MICROBIAL BIOSENSOR FOR ETHANOL DETERMINATION IN ALCOHOLIC BEVERAGES

L. Rotariu *, Camelia Bala and V. Magearu

abstract: A new microbial biosensor based on an immobilized yeast cells (*Saccharomyces ellipsoideus*) and Clark type oxygen electrode is described. Analytical determination is based on the respiratory activity of the microorganism in presence of the analyte. Response time of approximately 2 min. for steady-state method and 30 s. for initial slope method was registered. The calibration curve for ethanol was linear in the 3 to 50 mM range. This biosensor was used for selective determination of ethanol in the presence of glucose using a second Teflon membrane. The biosensor is practically specific to ethanol while the interference of glucose in determination of ethanol for a biosensor with dialysis membrane is about 30%. Selective biosensor was used to determine ethanol concentration in alcoholic beverages. A good correlation of the results between biosensor and spectrometric method with alcohol dehygrogenase was observed.

Introduction

Ethanol is very often used in the human nourishment although ethanol is not considered an aliment. Inside the human body ethanol is completely oxidised or can be partially eliminated trough skin, respiration or urine. Ethanol ingestion could affect nervous system, circulatory system or digestive system. Exceeding consumption could cause coma or even death. Alcoholmetrical determinations are from this point of view very important in clinical analysis [1].

In industry, determination of ethanol presents interest in preparing alcoholic beverages, in some biotechnological processes and in cosmetic industry. Ethanol could also represent a quality indicative for food when ethanol is produced in a process of food degradation.

Enzymatic methods for ethanol determination use only two enzymes alcohol oxidase (AOD) and alcohol dehydrogenase (ADH). Spectrometrical [2,3], chemiluminescence [4,5] or electrometrical methods were developed for AOD. Due to the lower selectivity of AOD this enzyme was used in realisation of HPLC detectors for alcohols [6,7].

ADH presents a better selectivity for ethanol and was used for enzymatic biosensor development. Spectrometric methods were also described based on colorimetric [3], fluorimetric [2] or chemiluminescence [4] NADH measurements.

---

* Department of Analytical Chemistry, Faculty of Chemistry, University of Bucharest, Sos. Panduri, No. 90, 762351-Bucharest - 5, Romania, Tel./Fax: ++ 40 21 410 48 88, E-mail: lrotariu@fx.ro

Analele Universităţii din Bucureşti – Chimie, Anul XII (serie nouă), vol. I-II, pag. 69–76

The main problems for ADH based biosensor is the high amount of coenzyme required, low stability of enzyme and the relatively high applied potential for amperometric determination [8÷11].

Mediators [12÷15] were also used for ethanol biosensors design with promising results.

Bacteria and yeasts are recognised as organisms, which metabolise very well all kind of substrates. Amperometric and potentiometric microbial and hybrid biosensor for sugars were previously reported [16,17] based on different types of oxygen electrode [18]. Based on microorganisms like *T. brassicae, S. cerevisiae, Acetobacter aceti or Acetobacter xylinnium*, different types of microbial biosensor for ethanol were developed using amperometric [19,20], potentiometric [21] or conductometric [22] detectors.

This work presents an amperometric microbial biosensor for determination of ethanol. Incubation with ethanol was used as a good method to improve the selectivity of a microbial biosensor for ethanol. A high selectivity was achieved by using a second Teflon membrane to cover the biocatalytical yeast layer.

Experimental

Materials

A yeast strain of *Saccharomyces ellipsoideus*, purchased from University of Galati (Romania), was used as a biocatalyst of the microbial sensor; all other reagents were of analytical grade. Standard solutions of ethanol were prepared in disodium phosphate - citric acid buffer 0.5 M.

Preparation of microbial electrode and assay procedure

Yeast cells were previously incubated in a medium containing 1.0 g/l KH_2PO_4, 3.5 g/l $(NH_4)_2SO_4$, 0.3 g/l $MgSO_4$, 0.1 g/l $CaCl_2$ and 1% ethanol, as unique carbon source. Incubation of the cells on a medium based on the substrate, that the biosensor will be realised for, is a well-known method to improve the selectivity of the selected cells. The *S. ellipsoideus* cells were maintained for 12 hours at 30 ^oC, under continuing oxygenation of the cells suspension. After centrifugation, solid deposit were suspended in distilled water and centrifuged again. The cell mass obtained was suspended in 0.9% NaCl solution and successive dilutions were realised (absorbance between 0.04 and 0.4 at 660 nm). 0.5 ml of each suspension was filtered through a dialysis membrane. After drying, each membrane was kept at 4^oC before utilisation.

