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Application of multistage continuous fermentation for production of fuel alcohol by very-high-gravity fermentation technology

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A fermentation system to test the merging of very-high-gravity (VHG) and multistage continuous culture fermentation (MCCF) technologies was constructed and evaluated for fuel ethanol production. Simulated mashes ranging from 15% to 32% w/v glucose were fermented by *Saccharomyces cerevisiae* and the dilution rates were adjusted for each glucose concentration to provide an effluent containing less than 0.3% w/v glucose (greater than 99% consumption of glucose). The MCCF can be operated with glucose concentrations up to 32% w/v, which indicates that the system can successfully operate under VHG conditions. With 32% w/v glucose in the medium reservoir, a maximum of 16.73% v/v ethanol was produced in the MCCF. The introduction of VHG fermentation into continuous culture technology allows an improvement in ethanol productivity while producing ethanol continuously. In comparing the viability of yeast by methylene blue and plate count procedures, the results in this work indicate that the methylene blue procedure may overestimate the proportion of dead cells in the population. Ethanol productivity (Yps) increased from the first to the last fermentor in the sequence at all glucose concentrations used. This indicated that ethanol is more effectively produced in later fermentors in the MCCF, and that the notion of a constant Yps is not a valid assumption for use in mathematical modeling of MCCFs. *Journal of Industrial Microbiology & Biotechnology* (2001) **27,** 87–93.

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

Introduction

The production of ethanol by fermentation using *Saccharomyces cerevisiae* is the most economical way to produce ethanol. Worldwide production of ethanol in 1999 reached 24×10^9 l/year [5]. In North America, fuel and industrial grade ethanol is produced at a rate of 7.0×10^9 l/year by both continuous and batch fermentation [8].

One technology that is significantly changing industrial ethanol production is very-high-gravity (VHG) fermentation. In VHG fermentation, mashes with greater than 27 g dissolved solids/100 g mash can be batch-fermented with all substrates present at zero time and without the use of conditioned or genetically modified *Saccharomyces* yeasts. This technology has led to production of 23.8% v/v ethanol in the laboratory from wheat mash containing 38% w/v dissolved solids [23], and it is gradually being applied to industry where goals of 15–16% or more alcohol in some locations are being set in order to lower costs [9]. 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 remarkably acceptable quality when diluted to the alcohol concentration of commerce [4,15]. Every major brewery in the world is producing or experimenting with production of beers from higher gravities than were heretofore considered possible. VHG technology has also helped to explain stuck and sluggish fermentations in all of the alcohol industry but especially in white wine manufacture [11]. Numerous advantages exist through the use of VHG technology including: reduction in the level of contaminating (yield-reducing) bacteria, increase in ethanol concentration [1], potential increases in fermentor space (~30%) due to possible removal of insoluble grain residuals and decreases in costs at all stages of production [24].

The primary disadvantage of batch ethanol fermentation is that significant downtime (cleaning, sanitizing, filling) between runs is necessary. This represents a major loss of productivity. Continuous culture fermentation provides advantages over batch fermentations including: 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 [3,13,20]. Unfortunately, continuous fermentations are more susceptible to long-term bacteriological problems. For this reason, both manufacturing processes are widely used worldwide.

The purpose of this study was to evaluate the performance of *S. cerevisiae* in fuel ethanol fermentations where VHG and MCCF technologies were merged into a single fermentation system. To our knowledge, this is the first study where the advantages of VHG technology have been adapted for use in multistage continuous culture.

Materials and methods

The multistage fermentation system

Five Bioflo III fermentors (New Brunswick Scientific, New Brunswick, NJ) were connected in series to produce a multistage continuous culture system simulating typical industrial designs as depicted in Figure 1. Each fermentor was raised 10 cm from the

