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Determination of ethanol in acetic acid-containing samples by a biosensor based on immobilized *Gluconobacter* cells

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Abstract. *Reshetilov AN, Kitova AE, Arkhipova AV, Kratasyuk VA, Rai MK. 2012. Determination of ethanol in acetic acid containing samples by a biosensor based on immobilized* Gluconobacter *cells. Nusantara Bioscience 4: 97-100.* A biosensor based on *Gluconobacter oxydans* VKM B-1280 bacteria was used for detection of ethanol in the presence of acetic acid. It was assumed that this assay could be useful for controlling acetic acid production from ethanol and determining the final stage of the fermentation process. Measurements were made using a Clark electrode-based amperometric biosensor. The effect of pH of the medium on the sensor signal and the analytical parameters of the sensor (detection range, sensitivity) were investigated. The residual content of ethanol in acetic acid samples was analyzed. The results of the study are important for monitoring the acetic acid production process, as they represent a method of tracking its stages.

Key words: *Gluconobacter*, biosensor, ethanol, acetic acid

Abstrak. *Reshetilov AN, Kitova AE, Arkhipova AV,. Kratasyuk VA, Rai MK. 2012. Penentuan etanol dalam sampel yang mengandung asam asetat dengan biosensor sel* Gluconobacter *yang diimobilisasi. Nusantara Bioscience 4: 97-100.* Sebuah biosensor berdasarkan bakteri *Gluconobacter oxydans* digunakan untuk mendeteksi etanol pada sampel yang mengandung asam asetat. Pengukuran dilakukan dengan elektroda Clark berdasarkan biosensor amperometrik. Uji ini diharapkan berguna untuk mengendalikan produksi asam asetat dari etanol dan menentukan tahap akhir proses fermentasi. Pengaruh pH pada stabilitas pengukuran dipelajari. Berbagai jenis larutan bufer (sitrat, Tris maleat, natrium fosfat) diuji untuk memilih varian optimal, yang merupakan bufer fosfat dengan pH dalam kisaran 6 sampai 7 unit. Sampel yang dianalisis dengan asam asetat pada konsentrasi sesuai dengan fermentasi selesai (9%) diencerkan 80 kali. Sensor tes etanol diaktifkan dalam kisaran 0,0125-2,00 mM (0,0006-0,0092%). Kandungan etanol dalam sampel komersial asam asetat dari berbagai produksi dinilai. Hasil dari penelitian ini penting untuk memantau proses produksi asam asetat, karena mereka mewakili metode pelacakan tahapannya.

Kata kunci: *Gluconobacter*, biosensor, etanol, asam asetat

INTRODUCTION

Bacteria of the genus *Gluconobacter* are widely used in various biotechnological processes, in particular, in production of vinegar and acetic acid from alcohol-containing products. At the initial stage of the fermentation process, the bioreactor contains a maximum amount of ethanol, which decreases as the content of acetic acid goes up. The decrease of ethanol concentration down to a certain level indicates the completion of the process. A real fermentation process (modeling, measurement and control) of acetic acid production by the repeated batch method using *Acetobacter* strains is given in Hekmat and Vortmeyer (1992). The fermentation was controlled by such parameters as acetic acid concentration, ethanol concentration and optical density of cell suspension. According to the data of the work, the uptake of ethanol and accumulation of acetic acid had close-to-linear dependences. Accumulation of acetic acid and uptake of ethanol were mutually dependent. Thus, the initial ethanol concentration of 30±5 g/L was totally utilized within 25 h, and the initial level of acetic acid increased from 10±5 g/L up to 95±5 g/L. The content of ethanol was determined by a gas sensor, and acetic acid was assayed by a gel-based pH electrode.

There are various biosensor approaches to detection of ethanol and acetic acid, based on the use of enzymes or microbial cells (Tkac et al. 2002; Wang et al. 2006). They enable monitoring the formation of acetic acid by the content of ethanol in the fermentation medium. Still, approaches based on microbial biosensors making possible ethanol assays in the presence of acetic acid have not been described.