The oxygen sensor MF-2100 consists in a platinum electrode, and an Ag/AgCl electrode, as reference electrode, covered with an oxygen permeable membrane. Internal electrolyte is based on a 0.1 M KCl.

Amperometric measurements were realised using an electrochemical workstation BAS 100B/W (Bioanalytical System, USA West Lafayette), at a applied potential of -650 mV, data display and recording were supported by BAS electrochemical software version 3.2. Spectrometric measurements were performed with JASCO UV-VIS 540.

The immobilised microorganisms were placed on the oxygen membrane and covered with a dialysis membrane and fixed with a rubber ring.

All the determinations were realised in a 10 ml measuring cell and all the solutions were previously saturated with oxygen. Before every determination, the biosensor was kept in oxygen saturated phosphate buffer solution. After the output signal of the microbial sensor became stable, the sensor was removed in a buffered standard solution of ethanol (saturated with oxygen).

The current decrease indicates that ethanol passes through the membrane and it is assimilated by the immobilized yeast cells. Oxygen consumption due to respiratory activity of the microorganism caused a decrease in dissolved oxygen concentration around the membrane and consequently brought about the decrease in output signal. The decrease of oxygen concentration was taken as the measure of ethanol concentration.

Decrease of the oxygen concentration around the oxygen electrode is measured and correlated with ethanol concentration from the sample.

In principle, there are two possibilities of measuring biosensor response: a) endpoint determination (steady-state method) and b) kinetic measurements (initial slope method).

For selective determination of ethanol in the presence of glucose was added a second Teflon membrane, which is permeable only for ethanol.

Results and Discussion

Optimisation of the microorganisms concentration in the biocatalytical layer

The membranes with immobilised yeast were tested on a 10 mM ethanol solution. Response curves were registred as a difference between steady state signal and base signal in oxygen-saturated buffer is represented in Fig. 1.

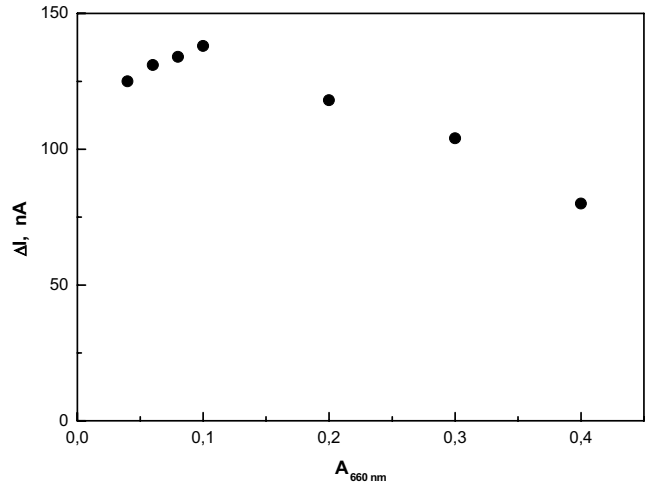

Fig. 1: *Optimisation of yeast cell concentration (temperature* 25°C*, p*H=7.00*, ethanol* 10 mM*).*
Each point represents the average of 3 determinations.

Low immobilized cell concentrations do not modify the base line of the biosensor in the presence of ethanol solution. Increasing the cell concentration on the surface of membrane leads to a very low base signal for microbial biosensor and signal decrease rapidly to zero in the presence of ethanol in the sample solution. In extremis, for very high concentration of yeast cells, oxygen electrode could not detect dissolved oxygen around the biocatalytical membrane. Membranes realised by deposing 0.5 ml of a cell suspension with absorbance of 0.1 showed the maximum response. These membranes have been used for further experiments.

Response curve of microbial biosensor

When the microbial sensor was immersed in the oxygen saturated buffer solution the output current of the sensor became stable within 5 min. After the steady current was obtained, the sensor was transferred to solutions containing different concentrations of ethanol in the range of 5÷20 mM. The output current began to decrease and the minimum current was observed within 2 min. Kinetic measurements could be done in the first 30 s. when the response curve is linear.