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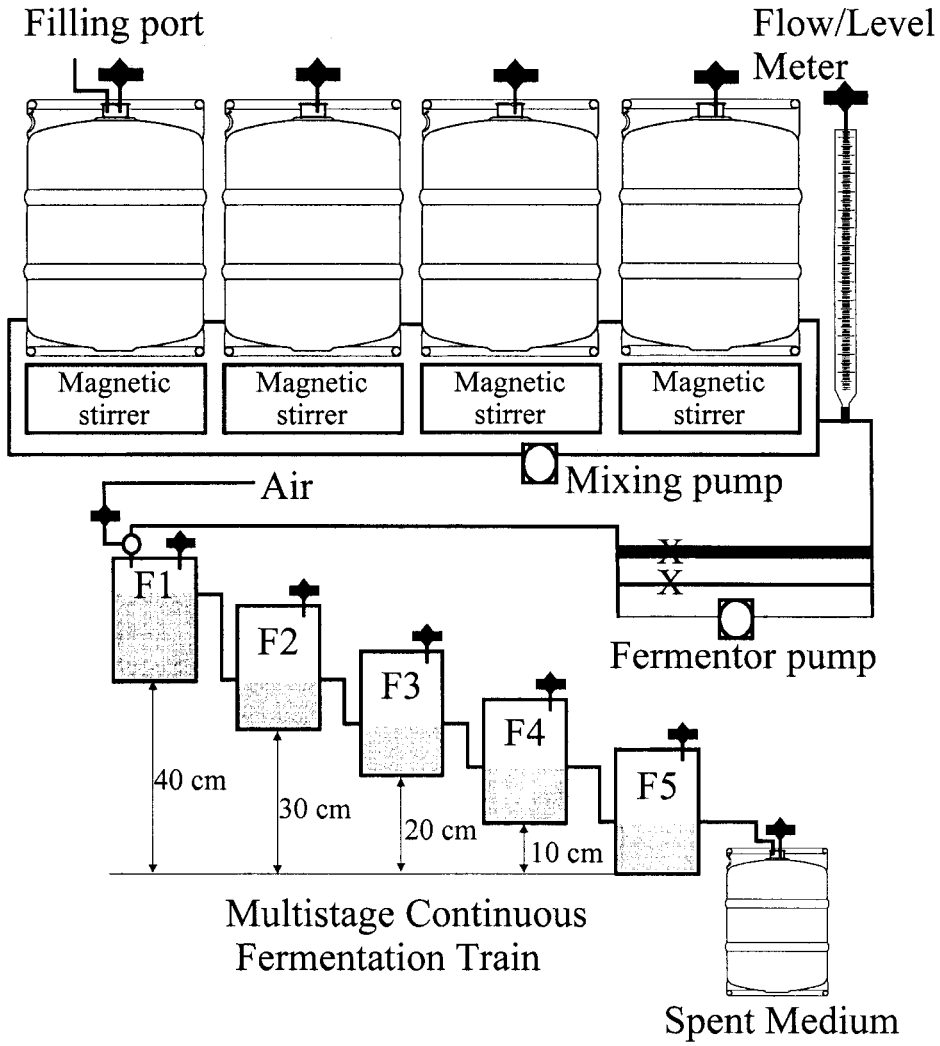

Figure 1 Schematic diagram of constructed MCCF system.

previous fermentor to allow flow by gravity to move the fermentor contents (*via* two stainless steel ports with large internal bores, constructed and installed on each fermentor). Fermentor 1 (F1) had a working volume of 5.0 l, while fermentors 2–5 (F2–F5) had 2.8 l each. Fermentations were conducted at 28°C [12] with 100 rpm agitation. During fermentation, sterile air was supplied to the first fermentor at two standard liters per minute to reduce the possibility of medium backflow through the fermentor pump, preventing contamination of the medium reservoir (MR), and to allow the yeast to synthesize required membrane unsaturated fatty acids and sterols [18].

Medium reservoir (MR)

Four 59-l stainless steel kegs (Sabco Industries, Toledo, OH) were connected in series to provide a MR capacity of >200 l (Figure 1). Each keg was stoppered with a #9 butyl rubber stopper through which a short piece of stainless steel tubing was inserted, and an Acrodisk 50 filter (Gelman Sciences, Ann Arbor, MI) was attached to provide sterile venting. The entire stopper was tightened to the keg by appropriate clamps and silicone sealant. On one of the kegs, an additional port was added through the stopper to allow medium to be added to the multikeg 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 aseptically wrapped. The kegs were individually autoclaved for 30 min at 121°C, cooled overnight, positioned on magnetic stirrers and aseptically connected. Each keg contained a large magnetic stirbar for continuous homogenization of the contents. Fresh medium was added to the MR by aseptically connecting a keg containing the sterile medium to the multikeg MR, and pressurizing the new keg with sterile air at 3 psi to transfer the contents. The multikeg MR was mixed from the first to the fourth keg for 6 h using an external peristaltic mixing pump.

