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Determination of Cyanide Using a Microbial Sensor

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ABSTRACT

A microbial cyanide sensor was prepared, consisting of immobilized *Saccharomyces cerevisiae* and an oxygen electrode. When the electrode was inserted into a solution containing glucose, the respiration activity of the microorganisms increased. The change in the respiration activity is monitored with the oxygen electrode. When cyanide is added to the sample solution, the electron transport chain reaction of the respiration system in the mitochondria is inhibited, resulting in a decrease in respiration. The inhibition is caused by cyanide binding with respiration enzymes such as the cytochrome oxidase complex in the mitochondrial inner membrane. Therefore, the cyanide concentration can be measured from the change in the respiration rate. When the sensor was applied to a batch system at pH 8.0 and 30°C, the cyanide calibration curve showed linearity in the concentration range between 0.3 μM and 150 μM CN^-.

Index Entries: *Saccharomyces cerevisiae;* microbial biosensor; immobilized cell; cyanide determination; respiration inhibition.

INTRODUCTION

Cyanide monitoring in waste is required for environmental control in a wide range of industries. Cyanide, a deadly poison, is widely used in industrial applications, especially for electroplating and metal cleaning.

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Hydrogen cyanide (HCN) is formed as a waste product in coke ovens and in coal gasification, and is used as a fumigating agent to kill rodents and other pests in grain bins, buildings, and in the holds of ships. Owing to the acute toxicity of this chemical, animal feeds are regularly analyzed for cyanide introduced from the decomposition of cyanogenic glycosides commonly occurring in many plants. Cyanide may enter the environment from many sources, in the form of water or air pollution. Therefore, development of a rapid and simple monitoring technique for cyanide is desirable.

Conventional methods of cyanide detection rely on separation of cyanide, from the acidified sample, in the form of hydrogen cyanide gas which is collected in an alkaline solution. The cyanide ion concentration is subsequently determined by optical spectrophotometry and atomic absorption spectrometry. The widely-used calorimetric method, based on the Koning reaction, involves toxic reagents and is subject to many interferences (*1*). Argentometry, based on the titration of available cyanide with standard silver nitrate to form argentocyanide, has an inadequate detection limit (*2*). The commercially available cyanide electrode, which potentiometrically monitors the reaction of cyanide with AgI and AgS packed onto its surface to form argentocyanide, is vulnerable to interference from sulfide and iodide compounds (*3*).

Enzymatic assays have also been developed for detecting of cyanide. Fenong described a method for cyanide detection based on rhodanese, which catalyzes cyanide to thiocyanate in the presence of thiosulfate (*4*). The thiocyanate produced is then compelled with Fe^{2+} to produce a chromophore that absorbs strongly at 460 nm. Fenong also reported that the sulfite product in the above reaction had a bleaching action on the rosaniline dye.

Recently, development has focused on amperometric biosensors for a variety of analytes including substrates, cofactors, prosthetic groups, enzyme activities, antibodies/antigens, inhibitors, and activators. Enzyme thermistor using immobilized rhodanese and injectase have also been developed for measuring cyanide concentrations in blast furnace water (*5,6*), and a flowthrough biosensor system has been developed for measuring of free cyanide in brass plating and rinsing solutions (*7*). This system consists of an enzyme column—prepared by activated-glutaraldehyde immobilization of rhodanese on aminopropyl glass beads—combined with an enzyme electrode. This electrode was prepared by immobilizing sulfite oxidase via glutaraldehyde activation to form an enzyme column and sulfite immobilized on a preactivated nylon membrane, which was then attached to the surface of a hydrogen peroxide electrode.

Enzyme sensors are highly specific for substrates, but the enzymes employed are generally expensive and unstable.

Microbial biosensors are generally composed of immobilized microorganisms and a suitable transducer such as a photodetector, potentimetric or amperometric electrode, or thermistor (*8,9*). They are suitable for online process control and environmental monitoring. Microbial biosensors

have been developed for assaying biological oxygen demand (BOD), a value that is related to the total content of organic material in waste water (*10,11*).

In the case of microbial biosensors measuring respiration activity, changes in the respiration of microorganisms, caused by assimilation of the analyte, are detected by an oxygen electrode. From these changes, the analyte concentration is estimated.

