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Possible Developments in Microbial and Other Sensors for Fermentation Control

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Introduction

Industrial applications of biochemical and microbiological processes in fields such as the production of pharmaceuticals, food manufacturing, waste-water treatment and energy production are ever increasing; fermentation reactions have a very important role in such biotechnological processes.

Precise control of the systems involved is essential if complex and changeable reaction mixtures and broths are to be used on an industrial scale. Without such control, substrates and products, catalysts, pH and other variable factors cannot be kept at optimal levels. Rapid and sensitive on-line monitoring and control of such variable factors call for sensors specific to the substrates and products of fermentation, as well as measurement of the numbers of viable whole cells present in the fermentation broth. Although spectrophotometry and chromatography can be used for the determination of organic compounds, they are unsuitable for on-line measurements. Electrochemical monitoring of such compounds has distinct advantages: for example, test samples do not need to be optically clear and can be measured over a wide range of concentrations.

A biosensor can be defined as any transducer for temperature, pH, optical activity etc., for use in combination with a biologically active system, for example enzyme, whole cell or antigen-antibody (Mandenius and Danielsson, 1981), and many such biosensors (such as enzyme and microbial electrodes and thermistors, and affinity sensors) have been developed for the determination of organic compounds (Guilbault, 1976, 1980, 1982; Suzuki and Karube, 1981; Suzuki, Satoh and Karube, 1982; Mosbach, Mandenius and Danielsson, 1983; *see also* Chapters 4 and 7 in Volume 1 of this Series). Microbial sensors are those biosensors which are composed of immobilized micro-organisms and an electrochemical device. The assimilation of organic compounds by the micro-organisms can be determined from their respiratory activity, which can be monitored directly by an oxygen electrode (Karube *et al.*, 1977; Suzuki,

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Satoh and Karube, 1982). Such biosensors are particularly suitable for on-line measurements (Karube and Suzuki, 1983a) as they can give accurate and rapid responses suitable for automatic processes controlled by computer systems (Mandenius and Danielsson, 1981). This chapter describes some microbial sensors being developed in Japan, together with some other sensors which are not based on biological systems but which could be useful for the monitoring and control of fermentation processes.

Sensors for fermentation substrates

GLUCOSE SENSOR

The determination of glucose levels is an important prerequisite of fermentation process control. Enzyme electrodes have been used for the determination of glucose in both clinical and food analysis (Guilbault, 1976). However, because enzymes are often unstable and expensive, and also because they may be inhibited by compounds in the culture media, enzyme electrodes are not suitable for monitoring fermentation processes. However, because assimilation of glucose by micro-organisms, and the attendant respiratory activity, can be monitored with an oxygen electrode, a microbial sensor consisting of immobilized whole cells of *Pseudomonas fluorescens* and an oxygen electrode has been investigated and used for the continuous determination of glucose in molasses (Karube, Mitsuda and Suzuki, 1979).

Figure 1 is a diagrammatic representation of the microbial electrode sensor. The electrode consisted of double membranes, one layer of which was a bacteria–collagen membrane (40 μm thick) and the other an oxygen-permeable Teflon membrane (27 μm thick), together with an alkaline electrolyte, platinum cathode and lead anode. The double membranes were in direct contact with the platinum cathode and were tightly secured with rubber rings. The microbial sensor was inserted into a sample solution which was saturated with dissolved oxygen and was stirred magnetically while measurements were taken. The current was measured by a milliammeter and the signal was displayed on a recorder.

Typical response curves are shown in *Figure 2*. The current at zero time was that obtained in a sample solution saturated with dissolved oxygen. The bacteria began to utilize glucose in the sample solution when the sensor was placed in it. Subsequently, the bacteria in the collagen membrane started to use oxygen, and this caused a decrease in the dissolved oxygen on the membrane. As a result, the current produced by the sensor decreased markedly until a steady state was reached, indicating that the consumption of oxygen by the bacteria and the diffusion of oxygen from the solution to the membrane were in equilibrium; this occurred within 10 min at 30°C. The strength of the steady-state current depended on the concentration of glucose. When the sensor was removed from the sample and placed in a glucose-free solution, the current gradually increased, returning to the initial level within 15 min at 30°C. (Hereafter, 'current' means the steady-state current).

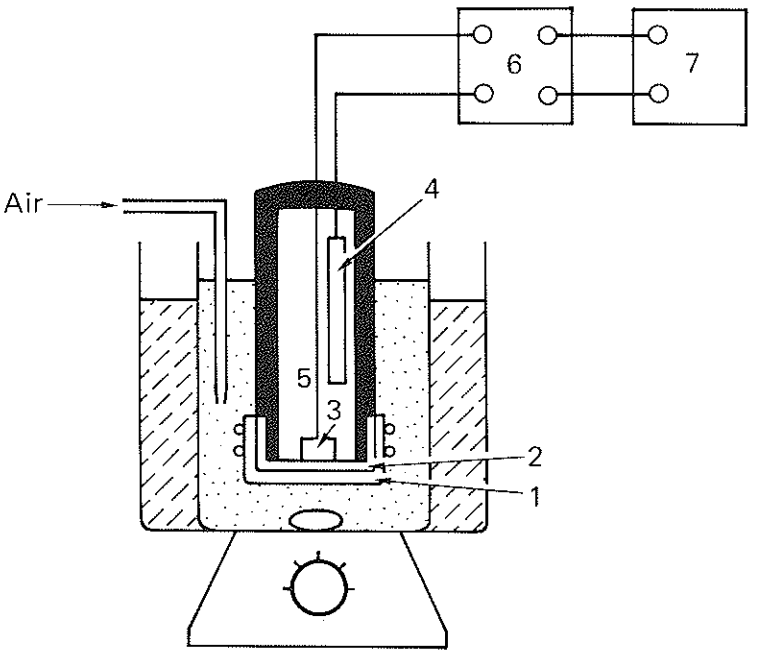

Figure 1. Diagram of the microbial sensor for glucose. 1, bacteria-collagen membrane; 2, Teflon membrane; 3, platinum cathode; 4, lead anode; 5, electrolyte (KOH); 6, ammeter; 7, recorder. Redrawn from Karube, Mitsuda and Suzuki (1979), courtesy of Springer-Verlag, Heidelberg.

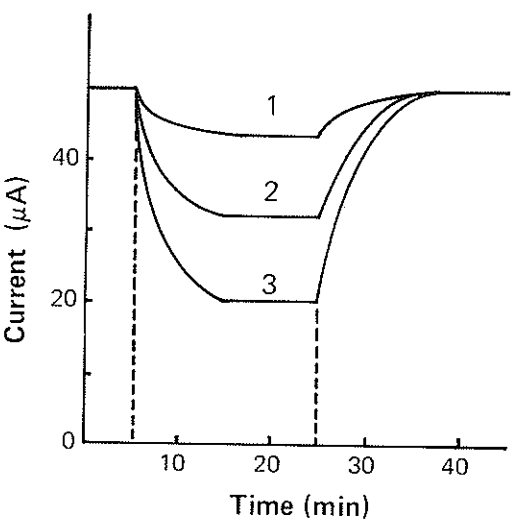

Figure 2. Response curves of the microbial sensor shown in *Figure 1*. Glucose concentrations were (1) 3·6 mg/*l*; (2) 10 mg/*l*; (3) 16 mg/*l*. Redrawn from Karube, Mitsuda and Suzuki (1974), courtesy of Springer-Verlag, Heidelberg.

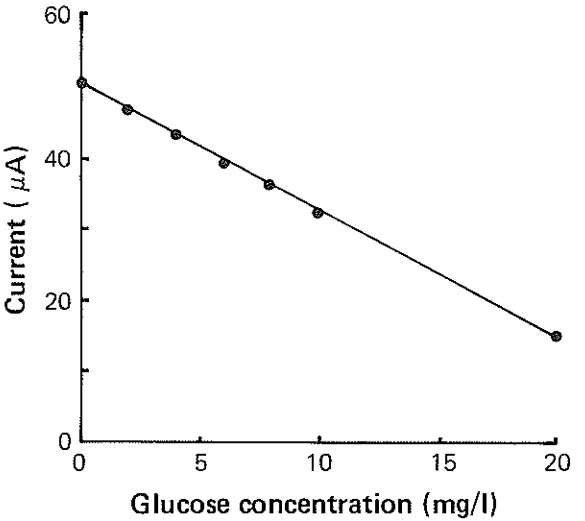

Figure 3. Calibration curve of the microbial sensor. Sample solutions (0·1 M phosphate buffer, pH 7·0) containing various amounts of glucose were employed for the experiments. Redrawn from Karube, Mitsuda and Suzuki (1979), courtesy of Springer-Verlag, Heidelberg.