Fermentor preparation

The five Bioflo III fermentors were autoclaved empty for 30 min with all parts and tubing ends clamped and aseptically wrapped. All Acrodisk 50 filters used on the fermentors, medium kegs and the reservoir were tested for water integrity as prescribed by Gelman Sciences. After autoclaving them, the five fermentors were aseptically connected to each other, to the MR and to the effluent keg.

Media formulation and preparation

The medium composition for all experiments was: 14–31.4% (w/v) cerelose (99.2% D-glucose) (Canada Starch, Etobicoke, ON), 2% (w/v) corn steep powder (Marcor Development, Hackensack, NJ) and 20 mM $(NH_4)_2HPO_4$ (Monsanto, Trenton, MI). Large volumes of media were prepared on a regular basis. A concentrated glucose component was autoclaved (40 l, 121°C for 50 min) separately from the concentrated corn steep powder and diammonium phosphate component (10 l, 121°C for 30 min) to prevent Maillard reactions. Once cooled, both components were aseptically combined to form the final medium.

Tests with a thermocouple installed in both the 59- and 12-l kegs (positioned at the geometric center) during autoclaving, and with *Bacillus stearothermophilus* spore vials (also positioned at the geometric center) (Difco, Detroit, MI), confirmed successful autoclaving.

Yeast master culture and storage

A pure culture of *S. cerevisiae* provided by Alltech (Nicholasville, KY) was inoculated into 100 ml of YEPD medium [7] in a 250-ml screw-capped sidearm flask and was grown overnight with shaking (150 rpm) at 30°C. Sterile glycerol was added to the culture to bring the final concentration of glycerol to 20%. The culture was then aseptically dispensed into a large number of sterile 1.8 ml cryogenic vials and stored at −70°C. These vials were gradually withdrawn over the course of the project for use.

Yeast inoculum preparation

One cryogenic vial containing yeast was thawed and aseptically added to a 1-l 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.

Fermentor inoculation and equilibration of the system

While the inoculum was being prepared, medium in the reservoir of the selected glucose concentration was pumped overnight into the fermentation train to fill each fermentor to its respective working volume. Agitation and cooling lines on each fermentor were turned on and set to the desired values. As well, the medium flowrate into F1 was selected and rechecked to ensure stability during the course of the experiment. The inoculum was then added to F1 and allowed to simultaneously inoculate (by overflow) all the fermentors in the system. The MCCF was allowed to run for an entire week to allow the yeast to reach steady state as confirmed by a less than 5% variation in glucose concentration in each fermentor over a 3-day sampling period. If any glucose was detected in F5 at steady state, the medium flowrate was reduced to F1 and the system was allowed to re-equilibrate for another week. This was repeated until the glucose concentration in F5 reached a minimum value (as close to zero as possible).

Measuring medium flowrate

The medium flow rate in the multistage continuous culture system was determined by a burette flowmeter. The medium flow rate was measured three times daily to ensure consistent flow. Adjustment was seldom required.

Fermentor sampling

Each fermentor was sampled using a pre-flushed sampling port present on the fermentor headplate. At a dilution rate of 0.2 h^{-1} with a working fermentor volume of 3 l, the time required for the fermentation system to replenish a 100-ml sample would be 10 min. This time would not seriously impact the steady state conditions achieved in the system.

A portion of the aseptically collected sample was used immediately for viability and microscopic evaluations, and the remaining amount was filtered through a 0.45-μm pore size Gelman membrane filter and frozen for future chemical analyses.

The length of time required to achieve steady state of the MCCF at each glucose concentration used (in one case 3 weeks of continuous operation) did not permit replication of experiments. However, for each experimental condition, medium formulations were strictly controlled, the MCCF was allowed to equilibrate for an extended period of time (minimum 1 week), triplicate samples were taken to confirm steady state and tentative steady states were sampled over a 3-day period to confirm equilibration.

