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# Measurement of Viable Cell Number in Mixed Culture Based on Microbial Respiration Rate

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# 미생물 호흡속도에 기초한 혼합배양중의 생균수 측정

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**Abstract** — A simple method to determine viable cell numbers of each species in mixed culture was developed. The oxygen uptake rate (OUR) equals to the product of the specific OUR and the size of the microbial population. In a mixed culture, the OUR is a result of the respiration activities of each sub-population. The OUR was determined from the slope of the linear relationship between time and the decrease of dissolved oxygen concentration when aeration was stopped. The specific OUR was calculated from the slope of the viable cell number versus OUR curve. These values for *C. lusitaniae* at 20 and 30℃ were $1.36\times10^{-9}$ and $3.90\times10^{-9}$ and those for *P. tannophilus* at 20 and 30℃ were $0.59\times10^{-9}$ and $1.86\times10^{-9}$ [(%/s)/(cells/m*l*)], respectively. Using these values, viable cell numbers were calculated after the OURs of mixed culture at two temperatures were measured. A good agreement between the viable cell numbers determined by this method and by plate count was obtained.

Determination of the amount of biomass in a system and its increase with time is required for the monitoring and controlling of fermentation processes. Several methods, such as hemacytometer counts, electronic particle counts (1) and colony counts have used for the determination of cell numbers in pure culture fermentations. However, the determination of the population of each species present during mixed culture is obviously more difficult than that of cell numbers in pure culture.

When only one species of microorganism of a mixed population is to be measured, enumeration techniques are usually the only option. Microscopic and biological counts can utilize visible differences in cell and colony morphology to distinguish between different sub-populations (2). In latter case, different media selective for single species may be used. Fluorescent antibodies offer a very specific means of identifying a particular microorganism applicable to microscopic counts (3) and flow microfluorometry (4). The Coulter Counter can only be used to resolve simple mixture where an appreciable difference in size of the each species present (5).

An alternative for quantitating amounts of each species is based on measurement of respiration rate. Boyles (6) developed an oxygen electrode chamber which provides a cheap and simple method of measuring specific growth rate. The value for specific growth rate can be determined graphically from the dissolved oxygen versus time trace. Matsunaga *et al.* (7) determined viable cell numbers with an electrode system composed of a membrane filter for retaining microorganisms and an oxygen electrode. The principle of the cell number deter-

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mination is based on sensing microbial respiration.

If two organisms have different respiration rates and if the dependence of respiration rate on temperature differs, then respiration rate measurement can be used to determine amounts of viable cells of each species. The system selected for experimental studies is a coculture of the yeasts *Pachysolen tannophilus* and *Candida lusitaniae* producing ethanol from a mixture of glucose, xylose and cellobiose. Microscopic examination and preliminary particle size analyses have shown both organisms to be approximately the same size, thereby eliminating particle size distribution measurements as a means of determining species populations. The respiration rates of each species at two different temperatures are measured with a dissolved oxygen probe and the viable cells are enumerated using the plate counts.

## Theory

The relationship between microbial oxygen uptake rate rate (OUR, respiration rate) and dissolved oxgyen concentration (DOC) is usually of the Michaelis-Menten type as shown in Fig. 1 (8). If the DOC is above a certain value termed $c_{crit}$, microorganisms consume dissolved oxygen with maximum rate. The maximum specific OUR of a microorganism is a constant value in defined environmental and non-limited growth conditions. The OUR per unit volume, Q, depends only on the size of the microbial population:

$$Q = Q_s \cdot N \tag{1}$$

where $Q_s$ is the maximum specific OUR and N is viable cell number. The viable cell concentration can be measured indirectly by measuring the OUR of a culture if the maximum specific OUR is known.

