Application of a biomass sensor system to determination of pyridoxine

Journal of Tokyo University of Fisheries, Vol. 84, No. 2, pp. 31–36, December 1997

APPLICATION OF A BIOMASS SENSOR SYSTEM TO DETERMINATION OF PYRIDOXINE*

Hideaki Endo*[1], Noriko Inada*[1], Masakazu Hoshi*[2], Tetsuhito Hayashi*[1] and Etsuo Watanabe*[1]

A biomass sensor system for viable yeast cells was prepared and applied to determine pyridoxine (PIN). The sensor consisted of an immobilized yeast (*Saccharomyces uvarum* 4288 ATCC9080) and Clark-type oxygen electrode. The determination is based on the respiratory activity of the microorganism. It was possible to determine the concentration of yeast cells in the range of 1.6×10^6–6.1×10^6 cells/m*l*. The sensor system was applied for determination of PIN. The output current reached plateau within 40 min and one assay could be completed within 60 min. Calibration curve was linear in the range of 0.1–0.5 ng/m*l* (relative error: ±5%).

Key words: Sensor, Pyridoxine, Electrode, Biomass

Introduction

Pyridoxine (PIN) is a main compound of vitamin B_6 and it plays an important role in the metabolism of living body. The determination of PIN is very significant in various research fields such as food and medical sciences. For determination of PIN, yeast microbiological method (Atkin *et al.*, 1943; Rabinowotz *et al.*, 1947; Fukui *et al.*, 1953) is the most popular among the established methods. This method was based on the measurement of growth rate (cell concentration) of yeast such as *Saccharomyces uvarum* in the presence of PIN, and the cell concentration was measured by spectrophotometry. However, existence of dead cells sometimes makes the results inaccurate, because spectrophotometer measures cell density regardless of their life or death. It is not possible to determine the concentration of only viable cells in a mixture of both viable and dead cells. To estimate the number of viable cells, colony counts method (Postgate *et al.*, 1969) have been used, but method is time-consuming and complicated.

On the other hand, several new methods such as electrochemical (Matsunaga *et al.*, 1984; Ramsay *et al.*, 1988), fluorescence (Zabryskie *et al.*, 1978; Armiger *et al.*, 1986), and ultrasonic (Zips *et al.*, 1978; Endo *et al.*, 1989) methods have been reported for the determination of microorganism concentration, and we have also developed a biomass sensor system for several strains of bacteria (Hoshi *et al.*, 1991). Our sensor system consists of an immobilized bacterial membrane and Clark-type oxygen electrode. The determination is based on the respiratory activity of bacterial cells. It was possible to determine the number of only viable microorganisms such as *Shewanella putrefaciens*, *Escherichia coli*, *Pseudomonas fluorescens*, and *Staphylococcus aureus*. Therefore this method could be applicable to measure the number of viable yeast cells for the determination of PIN.

In this study, we prepared a biomass sensor system for viable yeast cells, and applied it for the determination of PIN. The operational property of the sensor system was also examined.

* Received February 6, 1997.

*[1] Department of Food Science and Technology, Tokyo University of Fisheries, 5–7, Konan 4-chome, Minato-ku, Tokyo 108, Japan (東京水産大学食品生産学科).

*[2] Central Research Institute, Maruha Corporation, Wadai, Tsukuba-City, Ibaraki 300–42, Japan (マルハ株式会社中央研究所).

Materials and Methods

Materials

1) Reagents PIN and dialysis membranes were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Vitamin B_6 Assay Medium Base (VAMB) was obtained from Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan). The composition of the medium (1 *l*) was as follows; casamino acid: 8 g, inositol: 50 mg, thiamine hydrochloride: 500 μg, nicotinic acid: 5 mg, calcium pantothenate: 5 mg, biotin: 16 μg, KCl: 850 mg, glucose: 100 g, $CaCl_2$: 250 mg, $MgSO_4$: 250 mg, $MnSO_4$: 5 mg, KH_2PO_4: 1.1 g, $FeCl_3$: 5 mg, potassium citrate: 10 g, citric acid: 2 g. Peptone, malt extract and yeast extract were purchased from Difco Laboratories (Detroit, Michigan, USA). Other chemicals were obtained commercially and were of analytical reagent grade.

2) Standard solution of PIN For preparation of stock solution, 20 mg of PIN was dissolved in 25% ethanol aqueous solution and made up to 100 m*l*. The solution was diluted with distilled water to prepare standard solution in the range of 0.1–0.5 ng/m*l*.