Although the sensor showed a slight response to fructose, galactose, mannose and saccharose, no response to amino acids was observed. The specificity of the microbial sensor for glucose was therefore considered to be satisfactory.

Figure 3 shows the calibration curve of the microbial sensor. Using the 'steady state' method described above, a linear relationship was observed between the current and the concentration of glucose below 20 mg/ℓ. The minimum concentration for determination was 2 mg/ℓ. The current was reproducible with a coefficient of variation of ±6% when a sample solution containing 10 mg/ℓ of glucose was employed. The standard deviation was 0·6 mg/ℓ in 20 experiments.

The microbial sensor was able to determine glucose over the concentration range $10^{-4}-10^{-5}$ M, whereas ordinary enzyme electrodes had a range of $10^{-2}-10^{-4}$ M (Weibel *et al.*, 1973). The sensitivity of the microbial sensor was therefore better than that of the ordinary enzyme electrodes.

The microbial sensor was used to determine the concentration of glucose in molasses and gave results which were comparable with those obtained by the enzymatic method; the average coefficient of variation with the microbial sensor was ±10%. Long-term stability tests revealed no decrease in current output over a 2-week period and 150 assays.

ASSIMILABLE-SUGAR SENSOR

In the culture of micro-organisms in cane molasses, which contains various sugars, determination of the total assimilable sugars in the broth is important

for control of the fermentation process. However, conventional chemical methods are not completely reliable because unassimilable substances are also determined. Although various enzyme electrodes have been developed for the determination of sugars (Guilbault, 1976), in order to determine the total amount of these sugars many different kinds of enzyme electrode would be necessary. However, as discussed above, the expense and instability of enzymes generally precludes the use of enzyme electrodes for this purpose. A microbial sensor for total assimilable sugars using immobilized micro-organisms has therefore been investigated, using the same species of micro-organism as that being cultured in the fermentation. This method has been applied to the determination of total assimilable sugars in a fermentation broth for glutamic acid production. *Brevibacterium lactofermentum* was immobilized on nylon net and used as the microbial component of the sensor (Hikuma *et al.*, 1980a).

A maximum decrease in current (peak height) for a sample was normalized by the following equation in order to correct for variations in the current–concentration relationship of the microbial sensor:

$$\text{Normalized peak height} = \frac{\text{observed peak height}}{\text{peak height of the standard solution}} \qquad \text{Eq. (1)}$$

Figures 4 and *5* show calibration curves of the microbial sensor for assimilable sugars such as glucose, fructose and sucrose. Peak heights were normalized

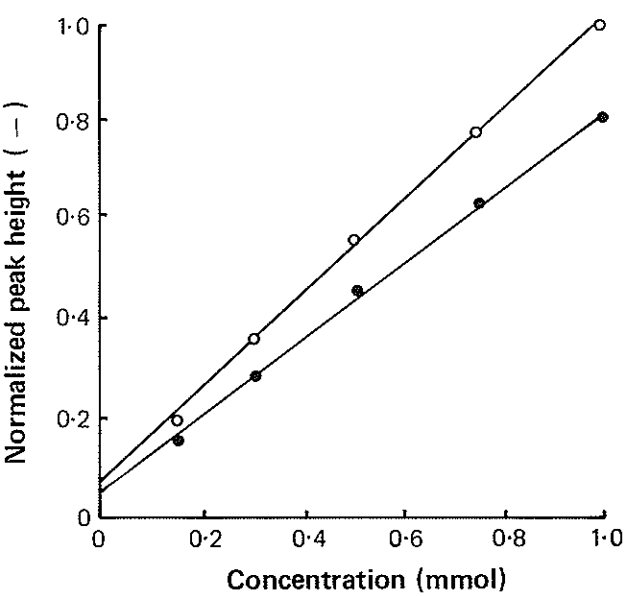

Figure 4. Measurement of assimilable sugars: current–concentration relationship of glucose (○) and fructose (●). A sample solution (0·21 ml) containing various amounts of glucose and fructose was injected into the system for 0·5 min. Redrawn from Hikuma *et al.* (1980a), *Enzyme and Microbial Technology* **2**, 234–238, by permission of the publishers, Butterworth & Co (Publishers) Ltd. ©

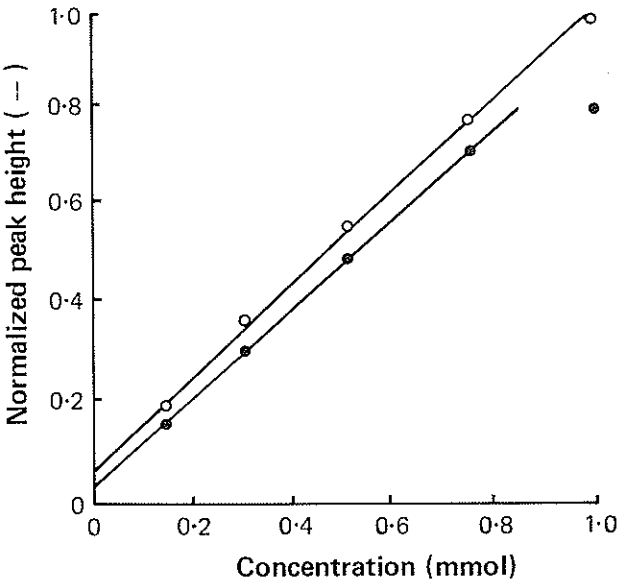

Figure 5. Measurement of assimilable sugars: current–concentration relationship of sucrose (●) and glucose (○). Redrawn from Hikuma *et al.* (1980a). *Enzyme and Microbial Technology* **2**, 234–238, by permission of the publishers, Butterworth & Co (Publishers) Ltd. ©

against the standard (1 mmol glucose) solution by equation 1. The calibration curve for glucose, fructose and sucrose can be represented respectively by the following equations derived by the method of least squares:

$$I_1 = 0{\cdot}07 + 0{\cdot}95C_1 \qquad \text{Eq. (2)}$$

$$I_2 = 0{\cdot}05 + 0{\cdot}77C_2 \qquad \text{Eq. (3)}$$

$$I_3 = 0{\cdot}03 + 0{\cdot}91C_3 \qquad \text{Eq. (4)}$$

where C is the concentration of sugar (mmol) in the flow cell, I is the normalized peak height and subscripts 1, 2 and 3 represent glucose, fructose and sucrose, respectively. The ratio of the sensitivity to glucose (180 mg/ℓ), fructose (180 mg/ℓ) and sucrose (360 mg/ℓ) was 1·00:0·80:0·92. The sensitivity of the microbial sensor for sugars corresponds to the amount of oxygen consumed by the immobilized organisms assimilating them. To examine the reproducibility of the microbial sensor, 0·8 mmol glucose was introduced into the system on 20 successive occasions and the coefficient of variation was ±2%.

The relationship between normalized peak height and the concentrations of glucose, other sugars and glutamic acid was examined. The sensor gave a weak response to sugars which may be utilized only slowly by the micro-organisms, and hardly responded at all to glutamic acid: the selectivity of the microbial sensor for assimilable sugars was thereby demonstrated to be satisfactory for monitoring the fermentation process.

When a mixture of assimilable sugars was applied to the system, the observed normalized peak height was compared with that calculated by the following equation:

$$I = 0{\cdot}15 + 0{\cdot}95C_1 + 0{\cdot}77C_2 + 0{\cdot}91C_3 \qquad \text{Eq. (5)}$$

where I is the normalized peak height and C_1, C_2 and C_3 are the concentrations of the assimilable sugars, as defined above. Equation 5 means that the response of the microbial sensor to a mixture of the assimilable sugars is equal to the algebraic sum of the responses to the individual assimilable sugars. The difference between the observed and the calculated values was within 8%. These results suggest that total assimilable sugars can be measured by this microbial sensor.