In a mixed culture, the oxygen uptake rate is a result of the respiration activity of each sub-population. The respiration rate of a mixed culture is the sum of the respiration rates of the individual species:

$$Q_m^\theta = \sum_{i=1}^{n} N_i \cdot Q_{s,i}^\theta \tag{2}$$

where $Q_m^\theta$ = respiration rate of mixed culture at temperature $\theta$,

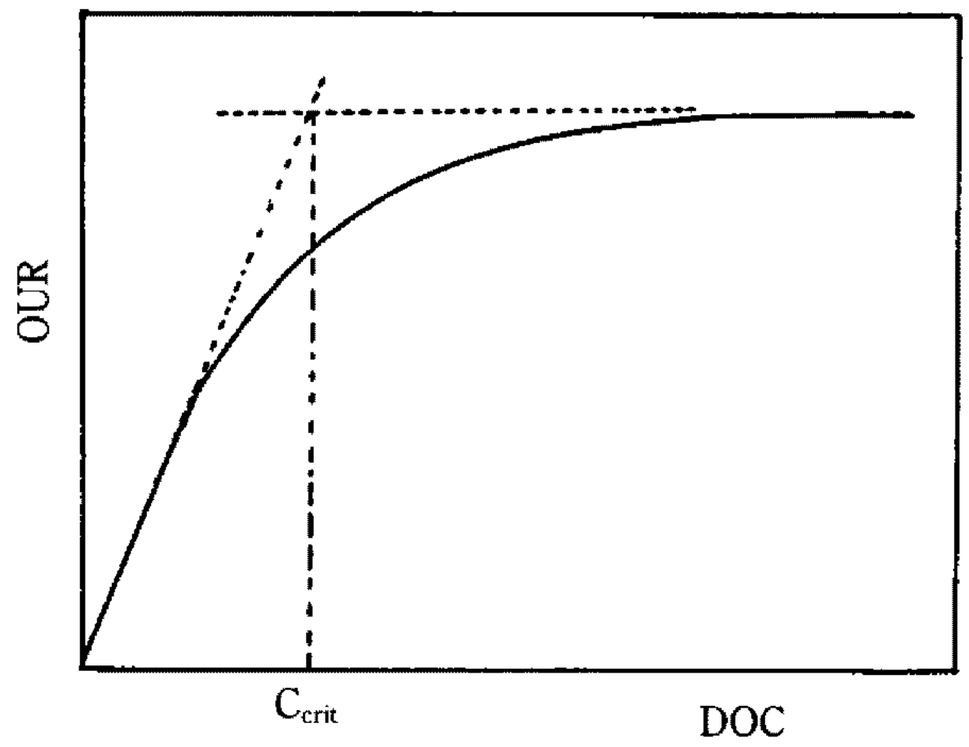


**Fig. 1. Effects of dissolved oxygen concentration on respiration rate.**

$Q_{s,i}^\theta$ = specific respiration rate of species i at temperature $\theta$,

$N_i$ = number of cells of species i, and

n = number of species

To determine amounts of each species present ($N_i$), n independent equations are needed. If the ratios of the $Q_{s,i}^\theta$ values are not constant with temperature, then measurements of the respiration rate for the mixed culture at n different temperatures will give the required number of equations. For two species, respiration rates measured at two temperatures will allow calculation of the number of viable cells of each species.

To use this method, the maximum specific oxygen uptake rates ($Q_s$) of each species are to be predetermined at different temperatures under the same condition. The dynamic method is used for OUR measurements by recording the DOC change of microbial cultures with a dissolved oxygen probe. The rate of the change of DOC is then equal to the OUR:

$$\frac{dC}{dt} = -Q \tag{3}$$

where C is dissolved oxygen concentration and t is time. Therefore, it is important that the DO concentration should be above the critical value during OUR measurement. The OUR can be calculated from the slope of the linear portion of DOC versus time trace. The slope of the linear relationship between the OUR and the viable cell number is the

maximum specific OUR.

## Materials and Methods

### Microorganisms and cultivation

*Pachysolen tannophilus* NRRL Y-2460 and *Candida lusitaniae* NRRL Y-5394 were used in this study. These were maintained on YM aga. slants. Inocula were grown aerobically in 125 m*l* flask containing 20 m*l* of growth medium (0.67% Yeast Nitogen Base plus 2.0% glucose) and 3 m*l* of the inocula were transferred to 100 m*l* of growth medium in 250 m*l* flask. the inoculated broths were incubated at 28℃ on a rotary shaker.