Microorganism and its cultivation

Saccharomyces uvarum 4288 ATCC9080 was used as a biocatalyst for the determination of PIN. The microorganism was cultivated in YMPD (Yeast extract–Malt extract–Peptone–Dextrose) solid-state medium which contained (g/*l*); yeast extract (3.0), malt extract (3.0), peptone (5.0), glucose (10.0), agar (20.0) and incubated at 30℃ for 20 hours.

Preparation of a biomass sensor

A biomass sensor was constructed with an immobilized microorganism membrane, Clark-type oxygen electrode (Able Co., Tokyo, Japan) and an oxygen permeable Teflon membrane (Fig. 1(a)). The oxygen electrode consisted of a platinum cathode (diameter: 11 mm), a lead anode, alkaline electrolyte (KOH), and an oxygen permeable Teflon membrane (thickness: 2.0 mil). To prepare the membrane with immobilized cells, several sheets of cellulose nitrate membranes (pore size: 0.45 μm, diameter: 13 mm, Advantec Toyo Ltd. (Tokyo, Japan)) were sterilized with steam at 115℃ for 20 min, and then 1.0 m*l* of the cell suspension was filtered through each membrane. One of the membranes was tightly set on a platinum cathode of oxygen electrode and covered with a dialysis membrane.

Apparatus and assay procedure

Figure 1(b) shows schematic diagram of a biomass sensor system for the determination of PIN. The system consisted of a biomass sensor, vessels of 0.05 M phosphate buffer (volume: 50 m*l*, pH 6.0) and YMPD medium solution which contained (g/*l*); yeast extract (3.0), malt extract (3.0), peptone (5.0), and glucose (10.0) (volume: 50 m*l*, pH 6.0), a water bath, and a recorder. Both phosphate buffer and YMPD medium solution were saturated with oxygen.

For the determination of PIN, one platinum loop of *S. uvarum* cultivated in YMPD solid-state medium was suspended in 100 m*l* of 0.9% NaCl solution to prepare inoculative cell solution. The cell suspension (0.5 m*l*) was inoculated into several test tubes containing 1.5 m*l* of different concentrations of PIN solution (0–0.5 ng/m*l*) and 2.0 m*l* of VAMB solution (conc.: 260 g/*l*). These test tubes were incubated at 30℃ for 15 hours. After the incubation, the culture broth was stirred and was kept in a micro-test tube for the measurement of cell concentration. One m*l* of the culture broth was filtered through the cellulose nitrate membrane and the membrane was tightly set on a platinum cathode of oxygen electrode as described above. The biomass sensor was immersed in the buffer vessel at first. After the output current of the sensor became stable, the sensor was taken out and immediately placed

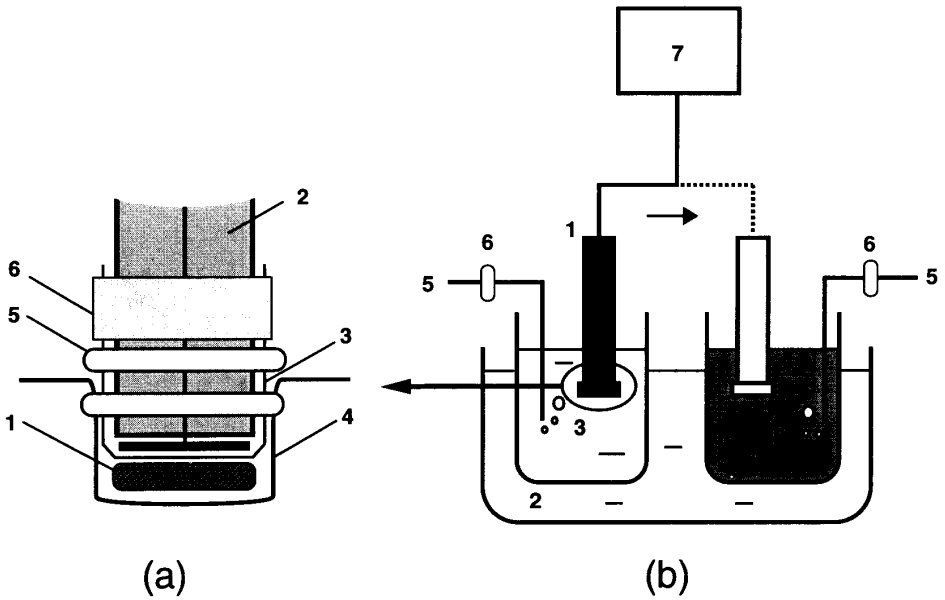

Fig. 1. Schematic diagram of the biomass sensor system.
a: Detector of biomass sensor
(1) membrane containing immobilized yeast; (2) oxygen electrode; (3) oxygen permeable teflon membrane; (4) dialysis membrane; (5) rubber ring; (6) parafilm
b: Biomass sensor system for PIN
(1) biomass sensor; (2) water bath; (3) vessel of 0.05 M phosphate buffer (pH 6.0); (4) vessel of YMPD solution; (5) air; (6) filter; (7) recorder.

