

Amperometric Biochemical Characterization of Isolated Fungal Strains

Sreenath Subrahmanyam,^{*,+,+++++} Narendran Kodandapani,^{++,} J. N. Ahamarshan,⁺⁺⁺ Balu Ranganathan,⁺ Kumaran Shanmugam,⁺ D. Jeyakumar,⁺⁺⁺⁺ and T. V. Subramanian⁺

⁺ Department of Chemical Engineering, A.C. College of Technology, Anna University, Madras, India

⁺⁺ Center for Ecological Science, Indian Institute of Science, Bangalore, India

⁺⁺⁺ Center for Biotechnology, A.C. College of Technology, Anna University, Madras, India

⁺⁺⁺⁺ Electrode and Electrocatalysis Division, Central Electrochemical Research Institute (CECRI), Karaikudi, India

⁺⁺⁺⁺⁺ Present address: Institute of BioScience and Technology, Cranfield University at Silsoe, Silsoe, MK 45 4DT, UK

e-mail: sri@cranfield.ac.uk; website: www.silsoe.cranfield.ac.uk/biotech

Abstract

The identification and biochemical characterization of microbial species by electrochemical methods have helped to develop microbial sensors that are rapid and effective. In this article, we present results of biochemical characterization of five fungi, the assimilation and biochemical characterization of various substrates and tolerance to selected inhibitors. The results are based on the respiratory rate and assimilatory profile of these fungi and are more rapid and reliable. The experimental results are discussed here with reference to individual fungus. Further, the advantages of the electrochemical assimilation for the biochemical characterization of the microbes have been discussed.

Keywords: Biochemical characterization, Fungi, Biosensor, Electrochemical assimilation, Amperometry

1. Introduction

A detailed investigation on the biochemical characteristics of the microorganisms under study is necessary to develop a biosensor for enhanced stability and selectivity. Microbial sensors for monitoring various pollutants have been developed [1], where the microorganisms are in direct contact with a transducer, converting the biochemical signal into an electrical response [2–6]. Microbial sensors comprising immobilized whole cells and an oxygen probe have been used for determination of assimilable sugars [7], acetic acid [8], alcohol [9], vitamins, antibiotics, peptides, enzyme activities, biochemical oxygen demand, cofactors, inhibitors for measurement of enzyme activities, and for determination of glutamic acid, based on the decarboxylation of glutamic acid by glutamate decarboxylase by measuring carbon dioxide produced, using carbon dioxide gas sensing probe [10]. Assimilation characteristics of various microorganisms such as molds, yeasts, bacteria, and actinomycetes have been tested with various substrates. Key metabolites or the rate of their overall metabolic reactions ATP, NADH, cytochrome content and DNA are suitable for describing the physiological state [11–18].

There have been reports on simultaneous measurement of two parameters, the content of proteins and nucleic acids and the determination of complex variables like biodegradable compounds and mutagenic substances in wastewater. It also has been found that a very low microbial loading of the biosensor is a prerequisite for a kinetically controlled respiration electrode and such sensors coupled with suitable immobilization of the microorganisms as well as thin membranes have to be used. The sensitivity of this type of sensor is mainly determined by the cell activity but not by diffusion limitation and the advantage is that one could attain response times of about 15 s by very low ‘microbe loading’ of the sensor [19–23]. In this article, we present the results of bioelectrochemical characterization of five fungi that have been isolated from areas where polyurethane

wastes are dumped. The technique that employs an electrochemical probe for study of biochemical characterization has many advantages to the conventional biochemical characterization that is studied based on the appearance of a ‘zone of inhibition’ on the microbial growth plates. The organisms’ potential to assimilate alcohols, carbohydrates, organic acids, amino acids, and their resistance to heavy metals and antibiotics have been discussed.

2. Materials and Methods

2.1. Materials

Potassium dihydrogen orthophosphate (AR grade) was purchased from Ranbaxy Laboratories, Bombay, India and all the other chemicals were purchased from Loba Chemie (AR grade) Bombay, India. All the analyses were done using Millipore water (deionized). Nitrocellulose membrane, (pore size 0.22 μm diameter) was purchased from Bioanalytical Systems Inc. USA.

2.2. Isolation and Identification of Fungi

The fungi were isolated from a polyurethane manufacturing industry in Vellore, India. These were identified at mycology division, Indian Agricultural Research Institute, Pusa, New Delhi, India. The names of the identified fungi are *Aspergillus niger*, *Aspergillus terreus*, *Fusarium oxysporum*, *Fusarium solani* and *Fusarium tamerii*.

