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Development of Continuous Fermentation Using Immobilized Yeast Cells
For Wine Cooler Process Development

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ABSTRACT

The continuous wine fermentation process, which employs a newly designed tapered column type bioreactor and immobilized yeast cells (Montrachet 522), was studied and its fermentation performance was compared with batch and suspended cell continuous wine fermentation systems.

It was found that a stable continuous culture fermentation process could be maintained for a period of 2–3 mo when the new bioreactor system packed with immobilized yeast cells was employed. The new bioreactor containing immobilized yeast cells performed significantly better than the suspended cell culture system or batch culture. The effluent wine from the continuous fermentor system contained 7.1% (v/v) ethanol and 0.18% (w/v) residual sugar at 0.01 h^{-1} dilution rate. The new continuous bioreactor system also gave 17–34 times higher maximum ethanol productivity compared to the conventional batch wine fermentation. At a low dilution rate, 0.01^{-1}, as high as 92% sugar to ethanol yield was achieved.

Based on the results obtained from this study, the possibility of developing a continuous wine cooler fermentation process was demonstrated. A two-stage continuous wine fermentation system may be designed and operated. The grape juice can be fed into the first-stage that is operated at about 0.2 h^{-1} dilution rate and the effluent from the first-stage is fed into the second-stage continuous fermentor operated at about 0.01 h^{-1} dilution rate. By doing so, a wine cooler can be

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produced continuously and efficiently, by employing the newly designed tapered column type bioreactor charged with the immobilized yeast cells.

Index Entries: Wine cooler; yeast; fermentation process; bioreactor.

INTRODUCTION

Continuous fermentation has been studied by many authors for different purposes for many years. Continuous fermentation could offer, in principle, considerable economic advantages, reducing both capital and operating costs and giving uniformity of product quality (*1–5*).

A high density cell culture system, such as immobilized yeast cells, increases the rate of fermentation and can be used in continuous mode of operation. Before the 1980s, continuous wine fermentation systems studied had focused on the recycling of cells and using flocculating strain of yeast for maintaining a high concentration of cells (*3,4*). At first for beer making, an idea of using immobilized yeast cells was demonstrated in 1971 (*6*). The new and improved immobilization procedures for the microorganisms have been developed from the late 1970s (*5,7–9*).

The use of immobilized yeast cell culture for wine making was first reported by Totsuka in 1980 (*10*). The best gels for the immobilization and entrapment of the yeast cells were found to be κ-carrageenan and calcium alginate with the measurement of heat generation owing to growth of immobilized yeast cells by Koga in 1985 (*11*). Continuous culture systems using immobilized yeast cells have been also applied to ethanol production (*5,12,13*). In this study, a novel bioreactor design of a tapered column type was employed as a continuous wine fermentation system. The continuous wine fermentor charged with immobilized cells was operated at 13°C for the purpose of reducing ethanol emission or loss to the atmosphere by evaporation at a higher temperature and, at the same time, retaining more volatile flavor components of wine during the fermentation process. A new conceptual design of continuous wine fermentation was evaluated by testing performances of two separate stages of wine fermentation: first, with the full-strength grape juice and, second, with the half-strength grape juice. Eventually, sequential two-stage continuous fermentation process technology could be developed based on the basic experimental data obtained from this kind of study.

In this study, our research focus was on fundamental process engineering aspects: (1) evaluation of the tapered column type new design of bioreactor system; (2) performance of continuous wine fermentation system using two different grape juice concentrations, one full strength and the other half-strength; (3) determination of fermentation kinetic parameters that will be essential to the process design; and (4) test of the operational stability of long-term continuous fermentation system, 2–3 mo

period; and (5) eventual optimization of continuous wine cooler fermentation in terms of productivity and operating conditions such as dilution rate.

MATERIALS AND METHODS

Yeast Strain and Media

The *Saccharomyces cerevisiae* strain 522 (Montrachet) from UC Davis, which is widely used by the California wine industry, was chosen for this study. Two kinds of media were used in these experiments. One of them was half-strength (12% sugar w/v) White Riesling grape juice that was prepared by adding equal volumes of sterile pure water and 50 ppm SO_2 before using (first series of experiments). Another was full-strength French Colombard grape juice having 22% sugar w/v and no SO_2 added (second series of experiment).

