Talanta 117 (2013) 366–370

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Development and characterization of microbial biosensors for evaluating low biochemical oxygen demand in rivers

Gab-Joo Chee [a,b,*]

[a] Department of Biochemical Engineering, Dongyang Mirae University, 62-160 Gocheok Guro, Seoul 152-714, Republic of Korea
[b] Institute of Biogeosciences, Japan Agency for Marine-Earth Science and Technology (JAMSTEC), 2-15 Natsushima, Yokosuka 237-0061, Japan

ARTICLE INFO

Article history:
Received 9 July 2013
Received in revised form
17 September 2013
Accepted 18 September 2013
Available online 25 September 2013

Keywords:
BOD
Biosensor
Artificial wastewater
Glucose
Glutamic acid

ABSTRACT

Five microorganisms were used to construct a biosensor for the evaluation of low biochemical oxygen demand (BOD) in rivers. Characterization and comparison of BOD biosensors were performed using two standard solutions: glucose and glutamic acid (GGA) and artificial wastewater (AWW). *Pseudomonas putida* SG10 demonstrated the best response when using AWW. *Trichosporon cutaneum* IFO10466, however, had an extremely poor response. When evaluating the biosensor response to each component of AWW, all of the microorganisms except *T. cutaneum* displayed the highest response to tannic acid. In a comparison of the two standard solutions for all the microorganisms, the biosensor responses of GGA were approximately three times higher than those of AWW were. In the BOD determination of environmental samples, the biosensor BOD values evaluated using AWW were slightly lower or equivalent to BOD_5 values, whereas the biosensor BOD values evaluated using GGA were considerably lower. These results suggest that GGA is suitable for the detection of high BOD in industrial wastewaters and factory effluents, while AWW is suitable for the detection of low BOD in rivers.

1. Introduction

Environmental pollution is inevitably increasing due to global economic expansion and industrial development. Environmental pollution is the introduction of contaminants, such as synthetic chemicals, pesticides, and heavy metals, into environmental systems. Biochemical oxygen demand (BOD) is one of the most widely used parameters for determining the presence of organic pollutants and is evaluated using aerobic microorganisms that require oxygen during the biochemical degradation of organic matter in water systems. The BOD_5 method was adopted in 1936 by the American Public Health Association Standard Methods Committee [1] and is defined as the biochemical oxygen demand of wastewaters and effluents measured over a period of five days at 20 °C. To avoid time-consuming tests, complicated procedures, and unsuitable monitoring processes, BOD biosensors have been developed. The biosensors require microorganisms with low selectivity and high biodegradation activity for a wide range of organic compounds. Therefore, it is critical to select an appropriate microorganism for the biosensor, such as *Arxula adeninivorans* LS3 [2,3], *Bacillus subtilis* [4], *B. subtilis* and *Bacillus licheniformis* [5], *Citrobacter* and *Enterobacter* sp. [6], *Enterobacter cloacae* [7], *Clostridium butyricum* [8], *Hansenula anomala* [9], *Klebsiella oxytoca* [10], *Lipomyces kononenkoae* [11], *Photobacterium phosphoreum* [12], *Pseudomonas putida* [13], *Pseudomonas fluorescens* [14], *Rhodococcus erythropolis* and *Issatchenkia orientalis* [15], *Saccharomyces cerevisiae* [16], *Serratia marcescens* LSY4 [17], *Torulopsis candida* [18], *Trichosporon cutaneum* [19–21], activated sludge [22], a mixture of microorganisms [23], multi-species culture (BOD-SEED) [24], or thermophilic bacteria [25]. The majority of microbial biosensors consist of an oxygen electrode and a biofilm of immobilized microorganisms that are isolated from wastewaters and activated sludge. BOD biosensors have been developed to detect high BOD in industrial wastewaters and factory effluents, which mainly consist of high concentrations of easily biodegradable organic compounds. In particular, *T. cutaneum* has been well studied as a bioelement of biosensors and was used in the first commercial BOD biosensor that was produced by Nisshin Electric Co. Ltd. (Japan) in 1983. BOD biosensors also have been developed for a more sensitive assay using multi-species [26], a mediator [27], and an optical fiber [28]. Estimated BOD values are variable and depend on the microorganisms and environmental samples. These biosensors, however, demonstrate poor responses for the detection of low BOD levels in rivers.