in YMPD medium solution. When a stationary current of the sensor was obtained, the current decrease was measured.

Conventional method for cell counting

A microorganism suspension was diluted with 0.9% NaCl solution to obtain a final cell number of 30–300 cells per 100 μl. *S. uvarum* cells were spread over the surface of YMPD solid-state medium. The plates were run in triplicate and were incubated for 40 h at 30°C. After incubation, the colonies on the plates were counted.

Results and Discussion

Response properties of a biomass sensor system

As preliminary experiments, cell concentrations of *S. uvarum* were determined by the biomass sensor system. The cells cultivated in YMPD solid-state medium were suspended in 0.9 % NaCl solution and adjusted to appropriate concentration of 10^6–10^7 cells/m*l*. Figure 2 shows typical response curve of the system. When the sensor was immersed in the buffer vessel, the output current of the sensor became stable within 10 min. After the stationary current was obtained, the sensor was transferred to YMPD medium solution. The output current began to decrease within 1 min and reached plateau within 40 min. This phenomenon indicated that various compounds in YMPD medium solution had passed through the cellulose nitrate membrane and was assimilated by the immobilized microorganism. The respiratory activity of the microorganism consumed dissolved oxygen around the membrane and consequently decreased the output current of the sensor. When the assimilation rate of the compounds in YMPD medium by microorganism and the diffusion rate of the

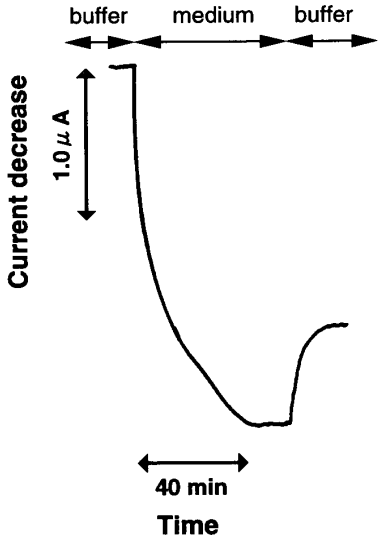

Fig. 2. Response curves of the sensor system for *Saccharomyces uvarum* 4288 ATCC9080. Cell concentration and temperature were 3.3×10^6 cells/m*l* and 30℃, respectively.

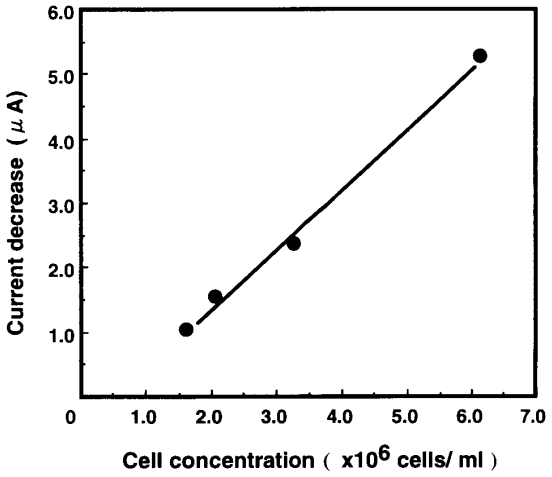

Fig. 3. Relationship between the cell concentration and the current decrease of the sensor. Temperature: 30℃.

dissolved oxygen attained equilibrium, original and stationary current was obtained. The difference in current decrease between the stationary currents obtained from buffer solution and from YMPD medium solution was used as the measurement of cell concentration. One assay could be completed within 60 min.

Figure 3 shows a relationship between the cell concentration and the current decrease of the sensor. The current decrease of the sensor increased with increasing the cell concentration. A good linear correlation was observed between them in the range of $1.6\times10^6-6.1\times10^6$ cells/m*l*. Thus the sensor system was useful for the determination of cell concentration of *S. uvarum*.