Effect of temperature

The respiration activity of the yeast cells depends on the presence of a carbon source and on the temperature. Fig. 2 shows the effect of temperature upon the response of microbial sensor. Temperature of 25°C was chosen as the working temperature. A lower base line for oxygen electrode, worst reproducibility and limited response range for sucrose was observed for higher temperatures.

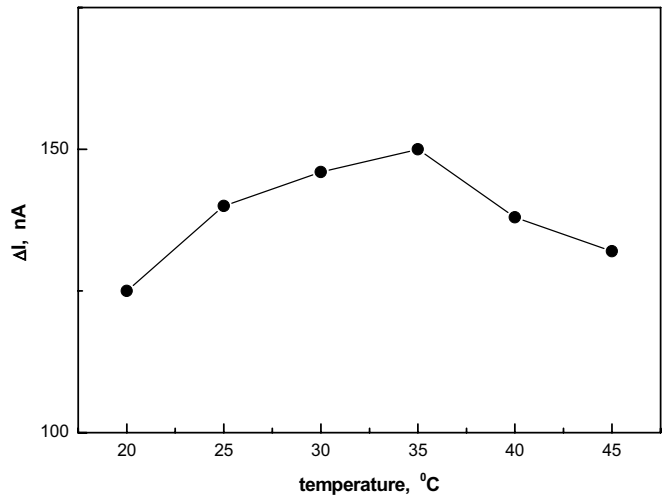

Fig. 2. *Effect of temperature on biosensor response (pH=7.00, ethanol 1 mM).*
Each point represents the average of 3 determinations.

Effect of *p*H

The effect of *p*H on the response of microbial biosensor was investigated in the *p*H range of 4÷8. The response to standard solution of 10 mM ethanol was recorded and no significant

differences were registered in this range. All further measurements were realised at the *p*H=7.00.

Calibration curve

Fig. 3 shows the calibration curves for ethanol using steady-state method under conditions of pH=7 and 25°C. Each point represents the average for 5 determinations. A linear relationship between the oxygen concentration decrease and concentration of ethanol was observed, up to 50 mM.

Parameters for linear regression (y=ax+b) are:

$$a = 43.6 \pm 4.6$$

$$b = 9.00 \pm 0.17$$

with the correlation coefficient of 0.9988 and standard deviation SD=8,22. The detection limit was 1.5 mM.

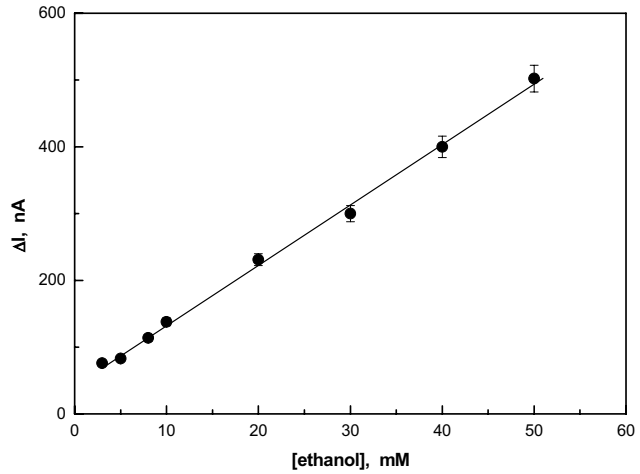

Fig. 3. *Biosensor calibration curve (steady state, temperature 25 °C, pH=7.00).*

An extension of the calibration range can be obtained using kinetic measurements. In this way a linear response was observed up to 80 mM ethanol.

Interferences

Selectivity of the microbial biosensor was study on some common organic compounds present in alcoholic beverages: methanol, propanol, iso-propanol, acetic acid and glucose. All measurements were performed with yeast cell immobilised on dialysis membrane (DM) at 25°C, *p*H=7.00 for concentrations of 10 mM for each compound.

Results presented in Fig. 4 show that only glucose interferes in the determination of ethanol. All the other organic volatile compounds do not interfere. Usually, interference of glucose is eliminated for almost all kind of biosensors by adding a second anti-interference enzymatic layer with GOD. In this case the substrate is a volatile compound and a gas permeable membrane could be used as anti-interference layer.

