

Application of L-lactate-cytochrome c-oxidoreductase for development of amperometric biosensor for L-lactate determination

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***Aim.** Development of amperometric biosensor based on L-lactate-cytochrome c-oxidoreductase (flavocytochrome b_2 , FC b_2) for lactate determination. **Methods.** All experiments were performed using the amperometric method of detection. The methods of electrochemical polymerization and immobilization in glutaraldehyde vapors were used for FC b_2 immobilization on the surface of electrodes. **Results.** The FC b_2 preparation, which demonstrated the best operational characteristics after immobilization in poly (3,4-ethylenedioxythiophene), was selected. The selectivity, operational and storage stability, and pH-optimum for operation of the created biosensor were determined. The analysis of L-lactate in the model solutions and wine samples was carried out using the developed biosensor. **Conclusion.** The FC b_2 -based biosensor due to its high stability can be effectively used for lactate determination in blood and other liquids containing no ethanol. After the selectivity optimization, the device can be also applied for wine analysis.*

Keywords: amperometric biosensor, flavocytochrome b_2 , L-lactate.

Introduction. To date, development of an amperometric biosensor for lactate analysis in wine-making is an important vital challenge. Numerous methods, traditionally used for the analysis of lactate concentration in wine (liquid chromatography, capillary electrophoresis, spectrophotometry and

fermentative method), often do not guarantee sufficient selectivity and sensitivity. Besides, these methods are hardly compatible with the wine technology because of complex and bulky equipment, necessity of sample pretreatment, laborious and durable analytical procedure [1].

Meanwhile, lactate is a substance to be thoroughly controlled at all stages of wine making since it indicates bacterial activity during must fermentation and, concurrently, determines quality and aroma of final product and wine stability at storage [1, 2].

An effective tool for the discrete and continuous lactate monitoring during wine production can be an amperometric biosensor – a device of small dimensions, simple and low-cost in usage, which requires neither sample pretreatment, nor complex equipment nor high-qualified maintenance staff [3]. An application of biosensors in food industry is indisputably advantageous due to their high sensitivity, a possibility of analysis of turbid solution, high compatibility with advanced microelectronic techniques, and small energy consumption [4]. Therefore, no wonder that biosensors are ever more widely applied in analysis of food quality, in particular, in wine production [5].

To develop amperometric biosensors intended for lactate determination in wine, most frequently NAD⁺-dependent lactate dehydrogenase or lactate oxidase (LOD) are used. An analysis of working characteristics of the biosensors based on these enzymes shows that the transducers with immobilized LOD have wider linear range and lower detection limit as compared with those based on lactate dehydrogenase. An essential advantage of oxidase biosensors is that there is no need in an exogenous cofactor for their functioning that considerably facilitates the analysis procedure [3]. Therefore, lactate amperometric biosensors based on immobilized LOD seem to be more promising for wine analysis.

Recently we have developed the immobilized LOD-based amperometric biosensor for quantitative lactate determination in wine [2]. It has perfect working characteristics: dynamic range of 0.008 – 1.0 mM and high selectivity. Its application in lactate analysis of wine and must was a success, the results correlated with those obtained by the traditional HPLC method.

However, this biosensor was disadvantageous due to low operational and storage stability: during first three hours of continuous work the sensor signal lost up to 70% of its initial value, and this level of activity was retained for next three days. Noteworthy, that 30% activity was sufficient for lactate analysis. Nevertheless, for optimization of lactate biosensor it was necessary to increase its stability.

An analysis of the published data showed that low operational stability is regularly inherent to the biosensors based on immobilized LOD. For instance, the oxidase biosensor for lactate determination in wine [6] demonstrated only 35% of initial activity after 150 measurements. In [7] the LOD sensor is described to lose half its activity just after the first measurement, and in 5-hour continuous operation it demonstrated 40% of the initial signal [8].

Decrease in the immobilized enzyme activity can be caused by a number of factors, i.e. temperature denaturation, proteolytic degradation, non-specific oxidation catalyzed by metals, changes of pH, solution ion strength, etc. [9]. Besides, electrode pollution with foreign proteins and other substances present in a tested solution can also be the reason [8]. Determination of the precise mechanism of enzyme inactivation and elaboration of a preventing procedure is an ideal tactic to solve the problem of increasing biosensor stability.

