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Development of a catalase based biosensor for alcohol determination in beer samples

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Abstract

An amperometric biosensor based on catalase enzyme for alcohol determination was developed. To construct the biosensor catalase was immobilized by using gelatin and glutaraldehyde on a Clark type dissolved oxygen (DO) probe covered with a teflon membrane which is sensitive for oxygen. The working principle of the biosensor depends on two reactions, which one is related to another, catalyzed by catalase enzyme. In the first reaction catalase catalyzes the degradation of hydrogen peroxide and oxygen is produced and also a steady-state DO concentration occurs in a few minutes. When ethanol added to the medium catalase catalyzes the degradation of both hydrogen peroxide and ethanol and this results in a new steady-state DO concentration. Difference for first and the last steady-state DO concentration occurred in the interval surface of DO probe membrane, which related to ethanol concentration, are detected by the biosensor. The biosensor response depends linearly on ethanol concentration between 0.05 and 1.0 mM with a detection limit of 0.05 mM and a response time of 3 min. In the optimization studies of the biosensor phosphate buffer (pH 7.0; 50 mM) and 35 °C were established as providing the optimum working conditions. In the characterization studies of the biosensor some parameters such as reproducibility, substrate specificity, operational and storage stability were carried out. Finally, by using the biosensor developed and enzimatic-spectrophotometric method alcohol concentration of some alcoholic drinks were determined and results were compared.

Keywords: Alcohol; Catalase; Enzyme electrode; Biosensors

1. Introduction

Ethanol induces some effects on the brain which lead to changes in behavior including motor incoordination, tolerance and dependence, aggression, and brain damage [1].

In the human body three metabolic pathways have been described for ethanol degradation. They involve in alcohol dehydrogenase system, microsomal ethanol oxidation system (MEOS) and catalase enzyme system. Approximately 2 or 3% ethanol is degraded by catalase enzyme system that exists in the peroxisomes. In this system,

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catalase uses hydrogen peroxide for the degradation of ethanol to acetaldehyde [2,3].

Sensitive, practical and economic measurement of ethanol is very important for clinical and forensic laboratories, food and beverages industry [4–6]. The development of biosensors is most promising in the progress of analysis of biologically active compounds. Biosensors have some advantages such as simple measurement procedure, short response time, sensitivity and selectivity. As a result of these important factors some biosensors based on alcohol dehydrogenase [7,8], alcohol oxidase [9,10], alcohol oxidase–peroxidase coupled enzyme system [11,12], microbial [13,14] and plant tissue material [15] have been developed for ethanol determination.

From the literature we can say that there are two biosensor which are very related to our study. One of them had used catalase enzyme immobilized for the characteristics alcohol determination. By using this biosensor ethanol was determined with a measurable concentration of 18 mM and the response time was within 30–90 s [16]. The other is a hydrogen peroxide sensor based on antagonism of peroxidase reaction to tyrosinase reaction using common substrate. The mechanism of this biosensor is nearly the same and related to the mechanism of our biosensor, except using two enzyme system [17].

In this study a biosensor based on catalase enzyme was developed for ethanol determination. The working principle of the biosensor depends on two reactions which one is related to another, catalyzed by catalase enzyme. According to the reactions given below hydrogenperoxide acts first substrate and ethanol acts second substrate for catalase enzyme.

$$H_2O_2 + H_2O_2 \overset{\text{Catalase}}{\rightleftarrows} 2H_2O + O_2 \quad (1)$$

$$CH_3CH_2OH + H_2O_2 \overset{\text{Catalase}}{\rightarrow} CH_3CHO + 2H_2O \quad (2)$$

When hydrogenperoxide is added to the reaction medium an increase occurs for dissolved oxygen (DO) concentration in the interval surface of DO probe membrane according to the first reaction and this results in a steady-state DO concentration in a few minutes. And then by adding ethanol to the reaction medium catalase catalyzes both first and second reaction. In this case, hydrogenperoxide is used for both first and second reaction. Sharing of hydrogenperoxide results in decrease for first steady-state DO concentration and a new steady-state DO concentration occurs. As a result, the difference between the first and the last steady-state DO concentration related to ethanol concentration are detected by the biosensor.

2. Materials and methods

2.1. Chemicals and apparatus

Catalase (E.C 1.11.1.6) from porcine liver and calf skin gelatin (225 bloom), glutaraldehyde (25%) and all other chemicals were purchased from Sigma Chemical Co. (USA). Ethanol, methanol and other alcohols used in the experiments were purchased from Merck (Darmstadt, Germany). All solutions used in the experiments were prepared just before their use.

