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Mediated amperometric biosensors for lactic acid based on carbon paste electrodes modified with baker's yeast *Saccharomyces cerevisiae*

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Abstract

Carbon paste electrodes modified with baker's yeast *Saccharomyces cerevisiae* (a source of flavocytochrome b_2) were investigated as amperometric biosensors for lactic acid. Phenazine methosulphate was used as a mediator. The optimal operational conditions of the electrodes were: an operating potential 0.0 V, solution pH 7.2, concentration of phenazine methosulphate in solution 0.2 mM. A linear range in the dependence of the current responses on the concentration of lactic acid was up to 1 mM. The suitability of the electrodes for determination of lactic acid in milk and dairy products such as kefir and yoghurt was tested. The yeast cells in the paste remained viable at least for 1 month.
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Keywords: Biosensor; *Saccharomyces cerevisiae*; Flavocytochrome b_2; Phenazine methosulphate; Lactic acid; Carbon paste

1. Introduction

Reliable and rapid monitoring of lactic acid is very important in clinical analysis, sports medicine and food industry. Amperometric biosensors for lactate usually employ enzymes such as NAD^+-dependent lactate dehydrogenase [1–3], lactate oxidase [4–7] and flavocytochrome b_2 [8–10]. All these enzymes catalyze the oxidation of L-lactic acid to pyruvic acid.

Flavocytochrome b_2 used for electrode modification was isolated from yeasts *Hansenula anomala* [8], baker's yeasts *Saccharomyces cerevisiae* [9] and, recently, from *Hansenula polymorpha* [10]. In the latter case, currents related to the direct electrochemical communication between the adsorbed flavocytochrome b_2 and graphite electrode surface were detected. However, these currents were much lower compared to those when free-diffusing redox mediators were present in the solution. The scheme of mediated electrocatalytic oxidation of lactic acid can be represented as follows:

$$\text{L} - \text{lactic acid} + \text{flavocytochrome } b_{2(ox)} \rightarrow \text{pyruvic acid} + \text{flavocytochrome } b_{2(red)} \quad (1)$$

$$\text{flavocytochrome } b_{2(red)} + \text{mediator}_{(ox)} \rightarrow \text{flavocytochrome } b_{2(ox)} + \text{mediator}_{(red)} \quad (2)$$

$$\text{mediator}_{(red)} \rightarrow e^- + \text{mediator}_{(ox)}, \quad (3)$$

where (1) and (2) are chemical reactions of flavocytochrome b_2 with lactic acid and mediator, respectively; (3) is the electrochemical oxidation of the reduced form of mediator. This reaction was used for amperometric detection of lactic acid.

Whole yeast cell-based biosensors mostly employ the respiratory activity of the yeasts coupled to oxygen or carbon dioxide electrodes to monitor the consumption or production of these gases [11–17]. To find alternatives to lactate biosensors based on isolated enzymes, electrodes modified with whole cells of *H. anomala* [18] or commercially available baker's yeasts [19,20] with various mediators were also investigated. Some research formerly was devoted to carbon paste electrodes containing dry baker's yeast as a source of NAD^+-dependent alcohol dehydrogenase [21]. These electrodes were found to be sensitive to primary alcohols in the presence of soluble NADH and mediator.

This paper presents our further investigation of carbon paste electrodes modified with commercially available baker's

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yeasts as amperometric biosensors for lactic acid using a mediator phenazine methosulphate (PMS). PMS (soluble or immobilized in various matrixes) and phenazine ethosulphate have been already employed as effective mediators for oxidation of flavocytochrome b_2 [10,22], NADH or NAD(P)H [23–28], phenols [29] at potentials close to 0 V. Sensitivity to lactic acid was tested using various configurations of the electrodes (yeast layer on the top of the electrode, yeast in the bulk carbon paste, PMS in solution or adsorbed on graphite). The suitability of yeast-modified electrodes for determination of L-lactic acid in milk and dairy products such as kefir and yoghurt was also tested.

