Voltammetric Determination of Ethanol by Using Mushroom (*Agaricus bisporus*) Tissue Homogenate-Based Biosensor

Mantar (*Agaricus bisporus*) Doku Homojenati Temelli Biyosensör Kullanarak Etanolün Voltametrik Tayini

Research Article / Araştırma Makalesi

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ABSTRACT

A new biosensor based on mushroom (*Agaricus bisporus*) tissue homogenate was developed for voltammetric determination of ethanol. Mushroom (*Agaricus bisporus*) was homogenated and immobilized onto the glassy carbon electrode via gelatine and cross-linked by glutaraldehyde to construct an electrochemical tissue homogenate based biosensor system. The homogenate contains alcohol oxidase (E.C 1.1.3.13) enzyme and it catalysis the degredation of ethanol to acetaldehyde and hydrogen peroxide. Assay principle of the biosensor based on the detection of reduction peaks of oxygen by using cyclic voltammetry at potentials between 0.0 and +0.7 V. To obtain a standard graphe for ethanol differential pulse (DP) method was used at potentials between +0.1 and +0.4 V. The electrode response depends linearly on ethahol concentration between 1.0 and 10.0 mM. Phosphate buffer (pH 7.0, 50 mM) and 30°C were obtained as the optimum conditions. In characterization studies of the biosensor some parameters such as; repeatability, substrate specificity, interference effects of some substances on the biosensor response were investigated. Finally, the proposed system was applied to ethanol detection in different wine and beer samples.

Key Words
Voltammetry, biosensor, tissue-homogenate, ethanol.

ÖZET

Bu çalışmada etanolün tayini için mantar (*Agaricus bisporus*) doku homojenatı temelli yeni bir biosensor geliştirildi. Mantar (*Agaricus bisporus*) homojenize edildi ve elektrokimyasal doku homojenatı temelli bir biyosensör sistemi geliştirmek için camsı karbon elektrot üzerinde jelatin ile immobilize edilip glutaraldehit ile çapraz bağlandı. Homojenat alkol oksidaz enzimi (EC 1.1.3.13) içerir ve bu enzim etanolün hidrojen peroksit ve asetaldehite yıkımını katalizler. Biosensörün ölçüm prensibi 0.0 ve +0.7 V potansiyel aralığında döngüsel voltammetri kullanılarak oksijenin indirgenme pikinin belirlenmesine dayanmaktadır. Etanol için standart grafik eldesinde +0.1 ve +0.4 V potansiyel aralığında diferansiyel puls (DP) metodu kullanıldı. Elektrodun cevabı 1.0 ile 10.0 mM etanol konsantrasyonu aralığında doğrusallık gösterdi. Optimum koşullar olarak pH 7.0 50 mM fosfat tamponu ve 30°C elde edildi. Biyosensörün karakterizasyonu çalışmalarında tekrarlanabilirlik, substrat spesifikliği ve bazı maddelerin biyosensör cevabı üzerine girişim etkileri gibi birtakım parametreler incelendi. Son olarak, geliştirilen sistem, farklı bira ve şarap örneklerinde etanol tayinine uygulandı.

Anahtar Kelimeler
Voltametri, biyosensör, doku homojenatı, etanol.

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INTRODUCTION

Biosensors are being developed for application in food, agriculture, biotechnology, medicine, environmental studies and the military. Modern technologies require sensors with distinguish advantages such as flexibility of design and choice of materials, low cost, and possibility of automation of the fabrication process. Enzymes are widely used in a variety of biosensors, bioassays, bioprocesses, bioremediation, and biofuel cells because of its specificity [1-3]. A key factor in the construction of a biosensor is the enzyme immobilization. Classical methods for immobilizing enzymes are physical adsorption, entrapment and covalent cross-linking, which have shortcomings such as the leakage and partially denaturation of the enzyme [4]. Although entrapment of purified enzymes in matrices has been performed, enzymes often fail to retain their native stability and activity upon immobilization; as a result, most of these immobilizing methods were neither simple nor stable [5]. Immobilized plant tissue materials have been used as a biocatalyst to develop different biosensors [6,7]. Biosensors developed using plant tissue materials in combination with transducers offer an alternative to other biosensors based on enzymes. In recent years, the usage of plant tissue based biosensors have also played very important role in biosensor applications and they have some advantages such as low cost, simplicity of construction, and not to be needed co-factor for enzyme regeneration [8-10]. In the construction of plant tissue biosensors especially young leaves, root tips, fruits and seeds of plants have been used in the biosensor construction [11].

