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Development and Application of a System for Analysis of Mixed Cultures of Microorganisms

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Abstract

Development and application of a system for real-time quantitative assessment of individual cell activities in a mixed culture system was investigated. This was based on a concept that the activities of individual cells in a mixed culture can be assessed if the cells are physically separated (in separate compartments) in a vessel while the culture conditions, including the broth components, are maintained the same in all the compartments during the cultivation. On this basis, three different apparatus (M-1, M-2, and M-3) were constructed using various types of membranes. In terms of mass transfer characteristics and membrane fouling, the M-3 apparatus was the most effective system for analysis of mixed cultures at high cell densities. With the M-3 apparatus, the interrelationships between two alcohol-producing strains (*Saccharomyces cerevisiae* and *Zymomonas mobilis*) under anaerobic and aerobic conditions were studied. Under anaerobic condition, except for possible competition for nutrients, there were no significant effects of the activities of one microorganism on the other. However, under aerobic condition, amensalism was observed because acetaldehyde that was produced by *Z. mobilis* inhibited the growth of *S. cerevisiae*.

Index Entries: Mixed cultures; cell activities; on-line monitoring; alcohol fermentation; acetaldehyde; aerobic cultures; anaerobic cultures.

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Introduction

The advantages of cultivating two or more microorganisms together (mixed culture) over cultivation of a single microorganism (pure culture) have been pointed out by many researchers *(1)*. In fermentation industries, mixed-culture systems have great potential for many processes involving more than one reaction step. With a mixed-culture system, many reaction steps can be done inside a single fermentor. For example, cheap raw materials such as starch can be hydrolyzed and converted to many useful metabolites in a mixed-culture system. However, industrial application of mixed cultures has been achieved only in very narrow areas such as in the production of traditional fermented food products and in wastewater treatment. This is partly because the various microorganisms present in a mixed-culture system usually have different optima culture conditions of pH, temperature, substrate, and oxygen concentrations. It is therefore difficult to determine the culture condition that will equally support maximum activities of the various microorganisms. A favorable culture condition for one microorganism may even be inhibitory for another microorganism within the system. Furthermore, although in most cases the activity of one microorganism affects (either positively or negatively) those of the other microorganisms, there is still no quantitative method of measuring the activities of the individual microorganisms within the mixed-culture system.

Efficient control of a fermentation process requires accurate on-line monitoring of the cell concentration. Monitoring of cell concentration is also quite important for determination of the effects of culture conditions on the activities of the individual microorganisms, as well as the interrelationships among the microorganisms within a mixed culture. In conventional mixed-culture systems, the colony-counting method using selective media is used *(2)*. However, in this method, experience and professional knowledge are required for determination of the appropriate selective medium. Also, since it takes some time before visible colonies are formed, the method cannot be used for on-line control of the process. Consequently, many alternative methods have been proposed for measurement of the individual cell concentrations in mixed-culture systems. These include the use of unique characteristics of the microorganisms such as cell morphology *(3,4)*, unique enzymes or metabolic products *(5,6)*, or selective staining *(7)*. However, the usefulness of these techniques is on a case-by-case basis, and it is difficult to obtain quantitative data with some of these methods. Thus, there is a need for a general method of accurately monitoring the concentrations of the individual cells in a mixed-culture system.

This study was aimed at developing a general method that can be used for analysis of mixed-culture systems, regardless of the types of the constituent microorganisms. An apparatus that enables accurate, quantitative, and real-time determination of the individual cell concentrations in a mixed culture was developed. As an example, this apparatus was used to analyze a mixed culture of ethanol-producing *Saccharomyces cerevisiae* and *Zymomonas mobilis* under both aerobic and anaerobic conditions.

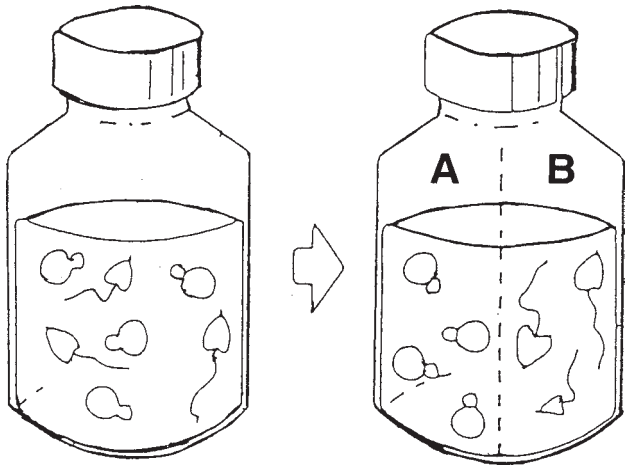

Fig. 1. The concept for development of a system for analysis of mixed-culture systems.