The sensor was tested in a fermentation broth of cane molasses used for the production of glutamic acid. A 30-fold dilution of the broth in tap water was used. The concentration of assimilable sugars in the broth was determined as glucose because the sensor had been calibrated with a standard glucose solution. For comparison, the total concentration of reducing sugars in the broth was determined by the conventional method. The latter gave higher results than the microbial sensor because the conventional method was affected by reducing substances other than the assimilable sugars, i.e. the microbial sensor had a greater specificity. The values obtained with the microbial sensor were accurate and reproducible with a coefficient of variation of ±4%. This sensor could be used for more than 10 days and 960 assays (Hikuma *et al.*, 1980a).

ACETIC ACID SENSOR

When micro-organisms are cultured with acetic acid as the carbon source, excess acetic acid inhibits growth, and the optimal concentration must therefore be maintained by on-line monitoring. Gas chromatography is unsuitable for on-line measurements, and therefore a microbial sensor comprising immobilized yeasts (*Trichosporon brassicae*), a gas-permeable Teflon membrane and an oxygen electrode has been investigated for the continuous determination of acetic acid in fermentation broths (Hikuma *et al.*, 1979a).

The microbial sensor itself comprised an oxygen electrode consisting of a Teflon membrane (50 μm thick), a platinum cathode, an aluminium anode and a saturated potassium chloride electrolyte. A porous membrane bearing the immobilized *T. brassicae* was fastened to the surface of the Teflon membrane and covered with a gas-permeable Teflon membrane: thus the micro-organisms were trapped between the two porous membranes. The microbial sensor system (*Figure 6*) consisted of a jacketed flow cell, a magnetic stirrer, a peristaltic pump, an automatic sampler and a current recorder.

The principle of this microbial sensor was similar to that described previously. The sample was kept at a pH well below the pK value for acetic acid (4·75 at 30°C) because acetate ions cannot pass through the membrane. Acetic

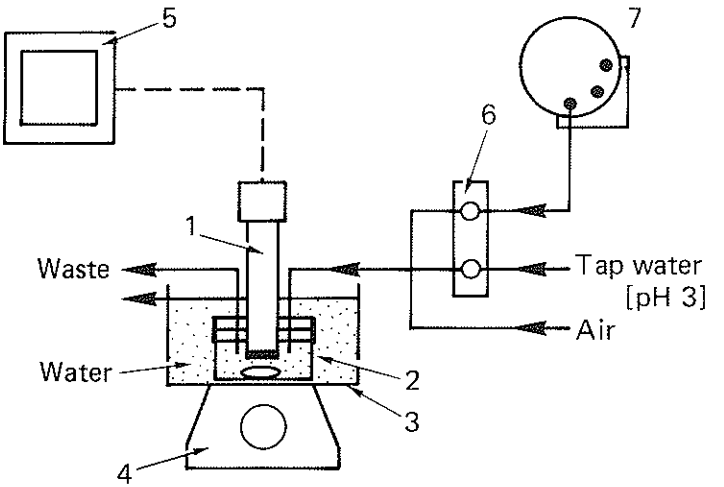

Figure 6. Acetic-acid sensor system. 1, Microbial electrode; 2, flow cell; 3, jacket; 4, magnetic stirrer; 5, recorder; 6, peristaltic pump; 7, sampler. Redrawn from Hikuma *et al.* (1979a) by permission of the publishers Elsevier Science Publishers B.V., Amsterdam.

acid from the sample passed through the membrane and was assimilated by the micro-organisms, so that the concentration of dissolved oxygen around the membranes decreased, and the current then decreased correspondingly until a steady state was reached; this steady-state current depended on the concentration of acetic acid in the sample. However, for the detection of acetic acid, because it takes a long time to reach the steady state, samples were passed into the flow cell for 3 min only. The maximum current decrease thus found was only 75% of that obtained by the steady-state method but the measurement could be done within 4 minutes. *Figure 7* shows the response curves obtained for acetic acid concentrations of 18, 36, 54 and 72 mg/ℓ by this method. The *total* time required for an assay of acetic acid was 30 min by the steady-state method and 15 min for the shorter method, which was therefore employed for the assay.

The calibration graphs obtained showed linear relationships between the current decrease and the concentration of acetic acid up to 72 mg/ℓ by the shorter method. The minimum concentration which could be determined was 5 mg/ℓ. The current difference was reproducible within ±6% for an acetic acid sample containing 54 mg/ℓ and the standard deviation was 1·6 mg/ℓ in 20 experiments.

With regard to the selectivity of the microbial sensor for acetic acid, it did not respond to volatile compounds such as formic acid and methanol, or to involatile nutrients such as glucose and phosphate ions. Although *Trichosporon brassicae* does utilize propionic acid, *n*-butyric acid and ethanol, these are not generally present in fermentations for glutamic acid, or are present in concentrations too low to affect the measurement of acetic acid.

The concentration of acetic acid in a fermentation broth which was producing glutamic acid was determined by the microbial sensor and also by

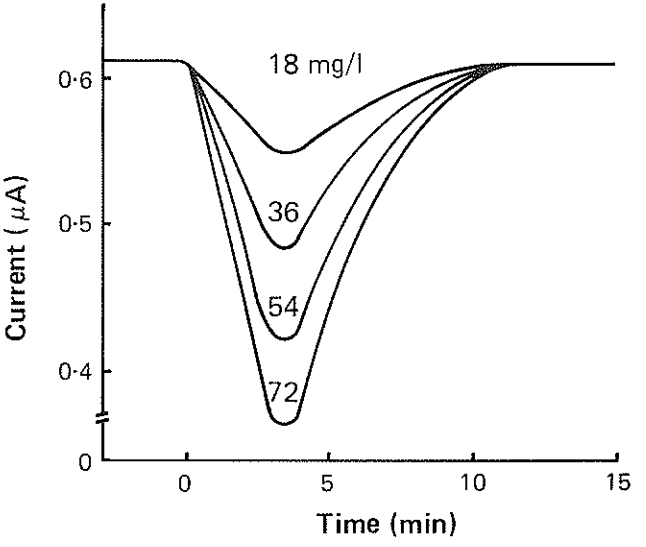

Figure 7. Response curve of the acetic acid sensor. Sample solution (2·4 ml) was passed into the flow cell for 3 min. Redrawn from Hikuma *et al.* (1979a) by permission of the publishers, Elsevier Science Publishers B.V., Amsterdam.

gas chromatography: good agreement was achieved, the regression coefficient being 1·04 for 26 experiments. The current output (0·29 – 0·25 μA) of the sensor was constant (within ±10% of the original values) for more than 3 weeks and 1500 assays. This microbial sensor for acetic acid in fermentation broths therefore appears to merit further investigation and development.

Sensors for cell populations

FUEL-TYPE ELECTRODE

The determination of microbial populations in fermenters is important for the control of fermentation processes. Several methods, such as haemacytometer counts (Pringle, 1975), electronic particle counts (Kubitschek, 1969), and colony counts (Postgate, 1969) have been used for the determination of cell numbers. However, most of these methods are time consuming and are not suitable for the continuous determination of cell numbers. Although turbidimetry is a simple method for monitoring the number of cells in a fermenter, sampling is necessary and a coloured or turbid broth is not suitable for this method.