Rapid detection and quantification of ethanol with high sensitivity, selectivity and accuracy is required in different fields, such as in medicine, biotechnology, food industry, forensic laboratories and clinical chemistry. The food, beverages and wine industry is very much interested in fast analytical methods to control fermentation processes and product quality [12,13]. Fast and quantitative determination of aliphatic short chain alcohols, especially methanol and ethanol, is of great interest in food, fermentation and wine industries as well as in clinical chemistry. Methanol is illegally used in the production of imitated spirits and wines. Intake of methanol will result in severe intoxication due to accumulation of toxic metabolites such as formic acid and formaldehyde [14]. Ethanol induces some effects on the brain which lead to changes in behavior including motor incoordination, tolerance and dependence, aggression, and brain damage [15]. A variety of methods and strategies had been reported for the determination of ethanol such as gas chromatography-mass spectrometry [16] and liquid chromatography [17]. These methods are relatively expensive, complex, and time consuming. Electrochemical biosensors, which combine specificity of enzymatic reactions with the sensitivity of amperometric detection, have proved to be very useful in solving these problems. These enzymatic biosensors are commonly based on the immobilization of either alcohol oxidase (AOX) or alcohol dehydrogenase (ADH) to an appropriate transducer [18, 19].

Different types of electrodes have been proposed for the detection of ethanol, namely, membrane electrode, carbon paste electrodes, and screen-printed electrodes. Carbon, in many respects, is an ideal electrode substrate due to its wide anodic potential range, low residual current, chemical inertness, and low cost. Glassy carbon and carbon paste are the most widely used carbon electrodes. Glassy carbon electrodes possess attractive electrochemical reactivity, good mechanical rigidity and negligible porosity [20].

The aim of the present study is to develop an inexpensive, sensitive and simple amperometric biosensor based on mushroom (*Agaricus bisporus)* tissue homogenate for ethanol determination.

MATERIALS AND METHODS

Chemicals

The mushrooms (*A. bisporus*) used in the biosensor preparation were purchased from a local market as fresh and culture vegetables. They were stored at 4°C until use. The 225 bloom calf skin gelatin and glutaraldehyde (25.0%) which were used in the immobilization, ethanol, methanol, 2-propanol, n-butanol, ascorbic acid, D-glucose

and also the other chemicals were purchased from Sigma, St. Louis, USA. All chemicals from commercial source were of analytical grade.

Apparatus

Cyclic Voltammetric and differential pulse measurements were carried out by using a PALM SENS electrochemical interface (PALM Instruments B.V, Netherlands). Experiments were carried out by using a three electrode system – a CHI 104 model glassy carbon working electrode, a CHI 111 model Ag/AgCl saturated reference electrode and a CHI 115 model platinum wire auxiliary electrode (Electrodes were purchased from CHI,USA). In the experiments Yellow line model magnetic stirrer (Germany), and pH meter (Hanna Inst.,Italy) for preparing buffer solutions were used. To clean the glassy carbon electrode a Sonicator (Ultrasonic LC30, Germany) was used. All solutions used in the experiments were prepared with ultra pure water obtained from Milipore Mili-Q system (Milipore Corp.,USA). All measurements were carried at constant temperature by using Thermostat (Nuve, Turkey).