Immobilization Procedure

From the fresh agar slant of the yeast strain 522, the yeast cells were transferred into 50 mL of original grape juice sterilized by membrane filtration (0.45 μm) and grown at 25°C. After about 2 d, the precultured cells were mixed thoroughly with 3% κ-carrageenan solution (vol. ratio 5:100). The κ-carrageenan (kindly provided by FMC Marine Colloid Division, Philadelphia, PA, USA) was dissolved, centrifuged to remove small insoluble particles,and then sterilized by autoclave. The inoculum contained more than 1×10^7 cells/mL. The yeast cells suspended in 3% κ-carrageenan solution were transferred by a peristaltic pump. The stream of droplets of the mixture was ejected from the syringe needles and immersed into sterilized 3% KCl solution by the pressure applied by sterile air (Fig. 1). The size of gel beads can be controlled between 1 to 4 mm in diameter by the conditions of the syringe needle size, the flow rate of solution, and the air flow rate. The yeast cells entrapped in the gel beads were reactivated by cultivating at 25°C in the fresh grape juice that was pasteurized. After about 1 wk, the cell concentration and the viable cell fraction in the reactivated gel beads were determined as approximately 1.2×10^8 cells/mL gel and 98% viability. The gel beads were also enlarged by about 10 to 20%. Throughout the immobilization, an aseptic technique was employed to prevent possible contamination.

Fermentor Designs and Configurations

The packed-bed type bioreactors charged with immobilized yeast cells are known to have the major problems in the production of ethanol. The evolution of large vol of carbon dioxide gas, the vol and flow rate of which increase with the axial distance from the bottom of the bioreactor, causes channeling, localized pressure build-up, structural damage to packing, flooding, and nonuniform distribution of substrate, product,

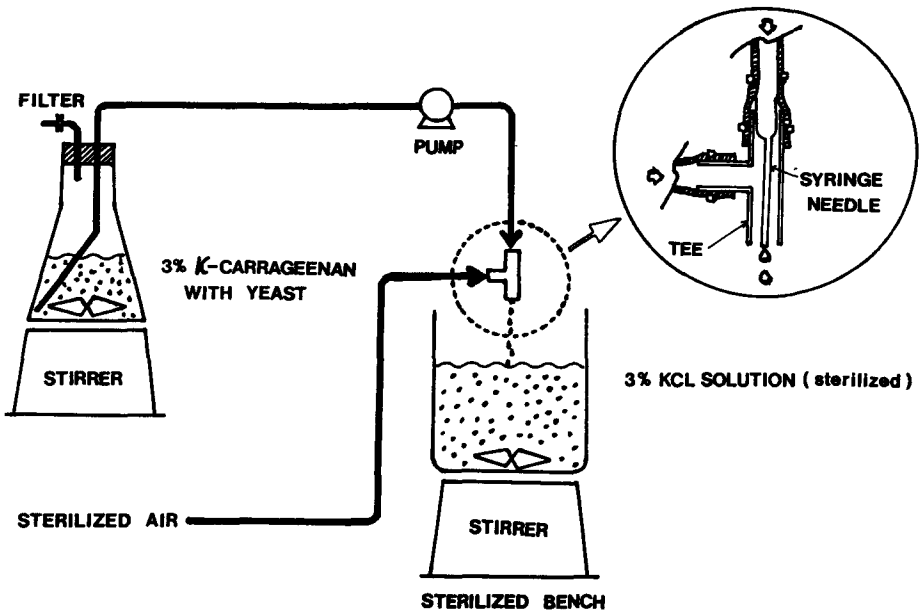

Fig. 1. Diagram illustrating the immobilization process.

viable cells, and CO_2 gas in the bioreactor. We found it extremely difficult to operate and control conventional cylindrical type of packed-bed bioreactor system for ethanol or wine fermentation.

