BACTERIA-BASED SENSOR FOR MONITORING GLYCEROL

Zhi H. LIU[a1], Meng L. WEN[a2,*], Yun YAO[a3], Nan H. SHI[a], Shi Q. LIU[b] and Ming QIAO[b1]

[a] *Department of Chemistry, Yunnan University, Kunming, Yunnan 650091, PR China; e-mail: [1] zhihualiu@yeah.net, [2] mlwen@ynu.edu.cn, [3] yun_yao@netease.com*

[b] *Provincial Key Laboratory of Fermentation Engineering, Yunnan University, Kunming, Yunnan 650091, PR China; e-mail: [1] qiaoming@ynu.edu.cn*

Received January 13, 1999
Accepted June 11, 1999

A new bacteria-based sensor for determination of glycerol was reported, in which mutant bacteria induced from the strain of *Bacillus subtilis* AS1.398 was used as a biocatalyst. The calibration graph of the proposed sensor was linear in the range of 1–10% (w/v) glycerol and the correlation coefficient was 0.9946. Lifetime of the sensor was more than two weeks. There was no significant interference from substances commonly coexisting in the fermentation broth except acetic acid. The sensor would be useful for monitoring glycerol during fermentation due to its linearity, selectivity, and lifetime. The bacteria could be used as a catalytic enzyme membrane which can reproduce itself in proper matrix.

Key words: Glycerol determination; Microbial membrane electrode; Induced mutant; *Bacillus subtilis* AS1.398; Biocatalysis.

The determination of glycerol is crucial due to its wide uses in various fields[1]. The official standard methods for determination of free glycerol in soaps, detergents, cosmetics, *etc.* were based on its oxidation by peroxidates. The formic acid produced by the oxidation rection could be titrated with standard sodium hydroxide[2]. The glycerol produced by fermentation could also be determined spectrophotometrically in which the chromotropic acid reaction was employed[3,4]. However, all these procedures above are not suitable for process monitoring.

Over last two decades, biosensors have become an attractive tool for fermentation process monitoring because of its high sensitivity and selectivity[5,6]. Concerning glycerol, various enzymes related to glycerol, such as glycerol dehydrogenase[7–15], glycerol kinase and glycerol-3-phosphate oxidase[16–20], and glycerol oxidase[21], were employed to construct biosensors in which the transducers could be electrochemical or spectrophotometrical. These enzyme-based biosensors were sensitive, selective, and elegant, but

much effort had to be made to isolate and purify the enzyme; also, their lifetime was limited. As alternative biocatalytic materials, immobilized bacterial cells are available for the construction of biosensors. Their major advantages are that a complete, nature-optimized enzymatic pathway is used and the increased stability of an enzyme is obtained because it is maintained in its natural environment[5,6,22]. In particular, the selectivity of bacteria-based biosensor could be greatly enhanced by preincubating the microorganism with desired substrates[23]. Although many induced bacetria-based sensors have been developed and applied to process control[6,23–25], there are no reported data on this type of bacterial sensor for glycerol. Here we report a new induced bacteria-based sensor for determination of glycerol.

EXPERIMENTAL

Materials

Apparatus. A shaker (WDP microbial culturing box, Optics, Machinery & Electricity Tech Co. Ltd., Shandong University, Jinan, PR China) was used to culture the bacteria. The growth of bacteria was observed and the bacteria were counted under a microscope (Zeiss, Germany). Oxygen-consuming rate and pH measurements were performed on a Model LM-1 digital oxygen analyzer combined with oxygen electrodes (Institute of Metallurgy, Chinese Academy of Sciences, Shanghai, PR China) and a Model-901 microprocessor ion analyzer (Orion Research Inc., Cambridge, MA, U.S.A.), respectively. The standard procedure reference for determination of glycerol was carried out with a spectrophotometer (Model 721-100, Third Analytical Instrument Factory, Shanghai, PR China). A super-thermostatic bath (CS501, Chongqing Experimental Instrument Factory, Chongqing, PR China) was used. The oxygen-based sensor system was described previously[26,27].