A standard solution is also extremely important in BOD biosensors. However, characterization of a standard solution for the calibration of BOD biosensors has not been extensively investigated. BOD biosensors have generally used glucose and glutamic acid (GGA) as a standard solution for calibration, which

* Correspondence address: Department of Biochemical Engineering, Dongyang Mirae University, 62-160 Gocheok Guro, Seoul 152-714, Republic of Korea.
Tel.: +82 2 2610 5193; fax: +82 2 2610 1858.
E-mail addresses: cheegj08@dongyang.ac.kr, jesuschee@gmail.com

0039-9140/$ - see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.talanta.2013.09.031

was adopted from the conventional BOD_5 test method [1] because the GGA solution is suitable for the estimation of high BOD in industrial wastewaters and factory effluents. Alternative standard solutions including the Organization for Economic Co-operation and Development (OECD) synthetic sewage and artificial wastewater (AWW) have also been used. OECD synthetic sewage has generally been adopted to evaluate high BOD in industrial wastewaters and factory effluents, similar to the GGA standard solution [26], while AWW has been used to estimate low BOD in rivers [29–33].

Many microorganisms have been used in biosensors for the BOD determination of industrial wastewaters, but not for the determination of low BOD in rivers. In this study, the following five microorganisms were characterized and evaluated for the determination of low BOD in rivers: *P. putida* SG10, *P. fluorescens* IAM12022, *P. putida* IAM1236, *B. subtilis* IAM12118, and *T. cutaneum* IFO10466. In addition, GGA and AWW were used to investigate the characterization of a standard solution for calibration.

2. Materials and methods

2.1. Bioelements, media, and cultures

The bioelements utilized in this study were as follows: *P. putida* SG10, *P. putida* IAM1236, *P. fluorescens* IAM12022, *B. subtilis* IAM12118, and *T. cutaneum* IFO10466. Media adopted were a nutrient broth and an artificial wastewater medium. The composition of 1 L of artificial wastewater medium was as follows: nutrient broth, 2000 mg; nitrohumic acid, 84.92 mg; gum arabic, 93.90 mg; sodium ligninsulfonate (NaLS), 48.54 mg; tannic acid, 83.50 mg; linear alkylbenzene sulfonate (LAS), 18.84 mg and pH 7.0 [32]. Cell cultures were carried out aerobically at 30 °C with shaking at 170 rpm, and initial incubation used a nutrient broth.

2.2. Biofilms and BOD sensors

The biofilms were prepared by immobilizing bioelements on a porous cellulose nitrate membrane (20 mm diameter, 0.45 μm pore size, Advantec, Japan) as described previously [32]. Calculated culture broths (wet cells 40 mg) were dropped on the cellulose nitrate membrane, and then adsorbed onto the membrane using the suction of an aspirator. The biofilms were washed with 10 mM phosphate buffer.

An oxygen electrode with a biofilm is schematically shown in Fig. 1. The biofilm was placed on the PTFE membrane of the oxygen electrode and fixed in place using 200-mesh nylon and an O-ring. The oxygen electrode with the biofilm was linked to both a digital multimeter and an electronic recorder.

2.3. Standard solutions of AWW and GGA

In this study, AWW and GGA were used as the two standard solutions. The BOD_5 test method [1] and most BOD biosensors have used GGA as the standard solution for calibration. GGA solution was prepared according to the Japanese Industrial Standard, in which a mixture of 150 mg/L glucose and 150 mg/L glutamic acid is equivalent to a BOD_5 value of 220 mg/L with a standard deviation of 10 mg/L [34]. AWW was employed as a standard solution for the biosensor to evaluate BOD in rivers, especially for low BOD sources such as drinking water. The constituents of AWW solution per liter are as follows: nitrohumic acid, 4.246 mg; gum arabic, 4.696 mg; NaLS, 2.427 mg; tannic acid, 4.175 mg; LAS, 0.942 mg. The AWW solution is equivalent to a BOD_5 and COD_{MN} value of 3.7 mg/L and 5.89 mg/L, respectively [32].

