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Determination of Microbial Populations with Piezoelectric Membranes

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The ultrasonic determination of microbial population was carried out by using a new apparatus composed of two piezoelectric membranes. When the apparatus was immersed in a medium containing cells and an arbitrary voltage was charged on a piezoelectric membrane, an output voltage was obtained from the other membrane. The output voltage increased with increasing cell numbers. The linear relationships between the output voltage and microbial populations of *Saccharomyces cerevisiae, Bacillus subtilis,* and *Klebsiella* sp. were observed, and the output voltage was reproducible with a relative error of 6%. Furthermore, the cell population of *S. cerevisiae* in a fermentor could be continuously determined with this apparatus.

The determination of microbial population in a fermentor is very important for the regulation of fermentation processes. Several methods, such as hemacytometer count (6) and colony count (5), have been used for the determination of cell population in fermentation broths. These methods are primitive, and their relative errors are quite large. The turbidimetric method is conventional and is used for the determination of cell population in industrial scale. However, this method cannot be applied to a colored medium, and for very turbid broths, a dilution treatment is inevitable.

In recent years, the impedance measurement of culture media was performed to determine cell population because microorganisms produce many kinds of ionic compounds, such as organic acids and nucleic acids, during the growth. This method is applicable to the determination of a small concentration of microorganisms. Other electrochemical methods for the determination of cell population have been developed (11, 12), but they are troublesome when some disturbances, such as various electochemically active components, exist in culture broths. Therefore, a direct and convenient method for the determination of microbial population is still required in fermentation industries.

The measurement of sludge concentration in wastewaters was performed with an ultrasonic method (3). Ultrasonic waves can transmit into sewages, and the velocity of waves changes as the solution components change. A new membrane called a piezoelectric membrane, which consists of a polyacetal resin, chlorinated polyethylene, and $Pb(Zr \cdot Ti)O_3$, has been developed as an oscillator. One advantage of this membrane is its flexibility, and it can also be cut into any shape easily.

In this paper, we describe the development of an apparatus for the determination of cell populations with the piezoelectric membrane. It was applied to the continuous monitoring of microbial population in a fermentor. Furthermore, the apparatus was applied to the continuous determination of the *Saccharomyces cerevisiae* population in media.

MATERIALS AND METHODS

Materials. Peptone (from casein) and meat extract were purchased from Kyokuto Pharmaceutical Co. (Tokyo, Japan). Yeast extract was bought from Difco Laboratories (Detroit, Mich.). Cane molasses was given by a sugar factory. Other reagents used for experiments were commercial or laboratory grade. Deionized water was used for all procedures.

Microorganisms. *S. cerevisiae, Bacillus subtilis* FERM-P no. 2040, and *Klebsiella* sp. were used for the determination of cell population in media.

Culture of microorganisms. The broth for cultivation of *S. cerevisiae* contained 4% glucose, 1 % peptone, 0.5% yeast extract, 0.5% KH_2PO_4, and 0.1% $MgSO_4$. The pH of the broth was adjusted to 7.0 with 1 N NaOH. The broth was sterilized for 20 min at 120°C. *S. cerevisiae* was cultured aerobically in a Sakaguchi flask containing 80 ml of the medium for 20 h at 30°C. The microorganisms in the medium were harvested by centrifugation (8,000 × *g* for 15 min at 5°C). The bacteria collected were then washed several times with 0.9% saline.

B. subtilis was cultivated aerobically at 30°C for 24 h in 80 ml of the medium containing 5% soluble starch, 1% meat extract, 1% peptone, 0.1% yeast extract, 0.5% NaCl, 0.02% $CaCl_2$, and 0.01% $MgSO_4 \cdot 7H_2O$ (pH 7.0).

Klebsiella sp. was cultured for 24 h at 30°C in 80 ml of a medium containing 1% meat extract, 1% peptone, and 0.5% NaCl (pH 7.2).

Apparatus. The apparatus used for the measurement of cell populations is shown in Fig. 1 (the distance between the two piezoelectric membranes was 2.5 mm). The surface of the piezoelectric membrane (1.5 by 2.0 cm; thickness, 0.2 mm) was coated by an epoxy resin adhesive (thickness, ca. 1 mm) for electrical insulation. The piezoelectric membrane was given by Mitsubishi Petrochemical Co. (Tsuchiura, Ibaragi, Japan). The apparatus was put in a medium (50 ml), and an arbitrary voltage was charged to the piezoelectric membrane by an oscillator (model VK-301a, TOA Electrics Ltd., Tokyo, Japan). The ultrasonic waves generated were transmitted in the medium, and they vibrated the other piezoelectric membrane. The output voltage generated was measured with an AC voltmeter (model DMM 126A, TOA Electrics Ltd.) and the signal was displayed on a recorder (model SP-J5C, Riken Denshi Co., Tokyo, Japan).

