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An amperometric microbial biosensor development based on *Candida tropicalis* yeast cells for sensitive determination of ethanol

Erol Akyilmaz*, Erhan Dinçkaya

Department of Biochemistry, Faculty of Science, Ege University, 35100 Bornova-Ýzmir, Turkey

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Abstract

Different branchs of industry need to use ethanol in their production and some progress and not only the industry also to determine ethanol sensitively, accurately, fast and economical is very important.

For the sensitive determination of ethanol a new amperometric biosensor based on *Candida tropicalis* cells, which contains alcohol oxidase enzyme, immobilized in gelatin by using glutaraldehyde was developed. In the study, before the microbial biosensor construction *C. tropicalis* cells were activated and cultured in a culture medium. By using gelatine and glutaraldehyde (0.1%) *C. tropicalis* cells obtained in logarithmic phase were immobilized and fixed on a pretreated teflon membrane of a dissolved oxygen probe. Ethanol determination is based on the assay of the differences on the respiration activity of the cells on the oxygenmeter in the absence and the presence of ethanol. The microbial biosensor response was depend linearly on ethanol concentration between 0.5 and 7.5 mM with 2 min response time. In the optimization studies of the microbial biosensor the most suitable microorganism amount were found as 4.42 mg cm^{-2} and also phosphate buffer (pH:7.5; 50 mM) and 30 °C were obtained as the optimum working conditions. In the characterization studies of the microbial biosensor some parameters such as substrate specificity, interference effects of some substances on the biosensor response, operational and storage stability were carried out.

Keywords: *C. tropicalis*; Microbial biosensor; Ethanol; Biosensor; Alcohol oxidase

1. Introduction

In the development of biosensors some biologically active materials such as enzymes, cells, plant and animal tissue have been mostly used. Enzymes are mostly used in the biosensor construction due to their high specific activities and analyte sensitivities. Therefore, most of the enzymes used in the biosensor applications are unstable and so expensive for routine analysis of the target analytes. Ethanol is one of these target analytes and ethanol assay is very important and necessary in different industries and biotechnological process such as production of alcoholic beverages, foodstuffs, cosmetic and pharmaceutical products (Boujtita et al., 1996, 2000). And also the measurement of ethanol for the diagnosis and treatment of various disorders is very important (Liden et al., 1998).

For the determination of alcohol some biosensors based on alcohol oxidase (Guilbault et al., 1983; Morales et al., 1998; Patel et al., 2001), alcohol oxidase-peroxidase coupled system (Johansson et al., 1993), alcohol dehydrogenase (Cai et al., 1997; Park et al., 1999) and plant tissue (Akyilmaz and Dinçkaya, 2000) were developed. By using *T. brassicae* (Karube et al., 1980), *C. vini* (Mascini et al., 1989) *G. oxydans* (Reshetilov et al., 1998) *Acetobacter aceti* (Kitagawa et al., 1987) *G. suboxydans* (Kitagawa et al., 1987) and *G. oxydans and P. methanolica* (Reshetilov et al., 2001) microorganisms microbial alcohol biosensors were also developed. Therefore, there is no study to developed a microbial alcohol biosensor based on *C. tropicalis*. This microorganism exhibits a specific alcohol oxidase activity and this enzyme has broad substrate specificity especially for short-chain primary alcohols. In addition, there are some advantages to use yeasts in the biosensor construction such as speed of growth, easy manipulation and growth on a variety of different carbon sources. Yeasts are particularly robust with a wide physicochemical tolerance, e.g. pH, temperature and ionic strength and tough cell walls.

* Corresponding author. Tel.: +90-232-38840001774; fax: +90-232-3888264.
E-mail address: akyilmaz@sci.ege.edu.tr (E. Akyilmaz).

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This paper describes a microbial biosensor for selective and not time-consuming ethanol determination based on *C. tropicalis* immobilized in gelatin by using a cross-linking agent glutaraldehyde on a Clark type dissolved oxygen probe.

Measurements were carried out by standard curves which were obtained by the determination of respiration activity of microorganism or consumed oxygen level reletad to ethanol concentration injected to the reaction medium.

2. Material and methods

2.1. Microorganism and chemicals

C. tropicalis was obtained from JCM (Japan Collection of Microorganism, The Institute of Physical and Chemical Research, RIKEN-JAPAN) in a lyophilized form. Calf skin gelatin (225 bloom), glutaraldehyde (25%) and all other chemicals were purchased from Sigma[1] Chemical Co., USA. All solutions used in the experiments were prepared just before their use.

2.2. Apparatus

In this study, a YSI Model 57 oxygenmeter, YSI 5739 Model dissolved oxygen (DO) probes (with YSI 5740 model cable) (YSI Co., Yellow Springs, USA), high sensitive teflon membranes (0.0005 in. thick) for oxygen, Hettich Universal 30 RF Model Centrifuge (Germany), Pharmacia LKB Novaspec II Spectrophotometer (UK), Stuart Scientific Linear Shaker Bath SBS 35 (UK), Sonifier B-12 (Bronson Sonic Power Company, Danburg/Connecticut-USA), and Ultra-thermostat (Colora, FRG) were used.