The tapered column type of novel design bioreactor employed in this study provided a satisfactory operation. The increasing vol of CO_2 gas, accumulated along the axial distance, can be accommodated by the tapered column type of bioreactor design without any serious problems confronted when conventional cylindrical type of the bioreactor system was used. The increasing cross-sectional area with height reduces the pressure drop and counterbalances the increasing pressure build-up owing to accumulation of CO_2 gas (Fig. 2). For the first run, 500 mL activated immobilized yeast gel beads were packed into the 1000 mL working vol tapered column bioreactor made of the cone shaped polycarbonate vessel with a 15° tapered angle. Similarly, 2000 mL working vol tapered column bioreactor made of glass with about a 10° tapered angle was packed with 1500 mL of the activated immobilized yeast gel beads from the second run. Both tapered column bioreactors were set up in a constant 13°C room temperature and the reservoir of grape juice was kept in a 0°C cold room. A schematic diagram of the experimental set-up is shown in Fig. 2.

In parallel with the immobilized cell fermentation experiment, suspended yeast cell culture system (1400 mL working vol Bioflo system, New Brunswick Scientific Co., USA) was operated at 400 rpm stirring speed and 18°C constant temperature for comparison purposes (Fig. 3).

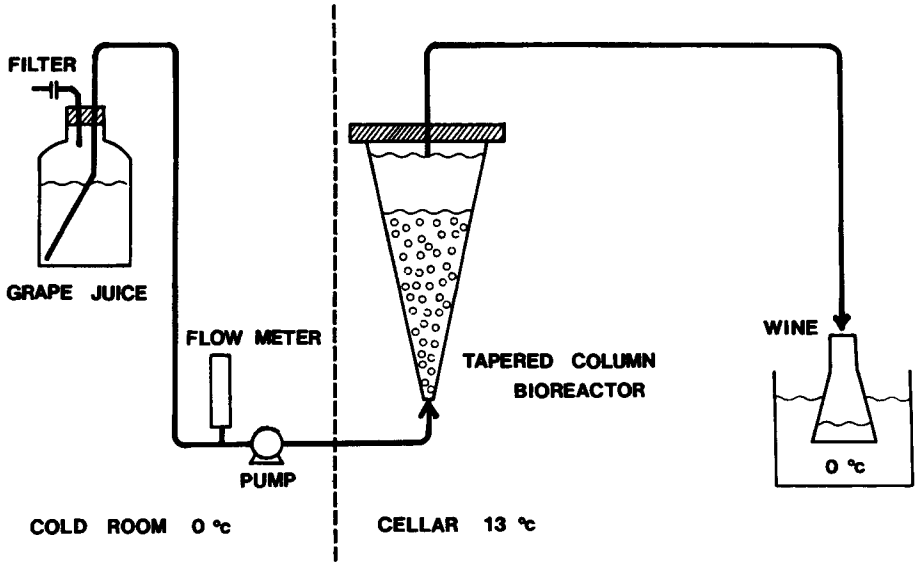

Fig. 2. Experimental set-up showing the newly designed tapered-column type bioreactor system and continuous wine fermentation using immobilized yeast cells.

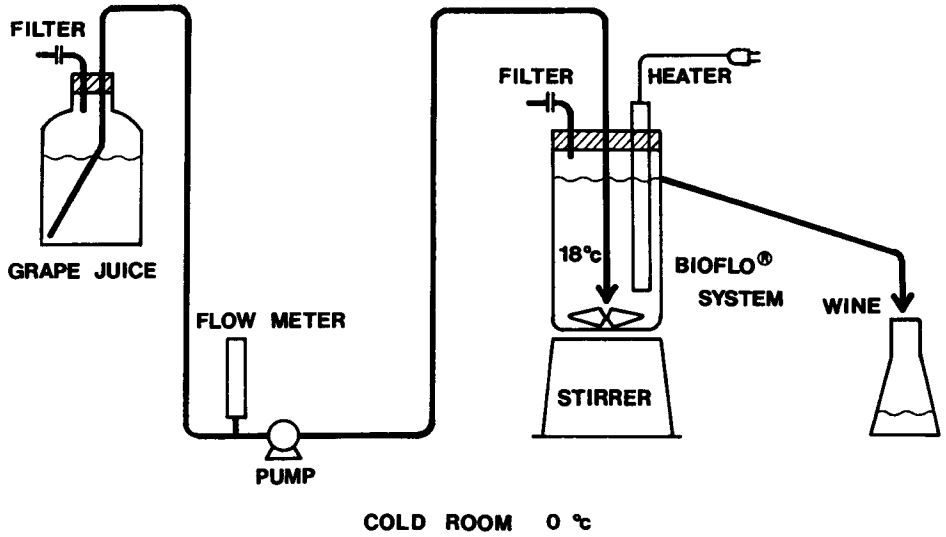

Fig. 3. Experimental set-up showing continuous suspended cell culture fermentation system.