2.3. Culture of Fungi

The fungi were cultured on 12 mL of the slant culture medium in the test tube of size 15 mm \times 160 mm at 27 $^{\circ}\text{C}$ for 2 days prior

to assimilation tests. The fungi were grown in Czapek-Dox media [KH_2PO_4 (1 g), NaNO_3 (2 g), MgSO_4 (0.5 g), KCl (0.5 g), FeSO_4 (0.01 g), sucrose (30 g) and agar (15 g) in 1 liter of distilled water at pH 7.2].

2.4. Fabrication of the Oxygen Electrode

A dissolved oxygen (DO) probe was fabricated using a gold cathode (area 0.03 cm^2), a platinum counter electrode and a Ag/AgCl reference electrode. The gas permeable membrane used in the probe was purchased from Century Instruments Co., Chandigarh, India. The electrochemical probe employed for the studies on assimilation and biochemical characterization, was fabricated by coupling the immobilized membrane with the DO probe with the aid of dialysis tube. A Wenking potentiostat (model POS 88, Japan) was used with a Rikadenki $X\text{-}Y\text{-}t$ recorder (Japan).

2.5. Assembly of the Fungal Electrode

The fungi sensor fabricated for assimilation tests is given in Figure 1. Nitrocellulose membrane (pore size $<0.25\text{ }\mu\text{m}$) procured from Millipore company was used as the matrix for immobilization. The fungi were immobilized by physisorption, after which the membrane was washed thoroughly with phosphate buffer (pH 7.2, conc. 100 mM at a temperature of $27\text{ }^\circ\text{C}$) to remove the loosely bound fungi on the membrane. A fungal dispersion (harvested during stationary growth phase) containing 0.2 g of wet weight of the fungus/ cm^3 was used for immobilization. The membrane retaining the fungus was placed on the Teflon membrane of the oxygen electrode so that the fungi were trapped between the two membranes. The membranes were covered with a nylon net and fastened with rubber rings. The immobilized membranes were stored at $4\text{ }^\circ\text{C}$ in phosphate buffer (pH 7.2, conc. 100 mM) when not in use.

2.6. Calculation of Oxygen Reduction Current

The oxygen reduction current (I) of the assembled sensor was obtained by applying a potential of -0.6 V to the gold cathode with reference to Ag/AgCl electrode. The solution was stirred

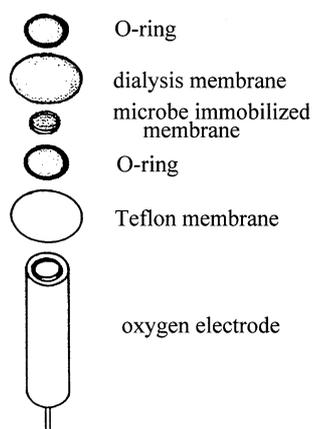


Fig. 1. Fabrication of the microbial electrode as described by Karube and Suzuki has been used [26].

and the current was recorded as steady-state current (I_s), after the addition of the analyte, a new steady state is now obtained (I_b) which is recorded. The change in the oxygen reduction current (ΔI), was calculated as ($\Delta I = I_b - I_s$).

3. Results and Discussion

3.1. Principle of Assimilation and Response of the Fungal Electrode

The initial current at zero time (i_0) was obtained with buffer solution saturated with DO, which indirectly shows the endogenous respiration of the fungi. The substrate was injected into the system and permeated through the porous nitrocellulose membrane, prior to being assimilated by the immobilized fungi. The enhanced assimilation results in increase in the respiratory activity of the fungi resulting in a decrease of DO at the fungal membrane/dissolved oxygen sensor interface. Hence, the current of the electrode decreased markedly with time until a steady state was reached since the respiration of the fungi was activated. The steady state indicated the diffusion of O_2 from a sample solution and that the O_2 consumption by fungi is in equilibrium. The steady-state current depends on numerous factors such as concentration of substrate, the assimilation activity of the immobilized fungi and the rate of metabolism of substrate.

3.2. Results on Assimilation of Substrates

The assimilation of amino acids, carbohydrates, organic acids and alcohols were studied for the fungi. Heavy metals and

Table 1. Electrochemical measurement of assimilation of amino acids by isolated fungi, concentration of amino acids, 2 mg/mL, in pH 7.2, 100 mM, temperature $27\text{ }^\circ\text{C}$. Values show the oxygen reduction current as explained in Section 2.6. Results shown are average of three measurements.