Materials and Methods

Basic Principles

We considered that a general method of accurate on-line determination of the individual cell concentration in a mixed-culture system can be developed if the following requirements are met:

1. The cells are physically separated from each other within the reactor. In other words, the reactor should be divided into compartments and each constituent microorganism cultivated within a separate compartment.
2. The culture conditions (pH, temperature, broth composition, and so on) are maintained the same in all the compartments throughout the cultivation. This means that a concentration gradient with respect to any of the media components should not develop in the course of fermentation. Since the activities of the cells (and consequent changes in the composition of the broth) affect each other, the data obtained with the conventional (uncompartmentalized) reactor can only be reproduced with the compartmentalized reactor if the broth composition and other fermentation conditions are maintained the same in all the compartments during the cultivation.

Figure 1 gives an example of an apparatus for a mixed culture of two microorganisms, in which a cultivation vessel is divided into two compartments by a membrane. The membrane allows free passage of the broth components, but each microorganism is retained within a compartment. Consequently, the broth composition remains the same in both compartments while the activities of the individual cells (cell concentrations) can be separately monitored. By increasing the number of compartments, a system for three or more microorganisms can be constructed.

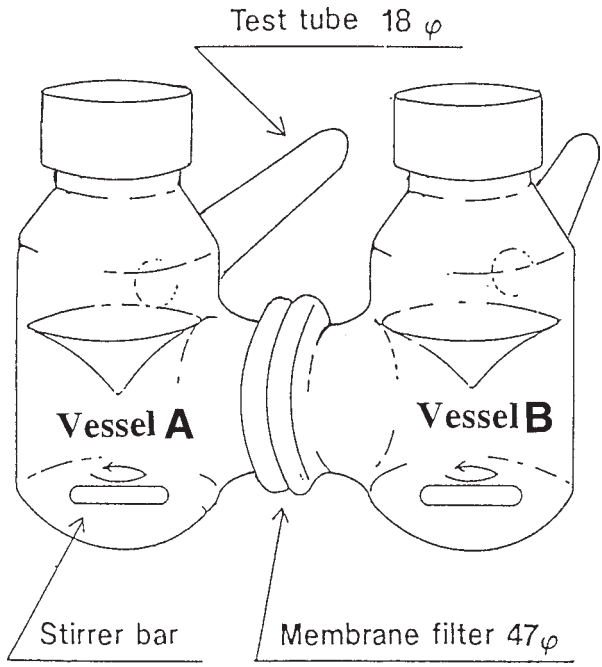

Fig. 2. A schematic diagram of the M-1 apparatus.

Based on the principle we have outlined, three types of apparatus (M-1, M-2, and M-3) for mixed culture of two strains of microorganisms were investigated using various types of membranes. Their basic characteristics are described next.

M-1 Apparatus

The M-1 apparatus is the simplest system that would permit cultivation of two different microorganisms, physically separated from each other, in the same cultivation broth (Fig. 2). An important factor in this system is how to overcome the diffusion limitation that may lead to gradient in the concentration of the broth components between the compartments. As shown in Fig. 2, two 300-mL vessels are separated by a membrane (47 mm diameter) that allows free diffusion of the broth components. Each vessel is equipped with a spectrophotometer cell at the upper part so that the density of the broth in each vessel can be determined at any time. The suitability of cellulose acetate (Advantec, Tokyo; 0.45-µm pore size, 125-µm thickness, 68% porosity), cellulose nitrate (Advantec; 0.45-µm pore size, 145-µm thickness, 78% porosity), NAL (Nihon Pore, Tokyo; material: nylon 66, 0.2-µm pore size, 150-µm thickness, 80% porosity), and DTTP (Millipore, Tokyo; material: polycarbonate, 0.6-µm pore size, 10 µm ± 5% thickness, 5–10% porosity) membranes in maintaining homogeneous concentrations of the broth components between the vessels were investigated.