### Apparatus

The apparatus for measuring OUR was consisted of the DO probe and two glass chambers (Fig. 2). One chamber was used for the measurement of DO concentration change and the other was used as accessory chamber. The measuring chamber was closed with rubber stopper to prevent oxygen transfer into the liquid sample during OUR measurement. The liquid sample was agitated by magnetic stirrer. The temperature of the sample was controlled by circulation of water from constant temperature circulating bath. The probe response was monitored with a recorder.

### Calibration of probe

The DO probe was calibrated with water saturated with air and nitrogen for 100% and 0% saturation, respectively. The stirring intensity during the calibration was the same as that of OUR measurement. The probe calibrations were conducted at two different measuring temperatures.

### Measurement of oxygen uptake rate (respiration rate)

A sample of yeast culture broth was centrifuged at 7000×g for 10 minutes, and yeast biomass was resuspended in a glucose solution (20 g/*l*) of the measuring temperature. The yeast suspension was aerated for 15~20 min at the measuring temperature and diluted with glucose solution if necessary. After then, it was transferred into the measuring chamber which was already reached to constant temperature. During the preparation of yeast sample, the probe was held in the accessory chamber of the measuring temperature. The probe was inserted into the measuring chamber and magnetic stirring was started at the predetermined speed. The probe response was recorded, and OUR was calculated from the slope of the linear portion of the probe trace. The OURs of pure and mixed cultures were measured at two different temperatures, 20 and 30 ℃.

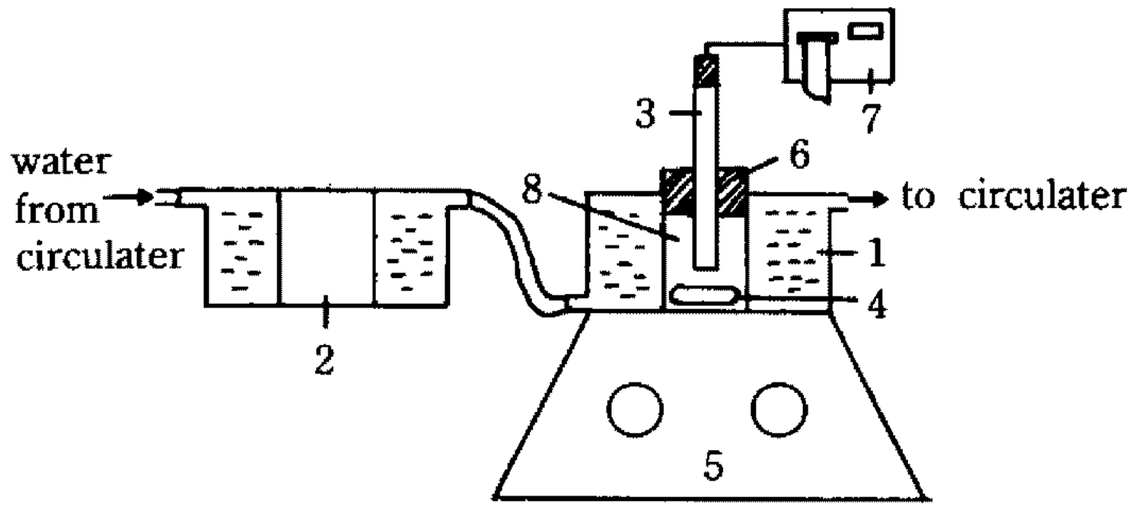


**Fig. 2. A schematic diagram of the apparatus for measurement of oxygen uptake rate.**
1. measuring chamber; 2. accessory chamber; 3. DO probe; 4. magnetic bar; 5. magnetic stirrer; 6. rubber stopper; 7. recorder; 8. sample

### Viable cell concentrations

Viable cell counts for pure cultures were determined by spreading known dilutions of fermentation broth on YM agar plates, followed by incubation at 30℃. For mixed cultures, cell counting was performed on a different medium for each species. *C. lusitaniae* is reported to be unable to assimilate nitrate(9) whereas *P. tannophilus* can(10). Therefore, a yeast carbon base medium with nitrate added was used for selectively culturing *P. tannophilus* for plate counts. Counts for *C. lusitaniae* were taken using YM agar plates.

