH control - where *L. paracasei* needs only to adapt once. Consequently, since *L. paracasei* cannot multiply to its full potential and its metabolic pathways may be in a constant state of "uncertainty", the growth of *S. cerevisiae* can thus increase. More research is needed to explore this possibility further.

#### 4.6.2.3 Recovery of ethanol.

Figure 4.55 represents the normalized ethanol concentrations produced by *S. cerevisiae* in F1 in the MCCF (previously equilibrated with *S. cerevisiae* and *L. paracasei* at a controlled pH of 5.5) with penicillin G added continuously at 2475 U/L or in pulses of differing frequencies and concentrations. As expected, since the initial viable numbers of *S. cerevisiae* in all penicillin G treatments were different, the initial concentrations of ethanol also varied. The initial concentration of ethanol for most penicillin G treatment conditions was between 35-38 g/L and overlapped the 37 g/L concentration obtained in the MCCF without *L. paracasei* (Fig. 4.13) - even when *L. paracasei* was at a maximum ( $> 4 \times 10^9$  CFU/ml) in the penicillin G treatments at 0 h. Curiously, as penicillin G addition was initiated for these conditions, the concentration of ethanol increased up to ~ 50 g/L in some treatments - which represents a 35% increase in ethanol concentration as compared to the 37 g/L achieved in the MCCF without *L. paracasei*. An increase in ethanol concentration was also achieved when *L. paracasei* and *S. cerevisiae* were allowed to equilibrate to a pH of 3.2 without any penicillin addition (Fig. 4.31). Here an increase of 13% is seen in the presence of *L. paracasei* as compared 37 g/L achieved in the MCCF without *L. paracasei*. Thus, for some reason, a higher yield of ethanol is achieved in conditions where penicillin G is used instead of pH to control viable numbers of *L. paracasei*. The most likely explanation to account for an increase in ethanol concentration under pH or penicillin G experiments is that, under these conditions, *L. paracasei* is providing additional nutrients to *S. cerevisiae* that could not normally be used by the yeast.

An examination of ethanol concentration between penicillin G treatment conditions reveals that all treatments led to increased amounts of ethanol. On average, the constant addition of 2475 U/L and the six hour pulsed addition at 2475 U/L resulted in a 40% increase in ethanol concentration - a result of the four log decrease in the viable numbers of *L. paracasei*. Although the level of *L. paracasei* decreased by more than 3 fold between six h pulsed additions and continuous additions of penicillin G at 2475 U/L,



**Figure 4.55** Ethanol concentration (normalized) over time in the MCCF ( $D = 0.066 \text{ h}^{-1}$ , 260 g/L glucose in the medium reservoir) with various frequencies of addition and concentrations of penicillin G after prior equilibration of the MCCF with both *L. paracasei* and *S. cerevisiae* at a controlled pH of 5.5 (■, constant addition of penicillin G at a concentration of 2475 U/L; ●, six hour pulsed additions of penicillin G at an overall average concentration of 2475 U/L; ▲, six hour pulsed additions of penicillin G at an overall average concentration of 1237 U/L; ◆, six hour pulsed additions of penicillin G at an overall average concentration of 618 U/L; □, 12 hour pulsed additions of penicillin G at an overall average concentration of 2475 U/L; ○, 18 hour pulsed additions of penicillin G at an overall average concentration of 2475 U/L; △, 24 hour pulsed additions of penicillin G at an overall average concentration of 2475 U/L).

there was no practical difference in either the final ethanol concentration or the relative recovery of ethanol when either condition was used.

#### **4.6.2.4 Production of lactic acid.**

Figure 4.56 represents the lactic acid concentration over time in F1 in the MCCF (previously equilibrated with *S. cerevisiae* and *L. paracasei* at a controlled pH of 5.5) when penicillin G was added continuously at 2475 U/L or in pulses of differing frequencies and concentrations. Fermentors with different concentration of penicillin G at time 0 had different initial concentrations of lactic acid. This was due to the different concentrations of viable *L. paracasei* in the penicillin G treatments as the equilibration of *S. cerevisiae* and *L. paracasei* (before addition of penicillin G) showed different steady states of viable bacteria. The reason for the initial difference in viable numbers is not known at this time. As expected (despite the initial difference in lactic acid concentration), the lactic acid concentration in all penicillin G treatment conditions decreased to a concentration of  $\sim 0.4$  g/L. This concentration of lactic acid matches the concentration of lactic acid that is normally found in the medium formulation used in this work, and thus no net production of lactic acid is seen over the penicillin G control even when viable numbers of *L. paracasei* were as high as  $1 \times 10^7$  CFU/ml. Any strategy which decreases lactic acid concentration is of benefit to fuel alcohol producers since a lower inhibition of *S. cerevisiae* occurs as a result of the lower concentration of lactic acid, and less substrate is used in the production of lactic acid.

#### **4.7 Mathematical modeling of MCCF fermentations.**

Many unstructured mathematical models exist for the prediction of biomass (X), substrate (S), and product (P) concentrations during the course of yeast-based fermentations. The unstructured models that have attracted the most attention in the published literature have been outlined and discussed in Section 2.4.1. For most of these

models, the authors have demonstrated a close relationship between experimental data and the data generated from their constructed models. Published information however is lacking on whether these and other unstructured models can be used to accurately predict X, S, and P concentrations in experiments other than those which were used to create the models. Marin (1999) examined and compared in detail many unstructured models used to predict yeast fermentations and concluded that a "universal" model does not exist. However, his examination (for the most part) was based on comparisons of the equations and the parameters used to make up the equations and not on a comparison of data generated from each of the models using identical parameter values. To complicate matters further, although most of the parameters used in unstructured models are well defined and considered "universal", the applicability of these parameter values has not been performed from model to model and raises the question of how accurately each model predicts experimental data using "universal" values. As well, the applicability of the models at different substrate concentrations and dilution rates when the values of constants are preserved across such conditions has not been researched. All of these models rely on a number of parameters which have standard definitions. Some of these parameters are "universal" ( $\mu_m$ ,  $K_s$ ) and appear in all the models discussed. Other parameters ( $K'_s$ ,  $K_{si}$ ,  $K'_{si}$ ,  $Y_{xs}$ ,  $Y_{ps}$ ,  $P_m$ ,  $P'_m$ ,  $v_m$ ) appear in most of the models while some parameters ( $X_m$ ,  $X_v$ ,  $m_s$ ) appear only in specific models.