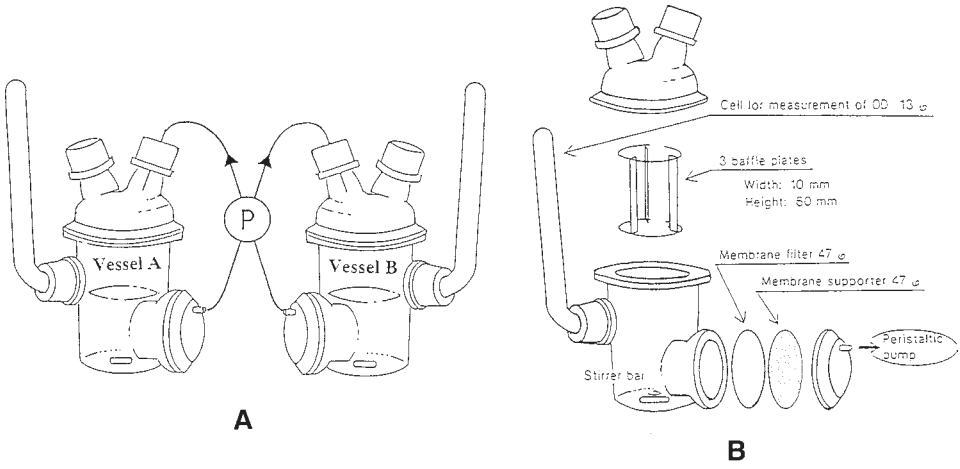

Fig. 3. Diagrams of the M-2 apparatus. **(A)** The experimental setup; **(B)** various parts of a vessel.

M-2 Apparatus

For the M-2 apparatus, two glass vessels, each with a working volume of 300 mL, were connected together by silicon tubes. A tube for pumping out the broth was connected to each vessel through a membrane unit consisting of a membrane (a cellulose acetate membrane; 0.45-µm pore size, 125-µm thickness, 78% porosity, 47 mm diameter), a membrane supporter, and exit port. A pump was used to circulate the broth filtrate between the two vessels. As shown in Fig. 3, the filtrate from one vessel is pumped into the second vessel from the top. Thus, the rate of nutrient exchange between vessels A and B is quite fast, thereby preventing concentration gradient between the two vessels. During the circulation, the liquid flow was perpendicular to the membrane (dead-end type). To ensure that complete mixing was achieved within a short time, a magnetic bar (2 cm long) was used for broth mixing (480 rpm) and a baffle with three plates (60 × 10 mm) was inserted into each vessel. Each vessel was also equipped with a spectrophotometer cell for measurement of the optical density (OD) of the broth.

M-3 Apparatus

As in the case of the M-2 apparatus, a pump was used to circulate the broth filtrate between the two vessels in the M-3 apparatus. The working volume of each vessel was 300 mL. However, as shown in Fig. 4, M-3 differs from M-2 in that the membrane unit is located at the lower part of the vessel. A magnetic bar (2 cm long) on the membrane is used for broth mixing (480 rpm), thus causing the broth to circulate parallel to the membrane. This results in a cross flow filtration. Furthermore, although the same cellulose acetate membrane was used, the effective surface area of the membrane was 3.7 times (90 mm diameter) larger than the one used for the

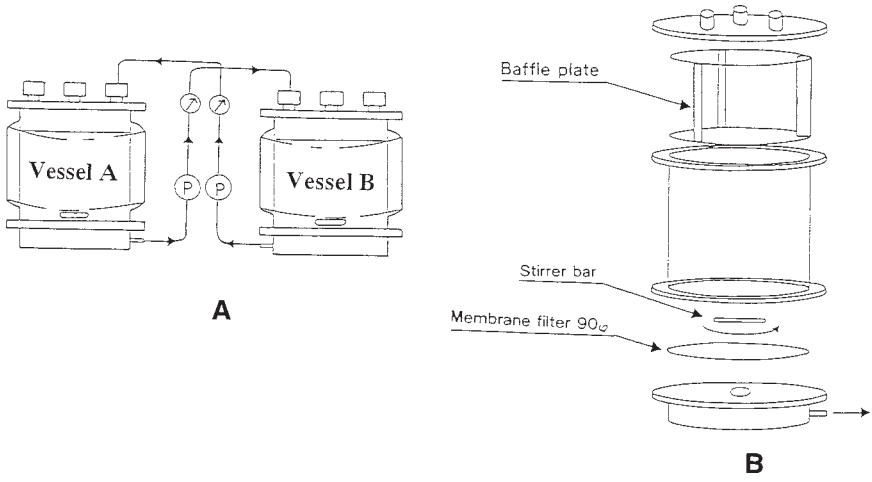

Fig. 4. Diagrams of the M-3 apparatus. **(A)** The experimental setup; **(B)** various parts of a vessel.

M-1 apparatus. Although the M-3 apparatus can be operated at a dilution rate of 3 volumes per volume per hour (vvh), most experiments were done at 2 vvh to avoid membrane damage during each experiment.