## Results and Discussion

### DO probe dynamics

The objective of the probe dynamic investigation was to find out the effects of the stagnant liquid film of outside of the membrane and the membrane on the probe response. The experiments were con-

ducted by changing the oxygen concentration in step way at room temperature. The response time of the DO probe is the time required to reach a certain fraction of steady-state current to a step change in oxygen concentration. The response time corresponding to 62.3% of the steady-state current is so-called disssolved oxygen time constant. The time constant of the probe was determined by changing the composition of the gas phase from air to nitrogen, and this value was 23.5 sec. The time constants of the probe in the liquid phase, either water or yeast broth saturated with air, was replaced to nitrogen were found to be 24.1 and 24.0 sec, respectively.

Two conclusions could be drawn from the above results. First, the values of time constants indicated that the time delay of the probe response due to the stagnant liquid film might be neglected compared to the effects of the probe. Second, the effects of the stagnant liquid film in water and yeast culture broth were the same. Since the experiments were performed at sufficiently high stirring intensity, the effects of the stagnant liquid film on the overall system dynamics might be constant and considered to be inherent to the probe dynamics.

Because of the significant time constant of the probe, a difference between observed and actual dissolved oxygen tension could be expected. However, this difference may be negligible for the linear portion of the oxygen uptake curve if the proper operating conditions (stirring intensity) are chosen (11).

**Effect of growth conditions on the yeast respiration**

Yeasts grown on glucose anaerobically lose their respiration capacities. When yeast grown anaerobically is exposed to aerobic conditions, its respiration activity developes. To find out the time needed for restoration of the yeast respiration, we measured the OURs of the yeast culture broths at different times after being exposed to full aerobic conditions at 20 and 30℃. The typical results are shown in Fig 3. The OUR's of yeasts reached at the maximum values approximately 15~20 min after the aeration of yeast culture broths. Therefore, the yeast samples were preaerated for at least 15 min before measuring OUR.