Substrates	Fungi				
	<i>Fusarium solani</i>	<i>Fusarium oxysporum</i>	<i>Fusarium tamerii</i>	<i>A. niger</i>	<i>A. terreus</i>
<i>Amino Acids</i>					
DL-Alanine	213	20	60	8	80
DL-2-Amino- <i>n</i> -butyric acid	213	5	–	–	–
L-Arginine HCl	26	8	40	2	100
DL-Aspartic acid	186	10	53	4	66
L-Cysteine	–	5	26	–	33
L-Glutamic acid	79	5	32	4	60
Glycine	106	10	24	–	60
Histidine HCl	53	5	16	4	60
DL-Isoleucine	106	5	16	4	100
L-Leucine	106	7	40	4	60
L-Lysine HCl	26	2	12	4	40
DL-Methionine	79	5	10	2	100
DL- β Phenylalanine	373	6	30	–	360
L-Proline	186	4	60	–	200
DL-Serine	79	7	64	2	150
DL-Threonine	106	6	24	6	150
DL-Tryptophan	26	–	30	4	50
L-Tyrosine	106	7	50	2	300
DL-Valine	79	7	20	6	350

antibiotics were studied for their inhibitory concentrations. Results of electrochemical measurement of assimilation of amino acids are presented in Table 1. Though sucrose was used in the media, glucose was used as the standard substrate for all the organisms, because assimilation studies for different microbes by various groups have been performed with glucose as a standard substrate and it is hence easy to compare the results. *Fusarium solani* showed the highest response among the fungi studied. The assimilation of glucose was found to be lower, as seen from the ΔI values recorded for *Aspergillus niger*. The other fungi recorded good responses for glucose. These results showed that the oxygen consumption varied among the fungi. *Fusarium solani* had excellent responses for all the amino acids, however, the fungus could not assimilate ornithine HCl, even at concentrations of 5 ppm. *Aspergillus niger* was not able to assimilate DL- β -phenyl alanine and L-proline at concentrations of 2 ppm and 1 ppm, respectively. *Aspergillus terreus* showed good signals for all the amino acids except for DL-2-amino-*n*-butyric acid. In general none of the fungus studied had an unusually high assimilation for any of the amino acids studied.

Table 2. Electrochemical measurement of assimilation of alcohols, organic acids and carbohydrates by isolated fungi. Substrate concentration at the time of injection onto the fungal electrode was 2 mg/mL, in pH 7.2, 100 mM, temperature 27 °C (Section 2.6). Results shown are average of three measurements.

Substrates	Fungi				
	<i>Fusarium solani</i>	<i>Fusarium oxysporum</i>	<i>Fusarium tamerii</i>	<i>A. niger</i>	<i>A. terreus</i>
<i>Organic acids</i>					
Fumaric acid	133	8	24	4	–
Citric acid	33	6	16	–	–
Acetic acid	333	20	120	25	260
Lactic acid	233	20	70	25	140
Maleic acid	–	5	–	5	–
Tartaric acid	26	20	–	6	–
Ascorbic acid	33	13	10	4	60
Formic acid	39	10	30	10	325
<i>Alcohols</i>					
Methyl alcohol	166	25	20	15	10
Ethyl alcohol	233	40	60	480	60
Propyl alcohol	99	20	60	5	10
Isobutyl alcohol	–	50	80	50	70
Isoamyl alcohol	–	50	40	35	130
Sorbitol	–	–	140	–	–
Mannitol	133	10	40	25	30
Glycerol	–	30	40	10	–
<i>Carbohydrates</i>					
Glucose	46	214	360	120	300
D-Mannose	79	100	60	–	50
D-Maltose	6	20	30	40	50
Sucrose	126	150	40	25	100
Lactose	–	–	–	–	–
D-Arabinose	53	50	4	20	25
Raffinose	39	–	4	25	40
D-Fructose	26	50	15	40	60
L-Arabinose	33	50	10	60	–
Trehalose	53	10	5	25	20
Cellobiose	26	15	25	25	20
D-Galactose	33	50	25	25	25
D-Melibiose	39	–	40	50	50
Dextrose	159	20	20	75	75

Table 2 presents electrochemical a measurement of assimilation of alcohols, organic acids and carbohydrates. As expected none of the fungi was able to assimilate lactose, however, surprisingly, a very weak signal was obtained for *Fusarium tamerii* confirming the assimilation of lactose at a concentration of 20 ppm. Even a lactose concentration as low as 4 ppm was high for assimilation by *Aspergillus niger*. It is worth mentioning that lactose as a substrate is very difficult to be assimilated by organisms. *Fusarium solani* showed best responses to carbohydrates. *Aspergillus niger* was not able to assimilate D-melibiose at low concentrations and weak responses were obtained for other fungi. *Aspergillus terreus* could not assimilate L-arabinose. *Fusarium oxysporum* and *Aspergillus niger* were able to assimilate malic acid, but only weak signals were obtained by these two fungi confirming low levels of assimilation. *Fusarium solani*, and *Aspergillus niger* showed good responses to acetic acid and alcohol, respectively, and hence they were successfully exploited for the development of microbial sensors [24, 25].