A careful examination of each model in Section 2.4.1 yields information concerning the mathematical nature and type of inhibition that influences the yeast growth and ethanol production. However, this examination does easily yield information on how well the equations predict experimental steady states. Only by performing the necessary calculations can one conclude if the model accurately predicts experimental data. An important practical use of a mathematical model would be to predict the X, S, and P concentrations at steady state (and also, if possible, the course of fermentation) in a multistage continuous fermentation under VHG conditions. To this end, the unstructured mathematical models outlined in Section 2.4.1 were modelled using identical parameter values for each model. The following parameter values (and their sources) were used

(where applicable) in all models:

$\mu_m$	0.77 h <sup>-1</sup>	(Batch data, Section 3.4.2)
$K_s$	0.64 g/L	(Batch data, Section 3.4.2)
$K_{si}$	84.25 g/L	(Batch data, Section 3.4.2)
$P_m$	102.57 g/L	(Kalmokoff and Ingledew, 1985)
$P'_m$	165.69 g/L	(Kalmokoff and Ingledew, 1985)
$Y_{xs}$	0.05 g/g	(MCCF, Section 4.2.9)
$Y_{ps}$	0.406 g/g	(MCCF, Section 4.2.9)

In cases where parameter values were not known or a parameter was unique to a specific model, the Excel "Solver" function was used to determine values for the unknown parameters that resulted in calculated steady state values that most closely matched actual steady state values in F1 (Section 4.2). This analysis permits one to compare the accuracy of the data generated from each model to actual experimental data, to compare the generated data between models, to compare the accuracy of the data predicted for fermentors F2-F5 to actual experimental data, and to compare the data generated from each model to actual experimental results at different medium reservoir glucose concentrations (where only the dilution rate and the glucose concentration were changed in each model). In addition, the model that most closely matches the experimental steady state data in Section 4.2 can be potentially used as a basis for predicting steady states in multistage fermentation and also serve as basis for future research in modelling of multistage fermentations.

#### **4.7.1 Accuracy of different unstructured mathematical models.**

Table 4.6 shows the accuracy of predicted model biomass concentration data as compared to actual MCCF experimental data in Section 4.2. All models were calculated with the standard set of constants listed on page 235. For each model, any unknown constants were determined by "Solver" in Excel. Each cell in the table shows the %

**Table 4.6** Accuracy of different unstructured mathematical models in predicting steady state experimental MCCF biomass (X) concentrations at different medium reservoir glucose concentrations, and in different fermentors.

MR glucose concentration (g/L)	Fermentor	Mathematical Model							
		Aiba <i>et al.</i> (1968)	Dourado <i>et al.</i> (1987)	Ghose and Tyagi (1979a)	Jarzebski <i>et al.</i> (1989)	Lee <i>et al.</i> (1983)	Luong (1985) and Levenspiel (1980)	Monod (1949)	Sevely <i>et al.</i> (1980)
152	F1	-33.09	ns <sup>1</sup>	0	-87.52	-33.11	56.83	56.45	-100
	F2	-19.76	ns	-44.7	-88.06	-30.61	-100	16.68	-100
	F3	-7.26	ns	-57.11	-89.95	-30.19	-100	-2.01	-100
	F4	-12.42	ns	-64.72	-89.79	-30.07	-100	-12.37	-100
	F5	-5.94	ns	-65.09	-89.05	-18.94	-100	-6.04	-100
191	F1	34.31	ns	-1.64	-84.99	-6.89	112.75	112.62	-100
	F2	33.43	ns	-52.29	-88.38	-19.92	-100	37.79	-100
	F3	17.43	ns	-62.17	-89.96	-21.01	-100	17.38	-100
	F4	22.59	ns	-63.04	ns	-9.76	-100	22.58	-100
	F5	1.27	ns	-70.96	ns	-20.75	-100	1.29	-100
225	F1	76.06	ns	-74.33	-85.47	-0.79	142.26	138.94	-100
	F2	74.74	ns	-70.45	-88.02	-5.34	-99.94	75.46	-100
	F3	52.85	ns	-72.04	ns	-5.96	-99.49	52.81	-100
	F4	41.15	ns	-72.05	ns	-6.03	-99.95	41.07	-99.98
	F5	21.75	ns	-74.25	ns	-14.57	-99.96	21.71	-99.98
254	F1	131.44	ns	-83.74	-86.385	5.69	126.63	160.11	-100
	F2	78.75	ns	-82.87	ns	-7.47	-99.56	78.79	-100
	F3	60.35	ns	-78.73	ns	-6.61	-99.6	60.43	-99.98
	F4	48.37	ns	-75.86	ns	-7.53	-99.63	48.41	-99.96
	F5	43.83	ns	-73.59	ns	-6.56	-99.64	43.83	-99.94
312	F1	104	ns	-91.99	ns	-15.18	-1.3	104.48	-99.96
	F2	130.79	ns	-93.32	ns	15.53	-81.01	131.02	-99.92
	F3	144.94	ns	-75.71	ns	31.61	-79.87	145.15	-99.87
	F4	146.9	ns	-66.83	ns	36.26	-79.73	146.93	-99.84
	F5	163.61	ns	-61.07	ns	46.78	-78.37	163.4	-99.82

Accuracy (% deviation from actual value): □ 0-1, □ 1-50, ■ 50-100, ■ >100

<sup>1</sup> no solution

deviation from the actual experimental value and is shaded to reflect the magnitude of the deviation. None of the unstructured mathematical models predicted the biomass concentration in all of the test conditions to an accuracy of  $< 1\%$ . Furthermore, only the Ghose and Tyagi model predicted one of the experimental steady state values without any deviation (F1, 152 g/L). The model by Dourado was surprisingly unable to provide any values for any of the MR glucose concentrations even if "Solver" was utilized to determine the values of unknown parameters for sugar concentrations other than 152 g/L. This was puzzling since Dourado *et al.* (1987) had applied their equations to multistage fermentations with *S. cerevisiae* and showed an accurate trend of predicted data that followed experimental data. The reasons for the lack of values from the Dourado model in Table 4.6 is not known. The Jarzebski model also failed to provide results in some fermentors (particularly as the MR glucose concentration was increased). As in the Dourado model, no explanation can be found for the lack of values. The model by Sevely *et al.* (1980) showed the worst accuracy of prediction. Most of the biomass concentration predicted had a deviation that was essentially 100%. Thus, this model has limited prediction ability for fermentations in MCCF. In contrast, using the same parameter values shared by all models, the model by Lee *et al.* (1983) most accurately predicted the biomass concentration in the MCCF in all fermentors and at all MR glucose concentrations. At worst, the model predicted that the biomass concentration was 46.78% of the actual experimental value in F5 at a 312 g/L concentration of glucose. Most of the predicted values deviated from the actual experimental values by 1-50% and only one predicted value had a deviation of  $< 1\%$ .