2.3. Culture medium of the microorganism

Lyophilized *C. tropicalis* were first activated in a yeast medium agar (pH 6.2) containing glucose ($10.0\,g\,l^{-1}$), peptone ($5.0\,g\,l^{-1}$), yeast extract ($3.0\,g\,l^{-1}$), malt extract ($3.0\,g\,l^{-1}$) and agar ($20.0\,g\,l^{-1}$) for 24 h at 25 °C. After this process *C. tropicalis* cells were inoculated into 50 ml of growth medium, that is containing the same substances of culture medium of the cells given below except ethanol, for 24 h at 25 °C and 200 rpm. Finally, the cells were incubated in 50 ml of the culture medium containing yeast extract ($3.0\,g\,l^{-1}$), $Na_2HPO_4{\cdot}2H_2O$ ($3.0\,g\,l^{-1}$), $NaH_2PO_4{\cdot}2H_2O$ ($3.0\,g\,l^{-1}$), $(NH_4)_2SO_4$ ($10.0\,g\,l^{-1}$), $MgSO_4{\cdot}7H_2O$ ($0.5\,g\,l^{-1}$), $CaCl_2$ ($0.1\,g\,l^{-1}$), peptone ($0.5\,g\,l^{-1}$), glucose ($5.0\,g\,l^{-1}$), KCl ($1.0\,g\,l^{-1}$) and ethanol ($30\,ml\,l^{-1}$) for 24 h at 25 °C and 200 rpm. During the incubation period protein and alcohol oxidase activity assay of the cells were done in every hour by using Lowry method (Lowry et al., 1951) and modified Sigma alcohol oxidase activity assay procedure (Sigma). The experiments showed that the best results for both protein and alcohol oxidase activity were obtained when *C. tropicalis* cells on the 14th hour of incubation period (in log phase) was used.

2.4. Preparation of the microbial biosensor

For the preparation of the microbial biosensor *C. tropicalis* cells in log phase were removed from the culture medium and lyophilized. For this purpose, the cells were removed from the culture medium by centrifugation at 2500 rpm for 10 min at 4 °C. The pellet was washed with phosphate buffer (pH 7.5, 50 mM) and centrifuged again at 2500 rpm and 4 °C for 10 min. Finally, the cells were taken into 5.0 ml of phosphate buffer (pH 7.5, 50 mM) and lyophilized.

In the microbial biosensor construction to prepare the bioactive layer of the biosensor the lyophilized *C. tropicalis* cells ($4.42\,mg\,cm^{-2}$) and gelatin ($4.42\,mg\,cm^{-2}$) were mixed and dissolved in 300 μl phosphate buffer (pH 7.5, 50 mM) at 38 °C. 200 μl of the solution was spread over the teflon membrane on dissolved oxygen probe, and then the bioactive layer was allowed to dry at 4 °C for 30 min. After that, the bioactive layer was treated with a cross-linking agent, glutaraldehyde (0.1%) (in phosphate buffer; pH 7.5, 50 mM) for 3 min to immobilize the cells on the surface of the dissolved oxygen probe membrane.

2.5. Ethanol assay procedure of the microbial biosensor

The function of the microbial biosensor can be described at two stages, first diffusion of oxygen from the air-saturated reaction medium through the teflon membrane. The membrane containing the microorganisms, and it is reduced at the cathode. A small proportion of the oxygen is consumed by the microorganisms. The steady-state current represents the oxygen diffusion through the composed membrane and reflects the endogenous respiration of the microorganisms. Second, ethanol is added to the reaction medium and it permeates through the teflon membrane, is taken up by *C. tropicalis* cells and subsequently degraded. These processes are caused by an increase of respiration activity resulting in a decrease in the dissolved oxygen concentration and the current decreases until a new steady-state is reached.

Respiration activity of the cells was determined by using a Clark type oxygenmeter (YSI Model 57, Yellow Springs Instruments Co., USA) contained an oxygen probe (YSI 5739 with a YSI 5740 cable). All the measurements were done at 30 °C by using a thermostatic reaction cells and the oxygen saturated phosphate buffer (50 mM, pH 7.5). The microbial biosensor prepared was placed into a reaction cell contained phosphate buffer (pH 7.5, 50 mM) which was saturated with oxygen, to obtain a steady-state baseline for dissolved oxygen concentration on the oxygenmeter in the

[1] Sigma Product No. A0438, A2404 and A6941, Enzymatic assay of alcohol oxidase (EC 1.1.3.13), Technical Service, Sigma, Deisenhofen, Germany.