Application of biomass sensor for determination of PIN

The biomass sensor system was applied for the determination of PIN. *S. uvarum* was cultivated in a test tube containing PIN and VAMB solutions, and then PIN concentration was measured with the sensor system. The response curve was given in Fig. 4. The output current reached plateau in 40 min and one assay could be completed in 60 min (relative error: ± 5%) as well as earlier result in Fig. 2. It was possible to determine the cell concentration of the organism cultivated in test tube containing PIN.

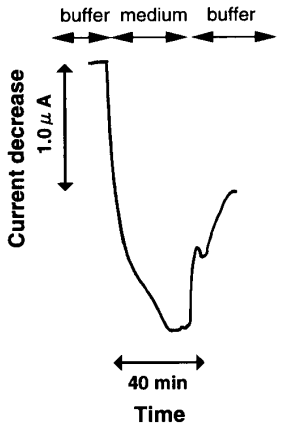

Fig. 4. Response curves of the sensor system for pyridoxine at 30℃. PIN concentration: 0.2 ng/m*l*.

In general, the response of the biosensor was readily influenced by analytical conditions. For this reason, it is necessary to establish favorable assay conditions to enhance the sensitivity of the sensor. Figure 5 shows the effect of temperature of the buffer and YMPD medium solutions on the current decrease of the sensor. The current decrease expanded with increasing temperature and it reduced again at 45℃. We assumed that the

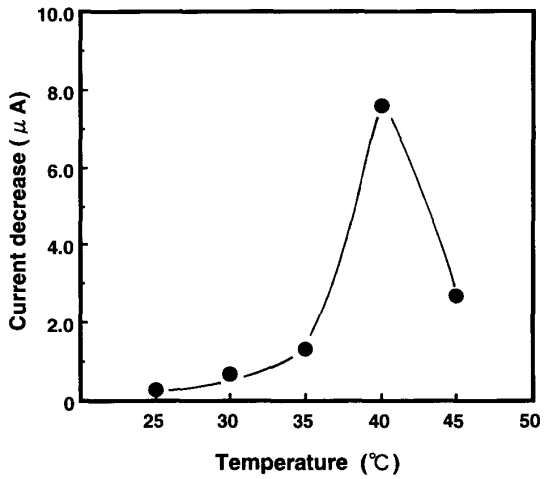

Fig. 5. Effect of temperature on the current decrease of the sensor. PIN concentration: 0.2 ng/ml.

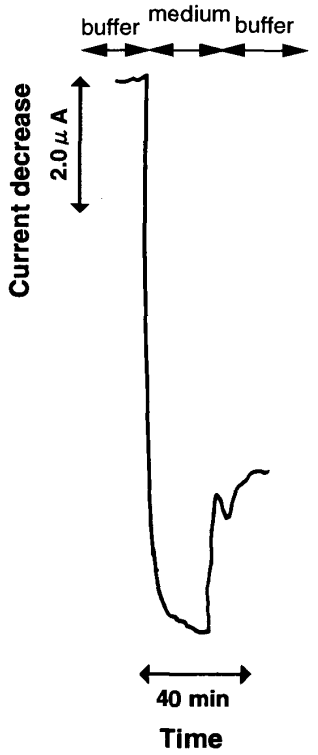

Fig. 6. Response curves of the sensor system for pyridoxine at 40℃. PIN concentration: 0.2 ng/ml.

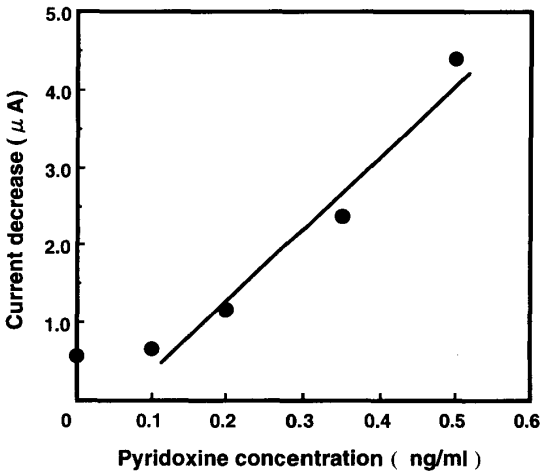

Fig. 7. Relationships between the current decreases of the sensor and PIN concentration. Temperature: 30℃.

metabolism of microorganism was temporarily facilitated by increase of temperature but the respiratory activity declined at higher temperature. Higher response was obtained at 40℃ and the response curve of the sensor in this condition was shown in Fig. 6. The output current reached plateau within 30 min and one assay could be completed within 40 min. Higher response is ordinarily preferable for the operation of the sensor, however, the response of the sensor became unstable at 40℃ (relative error: ±30%) compared to the condition at 30℃. It might have been caused by a damage of cell due to the increasing temperature during the assay. Moreover the response of the oxygen electrode might become unstable at higher temperature due to the decreasing solubility of oxygen. Therefore, operation at 30℃ was thought to be suitable for the system, since the optimum temperature of the cultivation for this microorganism was 30℃.