Determination of cell population. The number of *S. cerevisiae* cells was determined with a hemacytometer (Kayagaki Rika Kogyo Co., Ltd., Tokyo, Japan) and a microscope (model GC; Olympus Kogyo Co., Ltd., Tokyo, Japan). The numbers of *B. subtilis* and *Klebsiella* sp. cells were measured by the colony-counting method after incubation for 48 h at 37°C.

RESULTS

Response of the apparatus. When the piezoelectric membrane system (input voltage = 5.1 V) was immersed in the suspension of *S. cerevisiae,* 20 to 100 mV of alternating current potential was measured as an output voltage. The output voltage gradually increased with increasing cell population in preliminary experiments. The standard deviation of the determination was about 10% when a reaction medium of the same cell population (5×10^7 cells per ml) was used for experiments.

At first, the effect of the ultrasonic frequency of the output voltage was investigated with an *S. cerevisiae* suspension. Wet cells (0.2 g) were suspended in 50 ml of 50 mM phosphate buffer (pH 7.0, 5.5×10^7 cells per ml). The experiment was performed at 26.0 ± 0.5°C. The output voltage of the system increased linearly with increasing ultrasonic frequency (Fig. 2). It shows that the piezoelectric membrane does not have an intrinsic resonance frequency (the specific frequency at which a piezoelectric material resonates) in this frequency range. The frequency applied to the system was fixed at 40 kHz in subsequent experiments.

The effect of buffer concentration on the output voltage was investigated because the ionic strength seemed to play an important role in output voltage. The sound velocity usually increases with increasing concentrations of electrolytes (10). Therefore, the phosphate buffer concentration should affect the output voltage of the system (Fig. 3). The experimental conditions were the same as those in Fig. 2, except for the buffer concentration used. The output voltage of the system did not change much. A slight increase in the output voltage with high buffer concentrations was observed. When other electrolyte solutions such as KCl and Na_2CO_3 were used, a similar phenomenon was observed. The details of these phenomena are still unknown.

The pH of a solution of amino acids or proteins affects the sonic properties because of the change in net charge and degree of hydration (8). Therefore, the effect of the pH on the output voltage was investigated under the same conditions described above, except for the pH of the buffer used. Below pH 7.0, the output voltage

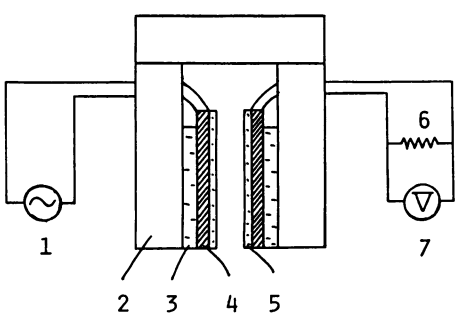

FIG. 1. *Apparatus used for the determination of microbial population: 1, oscillator; 2, plastic plate; 3, silicon rubber; 4, piezoelectric membrane; 5, epoxy resin adhesive; 6, resistance (100 kΩ); and 7, alternating current voltmeter.*

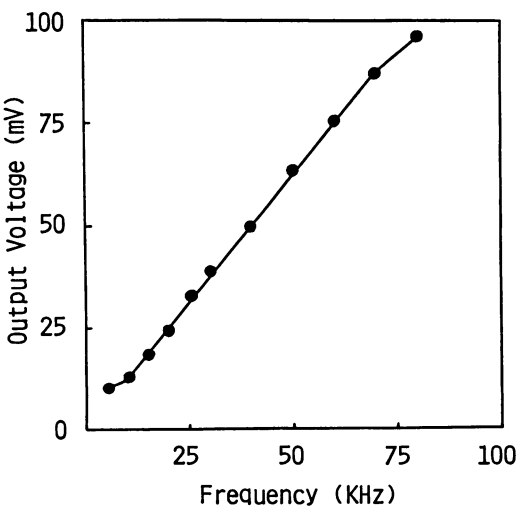

FIG. 2. *Relationship between the output voltage and ultrasonic frequency. The experiments were performed under the conditions described in the text.*