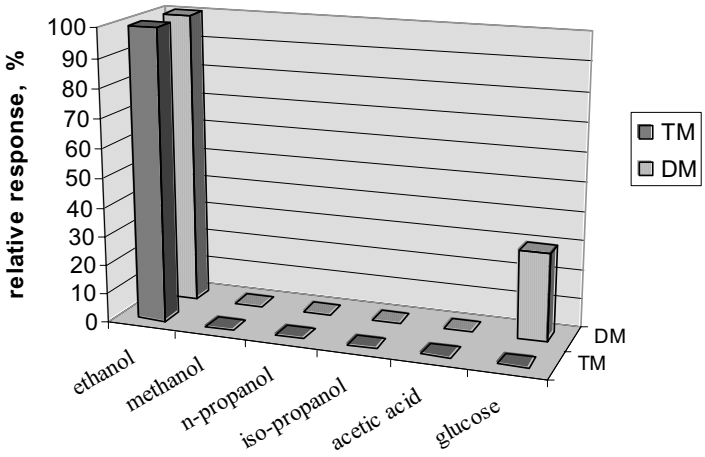

Fig. 4. *Selectivity of the microbial biosensor for ethanol.* (10 mM, 25^{0}C, *p*H=7).

Using a Teflon membrane (TM) the microbial biosensor responds selectively to ethanol in the presence of all the other compounds tested (Fig. 4.). Use of this membrane affect the response time of the biosensor, which increases to approximately 5÷6 min. Independency from the external factors as *p*H, contamination, represent positive effects of separation of the biocatalytical layer from the sample solution.

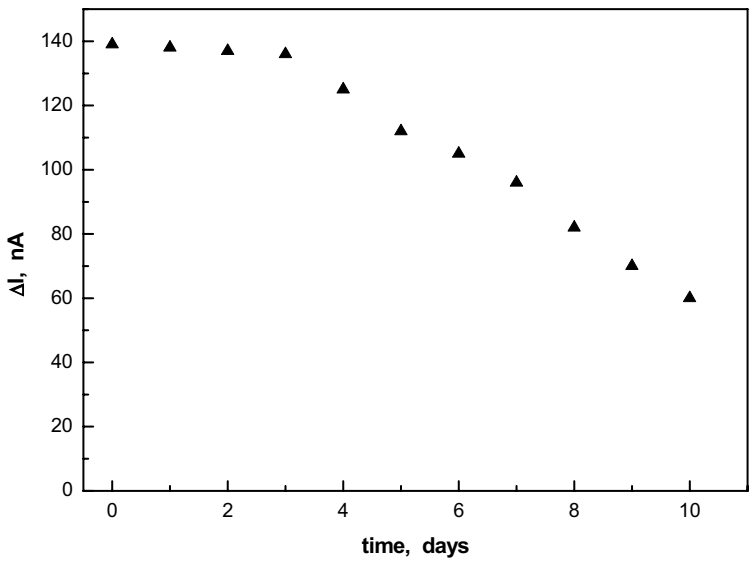

Fig. 5. *Biosensor stability* (*temperature* 25 °C, *p*H=7.00, *ethanol* 10 mM).
Each point represents the average of 3 determinations.

Stability

Biosensor stability was tested on a 10 mM sucrose solution, *p*H=7.00, 25°C, for 10 days with 5 determination per day (Fig. 5). In between the sensors was kept in buffer solution at 4°C. After 5 days, the signal decreases to 80% from initial value in the first day and reach 50% after 9 days.

Ethanol determination in alcoholic beverages

Microbial biosensor was used to determine ethanol concentration in alcoholic beverages. Different dilutions between 40 and 500 times were performed. Results were compared with the enzymatic spectrometric method (Table 1).

The correlation coefficient of experimental data of 0.9983 shows a good correlation between biosensor and spectrometric method. The variation coefficient was no more than 5% for biosensor and 2.5% for spectrometric method, for determination realised in the same day.

Table 1. Comparative results of ethanol determination from alcoholic beverages.

Sample	Biosensor		Spectrometric method	
	conc. (M) [a]	conc. % vol	conc. (M) [a]	conc. % vol
beer 1	0.87	4.00	0.88	4.05
beer 2	1.95	8.90	2.01	9.25
black bere	2.17	10.00	2.08	9.57
vodka	6.95	31.90	7.06	32.50
cognac	8.69	40.00	8.81	40.50
palinca 1	10.86	50.00	10.60	48.70
palinca 2	13.00	59.80	13.24	60.90

[a] 5 determinations were realised for each sample

Conclusions

A microbial biosensor for ethanol based on yeast cell of *S. ellipsoideus* was realised and characterised. Optimum working conditions were *p*H=7 and temperature of 25°C. A fast response of about 2 min. was registered for yeast immobilised on dialysis membrane and about 7 min. for selective biosensor with yeast immobilised on Teflon membrane.

