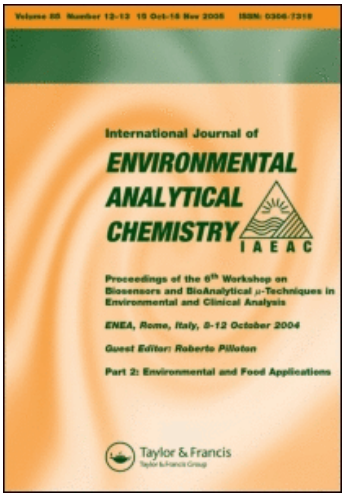

International Journal of Environmental Analytical Chemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713640455

A Bod Sensor Using *Klebsiella Oxytoca* AS1

A. Ohki[a]; K. Shinohara[a]; O. Ito[a]; K. Naka[a]; S. Maeda[a]; T. Sato[b]; H. Akano[b]; N. Kato[b]; Y. Kawamura[b]
[a] Department of Applied Chemistry and Chemical Engineering, Faculty of Engineering, Kagoshima University, Korimoto, Kagoshima, Japan [b] Nakano Central Research Institute, Nakamura-cho, Handa, Japan

To cite this Article Ohki, A. , Shinohara, K. , Ito, O. , Naka, K. , Maeda, S. , Sato, T. , Akano, H. , Kato, N. and Kawamura, Y.(1994) 'A Bod Sensor Using *Klebsiella Oxytoca* AS1', International Journal of Environmental Analytical Chemistry, 56: 4, 261 — 269
To link to this Article: DOI: 10.1080/03067319408034106
URL: http://dx.doi.org/10.1080/03067319408034106

Intern. J. Environ. Anal. Chem., Vol. 56, pp. 261-269
Reprints available directly from the publisher
Photocopying permitted by license only

A BOD SENSOR USING *KLEBSIELLA OXYTOCA* AS1

A. OHKI, K. SHINOHARA, O. ITO, K. NAKA and S. MAEDA

Department of Applied Chemistry and Chemical Engineering, Faculty of Engineering, Kagoshima University, 1–21–40, Korimoto, Kagoshima 890, Japan

T. SATO, H. AKANO, N. KATO and Y. KAWAMURA

Nakano Central Research Institute, 2–6, Nakamura-cho, Handa 475, Japan

(Received in final form, 23 August, 1993)

A BOD sensor using *Klebsiella oxytoca* AS1 was investigated with special emphasis on the effect of cell properties, such as the number, and growth phase, of immobilized cells, on the sensor response. The sensor response was almost independent of the cell number in the case of low BOD solutions, whereas the response increased with increasing cell number when high BOD solutions were used. The growth phase of the cells immobilized in the membrane affected the sensor response, especially when the membrane had been stored at 30 °C for long time. Cells immobilized at the beginning of the stationary growth phase were the most effective in terms of both the sensitivity and preservability. The response of *K. oxytoca* sensor to various substrates, including sugars and amino acids, was similar to that of a *Trichosporon cutaneum* sensor. The *K. oxytoca* sensor showed a higher resistance to some toxic substances, such as phenol, compared to *T. cutaneum* sensor.

KEYWORDS: Biochemical oxygen demand, sensor, *Klebsiella oxytoca* AS1, sensitivity, preservability.

INTRODUCTION

Biochemical oxygen demand (BOD) is a common parameter indicating organic loading in wastewater. The conventional and authorized method for the determination of BOD is the "5-day method", which needs a long time (5 days) to obtain results.[1] A microbial sensor using a yeast, *Trichosporon cutaneum*, has been developed for the rapid determination of BOD, [1–4] and is nowadays on the market. However, recently the yeast proved to be a major etiologic agent of Japanese summer-type hypersensitivity pneumonitis.[5–8] Further, the sensor has a disadvantage in that its response is occasionally affected by the coexistence of toxic substances. Therefore, it was considered to be important to search for other microorganisms for BOD sensor which are more harmless and have comparable or greater sensitivity than that of *T. cutaneum*.