The aim of the work was to assess the possibility of assaying ethanol in acetic acid-containing samples by a biosensor based on *G. oxydans* bacteria and, for

comparison, on alcohol oxidase. We assumed that this assay could be useful for controlling acetic acid production from ethanol and determining the final stage of the process. In spite of a significant dilution of the analyzed initial sample containing acetic acid, pH of the basic solution inevitably changes, which can affect the biosensor signal. A comprehensive theoretical calculation of the model, including pH changes and biosensor responses in this process, appears to be complex, due to which an experimental verification was used. Special attention was paid to the case simulating the final stage of acetic acid production, when the content of ethanol in the sample is low (from 1 down to 0.1%), and the concentration of acetic acid is high (of the order of 10%). The purpose of the work was also to choose the type of buffer solution and its effect on the stability of the bioreceptor, as well as to study the parameters of the sensor when the optimal type of buffer was used. Commercial solutions of acetic acid were taken as an approximation to the applied aspect of the problem. Publications on the subject have not investigated the issue in this statement of the problem.

MATERIALS AND METHODS

A microbial biosensor based on cells of *Gluconobacter oxydans* VKM B-1280 (purchased from All-Russian Collection of Microorganisms) and an enzyme sensor based on alcohol oxidase (isolated from *Hansenula polymorpha* NCYC 495 ln, activity 14 units/mg) (Ashin et al. 2004) were used for the ethanol assay.

The strain *G. oxydans* VKM B-1280 was grown on a nutrient medium containing (g/L): sorbitol, 100; yeast extract, 10. The cells were grown for 18 h on a shaker (200 rpm, 28°C) in 750 mL Erlenmeyer flasks containing 100 mL growth medium. Biomass was separated by centrifugation at 10,000 *g* for 5 min and washed twice with sodium phosphate buffer (30 mM, pH 6.6).

In formation of the microbial biosensor, cells were immobilized by their physical sorption on Whatman GF/A glass fibre filters. For this, 1 mg biomass was applied on a filter and dried for 20 min at room temperature (frozen biomass was used; during the preparation to immobilization, cells were preliminarily unfrozen).

Alcohol oxidase was immobilized in a layer of DEAE dextran on nitrocellulose membranes activated with benzoquinone (Zaitsev et al. 2007).

The bioreceptor (enzymes or cells immobilized on membranes) 3×3 mm^2 in size was fixed on the measuring surface of a Clark oxygen electrode (Kronas Ltd., Russia). The rate of stirring the solutions by a magnetic stirrer was 400 rpm. The measurements were carried out in an open cuvette (volume, 2 mL) by an IPC2L galvanostat/ potentiostat (Kronas Ltd, Russia) connected to a computer. A 100-µL sample of a required concentration was introduced into the cuvette. The sample was diluted 1:20. The measurements were done at room temperature. A 30 mM sodium phosphate buffer, pH 6.6, was used as a basic solution. The registered parameter was the maximum rate of signal change (nA/s).

The pH dependences were studied using the following buffer solutions: sodium phosphate buffer, MacIlvaine's citrate phosphate buffer and a buffer containing Tris (hydroxy-methyl) aminomethane maleate (Tris maleate). The molarity of the buffer solutions was 30 mM.

Samples simulating the main stages of the fermentation process were used for the analysis: 10% ethanol, the onset of the process; 5% ethanol in 5% acetic acid, the midpoint of the process; 1% ethanol in 9% acetic acid and 0.1% ethanol in 9% acetic acid, the completion of the process.

RESULTS AND DISCUSSION

Choice of the type of buffer solution

Various types of buffers (sodium phosphate, MacIlvaine's citrate phosphate, Tris-maleate) were used. It was assumed that buffer solution components could affect in different ways the stability of cells to pH changes. The concentrations of ethanol and acetic acid in the initial sample were, respectively, 0.1% (22 mM) and 9% (1.5 mM). This content of acetic acid corresponds to a minimum concentration of acetic acid in the fermentation medium, at which the process is terminated. For measurements, the sample was diluted 80-fold.