One of the methods of improvement of enzyme stability is a direct site-specific mutagenesis which results in higher enzyme lipophilicity or replacement of its catalytic groups to the sites more impenetrable to environmental effects [9]. It is evident that this approach is rather laborious and long-term and is effective only if the enzyme production is self-dependent.

Another method to increase biosensor stability is stabilizing additions, such as polyelectrolytes, osmolytes, alcohols, proteins-shaperonines, sugars, salts or nanoparticles of gold, zinc oxide, ferric oxide Fe₃O₄ [9 – 12]. These additions were shown to interact electrochemically with the enzyme, sometimes even to capsule it, thus changing the enzyme conformation and increasing its stability [9]. These stabilizers can be either introduced at the enzyme immobilization as a component of sensitive membrane, or added into the working buffer during measurements and storage of sensors.

Use of stabilizing substances seems to be an effective approach, however, the selection of optimal complex of stabilizers for a certain enzyme immobilized in a certain way is rather a time-consuming procedure. Besides, a complicated, multi-step immobilization of an enzyme together with stabilizers potentially decreases sensor-to-sensor response reproducibility; some salts-additions can cause an opposite effect, i.e.

enzyme destabilization [9]. Introduction of stabilizers into working solution at analysis of real liquids complicates the analytical procedure and probably can have an interfering effect on biosensor function.

According to the published data, the most essential reason of considerable drop of the activity of LOD, immobilized into sensitive membrane, is partial enzyme denaturation by hydrogen peroxide which, being generated in the reaction of lactate disintegration, oxidizes amino acids of the catalytic centre [8, 11]. Therefore an alternative way to improve stability of lactate transducer is using instead of LOD another enzyme, the functioning of which is not associated with generation of hydrogen peroxide.

The enzyme, able to be a biorecognition element of the lactate biosensor, is L-lactate-cytochrome c-oxidoreductase (EC 1.1.2.3; flavocytochrome b_2 (FC b_2) catalyzing electron transfer from L-lactate to cytochrome c in yeast mitochondria. FC b_2 can be extracted from *Saccharomyces cerevisiae*, *Hansenula anomala* or *Hansenula polymorpha* as a homotetramer, each subunit of which contains one molecule of flavine mononucleotide and one molecule of proheme IX [13]. FC b_2 functions *in vitro* in the presence of a number of synthetic acceptors of electrons that conditions the potential of this enzyme in analytical biotechnology. The investigations [14] show better FC b_2 storage stability (on the 5th day after immobilization the enzyme activity is half its initial value) and operational stability (50% of initial signal is registered after 6 hours of continuous work) as compared to these parameters for LOD.

Study on selectivity of the FC b_2 -based biosensors showed the absence of non-specific responses to malate, pyruvate, acetate, and isocitrate [13, 14]. As to the main wine components, ethanol and glucose, they, together with ascorbic acid and phenolic substances, essentially interfere in quantitative determination of lactate in wines and must [2]. This is why selectivity towards these substances is the greatest challenge, once the biosensor is developed to analyze lactate in wine materials.

This work was aimed at the development of lactate amperometric biosensor on the basis of immobilized flavocytochrome b_2 and evaluation of advantages and drawbacks of its application for wine analysis as com-

pared to the sensor based on immobilized lactate oxidase.

Materials and methods. *Materials.* Three preparations of the enzyme FC b_2 extracted from *Hansenula polymorpha* were used. Preparation FC b_2 №1 prepared in 40% ammonium sulphate (AS) had specific activity of 3 U/mg, protein concentration of 4.8 mg/ml; preparation №2 – in 80% AS; preparation №3 – specific activity 0.75 U/mg, protein concentration 13.9 mg/ml.

Monomer 3,4- ethylenedioxythiophene (EDT), production of Baytron M (Germany) and poly(ethylene glycol) MM = 1450, production of «Sigma» (Switzerland) were used as a matrix for the enzyme electrochemical polymerization.

Reagents $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, KH_2PO_4 , sodium lactate and potassium ferricyanide («Sigma», USA), hydrogen peroxide («Phargomed», Ukraine), glucose, L-ascorbic acid and bovine serum albumin (BSA) («Sigma-Aldrich Chimie S.a.r.l.», France), ethanol («Fluka», Germany), glutaraldehyde («Fluka», Switzerland), glycerol (Ukraine) were also used. All the reagents, both domestic and imported, were of analytical reagent grade and used as received without additional purification.