HPLC analysis

Lactic acid, glycerol, ethanol and glucose concentrations were determined by HPLC analysis as outlined by Narendranath *et al* [17]. The samples were thawed and diluted to the required extent with Milli-Q water. Aliquots (5 μl) of the diluted samples were mixed with equal volumes of 2.0% (w/v) boric acid (internal standard) and injected into an Biorad HPX-87H aminex column equilibrated at 40°C. The eluent 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 Waters Maxima 810 software.

Viability by membrane filtration

The membrane filtration procedure for viable cell counting was used [10]. Triplicate aliquots of an appropriately diluted fermentation sample were vacuum-filtered through sterile 0.45-μm, 47-mm grided GN-6 Gelman membrane filters aseptically placed on the filter base, rinsed with 5 ml of 0.1% sterile peptone water, placed onto YEPD plates and incubated at 27°C for 2 days.

Viable, dead and budding yeast cells by methylene blue

The methylene blue procedure outlined by Thomas and Ingledew was used [22]. A 1-ml aliquot of sample was diluted with an appropriate amount of Ringer's solution (with methylene blue) to give a total microscopic cell count of 400–500 (in 5 of 25 squares) in an improved Neubauer hemacytometer. Total, viable and dead yeast cells were enumerated in triplicate.

Yeast dry mass

The medium formulation used in the experiments complicated dry weight determinations since all media used contained some particulate matter. To overcome this, a MR sample was processed in addition to the fermentor samples. Triplicate 10-ml aliquots from each sample were filtered through 0.45-μm, 47-mm grided Gelman GN-6 membrane filters. These were weighed to four decimal places in desiccated, tared aluminum foil pans and then dried at 105°C for 2 h to constant weight, cooled in a desiccator and

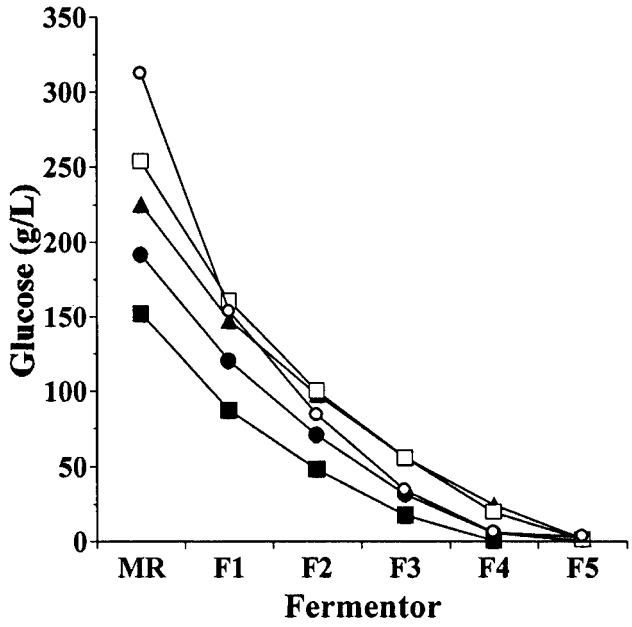

Figure 2 Glucose concentrations in each fermentor at steady state in the multistage continuous culture with the medium reservoir containing from 15.2% to 31.2% (w/v) glucose [■, 15.2% (w/v) glucose (D = 0.34, Flow = 15.87 ml/min); ●, 19.1% (w/v) glucose (D = 0.21, Flow = 9.80 ml/min); ▲, 22.5% (w/v) glucose (D = 0.16, Flow = 7.47 ml/min); □, 25.4% (w/v) glucose (D = 0.12, Flow = 5.60 ml/min); ○, 31.2% (w/v) (D = 0.05, Flow = 2.33 ml/min); MR = medium reservoir; F1–F5 = fermentors 1–5 in the MCCF system].

reweighed [2]. The yeast dry mass was determined by subtracting the insolubles of the MR sample (particulate matter) from the fermentor samples (yeast and particulate matter).