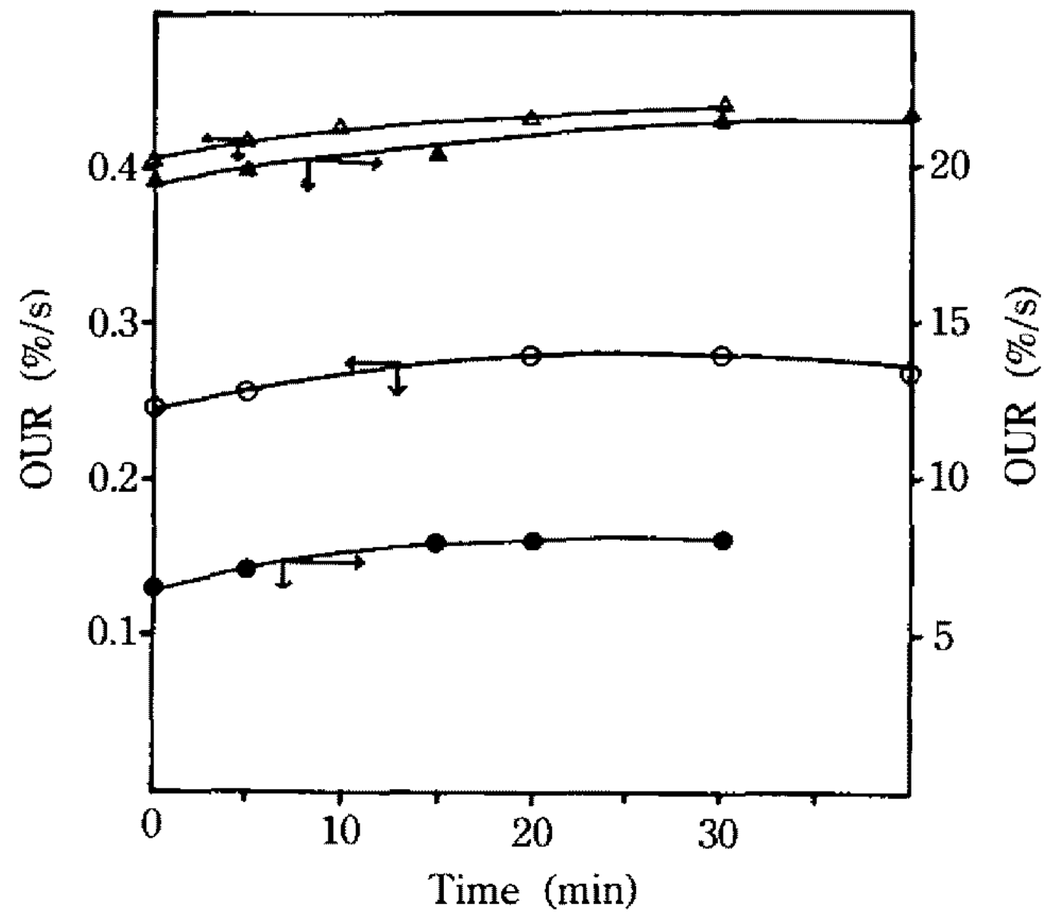


**Fig. 3. Restoration of yeasts respiration activity.**
○, *P. tannophilus* at 20℃ ; ●, *P. tannophilus* at 30℃ ; △, *C. lusitaniae* at 20℃ and ▲, *C. lusitaniae* at 30℃

Matsunaga *et al.* (7) reported that the steady-state current decreased with increasing glucose concentration and became constant above 300 mg/*l* when viable cell numbers were determined with an electrode system. To eliminate the effect of glucose concentration on the maximum OUR of yeasts, a glucose solution of 20 g/*l* concentration was used in all experiments. The calibration curves for yeasts were generated with cells of different ages.

**Determination of the maximum specific OUR's**

The oxygen uptake rate was calculated from the slope of linear portion of time (sec) versus % oxygen saturation curve. The relationships between the OUR and the viable cell concentration for *C. lusitaniae* and *P. tannophilus* of different ages at two temperatures were shown in Fig. 4 and 5, respectively. The slopes of the linear relationships represented the maximum specific OUR ($Q_s$) of the yeasts at each temperatures. The values of the maximum specific OURs for two yeasts at 20 and 30℃ are given in Table 1; these values were calculated using the least square analysis. The maximum specific OUR of *C. lusitaniae* was higher than that of *P. tannophilus*, and those values at 30℃ was higher than those of 20℃ for two yeasts as expected.