Evaluation of the Performance of the M-1, M-2, and M-3 Apparatus

Mass transfer between the two vessels and membrane fouling were used to assess the performance of the M-1, M-2, and M-3 apparatus using glucose solution as a model solution, and yeast cell suspension as a model cell suspension, respectively.

Glucose Transfer Between the Two Vessels

Three hundred milliliters of 30 g/L glucose solution was added to vessel A and an equal volume of distilled water was added to vessel B. The glucose solution in vessel A moves to vessel B by diffusion across the membrane (M-1) or by circulating the broth filtrate between the two vessels (M-2 and M-3).

Generally, mass transfer across membrane depends on the membrane permeability coefficient (k) and the effective surface area of the membrane (A). From changes in the glucose concentration in vessel A, the glucose transfer coefficient can be represented by Eq. 1:

$$\frac{dC_{Sa}(t)}{dt} = -kA[C_{Sa}(t) - C_{Sb}(t)] \quad (1)$$

where kA is the glucose transfer coefficient (h^{-1}), k is the membrane permeability coefficient ($h^{-1} \cdot cm^{-2}$), A is the effective surface area of the membrane (cm^2), $C_{Sa}(t)$ is the glucose concentration in vessel A at time t(g/L), and $C_{Sb}(t)$ is the glucose concentration in vessel B at time t(g/L).

At any point in time, it can be assumed that $C_{Sa}(t) + C_{Sb}(t) = C_{Sa}(0)$. Thus, Eq. 1 can be rearranged to give Eq. 2:

$$\frac{dC_{Sa}(t)}{C_{Sa}(t) - \frac{C_{Sa}(0)}{2}} = -2kA \cdot dt \tag{2}$$

Integration of Eq. 2 gives Eq. 3:

$$\ln \frac{C_{Sa}(t) - \frac{C_{Sa}(0)}{2}}{\frac{C_{Sa}(0)}{2}} = -2kA \cdot t \tag{3}$$

Percentage mixing (*De*), defined by Eq. 4, was used to assess the extent to which nutrients from one vessel had moved into the other at a given point in time:

$$De = \frac{C_{Sa}(0) - C_{Sa}(t)}{C_{Sa}(0) - C_e} \times 100 \tag{4}$$

where *De* is the percentage mixing and C_e is the equilibrium glucose concentration (g/L). Rearrangement of Eq. 4 results in Eq. 5, and Eq. 6 can be obtained from Eqs. 3 and 5:

$$1 - \frac{De}{100} = 1 - \frac{C_{Sa}(0) - C_{Sa}(t)}{C_{Sa}(0) - C_e} = \frac{C_{Sa}(t) - \frac{C_{Sa}(0)}{2}}{\frac{C_{Sa}(0)}{2}} \tag{5}$$

$$\ln\left[1 - \frac{De}{100}\right] = -2kA \cdot t \tag{6}$$

The values of *kA* for different membranes were calculated from the plot of $\ln[1 - (De/100)]$ against *t*.

Membrane Fouling

To establish an operation condition under which membrane fouling is minimized, the effects of medium circulation on membrane fouling in the M-2 and M-3 apparatus were investigated, using a suspension of commercial baker's yeast. The yeast suspension was added to vessel A and the medium was circulated through the membrane. Various initial cell concentrations and circulation rates were used, and the degree of membrane fouling was expressed as the ratio of the circulation rate after 2 h to the initial circulation rate (V/V_O). Effect of backflushing on membrane fouling was also investigated by varying the length and frequency of backflushing during broth circulation.

Microorganisms and the Cultivation Conditions

S. cerevisiae IFO 0309 and *Z. mobilis* IFO 13756 were used as representative yeast and bacteria cells, respectively. *S. cerevisiae* was subcultured monthly in the medium containing 10 g/L glucose; 5 g/L peptone; 3 g/L yeast extract; 3 g/L malt extract; and 15 g/L agar (pH 6.2). *Z. mobilis* was also subcultured monthly in a medium containing 10 g/L glucose; 5 g/L yeast extract; and 2 g/L $MgSO_4 \cdot 7H_2O$ (pH 6.8). Both cells were stored at 4°C. The medium used for the pre- and main cultures contained 30 g/L glucose; 8 g/L yeast extract; 8 g/L KH_2PO_4; 4 g/L $(NH_4)_2SO_4$; and 2 g/L $MgSO_4 \cdot 7H_2O$ (pH 6.0). An Erlenmeyer flask (500 mL) containing 100 mL of the culture medium was inoculated with 10 mL of the *Z. mobilis* stock cell suspension or two loops of *S. cerevisiae* slant culture and cultivated either statically (*Z. mobilis*) or at 220 rpm (*S. cerevisiae*). In both cases, the precultivation was done for 18 h at 30°C.