Results and discussion

Figure 2 illustrates the steady state glucose concentrations in the MR and in each of the five fermentors in the multistage continuous culture fermentation (MCCF) system when glucose concentrations were set from 15.2% to 31.2% (w/v). One of the objectives for these experiments was to determine what process parameters (primarily dilution rates) were needed to ensure complete utilization of glucose in the effluent of F5. This was determined experimentally for the glucose concentration set in each MR. Dilutions and flow rates are provided in the legend. Complete consumption of glucose is of great importance for industry in order to minimize wastage of substrate and maximize ethanol concentration and yield. In the MCCF, the yeast reaches a steady state or balanced growth where no net increases or decreases in cell numbers are seen. This steady state was achieved without cell recycle and without additions of yeast other than the original inoculation. The maximum glucose concentration remaining in F5 regardless of initial glucose concentration was 0.3% (w/v). This was with a starting 31.1% glucose concentration and a dilution rate (D) of 0.05 h^{-1}. All dilution rates in this work were calculated according to the conditions in F2 since all fermentors operating in the MCCF had the same volume as F2 except F1, which had approximately twice the volume and thus had a different D value. Industrial fermentation designs often use a first fermentor twice the volume as the other fermentors in order to maximize yeast cell growth earlier in the multistage system.

Figure 3 shows the relationship between the glucose concentration in the MR and the dilution rates used to achieve complete glucose consumption at the F5 outlet for each condition. A linear relationship with a high correlation exists between the two parameters. From a production point of view, the dilution rate for the MCCF can be adjusted to achieve total glucose consumption at any MR glucose concentration used. This provides the opportunity for a company to adjust the operation of the MCCF to handle fluctuations in incoming glucose concentrations. It is also seen from Figure 3 that the maximum glucose concentration that can be used in an MCCF of this type is ~32% (w/v). This shows that the MCCF system, as constructed and operated, is capable of VHG level fermentations (sugar concentrations near or exceeding 27 g of dissolved solids per 100 g of mash).

Figure 4 illustrates the individual steady state ethanol concentrations in fermentors when the glucose concentrations in the MRs were set from 15.2–31.2% w/v. As would be expected, steady state ethanol concentrations 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 31.2% glucose was provided. In other works, a maximum of 10.32% v/v ethanol in a single feed (D=0.05), four-stage, continuous, cascade, stirred tank fermentor system was produced utilizing 22.0% w/v glucose generated enzymatically from cellulosic bagasse [25]. For fuel alcohol plants producing ethanol by continuous culture, ethanol concentrations normally achieved are less than 14% v/v. The present work demonstrates that ethanol concentrations as high as 16.7% v/v can be achieved utilizing combined VHG and multistage continuous culture technologies. The ethanol concentration continued to increase in Figure 4 for all medium glucose levels from F1 to F5; however, the rates of ethanol production decreased from F1 to F5. This was probably due to the inhibition of ethanol production rate by ethanol, to the increasing glucose concentration in the

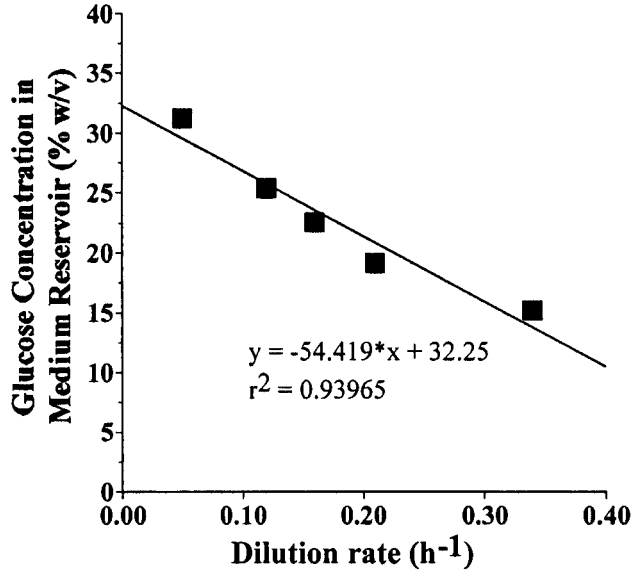

Figure 3 Dilution rates required in multistage continuous culture to ensure complete glucose utilization in F5 with increasing medium reservoir glucose concentrations.