Analytical Methods

Cell concentration was measured by direct cell counting with the hemacytometer slide (American Optics) under a microscope. The viable fraction of cells was estimated by the methylene blue staining method. The dry wt of cells was determined by the experimental standard calibration curve, in which the cell counts are plotted against the dry weight of cells.

Reducing sugar concentration was analyzed by the Bertrand method.

Ethanol concentration was determined by the AOAC method using a gas chromatograph (Hewlett-Packard Model #5720A), equipped with a FID detector.

RESULTS AND DISCUSSIONS

Operational Stabilities

The operational stability of the continuous bioreactor system was studied for the immobilized and suspended cell cultures and the profiles of cell concentration and viable cell fraction for immobilized and suspended cell cultures are shown in Fig. 4. Samples were taken every 1 or 2 d for a period of 60–90 d.

Immobilized yeast cell culture was run continuously for 60 d or longer and suspended cell culture for 90 d or longer. Both cell viabilities stayed above 90% without contamination. Total yeast cell counts inside the gel-

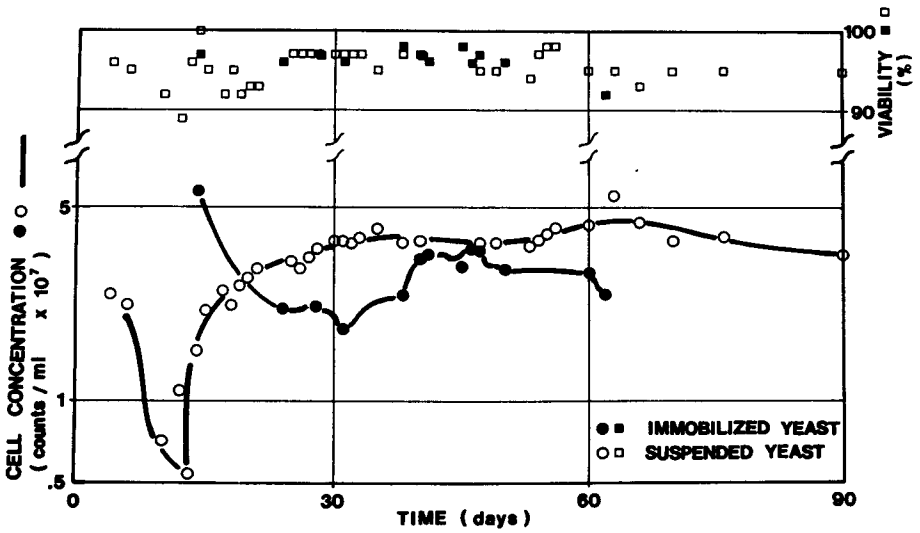

Fig. 4. Profiles of cell concentration and cell viability for both continuous fermentation systems: one with immobilized yeast cells and the other with suspended yeast cells.

bead containing immobilized cells were analyzed for samples taken at the beginning and the end of the experiment and the results are also shown in Table 1. The viable yeast cell counts were kept above 7×10^8 cells/mL gel beads after 2 and 3 mo without contamination. The vol of beads swelled about 10%.

As a function of dilution rate, the yeast cell concentrations for immobilized and suspended cultures were also monitored (Fig. 5). Immobilized cell culture was maintained above $2\text{–}3\times10^7$ cells/mL wine, although a slight decrease in cell concentration was observed with an increasing dilution rate. On the other hand, suspended cell culture showed a rapid wash out at a dilution rate greater than 0.01 h^{-1}.

It was found that a stable continuous culture fermentation process could be maintained for a period of 2–3 mo when immobilized yeast cells were used in combination with the new bioreactor design.