In our previous paper we briefly reported a BOD sensor using *Klebsiella oxytoca* AS1 which exhibited a high performance regarding preservability as well as the correlation of results with the conventional 5 day BOD method, in practical wastewater samples.[9]

In the present paper we report a BOD sensor using *K. oxytoca* AS1 in detail, and especially the effect of cell properties, such as the number of immobilized cells and their growth phase at immobilization, on the sensor response. Little information is available about the influence of culture process of microorganism on the response of sensor. It was considered that such information was required not only for BOD sensor but also for other sensors using intact microorganisms. Further, the response spectrum of *K. oxytoca* sensor as well as the influence of toxic substances were investigated; response spectrum means the extent of the kind of substrates which are responded by the sensor.

EXPERIMENTAL

Microorganisms

The isolation of *K. oxytoca* AS1 was described elsewhere.[10] *Klebsiella terrigena* JCM 1687 and *Trichosporon cutaneum* IFO 10466 were obtained from the Japan Collection of Microorganisms (Wako, Japan) and the Institute for Fermentation (Osaka, Japan), respectively.

Culture of microorganisms

The *Klebsiella* sp. bacteria were cultured under aerobic conditions in a peptone medium (100mL) containing 1 g peptone, 0.5 g NaCl, and 0.1 g yeast extract in an Erlenmeyer flask (300 mL) with a microporous silicone plug. The flask was shaken on a reciprocal shaker (100 strokes/min) at room temperature (24–26 °C). In the case of *T. cutaneum*, a medium (100 mL) containing 1 g peptone, 1 g beef extract, and 1 g glucose was used.

Immobilization of microorganisms

The microorganisms were separated by centrifugation and washed several times with sterilized water; 1 g (wet weight) of the microorganisms was resuspended in 200 mL of sterilized water. An aliquot of the suspension (0.4–1.5mL) was filtered through a porous acetylcellulose membrane (Millipore type HA, 0.45 μm pore size, 16 mm diameter, 150 μm thickness) attached with a doughnut-type scotch tape (both sides sticky) with suction. Another acetylcellulose membrane was attached, resulting in the immobilization of the microorganisms (ca. $0.5–2.2\times10^9$ cells) between the two membranes. The microbial membrane prepared was soaked in a buffered solution (5 mmol/L KH_2PO_4–Na_2HPO_4, pH 7.0) for 24 h (for 48 h; *T. cutaneum*) under air bubbling before use.

Assembly of the sensor

The microbial membrane was placed on the tip of an oxygen electrode (Model GU-S, Iijima Denshikogyo, Gamagoori, Japan) in a biosensor system with thermostated flow cell (30°C) (NA-S011, Nakano Vinegar Co., Ltd., Handa, Japan).

Procedure

Water saturated with dissolved oxygen was transferred into the flow cell at a rate of 4 mL/min. After the current output of the electrode reached a steady state, sample solutions were injected into the flow cell for 2.5 min. The current output of the oxygen electrode decreased and reached a steady state a few minutes after injecting the sample. The difference in the current output between the initial and steady state stages was measured.

Standard solution

A solution containing 150 mg/L each of glucose and glutamic acid was used as a BOD standard solution (BOD value: 220 mg/L) according to the Japanese Industrial Standard.[1]

Activity of free cell

An aliquot of the suspension of *K. oxytoca* (300 mL) precultured (flask culture) as mentioned above was placed in a peptone medium in a jar-fermenter (5 L; Sanki Seiki MB-C) of composition identical to that of the preculture. The culture was kept at 30 °C and stirred at 400 rpm under constant aeration of 5 L-air/min. Aliquots of the culture suspension were taken periodically, and analyzed for pH and absorbance at 660 nm in order to determine the growth of bacterial cells. Further, for each aliquot the rate of oxygen consumption per g-cell at 30 °C was measured using a digital oxygen electrode system (Rank Brothers Ltd., Cambridge, UK) under a condition: 1 mL of phosphate buffer (0.05 mol/L; pH 7.0), 0.5 mL of water, and 1 mL of the bacterial suspension (OD = 0.8) were mixed in a reaction cell, and then 0.5 mL of a BOD standard solution (220 mg/L) was added to it.