The effect of the type of buffer on the stability of cells is given in Figure 1. The range of investigated pH values for MacIlvaine's citrate phosphate buffer solution was 3.2-7 (curve 1). The bioreceptor enabled measurements without a loss of activity within the pH range of 3.2 to 6.4. At pH 7, the measurement error exceeded 10%.

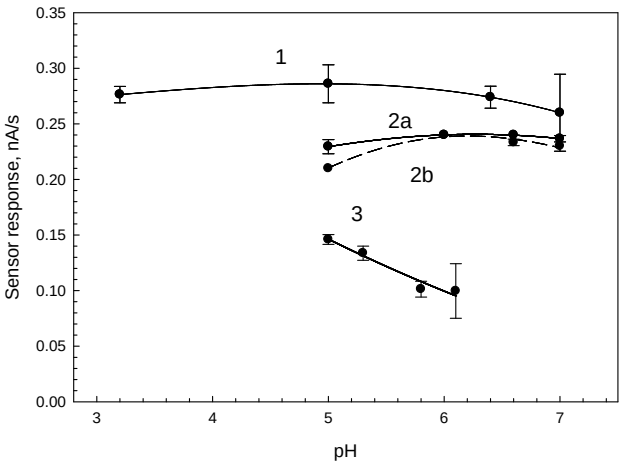

Figure 1. Dependences of the responses of a *G. oxydans*-based biosensor on pH of the buffer solution (1, citrate phosphate buffer; 2 (a,b), sodium phosphate buffer; 3, Tris-maleate buffer).

When using sodium phosphate buffer, the bioreceptor was stable within the pH range 5-7 in assaying ethanol samples (curve 2a). When, assaying samples containing ethanol and acetic acid (curve 2b), the sensor responses were stable within the pH range of 6-6.6. When using a buffer solution containing Tris-maleate, the range of

investigated pH values was 5-6.1 (curve 3). The responses were observed to be decreased at a pH rise.

The type of buffer solution affected the magnitude of sensor response. Thus, the highest response was obtained using a citrate buffer solution. However, in studies of sensor stability in citrate buffer the responses decreased in the first 6 h, which was not characteristic of other buffer types. Sodium phosphate buffer was chosen as an optimal basic solution and was used in further experiments.

Study of the major parameters of the sensor

The main analytical parameter of a biosensor is its calibration dependence. It was plotted as a function of ethanol concentrations in the measuring cuvette. Figure 2 (curve 1) presents the calibration dependence of a biosensor based on cells of the strain *G. oxydans* VKM B-1280. The range of assayed concentrations was 0.0125-2.00 mM (0.0006-0.0092%) (the final concentration of ethanol in the measuring cuvette is given). Sensor responses were recorded in a 30 mM sodium phosphate buffer solution with pH 6.6. The maximum sensitivity in the linear range was 1.2 (nA/s)/mM; the linear range of detection, 0.0125-0.5 mM (0.0006-0.023%). Curve 2 is a calibration curve for ethanol samples containing acetic acid as a background concentration (0.11% (18.7 mM), concentration in the cuvette; in the initial sample, the concentration was 9%). The linear range was 0.0125-1.25 mM (0.0006-0.06%) of ethanol; the sensitivity in the linear range, 1.2 (nA/s)/mM.

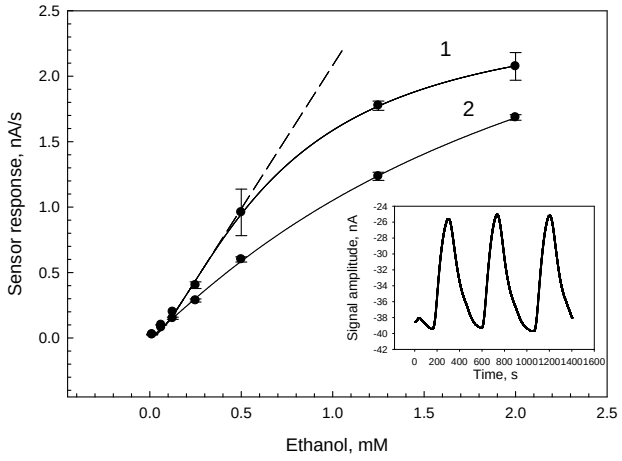

Figure 2. Calibration dependences of a biosensor based on the strain *G. oxydans* VKM B-1280 for detection of ethanol (1) and ethanol in the presence of acetic acid (2); the dashed line shows the maximum tangent slope at the initial segment of the curve. Insert, a typical shape of sensor response.