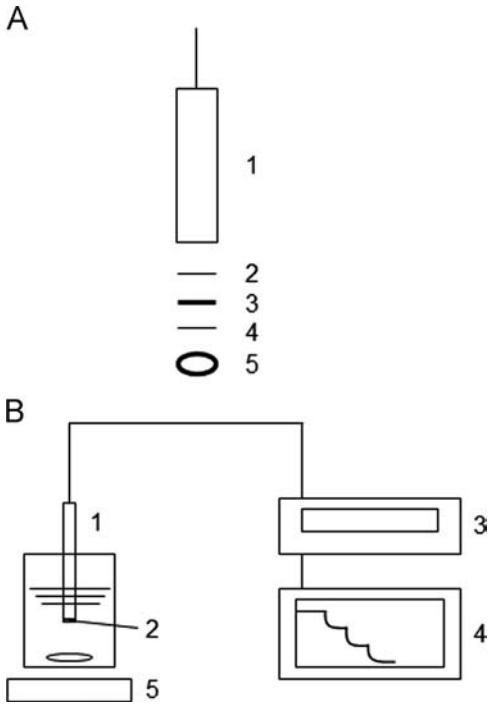

Fig. 1. Illustration of the set-up of the BOD biosensor. (A) An oxygen electrode with a biofilm-immobilized microorganism on a porous cellulose nitrate membrane. (1) oxygen electrode, (2) PTFE membrane, (3) biofilm, (4) 200-mesh nylon, (5) O-ring. (B) Biosensor system. (1) oxygen electrode, (2) biofilm, (3) multimeter, (4) recorder, (5) stirrer.

2.4. Experimental procedure

The BOD biosensor was inserted into the detection chamber containing 50 mL of 10 mM phosphate buffer (pH 7.0) solution saturated with air, while continuously stirring with a magnetic bar. The detection chamber was maintained at 30 °C using a thermostat. The response of the BOD biosensor was estimated by steady state current analysis. When the current of the oxygen electrode reached a steady state, the sample solutions were added to the 50 mL phosphate buffer solution. The current of the oxygen electrode decreased and reached a steady state in a few minutes. The response was recorded as the difference between the steady state current before and after addition of the sample.

3. Results

3.1. Biosensor responses of bioelements

The biosensor response of each bioelement was investigated using the AWW calibration solution (Fig. 2). The biosensor responses displayed a similar tendency to the growth of bioelements (data not shown). Of all the bioelements, the BOD biosensor with *P. putida* SG10 gave the best response; it exhibited a linear relationship below a BOD of 10 mg/L, and had a detection limit of 0.5 mg/L BOD. *P. fluorescens* IAM12022 had the second highest response. Although *B. subtilis* IAM12118 had a lower response compared to *P. putida* SG10, it had a much higher response compared to *T. cutaneum* IFO10466. Even though the biosensor with *B. subtilis* could obtain a response as low as 0.5 mg/L BOD, the response displayed low reproducibility. The linear range for the biosensor with *B. subtilis* was also narrower compared to the other bioelements, except for *T. cutaneum* IFO10466. As predicted by the growth rates (data not shown), *T. cutaneum* IFO10466 had a very

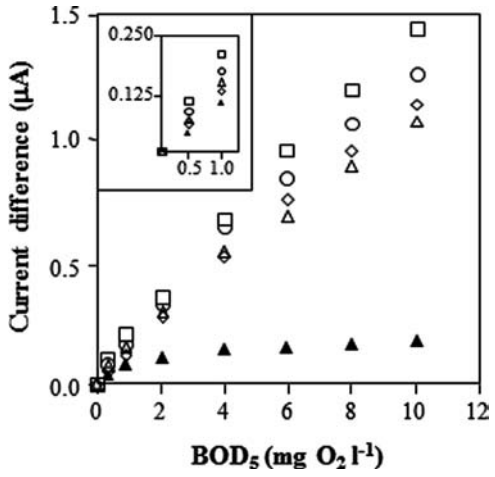

Fig. 2. Comparison of the biosensor response of each microorganism to the AWW solution. The biosensor responses below 1 mg/L BOD are described in the window. Each point is a mean of three experiments. (□) *P. putida* SG10; (○) *P. fluorescens* IAM12022; (◇) *P. putida* IAM1236; (△) *B. subtilis* IAM12118; (▲) *T. cutaneum* IFO10466.