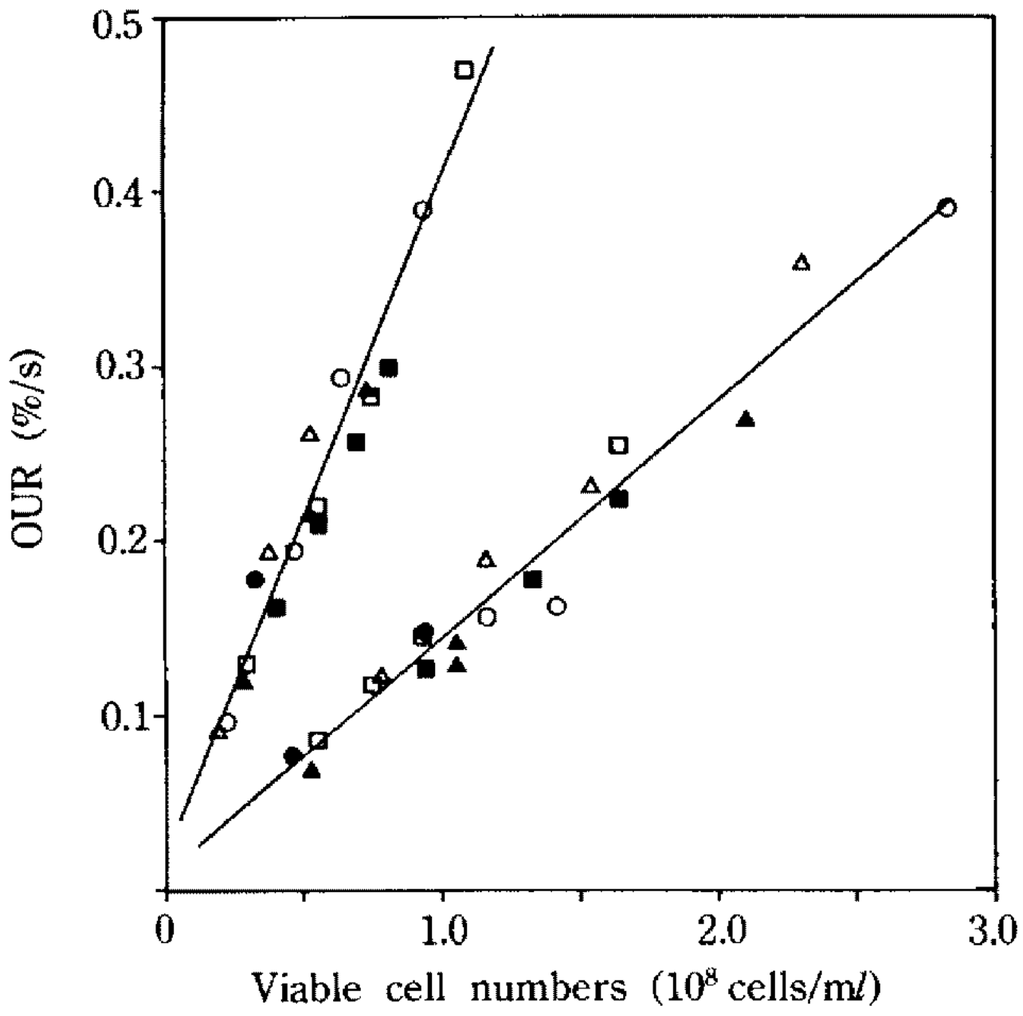


**Fig. 4. Relationship between the OUR and the viable cell number of *C. lusitaniae* incubated for different hours.**

■, 12 h; □, 23 h; ▲, 26 h; △, 49 h; ●, 77 h and ○, 108 h

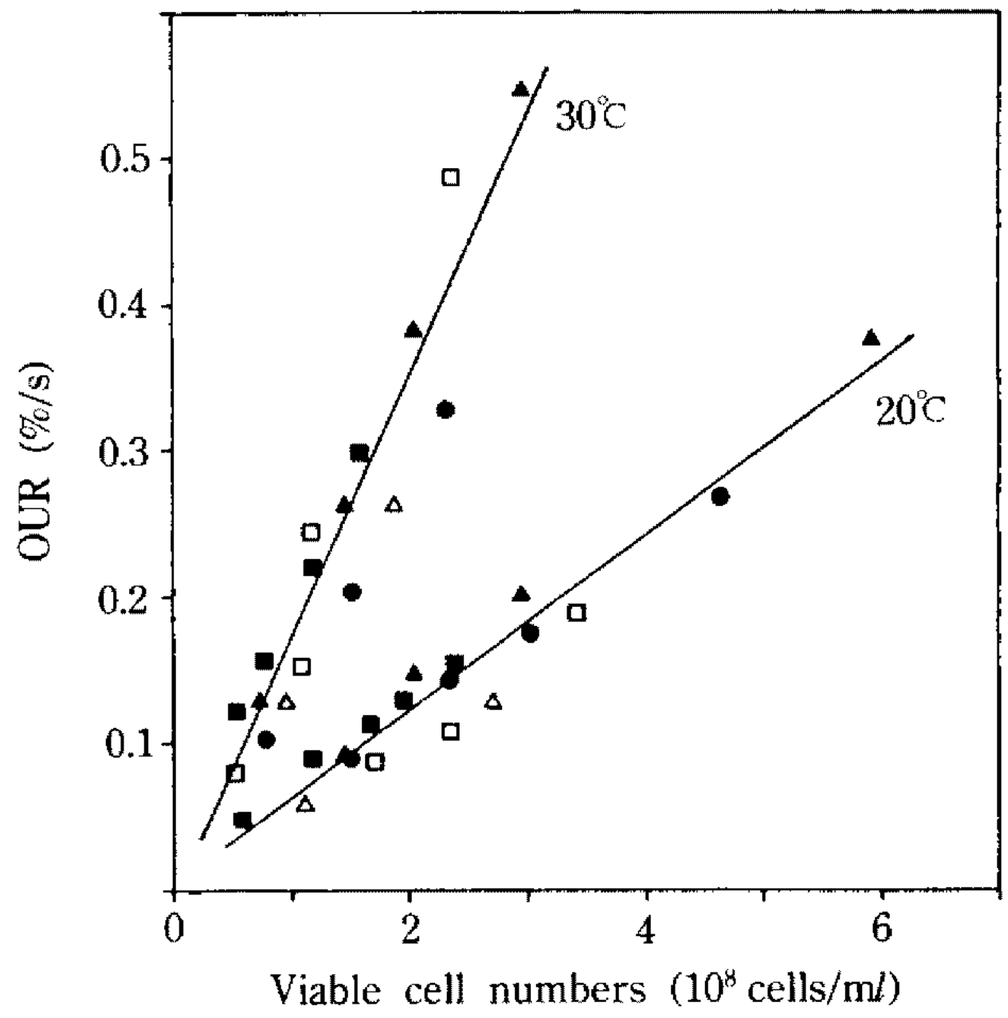


**Fig. 5. Relationship between the OUR and the viable cell number of *P. tannophilus* incubated for different hours.**

■, 14 h; □, 24 h; ▲, 26 h; △, 48 h and ●, 77 h

According to equation (2), viable cell numbers of the two yeasts in a mixed culture can be calculated from the total OURs measured at two temperatures only if the following condition is satisfied;

**Table 1. Maximum specific OURs of yeasts at 20°C and 30°C**

| Yeasts | Maximum specific OUR ($Q_s$) [(%/s)/(cell/ml)]×$10^9$ | | Ratio |
|---|---|---|---|
| | 20℃ | 30℃ | |
| *C. lusitaniae* | 1.36 | 3.90 | 2.87 |
| *P. tannophilus* | 0.59 | 1.86 | 3.17 |
| Ratio | 2.30 | 2.10 | |