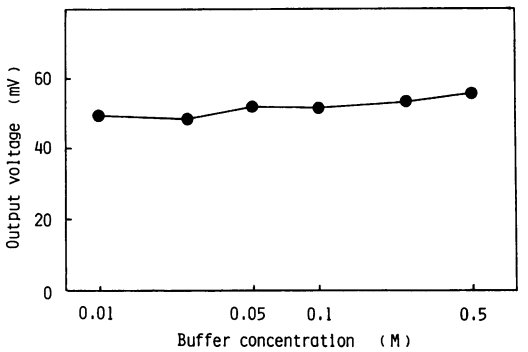

FIG. 3. *Effect of buffer concentration on the output voltage. The experiments were performed under the standard conditions, except for the phosphate buffer concentration used.*

slightly decreased with decreasing pH. Therefore, the pH of the medium does not play an important role in the generation of the output voltage in this system.

Sound velocity in a liquid gradually increases with increasing temperature up to approximately 70°C in water (10). The effect of temperature on the output voltage was therefore investigated under the conditions described above, except for the reaction temperature. The output voltage slightly increased with the increase in temperature in the range of 25 to 40°C, which is a standard temperature range for the fermentation of bacteria.

The density of the medium increases with the growth of cells. Therefore, the effect of the density of the solution on the output voltage without cells was investigated with the use of glycerol (Fig. 4). The output voltage gradually increased with increasing density of glycerol.

The compressibility of the medium will also change in the presence of cell growth. Therefore, the output voltage without cells was measured at 30°C with various solvents (Table 1 and Fig. 5). The output voltage rapidly decreased with increasing adiabatic compressibility (κ_s) below 4.0×10^{-11} cm^2/dyn and gradually decreased above it. The compressibility seemed to play an important role in the output voltage generation.

Calibration. Figure 6 shows the relationships between the output voltage and cell population. The appropriate amount of cells was suspended in 50 ml of the medium. The input voltage was 5.1 V. A linear relationship between the output voltage and cell population was observed in the range of 10^6 to 10^8 cells per ml. The measurements were performed at 30 ± 0.5°C. The output voltage was reproducible, with an average relative error of 6%, when a medium containing 1.5×10^7 *S. cerevisiae* cells per ml was used for the experiments. The system was also available for the determination of *S. cerevisiae* cell populations in the range of 10^8 to 10^{10} cells per ml, although the slope of the calibration curve was different from that for 10^6 to 10^8 cells per ml. This system could be applied not only to yeast cells, but also to gram-positive and gram-negative bacteria (Fig. 6).

Continuous determination of a microbial cell population of *S. cerevisiae* in a fermentor. The continuous monitoring of *S. cerevisiae* cell population during cultivation in the medium was performed by the piezoelectric membrane system (Fig. 7). The experimental conditions were as follows: temperature, 31 ± 0.5°C; rotation with magnetic stirrer, ca. 500 rpm; aeration with minipump, ca. 1 vol/vol/min; frequency of input, 40 kHz; input voltage, 5.1 V; and medium volume, 50 ml. The values determined by the system were slightly higher than those measured by hemacytometric and tur-

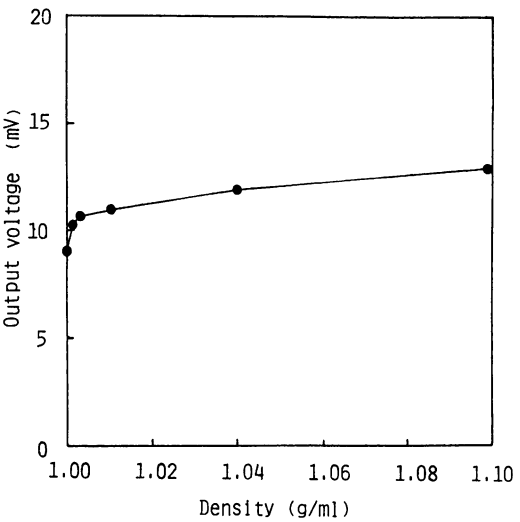

FIG. 4. *Effect of glycerol concentration on the output voltage. The experiments were performed under the conditions described in the text.*

TABLE 1. *Adiabatic compressibility of various solvents used*

Solvent	κ_s[a] ($\times 10^{-12}$ cm^2/dyn)
Acetone	97.8
Benzene	70.7
Dioxane	56.0
Ethanol	100.8
Ethyl acetate	89.9
Ethylene glycol	33.5
Glycerol	22.0
Water	44.1

[a] Adiabatic compressibilities (κ_s) of solvents at 30°C (11).