2. Experimental

Baker's yeasts *S. cerevisiae* from two different suppliers (SEMA, Panevezys, Lithuania and Pärmitehas, Estonia) were obtained from a local market. D,L-lactic acid was distilled before use. *N*-methylphenazonium methyl sulphate (or phenazine methosulphate) was obtained from Fluka. 0.1 M phosphate buffer contained additionally 0.1 M KCl. The values of pH were adjusted with KOH or HCl. All reagents were analytical grade. Raw milk was obtained from a local farm. Pasteurized milk, yoghurt and kefir were obtained from a local market. Before measurements, milk was stirred for 30 min. Both yoghurt and kefir were filtered through a paper filter.

Plain carbon paste was prepared by mixing 100 mg of graphite powder (Merck) with 50 μl of paraffin oil (Fluka). For adsorption of PMS, graphite powder was kept in 1, 5, 10 mM solutions of PMS for various periods of time (4 to 12 h) and then dried in the darkness at room temperature for 24 h.

A layer of the yeast on a surface of a plain carbon paste electrode was formed by dipping the electrode in suspensions of various concentrations (10, 50, 100, 200 mg) of the yeast in 2 ml of phosphate buffer at pH 7.2. The electrodes were allowed to dry at room temperature for 30 min and then covered with a dialysis membrane. Bulk yeast-modified pastes were prepared by mixing 40 mg of yeast with 60 mg graphite or PMS-modified graphite powder and 50 μl of paraffin oil. The paste was packed into an electrode body consisting of a plastic tube (2.9 mm diameter) with a copper wire serving as an electrode contact. After smoothing the surface with white filter paper, the electrode was covered with a dialysis membrane (Sigma-Aldrich).

Electrochemical measurements were performed in a three-electrode cell arranged with a magnetic stirrer. Carbon paste electrode served as a working electrode. Platinum wire and Ag/AgCl, 3 NaCl were, respectively, counter- and reference electrodes. All measurements were performed with BAS-Epsilon Bioanalytical system (West Lafayette, USA).

All experiments were carried out at room temperature at reduced light since PMS is light sensitive. For some experiments, solutions were deoxygenated by purging nitrogen for 20 min.

To test the viability of the cells in the carbon paste, 10 mg of the paste were suspended with stirring in 0.2 ml of sterile water. Primary culture of the yeast (4 mg) was also suspended for comparison. Both suspensions were seeded on the YEPD medium (1% yeast extract, 2% peptone, 2% glucose and 2.5% agar) [30,31]. The plates were kept for 3 days at 30 °C. The cloning was carried out until the separate colonies of the yeast were obtained. The resemblance of the cells was controlled by microscopy. The activity of the paste to lactic acid and the viability of the yeast cells were tested every seventh day.

3. Results and discussion

Six yeast samples (three for each supplier) were tested as sources of flavocytochrome b_2. The samples from Lithuanian and Estonian suppliers are denoted L1, L2, L3 and E1, E2, E3, respectively.

Flavocytochrome b_2 is known to catalyze the oxidation of L-lactate to pyruvate whereas D-lactate is a competitive inhibitor of lactate oxidation; pyruvate is known to be a competitive inhibitor for the oxidized enzyme at low concentrations [32]. A racemic mixture of D,L-lactic acid used for this as well as for our previous [20] investigation, nevertheless, was applicable to achieve stable and reproducible electrode responses.

The sensitivity of yeast-modified electrodes to lactic acid depended on the mode of immobilization of the yeast cells. All electrodes containing intact yeast cells on the surface of a plain carbon paste (PMS in solution) did not respond to the addition of lactic acid at potentials 0 and 0.1 V (the $E^{o\prime}$ of PMS in solution is about −0.1 V vs. Ag/AgCl, 3 M KCl at pH 7.4 [26]). Probably, the explanation of this could be that PMS did not penetrate through the yeast cell wall and did not reach the enzyme (flavocytochrome b_2 is known to be located in the inter-membrane space of yeast mitochondria [33]). All carbon paste electrodes with admixed yeasts were sensitive to lactic acid. Yeast cell walls were probably damaged during grinding with graphite powder, thus, the enzyme could be reached by PMS. Probable damage of the cell walls could also explain electrode responses to lactic acid when potassium ferricyanide was used as a mediator (unpublished results) since electrodes containing intact yeast cells did not show responses under the same conditions. Recent electrochemical investigation of redox activity in living yeast cells [34,35] revealed that the cell walls of the intact yeast *S. cerevisiae* could not be crossed by potassium ferricyanide.