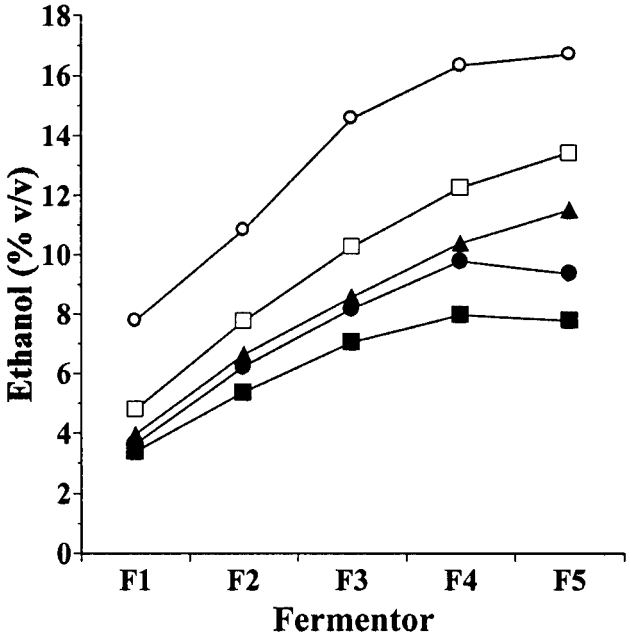

Figure 4 Ethanol concentrations in each fermentor at steady state in multistage continuous culture with the medium reservoir containing 15.2–31.2% (w/v) glucose [■, 15.2% (w/v) glucose ($D = 0.34$, Flow = 15.87 ml/min); ●, 19.1% (w/v) glucose ($D = 0.21$, Flow = 9.80 ml/min); ▲, 22.5% (w/v) glucose ($D = 0.16$, Flow = 7.47 ml/min); □, 25.4% (w/v) glucose ($D = 0.12$, Flow = 5.60 ml/min); ○, 31.2% (w/v) glucose ($D = 0.05$, Flow = 2.33 ml/min)].

fermentors resulting from the increased glucose concentration added to F1, to depletion of utilizable nitrogen in the medium and/or to the shift of biomass from actively growing to non-growing cells where the rate of ethanol production can be more than 30 times lower than in actively growing cells [19]. Under 15.2% and 19.1% glucose conditions, a small decrease in ethanol concentration from F4 to F5 was seen, which suggested that ethanol might be consumed in F5. This indicates that the dilution rate in the MCCF could have been increased for these conditions. Further evidence for this is found in Figure 2 where the glucose concentrations in F4 for these conditions were at very low levels — an increase in the dilution rates would have increased the flows through the MCCF (increased productivity) and increased the glucose levels in F4.

The yeast viabilities (by methylene blue) at steady state in each fermentor are seen in Figure 5. The general trend was a viability decrease from F1 to F5 under all conditions. In general, the slopes of the lines plotted decreased with increased MR glucose concentrations. More than 70% of the yeasts was still viable in F5 in the MCCF at all glucose concentrations used up to 25.4% w/v. This contrasts with a 34% viability seen in F5 at 31.2% initial glucose (a 59.8% decrease). Presumably, the yeasts under 31.2% glucose conditions were experiencing stressful conditions. The low viability in this condition is alarming since only 35% of the cells was assumed to produce ethanol in the latter stages in the MCCF.

Figure 6 illustrates the viable yeast concentrations at steady state in colony-forming units (cfu) per milliliter (by microbiological plating) in each fermentor with incoming glucose concentrations set from 15.2% to 31.2% (w/v). The data for the 15.2–25.4% MR conditions showed an initial increase (F1–F2) in cell numbers followed by no further increases (F2–F5). Since the yeasts are producing ethanol in all the fermentors in the MCCF and there are no net increases in yeast numbers from F2 to F5, one would expect that the ethanol productivity (gram of ethanol produced per gram of glucose consumed) had increased since glucose would not be needed for an increase in the cell multiplication rate (to cause an increase in the steady state yeast numbers). This hypothesis was confirmed in the present work for all glucose conditions in the MR — ethanol productivity (Yps) increased from F1 to F5 at all glucose conditions. The largest increased occurred at 25.4% glucose where ethanol productivity increased from F1 (0.405 g ethanol/g glucose consumed) to F5 (0.503 g ethanol/g glucose consumed). 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. In chemical engineering circles, the notion that ethanol productivity (Yps) is a constant in fermentation experiments (and/or mathematical modelling) is not correct. The present work shows that Yps must be experimentally derived on an experiment by experiment basis in order to accurately determine correct values. An interesting observation is seen when the data in Figures 5 and 6 are compared. According to the data collected using methylene blue (Figure 5), the viability of yeast in all conditions decreased, and yet in Figure 6, the yeast viability (by plating) for most of the conditions increased slightly from F1 to F2, and then remained constant from F2 to F5 (this observation remained even when both figures were compared on a logarithmic basis). When the data in Figure 6 are compared to data showing total cell counts (not shown), the same trends in data are observed in both plots

