# ETHANOL MICROBIAL SENSOR BASED ON *GLUCONOBACTER XYLINUS* IMMOBILIZED ON EGGSHELL MEMBRANE

# CẢM BIẾN SINH HỌC XÁC ĐỊNH ETHANOL TRÊN CƠ SỞ *GLUCONOBACTER XYLINUS* CỐ ĐỊNH TRÊN MÀNG VỎ TRỨNG

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**ABSTRACT**

*A microbial biosensor using a pH meter and Gluconacetobacter xylinus -immobilized eggshell membrane was developed. The microbial biosensor responds linearly to ethanol in the range 25–250 mM with a response time of 50 s. The optimal loading of bacterial cells on the biosensor membrane was 30 mg (wet weight). The optimal initial pH for the microbial biosensor was pH 7.0. The biosensor was applied to determine the ethanol contents in various alcohol beverages and the results were compared to that obtained by a distillation-titration method. The proposed microbial biosensor is cost-effective, fast and easy to operate device to determine the ethanol content in simple alcohol beverages.*

***Keywords:*** *Gluconacetobacter xylinus; microbial biosensor; etanol; eggshell membrane.*

**TÓM TẮT**

*Một cảm biến vi sinh sử dụng máy đo pH và Gluconacetobacter xylinus cố định trên màng vỏ trứng đã được chế tạo. Cảm biến này cho tín hiệu tuyến tính với etanol trong khoảng 25-250 mM trong thời gian 50 s. Lượng tế bào vi khuẩn được cố định tối ưu trên màng cảm biến là 40 mg (trọng lượng ướt). pH ban đầu tối ưu là 7.0. Cảm biến sinh học này đã được sử dụng để xác định hàm lượng etanol trong các mẫu đồ uống có cồn khác nhau, và các kết quả này được so sánh với phương pháp chưng cất – chuẩn độ.Cảm biến vi sinh được đề xuất này có hiệu quả về mặt chi phí, cho kết quả nhanh và dễ sử dụng để xác định hàm lượng etanol trong các mẫu đồ uống chứa cồn có thành phần đơn giản.*

***Từ khóa***: *Gluconacetobacter xylinus; cảm biến vi sinh; ethanol; màng vỏ trứng.*

## 1. INTRODUCTION

Fast and accurate measurement of ethanol is necessary in many different industries and processes such as in alcohol beverages, food, cosmetic, and pharmaceutical products [1]. In addition, the measurement of ethanol is particularly important for breath alcohol level determination for drivers [2]. Common methods including gas-diffusion flow injection analysis system [3], electroanalysis [4], infrared (IR) [5], direct injection gas chromatography (GC)/flame ionization detection (FID) [6], HPLC[7], FT-near infrared (NIR) spectrometry, and FT-Raman spectrometry [8] have been used. However, these methods require not only complex and expensive instruments but also laborious extraction and separation pretreatments.

Therefor it is necessary to develop a simple, fast and cost-effective method for the determination of ethanol with acceptable accuracy and sensitivity.

Enzymes are the preferred choice in biosensor construction due to their high specific activities and analyte sensitivities. For example, biosensors based on alcohol oxidase [9], alcohol oxidase-peroxidase coupled system [10], alcohol dehydrogenase [11] have been developed. Unfortunately their uses in biosensor construction are still limited by the, time-consuming and costly enzyme purification procedures. In addition, multiple enzymes or cofactor/coenzyme are often required to generate measurable products.

Thus, microorganisms have been proposed as an alternative to these enzymes. Recently a microbial ethanol sensor for alcohol beverages has been developed [12]. Since cells containing enzymes and cofactors can react with different substrates, it is possible for them to detect a large number of substances. If the whole cells containing active enzymes instead of the purified enzymes are used, simpler and more cost-effective biosensors can be developed. Another advantage of the whole cells is that enzymes are usually more stable in their natural cell environment [13]. Furthermore, microbial biosensors are easy to operate and have short response times which are very suitable for online and field monitoring [14]. To our knowledge, there is no report on microbial ethanol biosensor using *Gluconacetobacter xylinus*. *Gluconacetobacter xylinus,* a rod-shaped, strictly aerobic gram-negative bacterium, is well-known for the ability to produce bacterial cellulose. This microorganism possesses the advantages of fast growth rate and ease of manipulation. It is also very robust as it can tolerate a wide range of physicochemical conditions such as pH, temperature.