In the study, a YSI Model 57 oxygenmeter, YSI 5739 Model DO probes (with YSI 5740 model cable)(YSI Co., Yellow Springs, USA), high sensitive teflon membranes (0.0005 in. thick) for oxygen and Ultra-thermostat (Colora, FRG) were used for constant temperature in the experiments.

2.2. Preparation of the biosensor

First of all DO probe was covered with high-sensitive teflon membrane by using an O-ring and then teflon membrane which is selective for oxygen was pretreatment with 0.5% SDS (sodiumdodecylsulfate) in phosphate buffer (50 mM, pH 7.5) to reduce the tension on the membrane surface. After that, catalase enzyme (9333 U ml^{-1}) and gelatin (66.6 mg ml^{-1}) were mixed in phosphate buffer (300 μl) at 38 °C for a few minutes to dissolve the mixture. 200 μl of the solution was spread over the DO probe membrane surface and allowed to dry at 4 °C for 1 h and then the biosensor was crosslinked with glutaraldehyde (2.5%) in phosphate buffer (50

mM, pH 7.5) so a bioactive layer was formed on the DO probe.

2.3. Measurements

In the reaction (1) catalase converts hydrogenperoxide to oxygen and water, DO concentration increase and as a result of this a steady-state (1) DO concentration occurs. When ethanol added to the medium catalase catalyzes both first and second reaction and using of hydrogenperoxide by second reaction occurs a difference in the steady-state (1) DO concentration and a new steady-state (2) DO concentration occurs in a few minutes. We can detect the differences between steady-state (1) and steady-state (2) DO concentration related to ethanol concentration on the oxygenmeter. All the measurements were done at 35 °C by using a thermostatic reaction cells and the oxygen saturated phosphate buffer (50 mM, pH 7.0) was used in all experiments.

2.4. Application

For the determination of ethanol concentration in alcoholic drinks the biosensor developed and modified enzymatic-spectrophotometric method based on alcohol oxidase–peroxidase enzymes (Sigma Product No. A0438, A2404 and A6941, enzymatic assay of alcohol oxidase (EC 1.1.3.13)) were used. For this purpose three beer samples contained different alcohol concentration were used. The results obtained by two methods were compared.

3. Results and discussion

3.1. Optimization of the biosensor

3.1.1. Effect of the enzyme activity on the biosensor response

To detect the effect of the enzyme activity on the biosensor response different enzyme amounts were used in the construction of the biosensor. For this purpose three biosensors which contain 826, 1652 and 8260 U cm^{-2} activity of catalase, were prepared by immobilizing with gelatin (11.8 mg cm^{-2}) and glutaraldehyde (2.5%) (Fig. 1).

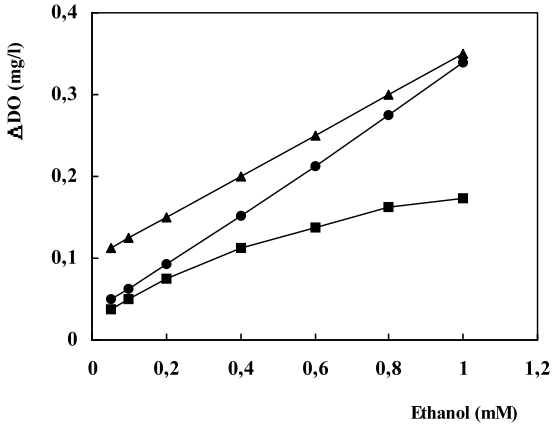

Fig. 1. The effect of enzyme amount on the biosensor response (phosphate buffer; pH 7.0; -▲-▲-, 8260 U cm^{-2}; -●-●-, 1652 U cm^{-2}; -■-■-, 826 U cm^{-2}; the percentage of glutaraldehyde and gelatine amount were kept constant at 2.5% and 11.8 mg cm^{-2}, respectively).

As illustrated in the figure when the biosensors contained 1652 and 8260 U cm^{-2} activity of catalase were used ethanol can be detected with a detection limit of 0.05 mM. Increase in the catalase activity from 826 to 1652 U cm^{-2} results in a positive effect for the linearity and also higher biosensor responses. Therefore, increase in activity from 1652 to 8260 U cm^{-2} didn't give any clear effect on the biosensor response. In this case it can be said that, results obtained from experiments showed that the most suitable biosensor responses were obtained for the biosensor contained 1652 U cm^{-2} activity. Although the highest biosensor responses were obtained when 8260 U cm^{-2} activity was used, both it was more economic and almost gave the same responses 1652 U cm^{-2} activity was chosen for the most suitable enzyme activity.