RESULTS AND DISCUSSION

Effect of the number of immobilized cells

Figure 1 shows the effect of the number of immobilized cells on the sensor response (current difference). When the current differences are plotted against BOD in the data of Figure 1, straight lines (calibration curves) are obtained in all cases of cell numbers.

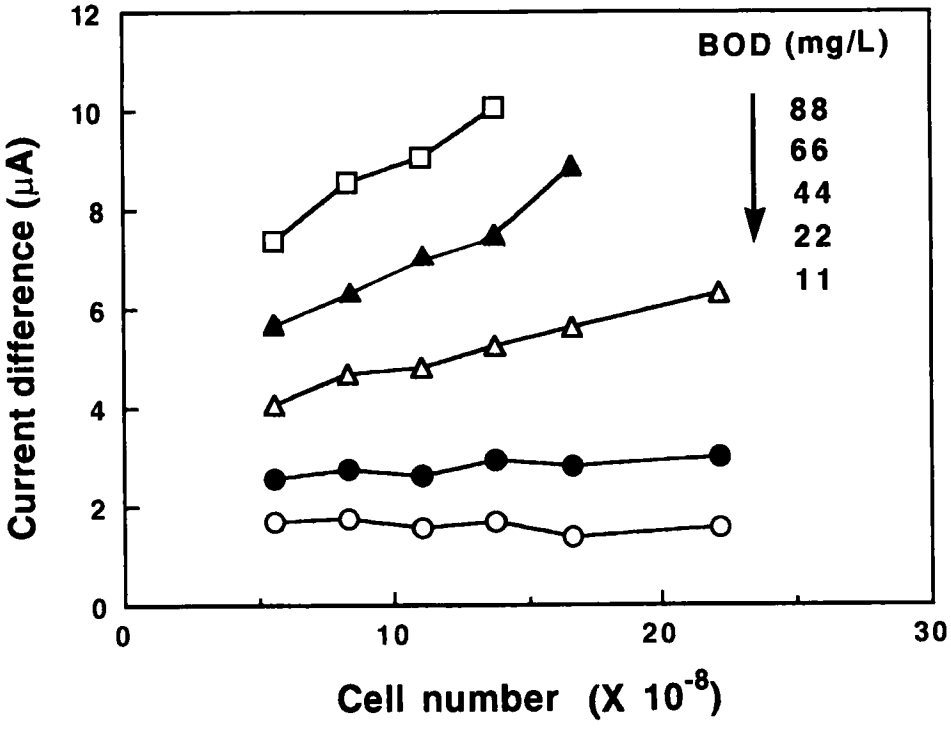

Figure 1 Effect of the number of immobilized cells. The sensor response was measured by use of the bacterial membrane of each immobilized cell number, when various concentrations of BOD solutions were injected. The culture time of cells was 20 h.

In the cases of low concentrations of BOD solutions, the sensor responses were almost independent of the cell number. The responses, however, increased with increasing cell number when high BOD solutions were used. In the former cases only a part of cells are involved in ingesting the organic compounds in BOD solutions when large numbers of cells are used; the organic compounds are considered to be entirely consumed on halfway of permeation through the membrane.

The response of the sensor was enhanced when large numbers of cells were immobilized in the membrane. However, at high concentrations of BOD, the assay was not favorably carried out because the oxygen dissolved in the solution was completely consumed. Therefore, the optimum number of immobilized cells is found to be around 1.0×10^9.