For the main culture, the two vessels were filled with 300 mL of the culture medium, inoculated with 3 mL of the preculture broth and cultivated at 30°C. In the case of aerobic culture, the rotation speed of the magnetic bar was 220 rpm, and the aeration rate (with ordinary air) was 50 mL/min. During the anaerobic culture, nitrogen gas was used for aeration at 50 mL/min. The eccentricity of the shaker was 7 cm.

Suitability of M-3 Apparatus for Analysis of Mixed-Culture Systems

Anaerobic batch cultivation of *S. cerevisiae* was performed in the M-3 apparatus. Vessel A contained both the medium and the yeast cells whereas vessel B contained only the medium. The broth circulation rate was 2 vvh and backflushing was done for 24 s every 6 min of circulation. As a control, anaerobic batch cultivation of *S. cerevisiae* in a glass vessel without the membrane filter unit (vessel C) was also done. The same experiment was repeated with *Z. mobilis*.

Use of M-3 Apparatus for Analysis of Mixed Cultures of *S. cerevisiae* and *Z. mobilis* Under Anaerobic and Aerobic Cultivation Conditions

The M-3 apparatus was used to study the interrelationship between *S. cerevisiae* and *Z. mobilis* under anaerobic cultivation conditions. The anaerobic condition was achieved by sparging with nitrogen gas at a rate of 50 mL/min. Both vessels A and B were filled with 300 mL each of the culture medium. *S. cerevisiae* was inoculated into vessel A whereas *Z. mobilis* was inoculated into vessel B. In both cases, the preculture was done for 18 h and the inoculum volume was 20 mL. The medium circulation rate was 2 vvh and backflushing was done for 24 s every 6 min of circulation. The temperature was controlled at 30°C throughout the cultivation. As a control experiment, a pure culture of each strain was done. A single vessel without a membrane was inoculated with 10 mL of either of the cell strains, and except for medium circulation, other cultivation conditions were as described for the mixed culture.

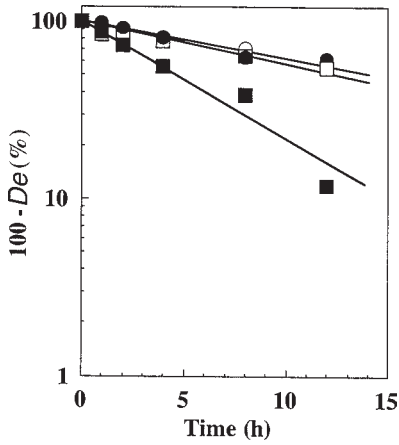

Fig. 5. Effect of membrane characteristics on *kA* values in the M-1 apparatus. The membranes used were cellulose acetate with a 0.45-µm pore size (○), cellulose nitrate with a 0.45-µm pore size (●), NAL with a 0.2-µm pore size (□), and DTTP with a 0.6-µm pore size (■).

A mixed culture of *S. cerevisiae* and *Z. mobilis* under aerobic condition was also studied, using the M-3 apparatus. The experimental conditions for both the mixed and pure cultures were the same as described for the anaerobic culture except that ordinary air was used in place of nitrogen gas for aeration.

Analytical Methods

The cell concentrations were expressed in unit optical densities (UODs) at 580 nm, using a spectrophotometer (Specronic 20A, Shimadzu, Tokyo, Japan). Glucose was measured enzymatically with a glucose test kit (Wako Pure Chemical, Osaka, Japan). Concentrations of ethanol and acetaldehyde were measured by gas chromatography with flame ionization detectors after removing cells by centrifugation *(8)*.

Results and Discussion

Mass Transfer in the Systems

Figure 5 shows the relationship between ln(100-*De*) and *t* for different types of membranes when 3% glucose was added to vessel A of the M-1 apparatus. From the slopes of the curves, the *kA* values for cellulose acetate, cellulose nitrate, NAL, and DTTP membranes were 2.3×10^{-2} (h^{-1}), 2.3×10^{-2} (h^{-1}), 2.5×10^{-2} (h^{-1}), and 7.3×10^{-2} (h^{-1}), respectively. This shows that the rate of glucose transfer by simple diffusion from vessel A to B was low regardless of the membrane type. Thus, even with a membrane of high permeability coefficient (*k*), it is extremely difficult to obtain the mass transfer rate necessary to maintain the same culture conditions in vessels A and B of