The respiratory chain in the inner mitochondrial membrane contains three major enzyme complexes through which electrons pass, from NADH to O_2. In the native membrane, the mobile electron carriers, ubiquinone and cytochrome-*c*, complete the electron-transport chain by shuttling between the enzyme complexes. The path of electron flow is NADH, NADH dehydrogenate complex, ubiquinone, b-c_1 complex, cytochrome-*c*, cytochrome oxidase complex, and molecular oxygen (O_2).

Cyanide compounds such as potassium cyanide and sodium cyanide bind with heme-*a* in cytochrome-*a* and cytochrome-a_3, which are included in the cytochrome oxidase complex. The resultant inactivation of heme-*a* causes a decrease in the respiration. The cyanide induced decrease in respiration activity causes a decrease in oxygen consumption. With an oxygen electrode, the cyanide concentration can be measured from the change in the oxygen consumption of the immobilized cells. This paper reports on the development of a microbial biosensor for the determination of cyanide. The effect of immobilized microbe loading, reagents, reaction temperature, and buffer pH were investigated. By examining the effects of these variables, a set of experimental conditions was optimized.

MATERIALS AND METHODS

Reagents

Potassium cyanide, potassium phosphate, Tris(hydroxymethyl) amino methane, and glucose were purchased from Wako Chemical (Kyoto, Japan). Peptone, yeast extract, malt extract, and nutrient broth were purchased from Difco (Detroit, MI). All reagents used were of analytical grade, and the water used was deionized or distilled.

Strain and Cultivation

Micrococcus luteus IFO 3342, *E. coli* IFO 14249, *Pseudomonas aeruginosa* IFO 1095, *Saccharomyces cerevisiae* IFO 0337, and *Trichosporon cutaneum* IFO 10466 were obtained from IFO Culture Collection, Institute for Fermentation, Osaka. *Bacillus subtilis* IAM 1069 was obtained from IAM Culture Collection, Institute for Molecular and Cellular Biosciences, University of Tokyo. *B. subtilis* IAM 1069, *M. uteus* IFO 3342, *E. coli* IFO 14249, and *P. aeruginosa* IFO 1095 were cultivated in an N medium consisting of 8 g/L nutrient broth. Cultivation was done in N medium aerobically at 38°C for

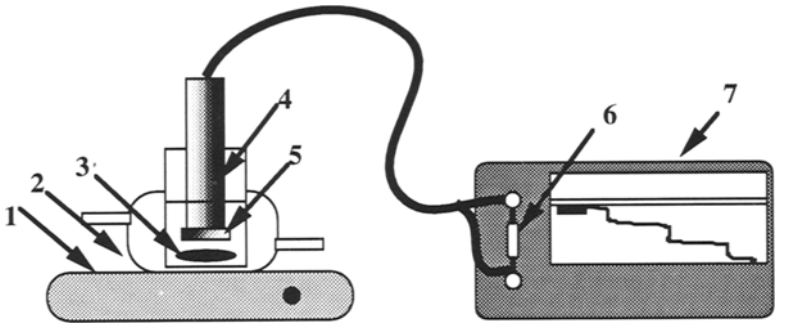

Fig. 1. Schematic diagram of the microbial cyanide biosensor. 1, stirrer; 2, thermostatic water bath; 3, magnetic stirrer; 4, oxygen electrode; 5, biomembrane; 6, 10-kΩ resistor; 7, recorder.

one day. *S. cerevisiae* IFO 0337 and *T. cutaneum* IFO 10466 were cultivated in YM medium consisting of 10 g/L glucose, 5 g/L peptone, 3 g/L yeast extract, and 3 g/L malt extract at pH 5.8. Cultivation was done in YM medium at 28°C for 1 d. Cells were harvested by centrifugation at 5000 rpm for 10 min and washed twice in 0.01*M* Tris-HCl buffer (pH 6.0). The supernatant was discarded and the resulting pellet was used for the immobilized cells.

Construction of the Microbial Sensor

A suspension of cells onto a porous cellulose nitrate membrane (pore size: 0.45 μm, Tokyo Roshi Kaisha, Tokyo, Japan) while applying gentle suction from a water pump. The microorganisms were trapped between two porous membranes as previously described (*12*). The membrane incorporating the immobilized microbes was placed on the teflon membrane cover of a Clark-type oxygen electrode (Type U-1, ABLE, Tokyo, Japan) and covered with a protective layer of 200-μm nylon mesh.