The impedance measurement of culture media has been proposed as a method for cell number determination. Nutrients are converted by bacteria to various charged metabolites, such as organic acids (Handley and Senyk, 1975): consequently the impedance of the medium increases with increasing culture time. This method is suitable for the determination of small numbers of bacteria. An electrochemical method based on the potentiometric determination of hydrogen molecules also has been developed for the estimation of cell

numbers of hydrogen-producing bacteria such as Enterobacteriaceae (Wilkins, Stoner and Boykin, 1974; Wilkins, Young and Boykin, 1978). However, both electrochemical methods determine cell numbers indirectly from bacterial metabolites, and the results obtained are not always correlated with true cell numbers. Simple and continuous methods for the direct determination of cell populations are still required for fermentation control. In the microassay of vitamin B_1 (*see later*) an electrode composed of a platinum anode and a silver peroxide cathode has been used (Matsunaga, Karube and Suzuki, 1978a). Although the detailed mechanism of current generation in this process has not yet been elucidated, it has been suggested that most of the current generated is attributable to the direct transfer of electrons from reduced bacterial coenzymes to the electrode. This electrochemical system could also be used for the determination of microbial populations.

The use of such a system for the determination of populations of *Saccharomyces cerevisiae* and of *Lactobacillus fermentum*, two micro-organisms which are of importance in the food industry, was investigated (Matsunaga, Karube and Suzuki, 1979). The electrode system (*Figure 8*) comprises two similar

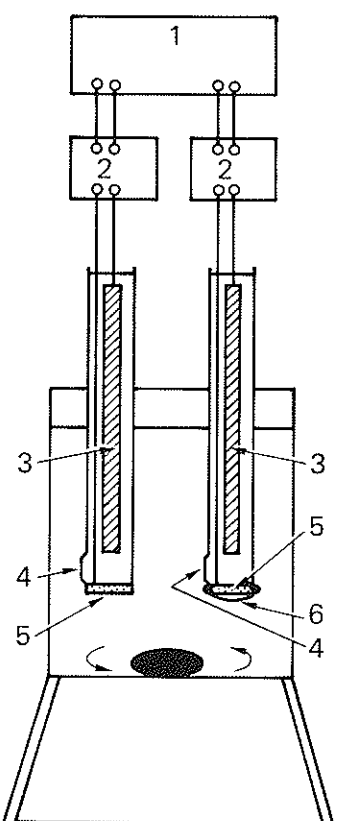

Figure 8. Diagram of the electrode system for the determination of a microbial population. 1, recorder; 2, ammeter; 3, cathode (Ag_2O_2); 4, anion exchange membrane; 5, anode (Pt); 6, cellulose dialysis membrane. Redrawn from Matsunaga, Karube and Suzuki (1979) courtesy of American Society for Microbiology.

electrodes, each consisting of a platinum anode (diameter 1·2 cm) and a silver peroxide cathode. One electrode is the determination electrode and the other (reference) electrode is covered with a cellulose dialysis membrane. The electrolyte is phosphate buffer (0·1M, pH 7·0). An anion exchange membrane is used as a separator.

Currents are obtained from both electrodes when they are inserted into a culture broth: that from the determination electrode (I_1) results from oxidation of micro-organisms as well as electroactive substances; that from the reference electrode (I_2) is attributable to oxidation of electroactive substances only, because micro-organisms cannot pass through the cellulose dialysis membrane. Consequently, the difference in the current from the two electrodes ($\Delta I = I_1 - I_2$) is proportional to the number of microbial cells in a culture broth, i.e. $I = I_1 - I_2 = kn$, where n is the number of micro-organisms and k is a constant.

When the electrodes were immersed in a culture broth containing 4×10^8 cells of *S. cerevisiae* per ml, high currents were obtained initially from both electrodes as the anodes polarized. Subsequently, the anode potentials became constant, the diffusion of micro-organisms and electroactive substances to the determination electrode became the rate-determining factor, and a steady-state current was achieved. The response time (i.e. the time taken for the current to reach a steady state) of the determination electrode was 10 min, whereas that of the reference electrode was 15 min. A linear relationship was seen between the current difference and the number of cells below 4×10^8 cells/ml. The current difference was reproducible with a coefficient of variation of ±5% when a medium containing 2×10^8 *S. cerevisiae* cells/ml was used.

The number of cells in a population of *Lactobacillus* was similarly determined by the electrode system and again a linear relationship was observed between the current difference and the number of cells measured by colony counting. This system is also applicable to anaerobic bacteria.

For comparison, the number of cells in a population of *S. cerevisiae* in a fermenter was determined repeatedly using the electrochemical method, and also by visual counting: it was found that the cell number plots measured by the latter method were similar to those resulting from the electrochemical method. Some slight deviation was noted when *S. cerevisiae* was in the exponential growth phase, and may have been attributable to budding of the yeast, as in fact many buds were visible on light microscopy at this time.

That only viable cells are determined by the electrochemical method is shown by the fact that the current differences between the determination and the reference electrodes decreased markedly when the micro-organisms were inactivated by heat. Thus measurement of the number of viable cells is not interfered with by non-viable cells and non-microbial particles.

POTENTIOSTATIC DEVICE

The system described above is not suitable for the continuous on-line determination of cell populations because the silver peroxide cathode is decomposed by the sterilization necessary. Another potentiostatic method for this purpose has therefore been investigated. This system also has a determination and a reference component, each of which consists of two platinum

electrodes (one working and one counter electrode) and a saturated calomel electrode (SCE). The surface of the anode of the reference system is covered with a cellulose dialysis membrane. For our experiments the three electrodes were connected through a potentiostat and it was found that the currents of both systems increased with increasing potential. The current difference also increased, and became constant above + 0·2 V; experiments therefore were performed at the anode potential of +0·2 V relative to the SCE (Matsunaga, Karube and Suzuki, 1980a).

When both systems were immersed in a culture broth of *B. subtilis* containing $1{\cdot}4 \times 10^9$ cells/ml, high currents were obtained initially, and then a steady-state current was attained. The response time (i.e. the time required for the current to reach a steady state) was 3–5 min for both systems; the steady-state current was measured 5 min after the system had been placed in the medium.

As before, the current difference between determination and reference systems was drastically reduced when the broth was sterilized for 10 min at 110°C, suggesting that the current difference between the two systems noted in unsterilized broth is caused by reaction of the living bacteria with the anode of the determination component.

A linear relationship was obtained between the current difference and the cell population of *B. subtilis* below $2{\cdot}0 \times 10^9$ cells/ml. The current difference was reproducible with a coefficient of variation of ±4%.

Figure 9 shows results of continuous determinations of the microbial population in a fermenter. The solid line represents the time course of cell population growth as determined by the electrochemical system, whereas the points represent cell numbers determined by colony counting (Postgate, 1969); the two sets of results were very similar. These measurements were repeated 20 times, and growth curves similar to those shown in *Figure 9* were obtained each time. The current difference was reproducible with a coefficient of variation of ±8%. These results suggest that the system can be used for more than 400 hours.

Electrodes must be sterilized if they are to be used for continuous on-line monitoring of cell numbers in industrial ferments. After sterilization for 10 min in an autoclave at 110°C, the systems were immersed in the broth containing $1{\cdot}4 \times 10^9$ cells/ml: the currents obtained did not differ from those observed before sterilization of the electrodes, even when the latter had been sterilized 30 times.

The minimum number of detectable cells was 10^8 cells/ml: as the saturation level of *B. subtilis* cells in culture was 4×10^9 cells/ml, the sensitivity of this system is sufficient for practical application. Cell numbers of anaerobic bacterial populations (*Lactobacillus fermenti*) and of yeast (*Saccharomyces cerevisiae*) can also be determined by this electrochemical system.