Growth curve and activity of free cell

Figure 2 shows the growth curve of *K. oxytoca* AS1 and pH change in the culture medium when the bacterium was cultured in a jar-fermenter. The growth turned into log phase after ca. 3 h lag phase, reaching stationary phase after 12 h culture. The pH in the

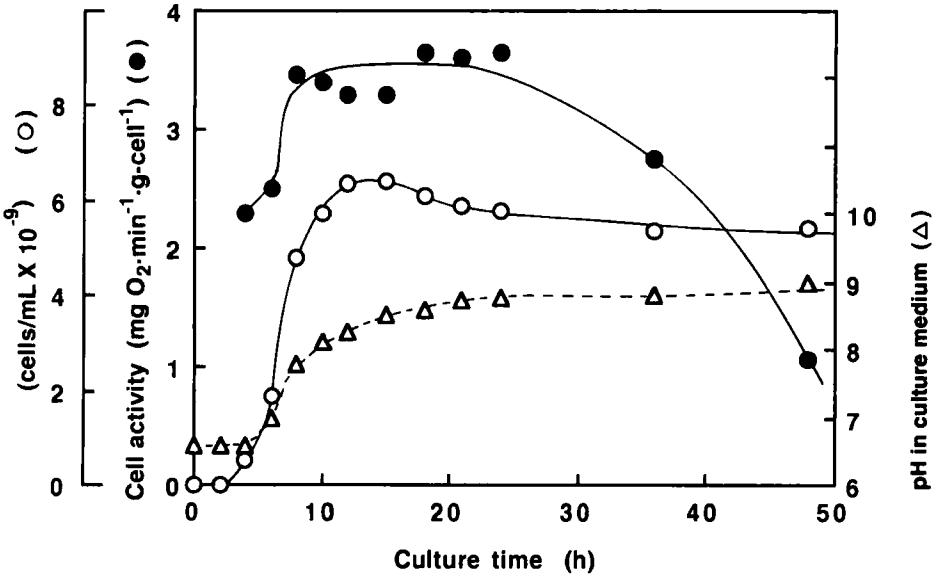

Figure 2 Growth curve, pH in culture medium, and free cell activity. Experimental conditions are described in the text.

medium gradually increased with a progress in culture, and reached ca. 9.0 after 20 h culture.

The activity of free cells at each growth phase was evaluated in terms of the rate of oxygen consumption when the bacteria ingested the organic compounds in a BOD solution in the jar-fermenter culture. The free cell activity gradually increased in course of log phase, reaching a constant value in the stationary phase. The activity, however, decreased after 20 h culture, being about one third of the maximum after 48 h culture. This is partly because of the increase in pH in the culture medium due to the production of ammonia with bacterial growth.

Effect of growth phase

When the bacterium was cultured in a flask with shaking, the growth became considerably slower compared to that in the jar-fermenter culture mentioned above (Figure 2), turning into log phase after ca. 5 h lag phase and reaching stationary phase after 18 h.

Figure 3 exhibits the effect of the growth phase (culture time) of the cells used in the membrane on the sensor response. The cells used in this experiment were prepared by the flask culture, and the number of cells immobilized in the membrane was set almost identical with each other (ca. 1.1×10^9 cells)

The response of the sensor was the largest by use of 20 h-cultured cell, which was at the beginning of stationary phase. However, the activity change observed in free cell at each phase did not so much reflect the sensor response. The assay of BOD was favorably