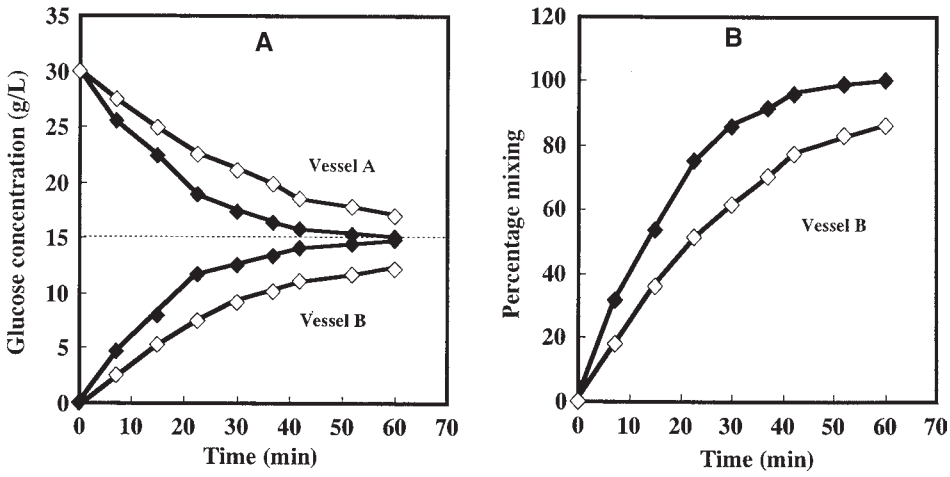

Fig. 6. Time courses of glucose transfer **(A)** and percentage mixing **(B)** in M-2 apparatus. The broth circulation rates were 1 vvh (◇) and 2 vvh (◆).

apparatus M-1. It was estimated that in order to achieve 80% transfer in 1 h, a *kA* value of 8.1×10^{-1} (h^{-1}) is required.

The M-2 apparatus was therefore constructed and the rate of glucose transfer from vessel A to B was investigated. As shown in Fig. 6A, a relatively high rate of glucose transfer from vessel A to B was achieved in the M-2 apparatus. The effects of medium circulation rate on the increase in percentage of glucose transfer with time is shown in Fig. 6B. At a circulation rate of 1.0 vvh, a *De* value of 80% was achieved in 50 min, and by increasing the circulation rate to 2.0 vvh, 80% *De* was attained in 25 min.

Attempts were made to increase the mass transfer between the two vessels by using various types of membranes (cellulose acetate, NAL, DTTP, and polyvinyl alcohol) with various pore sizes ranging from 0.2 to 3.0 μm, but no significant increase in *De* was observed (data not shown).

Membrane Fouling and Effect of Backflushing

When a yeast suspension was added to vessel A and the suspension was circulated across the membrane, membrane fouling was observed owing to cell adsorption to the membrane. Various methods of cleaning the membrane during the broth circulation were investigated. It was found that intermittent backflushing could be effective in cleaning the membrane and thus maintaining a stable flow rate over a long period of time.

Figure 7 shows the effects of the initial cell concentration on decrease in circulation rate in the M-2 and M-3 apparatus. Various concentrations of baker's yeast suspension were circulated at a rate of 1 vvh and 12 s of backflushing was done at 4-min intervals. The ratio of the circulation rate (after 2 h) to the initial circulation rate (V/V_O) was plotted against the initial cell concentration, as shown in Fig. 7. In the case of the M-2 apparatus, the circulation rate decreased sharply as the initial cell concentration was

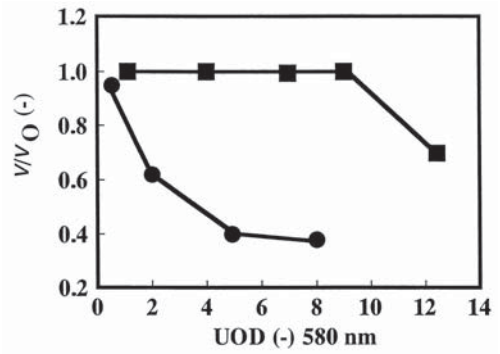

Fig. 7. Effect of initial cell concentration on the ratio of the flow rate after 2 h to the initial flow rate (V/V_O) in (●) M-2 and (■) M-3. The initial circulation rate was 1 vvh and 12 s of backflushing was done every 4 min.