ULTRASONIC DETERMINATION

A Japanese team (Hayakawa and Kori, 1972) has measured sludge concentration in waste waters by an ultrasonic method, employing the principle that the velocity of the ultrasonic waves changes as the components of the sludge

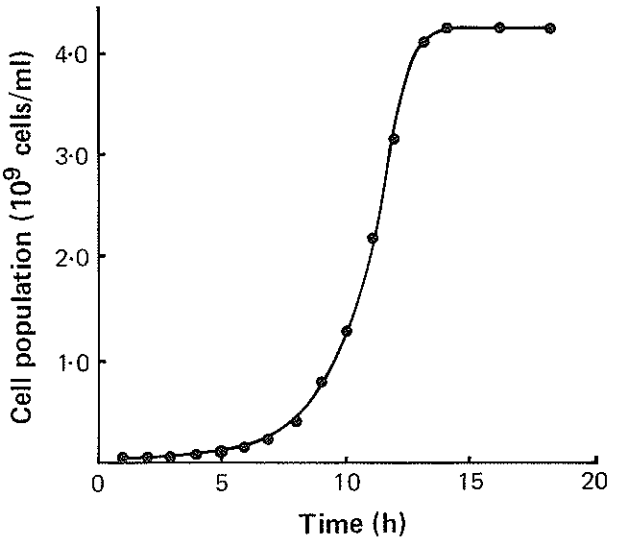

Figure 9. Continuous determination of cell populations. (—) Population determined by the electrochemical method; (●) population determined by colony counts. Redrawn from Matsunaga, Karube and Suzuki (1980a), courtesy of Springer-Verlag, Heidelberg.

change. For this type of experiment a piezoelectric membrane, which consists of a polyacetal resin, chlorinated polyethylene and Pb $(Zr{\cdot}Ti)O_3$, has now been developed as an oscillator. This new membrane is flexible and also can be cut easily into any shape.

An apparatus for the determination of cell populations with the piezoelectric membrane has been investigated in this laboratory (Ishimori, Karube and Suzuki, 1981) and has been used for continuous determination of a population of *Saccharomyces cerevisiae* in a fermenter.

The apparatus is shown in *Figure 10*, the distance between the two piezoelectric membranes being 2·5 mm. The surface of these membranes was coated with an epoxyresin adhesive for electrical insulation. The apparatus was placed in 50 ml of medium and an arbitrary voltage was applied to one piezoelectric membrane by an oscillator. The ultrasonic waves generated were transmitted through the medium and caused the other piezoelectric membrane to vibrate. The output voltage generated was measured.

When the system (input voltage 5·1 V) was immersed in the *S. cerevisiae* suspension, an output voltage of 20–100 mV was detected. In preliminary experiments the output voltage gradually increased with increasing cell population. The standard deviation of the determination was about 10% when a reaction medium of the same cell population (5×10^7 cells/ml) was used for successive experiments.

Initially, the effect of the ultrasonic frequency on the output voltage was investigated: the latter was found to increase linearly with increasing ultrasonic frequency, showing that the piezoelectric membrane does not have an intrinsic resonance frequency (the specific frequency at which a piezoelectric material

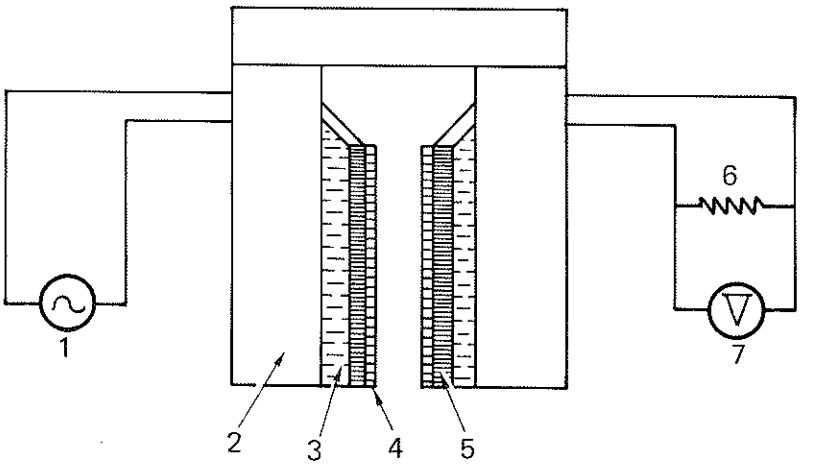

Figure 10. Apparatus used for the determination of a microbial population. 1, Oscillator; 2, plastic plate; 3, silicon rubber; 4, piezoelectric membrane; 5, epoxyresin adhesive; 6, resistance (100 kΩ); 7, alternating current voltmeter. Redrawn from Ishimori, Karube and Suzuki (1981), courtesy of American Society for Microbiology.

resonates) in this frequency range. The frequency applied to the system was maintained at 40 kHz in subsequent experiments.

When cells were suspended in 50 ml of the medium, with an input voltage of 5·1 V, a linear relationship between the output voltage and the cell population was observed in the range $10^6 - 10^8$ cells/ml. The measurements were made at $30 \pm 0{\cdot}5$°C. The output voltage was reproducible within ±6% when a medium containing $1{\cdot}5 \times 10^7$ *S. cerevisiae* cells/ml was used for the experiments. The system could also be used for the determination of *S. cerevisiae* populations in the range $10^8 - 10^{10}$ cells/ml, although the slope of the calibration curve differed from that for $10^6 - 10^8$ cells/ml. This system is applicable not only to yeast cells but also to Gram-positive and Gram-negative bacteria.

The values obtained by the piezoelectric system were slightly higher than those measured by haemacytometric and turbidimetric methods. The deviations were particularly great in the exponential growth phase and this may have been attributable to budding of *S. cerevisiae*, as was also the case with the fuel-type electrode, described earlier. However, a satisfactory degree of agreement between this method and the conventional ones was attained. The conductivity of the medium increased during cell growth, because of charges on cells, and electrolytes produced by bacteria.

This system can be used for the determination of cell populations in coloured media such as molasses. An additional advantage is that aeration, rotation and foaming of the medium during fermentation appeared to have little effect on the output voltage.

The apparatus was used for repeated determinations of cell numbers in the medium under the standard conditions, and was sterilized with an autoclave at 120°C for 10 min before each determination. There was little change in the output voltage until the apparatus had been used five times: on the sixth

occasion the output voltage suddenly disappeared, probably because electrical leakage caused by small cracks on the surface of the adhesive layer stopped the vibration of the piezoelectric membrane. In order to achieve satisfactory stability or reusability of this apparatus, therefore, other adhesives for coating and electrical insulation of the piezoelectric membrane must be investigated.

Sensors for products of fermentation

ALCOHOL SENSOR

When yeasts are cultured using sugar as a carbon source it is well known that ethyl alcohol as a by-product reduces the yield of whole cells per unit of sugar (Wang, Cooney and Wang, 1977). Similarly, in the culture of micro-organisms using methyl alcohol as a carbon source, the concentration of methyl alcohol must be maintained at the optimal level to avoid substrate inhibition (Reuss *et al.*, 1975). Although many enzyme electrodes for the determination of alcohols have been described (Guilbault, 1976), a more stable biosensor is preferable. The concentration of methyl alcohol has been estimated by analysis of the exhaust gas from a fermenter with a flame ion detector (Wang, Cooney and Wang, 1977). However, this method is not reliable because the values obtained are influenced by operational fermenter conditions such as the aeration and agitation rates.

In the knowledge that many micro-organisms utilize alcohols as carbon sources, and that this assimilation can be determined from their respiratory activity, a microbial sensor for alcohols has been devised by Hikuma *et al.* (1979b) using immobilized micro-organisms (yeasts or bacteria), a gas-permeable Teflon membrane and an oxygen electrode.

For the detection of ethyl alcohol, immobilized *Trichosporon brassicae* was used. The pH of the culture medium was 6·0 – 6·2. A linear relationship was observed between the current decrease when the sensor was placed in the alcohol-containing sample, and the concentration of ethyl alcohol below 22·5 mg/ℓ. The minimum concentration of alcohol for the determination was 2 mg/ℓ. The current difference was found to be reproducible within ±6% when a sample solution containing 16·5 mg/ℓ of ethyl alcohol was used. The standard deviation was 0·5 mg/ℓ in 40 experiments.

Under the test conditions used, with a pH above 6·0, the sensor did not respond to volatile compounds such as methyl alcohol, formic acid, acetic acid (cf. the use of *T. brassicae* to determine acetic acid at pH 3·0, page 319), propionic acid, and other nutrients such as carbohydrates, amino acids and ionic compounds. As the microbial sensor was covered with a gas-permeable membrane, only volatile compounds can permeate the membrane. However, *T. brassicae* does not utilize methyl alcohol, and the selectivity of the microbial sensor for ethyl alcohol was considered to be satisfactory.