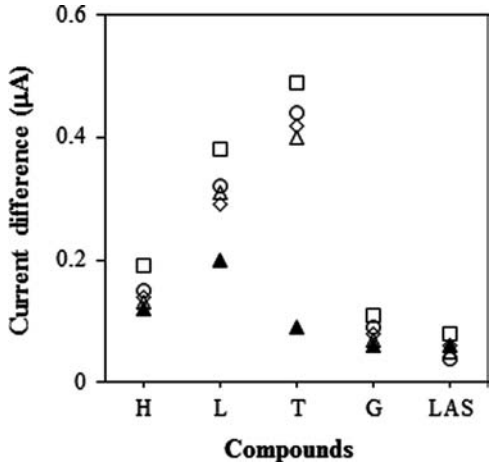

Fig. 3. Comparison of the biosensor response of each microorganism to each compound in AWW. (□) *P. putida* SG10; (○) *P. fluorescens* IAM12022; (◇) *P. putida* IAM1236; (△) *B. subtilis* IAM12118; (▲) *T. cutaneum* IFO10466. H: nitrohumic acid; L: sodium ligninsulfonate; T: tannic acid; G: gum arabic; LAS: linear alkylbenzene sulfonate.

poor response, and the difference in the current barely changed over the investigated range of BOD.

The response time of biosensors was measured using a steady-state method. The response time generally depends on the concentration and compound content of the samples to be measured. The response time of the biosensors was less than 15 min. In particular, the response time at below 2 mg/L BOD took less than 2 min. The response time of each bioelement was not significantly different in the measuring range. The stability of the biosensors was carried out at 1 mg/L BOD of AWW. *P. putida* SG10 had a constant response over a period of 10 days within ± 10% fluctuation. *P. fluorescens* IAM12022 had a similar response as *P. putida* SG10, but *T. cutaneum* IFO10466 did not.

3.2. Biosensor response of bioelements to each compound

Fig. 3 shows the comparison of the biosensor response to each compound (at 10 mg/L) contained in the AWW solution. The trend of the biosensor responses was similar to those of the calibrations, as shown in Fig. 2. *P. putida* SG10 had the highest response to all the compounds, and *T. cutaneum* IFO10466 displayed the lowest values, except for LAS. In particular, the biosensor response of *P. putida* SG10 was about two-fold for lignin and five-fold for tannin, compared to that of *T. cutaneum* IFO10466. On the other hand, only *T. cutaneum* had the highest response to lignin, not to tannic acid. Of all the compounds, *P. putida* IAM1236 had a lower response than *B. subtilis* IAM12118 to only lignin. The biosensor responses to lignin and tannic acid, which make up 40% of the contents of the AWW calibration solution, were the highest. These results suggest that BOD levels of real samples such as rivers are more accurately evaluated.

3.3. Comparison of the biosensor response with GGA and AWW

BOD biosensors need a standard solution for calibration to enable a comparison to the BOD_5 method. To compare the standard solutions, GGA and AWW solutions were selected as a reference for the BOD_5 method.

Fig. 4 shows the biosensor responses of five bioelements when using GGA and AWW solutions. GGA is the standard solution for calibration of the conventional BOD biosensor and the BOD_5 method [1].

At a BOD of 2 mg/L, the biosensor response of *P. putida* SG10 was 0.24 μA for AWW and 0.78 μA for GGA. The biosensor response in GGA was approximately three-times higher than that in AWW. The response ratio of AWW to GGA showed a similar tendency in *P. fluorescens* IAM12022, *P. putida* IAM1236, and *B. subtilis* IAM12118. Surprisingly, the biosensor response of *T. cutaneum* IFO10466 in GGA was approximately 12-times higher than that in AWW, and was similar to the current level of *P. putida* SG10. The strain IFO10466 had relatively high activity in comparison with the other elements in GGA. These results are consistent with previous findings that GGA is readily degraded by microorganisms [35,36]. As shown in Fig. 4, the responses of GGA were considerably higher than those of AWW were for all the bioelements. Therefore, AWW was considered a more suitable calibration solution than GGA when evaluating low BOD in rivers, which contains biopersistent organics.