3.3. Results on Response on Inhibitory Concentrations of Selected Heavy Metals and Antibiotics

Table 3 presents the response for inhibitory concentrations of selected heavy metals and antibiotics. *Aspergillus niger* and *Aspergillus tamerii* showed better resistance to selected heavy metals and antibiotics in relation to the other fungi studied. *Fusarium solani*, *Fusarium tamerii* and *Aspergillus terreus*, had high resistance of 30 ppm for cadmium. *Aspergillus terreus* had a very high resistance of 80 ppm for cobalt. All the fungi studied, showed low resistance to mercury, with maximum resistance shown by *Fusarium oxysporum*. *Fusarium tamerii* had a resistance as high as 90 ppm for lead. *Fusarium solani* had high resistance to mercury with 50 ppm as concentration.

Table 3. Electrochemical measurement of resistance of fungi to heavy metals and antibiotics. Stock solution of substrate concentration was 200 ppm, in pH 7.2, 100 mM, temperature 27 °C (Section 2.6). Results shown are average of three measurements.

	Fungi				
	<i>Fusarium solani</i>	<i>Fusarium oxysporum</i>	<i>Fusarium tamerii</i>	<i>A. niger</i>	<i>A. terreus</i>
<i>Heavy metals</i>					
Cadmium	30	–	30	11	30
Cobalt	10	40	60	20	80
Manganese	20	40	40	30	60
Strontium	220	40	40	30	13
Nickel	50	20	40	30	80
Zinc	–	–	–	–	30
Copper	30	50	50	50	40
Chromium	–	20	40	60	90
Iron	40	20	60	40	20
Mercury	20	30	20	10	10
Lead	–	–	90	40	20
Magnesium	50	60	–	–	–
<i>Antibiotics</i>					
Gryseofulvin	15	3	14	3	21
Mycoderm	12	8	19	6	16
Clotrimoxazole	3	4	15	9	12
Ketagonazole	3	3	5	3	8

Table 4. C represents the results obtained by conventional technique, S represents the results obtained by electrochemical assimilation technique. Comparison between the data by conventional technique obtained by studying the 'zone of inhibition in plates' and the data obtained by electrochemical assimilation technique. Concentration of substrates used for study was 2 mg/mL, media used for fungal growth during this experiment was Czapek-Dox, the composition of which is mentioned in Section 2.3. ++: very positive, +: positive, -: negative, D+: delayed positive response, W: weak positive response, D-: delayed negative response, +-: variable response, X: result not available.

Substrate	Fungi									
	<i>Fusarium solani</i>		<i>Fusarium oxysporum</i>		<i>Fusarium tamerii</i>		<i>A. niger</i>		<i>A. terreus</i>	
	C	S	C	S	C	S	C	S	C	S
DL-Aspartic acid	+	++	+	+	+	+	D+	+-	+	-
Glycine	++	++	+	-	+	+	-	-	-	+
DL-Isoleucine	+	++	-	+-	-	+-	D+	+-	+	+
Glucose	++	++	+	+	+	+	+	+	+	+
Sucrose	-	+	-	+-	D+	+-	D+	+-	+	+
Formic acid	+	+	-	+-	+	+	-	+-	+	+
Ascorbic acid	-	+	-	+-	-	-	W	+-	-	+
Ethyl alcohol	++	++	-	+-	+	+	+	+	+	+
Methyl alcohol	+	+	-	+-	-	+-	+	+	+	+
TDA	D+	+-	-	X	D+	+-	-	+-	W	+-
Cadmium	D-	+-	D-	X	W	+-	+	+	+	++
Copper	D+	+-	W	+-	-	+-	++	++	+	++
Mercury	D+	+-	D+	+-	D-	+-	++	++	+	++
Gryseofulvin	-	+-	-	+-	+	+	+	++	+	++
Mycoderm	-	+-	-	+-	-	+-	+	++	-	+-

Aspergillus terreus showed good resistance of 21 ppm for gryseofulvin and 16 ppm for mycoderm. A high resistance of 19 ppm of mycoderm and 15 ppm of clotrimoxazole was recorded for *Fusarium tamerii*. *Aspergillus terreus* showed 8 ppm as maximum resistance limit for ketagonazole. In summary, none of the fungi showed remarkable resistance to inhibitory concentrations of either heavy metals or antibiotics.