In general for most of the models, the accuracy of predicted concentrations from F1-F5 at each MR glucose concentration did not change. The Aiba model at the 225 and 254 g/L MR glucose concentrations showed the greatest change in accuracy. At the 254 g/L MR glucose concentration, the Aiba model predicted data that resulted in a deviation from actual experimental data of 131.44% in F1 that decreased to  $< 50\%$  in F4 and F5. The accuracy of predicted concentrations in some models changed as the MR glucose concentration was changed. As the MR glucose concentration increased, the Aiba model

predicted data in F1 to F5 that resulted in larger deviations. At the 152 g/L MR glucose concentration, the predicted biomass concentration in the Aiba model deviated by a maximum of 33% while at the 312 g/L MR glucose concentration, the deviation from experimental data was greater than 100%. In the Luong and Levenspiel model, the accuracy of predicted values in F1 to F5 increased as the MR glucose concentration increased. At the 152 g/L concentration of glucose, the % deviation from actual experimental data was, for the most part, 100% which decreased to below 81% at 312 g/L.

Table 4.7 shows the accuracy of predicted model substrate (glucose remaining) concentration data as compared to actual MCCF experimental data in Section 4.2. In general, the accuracy of substrate (glucose) prediction was poorer for all models than the predictions made for biomass (Table 4.6) and product (Table 4.8). The models which predicted substrate concentration with the most accuracy was the Aiba and the Ghose and Tyagi models. The Aiba model most accurately predicted substrate concentration in all fermentors at lower MR glucose concentrations while the Ghose and Tyagi model most accurately predicted substrate concentration at higher MR glucose concentrations. The Lee *et al.* model, which most accurately predicted biomass concentration in Table 4.6, overestimated the substrate concentration in most conditions. Many test conditions in the Lee *et al.* model resulted in an accuracy which deviated by over 500% - one condition deviated from actual experimental data by 9490%! The models which showed the poorest accuracy of prediction for substrate were the Luong and Levenspiel, Lee *et al.*, and the Monod models. Although some of the models predicted substrate concentration better than others, none of the models tested were satisfactory in predicting in all cases the substrate concentration in the MCCF at different MR glucose concentrations and in different fermentors. The prediction accuracy in the Aiba and the Lee *et al.* models could be improved. One potential area of improvement in both models would be to modify the equation defining substrate utilization. In both models, the equation governing substrate utilization (Equation 2.27 for the Aiba model and Equation 2.29 for the Lee *et al.* model) is linked exclusively to growth which means that substrate

**Table 4.7** Accuracy of different unstructured mathematical models in predicting steady state experimental MCCF substrate (S) concentrations at different medium reservoir glucose concentrations, and in different fermentors.

MR glucose concentration (g/L)	Fermentor	Mathematical Model							
		Aiba <i>et al.</i> (1968)	Dourado <i>et al.</i> (1987)	Ghose and Tyagi (1979a)	Jarzebski <i>et al.</i> (1989)	Lee <i>et al.</i> (1983)	Luong (1985) and Levenspiel (1980)	Monod (1949)	Sevely <i>et al.</i> (1980)
152	F1	0	ns <sup>1</sup>	-98.84	-0.8	-0.04	-89.71	-99.76	-0.04
	F2	-1.2	ns	-99.98	18.08	28.09	-100	-100	6.42
	F3	-52.31	ns	-100	72.08	153.19	-100	-100	-11.45
	F4	-62.65	ns	-100	775.19	6984.06	-100	-100	-100
	F5	-0.1	ns	-100	0	0	-100	0	0
191	F1	-41.07	ns	-99.15	-18.92	-10.39	-96.78	-99.91	-28.06
	F2	-91.4	ns	-99.98	-24.75	13.51	-100	-100	-59.6
	F3	-99.81	ns	-100	-62.38	100.67	-100	-100	-100
	F4	-100	ns	-100	ns	833.095	-100	-100	-100
	F5	-100	ns	-100	ns	9490.44	-100	-100	-100
225	F1	-59.61	ns	-95.67	-26.42	-10.67	-10.17	-99.94	-39.78
	F2	-98.99	ns	-99.93	-45.92	6.02	-100	-100	-85.25
	F3	-99.98	ns	-100	ns	55.46	-100	-100	-100
	F4	-100	ns	-100	ns	207.88	-100	-100	-100
	F5	-100	ns	-100	ns	3627.18	-100	-100	-100
254	F1	-82.46	ns	-76.94	-33.13	-5.89	245.39	-99.96	-52.11
	F2	-99.83	ns	-99.64	ns	22.23	-100	-100	-100
	F3	-100	ns	-100	ns	91.05	-100	-100	-100
	F4	-100	ns	-100	ns	391.93	-100	-100	-100
	F5	-100	ns	-100	ns	7318.32	-100	-100	-100
312	F1	-99.54	ns	-28.53	ns	19.2	547.1	-99.98	-100
	F2	-100	ns	-97.57	ns	85.28	-100	-100	-100
	F3	-100	ns	-99.93	ns	326.09	-100	-100	-100
	F4	-100	ns	-100	ns	2311.8	-100	-100	-100
	F5	-100	ns	-100	ns	4468.81	-100	-100	-100

Accuracy (% deviation from actual value): □ 0-1, ▤ 1-50, ▥ 50-100, ▦ >100

<sup>1</sup> no solution

is only consumed with yeast cells that are multiplying. However, it is well known that yeast cells can consume substrate and produce alcohol when not multiplying. This fact is reflected in the model by Sevely (Equation 2.20) where the consumption of substrate is dependant on growth and also on the production of ethanol. These changes may increase the accuracy of prediction of substrate in both these models but as each set of equations in each model are interdependent, one cannot easily foresee how these changes would affect the final predicted concentrations once the models are run.