3.4. Analysis of environmental samples

Environmental samples from rivers were evaluated by the biosensor with *P. putida* SG10 using GGA and AWW calibration

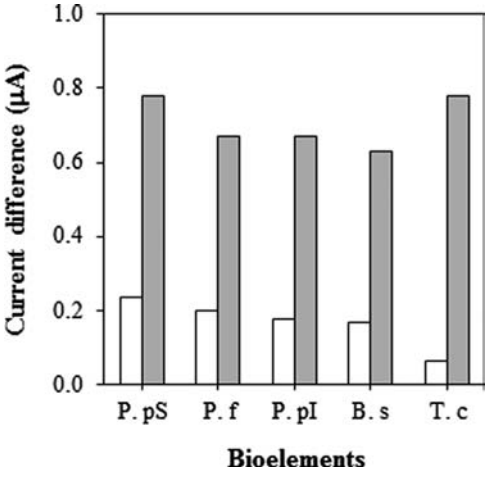

Fig. 4. Comparison of the biosensor response of each bioelement when using GGA and AWW calibration solutions. The biosensor response was determined at pH 7.0 and 30 °C. Each point is a mean of three experiments. P. pS: *P. putida* SG10; P. f: *P. fluorescens* IAM12022; P. pI: *P. putida* IAM1236; B. s: *B. subtilis* IAM12118; T. c: *T. cutaneum* IFO10466. (□) AWW; (■) GGA.

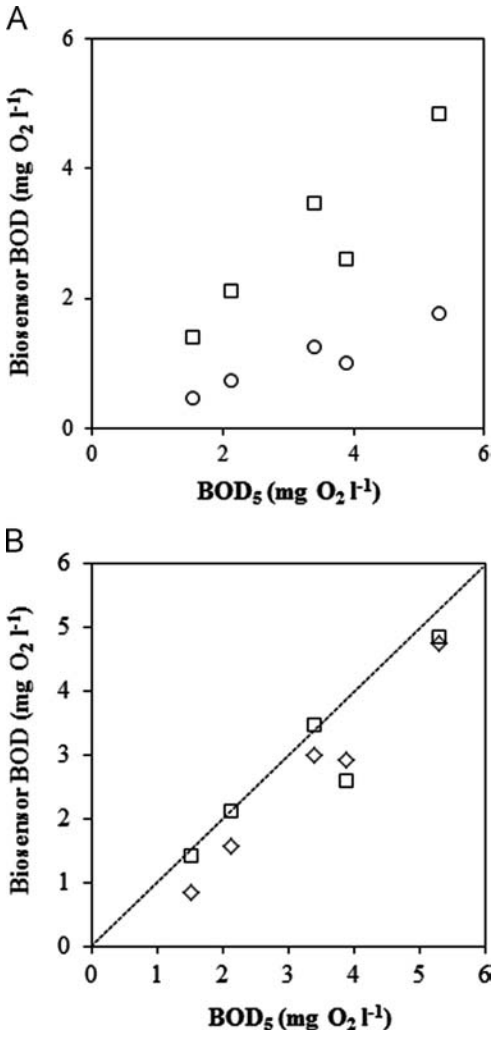

Fig. 5. Assay of environmental samples. (A) Comparison of the biosensor BODs and BOD_5 values to environmental samples evaluated using GGA (○) and AWW (□) calibration solutions. (B) Correlation between BODs and BOD_5 of *P. putida* SG10 (□) and *P. fluorescens* IAM12022 (◇). The dotted line is the calculated slope, $r=1$.

solutions (Fig. 5A). The BODs evaluated using AWW were similar to values of BOD_5, and the values were approximately two- to three-times higher than those evaluated using GGA. These results were similar to the trend observed for the biosensor response to the two standard solutions, as shown in Fig. 4. Fig. 5B shows BOD values evaluated by the biosensor with *P. putida* SG10 and with *P. fluorescens* IAM12022 for various environmental samples. BOD values of *P. putida* SG10 were very close to the calculated slope line, but were not close for *P. fluorescens* IAM12022.

4. Discussion

BOD, unlike chemical analyses such as COD and TOC, is a method evaluated by a microbial ecosystem and is used by the APHA [1]. BOD analysis directly represents the influence of substrates on natural ecosystems. Therefore, microorganisms play a key role in BOD analysis. BOD biosensors have been developed to use an infinite number of various microorganisms and to use GGA as the standard solution for calibration. BOD biosensors might be restricted by limited detection capacity for a broad spectrum of organic pollutants. The microorganisms generally display high catabolism in industrial wastewaters and factory effluents. However, rivers differ in their components compared to industrial wastewaters and factory effluents. Rivers contain biopersistent organic compounds like humic acid, lignin, and tannic acid. Five bioelements were used to evaluate the response of the BOD biosensor; *P. putida* SG10 had the highest response, whereas *T. cutaneum* IFO10466 had a very low response due to poor assimilation to organic compounds (Fig. 2).