Cleaning Procedure of the Working Electrode

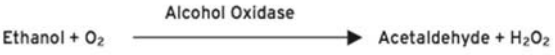
Prior to coating, the glassy carbon electrode surface was polished with alumina slurries on microfiber cloth to obtain a mirror surface. After that it was thoroughly rinsed with water and sonicated first in absolute ethanol and then in bidistilled (double, deionize, triple) water for 10 min. to remove adsorbed particles. After all, electrode was thoroughly rinsed and checked if it was clean enough for immobilization or not by measuring CV (3 cycles, potential between -1.0 V and +1.0 V) in 0.1 M nitric acid.

Preparation of the Biosensor

Mushroom (*A. bisporus*) tissue (200 mg) was homogenized in the phosphate buffer (200 µl, pH 7.0; 50 mM) by using a glass homogenizer. Then this homogenate (100 µl) and gelatin (5.0 mg) were mixed and incubated at 38°C for 5 minutes to dissolve the gelatin. 20 µl of the final solution was carefully spread over the glassy carbon (GC) electrode and allowed to dry at 4°C for 1 hour. At the end of the time the glassy carbon electrode cross-linked by glutaraldehyde (2.5%) for 4 min. to construct an electrochemical biosensing system for ethanol detection.

Measurement Procedure

The response current was marked with the change value between the steady-state current and background current. Measurements were carried out by noting the decrease of dissolved oxygen concentration in relation to substrate concentration added into the reaction cell . The biosensor based on mushroom tissue homogenate was put into the thermostatic reaction cell containing working buffer (pH 7.0, 50 mM potassium phosphate buffer) and the magnetic stirrer was set at a constant speed. Stirrer turned off 3 minutes later and substrate was injected into the reaction cell. The dissolved oxygen concentration started to decrease and it reached a constant dissolved oxygen concentration a few minutes later due to the enzymatic reaction given below.



The biosensor response was defined as the decrease in dissolved O_2 concentration, which was linearly related to the ethanol concentration in the standard solutions. Alcohol oxidase is responsible for the oxidation of low molecular weight primary alcohols to the corresponding aldehyde, using molecular oxygen (O_2) as the electron acceptor. Thus alcohol is oxidized to aldehydes and O_2 is reduced to H_2O_2. Due to the strong oxidizing character of O_2 the oxidation of alcohols by alcohol oxidase is irreversible. Alcohol oxidase based ethanol biosensor developed so far is based on the monitoring of O_2 consumption.

RESULTS AND DISCUSSION

Cyclic voltammograms (CV) obtained from the experiments

Cyclic voltammograms are the most effective factor to detect the electrochemical changes result from the enzymatic reaction in the biosensor studies. The voltammograms obtained from the experiments were recorded using modified glassy carbon electrode. The CVs of 1.0, 2.5, 5.0, and 10.0 mM solution of ethanol were obtained in the

potential range between (0.0) and (+ 0.7) V by using phosphate buffer (pH 7.0; 50 mM). Figure 1 shows the results obtained from the experiments.

As can be seen in Figure 1, electrochemical reduction of oxygen on the surface of modified electrode in pH 7.0 phosphate buffer shows a relationship between concentration of substrate (ethanol) and current value. Decreases in currents were obtained especially at potentials between 0.4 and 0.7 V due to increased concentrations of ethanol. The results obviously represent the catalytic action of alcohol oxidase on the the surface of the modified electrode

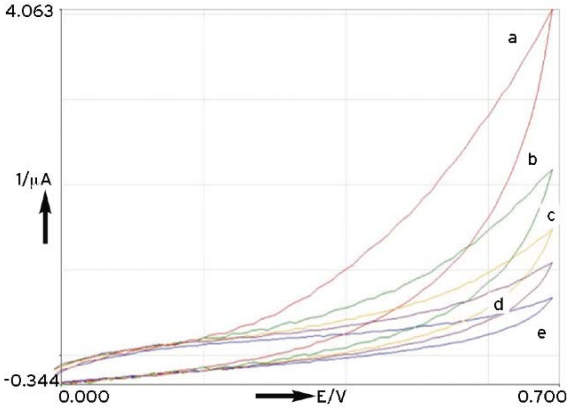


Figure 1. CVs of the mushroom (*Agaricus bisporus*) tissue homogenated based biosensor. (Phosphate buffer; pH 7.0, 50 mM, T: 30°C) (from a to e: baseline, 1.0, 2.5, 5.0, and 10.0 mM ethanol). The amount of homogenate, gelatine and percentage of glutaraldehyde were kept constant at 200.0 mg, 5.0 mg, and 2.5%, respectively. Scan rate is 25 mVs^{-1} and potentials referred to Ag/AgCl reference electrode.