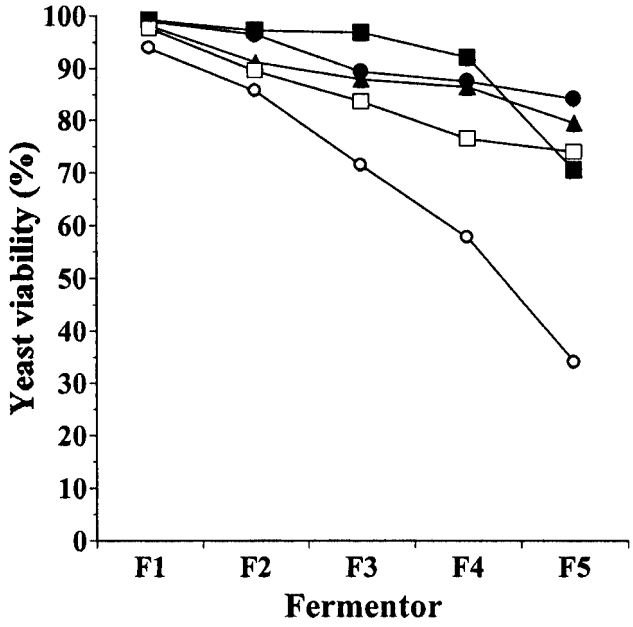

Figure 5 Viability (assessed by methylene blue) of *S. cerevisiae* at steady state in each fermentor in multistage continuous culture with the medium reservoir containing 15.2–31.2% (w/v) glucose [■, 15.2% (w/v) glucose ($D = 0.34$, Flow = 15.87 ml/min); ●, 19.1% (w/v) glucose ($D = 0.21$, Flow = 9.80 ml/min); ▲, 22.5% (w/v) glucose ($D = 0.16$, Flow = 7.47 ml/min); □, 25.4% (w/v) glucose ($D = 0.12$, Flow = 5.60 ml/min); ○, 31.2% (w/v) glucose ($D = 0.05$, Flow = 2.23 ml/min)].

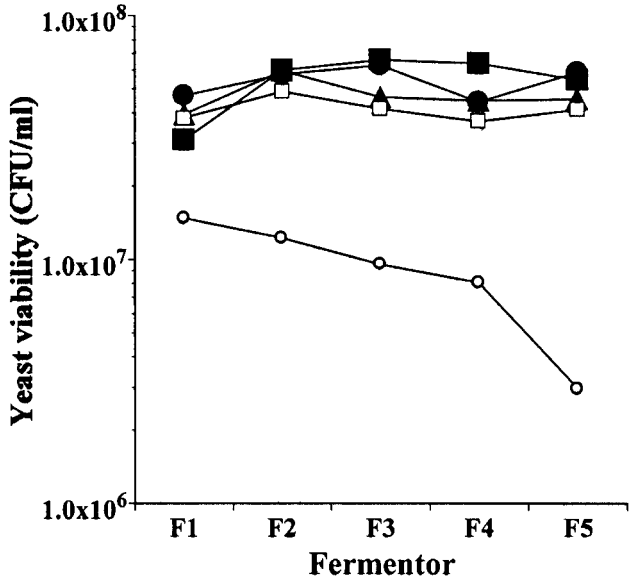

Figure 6 Viability (cfu/ml) of *S. cerevisiae* at steady state in each fermentor in multistage continuous culture with the medium reservoir containing 15.2–31.2% (w/v) glucose [■, 15.2% (w/v) glucose (D = 0.34, Flow = 15.87 ml/min); ●, 19.1% (w/v) glucose (D = 0.21, Flow = 9.80 ml/min); ▲, 22.5% (w/v) glucose (D = 0.16, Flow = 7.47 ml/min); □, 25.4% (w/v) glucose (D = 0.12, Flow = 5.60 ml/min); ○, 31.2% (w/v) glucose (D = 0.05, Flow = 2.23 ml/min)].