The biosensor responses in GGA and AWW calibration solutions were evaluated by the bioelements (Fig. 4). The bacteria had more active catabolism in GGA than in AWW; therefore, the responses were higher when using the GGA solution. These results suggest that environmental samples such as rivers are evaluated as lower BOD values rather than real BOD values by biosensors when using GGA as the standard solution, which was revealed in the evaluation of the environmental samples, as shown in Fig. 5A. Therefore, GGA may be not suitable as the standard solution for BOD detection in rivers. Other solutions for calibration are the Organization for Economic Co-operation and Development (OECD) synthetic wastewater [37,38], alanine and glutamic acid [39], hydroxybutyric acid [26], and D-glucose [40]. The relationship between OECD synthetic wastewater and GGA is linear with a coefficient (R^2) of 0.9927, although the biosensor has a biofilm that contains seven kinds of microorganisms isolated from activated sludge [41]. The synthetic wastewater might be a more suitable calibration solution for food and fermentation industry because it is synthetic sewage feed.

In this study, the employed microorganisms indicated very high responses when using the GGA calibration solution. The measuring ranges of the microorganisms are as follows: 15–200 mg/L BOD for *P. fluorescens* [14], 1–40 mg/L BOD for *P. putida* [13], 2–22 mg/L BOD for *B. subtilis* [4], and 4–100 mg/L BOD for *T. cutaneum* [36]. Of these microorganisms, *T. cutaneum* has been developed as a commercial BOD biosensor because of its wide measurement range and relative suitability to industrial wastewaters [33]. As shown in Fig. 2, however, the BOD biosensor with *T. cutaneum* resulted in a nearly flat response over 4 mg/L BOD when using the AWW calibration solution. In the BOD detection of environmental samples shown in Fig. 5A, the ratio of the biosensor response to BOD_5 was 0.67–1.02 for AWW and 0.26–0.37 for GGA. The biosensor response using GGA was extremely low compared with BOD_5. These results suggest that AWW as a standard solution is more suitable to evaluate low BOD in rivers. In BOD analyses by biosensors, wastewaters containing a high concentration of easily biodegradable compounds generally provide more accurate BOD values than those containing a low concentration and/or biopersistent organics.

5. Conclusions

To evaluate low BOD in rivers, five microorganisms and two standard solutions were used in a biosensor. Of the five microorganisms, *P. putida* SG10 had the best response when using the AWW calibration solution, whereas *T. cutaneum* had a very poor response.

In the analysis of environmental samples using two standard solutions, AWW was described as a suitable calibration solution for the detection of low BOD in rivers, whereas GGA was described as a suitable calibration solution for the detection of high BOD in industrial wastewaters. BOD analyses are affected by some substrates and the microorganisms present in environmental samples.

References

[1] APHA, Standard Methods for the Examination of Waters and Wastewater, 16th ed., American Public Health Association, Washington, DC, 1986.