Measurement System

The measurement was made in a batch reaction cell. A schematic diagram of the measurement system is shown in Fig. 1. The sensor was placed in a thermostatically-controlled, circulating water jacket containing a total volume of 30 mL of buffer solution. The buffer was stirred magnatically during the measurement. A 10-kω resistor was connected in parallel with the oxygen electrode. The current output of the oxygen electrode was measured with an electronic polyrecorder (Type EPR-151A, TOA Electronics, Tokyo, Japan). When the output current became stable, the sample solution was injection into the sensor cell, and the current change was recorded.

Table 1
The Relative Respiration Activities and Responses of Several Microorganisms to Cyanide

Strain no.	Name	Respiration activity, %	Responses to cyanide, %
IFO 0337	*S. cerevisiae*	100	100
IFO 10466	*T. cutaneum*	60	100
IFO 142249	*E. coli*	70	50
IAM 1069	*B. subtills*	5	—
IFO 3342	*M. luteus*	5	—
IFO 1095	*P. aeruginosa*	5	—

—, very weak response.

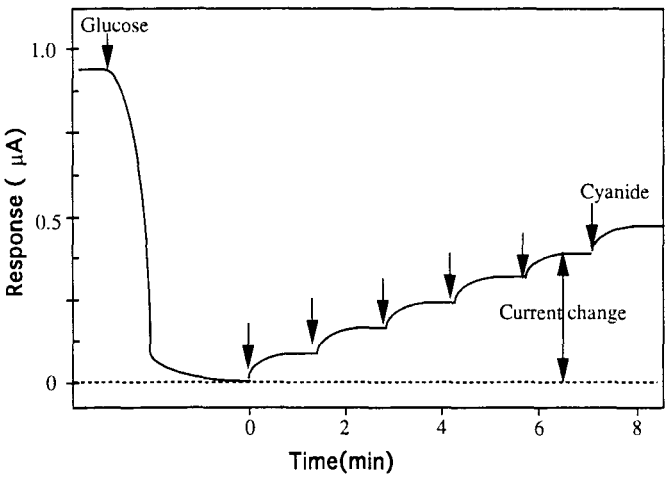

Fig. 2. Response curve of *S. cerevisiae*. Buffer: 0.01*M* Tris-HCl, pH 8.0. T = 30°C. Each addition step corresponds to an increase in cyanide concentration of 7.5 μM.

RESULTS AND DISCUSSION

Selection of Microorganisms for Detection of Cyanide

When estimating measuring cyanide concentration from the inhibition of respiration, the higher the respiration activity of the microorganism, the wider the measurement range of cyanide. In addition, the microorganism used cyanide must be suitable for immobilization. Several microorganisms were immobilized on the oxygen electrode, and their respiration activity was determined by adding 100 mg/L of glucose, followed by addition of 15 μM of cyanide. The response of each electrode was determined and compared with the others (Table 1). *S. cerevisiae* exhibited greater respiration activity than the other microbes tested and had a higher sensitivity to cyanide. Therefore, *S. cerevisiae* was immobilized for the microbial cyanide sensor. Figure 2 shows the typical response curve of

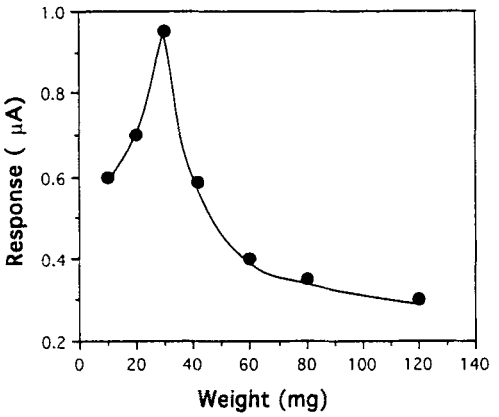

Fig. 3. Effect of microorganism mass on the response to glucose. Buffer: 0.01*M* Tris-HCl, pH 6.0, T = 30°C.

S. cerevisiae. The response of the microbial sensor to cyanide was determined by calculating the difference between the current before and after the addition of cyanide. Less than 2 min were required for the current from the microbial sensor to stabilize following addition of cyanide to the reaction vessel.

Mass of Immobilized Organisms

The effect of the immobilized mass of *S. cerevisiae* on the response of the microbial cyanide sensor was investigated. Wet cells (10–150 mg) were immobilized on the oxygen electrode and 100 mg/L glucose was added. The current response of the oxygen electrode is shown in Fig. 3. A maximum response was observed for 30 mg of immobilized *S. cerevisiae*. Therefore, 30 mg of wet cells were immobilized on the oxygen electrode for later analyses.