(with the exception of the 31.2% MR condition). Taken together, these two 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 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 h) and "non-viable" (exponential and stationary phase cells heat-treated at 70°C for 2–4 h in water) states. Those authors concluded that the plate count method is the most reproducible method of determining the numbers of viable and dead cells [21].

Figure 7 illustrates the individual yeast dry weights at steady state in fermentors with initial glucose concentration set from 15.2% to 31.2% (w/v). The biomass concentration for all conditions, except at 31.2%, continually increased from an average F1 of 4.73 g/l to an average F5 of 8.89 g/L (a 4.17 g/l increase in biomass). These dry weight results would initially indicate that the yeast numbers are increasing across the MCCF. However, the yeast cell numbers by plating on YEPD (Figure 6) remain constant over the same conditions where dry weights increase in Figure 7 (even when both charts were compared on a logarithmic basis). The only explanation to account for a constant yeast cell number, 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 ~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 while few changes were seen in yeasts grown in batch [6]. 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 7, the biomass in the fermentors with 31.2% w/v 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 decrease seen for the 31.2% condition in Figure 6), 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 last reason is found in work showing that cell volumes of *S. cerevisiae* decreased as the osmolality of NaCl increased [16].

It seems clear from this work that a coupling of VHG fermentation to MCCF is feasible. Currently, MCCFs are utilized in a number of fuel ethanol continuous production plants in North America and produce ~12% v/v ethanol (personal communication). The 17% v/v ethanol concentration shown in this work represents a 42% increase in ethanol production over what is currently produced. In an industry where profit margins can be very low [14], any technique that can increase ethanol production should be seriously considered. In addition, a serious examination of the merger of VHG and multistage continuous technologies is lacking in published work and is needed to allow further exploration in this area. Although, at present, maximum alcohol concentrations do not approach the values over 20% v/v achievable in batch laboratory fermentations, economics of alcohol production and purification can be improved, and the increased alcohol may

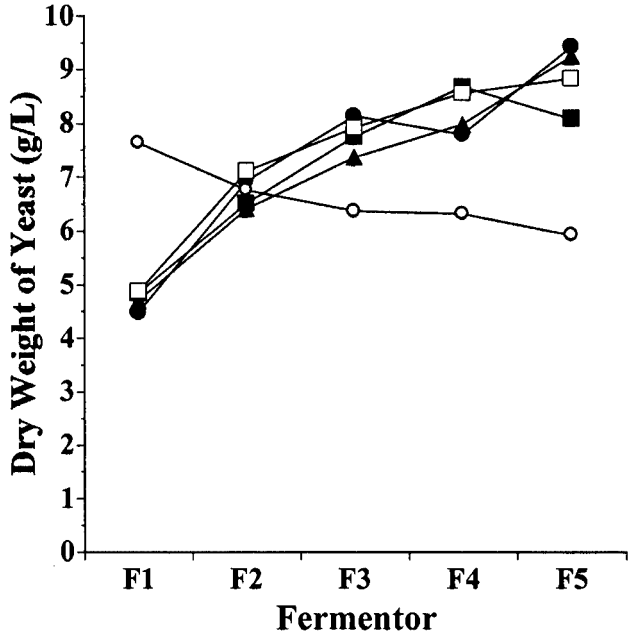

Figure 7 Dry weights of *S. cerevisiae* at steady state in each fermentor in multistage continuous culture with the medium reservoir containing 15.2–31.2% (w/v) glucose [■, 15.2% (w/v) glucose (D = 0.34, Flow = 15.87 ml/min); ●, 19.1% (w/v) glucose (D = 0.21, Flow = 9.80 ml/min); ▲, 22.5% (w/v) glucose (D = 0.16, Flow = 7.47 ml/min); □, 25.4% (w/v) glucose (D = 0.12, Flow = 5.60 ml/min); ○, 31.2% (w/v) glucose (D = 0.05, Flow = 2.23 ml/min)].

also aid in control of bacterial contamination so often seen in such systems. Modifications to the base multistage system such as cell recycle and/or simultaneous saccharification and fermentation may permit higher ethanol concentrations to be reached.

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