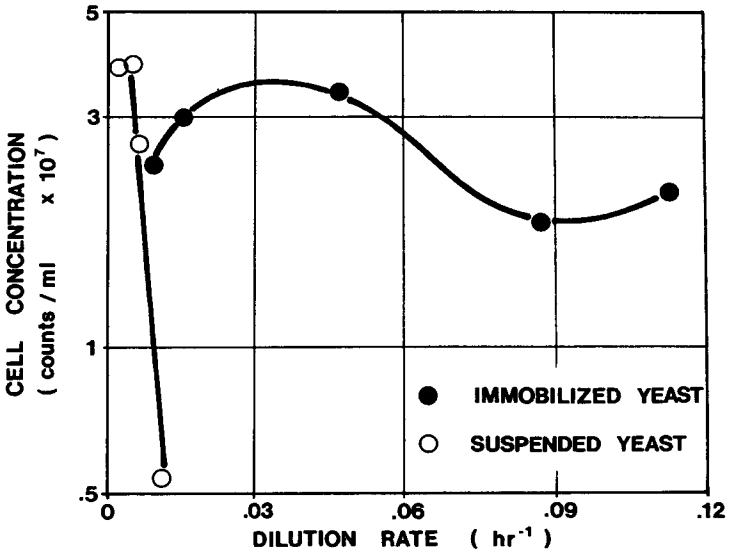

Fig. 5. Profiles of cell concentration as a function of dilution rate for both immobilized and suspended yeast cell cultures.

Table 1
Cell Concentrations and Viability of Yeast

Continuous fermentations using immobilized cells	Period of culture	Total yeast counts and viability: Cells/mL gel beads, at the start	Total yeast counts and viability: %, at the end
1st run	(62 d)	1.2×10^8 (98 d)	8.0×10^8 (93 d)
2nd run	(103 d)	7.8×10^8 (99 d)	1.8×10^9 (63 d)

Fermentation Performances

The progress of continuous fermentations is depicted in Fig. 6. The reducing sugar concentration and ethanol concentration in wine for immobilized and suspended cell cultures as a function of time are shown in Fig. 6. A significant improvement of fermentation performance was observed with the tapered column bioreactor system charged with the immobilized yeast cells when compared with the suspended cell culture system.

The tapered column bioreactor with immobilized yeast cells fermented almost completely the half-diluted grape juice to wine, which contained 7.1% v/v alcohol and 0.18% w/v residual sugar at a 0.009 h^{-1} dilution rate. On the other hand, suspended yeast cell culture could ferment to only about half as much ethanol concn. at a 0.0021 h^{-1} dilution rate.

Again, the immobilized yeast cells in the newly designed bioreactor system performed better compared to the suspended cell fermentation system.

Effect of Dilution Rate on EtOH Concentration and Productivity

Ethanol concentration of wine and ethanol productivity, defined here as mL EtOH produced/L fermentor/h, for immobilized and suspended cell cultures are shown in Fig. 7.

The ethanol concentration decreases as dilution rate increases for all continuous fermentations using immobilized and suspended yeast cell cultures. The ethanol concentration for immobilized yeast cell culture is much higher than that for suspended cell culture at the same dilution rate of 0.01 h^{-1}, mainly owing to higher cell concentration in the immobilized yeast cell fermentor system.

The ethanol productivities for immobilized yeast cell cultures go through maxima at about dilution rates, 0.06^{-1} (for the first series of experiment) and 0.12 h^{-1} (for the second series of experiment), and could be maintained relatively high about 1.5 mL EtOH/L/h (first run) and more than 3.0 mL EtOH/L/h (second run). The ethanol productivity in the second run is about 2 to 4 times higher than the first run in the dilution rate range of 0.03–0.12 h^{-1}. The difference between the results of the first and second series of runs of continuous immobilized cell cultures was attributable to the relative packing volume of gel-beads (50 and 75% of bioreactor vol, respectively), substrate concentration (12 and 22% w/v sugar, respectively), grape juice variety (White Riesling and French Colombard, respectively), SO_2 content (50 ppm and none, respectively), viable yeast cell count (7.4×10^8 and 1.1×10^9 cells/mL gel beads, respectively), and mean diameter of gel bead (1.73 and 1.53 mm, respectively). All of these parameters and factors are important to the fermentation performance and ethanol productivity in the continuous immobilized cell fermentation system. The ethanol productivity for continuous suspended yeast cell culture was found to be much lower, about 0.08 mL EtOH/L/h, indicating