Measurement. All electrochemical experiments were performed by the traditional three-electrode system in which screen-printed electrode SensLab («SensLab GmbH», Leipzig, Germany) combined all three electrodes, i.e. platinum working, auxiliary, and referent.

Platinum screen-printed electrodes SensLab were tested regarding reproducibility and operational capacity within the range from 0 to +600 mV (potential scan rate of 20 mV/s). Cyclic voltamperometry was carried out by the potentiostat PalmSens (Palm Instruments BV, Netherlands). The amperometric device used in the work consisted of potentiostat PalmSens, electrochemical cell and computer (general view - in Fig. 1).

The cyclic voltammogram was obtained using a platinum electrode SensLab with FC b_2 immobilized in the working buffer at addition of 2 mM potassium ferricyanide and 1 mM lactate (Fig. 2). As seen, an insertion of the substrate and the mediator into the working cell results in generation of oxidizing current and increase of transducer signal. We chose the potential of +450

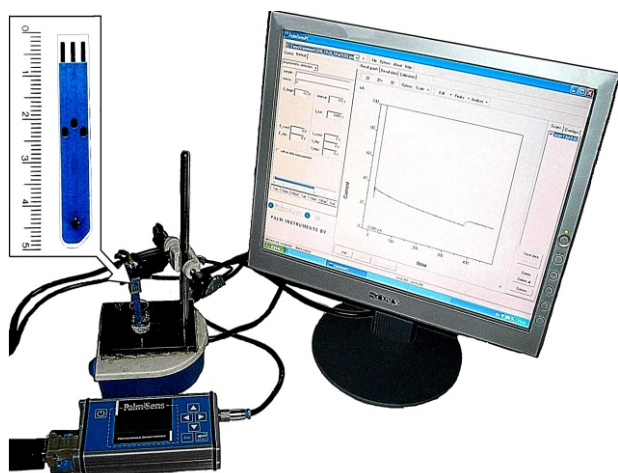


Fig. 1. General view of amperometric device with potentiostat PalmSens.

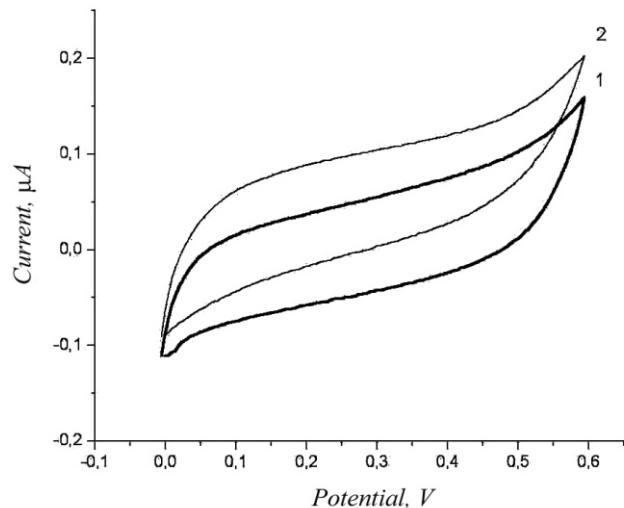


Fig. 2. Cyclic voltamperogram obtained by commercial screen-printed platinum electrodes SensLab with immobilized FC b_2 in phosphate buffer (1) and at addition of 2 mM potassium ferrocyanide and 1 mM lactate(2).

mV as a working parameter since it is the value of redox potential of potassium ferricyanide [15].

Amperometric measurement was carried out by the potentiostat PalmSens in a 5-ml electrochemical cell at a constant potential.

FC b_2 immobilization by electrochemical polymerization in polymer EDT. Electrochemical polymerization as a method of enzyme immobilization is thoroughly described in [2]. For electrochemical polymerization, the component mixture, consisting of 10^{-2} M

3,4- ethylenedioxythiophene (EDT), 10^{-3} M polyethylene glycol, and 3 μ l FC b_2 solution, was prepared in 20 mM phosphate buffer, pH 6.2.

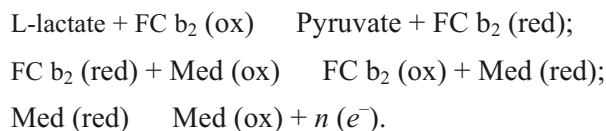
EDT was polymerized at the potential from + 0.2 V to + 1.5 V, applied for 15 cycles at the scan rate of 0.1 V/s.