Reagents. The reagents for isolation of the microorganism were chemically pure. The glycerol for microorganism cultivation was of industrial grade. The poly(vinyl alcohol) (PVA) was obtained from Sigma (average molecular weight 70 000–100 000). Deionized water (1 MΩ) was used in the preparation of phosphate buffer solution (PBS). All other solutions were prepared with the PBS. A 50% glycerol solution was standardized spectrophotometrically by chromotropic acid after the oxidation of glycerol to formaldehyde with periodates[3,4]. Standard solutions of 1–10% glycerol were prepared by serial dilution of the above standard solution.

Construction of the Biosensor

Culture of bacteria. The induced mutant from strain *Bacillus subtilis* AS1.398, which was isolated from wild strain, was grown with culture A (peptone 1%, yeast extracts 0.3%, glycerol 10%, K_2HPO_4 0.5%, agar 2%, salt solution 0.25 ml, pH 6.5). Then the induced strain was cultured for at least 5 generations with addition of 5% of glycerol while the other nutrients were the same. Its characteristics remained stable. All the cultures were operated at 32 °C, 2 500 rpm for 36 h when the rate of respiration was at the maximum. The response

rates ($\Delta E/\Delta t$) of the sensor were determined under the conditions of 5% of glycerol, 32 °C, and in pH 6.99 PBS buffer. After the bacteria were harvested, the cells were counted under the microscope. The number of bacteria was $4.55 \cdot 10^{10}$/ml suspension.

Immobilization of bacteria with poly(vinyl alcohol) embedding method. A culture dish (i.d. 9 cm) containing 1 g PVA powder and 5 ml deionized water was put into a pressure cooker and heated to 121 °C, at $1.5199 \cdot 10^5$ Pa for 30 min. Then 5 ml of microorganism suspension and 0.01 g of peptone were added into the cooled dish. After the mixed solution (PVA 9%, peptone 1%, microorganism suspension 50%, $2.175 \cdot 10^{12}$) was dried in a vacuum dryer for 24 h at room temperature, the living cells were embedded in the PVA membrane (*ca* 400 μm thick).

Construction of the sensor. The sensor system was shown previously[26,27]. The bacteria membrane was held in place mechanically with a Nylon net (200 mesh). The electrode was soaked in PBS buffer (pH 6.99), while bacterial membranes were stored and reproduced in a solution of 2.5% glycerol at room temperature. The sensor was washed with deionized water and PBS before each measurement.

Procedure

PBS buffer (5 ml) was added into the cell at a constant temperature (32 °C) and the sensor was inserted into the cell. When the oxygen analyzer reaches the first steady state, a series of glycerol samples were injected into the cell. The value of ΔE (mV) could be recorded from the screen of the digital oxygen analyzer when second steady state was reached (30–120 min were required, depending on the concentration of glycerol in the cell). By adding a series of standard solutions of glycerol in PBS buffer at pH 6.99, the calibration curve with ΔE *vs* [glycerol] was plotted. The unknown concentration was determined from the calibration curve.

RESULTS AND DISCUSSION

Response Characteristics and Lifetime of the Sensor

Usually the separation of glycerol from the broth is very tedious and time-consuming. A biosensor that could be used to determine directly the glycerol in fermentation broth without several pretreatment steps was promising. The linear response range of this sensor was 1 to 10% of glycerol, which was matched to the concentration range of crude glycerol. Figure 1 shows the calibration plot for the proposed sensor. The regression equation was $\Delta E = 11.25$ [glycerol] + 6.54, the correlation coefficient r being 0.9946. A serious problem for the microbial sensor was usually its instability, which resulted in a poor reproducibility of the results. Hence, a calibration curve was made for each determination.