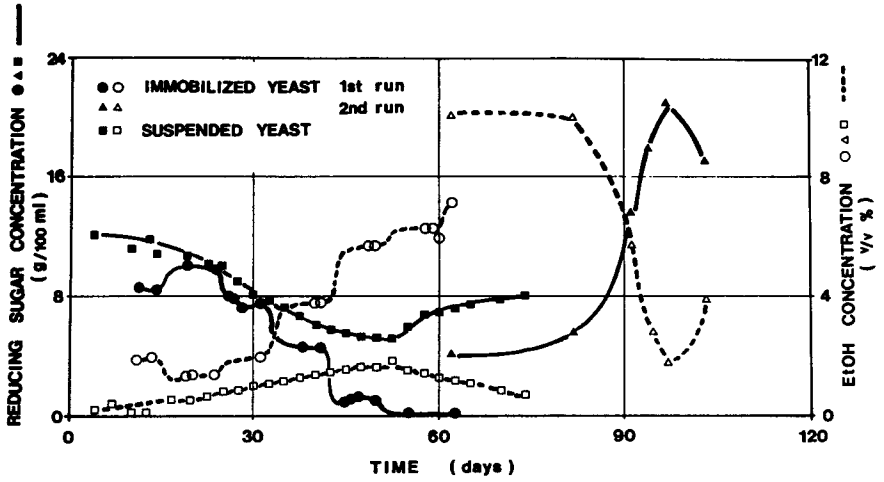

Fig. 6. Profiles of reducing sugar concentration and ethanol concentration for both the immobilized and suspended cell cultures. Two series of experiments with immobilized cells were run: the first series with a full-strength grape juice and the second series with half-strength grape juice. These two series of experiments simulated the first-stage and second-stage continuous fermentation system.

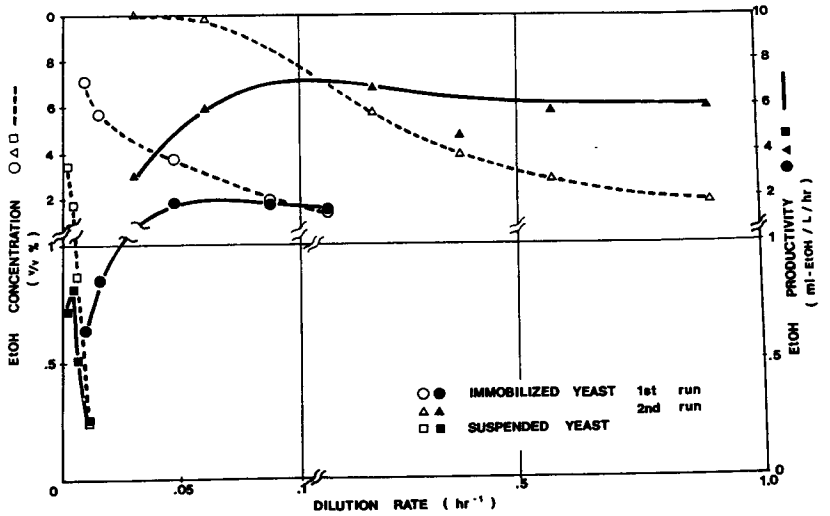

Fig. 7. Profiles of ethanol concentration and ethanol productivity as a function of dilution rate for both immobilized and suspended cell cultures.

that it was inferior to the conventional batch wine fermentation. The ethanol productivity for conventional batch wine fermentation is estimated to be about 0.20 to 0.40 mL EtOH/L/h in general. Thus, the continuous immobilized yeast cell culture, that gave 17–34 times higher productivity as compared with the conventional batch fermentation, was determined to be far superior to the conventional batch wine fermentation process in terms of ethanol productivity. The ethanol productivity was also 1.6–3.2 times higher at or near the optimal dilution rate of 0.01 h^{-1}.