[2] C. Chan, M. Lehmann, K. Tag, M. Lung, G. Kunze, K. Riedel, B. Gruendig, R. Renneberg, Biosens. Bioelectron. 14 (1999) 131.
[3] M. Lehmann, C. Chan, A. Lo, M. Lung, K. Tag, G. Kunze, K. Riedel, B. Gruendig, R. Renneberg, Biosens. Bioelectron. 14 (1999) 295.
[4] K. Riedel, R. Renneberg, M. Kuhn, F. Scheller, Appl. Microbiol. Biotechnol. 28 (1988) 316.
[5] T.C. Tan, K.G. Neoh, F. Li, Sensors Actuators B 8 (1992) 167.
[6] E. Galindo, J.L. Garcia, L.G. Torres, R. Quintero, Biotechnol. Tech 6 (1992) 399.
[7] P. Villalobos, C.A. Acevedo, F. Albornoz, E. Sanchez, E. Valdes, R. Galindo, M.E. Young, Bioprocess Biosyst. Eng. 33 (2010) 961.
[8] I. Karube, T. Matsunaga, S. Suzuki, J. Solid-Phase Biochem. 2 (1977) 97.
[9] J. Kulys, K. Kadziauskiene, Biotechnol. Bioeng. 22 (1980) 221.
[10] A. Ohki, K. Shinohara, O. Ito, K. Naka, S. Maeda, J. Environ. Anal. Chem. 56 (1994) 261.
[11] M. Reiss, A. Tari, W. Hartmeier, Bioengineering 9 (1993) 87.
[12] C.K. Hyun, E. Tamiya, T. Takeuchi, I. Karube, N. Inoue, Biotechnol. Bioeng. 41 (1993) 1107.
[13] Y.-R. Li, J. Chu, Appl. Biochem. Biotechnol. 28 (1991) 855.
[14] N. Yoshida, K. Yano, T. Morita, S.J. McNiven, H. Nakamura, I. Karube, Analyst 125 (2000) 2280.
[15] K. Riedel, in: G. Ramsay (Ed.), Commercial Biosensors: Applications to Clinical, Bioprocess, and Environmental Samples, Wiley, New York, 1998, p. 267.
[16] K.S. Seo, K.H. Choo, H.N. Chang, J.K. Park, Appl. Microbiol. Biotechnol. 83 (2009) 217.
[17] M.-N. Kim, H.-S. Kwon, Biosens. Bioelectron. 14 (1999) 1.
[18] S. Sangeetha, G. Sugandhi, M. Murugesan, V.M. Madhav, S. Berchmans, R. Rajasekar, S. Rajasekar, D. Jeyakumar, G.P. Rao, Electroanalysis 8 (1996) 698.
[19] M. Hikuma, H. Suzuki, T. Yasuda, I. Karube, S. Suzuki, Appl. Microbiol. Biotechnol. 8 (1979) 289.
[20] I. Karube, S. Mitsuda, T. Matsunaga, S. Suzuki, J. Ferment. Technol. 55 (1977) 243.
[21] Z. Yang, H. Suzuki, S. Sasaki, I. Karube, Appl. Microbiol. Biotechnol. 46 (1996) 10.
[22] Y. Sakai, N. Abe, S. Takeuchi, F. Takahashi, J. Fermen. Bioeng. 80 (1995) 300.
[23] S.E. Strand, D.A. Carlson, JWPCF 56 (1984) 464.
[24] T.C. Tan, C. Wu, Sensors Actuators B 54 (1999) 252.
[25] I. Karube, K. Yokoyama, K. Sode, E. Tamiya, Anal. Lett. 22 (1989) 791.
[26] J. Jung, S. Sofer, F. Lakhwala, Biotechnol. Tech. 9 (1995) 289.
[27] N. Yoshida, J. Hoashi, T. Morita, S.J. McNiven, H. Nakamura, I. Karube, J Biotechnol. 88 (2001) 269.
[28] C. Preininger, I. Klimant, O.S. Wolfbeis, Anal. Chem. 66 (1994) 1841.
[29] G.-J. Chee, Y. Nomura, K. Ikebukuro, I. Karube, Biosens. Bioelectron. 15 (2000) 371.
[30] G.-J. Chee, Y. Nomura, K. Ikebukuro, I. Karube, Biosens. Bioelectron. 22 (2007) 3092.
[31] G.-J. Chee, Y. Nomura, K. Ikebukuro, I. Karube, Biosens. Bioelectron. 21 (2005) 67.
[32] G.-J. Chee, Y. Nomura, I. Karube, Anal. Chim. Acta 379 (1999) 185.
[33] Y. Nomura, G.-J. Chee, I. Karube, Field Anal. Chem. Technol. 2 (1998) 333.
[34] JIS, Japanese Industrial Standard Committee Testing Methods for Industrial Waste Water, JIS K0102, Japanese Standards Association, Tokyo, 1993.
[35] E. Praet, V. Reuter, T. Gaillard, J.-L. Vasel, Trends Anal. Chem. 14 (1995) 371.
[36] K. Riedel, K.P. Lange, H.J. Stein, M. Kuhn, P. OttF. Scheller, Water Res. 24 (1990) 883.
[37] J. Liu, L. Bjornsson, B. Mattiasson, Biosens. Bioelectron. 14 (2000) 883.
[38] OECD, Activated sludge, respiration inhibition test, Test Guideline no. 209, Organization for Economic Cooperation and Development Guideline for Testing of Chemicals, OECD, Paris, 1984.
[39] J.L. Marty, D. Olive, Y. Asano, Environ. Technol. 18 (1997) 333.
[40] K. Riedel, M. Lehmann, K. Tag, R. Renneberg, G. Kunze, Anal. Lett. 31 (1998) 1.
[41] C. Liu, C. Ma, D. Yu, J. Jia, L. Liu, B. Zhang, S. Dong, Biosens. Bioelectron. 26 (2011) 2074.