The interference of glucose in ethanol determination is significant even after yeast incubation with ethanol before biosensor preparation. Use of the Teflon membrane allows to prepare a highly selective biosensor for ethanol.

Linear response was recorded in the range of 3÷50 mM ethanol. Improve of the sensitivity is object of further studies.

Comparable sensitivity and good correlation with enzymatic spectrometric method were observed.

REFERENCES

1. Cotrău, M. and Proca, M. (1988) **Toxicologie analitică**, Ed. Medicală, Bucureşti.
2. Forster, E., Silva, M., Otto, M. and Perez-Bendito, D. (1993) *Anal. Chim. Acta* **274**, 109-16.
3. Chung, S.Y., Vercellotti, J.R. and Sanders, T.H. (1995) *J. Agr. Food Chem.* **43**(6), 1545-8.
4. Nabi, A. and Wordsfold, P.J. (1986) *Talanta* **111**, 1-7.
5. Xie, X., Subiman, A.A., Guilbault, G.G. and Yang, Z. (1992) *Anal.Chim. Acta* **266**, 325-9.
6. Marko-Varga, G., Johansson, K. and Gorton, L. (1994) *J. Chromatogr. A.* **660**(1-2), 153-67.
7. Lohanson, K., Jonsson-Pettersson, G., Gorton, L., Marko-Varga, G. and Csoregi, E. (1993) *J. Biotechnol.* **31**, 301-16.
8. Miertus, S., Katrlik, J., Pizzariello, A., Stredansk´y, M., Svitel, J. and Svorc, J. (1998) *Biosens. Bioelectron*. **13**(7-8), 911-23.
9. Gorton, L., Dominguez, E., Hahn-Hagerdel, B., Lan, H.L., Okamoto, Y., Hale, P.D. and Skutheim, T.A. (1993) *Anal. Chim. Acta* **249**, 43.
10. Katrlik, J., Svorc, J., Stred'ansky, M. and Miertus, S. (1998) *Biosens. Bioelectron.* **13**(2), 181-91.
11. Geng, L., Boguslavsky, L.I., Kovalev, I.P., Sahni, S.K., Kalash, H., Skotheim, T.A., H., S.L. and Laurinavicius, V. (1996) *Biosens. Bioelectron.* **11**(12), 1267-75.
12. Pandey, P.C., Upadhyay, S., Upadhyay, B.C. and Pathak, H.C. (1998) *Anal. Biochem.* **260**(2), 195-203.
13. Lorenzo, E., Pariente, F., Hernandez, L., Tobalina, F., Darder, M., Wu, Q., Maskus, M. and Abruna, H.D. (1998) *Biosens. Bioelectron.* **13**(3-4), 319-32.
14. Leca, B. and Marty, J. (1997) *Biosens. Bioelectron.* **12**(11), 1083-8.
15. Castanon, M.J.L., Ordieres, A.J.M. and Blanco, P.T. (1997) *Biosens. Bioelectron.* **12**(6), 511-20.
16. Rotariu, L., Bala, C. and Magearu, V. (2000) *Rev. Roum. Chim.* **45**(1), 21-6.
17. Rotariu, L., Bala, C. and Magearu, V. (2002) *Anal. Chim. Acta* **458**(1), 215-22.
18. Rotariu, L., Bala, C., Fleschin, S. and Magearu, V. (1994) *Anal. Univ. Buc.*, Anul III (serie noua), 45-8.
19. Gonchar, M.V., Maidan, M.M., Moroz, O.M., Woodward, J.R. and Sibirny, A.A. (1998) *Biosens. Bioelectron*. **13**(9), 945-52.
20. Reshetilov, A.N., Lobanov, A.V., Morozova, N.O., Gordon, S.H., Greene, R.V. and Leathers**,** T.D. (1998) *Biosens. Bioelectron.* **13**(7-8), 787-93.
21. Korpan, Y.I., Gonchar, M.V., Soldatkin, A.P., Starodub, N.F., Sandrovski, A.K., Sibirnyi, A.A. and El'skaia, A.V. (1992) *Ukr. Biokhim. Zh.* **64**(3), 96-100.
22. Korpan, Y.I., Dzyadevich, S.V., Zharova, V.P. and El'skaya, A.V. (1994) *Ukr. Biokhim. Zh.* **66**(1), 78-82.