FC b_2 immobilization in glutaraldehyde (GA) vapour. Bioselective membranes were formed in the mixture of 3 μ l FC b_2 solution and 5% BSA (1:3) in 10 mM phosphate buffer, pH 7.2. The mixture was deposited on the working electrode surface. For polymerization, the transducers were placed for 10 min into saturated vapour of glutaraldehyde, whereupon they were dried in the air.

Lactate determination in model solutions. Measurement was carried out at room temperature in an open vessel at intensive stirring. 20 mM solution $\text{KH}_2\text{PO}_4 - \text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, pH 7.6, was used as a working buffer, 2 mM potassium ferricyanide – as a mediator.

Substrates concentration was changed by addition of defined aliquots of concentrated solutions. After each signal, the sensor was washed in a buffer solution until the base signal was stabilized.

Results and discussion. Operation of FC b_2 -based amperometric biosensors is based on the enzymatic reaction with mediator Med [3]:



The electrons, which are generated as a result of these reactions and density of which is proportional to the lactate concentration in the working cell, are registered by the amperometric transducer.

At the first stage of the research, FC b_2 was immobilized in GA vapour in the BSA membrane. To select the best enzyme preparation the laboratory prototypes were developed on the basis of three FC b_2 preparations different by the activity and presence of additives. The preparations 1 and 2 after immobilization in GA vapours appeared to be inactive and did not respond to lactate. The biosensor based on preparation 3 immobilized in GA vapours demonstrated low (12 nA) signals

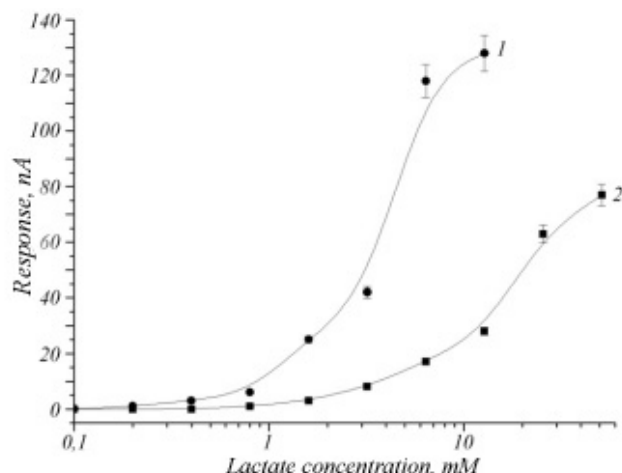


Fig. 3. Calibration curves of laboratory prototypes of amperometric biosensor obtained by FC b_2 electrochemical polymerisation in polymer PEDT: 1 - preparation 1, 2 - preparation 2. Measurement in 20 mM phosphate buffer, pH 7.6, potential of +450 mV vs reference electrode.

to lactate, its working dynamic range was 0.002 – 0.032 mM. Study on the biosensor selectivity showed that it gives practically no response to glucose, essential non-specific responses to other interfering substances, responses to ascorbic acid being negative. Considering that in the experiments interfering substrates were of the concentrations equal to maximal in wine [1], and lactate concentration corresponded to saturation of the biosensor calibration curve, we stated that the sensor based on preparation 3, immobilized in GA vapours, has insufficient selectivity and, thus, it is unusable for the lactate measurement in wine and must.

Examination of storage stability showed that the sensor activity in one day after immobilization was 18% of initial value; operational stability was completely lost in one hour of continuous work.

Since FC b_2 immobilization in GA vapours gave inadequate results, an alternative immobilization method was studied, i.e. electrochemical polymerization in PEDT. All the preparations, immobilized in this way, appeared to be active. Calibration curves of the laboratory prototypes with preparations 1 and 2 are presented in Fig. 3. The sensor based on preparation 3 demonstrated very small (no more than 11 nA) re-

sponses to lactate and working range within 0.001 – 0.06 mM substrate concentration.

To select the most effective variant of FC b_2 preparations, the developed biosensors using three of them, were compared regarding their operational dynamic ranges, limits of detectable lactate concentrations, selectivity, operational and storage stability.