Cyclic voltammograms of carbon paste electrodes containing baker's yeasts (CPBY) in phosphate buffer at pH 7.2 showed similar properties as described in [20], i.e., the growth of both anodic and cathodic currents in the potential region −0.6 to +0.2 V (probably due to swelling of the lipid–protein-based carbon paste interface). Constant voltammograms of CPBY (at a sweep rate 10 mV/s) could be recorded after 6 to 16 potential scans. Cyclic voltammograms of CPBY (sample E1) carbon paste electrode and a plain carbon paste electrode in non-deoxygenated phosphate buffer at pH 7.2 are shown in Fig. 1 (solid and dashed lines, respectively).

The increase in the cathodic current below −0.2 V recorded for a plain carbon paste electrode is related to the reduction of oxygen and could be eliminated by continuous cycling in the deoxygenated buffer [20]. Cathodic currents

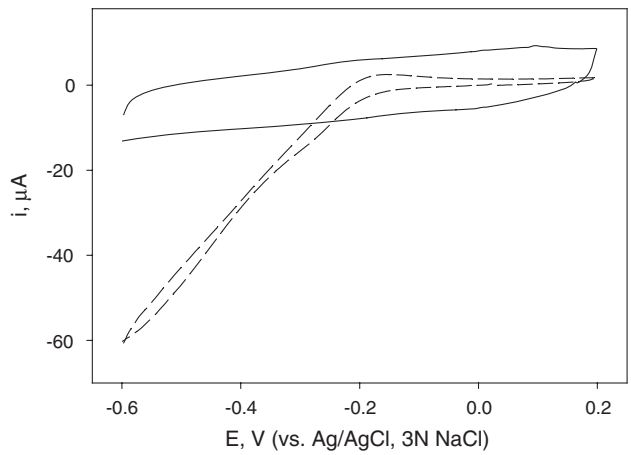

Fig. 1. Cyclic voltammograms of a plain carbon paste (dashed line) and CPBY (the sixth scan, solid line) in non-deoxygenated phosphate buffer pH 7.2 at a potential scan rate 2 mV/s.

below −0.2 V were not observed for freshly prepared CPBY.

Cyclic voltammograms of a plain carbon paste electrode and CPBY (sample E1) in PMS solution are shown in Fig. 2.

In the first cycle of the CPBY in 0.2 mM PMS in phosphate buffer at pH 7.2 (Fig. 2, dashed line), only one redox process related to oxidation–reduction of PMS with the $E^{o\prime}$ of about −0.14 V could be observed. During the prolonged cycling, the peak currents increased with each subsequent scan, the $E^{o\prime}$ shifted to a more positive potential of about −0.09 V (determined from the sixth cycle) and an additional pair of peaks appeared (Fig. 2, solid line). The $E^{o\prime}$ of this latter redox process was about −0.4 V. The values of peak separation (ΔE_p) at a sweep rate 2 mV/s were about 0.09 and 0.06 V, respectively. At higher potential sweep rates (10, 20 mV/s), the $E^{o\prime}$ practically did not changed whereas ΔE_p increased indicating lower reversibility of the redox processes. Cyclic voltammograms were almost identical in both non-deoxygenated and deoxygenated buffers. When the CPBY were transferred to phosphate buffer without PMS, voltammetry showed the same two redox processes, however, with lower peak current (i_p) values indicating that some amount of PMS was adsorbed onto the surface of electrode. However, the adsorption was not strong since during repeated cycling peak currents of both redox pairs gradually decreased. Only one electrochemical process with the $E^{o\prime}$ about −0.15 V was initially also observed at a plain carbon paste electrode. Cyclic voltammogram with an additional poorly defined pair of peaks with the $E^{o\prime}$ about −0.4 V (Fig. 2, dotted line) was recorded after prolonged cycling.

Voltammetry of carbon paste electrode prepared from graphite with adsorbed PMS revealed the redox process with the $E^{o\prime}$ at about −0.35 V (Fig. 3, dashed line).