3.4. Comparison Between Results of Substrate Assimilation by Electrochemical Method and Conventional Method

Results of the substrate assimilation by 'electrochemical assimilation technique' were compared with conventional result obtained by studying the 'zone of inhibition on plates' (Table 4). The results obtained by the 'electrochemical assimilation technique', were similar to that obtained using the conventional method (zone of inhibition in plates), However, small differences in the results could be attributed to the fact that conventional technique gives only slow rates of assimilation by the fungi, and the 'amperometric technique' is fast and registers changes in the oxygen consumption during a very short time. *Fusarium solani* showed good response for alcohols, and ΔI value for ethanol was found to be very high among the alcohols in particular. *Aspergillus terreus* was not able to assimilate mannitol and others showed only a feeble response. All the interpretation of the values for resistance and inhibitory concentrations are based on the definition mentioned here. Resistance is the ability of the fungi to withstand the concentration of the antibiotic or heavy metal under study and thrive on substrate. This differs from one fungal strain to the other. Inhibitory concentration is the minimal concentration of the antibiotic or heavy metal that would inhibit the growth of the fungi. Readers are reminded that there are a number of definitions for these two terms and these cannot be used interchangeably.

3.5. Optimization of pH and Temperature for Fungal Growth

pH values and temperature for the growth of fungi were optimized using the same technique. The optimal temperature for *Fusarium oxysporum* and *Aspergillus niger* was found to be between 33 and 35 °C. *Fusarium terreus* had an optimum temperature of 27 °C for growth. *Aspergillus terreus* and *Fusarium solani* show maximum assimilation of glucose between 28 and 32 °C giving us the optimal temperature for growth of these fungi (Fig. 2). The optimal pH values for the growth of these fungi vary from pH 6 to pH 8, as can be seen in Figure 3.

4. Conclusions

Biochemical characterization is mandatory for new microbial strains that are isolated. The conventional methods adopted are both, time-consuming and imprecise. The advantage of this method is that we can quantify the substrate assimilation to very

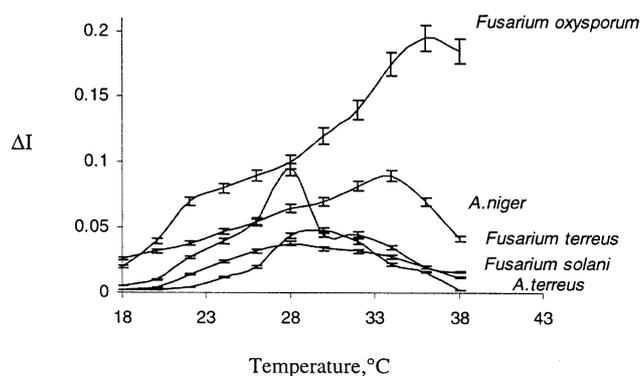


Fig. 2. Optimal temperature for the growth of the fungi, phosphate buffer pH 7.2 was used, conc. 100 mM.

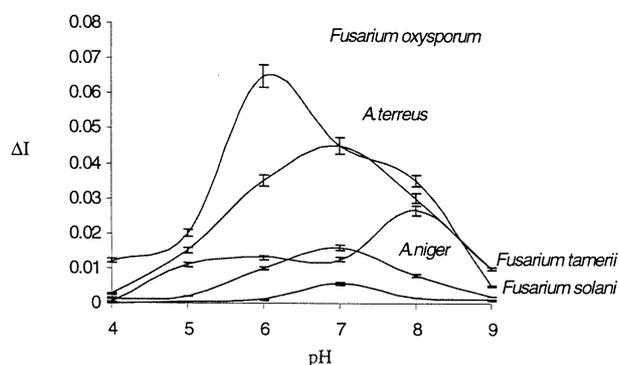


Fig. 3. Optimal pH for the growth of the fungi, phosphate buffer used, conc. 100 mM.

low levels that is either very difficult to achieve or in most cases impossible. That is one of the reasons why we believe that this method is more precise in comparison to conventional methods of biochemical characterization. We have endorsed the fact that biochemical characterization of microbes through rapid electrochemical methods using the amperometric principles is both sensitive and fast with an added advantage of its ability of quantitative registration at very low signals. Based on selective assimilation of the organisms, a potential organism for development of specific sensor could also be screened for further study, for example, *Fusarium solani*, and *Aspergillus niger* showed good responses to acetic acid and alcohol, respectively, and hence they were successfully exploited for the development of microbial sensors [24, 25]. These results could be used to compare different strains, mutant transformants of one and the same species.