Eggshell membrane is mainly composed of biological molecules and protein fibers. The net-veined structure and the gas-permeable hydrophilic property of eggshell membrane provide an excellent biological microenvironment for the cells to survive and maintain its enzymatic activity. In addition, eggshell membrane is a cheap and easily available biomaterial as compared to other existing artificial membranes. The main objective of this work is to develop a simple microbial biosensor based on *Gluconacetobacter xylinus* for fast determination of ethanol. The bacterial cells were immobilized by glutaraldehyde on an eggshell membrane which was subsequently positioned on a pH electrode for biosensing. Our proposed method is relatively accurate and has short response time. The detection principle is based on the production of proton by the bacteria upon exposure to ethanol. It has was applied to determine the ethanol contents in various alcohol samples with acceptable accuracy.

## 2. MATERIALS AND METHODS

### 2.1 Materials

Ethanol (> 99.5% v/v) and 25% (w/w) glutaraldehyde solution in water were purchased from Xilong Scientific Co., Ltd, China). All the solutions were freshly prepared. All reagents of analytical-reagent grade were used without further purification. Fresh eggs and beers were purchased in local supermarkets.

### 2.2 Methods

#### *2.2.1. Cell culture*

*Gluconacetobacter xylinus* was isolated from a bacterial flora in vinegar samples from local market. These samples were activated in a medium containing bean sprout extract 10% (w/v), glucose 2% (w/v) and pepton 1% (w/v),

and then inoculated and isolated using a medium containing glucose 1% (w/v), yeast extract 0.4% (w/v), pepton 0.2% (w/v), Bromocresol green 0.02% (w/v), agar 2% (w/v), ethanol 2% (v/v).

Bacteria separated from the formed cellulose layer were grown in a medium containing 1% (w/v) glucose, 1% (w/v) yeast extract, 0.3% (w/v) peptone, 2% (v/v) ethanol. The bacteria were collected by centrifugation at 8,000 rpm for 5 min and washed twice with 50 mM phosphate buffer (pH 7.0). Under microscope they showed short rod forms and exist in singular individuals or in pairs. They then were submitted for identification by 16S gene sequencing at Nam Khoa Service and Trade Co., Ltd (Vietnam) and were concluded to be a strain of *Gluconacetobacter xylinus*.

### *2.2.2. Preparation of microbial biosensor*

An eggshell membrane was carefully peeled from a fresh eggshell. It was cleansed with large amounts of distilled water. The membrane was placed in a clean watch glass and cut into a circle of about 20 mm diameter. 100 µL of water containing 10 mg wet bacteria cells was spread over the eggshell membrane and dried at 4 $^{o}$C for 30 minutes. 100 µL of 2.5% (w/w) glutaraldehyde solution was spread onto the membrane as a cross-linking agent. A glass rod was used to gently spread the glutaraldehyde solution thoroughly on the membrane. After 120 min of drying, the bacterial cell-immobilized eggshell membrane was then immersed distilled water to remove excess reagents. After washing, the membrane was positioned on the surface of a pH electrode and kept in steady position by an *O*-ring.

### *2.2.3. Measuring ethanol concentration*

The pH electrode with immobilized bacteria on eggshell membrane was immersed to a beaker with distilled water. Various volumes of standard or sample ethanol solution were injected into the water in constant stirring to obtain predetermined concentration of ethanol. The pH change was captured manually and processed using Excel software. Using the definition of pH as –log [$H^+$], the changes of $H^+$ concentrations after 50 s after injecting ethanol were calculated and used as analytical signal.