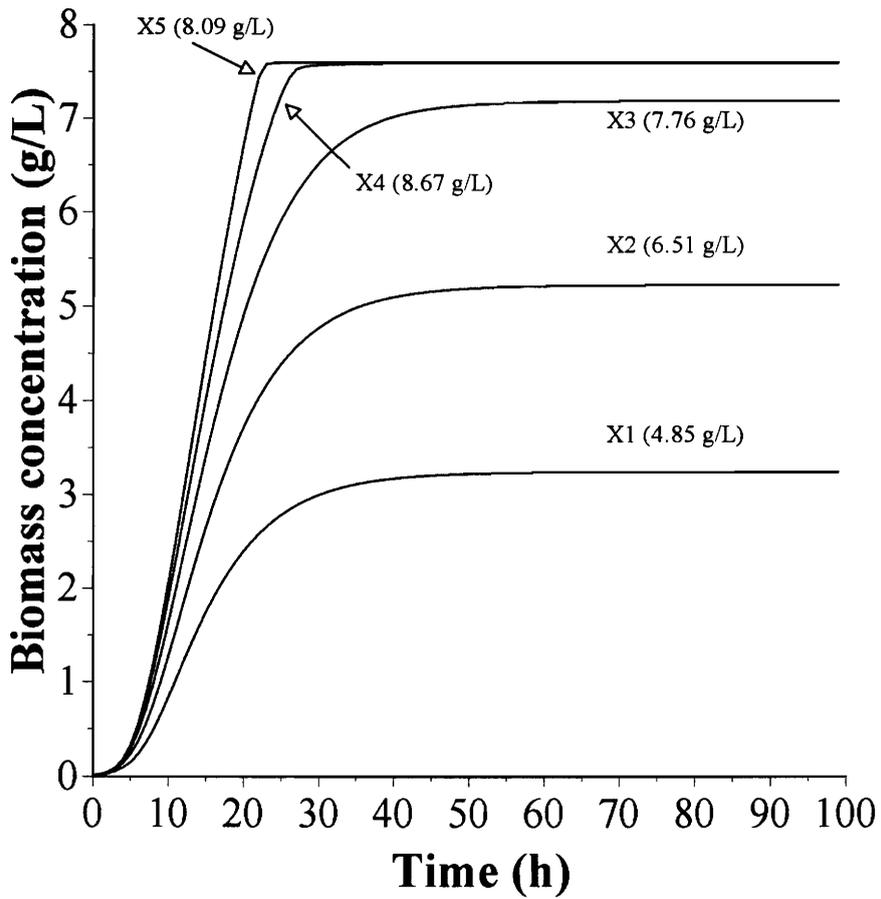
Table 4.8 shows the accuracy of predicted model product (ethanol) concentration data as compared to actual MCCF experimental data in Section 4.2. In contrast to the predicted concentrations of biomass and substrate, all of the models were comparatively better in predicting product concentration data than biomass or substrate concentrations from the MCCF experimental data. The models which best predicted ethanol concentrations were the Aiba and the Lee *et al.* models. The Lee *et al.* model consistently predicted ethanol concentrations that resulted in deviations from experimental data that were below 50%. The Aiba model, for the most part, also showed the same amount of deviation. Further refinements to the equations governing ethanol production in both models (Equation 2.26 for the Aiba model and Equation 2.30 for the Lee *et al.* model) may lead to more accurate prediction. These equations link ethanol production exclusively to growth. As ethanol is also made by non-proliferating cells, an additional term in these equations that incorporates ethanol production by non-proliferating cells would be the most obvious place for improvement. The equation governing ethanol production in the Sevely model (Equation 2.21) is the only model listed which attempts to include a term for ethanol production other than what is produced by proliferating cells. As was discussed earlier, changes to one equation in a model affects all the other equations in a model since the equations are not operating in isolation, but are interdependent. Thus, only by re-running a model that has any modifications can one see what effect the modifications have on predicted values.

From the data presented in Tables 4.6, 4.7, and 4.8, the model which best appeared to predict the data in the MCCF is the Aiba model. The accuracy of prediction

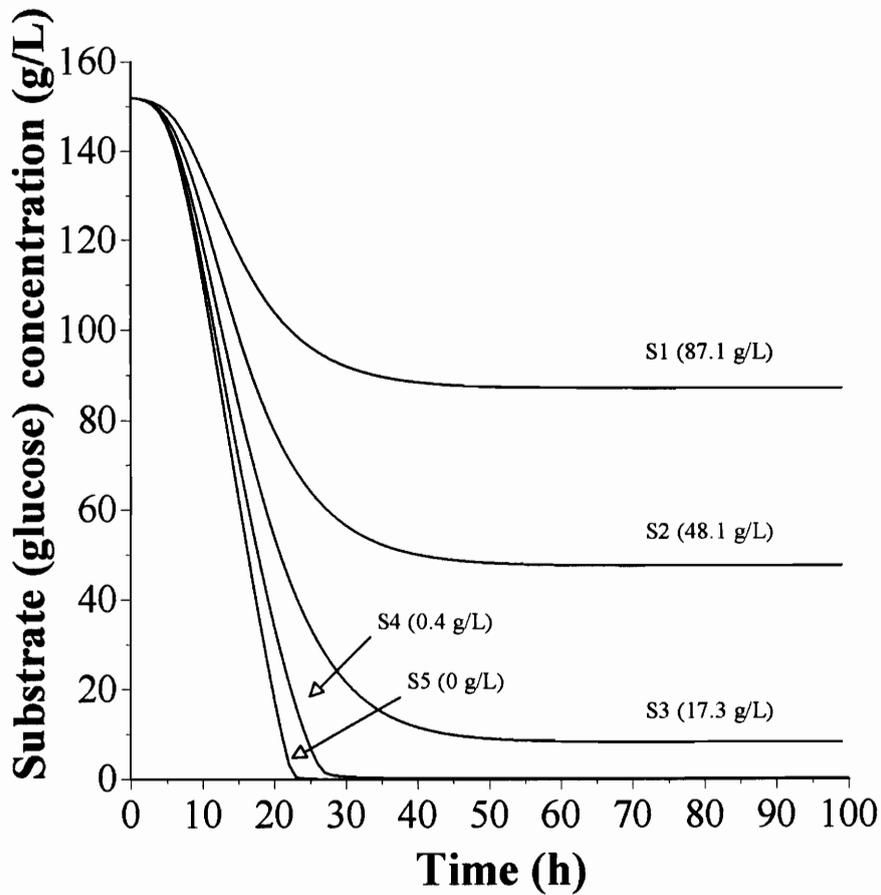
in this model was highest at lower MR concentration of glucose which decreased (particularly for biomass) as the glucose concentration was raised. A very good agreement between predicted values and experimental data was found for product concentrations at the 152 g/L concentration of glucose. The largest deviation from experimental values was 5% which makes the model more attractive for use in industry. At worst, the model predicted a deviation of 163.61% for biomass from actual steady state experimental data in F5 at a glucose concentration of 312 g/L. While this magnitude of deviation is small as compared to other models tested, the model requires further refinement in order for it to more accurately predict production steady states in industry. Further potential refinements to the Aiba model (other than what was discussed earlier) are possible. The determination of constants used in the model can be made for each fermentor in the MCCF rather than applying identical constant values across all fermentors. Since each fermentor produces unique steady state values for X, S, and P, it is reasonable to assume that the values for constants would be different in each fermentor.