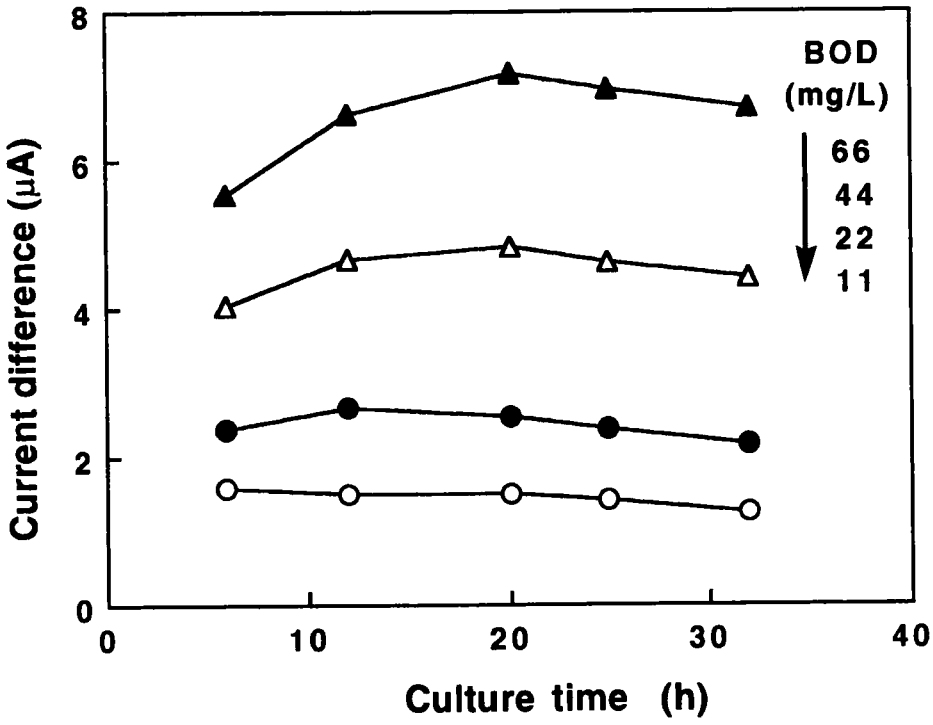

Figure 3 Effect of growth phase. The sensor response was measured by use of the membrane immobilizing the cells (ca. 1.1×10^9 cells) which had been cultured for each time noted, when various concentrations of BOD solutions containing 0.05 mol/L KH_2PO_4–Na_2HPO_4 (pH 7.0) were injected. The membrane was immediately used after the preparation.

carried out by using the cells of 12 h–32 h cultured. Even in the case of 6 h-cultured cell, the calibration curve was a straight line up to 44 mg/L of BOD, deviating a little from linearity only at 66 mg/L of BOD. This is explained by the picture that the response is for the most part determined by other factors, such as diffusion of oxygen and organic compounds.

After the bacterial membrane had been stored in an incubator at 30°C for 10 days, the assay of BOD was also carried out. As seen in Figure 4, the influence of the bacterial growth phase on the sensor output becomes remarkable compared to the case of immediate use (Figure 3). The assay of BOD was favorably performed only by use of 20 h-and 25 h-cultured cells; these cells were around at the beginning of stationary phase, showing high free cell activity as mentioned above. The use of 6 h- and 12 h-cultured cells, however, resulted in a failure of BOD assay. Consequently, it is proved that the use of the cells of high activity is important in order to keep the membrane efficiency for long time.