Self-reproduction of the bacterial cells in the immobilized PVA membrane was observed as the clear culture fluid for storing the sensor became turbid overnight. When continually used, the signal of the sensor was cha-

otic and analytically unusable on the 17th–18th day; at the same time, the change in turbidity of the solution was not so apparent. On the 18th day, the regression equation was ΔE = 8.30 [glycerol] + 15.25, with r = 0.9896. However, the value of ΔE decreased sharply and was unsuitable for too high or too low concentrations. Although the lifetime of the biosensor was about 18 days, it was sufficient for monitoring the glycerol fermentation since its cycle was usually ten days.

The rather long response time of the sensor may be a result of the assimilation of glycerol by several catalytic enzymes located within the immobilized *Bacillus subtilis* AS1.398 which is relative slow.

Effect of pH and Temperature

All the determinations were performed in a PBS buffer because the bacterial membrane was stored and reproduced in PBS solution of glycerol. In addition, the enzyme system has a mild activity in PBS solution. Figure 2 shows the variation in sensor response with a change in pH at 32 °C and a substrate at 2.5% glycerol. Unlike optimum pH for other catalyses, the optimum pH of the enzyme system within the induced mutant from *Bacillus subtilis* AS1.398 is acidic, as shown in Fig. 2. Although the signal raised up at low pH, pH 6.99 was chosen as the working pH to match the pH of crude

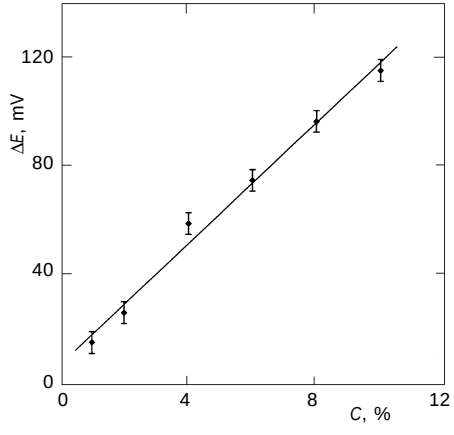

FIG. 1
The calibration graph of biosensor for determination of glycerol. Conditions: 5 ml of PBS, pH 6.99, 32 °C; y = 11.249x + 6.5452, R^2 = 0.9893

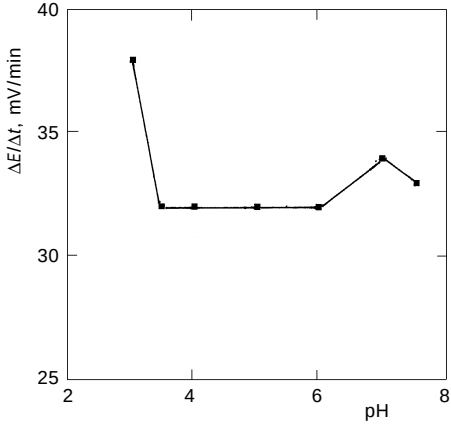

FIG. 2
Dependence of the rate ($\Delta E/\Delta t$) on pH (PBS, 32 °C, [glycerol] = 2.5%)

glycerol usually between 5 and 7. Thus, crude glycerol could be determined directly by proposed sensor without further pH adjustment.

The activity of the enzyme system increased with temperature and reached a maximum at 32 °C, as shown in Fig. 3. This was the optimum temperature, based on the fact that the enzyme system activity persists and is stable. Also, this was the best growth temperature for this microorganism.