It was revealed that the biosensor with preparation 3 immobilized in PEDT demonstrated considerably lower, as compared to other preparations, detectable limit (1 μ M lactate), though its dynamic range was short, and the signal did not exceed 11 nA, which restrict its application for the lactate analysis in real liquids. The sensors based on preparations 1 and 2 are featured by wide dynamic range (0.2 – 6.4 mM and 0.8 – 25.6 mM lactate, correspondingly) the signal up to 70 – 130 nA. At the same time, their limit of detectable substrate concentration is rather high, which makes it difficult to analyze wines with low lactate content.

The results of examination of operational stability (Fig. 4a) show that the sensor with preparation 1 loses 40% of its initial activity over the first hours of continuous operation, thereafter the signal practically does not change, i.e. the response is highly reproducible. The reason of activity decrease can be a segregation of weakly adherent parts of the sensitive membrane from the electrode surface and/or wash-out of poorly immobilized enzyme molecules into the solution [8]. Operational stability at using preparation 2 is worse, sensor activity decreases throughout the whole period of continuous work which results in poor signal reproducibility. Operational stability of the biosensor with preparation 3 which completely loses its initial activity during three hours is unsatisfactory.

Storage stability (Fig. 4b) of the biosensor with preparation 3 is also very low; its activity is completely lost over 5 days. Stability of sensor with preparations 1 and 2 is better, they demonstrated about 30% initial signal in 90-day storage.

Further work was aimed at examination of selectivity of amperometric biosensors based on three FC b_2 preparations. The responses were obtained as lactate (in concentrations corresponding to a higher limit of the dynamic range of each transducer) and main interfering substances, i.e. ethanol, glycerol, glucose, ascor-

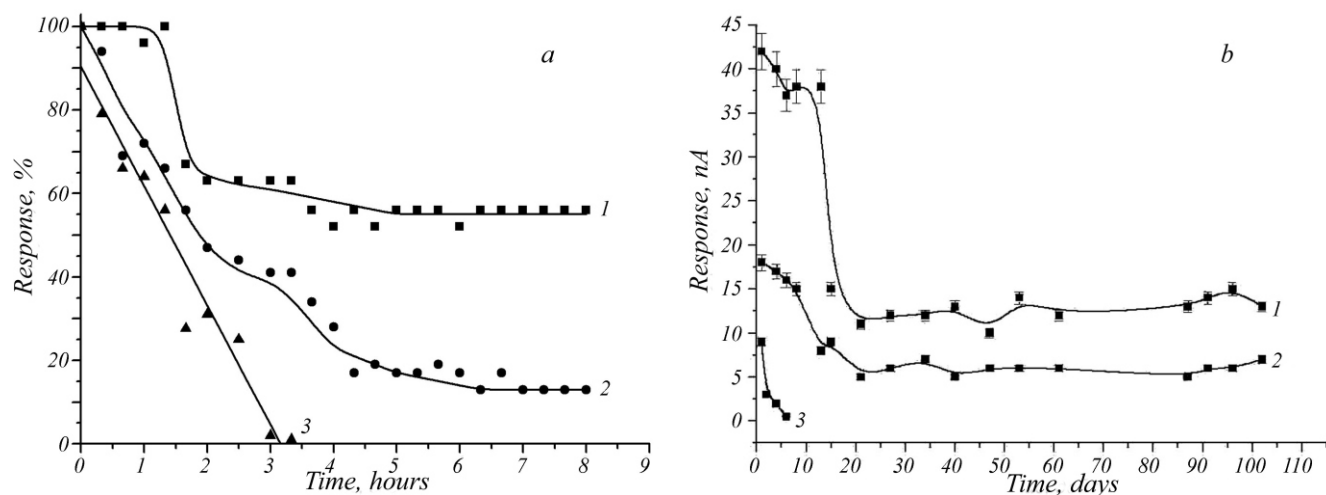


Fig.4. Operational (a) and storage (b) stability of amperometric biosensor based on FC b_2 immobilized in polymer PEDT: 1 - preparation 1; 2 - preparation 2; 3 - preparation 3. Final concentration of lactate in measurement cell: 3.2 mM - for preparations 1 and 2; 0.032 mM - for preparation 3. Measurement in 20 mM phosphate buffer, pH 7.6, potential of +450 mV vs reference electrode.

Table 1.