The second redox process with the $E^{o\prime}$ about −0.07 V (Fig. 3, solid line) appeared when the paste contained both graphite with adsorbed PMS and baker's yeast. The addition of baker's yeast to PMS-modified graphite also exhibited the promotion of oxidation/reduction of the mediator as it was observed in our previous investigation [20]. Former studies of the electrochemistry of PMS adsorbed onto solid graphite electrode [24] or adsorbed onto Reinecke salt and admixed to graphite powder and epoxy resin [26] showed that in both these cases only one electrochemical process was observed with the $E^{o\prime}$ values of −0.16 V (vs. SCE) at pH 7 and −0.122 V (vs. Ag/AgCl, 3 M KCl) at pH 7.2, respectively. The values of the $E^{o\prime}$ of adsorbed PMS were similar to those of the soluble PMS. However, cyclic voltammetry of carbon paste electrodes containing PMS adsorbed onto zirconium phosphate [27] revealed two redox processes: the first one with the $E^{o\prime}$ of 0.1 V (shifted to more positive value compared to that of the soluble PMS) at pH 7 and an additional one with the $E^{o\prime}$ of −0.08 V (vs. SCE) probably due to some differences in the interaction of PMS and/or products of photochemical decomposition of PMS with zirconium phosphate. Redox process at more negative potential could probably be related to the product of PMS decomposition probably such as phenazine (the $E^{o\prime}$ of phenazine is about −0.48 V vs. SCE at pH 7).

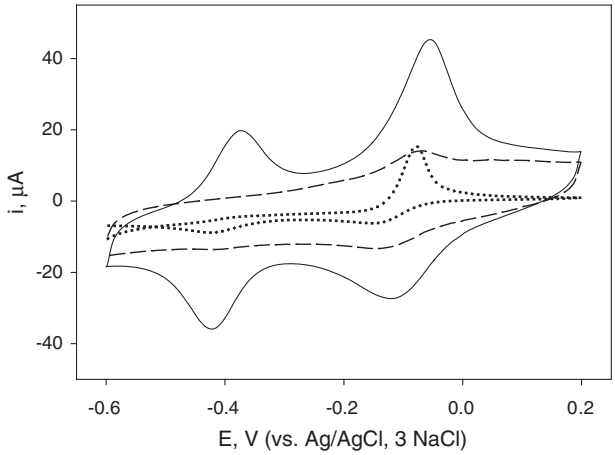

Fig. 2. Cyclic voltammograms of CPBY (dashed line and solid line for the first and the sixth scans, respectively) and a plain carbon paste electrode (the sixth scan, dotted line) in 0.5 mM PMS solution pH 7.2 at a potential scan rate 2 mV/s.

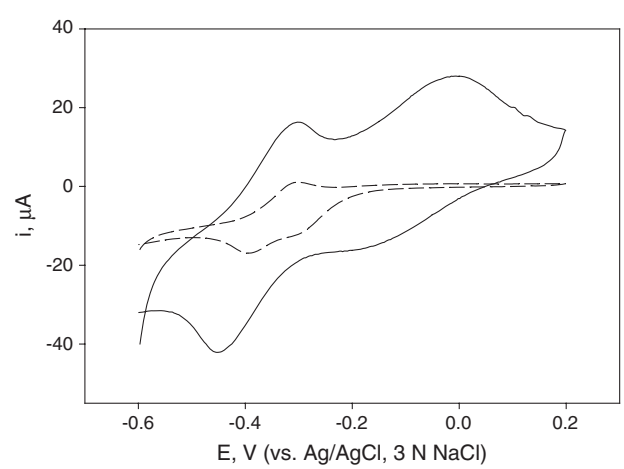

Fig. 3. Cyclic voltammograms of carbon paste electrodes prepared from graphite with adsorbed PMS (dashed line) and CPBY (the sixth scan solid line) in non-deoxygenated phosphate buffer pH 7.2 at a potential scan rate 2 mV/s.