Selectivity and Recovery

Selectivity was determined by a separate solution method, in which two solutions with equal concentration were chosen. First, the biosensor was in-

TABLE I
The value of $\Delta E_I/\Delta E_0$ for interfering substances *vs* glycerol at 32 °C, in PBS of pH 6.99; ([Interfering substance] = [glycerol] = 2.5%)

Substance	$\Delta E_1/\Delta E_0$ [a]	Substance	$\Delta E_1/\Delta E_0$ [a]
D-Glucose	0.1356	D,L-Proline	0.1186
Maltose	–0.0339	D,L-Alanine	0.0508
Sucrose	–0.1356	D,L-Tryptophan	0.0339
Urea	0.1356	$MgCl_2 \cdot 6\ H_2O$	–0.1017
Citric acid	0.0848	1-Propanol	–0.0169
D,L-Malic acid	0.0000	Ethanol	–0.0508
D,L-Tyrosine	0.1186	Formaldehyde	0.0847
Acetic acid	0.2033		

[a] $\Delta E_I = \Delta E$ of the interfering substance, $\Delta E_0 = \Delta E$ of glycerol.

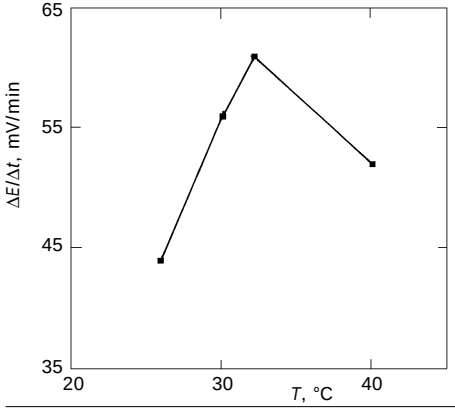

FIG. 3
Dependence of the rate ($\Delta E/\Delta t$) on temperature (PBS, pH 6.99, [glycerol] = 5%)

serted into a sample solution and the ΔE_0 was obtained; then inserted into a solution containing only interference substance and the ΔE_I was obtained. Comparing the ΔE_0 with ΔE_I, the relative response could be determined. Based on this method, possible interference from various substances coexisting in the broth, such as amino acids, urea, organic acids, glucose and disaccharides (maltose and sucrose) and ethanol, was taken into account. The results are shown in Table I. Except for the significant interference from acetic acid, relative response from glucose, urea, tyrosine and proline could be neglected because the concentration of these compounds is normally lower than that of glycerol in crude glycerol. The respiration of *Bacillus subtilis* AS 1.398 is possibly hindered by glucose, sucrose, $MgCl_2 \cdot 6\ H_2O$, 1-propanol and ethanol. Thus negative signal is obtained. The recovery was measured at two different concentrations (Table II).

TABLE II
The recovery for determination of glycerol at 32 °C, in PBS of pH 6.99

C, %	1	2	3	4	5	Average recovery, %	RSD[c], %
2.5	97.63	111.85	101.85	90.51	86.96	97.76[a]	10.0
	113.60	120.00	118.00	132.40	117.20	120.24[b]	6.0
5.0	93.26	98.59	95.04	96.82	80.81	92.86[a]	7.6
	105.80	114.00	106.20	106.80	108.40	108.24[b]	3.1

[a] Microbial electrode; [b] spectrophotometrically with chromotropic acid[4]; [c] relative standard deviation (RSD) is the sample standard deviation divided by sample mean.

Conclusions

The proposed biosensor could be useful for monitoring glycerol fermentation process in terms of its linearity, lifetime, and selectivity. The bacteria can be used as a catalytic enzyme membrane, which can reproduce itself in proper matrix. The application of the proposed biosensor to fermentation process monitoring is being investigated.

The authors gratefully acknowledge support from the Natural Science Fund of Yunnan (grant No. 96B008M) and the Science Fund of the Yunnan Education Department (grant No. 9512062).