#### **4.7.2 MCCF modeling using the Aiba model.**

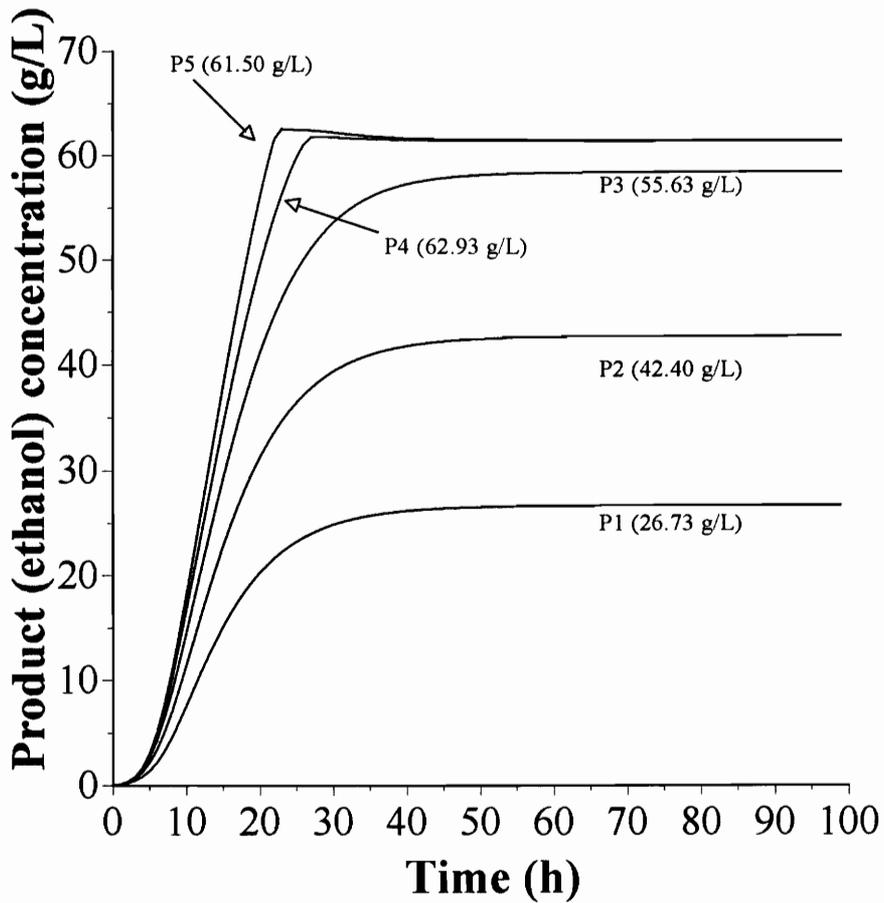
The Aiba model can be used to predict the X, S, and P concentrations during the course of fermentation and are depicted in Figures 4.57, 4.58, and 4.59 respectively. The model was fitted with data obtained at a run with a dilution rate of  $0.34 \text{ h}^{-1}$  and a MR glucose concentration of 152 g/L which provided the most accurate predictions. Similar calculated runs could be performed for the other concentrations of glucose used in the MCCF. For each predicted trendline, the corresponding steady state experimental concentration is indicated in parenthesis. As expected the predicted values for X, S, and P follow the trends that are normally seen for fermentations involving yeast. The Aiba model predicted in Figures 4.57, 4.58, and 4.59 that an overall steady state for X, S, and P would be reached in  $\sim 80$  hours. In reality, steady state was achieved in the MCCF (for a dilution rate of  $0.34 \text{ h}^{-1}$  and a MR glucose concentration of 152 g/L) in seven days (168



**Figure 4.57** Predicted biomass concentration in all fermentors in the MCCF based on the Aiba model. The model was run at a MR glucose concentration of 152 g/L and a dilution rate of  $0.34 \text{ h}^{-1}$ . Values in parenthesis indicate actual experimental steady state values in the MCCF.



**Figure 4.58** Predicted substrate (glucose) concentration in all fermentors in the MCCF based on the Aiba model. The model was run at a MR glucose concentration of 152 g/L and a dilution rate of 0.34 h<sup>-1</sup>. Values in parenthesis indicate actual experimental steady state values in the MCCF.



**Figure 4.59** Predicted product (ethanol) concentration in all fermentors in the MCCF based on the Aiba model. The model was run at a MR glucose concentration of 152 g/L and a dilution rate of 0.34 h<sup>-1</sup>. Values in parenthesis indicate actual experimental steady state values in the MCCF.

h). Thus, the Aiba model predicts faster kinetics (~2 fold) than what is seen in the experimental MCCF. This limits the usefulness of this model in the present work to be used as a benchmark during the course of a MCCF fermentation. The ability to predict real-time X, S, and P concentrations would be of great utility in industry. Fermentation data could be compared to predicted data to see if any deviations exist and could indicate a problem early on in the fermentation. Proactive measures could then be made to correct the fermentation at an early stage. Further refinements to the Aiba model may allow closer agreement in the fermentation time course of an MCCF.

## 5 CONCLUSIONS

It seems clear from this work that a coupling of very high gravity (VHG) fermentation to multistage continuous culture fermentation (MCCF) systems is feasible. Currently, multistage continuous culture fermentations are utilized in a number of continuous fuel ethanol production plants in North America and produce 10 - 12% v/v ethanol (personal communication). The 17% v/v ethanol concentration shown in this work represents a 42% increase in ethanol production over the maximum amount currently produced. Approximately 80% of the theoretical maximum ethanol conversion was achieved in the MCCF across all medium reservoir (MR) conditions and fermentors. Modifications to the base multistage system such as cell recycle and/or simultaneous saccharification and fermentation (SSF) may permit higher ethanol concentrations to be reached.

Glucose was almost totally consumed within the MCCF at all MR glucose concentrations. A maximum of 0.97% glucose remained unused by F5 in all conditions. The MCCF can be operated with incoming glucose concentrations up to 320 g/L which indicates that the MCCF system is capable of operating under VHG conditions.

Yeast cell numbers (total and viable) for most MR conditions increased in the MCCF from F1 to F2 and then remained nearly constant for the rest of the fermentors. With 312 g of glucose /L, the cell number attained was only half that observed with lower glucose concentrations. This was the only condition that showed a constant decrease in the number of viable cells. More than 70% of the yeast remained viable by F5 in all MR conditions except with the highest glucose concentration (312 g/L) where only 35% of the yeast were viable.