Optimization of the Biosensor

Effect of the amount of mushroom (*Agaricus bisporus*) tissue homogenate on the biosensor response

To detect the effect of the alcohol oxidase activity on biosensor response 100 mg, 200 mg and 300 mg mushroom tissues were used in construction of the biosensors. Although the linearity of the 100 mg tissue homogenate seems good, it gives lower responses than the others for ethanol detection. 300 mg tissue homogenate gives the highest responses but in fact the most sensitive results can be obtained from 200 mg tissue homogenate. Increasing the mushroom tissue amount from 200 mg to 300 mg did not give any clear effect on the biosensor response. A small slope means a small and insensitive biosensor signal so it can be easily said that the most suitable biosensor responses were obtained for the biosensor contained 200 mg tissue homogenate.

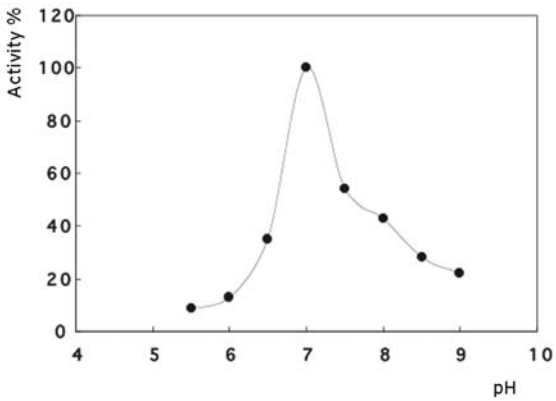


Figure 2. The effect of pH on the biosensor response (pH: 5-6, citrate buffer; pH: 6.5-8.0, phosphate buffer; pH: 8.5-9.0, glycine buffer. Concentration of all buffers is 50 mM). The amount of the mushroom tissue, the percentage of glutaraldehyde, gelatin amount and temperature were kept constant at 200 mg, 2.5%, 5 mg and 30°C, respectively.

Effect of pH

In order to investigate the optimum pH of the biosensor, different pH values between 5.5 and 9.0 were used in the experiments. For this purpose 50 mM concentration of citrate (pH 5.5 – 6.0), phosphate (pH 6.5 – 7.0 – 7.5 – 8.0) and glycine (pH 8.5 – 9.0) buffers were used. According to optimization studies of the biosensor, the optimum pH was obtained to be 7.0. Figure 2 shows the results obtained from the experiments.

Effect of Temperature

For determination of temperature effect on the biosensor response, the experiments were carried out between 15 and 40°C. According to the results the highest response of the biosensor was observed at 30°C. Below and above 30°C, decreases in the biosensor responses were recorded because of the loss in activity of the enzyme found in the homogenate. Furthermore when the temperature increases, dissolved oxygen amount in water that is needed for enzyme activity decreases.

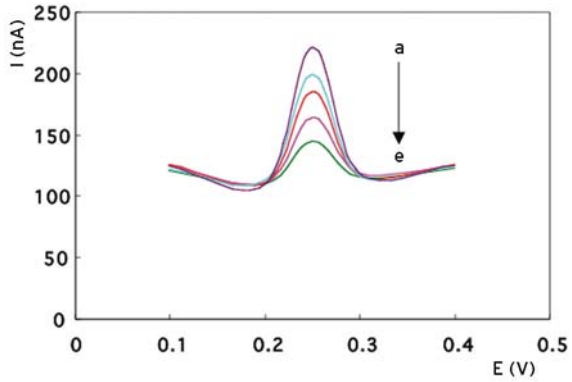


Figure 3. Differential pulse voltammograms obtained for different ethanol concentrations. (Phosphate buffer; pH 7.0, 50mM, T: 30°C) (from a to e: 1.0, 2.5, 5.0, 7.5 and 10.0 mM ethanol). The amount of homogenate, gelatine and percentage of glutaraldehyde were kept constant at 200.0 mg, 5.0 mg, and 2.5%, respectively.