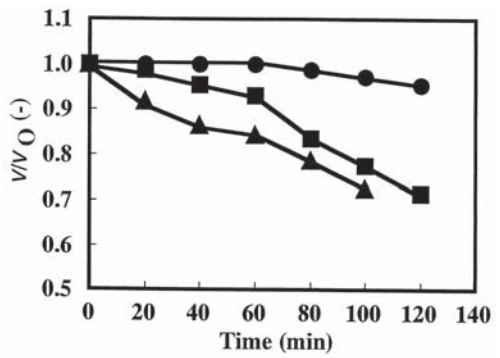

Fig. 8. The effects of backflushing conditions on the circulation rate in the M-3 apparatus. The OD of the cell suspension was 20 (UOD) and the initial circulation rate was 2 vvh. The backflushing conditions were (■) 12 s every 4 min, (▲) 12 s every 6 min, and (●) 24 s every 6 min.

increased. The circulation rate decreased to <90% of the set value even with a very low initial cell concentration of 0.8 (UOD). On the other hand, the circulation rate remained higher than 90% of the set value even when an initial cell concentration of 10 (UOD) was used in the M-3 apparatus.

The effects of backflushing conditions on the circulation rate when the initial cell concentration was increased to 20 (UOD), was investigated using the M-3 apparatus. This value was considered the maximum cell concentration obtained in most batch cultures. The results (Fig. 8) showed that even with a very high cell concentration of 20 UOD and circulation rate of 2 vvh, 24 s of backflushing at 6-min intervals was enough to prevent membrane fouling.

Suitability of M-3 Apparatus for Analysis of Mixed-Culture Systems

Figure 6 shows that it took about 50 min to achieve an equilibrium concentration of glucose in the two vessels of the M-3 apparatus. An experi-

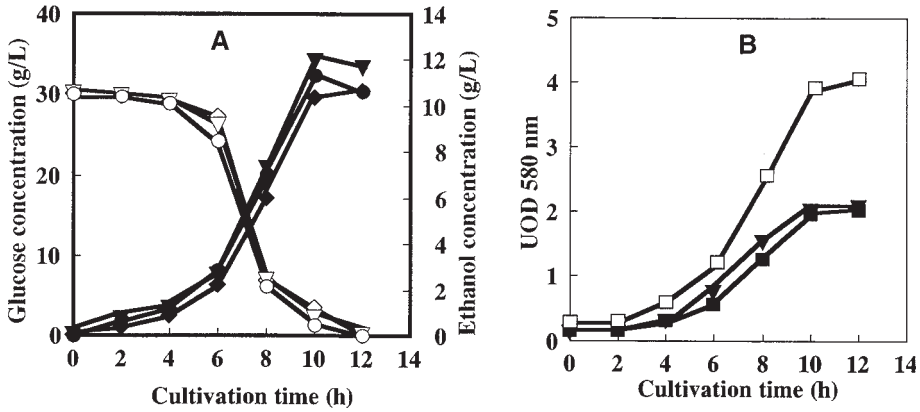

Fig. 9. Changes in **(A)** glucose (○, ◇, ▽) and ethanol (●, ◆, ▼) concentrations in vessels A, B, and C, respectively; and **(B)** cell growth in vessels A (□), C (▼), and A/2 (■) during anaerobic cultivation of *S. cerevisiae* in the M-3 apparatus.

ment was therefore conducted to determine whether this rate of mass transfer between the two vessels would affect the performance of M-3 as an apparatus for analysis of mixed-culture systems (i.e., if the growth rate of the cells in one vessel would be limited by the rate of nutrients transfer from the second vessel).

Figure 9A shows time courses of glucose and ethanol concentrations in each of the vessels when *S. cerevisiae* was inoculated in only vessel A. Although there was about a 20-min time lag in the concentrations of glucose and ethanol between vessels A and B, the fermentation pattern was quite similar to that observed in vessel C. At the end of the cultivation, it was found that about 10 mL of the culture broth was retained inside the tubing and the membrane unit in the M-3 apparatus. This is a possible reason for the slight differences observed between the cell concentrations in vessels A and C. Since the total amount of nutrients utilized by the cells in vessel A is two times (total volume of the medium in vessels A and B = 600 mL) higher than that in vessel C (300 mL), the cell concentration in vessel A would be twice that in C. This was consistent with the experimental results (Fig. 9B). Thus, it can be assumed that the rate of mass transfer between vessels A and B is sufficient and does not limit the growth of the cells. Similar results were also obtained when *Z. mobilis* was cultivated under the same experimental conditions.