Ethanol fermentations were conducted in batch, single-stage continuous stirred tank reactor (CSTR), multistage CSTR, and in a *Lactobacillus*-contaminated fermentor that corresponded to the first fermentor in the multistage CSTR system. Using a glucose concentration of 260 g/L in the medium, the highest ethanol concentration reached was in batch (116 g/L), followed by the multistage CSTR (106 g/L), and the single-stage CSTR continuous production systems (60 g/L). The highest ethanol productivity at this sugar concentration was achieved in the multistage CSTR system where a productivity of 12.7 g/L/h was seen. The other fermentation systems in comparison did not exceed an ethanol productivity of 3 g/L/h. Thus, ethanol fermentation in multiple stages (having a total equivalent working volume of a single stage) permits a much higher ethanol productivity (over 4x) than can be achieved in single-stage CSTR or a batch fermentation.

*Lactobacillus paracasei* was introduced as a contaminant into a multistage continuous culture ethanol fermentation at ratios of 1:100, 1:1, and 70:1 with *S. cerevisiae*, but failed to overtake the yeast as is normally seen in industry. 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  $\sim 3 \times 10^7$  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 prior to bacterial inoculation. However, *L. paracasei* decreased steadily from its initial inoculation level of  $\sim 2.2 \times 10^9$  CFU/ml and stabilized at  $3.7 \times 10^5$  CFU/ml after ten 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 from the fermentation train. However, when the pH was controlled at values higher than pH 4, changes in steady states were immediately seen. Ethanol concentrations decreased by 44% after four days at a pH of 6.0. This coincided with an increase in *L. paracasei* to  $> 10^{10}$  CFU/ml, and a 4 fold increase in lactic acid concentration to 20 g/L. The viable numbers of *S. cerevisiae* also continually decreased during this time from its steady state

value by 83%. This decrease was most likely due to the production of lactic acid by *L. paracasei* (yeast is sensitive to ~5 g/L) and due to depletion of nutritional components by competition by *L. paracasei*. It was not possible in this work to separate the effects of both mechanisms on the growth of *S. cerevisiae* or to confirm the magnitude of inhibition by both mechanisms.

Pure lactic acid was added to batch VHG fermentations and to continuous VHG fermentations equilibrated to steady state with *Saccharomyces cerevisiae*. In continuous fermentations, a 53% reduction in growth of *S. cerevisiae* was seen at an undissociated lactic acid concentration of 3.44% w/v, and a 99.99% reduction at 5.35% w/v. The difference in yeast cell number in these fermentations was probably not due to pH since the pH changed a maximum of only 0.2 units from the control. Similar fermentations performed in batch showed that growth inhibition was nearly identical which indicates that the apparently high resistance of *S. cerevisiae* to lactic acid in continuous VHG fermentations is not due to differences in culturing. Although the total amount of ethanol decreased from 48.7 to 14.5 g/L when 4.74% w/v undissociated lactic acid was added, the specific ethanol productivity increased ~3.2 fold from  $7.42 \times 10^{-7}$  to  $24.0 \times 10^{-7}$  g ethanol per L medium per CFU which indicated that lactic acid stress actually improved ethanol production on a viable cell basis.

The level of contamination by *L. paracasei* in the MCCF was reduced by the application of pH control. In MCCF fermentations where the pH was controlled from 4.0 to 6.0, the viable numbers of *L. paracasei* was reduced from a maximum of  $7.12 \times 10^9$  CFU/ml (pH 5.5) to  $5.41 \times 10^8$  (pH 4.0). The viable number of *S. cerevisiae* in these fermentations showed an inverse relationship to the viable number of *L. paracasei* -  $2.3 \times 10^7$  CFU/ml of yeast were seen at pH 4.0 while  $\sim 1 \times 10^7$  CFU/ml was seen as the pH reached 6.0. At a pH of 4.0, the viable number of *L. paracasei* was approximately 200 fold higher than that of *S. cerevisiae*. It was determined in batch that the growth of *S. cerevisiae* is not sensitive to pH when the pH was between 4.0 and 6.0. Thus, the

decrease in viable numbers of *S. cerevisiae* must be directly due to the effects caused by the growth and lactic acid production of *L. paracasei*. In these fermentations, the maximum ethanol concentration achieved was at pH 4.0 in F5 where 105 g/L was produced and is identical to the ethanol concentration achieved in the uncontaminated control. As the pH was increased from 4.0, the concentration of ethanol leaving the MCCF as compared to the uncontaminated control was: 93% (pH 4.5), 86% (pH 5.0), 86% (pH 5.5), and 77% (pH 6.0). It seems clear that, in a MCCF contaminated with *L. paracasei*, controlling the pH to 4.0 in only the first fermentor allowed the MCCF to produce ethanol at the same concentration as in the control and is an effective way to regain ethanol concentration when the MCCF is contaminated by *L. paracasei*. This has been empirically seen in industry where the technique is often used to eliminate the effects of a high bacterial population.

The level of contamination by *L. paracasei* in the MCCF was also reduced by the introduction of penicillin G in the MCCF. Constant as well as variable concentrations of penicillin G were tested to determine their effectiveness at controlling contamination by *L. paracasei*. All additions of penicillin G decreased the viable number of *L. paracasei* by at least 3 log units. It is clear that the viable number of *L. paracasei* decreased to a greater extent when additions of penicillin G were pulsed at a six hour frequency at an overall average concentration of 2475 U/L as compared to continuous addition at 2475 U/L. A 3.5 fold difference in viable numbers is apparent between the two conditions. Changes in the frequency of antibiotic addition did not appear to effect the viable numbers of *L. paracasei*. Thus, by simply changing the mode of addition of penicillin G from continuous to pulsed (where the overall average concentration is identical in both treatments), a lower level of contamination can be realized. As expected, the addition of penicillin G to the F1 fermentors, regardless of the mode of addition, did not cause a decrease in viability of *S. cerevisiae*. Rather, viable numbers of *S. cerevisiae* increased for all treatment conditions and in one case (12 hour pulsed additions at 2475 U/L) increased by nearly 4x. An examination of ethanol concentration between penicillin G treatment

conditions reveals that all penicillin G treatments led to recovery of ethanol. On average, the constant addition of 2475 U/L and the six hour pulsed addition at 2475 U/L resulted in a 40% increase in ethanol concentration over MCCF fermentations where *L. paracasei* was present. The control of *L. paracasei* in the MCCF appears to be more effective when antibiotic is added rather than when the pH is lowered. The viable number of *L. paracasei* reached a steady state value of  $3.7 \times 10^5$  CFU/ml when the pH of the MCCF "naturally" dropped to 3.2 whereas the viable number of *L. paracasei* decreased to  $1.02 \times 10^5$  CFU/ml with six hour pulsed additions of penicillin G at 2475 U/L. Thus, a 3.2 fold decrease in viable numbers of *L. paracasei* was obtained with pulsed additions of an antibiotic as compared to the "natural" conditions found in MCCF. Clearly the mode of addition of an antibiotic holds promise for more effective control of contaminants in fuel alcohol fermentations. Further research is needed to further explore the effects of frequencies and concentrations of other effective antibiotics which can be used in this industry.