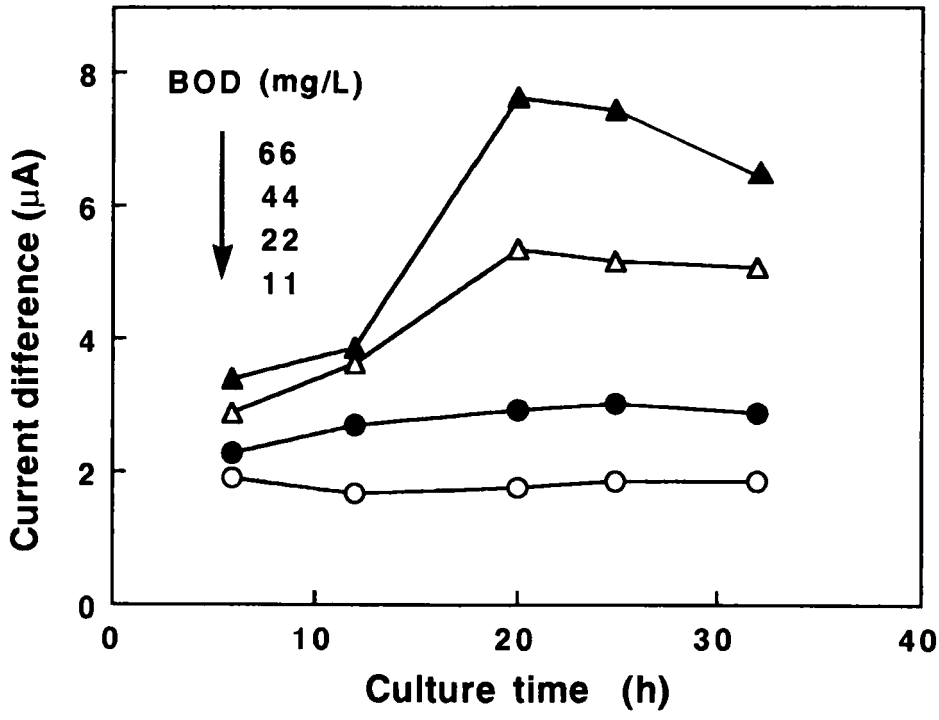

Figure 4 Effect of growth phase. Experimental procedure was the same as those in Fig. 3 except that the membrane prepared was kept at 30 °C in an incubator for 10 days and then used.

BOD of various substrates

BOD values of various substrates were determined by using *K. oxytoca* AS1, *T. cutaneum* IFO 10466, and *K. terrigena* JCM 1665 (Table 1). In API 50 CH test of *K. oxytoca* AS1, acid production was observed in all cases of the sugars indicated in Table 1. However, the *K. oxytoca* sensor scarcely responded to some of those sugars. The response behavior of the *K. oxytoca* sensor toward each substrate in Table 1 is approximately similar to that of a *T. cutaneum* sensor; the yeast (IFO 10466) now is being provided for special use of BOD assay. Regarding the response to such disaccharides as sucrose and lactose, the *K. oxytoca* sensor is superior to *T. cutaneum* sensor, while the latter surpasses the former in the response to D-(-)-arabinose. The both sensors exhibited similar responses to amino acids, such as glutamic acid, aspartic acid, and glycine.

K. terrigena has a very similar profile of acid production from various sugars to that for *K. oxytoca*. However, the sensor using the bacterium showed a different result from that of a *K. oxytoca* sensor. The *K. terrigena* sensor exhibited higher responses to such sugars as glucose, fructose, and maltose. However, regarding other sugars and amino acids, the responses were much lower than those of *K. oxytoca* and *T. cutaneum* sensors.

Table 1 BOD of various substrates.

	Sensor BOD (g/g)[a]		
	K. oxytoca AS1	*T. cutaneum IFO 10466*	*K. terrigena JCM 1665*
Sugars			
Glucose	1.06	0.85	1.53
Fructose	1.04	0.50	1.37
D-Mannitol	1.03	0.10	1.46
Maltose	0.53	0.54	0.15
Sucrose	0.45	0.04	0.18
Trehalose	0.31	0.41	0.60
Lactose	0.30	0.06	0.07
Gluconic acid	0.10	0.08	0.06
Soluble starch	0.06	0.02	0.04
D-Arabinose	0.05	0.34	b)
D-Xylose	0.06	0.11	b)
L-Arabinose	b)	b)	b)
Arabitol	b)	b)	b)
Melibiose	b)	b)	b)
Amino acids			
Glutamic acid	0.56	0.54	0.18
Aspartic acid	0.43	0.38	0.10
Glycine	0.35	0.45	0.03

a) This denotes oxygen consumption (g) of microorganisms when 1 g of substrate was ingested.
b) very small (< 0.03).

Influence of toxic substances

The influence of toxic substances on the sensor response was investigated (Table 2). Each BOD solution containing a toxic substance was injected, the concentration of the toxic substance being 5–10 times higher than that of the Japanese industrial wastewater standards. As shown in Table 2, the sensor response was not much influenced by the presence of those toxic substances (CN^-, Cr(VI), Hg(II), As(III), Cu(II), Zn(II), Pb(II), phenol) at these concentrations.