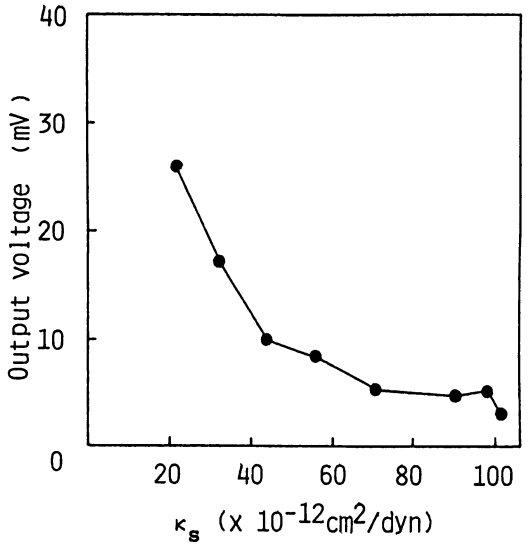

FIG. 5. *Relationship between the output voltage and adiabatic compressibility (κ_s). The experiments were performed under the conditions described in the text.*

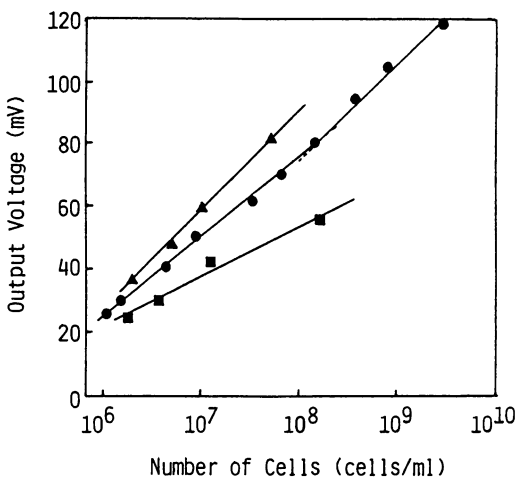

FIG. 6. *Relationship between the output voltage and the cell number. The experiments were performed under the conditions described in the text. Symbols: ●, S. cerevisiae; ▲, B. subtilis; and ■, Klebsiella sp.*

bidimetric methods. The deviations were especially large in the logarithmic phase of bacterial growth. This may be caused by the budding of *S. cerevisiae*. However, desirable agreement was obtained between this method and the conventional ones. Additionally, the conductivity of the medium increased during cell growth (Fig. 8). This was caused by electrolytes produced by bacteria and by charges on cells.

Colored media, such as molasses, are used for fermentation of microorganisms. Therefore, the system was applicable to the continuous determination of *S. cerevisiae* cell populations in cane molasses. The medium used contained 5% molasses (pH 7.0). The experimental conditions were the same as those of Fig. 7, except for the reaction temperature (30 ± 0.5°C) and input voltage (4.0 V). A similar good agreement with hemacytometric and turbidimetric methods, as

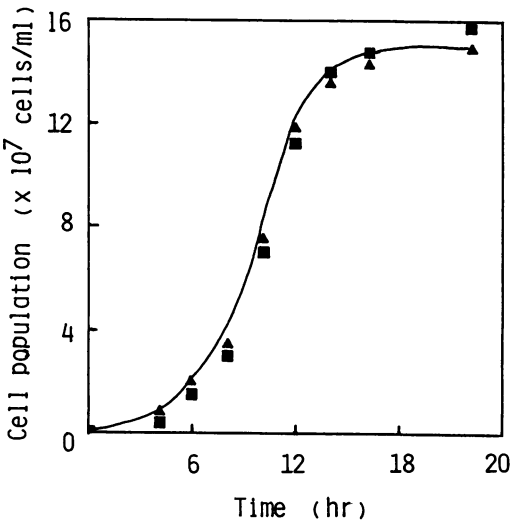

FIG. 7. *Time course of the cell population of S. cerevisiae in the medium (pH 7.0). The solid line indicates the cell population determined by the apparatus proposed. ■ and ▲, Cell population determined by the conventional turbidimetric and the hemacytometric method, respectively.*