## 3. RESULTS AND DISCUSSIONS

### 3.1. Effect of the amount of microorganisms

The response of a microbial biosensor is expected to depend on the amount of immobilized microorganisms. Therefore, various amounts of microorganism (10–50 mg) were used to prepare the biosensor eggshell membranes.

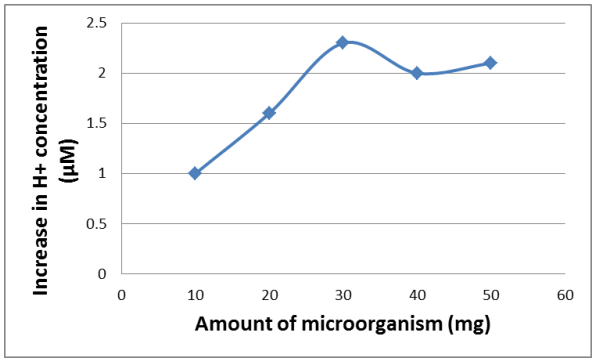


***Figure 1.*** *Effect of the amount of immobilized microorganism*

Figure 1 displays the effect of the amount of bacteria on the biosensor response upon exposure to 25 mM ethanol. Increasing the amount of immobilized bacteria on the eggshell membrane increased the biosensor response. However, too many microorganisms will lower the response and lengthen the response time since the biosensing layer is thicker. The optimal amount of microorganism is 30 mg as it produces the highest response to ethanol.

### 3.2. Effect of concentration of glutaraldehyde

Glutaraldehyde has been a useful cross-linker to immobilize biological molecules such as enzymes and bacterial cells on solid substrates. Various concentrations of glutaraldehyde (1.0–3.5% w/w) were used to immobilize the microorganisms (30 mg wet weight) on the eggshell membranes and their responses to 1.00% ethanol.

Figure 2 displays the effect of glutaraldehyde concentration on biosensor response. The response increases with the increase in concentration of glutaraldehyde until it reaches the highest at 2.5% w/w. Further increase in concentration of glutaraldehyde causes the decrease in response to ethanol, attributing to deactivation of the cells. As such, 2.5% w/w glutaraldehyde was chosen for this work.

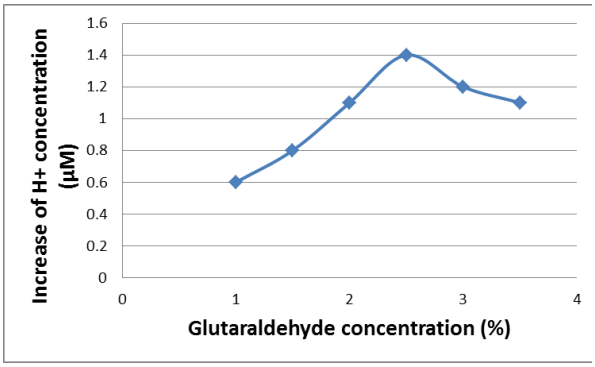


***Figure 2.*** *Effect of concentration of glutaraldehyde*

### 3.3. Effect of initial pH

Because solution pH affects essentially functionality of enzymes and microorganism, the biosensor was tested with various initial pH of ethanol solution. A buffer was not used because it will stabilize the pH of solution.

Figure 3 shows that higher values of initial solution pH (lower initial concentration of $H^+$) produce higher analytical signals. Howerver, values of pH higher than 7.0 reduce the responses, possibly due to the deactivation of enzymes at these pH.