The responses of CPBY to lactic acid in PMS solutions (0.1, 0.2, 0.5, and 1 mM) at various pH values (in the pH range 4–9) were tested at operating potentials −0.3, −0.2, −0.1, 0.0 and +0.1 V. All electrodes responded to lactic acid at potentials higher than −0.1 V, however, with different sensitivity and reproducibility of the results. Electrodes also differed in time needed to achieve the steady states of the background currents (10 to 40 min) before the measurements, in the values of the background currents (0.005 to 0.2 μA) and in the i_p values of the cyclic voltammograms. The concentration of PMS had a great influence on the reproducibility and stability of the responses. At high concentration of PMS (1 mM), the electrode responses decreased gradually with repeated additions of lactic acid. The reproducibility was improved by lowering the concentration of the mediator. The dependence of current responses on solution pH was similar to that observed for baker's yeast modified electrodes [20], for carbon paste electrodes modified with yeast *H. anomala* and various mediators [18] or for with flavocytochrome b_2, lipid and cytochrome *c* as a mediator [9]. The highest responses were obtained in the pH range 7 to 8. The most optimal operational conditions were determined to be as follows: an operating potential 0.0V, concentration of PMS 0.2 mM, solution pH 7.2. To obtain reproducible current responses, CPBY had to be kept at an open circuit for 30 min in 0.2 mM PMS solution. After that, the electrodes were poised at an operating potential 0.0V until the steady states of the background currents were achieved (10 to 30 min). The responses started after 5 to 10 s after the injection of lactic acid and achieved the steady states within 1 min. The apparent Michaelis constant (K_M') and the maximum value of the current (i_{max}) were obtained from Lineweaver–Burke plot (i^{-1} vs. [D,L-lactic acid]$^{-1}$). The results for six yeast samples are presented in Table 1. The dependence of the current responses of CPBY (sample L1) on the concentration of lactic acid is shown in Fig. 4. As racemic mixture of D,L-lactic acid contains equal amounts of D- and L-lactic acid and flavocytochrome b_2 oxidizes only L-lactic acid, actually the concentrations should be twice lower. But since a mixture was used, the results presented in Table 1 and Fig. 4 refer to D,L-lactic acid.

A linear range in the dependence of current responses on concentration of lactic acid for all electrodes was up to 1 mM (with R^2 value not lower than 0.998).

The loss in sensitivity of the electrodes during one-day measurements (60 to 70 injections of 50 μl of 0.05 M of lactic acid to 15 ml of buffer solution) did not exceed 10% of the initial value. After keeping the electrodes that have been in use overnight (dry or in phosphate buffer), further loss in sensitivity was observed (50% to 85%) whereas electrodes prepared from the pastes after 1 month of storage at room temperature showed 85% to 100% of the initial sensitivity.

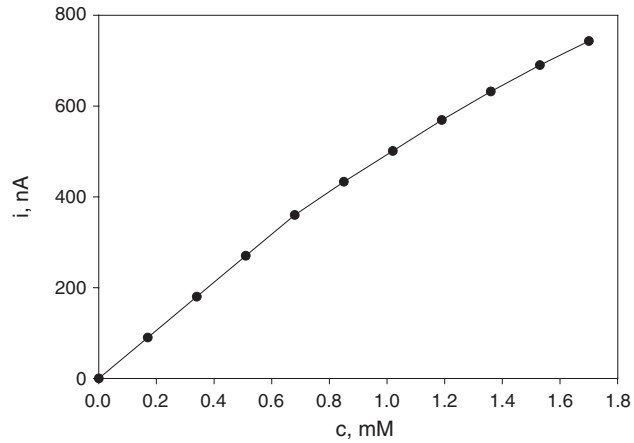

Fig. 4. Dependence of current responses of CPBY (sample L1) to D,L- lactic acid in 0.2 mM PMS solution at an operating potential 0.0 V.

All electrodes prepared from graphite with adsorbed PMS and baker's yeast also responded to lactic acid. However, the stability and the reproducibility of the responses were much lower compared to those of CPBY when the mediator was in the solution. Prolonged adsorption time and higher PMS concentration did not increase the stability of the current responses. The signals decreased with repeated measurements. Cyclic voltammograms of the electrodes that have already lost the sensitivity to lactic acid showed no peaks related to the oxidation/reduction of PMS. However, when transferred to the solution of PMS, the electrodes responded to lactic acid again.

Storage stability of the yeast-containing pastes depended markedly on the presence of the mediator. The loss of sensitivity of pastes prepared from graphite with adsorbed PMS was much faster compared to that without adsorbed mediator.