A bioreceptor based on *G. oxydans* cells was evaluated for the reproducibility of responses to samples containing ethanol and acetic acid (a sample contained 0.025% (22 mM) ethanol and 2.4% (375 mM) acetic acid, in the cuvette, the sample was diluted 20-fold). The coefficient of variation was 6%. The measurements were done in a sodium phosphate buffer solution of a 30 mM concentration. The response time was 120 s. The sensor enabled 6 measurements per hour.

Assay of samples simulating the acetic acid production process with various contents of ethanol in acetic acid solution

Information on the ethanol content is vital for the acetic acid production process to be optimal, as its decrease to a certain final level (0.1%) indicates the completion of the process. We analyzed samples simulating acetic acid production at various stages of the process.

The values of sensor responses to samples simulating various fermentation stages are given in Table 1. In the analysis of a sample containing 9% (1.5 M) acetic acid (concentration in the cuvette, 0.11% (18.7 mM)) and 0.1% (22 mM) ethanol (0.00125% (0.26 mM)), the pH value of the initial buffer solution equal to 6.6 decreased by unity. For subsequent assays, the dilution was increased by an order of magnitude, which in practice had no effect on pH of the buffer solution.

Table 1. Dependence of sensor responses on ethanol concentrations in a model sample.

Sample	pH of samples	Dilution of samples with buffer	pH of samples in measuring cuvette	Sensor response, nA/s
0.1% ethanol in 9% acetic acid	2.4	80-fold	5.5	0.200±0.005
1% ethanol in 9% acetic acid	2.4	800-fold	6.6	0.206±0.004
5% ethanol in 5% acetic acid	2.5	4000-fold	6.6	0.202±0.009
10% ethanol	5.0	8000-fold	6.6	0.200±0.019

Thus, to assess ethanol in assayed samples they should be diluted 80 times (final dilution in a cuvette) and more. With this dilution, pH of the basic buffer solution changes insignificantly.

Assay of ethanol in real samples

We estimated the residual ethanol in samples of vinegar produced by a microbiological method (Table 2). The correlation coefficient of the data obtained using a microbial sensor and enzyme (alcohol oxidase-based) sensor was 0.98.

Table 2. Content of residual ethanol in acetic acid.

Sample	Ethanol (mM)	
	G. oxydans	Alcohol oxidase
Apple vinegar (Egorye)	12.0±0.7	11.9±1.1
Apple vinegar (Abriko)	10.9±1.0	11.7±0.8
Wine vinegar (Baltimor)	16.6±1.2	14.9±1.1

High dilutions of the initial sample simulating the composition of the fermentation medium in acetic acid production were shown to practically buffer low levels of

pH. At these dilutions with the basic buffer solution, ethanol concentrations corresponded to the linear range of the biosensor based on *G. oxydans* cells. Insignificant pH changes caused by the presence of acetic acid did not affect the measurement results when using this type of biosensor. The buffer systems studied had different effects on the biosensor parameters, due to which an optimal (sodium phosphate) buffer solution was chosen.

CONCLUSION

The optimal conditions for operating a *Gluconobacter oxydans*-based biosensor in an acetic acid-containing medium were determined. Under these conditions, the biosensor was shown to be capable of assaying the content of ethanol in acetic acid-containing samples within the linear segment (0.0125-0.5 mM) of the calibration dependence. The data obtained indicate a possibility of monitoring the acetic acid production process by both microbial and enzyme biosensors. The enzyme biosensor having a higher selectivity as compared with the microbial biosensor can be used as a control for estimating the total content of alcohols.

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