In the case of the *T. cutaneum* sensor, the presence of phenol and CN^- in sample solutions affected the sensor response; especially the presence of phenol interfered with the assay of BOD, giving an extraordinary positive error of BOD. As shown in Table 3, when phenol was added to a BOD standard solution, the response was extraordinarily increased. Accordingly, the sensor cannot be applied to the BOD assay of wastewaters which contain even a trace amount of such a toxic substance.

In conclusion, *K. oxytoca* AS1 proved to be an excellent sensing microorganism for BOD with good resistance to toxic substances and a wide response range. The optimum conditions for the sensor were as follows: i) the number of immobilized cells is ca. 1.0×10^9 and ii) the growth phase of the cell is at the beginning of stationary phase. This provides new information for the development of biosensors using intact microorganisms.

Table 2 Influence of toxic substances on sensor response[a].

Toxic substance[b]	*mg/L*	*Response (%)*
Control		100
CN^-	10	95
Cr(VI)	5	94
Hg(II)	0.1	100
As(III)	10	94
Cu(II)	30	94
Zn(II)	25	102
Pb(II)	5	98
phenol	50	102

a) Toxic substances were added to a BOD solution (88 mg/L).
b) These were added as NaCN, $K_2Cr_2O_7$, $HgCl_2$, As_2O_3, $CuCl_2$, $Zn(NO_3)_2$, and $Pb(NO_3)_2$.

Table 3 Influence of phenol.

No.	*Substrate (mg/L)*		*Response (μA)*[b]	
	BSS[a]	*Phenol*	*K. oxytoca sensor*	*T. cutaneum sensor*
1	0	5	c)	2.8
2	0	10	c)	5.2
3	0	15	c)	7.2
4	88	0	10.2 (100)	6.1 (100)
5	88	2	10.4 (102)	6.8 (112)
6	88	5	10.4 (102)	8.4 (137)

a) BOD standard solution.
b) Parentheses show the percentage of the response to control (No. 4).
c) not detected.

Acknowledgement

The authors are grateful to the Nakano Foundation for support of this study.

References

1. Standard Methods 17th Edition (1989), American Public Health Association, Washington D.C.; Testing methods for industrial wastewater, JIS K0102 (1989) p47, Japanese Industrial Standards Committee, Tokyo, Japan.
2. M. Hikuma, H. Suzuki, T. Yasuda, I. Karube and S. Suzuki, *European J. Appl. Microbiol. Biotechnol.*, **8**, 289–297 (1979).
3. K. Riedel, R. Renneberg, M. Kühn and F. Scheller, *Appl. Microbiol. Biotechnol.*, **28**, 316–318 (1988).
4. K. Riedel, K. P. Lange, H. J. Stein, M. Kühn, P. Ott and F. Scheller, *Wat. Res.*, **24**, 883–887 (1990).
5. K. Shimazu, M. Ando, T., Sakata, K. Yoshida and S. Araki, *Am. Rev. Respir. Dis.*, **130**, 407–411 (1984).
6. K. Soda, M. Ando, K. Shimazu, T. Sakata, K. Yoshida and S. Araki, *Am. Rev. Respir. Dis.*, **133**, 83–87 (1986).
7. H. Yamasaki, M. Ando, T. Sakata, S. Araki and K. Onoue, *Am. Rev. Respir. Dis.*, **141**, 734–742 (1990).
8. M. Ando, T. Sakata, K. Yoshida, H. Yamasaki, S. Araki, K. Onoue and T. Shinoda, *J. Allergy Clin. Immunol.*, **85**, 36–44 (1990).
9. A. Ohki, K. Shinohara, K. Naka and S. Maeda, *Anal. Sci.*, **7**, (Supplement), 1061–1064 (1991).
10. S. Maeda, A. Ohki, K. Miyahara, K. Naka and S. Higashi, *Appl. Organometallic Chem.*, **6**, 415–420 (1992).