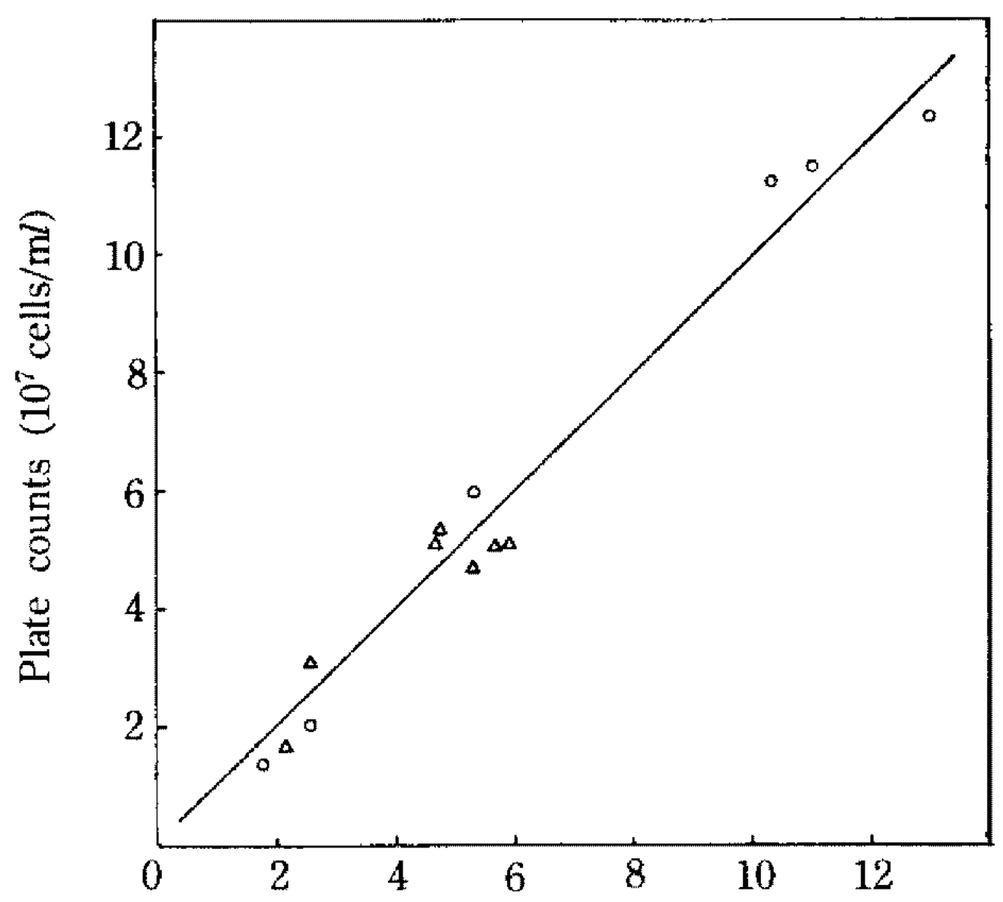


**Fig. 6. Comparison of viable cell number determined by the proposed method and by plate counts.**

○, *C. lusitaniae* and △, *P. tannophilus*

$$\frac{Q_{s,1}^{30}}{Q_{s,1}^{20}} = \frac{Q_{s,2}^{30}}{Q_{s,2}^{20}} \tag{4}$$

These ratios for *C. lusitaniae* and *P. tannophilus* were 2.87 and 3.17, respectively.

**Comparison of viable cell numbers obtained by two methods**

Samples were withdrawn periodically from the mixed culture of two yeasts growing on mixture of glucose, cellobiose and xylose, then the oxygen uptake rate (respiration rate) of a mixed culture ($Q_m$) was measured. When $Q_m$ values at each temperatures are obtained, we can get the following two equations according to equation (2);

$$Q_m^{20} = N_1 \cdot Q_{s,1}^{20} + N_2 \cdot Q_{s,2}^{20} \tag{5}$$

$$Q_m^{30} = N_1 \cdot Q_{s,1}^{30} + N_2 \cdot Q_{s,2}^{30} \tag{6}$$

where $Q_m^{20}$ and $Q_m^{30}$ are OURs of a mixed culture at 20 and 30℃, respectively. $Q_s$ values of each species at 20 and 30℃ are known values(Table 1) from the above two equations, viable cell numbers of each species in a mixed culture($N_1$ and $N_2$) can be calculated.

These calculated values based on respiration rate were compared to those obtained by plate counts using selective media. As shown in Fig. 6, a good agreement with the correlation coefficient of 0.98 was obtained.

## 요 약

혼합배양중의 각 미생물의 생균수 측정은 순수배양보다 훨씬 복잡하다. 특히 두 균주의 크기가 비슷한 경우에는 사용할 수 있는 방법이 더 제한된다. 본 연구에서는 두 균의 크기가 비슷한 경우에도 적용될 수 있는 간단한 생균수 측정방법을 개발하였다.

미생물 배양액의 산소흡수속도(OUR)는 세포수에 비례하며 이때의 비례상수인 최대 비산소흡수속도(maximum specific OUR)를 알고 있으면 배양액의 OUR을 측정함으로써 간접적으로 생균수를 구할 수 있게 된다. 혼합배양의 경우 산소흡수속도는 각 미생물의 호흡속도의 합이 되며, 각 미생물의 호흡속도가 서로 다르고 또한 온도의존성이 다르다면 호흡속도의 측정을 이용하여 각 생균수를 간접적으로 측정할 수 있다.

산소흡수속도는 시간에 따른 용존산소 농도의 변화를 DO probe를 이용하여 측정하여 구했으며, 최대 비산소흡수속도는 plate count에 의한 생균수와 OUR의 직선관계의 기울기에서 구했다. *C. lusitaniae*의 최대 비산소흡수속도는 20과 30℃에서 각각 $1.36\times10^{-9}$과 $3.90\times10^{-9}$, *P. tannophilus*는 20과 30℃에서 각각 $0.59\times10^{-9}$와 $1.86\times10^{-9}$[(%/s)/(cells/ml)]이었다. 이들 값을 이용하여 계산한 혼하배양액중의 두 효모의 생균수와 서로 다른 배지를 이용하여 plate count로 측정한 생균수와의 관계는 상관계수 0.98로서 비교적 잘 일치되었다.

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