Effect of Buffer and Concentration

The effect of buffer type and concentration on the sensor response was investigated. The respiration activity was determined by adding 100 mg/L of glucose. As shown in Fig. 4, 0.01*M* Tris-HCl buffer elicited a greater respiration activity than the other buffers. Therefore, 0.01*M* Tris-HCl buffer was used throughout the study.

Effect of pH and Temperature

Since the activity of bacteria is dependent on pH, the output of the microbial electrode may be affected by the solution pH. The respiration activity was determined by adding 100 mg/L of glucose. Figure 5 shows the response-pH curve at various pH values. A peak in response was observed at pH 6.0. Although bacterial respiration was observed to be repressed at higher pHs, an acid pH results in evaporation of cyanide in

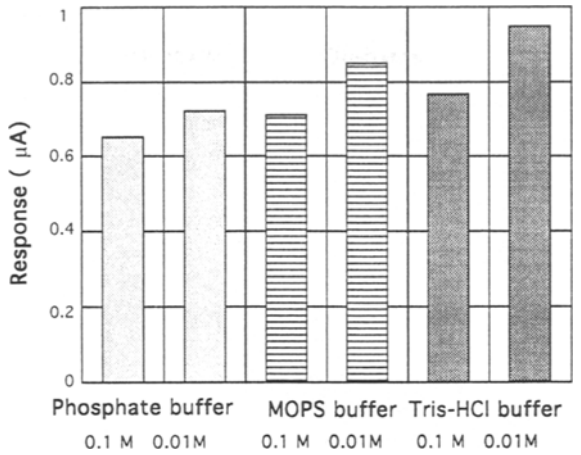

Fig. 4. Effect of buffer type and concentration on the response to glucose. Buffer: pH 6.0, T = 30°C.

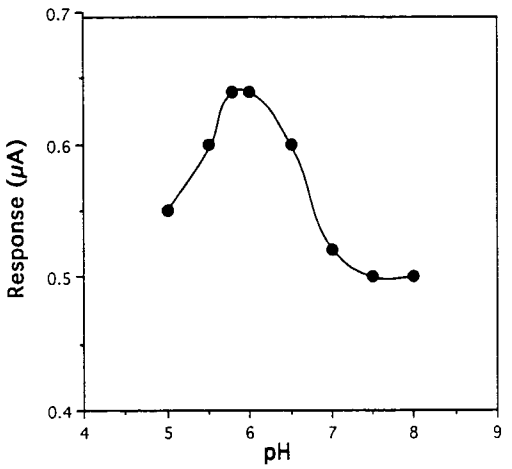

Fig. 5. Effect of pH on the response to glucose. Buffer: 0.01*M* Tris-HCl, T = 30°C.

the form of hydrogen cyanide gas. Therefore, a weak alkaline buffer—0.01*M* Tris-HCl buffer pH 8.0—was employed in this sensor.

The effect of temperature on the sensor response was also investigated. As shown in Fig. 6, significant change in respiration was not observed in the temperature range between 20 and 45°C. An operational temperature of 30°C was employed for the microbial cyanide sensor.

Amount of Glucose

In order to increase the measuring range of cyanide, the respiration activity must be increased by adding glucose. A calibration curve for glucose in 0.01*M* Tris-HCl buffer pH 8.0, 30°C, is shown in Fig. 7. Good linearity was observed in the concentration range between 10 and 150 mg/L. 150 mg/L of glucose was added to the cell prior to cyanide determination.

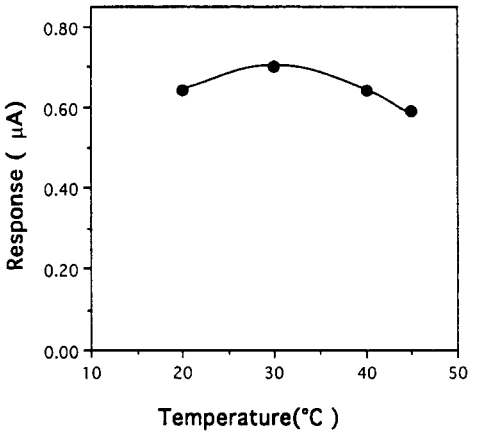

Fig. 6. Effect of temperature on the response to glucose. Buffer: 0.01*M* Tris-HCL, pH 6.0.