3.1.2. Effect of pH on the biosensor response

For the determination of the effect of pH value on the biosensor response different buffer systems were investigated. For this purpose 50 mM concentration of citrate (pH 4.0–5.5), phosphate (pH 6.0–8.0) and glycine (pH 8.5–10.0) buffers were

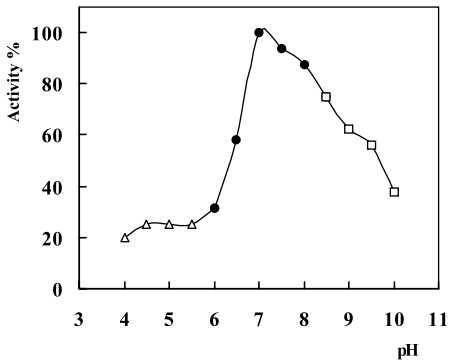

Fig. 2. The effect of pH on the biosensor response (-△-△-, citrate buffer; -●-●-, phosphate buffer; -□-□-, glycine buffer. Concentration of all buffers is 50 mM. The activity of catalase, the percentage of glutaraldehyde and gelatine amount were kept constant at 1652 U cm^{-2}, 2.5% and 11.8 mg cm^{-2}, respectively).

used in the experiments. The optimum pH value was obtained as 7.0 (Fig. 2).

3.1.3. Effect of temperature on the biosensor response

For determination of temperature effect on the biosensor response, the experiments were carried out between 15 and 45 °C. According to the results, the highest biosensor response was observed at 35 °C. Below and above 35 °C, decreases in the biosensor responses were recorded (Fig. 3).

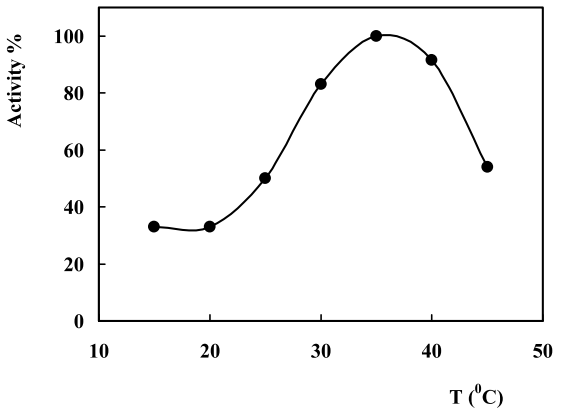

Fig. 3. The effect of temperature on the biosensor response (phosphate buffer; pH 7.0, 50 mM. The activity of catalase, the percentage of glutaraldehyde and gelatine amount were kept constant at 1652 U cm^{-2}, 2.5% and 11.8 mg cm^{-2}, respectively).

3.2. Analytical characteristics of the biosensor

3.2.1. Linear range of the biosensor

The results obtained for the determination of detection limits for ethanol were given in Fig. 4. When we consider the figure, the biosensor responses depend linearly on ethanol concentration between 0.05 and 1.0 mM with a ($y = 0.305 + 0.032$) and $R^2 = 0.9996$. The detection limit of the biosensor is 0.05 mM.

3.2.2. Reproducibility

To detect the reproducubility of the biosensor experiments were also investigated for 0.4 mM ethanol concentration ($n = 10$). From the experiments the average value ($\bar{x}$), the standard deviation (S.D.) and variation coefficient (C.V.) were calculated as 0.396 ± 0.00516 mM and 1.30%, respectively.

3.2.3. Substrate specificity

For investigation of substrate specificity of the biosensor 0.4 mM of ethanol and standards of various compounds such as methanol, *n*-propanol, *n*-butanol, *iso*-propanol, ethylenglycol, diethylenglycol and glucose were examined. The biosensor response obtained for ethanol was accepted as 100% and compared with the biosensor responses obtained for the other substances (Table 1). From the table except ethylenglycol and diethylenglycol

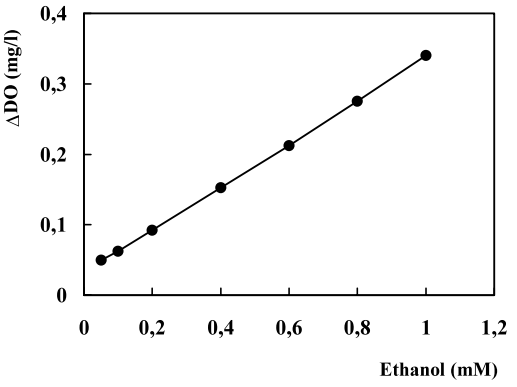

Fig. 4. Calibration curve of the biosensor (phosphate buffer; pH 7.0, 50 mM; $T = 35$ °C. The activity of catalase, the percentage of glutaraldehyde and gelatine amount were kept constant at 1652 U cm^{-2}, 2.5% and 11.8 mg cm^{-2}, respectively).