When the concentration of ethyl alcohol in fermentation broths was determined by the microbial sensor and by gas chromatography, satisfactory comparative results were obtained, with a correlation coefficient of 0·98 for 20 experiments. When ethyl alcohol solutions (5·5 – 22·3 mg/ℓ) were used to test

the long-term stability of the sensor, the current output was found to be almost constant for more than 3 weeks and 2100 assays.

An unidentified bacterium was used for the determination of methyl alcohol. As before, the microbial sensor consisted of immobilized bacteria (which used methyl alcohol in this case), a gas-permeable membrane, and an oxygen electrode. The determination was performed under the same conditions as those described for the ethyl alcohol sensor. A linear relationship was observed between the current decrease and the concentration of methyl alcohol (*Figure 11*), showing that the sensor may be of use for the determination of methyl alcohol.

GLUTAMIC ACID SENSOR

Large quantities of glutamic acid for use in the food industry are produced by fermentation, calling for rapid automatic measurement of glutamic acid in fermentation media. Although AutoAnalyzer-based enzymatic reactions can be used, this entails the consumption of expensive enzyme. Certain micro-organisms contain glutamate decarboxylase, which catalyses the decarboxylation of glutamic acid to produce carbon dioxide and amine: a microbial sensor for glutamic acid therefore has been devised incorporating immobilized *Escherichia coli*, as a source of glutamate decarboxylase activity, in conjunction with a CO_2-sensing electrode (for details *see* Hikuma *et al.*, 1980b). Preliminary experiments showed that *E. coli* did not evolve carbon dioxide under anaerobic conditions in the absence of glutamic acid, indicating that the normal respiration of the bacteria was depressed under anaerobic conditions and that any carbon dioxide produced by these bacteria under such conditions would result from the glutamate decarboxylase reaction. Nitrogen gas was

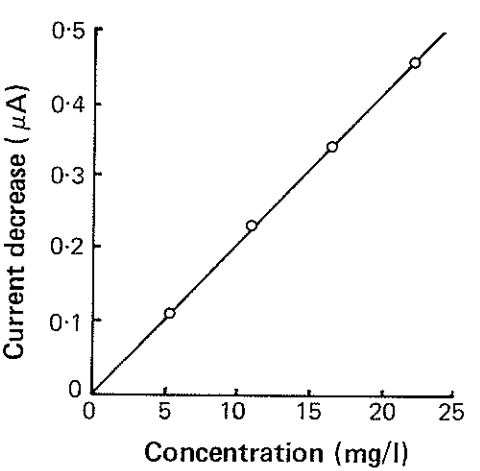

Figure 11. Calibration curve of the microbial sensor for methyl alcohol. Determination was carried out by the steady-state method. Redrawn from Hikuma *et al.*, (1979b), copyright © (1979) Biotechnology and Bioengineering. Courtesy of John Wiley & Sons, Inc.

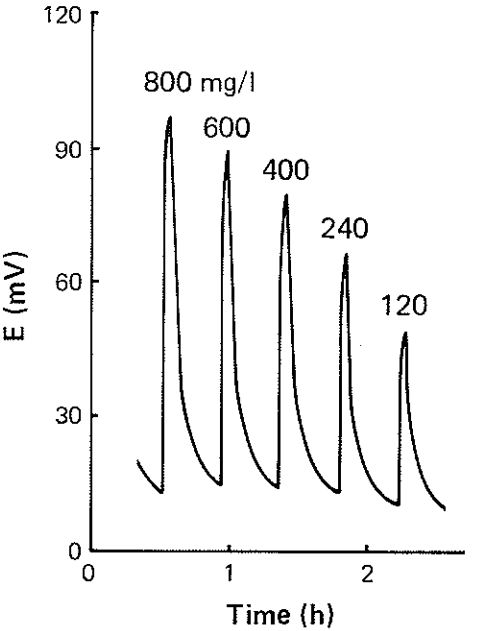

Figure 12. Response curves of glutamic acid sensor. Sample solution (3 ml) was injected for 3 min. Redrawn from Hikuma *et al.*, (1980b), by permission of the publishers, Elsevier Science Publishers B.V., Amsterdam.

passed through the flow cell in order to remove any dissolved oxygen from the buffer and sample solution. When the latter, containing glutamic acid, was injected into the system, glutamic acid permeated through a cellophane membrane to the immobilized micro-organisms and was metabolized by the micro-organisms to produce carbon dioxide. The enzyme reaction was carried out at pH 4·4, which was sufficiently below the pK_a value (6·34 at 25°C) of carbon dioxide to allow accumulation of carbon dioxide around the membranes. As a result, the potential of the CO_2-sensing electrode increased with time. Although a 5-minute injection period is necessary to obtain steady conditions, the assay can be done by using an injection period of 1–3 min and measuring the maximum potential, with little loss of sensitivity.

Figure 12 shows the response of the microbial sensor to various concentrations of glutamic acid. The plot of the maximum potential vs. the logarithm of the glutamic acid concentration was linear in the range 100–800 mg/ℓ. When replicates of a glutamic acid solution (400 mg/ℓ) were measured, the standard deviation was 1·2 mg/ℓ (20 experiments).

When this sensor was used to determine known concentrations of glutamic acid in a fermentation broth, satisfactory recovery data were obtained (99–103%) which were in good agreement with determinations by the AutoAnalyzer. The sensor was considered to be highly selective, stable and reproducible.

Potentiometric microbial electrodes for amino acids have also been investigated by Rechnitz and co-workers (Kobos and Rechnitz, 1977; Rechnitz *et al.*, 1977, 1978).

FORMIC ACID SENSOR

Formic acid is a commonly occurring intermediate of cellular metabolism, found in culture media, urine, blood and gastric juices, and as a product of many chemical reactions. It is now attracting attention as an intermediate of biomass conversion. Although selective spectrophotometric enzymatic methods involving formate dehydrogenase, malate dehydrogenase and tetrahydrofolic acid synthetase have been reported (Guilbault, 1976), these conventional methods are not suitable for on-line monitoring.

A fuel-cell type electrode consisting of a platinum anode and a silver peroxide cathode has been described for measuring the hydrogen produced from glucose by bacteria (Karube, Matsunaga and Suzuki, 1977). In addition, anaerobic bacteria such as *Escherichia coli*, *Clostridium butyricum* and *Rhodospirillum rubrum* produce hydrogen from formic acid. It should therefore be possible to determine formic acid by using *C. butyricum* and a fuel-cell type electrode. Such a specific microbial sensor, comprising immobilized *C. butyricum*, two gas-permeable Teflon membranes and a fuel-cell type electrode has been investigated (Matsunaga, Karube and Suzuki, 1980b). This sensor did not respond to non-volatile nutrients such as glucose, pyruvic acid and phosphate ions. Although volatile compounds such as acetic acid, propionic acid, *n*-butyric acid, methyl alcohol and ethyl alcohol can permeate the porous Teflon membrane to reach the immobilized bacteria, no current was obtained with these compounds because *C. butyricum* did not produce hydrogen from them. The microbial sensor, and also gas chromatography, were used to determine formic acid in an *Aeromonas formicans* culture medium. Good agreement was obtained between these methods, the regression coefficient being 0·98 for 10 experiments. The culture medium itself did not affect the current produced by the sensor.

METHANE GAS SENSOR

Methane is an attractive energy source and one of the chief components of natural gas. It is widely used as a fuel and, as is well known, can form explosive mixtures with air (5–14%). Rapid methods for the detection and determination of methane in air are therefore required in various fields such as coal mining and gas production. Methane is also produced by methanogenic bacteria, and world-wide interest has been aroused in the production of methane by the fermentation of biomass. Other bacteria which oxidize methane with the concomitant consumption of oxygen (Ribbons, 1975) can be used in a methane-sensor system. The characteristics of such a system have been investigated in this laboratory and the system has been used to determine methane in air (Karube, Okada and Suzuki, 1982).