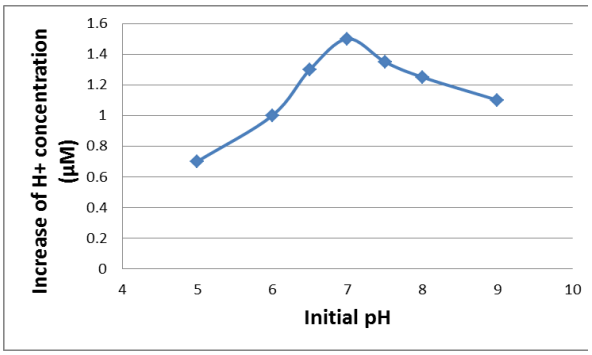


***Figure 3.*** *Effect of initial pH*

### 3.4. Analytical characteristics of the ethanol biosensor

In our study, an interval of 50 s was chosen as the response time because the concentration of $H^+$ increased linearly in this time interval for all experiments.

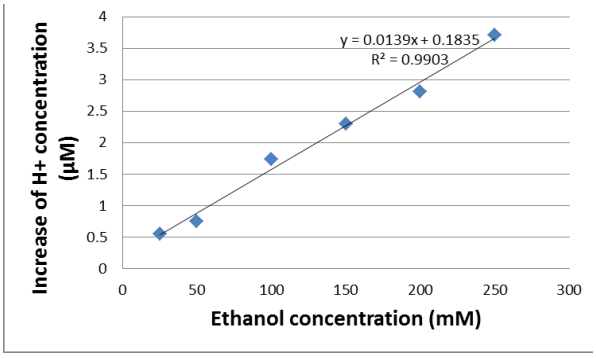


***Figure 4.*** *Calibration curve of the microbial ethanol biosensor*

Figure 4 shows the calibration curve of the biosensor in the range 25.0-300 mM ethanol with a linear regression equation: $\Delta[H^+] = 0.0139\ [\text{ethanol}]+0.1835$; $r^2 = 0.9903$, where the concentration of ethanol is in mM, while that of $H^+$ is in µM. The standard errors of the slope and *y*-intercept are 0.0011 and 0.0077, respectively. Satisfactory repeatability of the biosensor was confirmed with a relative standard deviation (RSD) of 4.0% which was obtained by subjecting the biosensor repetitively to a 25 mM ethanol at initial pH=7.0 for seven times. The

reproducibility of the bacterial cell-immobilized eggshell membrane was also assessed by preparing four different biosensor membranes under the same conditions in different batches. The responses of these four membranes were determined by exposing them to a 25 mM ethanol standard. The RSD of the responses was relatively high (7.2%), indicating that the procedure of preparation of the bacterial cell-immobilized eggshell membrane needs to be improved.

### 3.5. Alcohol sample analysis

***Table 1.*** *Comparison of two analytical method for some alcoholic beverages*

| Sample | Ethanol concentration (%, v/v) | | Error (%) |
|---|---|---|---|
| | Dilution-Biosensor | Distillation-Titration | |
| Beer 1 | 3.0 | 2.6 | 15 |
| Beer 2 | 3.8 | 3.2 | 19 |
| Vodka | 37 | 35 | 5.7 |

The determination of ethanol in commercial alcoholic drinks was carried out. All of them contained relatively high ethanol contents, so a dilution step for these samples was required. Each sample was diluted with diluted by distilled water to a concentration within the working range of the biosensor. pH of each sample was adjusted to 7 using NaOH before biosensor measurements. The accuracy of our biosensor method was also evaluated by comparison with a distillation-titration method with $K_2Cr_2O_7$.

Table 1 depicts the analytical results for three commercial alcohol beverages. There were relatively high differences (15% and 19%) between the two methods for in ethanol content of the beer samples. However, the difference was lower (5.7%) for vodka. The reason may lie in the complex composition of beers, which may interfere with the accuracy of the biosensor method. Further improvements should be focused on the selectivity of this microbial sensor.

## 4. CONCLUSION

This work demonstrates the feasibility of preparing a microbial biosensor based on a pH meter with a bacterial cell-immobilized eggshell membrane. Our biosensor exhibits fast response (50 s) to ethanol with relatively good repeatability. The advantages of our device are cost-effective, simple design and ease of operation. Future works should be directed to the immobilization procedure to improve reproducibility of eggshell membrane and enhancing selectivity of the device.

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