Table 1
Substrate specificity of the biosensor

Substrate	Activity (%)
Ethanol	100
Methanol	17
n-Propanol	50
n-Butanol	29
iso-Propanol	25
Ethylenglycol	0
Diethylenglycol	0
Glucose	17

the biosensor gave different responses for the other substances which were used for the substrate specificity of the biosensor. But all of them are not so effective as much as ethanol.

3.2.4. Storage stability

Storage stability experiments were made for 6 weeks storage period to detect decreases in the biosensor response. During this period enzyme used in the biosensor construction lost its activity as related to time. In order to determine the storage stability of the biosensor, measurements were carried out periodically every week. The biosensor prepared was actually used for only once a week and the other days of the week the biosensor was stored at +4 °C in a flask contained distilled water to prevent the gelatin-catalase bioactive layer from getting dry. After the storage period, it was determined that the remain activity of the biosensor was more than 78% of its initial activity (Fig. 5).

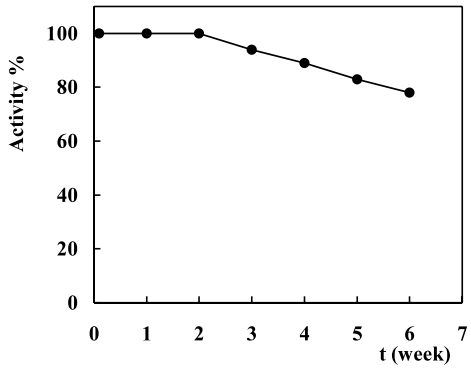

Fig. 5. Storage stability of the biosensor (phosphate buffer; pH 7.0, 50 mM; $T = 35$ °C. The activity of catalase, the percentage of glutaraldehyde and gelatine amount were kept constant at 1652 U cm^{-2}, 2.5% and 11.8 mg cm^{-2}, respectively).

3.3. Determination of ethanol concentration in beer samples

By using both the biosensor developed and enzymatic-spectrophotometric method ethanol level in the beer samples containing different ethanol concentration (3.0, 4.0, and 5.0%) were determined for ($n = 5$) analysis. For this purpose because of the biosensor have a linear range between 0.05 and 1.0 mM ethanol concentrations the beer samples were diluted by taking into consideration their ethanol level contained. After dilution each samples contained 0.4 mM concentration of ethanol. Dilution procedure also minimized the interference effects of substances which would come from containing of the samples such as carbohydrates, proteins, extract and aromatic substances. The analytical results obtained for two methods are given in Table 2. According to the results it can be said that results related to ethanol level in the beer samples obtained by using the biosensor were found to be more sensitive in comparison to the results of enzymatic-spectrophotometric method and also standard deviations and variation coefficients of the biosensor are better than enzymatic-spectrophotometric method's. In addition, the enzymatic-spectrophotometric method needs alcohol oxidase, peroxidase enzymes and some additional chemicals so it can be seen as an expensive method for routine analysis of ethanol.

If we consider the results obtained from the biosensor it is obvious that the biosensor developed based on catalase enzyme was found to be more advantageous for some parameters in comparison to other methods known based on alcohol oxidase, alcohol oxidase–peroxidase couple enzyme system and alcohol dehydrogenase enzyme. The novel biosensor is more suitable and economic especially for routine ethanol analysis because it is sensitive and its response time, operational and storage stability and also reproducibility are very good. By using the biosensor more than 50 measurements were done. Moreover, the high cost of alcohol oxidase, alcohol dehydrogenase

Table 2
Determination of ethanol concentration in the beer samples by using the biosensor and enzymatic-spectrophotometric methods

Sample	Ethanol (%) reported[a]	Ethanol (%) found[b]	$n = 5$	
			S.D.	C.V.%
Catalase biosensor				
A	3.0	2.96	±0.00223	1.10
B	4.0	3.95	±0.00230	1.14
C	5.0	4.97	±0.00370	1.84
Enzymatic-spectrophotometric method				
A	3.0	3.13	±0.0618	1.97
B	4.0	4.11	±0.0980	2.01
C	5.0	5.19	±0.0983	1.92

[a] Reported by manufacturers.
[b] The average of five analysis.

and peroxidase enzymes used in the construction of the alcohol biosensor is a factor that limits widespread use of these type biosensor for routine purpose. However, the immobilization of the enzymes on the teflon membrane surface with gelatine and glutaraldehyde reduces the cost of the procedure, considerably.

Furthermore if we consider substrate specificity, standard deviation and variation coefficient results of the biosensor it can be said that the biosensor can be used easily and reliable for the selective determination of ethanol in the alcoholic drinks.

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