Figure 7 shows the relationships between the current decreases of the sensor and PIN concentrations. When the concentration of PIN was 0 ng/ml, a small response was observed. This response was due to the respiratory activity of the cells inoculated to the test tube at the preparation of the standard sample. Calibration curve was linear in the range of over 0.1 ng/ml and under 0.5 ng/ml as shown in the figure. One assay could be completed within 60 min. Although the membrane with the immobilized cells of the electrode needed to be renewed for each measurement, the membrane could be easily exchanged by using a rubber ring (Fig. 1(a)).

In conclusion, this sensor system could be used for the determination of PIN, and it has the possibility to analyze other compounds of vitamin B_6 such as pyridoxal and pyridoxamine. Further studies in our laboratory are towards the reduction of the assay time, and the application of the system for food analysis.

References

Armiger, W. B., Forro, J. F., Montalvo, L. M., Lee, J. F. and Zabryskie, D. W. 1986. The interpretation of on-line process measurements of intracellular NADH in fermentation processes. Chem. Eng. Commun., **45**: 197–206.

Atkin, L., Schultz, A. S., Williams, W. L. and Frey, C. N. 1943. Yeast microbiological methods for determination of vitamins. Analytical Edition, **15**: 141–144.

Endo, H., Sode, K., Ogura, K., Ohya, K. and Karube, I. 1989. Determination of microbial concentration with a piezoelectric gum sensor. J. Biotechnol., **12**: 307–316.

Fukui, S., Kishibe, T. and Tani, Y. 1953. The microbiological assay of vitamin B_6 (I); Determination of total vitamin B_6 by *Saccharomyces carlsbergensis* and its application to several food materials. Vitamins, **6**: 436–439.

Hoshi, M., Nishi, H., Hayashi, T., Okuzumi, M. and Watanabe, E. 1991. Development of biosensor for the determination total viable bacteria cell count. Nippon Suisan Gakkaishi, **57**: 281–285.

Matsunaga, T. and Namba, Y. 1984. Detection of microbial cells by cyclic voltammetry. Anal. Chem., **56**: 798–801.

Postgate, J. R. 1969. Viable counts and viability. Methods Microbiol., **1**: 611–621.

Rabinowotz, J. and Snell, E. E. 1947. Extraction procedures for the microbiological determination of vitamin B_6. Anal. Chem., **19**: 277–280.

Ramsay, G. and Turner, A. P. F. 1988. Development of an electrochemical method for the rapid determination of microbial concentration and evidence for the reaction mechanism. Anal. Chim. Acta., **215**: 61–69.

Zabryskie, D. W. and Humphrey, A. E. 1978. Estimation of fermentation biomass concentration by measuring culture fluorescence. Appl. Environ. Microbiol., **35**: 337–343.

Zips, A. and Faust, U. 1989. Determination of biomass by ultrasonic measurements. Appl. Environ. Microbiol., **55**: 1801–1807.

バイオマスセンサーを応用したピリドキシンの定量

遠藤英明・稲田典子・星　昌和・林　哲仁・渡辺悦生

バイオマスセンサーシステムをピリドキシン (PIN) の定量に応用した。本システムは，*Saccharomyces uvarum* 4288 ATCC9080 の固定化微生物膜とクラーク型酸素電極より構成され，菌体の呼吸活性を測定する原理に基づいている。バイオマスセンサーとしては，酵母菌体量が 1.6×10^6～6.1×10^6 cells/ml の範囲で測定が可能であった。次にこのシステムを PIN の計測に応用したところ，センサーの出力電流値は 40 分以内で定常になり，一検体の分析所要時間は約 60 分であった。また PIN は，0.1～0.5 ng/ml の濃度範囲で測定が可能であり，測定値の相対誤差は ±5% であった。

キーワード：センサー，ピリドキシン，電極，バイオマス