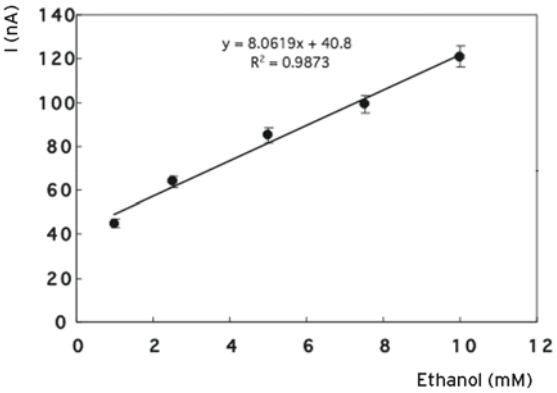


Figure 4. Calibration curve of the biosensor. (Phosphate buffer; pH 7.0, 50 mM). The amount of the mushroom tissue, the percentage of glutaraldehyde, gelatin amount and temperature were kept constant at 200 mg, 2.5%, 5 mg and 30°C, respectively.

Analytical Characteristics

Differential Pulse Voltammograms and Linear Range

To obtain a standard curve for ethanol differential pulse (DP) method was used at potentials between +0.1 and +0.4 V. Voltammograms were shown in Figure 3. According to the voltammograms there is a relationship between concentration of ethanol and current. From the experiments a linearity for the biosensor was obtained in concentration range between 1.0 and 10 mM ethanol (Figure 4). Detection limit of the biosensor was obtained to be 0.69 mM.

Repeatability of the Biosensor

Repeatability of the biosensor was also studied for ethanol concentration of 5.0 mM (n = 5) under the optimum working conditions. According to the results obtained from the experiments the average value (X), the standard deviation (SD) and coefficient of variation (CV %) were found to be 5.023 mM, $\pm$ 0.24 and 4.80 %, respectively.

Sample Application

The biosensor was used for the determination of ethanol in alcoholic beverages. Because of the biosensor have a linear range between 1.0 mM and 10.0 mM, normal beer (5.0 % alcohol), dark beer (6.1 % alcohol), light beer (3.0 % alcohol) and red wine (13.5 % alcohol) were diluted to contain 5.0 mM alcohol concentration in the reaction medium. Samples were studied in triplicate. Results obtained from the experiments can be seen in Table 1.

Ethanol detection especially in alcoholic beverages is more complicated because a greater number of interfering substances could be present. These results seem good for an easy, routine and economic analysis of ethanol by using the tissue-homogenate based biosensor.

CONCLUSION

A biosensor based on mushroom (*Agaricus bisporus*) tissue homogenate was developed for

Table 1. Sample application of the biosensor for alcoholic beverages.

Sample	Alcohol Concentration, Diluted (mM)	Alcohol Concentration, Found (mM)	Recovery %
Red Wine	5.0	5.20	104.0
Dark Beer	5.0	4.32	86.4
Normal Beer	5.0	4.89	97.8
Light Beer	5.0	4.51	90.2

the voltammetric determination of ethanol. A plant tissue was used in the construction of the biosensor because it is simple to prepare, and cofactor is not needed for enzyme regeneration. In addition, it demonstrates a considerable serious economic advantage. Linear range for ethanol and also detection limit of the biosensor is very well so with all these features mushroom (*Agaricus bisporus*) tissue homogenate based biosensor offer an alternative to other biosensors based on isolated enzymes.

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