A number of unstructured mathematical models were applied to the fermentations in the MCCF to determine the accuracy of each to actual experimental data and to predict the course of fermentation in a MCCF. None of the unstructured mathematical models predicted the biomass, substrate, or product concentrations in all of the test conditions to an accuracy of < 1%. Using identical parameter values in all models, the model by Lee *et al.* (1983) most accurately predicted the biomass concentration in the MCCF in all fermentors and at all MR glucose concentrations. At worst, the model predicted that the biomass concentration was 46.78% of the actual experimental value in F5 at a 312 g/L concentration of glucose. Most of the predicted values deviated from the actual experimental values by 1-50% and only one predicted value had a deviation of < 1%. The models which predicted substrate concentration with the most accuracy were the Aiba and the Ghose and Tyagi models. The Aiba model most accurately predicted substrate concentration in all fermentors at lower MR glucose concentrations while the Ghose and Tyagi model most accurately predicted substrate concentration at higher MR glucose

concentrations. The models which best predicted ethanol concentrations were the Aiba and the Lee *et al.* models. The Lee *et al.* model consistently predicted ethanol concentrations that resulted in deviations from experimental data that were below 50%. The Aiba model, for the most part, also showed the same amount of deviation. In general, the model which best predicted biomass, substrate and product concentrations in the MCCF was the Aiba model. The accuracy of prediction in this model was highest at lower MR concentration of glucose which decreased (particularly for biomass) as the glucose concentration was raised. A very good agreement between predicted values and experimental data was found for product concentrations at the 152 g/L concentration of glucose. The largest deviation from experimental ethanol values was 5% which makes the model more attractive for use in industry. At worst, the model predicted a deviation of 163.61% for biomass from actual steady state experimental data in F5 at a glucose concentration of 312 g/L. While this magnitude of deviation is small as compared to other models tested, the model requires further refinement in order for it to more accurately predict production steady states in industry. In predicting the course of fermentation in the MCCF, the Aiba model predicted fermentation trends that are normally seen in continuous fermentations. However, the Aiba model predicted that the time required to reach steady state in the MCCF was 80 h which was approximately 2x faster than the actual time required. Further refinements to the Aiba model may permit a more accurate prediction of the course of fermentation in the MCCF.

## 6 APPENDIX

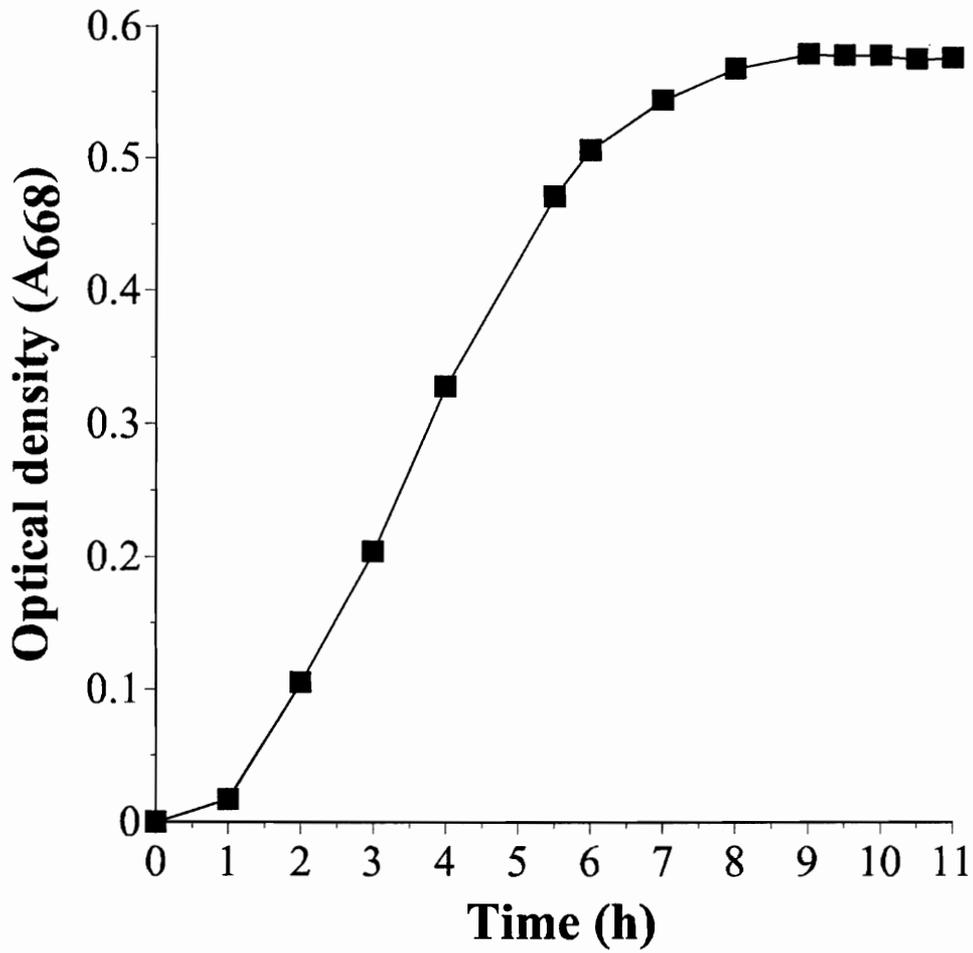
### 6.1 Mixing time in medium reservoir with new additions of medium.