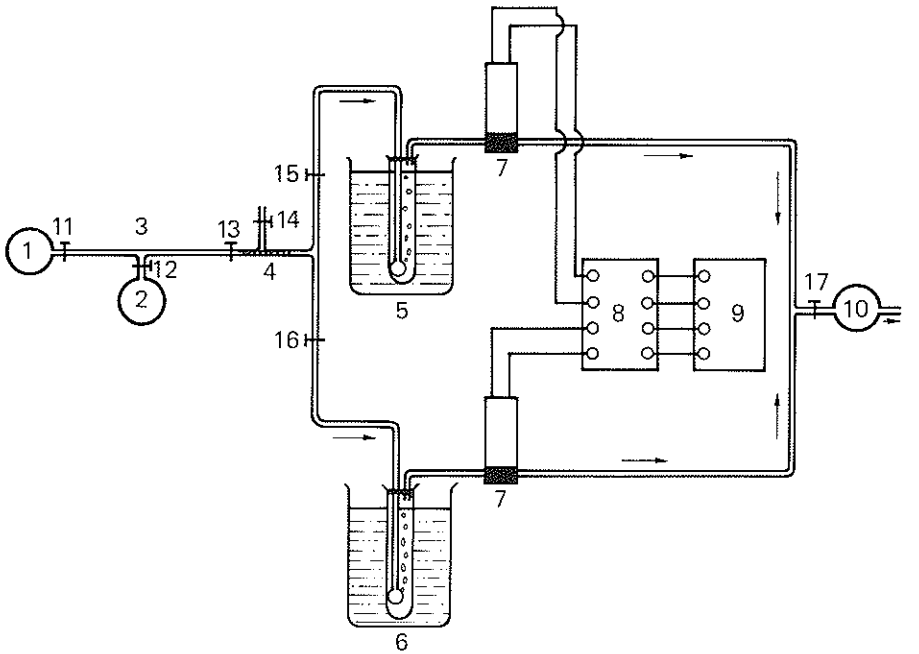

Figure 13. Diagram of microbial sensor system for methane. 1, vacuum pump; 2, sample gas bag; 3, gas sample line; 4, cotton filter; 5, control reactor; 6, methane-oxidizing bacteria reactor; 7, oxygen electrode; 8, amplifier; 9, recorder; 10, vacuum pump; 11–17, glass stopcocks. Redrawn from Karube, Okada and Suzuki (1982), by permission of the publishers, Elsevier Science Publishers B.V., Amsterdam.

The system comprised two oxygen electrodes, two reactors, an electrometer and a recorder (*Figure 13*). The reactors contained the culture media, one with and one without the bacterium *Methylomonas flagellata*. The oxygen electrodes consisted of a Teflon membrane, a platinum cathode, a lead anode and a sodium hydroxide electrolyte. The electrodes were fixed to custom-made Teflon flow-through cells, and the system was connected using glass and Teflon tubing. Two vacuum pumps were used, one to evacuate the gas-sample tube and the other to transport the sample gas through the system. The flow rate of the sample gas through the reactors was controlled (80 ml/min) with the glass valves. The cotton filter removed other micro-organisms in the gas sample and prevented contamination of the two reactors and gas lines. The latter were designed to maintain symmetry between the measuring and reference flows. The flow rates in each line were balanced by adjusting valves 15 and 16 (*Figure 13*). The reactors were maintained at 30°C ± 0·1°C.

When the sample gas containing methane entered the reactor, methane was assimilated by the micro-organisms with consumption of oxygen, so that current from the oxygen electrode decreased to a minimum steady state. As the system contained two oxygen electrodes, the maximum difference between the currents depended on the concentration of methane in the sample gas. When pure air was again passed through the reactors, the current returned to its initial

level within a minute. The response time required for the determination of methane was less than a minute, and the total time required for methane assay was 2 minutes.

Calibration graphs for the system were strictly linear for methane concentrations in the range 0–6·6 mmol, the current difference ranging from 0 to 0·35 μA. The minimum concentration for determination was 5 μmol. The current difference measured for the same sample (0·66 mmol) was reproducible within 5% and the S.D. of the current was 9·40 nA in 25 experiments.

This microbial sensor system was applied to the determination of methane in air samples that were also analysed by conventional gas chromatography and, over the range 0·2–3·5 mmol methane in air, the correlation coefficient between the results of the two methods was 0·97: the minimum measurable concentration is 3 mmol by gas chromatography with a flame ionization detector, and 5 mmol with this microbial sensor. The sensor employing *M. flagellata* therefore warrants further development for rapid on-line determination of methane.

Microbioassays

ELECTROCHEMICAL MICROBIOASSAY OF VITAMIN B_1

As some bacteria require specific nutrients for their growth, bioassay of amino acids, vitamins and antibiotics is feasible using micro-organisms such as *Lactobacillus* spp. and *Streptococcus* spp. Microbioassays of biologically active materials usually involve turbidimetric or titrimetric methods, but these require lengthy incubation periods: the turbidimetric method requires bacterial culture for more than 16 h and is unsuitable for coloured samples, while the titrimetric method requires incubation for more than 72 h at 30°C (Berg and Behagel, 1972).

An electrode system involving *Lactobacillus fermentum* ATCC 9338 for the microbioassay of vitamin B_1 has been investigated in this laboratory (Matsunaga, Karube and Suzuki, 1978a). The sensor system, which incorporated a silver peroxide cathode, a platinum anode and phosphate buffer electrolyte, is shown in *Figure 14*. An anion-exchange membrane was used to separate the electrochemical cell from the culture broth.

For calibration, 5 ml of the double-strength basic medium and 5 ml of a sample solution containing vitamin B_1 were sterilized for 15 min at 120°C, after which 50 μl of bacterial suspension was added to the medium containing vitamin B_1. After incubation for 6h at 37°C the electrode system was inserted in the culture broth and the current was measured. The current increased with increasing incubation time, the maximum being obtained at the mid-point of the exponential growth phase. There was no increase in current from the medium in the absence of vitamin B_1. The time required to obtain the maximum current decreased with increasing amount of the bacterial suspension injected. The minimum time for microbioassay was 6 h when 5×10^{-6} g wet cells per ml were injected into the incubation medium. Further increase of

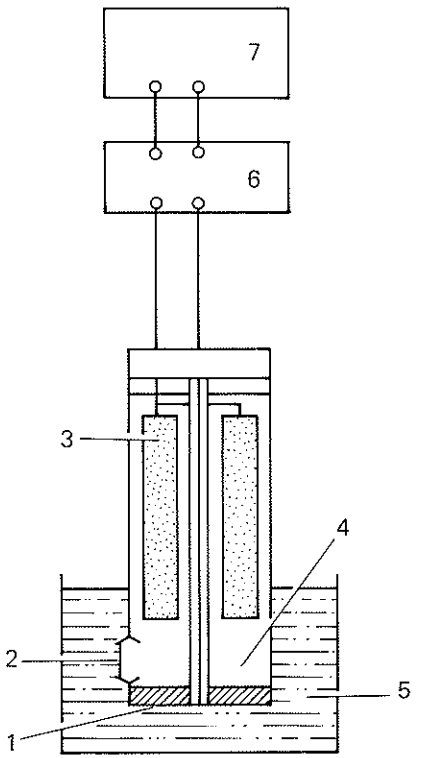

Figure 14. Diagram of the electrode for electrochemical microbioassay. 1, anode (Pt); 2, anion-exchange membrane; 3, cathode (Ag_2O_2); 4, electrolyte (0·1 M phosphate buffer); 5, sample solution (medium, vitamin B_1, bacteria); 6, ammeter; 7, recorder. Redrawn from Matsunaga, Karube and Suzuki (1978a), by permission of the publishers, Elsevier Science Publishers B.V., Amsterdam.

the amount injected did not shorten the incubation time: for further work, therefore, an amount of 5×10^{-5} g/ml was used.

The culture medium for microbioassay was incubated for 6 h at 37°C before the electrode was inserted: the open-circuit anode potential was 420 mV vs. a saturated calomel electrode (SCE) and the cathode potential was 220 mV vs. SCE. As the anode was polarized, a high current was obtained initially. The anode potential subsequently became constant, diffusion of the electroactive substances produced became the rate-determining factor, and a steady-state current was obtained; this occurred within 15 min in all cases.