REFERENCES

1. Zhuge J., Fang H. Y., Zhuge B.: *Gongye Weishengwu* **1997**, *27*, 34; *Chem. Abstr.* **1998**, *128*, 242909.
2. Hautfenne A.: *Pure Appl. Chem.* **1982**, *54*, 1257.
3. Zhuge J.: *Analysis of Fermentation Glycerol*. Wuxi Light Industry University, Wuxi, PR China 1994 (in Chinese).
4. Zhang Y. J., Jing F. M.: *The Collection of Analysis of Soap, Glycerol and Common Raw Materials*, p. 206. Chinese Light Industry Press, Beijing, PR China 1995 (in Chinese).
5. Cass A. E. G.: *Biosensor: A Practical Approach*. Oxford University Press, Oxford 1990.
6. Riedel K., Reinnehere R., Wollenberger U., Kaiser G., Scheller F. W.: *J. Chem. Technol. Biotechnol.* **1989**, *44*, 85.
7. Ince J. E., Knowles C. J.: Eur. 353 049; *Chem. Abstr.* **1989**, *112*, 213088.
8. Akira N., Fukaya M., Okumura H., Kawamura Y.: Eur. 400 918; *Chem. Abstr.* **1991**, *115*, 109958.
9. Matsumoto K., Matsubara H., Hamada M., Doi T., Osajima Y.: *Agric. Biol. Chem.* **1991**, *55*, 1055.
10. Matsumoto K., Matsubara H., Hamada M., Osajima Y.: *Nippon Shokughin Kogyo Gakkaishi* **1991**, *38*, 699; *Chem. Abstr.* **1991**, *115*, 278005.
11. Ueda S., Takahashi M., Misaki H.: PCT Int. Appl. WO92 20 818; *Chem. Abstr.* **1992**, 118, 76593.
12. Kittsteiner-Eberle R., Ogbomo I., Schmidt H. L.: *DECHEMA Biotechnol. Conf.* **1992**, *5 (Part A)*, 497; *Chem. Abstr.* **1993**, *119*, 201783.
13. Adachi K., Matsushita K.: Japan Kokai Tokkyo Koho JP 05 227 954; *Chem. Abstr.* **1992**, *119*, 248180.
14. Prodromidis M. I., Stalikas C. D., Tzouwara-Karayanni S. M., Karayannis M. I.: *Talanta* **1996**, *43*, 27.
15. Laurinavicius V., Kurtinaitiene B., Gureviciene V., Boguslavsky L., Geng L., Skotheim T.: *Anal. Chim. Acta* **1996**, *330*, 159.
16. Montesinos J. L., Alonso J., Delvalle M., Lima J. L. F. C., Poch M.: *Anal. Chim. Acta* **1991**, *254*, 177.
17. Merchie B., Girard A., Maisterrena B., Michalon P., Couturier R.: *Anal. Chim. Acta* **1992**, *263*, 85.
18. Montoya A., March C., Mocholi A., Abad A., Manclus J. J., Ferrero J. M.: *Sens. Actuators B* **1993**, *16*, 429.
19. Laurinavichyus V. A., Tsitsenene R. A., Kurtinaitene B. S., Meshkis R. Yu., Martsinkyavichene L. Yu.: *J. Anal. Chem.* **1995**, *50*, 1207.
20. Rank M., Gram J., Nielsen K. S., Danielsson B.: *Appl. Microbiol. Biotechnol.* **1995**, *42*, 813.
21. Aoyama N., Tsuda M., Shimizu Y., Sekine S., Tatano T.: Eur. 411 604; *Chem. Abstr.* **1991**, *115*, 25543.
22. Rechnitz G. A.: *Science* **1981**, *214*, 287.
23. Riedel K., Reinneberg R., Scheller F. W.: *Anal. Lett.* **1990**, *23*, 757.
24. Suzuki M., Lee S., Fujii K., Arikawa Y., Kubo I., Kanagawa T., Mikami E., Karube I.: *Anal. Lett.* **1992**, *25*, 973.
25. Beyersdorf-Radeck B., Karlson U., Schmid R. D.: *Anal. Lett.* **1994**, *27*, 285.
26. Tai G. Y., Wen M. L., Wang C. Y.: *Microchem. J.* **1996**, *53*, 152.
27. Zhao Y. B., Wen M. L., Liu S. Q., Liu Z. H., Zhang W. D., Yao Y., Wang C. Y.: *Microchem. J.* **1998**, *60*, 201.