Use of M-3 Apparatus for Analysis of Mixed Cultures of S. cerevisiae *and* Z. mobilis *Under Anaerobic and Aerobic Cultivation Conditions*

Figure 10 shows time courses of *Z. mobilis* and *S. cerevisiae* growth in anaerobic and aerobic mixed cultures. In both cases, glucose was completely consumed and the experiments were stopped at 9 h. Under anaerobic condition, the final UODs of *Z. mobilis* and *S. cerevisiae* in the mixed

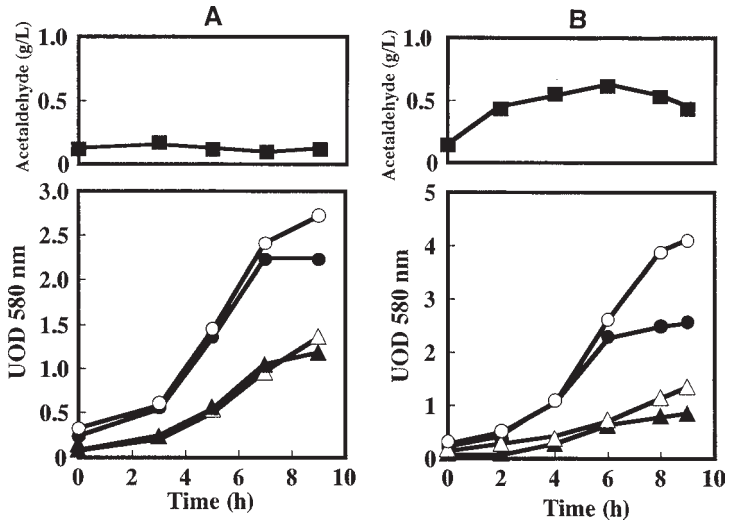

Fig. 10. Anaerobic **(A)** and aerobic **(B)** cultivation of *S. cerevisiae* and *Z. mobilis* under pure (open symbols) and mixed (closed symbols) cultures. (○, ●), *S. cerevisiae*; (△, ▲) *Z. mobilis*; (■) acetaldehyde.

culture were 1.1 and 2.3, respectively (Fig. 10A). In the pure-culture systems, the growth rates for the two cell strains were almost the same as those obtained in the mixed culture. As shown in Fig. 10A, the final UODs in the pure culture were 1.3 for *Z. mobilis* and 2.7 for *S. cerevisiae*. These results show that in an anaerobic mixed culture of these two microorganisms, apart from possible competition for nutrients, there are no significant inhibition or promotion effects of one microorganism on the other.

Figure 10B shows time course for the growth of *Z. mobilis* and *S. cerevisiae* in aerobic mixed and pure cultures. In pure cultures, the final UODs for *S. cerevisiae* and *Z. mobilis* were 4.2 and 1.3, respectively. In the mixed culture, the final UOD of *Z. mobilis* was 0.9, which is only slightly lower than the value obtained in a pure culture. On the other hand, the final UOD of *S. cerevisiae* was only 2.6 in the mixed culture as compared with 4.2 in the pure culture. Since under anaerobic condition there was no significant effects of the activities of one cell on the other, the inhibition of *S. cerevisiae* growth in aerobic mixed culture may be owing to the inhibitory products of *Z. mobilis*. It has been reported that under aerobic condition, both the cell yield and ethanol production by *Z. mobilis* decrease while acetic acid and acetaldehyde are accumulated *(9–11)*. Acetaldehyde inhibits the growth of both *Z. mobilis* and *S. cerevisiae*.

As shown in Fig. 10A,B, the acetaldehyde concentrations in the broth during batch cultivation under anaerobic and aerobic conditions were 0.1 and 0.6 g/L, respectively. We have already reported that under aerobic condition, acetaldehyde production increased as the volumetric oxygen transfer coefficient (k_La) was increased and that the inhibitory effect of acetaldehyde on the growth of *S. cerevisiae* increased with an increase in the

acetaldehyde concentration *(11).* Although the growth of *S. cerevisiae* was inhibited in a medium containing 0.5 g/L of acetaldehyde, the same concentration of acetaldehyde had no significant effect on the growth of *Z. mobilis* (data not shown).

These results show that under aerobic mixed culture of *S. cerevisiae* and *Z. mobilis*, the growth of *S. cerevisiae* is inhibited by acetaldehyde, which is produced *by Z. mobilis*. Thus, by using the M-3 system, the amensalism between *S. cerevisiae* and *Z. mobilis* in a mixed culture under aerobic condition has been identified. This system can be applied for analysis of various mixed-culture systems. Furthermore, since on-line monitoring of the individual cell concentrations is possible, it can be used for optimization of various mixed-culture systems.

Acknowledgment

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