5. Acknowledgement

SS thanks the Council of Scientific and Industrial Research, CSIR, Government of India, for a Senior Research Fellowship.

6. References

- [1] K. Riedel, R. Renneberg, U. Wollenberger, G. Kaiser, F. Scheller, *J. Chem. Technol. Biotechnol.* **1989**, *44*, 85.
- [2] K. Riedel, M. Kuhn, F. Scheller, *Biophysics* **1985**, *107*, 189.
- [3] C.A. Corcoran, G.A. Rechnitz, *Trends Biotechnol.* **1985**, *3*, 92.
- [4] U. Wollenberger, F. Scheller, P. Att, *Anal. Lett.* **1980**, *13*, 1201.
- [5] R. Renneberg, K. Riedel, P. Liebs, F. Scheller, *Anal. Lett.* **1984**, *17*, 349.
- [6] R. Renneberg, K. Riedel, F. Scheller, *Appl. Microbiol. Biotechnol.* **1985**, *21*, 180.
- [7] M. Hikuma, H. Obana, T. Yasuda, *Enzyme Microbiol. Technol.* **1980**, *2*, 234.
- [8] M. Hikuma, H. Suzuki, T. Yasuda, I. Karube, S. Suzuki, *Eur. J. Appl. Microbiol. Biotechnol.* **1979**, *8*, 289.
- [9] M. Hikuma, T. Kubo, T.Y. Yasuda, I. Karube, S. Suzuki, *Biotech. Bioeng.* **1979**, *21*, 1845.
- [10] S. Suzuki, I. Karube, *Appl. Biochem. Bioeng.* **1981**, *3*, 145.
- [11] A. Heller, *Current Opinion in Biotechnology* **1996**, *7*, 50.
- [12] J. Kathryn, S. Asala, M. Swartz, J.W. Anderegg, D.C. Johnson, *Electroanalysis* **2000**, *12*, 731.
- [13] I.A. Borisov, A.V. Lobanov, A.N. Reshetilov, B.I. Kurganov, *Prikl. Biokhim. Mikrobiol.* **2000**, *36*, 254.
- [14] A. Romani, M. Minunni, N. Mulinacci, P. Pinelli, F.F. Vincieri, M. Del Carlo, M. Mascini, *J. Agric. Food. Chem.* **2000**, *48*, 1197.
- [15] A.P.F. Turner, *Current Opinion in Biotechnology* **1994**, *5*, 49.
- [16] R.P. Baldwin, *J. Pharm. Biomed. Anal.* **1999**, *19*, 69.
- [17] Yun-Ying Liao, Tse-Chuan Chou, *Electroanalysis* **2000**, *12*, 89.
- [18] K. Stuijk, *Electroanalysis* **2000**, *12*, 1, 55.
- [19] I. Karube, Y. Nomura, Y. Arikawa, *Trends Anal. Chem.* **1995**, *14*, 295.
- [20] H. Suzuki, E. Tamiya, I. Karube, *Electroanalysis* **1995**, *6*, 299.
- [21] I. Karube, M. E. Sang Mok Chang, *Biosensor Principles and Applications*, Marcel Dekker Inc., New York **1994**, p. 267.
- [22] I. Karube, K. Yokoyama, *Proc. NATO Advanced Research Workshop*, Brixen, Italy **1993**, 281.
- [23] I. Karube, K. Yokoyama, *Biotechnology Current Progress*, **1992**, 1.
- [24] S. Subrahmanyam, K. Shanmugam, V.M. Madhav, M. Murugesan, T.V. Subramanian, D. Jeyakumar, *Electroanalysis* **2000**, *12*, 1051.
- [25] S. Subrahmanyam, K. Shanmugam, M. Karunakaran, V.M. Madhav, T.V. Subramanian, D. Jeyakumar, *Electroanalysis*, in press.
- [26] I. Karube, M. Suzuki, in *Biosensors: A Practical Approach* (Ed: A.E.G. Cass), **1990**, 155.