Experiments related to the investigation of the viability of the yeast cells (samples E1, E3) showed that both paste and control probe suspensions gave almost equal number of colonies within the limits of the reliability (±10%). Thus, the yeast cells did not lose their viability during preparation of the paste. No significant drop of the yeast cell viability was observed in the period of 28 days. This phenomenon probably

Table 1
Characteristics of baker's yeast-modified carbon paste electrodes at an operating potential 0.0 V and phenazine methosulphate concentration 0.2 mM in phosphate buffer at pH 7.2

Sample	Current response to 0.17 mM D,L-lactic acid (nA)	K_M'(mM)	i_{max} (μA)
L1	114	24.1	16.1
L2	68	5.9	2.4
L3	76	7.1	3.3
E1	92	3.6	2.1
E2	91	7.4	4.1
E3	97	4.6	2.6

Table 2
Concentrations of L-lactic acid in milk dairy products (diluted 300 times) determined by using baker's yeast (L3 or E3)-modified carbon paste electrode at an operating potential 0.0 V and a soluble mediator phenazine methosulphate (0.2 mM in phosphate buffer pH 7.2)

Days	L-lactic acid in kefir (mM)	L-lactic acid in yoghurt (mM)
1	0.42±0.03	0.37±0.03
2	0.52±0.01	0.41±0.02
3	0.52±0.01	0.44±0.03
4	0.54±0.03	0.46±0.01

Gradual increase of the amount of L-lactic acid with time was observed.

refers to the conservation of the yeast cells in the paste and needs further investigation. It was also observed that when the pastes contained adsorbed PMS (or Prussian Blue [20]) the loss of sensitivity to lactic acid was much faster: after 3 weeks the current responses were less than 40% of the initial. Besides, cyclic voltammetry at electrodes prepared from non-sensitive pastes showed reduction currents below −0.2 V (whereas for electrodes that did not contain adsorbed mediators these currents did not appear after 1 month.) Viability tests showed much lower amounts living cells.

CPBY were tested for the sensitivity to glucose, ethanol (common yeast metabolites), ascorbic acid and acetaminophen in 0.2 mM PMS solutions at pH 7.2. The electrodes were not sensitive to the injection of 0.17 mM of glucose, ethanol and acetaminophen at an operating potential 0.0 V. However, the electrodes remained sensitive to ascorbic acid. The response to 0.17 mM of ascorbic acid at CPBY (sample L2) was 190 nA that was more than 2 times higher than that at a plain carbon paste electrode (80 nA) under the same conditions.

CPBY were tested for suitability to determine lactic acid in milk and dairy products such as kefir and yoghurt that did not contain ascorbic acid. It has been observed that the amounts of L- and D-lactic acids in dairy products are influenced by lactic acid bacteria starter culture and conditions of manufacture [36–38]. 50 μl of milk or filtered kefir or yoghurt were injected into 15 ml of phosphate buffer at pH 7.2, i.e., the samples were diluted 300 times. Determination of low concentrations of lactic acid in milk (1 and 2 day) was complicated by detection limits of the electrodes (16–21 μM, calculated from the response ratio to noise 3 : 1) and probable electrode fouling with milk proteins when higher amounts of milk were injected (since electrode sensitivity after milk analysis was 20–25% lower compared to that before analysis). The fouling was practically avoided when kefir and yoghurt filtrates were used. The changes in the concentration of lactic acid were monitored for 4 days. The results are presented in Table 2 (each concentration is a mean value of three measurements).

4. Conclusions

Carbon paste electrodes modified by baker's yeasts *S. cerevisiae* in combination with soluble mediator phenazine methosulphate could be used for the determination of lactic acid at an operating potential 0.0 V (vs. Ag/AgCl, 3 N NaCl). The electrodes were suitable for the determination of lactic acid in the concentration range 0.03 to 1 mM. The advantages of the electrodes were the simple preparation from the cheap source of the enzyme flavocytochrome b_2 and stability of the prepared pastes. The disadvantages of the electrodes were long time to achieve reproducible results, high noise level, thus, high detection limit and sensitivity to ascorbic acid.

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