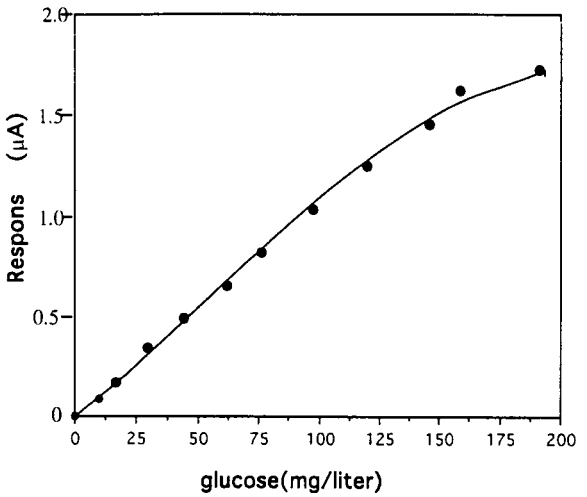

Fig. 7. Relationship between the response and the glucose concentration. Buffer: 0.01*M* Tris-HCl, pH 8.0, T = 30°C.

Sensor Calibration

A calibration curve for cyanide in 0.01*M* Tris-HCl buffer pH 8.0, 30°C is shown in Fig. 8. 150 mg/L of glucose was first added to the reaction cell, several concentrations of cyanide were added, and the response was calculated after the current of the electrode reached a steady state. The detection limit was 0.3 μM CN^- with a linear range extending to 150 μM. Good linearity could be observed in the concentration range.

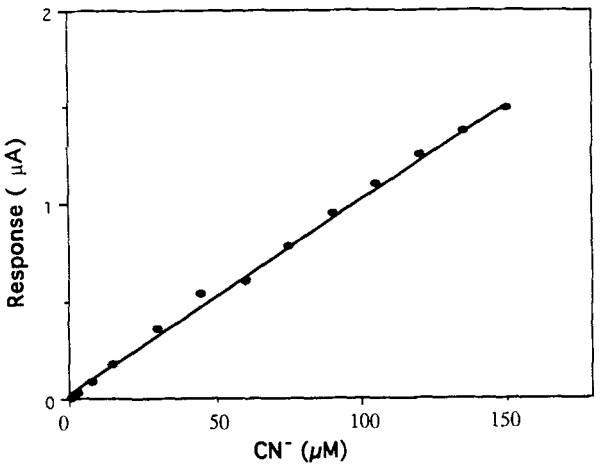

Fig. 8. Sensor calibration graph for cyanide ion concentration. Buffer: 0.01*M* Tris-HCl, pH 8.0, T = 30°C. Correlation coefficient: 0.997.

CONCLUSION

This research has shown that cyanide may be detected using *S. cerevisiae* immobilized on an oxygen electrode. Following glucose addition, the respiratory activity of the microorganism is increased facilitating measurement of cyanide with high sensitivity. The system was operated at 30°C, using a 0.01*M* Tris-HCl buffer at pH 8.0. The cyanide calibration curve showed linearity in the concentration range between 0.3 and 150 μM CN^-. The system could be used at room temperature, since no significant change in respiration was observed in the temperature range between 20 and 45°C.

Free cyanide is known to inhibit both rhodanese and sulfite oxidase (*13*) and, in addition, rhodanese is strongly inhibited by sulfite and sulfate anion. For enzymatic assays, including enzyme sensors, preliminary studies must therefore be performed using soluble enzyme to show that no inhibition owns over the desired concentration range.

The microbial cyanide biosensor developed here was more sensitive than the enzyme thermistor reported by Mattiasson (*5*) and the flow-through biosensor system of Groom (*7*). The colorimetric method offers a much lower detection limit (0.9 μM), but achievement of this level requires freshly prepared pyridine-HCl-barbiturate reagent and immediate absorbance measurement. The Orion cyanide electrode requires compensation for any possible interference from sulfide and iodide ions.

The microbial cyanide biosensor does not require preparation of immobilized enzymes or pretreatment. This attribute makes the sensor applicable to the on-line monitoring of cyanide and, possibly, further biosensors

can be developed for similar toxic chemicals. The work reported here is the first step toward development of a practical cyanide biosensor system for various environmental applications. The quantity of immobilized cells is increased in the column; the concentration range and the sensitivity of cyanide detection may be increased. Further investigations are being conducted to study the effects of the cell immobilization method, cell stability, and sensor reusability. It is hoped that, eventually, the microbial cyanide sensor may be used to assist in control and monitoring of river and lake environments.

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