To determine the mixing time required to homogenize contents of all four vessels in the medium reservoir (Fig. 3.1) when fresh medium was added, a test was performed with methylene blue dye. The medium reservoir was filled with water to a maximum working volume of 200 L. All the magnetic stirbars were turned on and the stirrers were set to a value where a vortex was visually observed in the open kegs. The first keg in the medium reservoir (keg with medium filling port) was clamped off on both ports and 0.5 g methylene blue ( $\Sigma_{\max} = 668 \text{ nm}$ ) was added to this keg. After one hour (to ensure that all the methylene blue had dissolved and was homogeneous) the clamps were removed from the keg, the external peristaltic pump was set to pump clockwise at a setting of 4. Five ml samples were removed from the fourth tank (closest to the 50 ml burette) at appropriate intervals and read at 668 nm.

The results from the fourth keg in the reservoir are depicted in Figure 6.1. After nine hours of mixing the methylene blue concentration stabilized at an  $A_{668}$  of  $\sim 0.58$ . This is the minimal time required to ensure complete mixing of the reservoir when the volume of the reservoir was  $\sim 200$  L. In cases where the volume of the reservoir was  $< 200$  L and fresh medium was added, the mixing procedure with the external pump was still performed for nine hours as additional mixing time was not detrimental to the contents of the reservoir.

### 6.2 Sterility confirmation for 40 L medium in stainless steel kegs.

To determine the proper length of time required to sterilize 40 L of medium in a 59 L Sabco stainless steel keg, a time-temperature profile was constructed for a 60 minute autoclave run. A thermocouple was positioned in the geometric center of the liquid in the keg along with a *Bacillus stearothermophilus* spore vial (Difco, Franklin



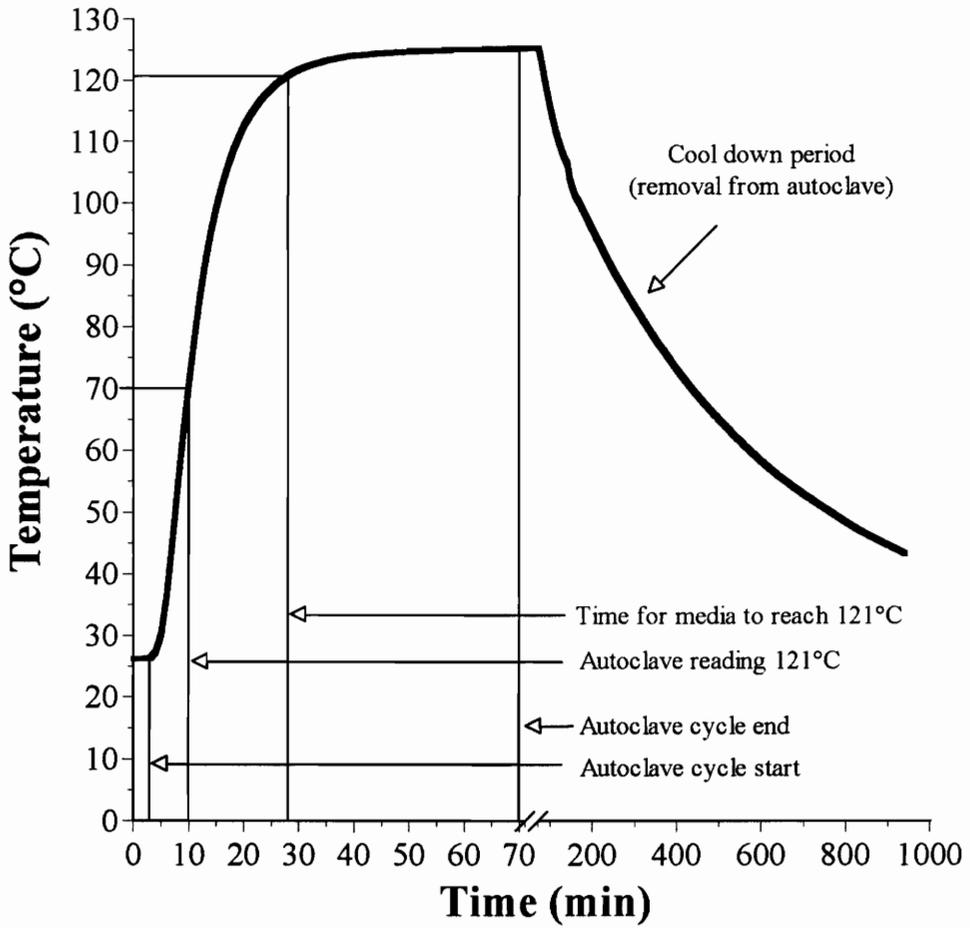
**Figure 6.1** Mixing time required to ensure homogenization of the 4-tank medium reservoir.

Lakes, NJ) to provide an additional sterility test. A time-temperature profile of the keg is typically depicted in Figure 6.2. After ~ five minutes of autoclaving, the autoclave LED control indicated that the vessel had reached 121°C and had begun its holding mode at 121°C. However, the contents of the keg had only reached 70°C ! It was not until ~25 minutes into the autoclave run that the contents of the keg reached 121°C. With the “standard” practice of having a 15-20 minute holding time at 121°C to sterilize liquids, the total autoclave time required for the keg would be 45 minutes. An additional five minutes was added to the autoclave time to add an additional measure of security. The autoclave time adopted for all media formulations in the 59 L kegs was therefore 50 minutes. Following the autoclave cycle, the keg was immediately removed from the autoclave and allowed to cool. It is evident in Figure 6.2 that the very long down time required to cool down the contents of the keg to a temperature where it could be safely managed required the autoclaved kegs to be cooled overnight before the contents could be mixed with the CSP fraction and added to the medium reservoir.

Sterility was also verified with the spore vial after a 24 hour and a one week incubation period at 56°C. Positive control vials (unheated) turned yellow after 24 hours (presence of viable *B. stearothermophilus*) while the heat-treated vials remained purple (no viable *B. stearothermophilus*) after one week incubation.

The autoclave time of 50 minutes as determined from Figure 6.2 pertains to conditions where the liquid to be autoclaved began at room temperature (~25°C). During the preparation of the glucose component in the 59 L kegs, the required crude glucose and water were mixed in a sanitized steam kettle and minimally heated (~50-60°C) to facilitate dissolution of the glucose (see Section 3.5.1.2). This higher initial temperature was not considered to seriously impact the autoclaving procedure and little browning of the sugar occurred (due to the planned absence of nitrogenous medium constituents at this stage).

Since the experiments required preparation of very large volumes of medium, two identical Eagle 3000 autoclaves (Steris, Mississauga, ON) were used in parallel to reduce the preparation time. The use of thermocouples and spore vial tests in both autoclaves



**Figure 6.2** Time-temperature profile for 40 L of a 34 g/100ml crude glucose solution autoclaved for one hour in a Sabco 59 L stainless steel keg.

yielded similar results.

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