A linear relationship was obtained between the steady-state current and the vitamin B_1 concentration below 25×10^{-9} g/ml. The steady-state current was reproducible within ±8% when a medium containing 25×10^{-9} g/ml of vitamin B_1 was used. The standard deviation was 1×10^{-9}g when various concentrations of vitamin B_1 were measured by this method. Formic acid contributed little to current generation, and lactic acid, acetic acid and carbon dioxide were not oxidized at the anode.

The relationship between the steady-state current and the amount of bacteria suspended in the culture medium is given by the equation:

$$I = 0{\cdot}21\ C + 0{\cdot}19 \qquad \text{Eq. (6)}$$

where I is the current density ($\mu A/cm^2$) and C is the concentration of bacteria ($\times 10^{-3}$ g wet cells/ml). The current increased linearly with increasing amount of bacteria. However, when the bacterial suspension was boiled for 15 min, the current decreased to the value which was obtained from the culture broth without bacteria, showing that only active bacteria contribute to current generation.

ELECTROCHEMICAL MICROBIOASSAY OF NICOTINIC ACID

Another method for the determination of nutrients, employing bacteria which produce mainly lactic acid as a metabolite, has been investigated. Nicotinic acid has been determined rapidly by using *Lactobacillus arabinosus* ATCC 8014 immobilized in 2% agar gel and a combined glass electrode to measure lactic acid produced (Matsunaga, Karube and Suzuki, 1978b). As the membrane potential of the glass electrode is proportional to the logarithm of proton activity in the solution, the lactic acid produced by the immobilized whole cells can be determined by the electrode. The potential increased with increasing concentration of nicotinic acid in the medium. The potential difference (ΔE_1) between the initial medium and the medium incubated for 1 h with immobilized bacteria, was calculated. The potential difference (ΔE_2) between the incubated blank and sample media was also calculated. The rate of production of lactic acid by immobilized bacteria in the media containing nicotinic acid was higher than that in the blank medium.

The potential difference (ΔE) between ΔE_1 and ΔE_2 was found to be proportional to the logarithm of the nicotinic acid concentration:

$$\Delta E = \Delta E_1 - \Delta E_2 = k\log[\text{nic}] \qquad \text{Eq. (7)}$$

where ΔE is a potential difference, [nic] is the nicotinic acid concentration and k is a constant.

The potential difference first increased linearly with increasing incubation time, but reached a plateau after incubation for 1 h, because both ΔE_1 and ΔE_2 then changed at the same rate. An incubation time of 1 h was selected for the assay of nicotinic acid.

The relationship between the potential difference and the logarithm of the nicotinic acid concentration was examined. A linear relationship was obtained for $5 \times 10^{-8} - 5 \times 10^{-6}$ g of nicotinic acid in the 1 ml aliquot added. The potential difference was reproducible with a coefficient of variation of $\pm 5\%$ when a medium containing 5×10^{-7} g of nicotinic acid per ml was employed; the standard deviation was 2×10^{-8} g/ml in 30 experiments.

To study the stability of the immobilized bacteria, they were stored in physiological saline at 5°C and used at 10-day intervals to assay nicotinic acid.

The potential difference obtained from each experiment was constant for 30 days.

ELECTROCHEMICAL MICROBIOASSAY OF NYSTATIN

Although many polyene antibiotics active against fungi, yeasts and other organisms have been isolated from the culture broth of *Streptomyces* spp., most are too toxic for clinical use; only nystatin and a few other polyene antibiotics have found practical clinical application (Hamilton-Miller, 1973).

The agar diffusion method is not entirely suitable for the microbioassay of these antibiotics, which consist of heterogeneous mixtures of closely related compounds which are poorly soluble in water, diffuse poorly in agar gel, are unstable in bright sunlight and produce zones of inhibition that may be neither clear nor proportional in size to the logarithm of the antibiotic concentration. Simple and rapid methods are therefore required for determination of polyene antifungal antibiotics.

It is thought that polyenes bind with the sterol present in the membranes of sensitive cells, leading to the formation of pores (Hamilton-Miller, 1973). The subsequent death of the micro-organism is preceded by the leakage of cellular materials. The death of the micro-organisms can be detected with an oxygen electrode. Using this principle, a yeast electrode comprising a membrane supporting immobilized yeast (*Saccharomyces cerevisiae*) attached to an oxygen electrode, a collagen membrane to prevent leakage of the yeast cells, and an oxygen electrode has been devised, and its use for the determination of nystatin has been investigated (Karube, Matsunaga and Suzuki, 1979).

The respiration of immobilized micro-organisms can be determined by the oxygen electrode, as described above. However, nutrients such as glucose in the sample solution affect the respiration of yeast cells, and such effects must be avoided. If sufficient nutrients are present in the solution, the electrode current is not affected by changes in nutrient concentration. The steady-state current decreases linearly with increasing glucose concentration, and becomes constant at concentrations exceeding 300 mg/ℓ; the rate of metabolism in the yeast cells may be rate-determining in this range, and the current depends on the total respiratory activity of the yeast cells. A buffer solution containing 500 mg/ℓ glucose was therefore used.

The total respiratory activity of yeast, and therefore the current, is affected by the number of yeast cells in the membrane. It was found that the current decreased with increasing number of cells but a linear relationship was obtained for $1 \times 10^7 - 4{\cdot}6 \times 10^7$ yeast cells. Consequently, a membrane containing $4{\cdot}5 \times 10^7$ cells was used thereafter for the electrode. When the yeast electrode was inserted into the glucose-buffer solution containing nystatin, a steady-state current was obtained and then the current began to increase giving a sigmoidal curve. When the electrode was inserted into the glucose-buffer solution not containing nystatin, no current increase was observed. In the presence of nystatin, the electrode current ultimately reached the level of the electrode in the absence of yeast. The rate of current increase is a measure of

the nystatin concentration, and is most easily measured as the linear slope at the mid-point of the sigmoidal curve.

The relationship between the response time and the nystatin concentration showed decreasing response times for increasing amounts of nystatin. As nystatin ($C_{47}H_{75}O_{17}$) is a large molecule (molecular weight 926), it diffuses only very slowly through the collagen membrane; therefore, several minutes are required to initiate the current increase. A pH of 4·5 is most suitable for nystatin determination with the yeast electrode.

The relationship between the rate of current increase and the nystatin concentration was linear below 54 units/ml. The rate of current increase had a standard deviation of 1·2 units/ml when a sample solution containing 27 units/ml of nystatin was analysed (30 experiments).

Conclusion

For rapid and precise monitoring of the various factors involved in fermentation reactions, it seems entirely sensible to employ the micro-organisms themselves, which are highly sensitive to changes in their environment. This chapter has touched upon some of the ways in which microbial sensors (i.e. immobilized micro-organisms in conjunction with an electrochemical device) might be employed to monitor levels of fermentation substrates, reaction products, nutrients and antibiotics. In addition, various properties of the bacteria (electron transfer from reduced bacterial coenzymes; density and/or compressibility) could be allied with different types of device (e.g. oxygen electrodes; piezoelectric membranes) to measure cell population.

This account has dealt almost entirely with those microbial sensors which are being investigated and developed in Japan for application to the monitoring of fermentation processes. Here, as in research laboratories world wide, work on these devices continues, and much remains to be done (for examples and reviews of recent work on various types of biosensor in Japan and in other countries, *see* Carr and Bowers, 1980; Wingard, Katzir and Goldstein, 1981; Suzuki, Satoh and Karube, 1982; Karube and Suzuki, 1983; Lowe, Goldfinch and Lias, 1983; Mosbach, Mandenius and Danielsson, 1983; Wingard, 1983; Aston and Turner, 1984; Neujahr, 1984). Because sensors which work well under laboratory conditions do not always succeed when placed in industrial media, basic research on sensors using not only micro-organisms now available, but also, possibly, new mutants with improved properties, must be backed up by developmental modification and refinement. Nevertheless, these and other types of biosensor which can be used in on-line systems continuously to record changes in biochemical and microbiological processes with the maximum accuracy and minimum delay, would appear to be indispensable for the efficient operation of numerous biotechnological processes, and should therefore amply repay further investigation.

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