Effect of Dilution Rate on Yield

The ethanol yields defined as the actual amount of ethanol produced divided by the theoretical amount of ethanol to be produced from sugar, for the immobilized (the first and second series of runs) and suspended yeast cells cultures as a function of dilution rate are shown in Fig. 8. The yield decreases as dilution rate increased for all immobilized and suspended yeast cultures. The yields for both immobilized yeast cell cultures (the first and second series of runs) are much higher than the suspended cell culture. The yield for the second run is 20% higher than the first run of immobilized yeast cell cultures in the dilution rate range between 0.06 and 0.12 h^{-1}. At a low dilution rate of 0.01 h^{-1} for the first run of immobolized yeast cell cultures, 92% yield was obtained.

Effect of Dilution Rate on Specific Rate of EtOH Production

Specific rates of EtOH production, defined as g-EtOH produced divided by g-yeast cells as dry wt/h, for immobilized (the first and second series of runs) and suspended yeast cell cultures as a function of dilution rates are shown in Fig. 8.

The specific rate of EtOH production for suspended yeast cell culture is much lower, below 0.05 g-EtOH/g-cell/h, but it goes up suddenly as dilution rate increased from 0.01 h^{-1}, because of a small amount of yeast cells near the washout condition. The specific rates of EtOH production of both immobilized yeast cell cultures go up as dilution rate increased and both pass through maxima of 0.1 g-EtOH/g-cell/h at 0.05 h^{-1} (the first run) and 0.4 g-EtOH/g-cell/h at 0.4 h^{-1}. The specific rate of EtOH production determined for the second run was four times higher than that of the first run.

Comparison of Kinetic Constant

The V_{max} values for immobilized yeast cell cultures were 0.11 g-EtOH/g-cell/h (the first run) and 0.43 g-EtOH/g-cell/h (the second run) as shown in Fig. 8. These values are lower than the value found by Hamamci (*14*), who found a value of 0.7 g-EtOH/g-cell/h with synthetic medium containing a high glucose concentration. This difference of the V_{max} value can

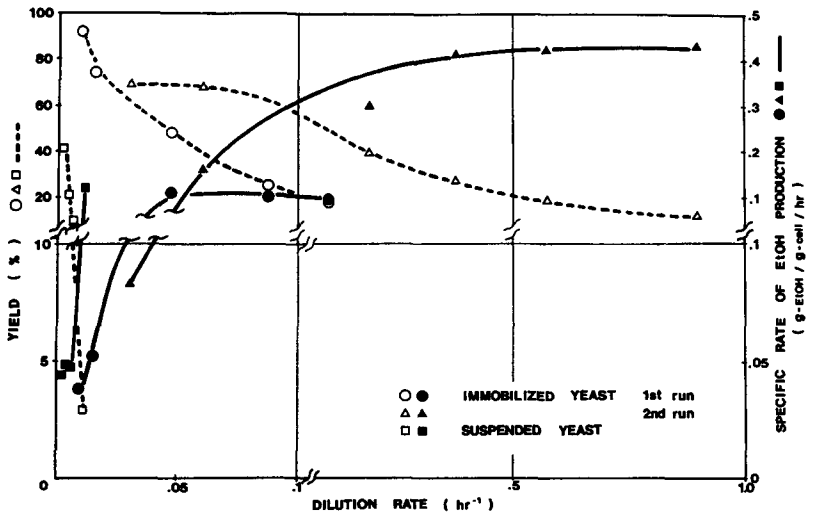

Fig. 8. Profiles of specific rate of ethanol production and ethanol yield for both immobilized and suspended cell cultures as a function of dilution rate.

be mainly caused by both differences in temperature (13°C vs 30°C), the medium, and total gel beads vol (75% of bioreactor vs 100%).

CONCLUSIONS

The tapered column bioreactor design was found to provide a satisfactory performance for the gas-liquid-solid, 3-phase system fermentation. In this study, optimal dilution rate for the half-diluted wine fermentation at 13°C was found to be 0.01 h^{-1} and also for half fermentation of original grape juice was selected to be 0.2 h^{-1}. For example, a two-stage continuous wine fermentation system may be designed using the new tapered column bioreactors packed with immobilized yeast cells. The first-stage and second-stage dilution rate can be maintained at 0.2 and 0.01 h^{-1}, respectively. Further studies will be required for the improvement of two-stage continuous wine fermentation process for development of industrial wine cooler process employing the new bioreactor system and immobilized yeast cells as demonstrated in this paper.

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