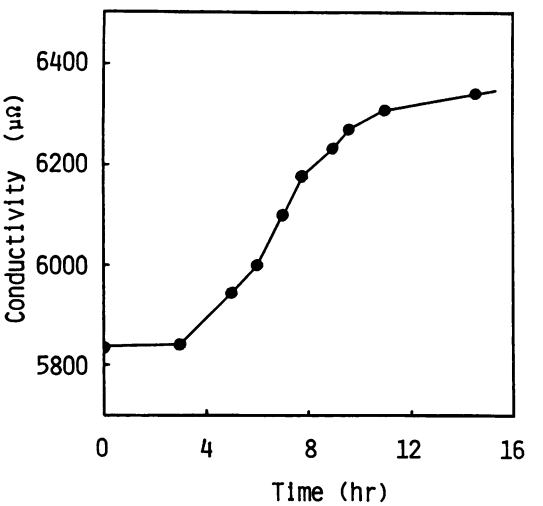

FIG. 8. *Time course of the conductivity of the medium. The experiments were performed under the same conditions as those in Fig. 7.*

shown in Fig. 7, was obtained with a relative error of 7%. This system could be used for the determination of cell populations in colored media. Additionally, little effect of aeration, rotation, and foaming of the medium on the output voltage was observed during fermentation.

Reusability of the apparatus. The apparatus was repeatedly used for the determination of the cell population in the medium under the standard conditions. The apparatus was sterilized with an autoclave at 120°C for 10 min each time. The output voltage did not change by a relative error of 6% until the apparatus was used five times. At the sixth time the apparatus was used, the output voltage suddenly disappeared, probably because the electrical leakage caused by the small cracks on the surface of the adhesive layer stopped the vibration of the piezoelectric membrane. Therefore, the stability or reusability of this apparatus was still not satisfactory.

DISCUSSION

The determination of cell population in media was performed by a system consisting of two piezoelectric membranes. The values determined by this system were in good agreement with those obtained by conventional turbidimetric and hemacytometric methods.

Many electrochemical methods based on a change of conductivity or impedance have been reported for the determination of cell population (1, 2, 4). The output voltage of this apparatus slightly increased with an increase in electrolyte concentration below 0.1 M (Fig. 3). It might be caused by the increase in conductivity of the solutions. Therefore, the output voltage was measured after the removal of cells by centrifugation (9,000 × g for 20 min) and filtration; it was then compared with the value obtained with the cell suspension. The output voltage of 55 mV that was observed in the cell suspension decreased to 15 to 20 mV in the supernatant and 7 to 9 mV in water. Therefore, some ionic compounds in the medium affected the output voltage, but cells mainly affected the output voltage.

The density of broth also increases with the growth of cells. Sound velocity (U) is generally related to the coefficient of adiabatic compressibility (κ_s) and the solution density (ρ) as shown in the following equation (10): $U = (\kappa_s \rho)^{-1/2}$. Any change in solution structure accompanied by a change in density or compressibility is reflected by a change in velocity. The output voltage gradually increased with increasing concentrations of glycerol, which increased the density of the solution (Fig. 4). According to the equation, the output voltage would decrease with increasing solution density if the output voltage were related to the sound velocity. However, the output voltage decreased with an increase in the adiabatic compressibility of the liquids (Fig. 5). Therefore, the output voltage seems to be related to the compressibility rather than to the sound velocity and the density. The conductivity of the medium would also play a part in the generation of the output voltage as described above.

Sound waves usually transmit faster in solids than in liquids (water at 0°C, 1,400 m/s; ice at 0°C, 1,850 m/s [7]), and less energy of sound waves is usually lost in solids than in liquids. The distance between the piezoelectric membranes of this system was so small (2.5 mm) that the solidity of the medium increased with the growth of cells. In other words, the compressibility of the medium decreased with the growth. Additionally, the increase in the conductivity of the medium with the cell growth might occur with the amplitude of sound waves. Consequently, the output voltage would increase with increasing cell population. The details are, however, still unclear.

Simple, rapid, and sterilizable sensors for the determination of cell population are required in fermentation industries. The system proposed here is very convenient, compact, and reproducible. Good agreement was obtained between this system and conventional methods. The continuous determination of cell population can be performed by the system proposed. However, the stability of this system was not sufficient because a leakage of electricity from the surface of the membrane occurred after five uses. High-frequency vibration and sterilization in an autoclave at 120°C seemed to cause small cracks on the surfaces of the adhesive layers. Therefore, other adhesives are required for coating and electrical insulation of the piezoelectric membrane. Further research in our laboratory is oriented toward the application of this system to other microorganisms and the solution of the problems described above.

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