Responses of laboratory prototypes of amperometric biosensors with different FC b_2 preparations to lactate (at saturation) and main interfering substances in wine

Preparation FC b_2 №	Response to, nA				
	Lactate	Ethanol, 15 mM	Glycerol, 1 mM	Glucose, 5 mM	Ascorbic acid, 0.13 mM
1	118	150	10	0,5	-3
2	63	53	7	3	0,5
3	11	40	9	7	-31

bic acid (in maximum concentrations actual for wines), were inserted into electrochemical cell (Table 1).

As seen, selectivity of preparation 3 is the worst; it gives, alike at immobilization in GA vapour, essential non-specific responses to main interfering substances (positive signals to ethanol, glycerol, glucose, and negative – to ascorbic acid) which even exceed the lactate signal. This can be caused by poor purification of the preparation. As to the preparations 1 and 2, they have better selectivity, practically do not respond to ascorbic acid, glucose and glycerol, though the ethanol signal is close to that to lactate.

The characteristics of amperometric biosensors based on three FC b_2 preparations immobilized in PEDT, are compared with those of the biosensor based on LOD immobilized in PEDT which we developed earlier (Table 2).

Taking into consideration the results obtained, the FC b_2 preparation 1 was chosen from three tested preparations as an optimal variant by dynamic range and stability for the development of amperometric lactate biosensor. Operational and storage stability of the transducer based on this preparation is better than of LOD-based biosensor. Nevertheless, its application in wine analysis was inefficient: because of poor selectivity the measurement results exceeded actual values of lactate concentration in wine by several times.

The essential non-specific signals of biosensors with immobilized FC b_2 can be explained by insufficient selectivity of FC b_2 or by relatively high working potential (+450 mV) which conditions the oxidation of interfering particles and, correspondingly, generation of the amperometric electrode response.

Table 2.

Comparative analysis of laboratory prototypes of amperometric biosensors with different FC b_2 preparations and LOD-based biosensor

Enzyme	Minimum measured lactate concentration, mM	Working dynamic range, mM	Operational stability (%) at continuous work during			Residual storage stability (%)		
			1 h	3 h	8 h	in 1 day	in 5 days	in 100 days
FC b_2 № 1 (14,4 U/ml)	0,2	0,2–6,4	96	63	56	100	93	36
FC b_2 № 2 (6,5 U/ml)	0,8	0,8–25,6	72	41	13	94	89	38
FC b_2 № 3 (10,4 U/ml)	0,001	0,001–0,064	64	2	0	30	10	–
LOD (39 U/mg)	0,008	0,008–1	89	33	25	30	18	–

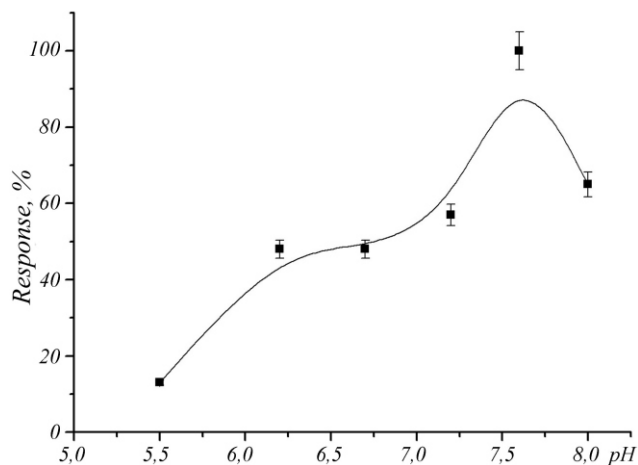


Fig. 5. Dependence of response of amperometric biosensor based on FC b_2 (preparation 1), immobilized in polymer PEDT, on pH of working solution. Concentration of lactate added into measurement cell - 6.4 mM. Measurement in 20 mM phosphate buffer, potential of +450 mV vs reference electrode.

Noteworthy, the LOD-based amperometric biosensor, developed by us, had high selectivity: it demonstrated small non-specific response only to ethanol in high concentrations nonrelevant for wines [2]. In contrast to the biosensor with immobilized FC b_2 , the LOD-based biosensor works at lower potential (+200 V), at which an effect of interfering substances on biosensor functioning considerably less.

At the last stage of the research pH-optimum for the biosensor with FC b_2 preparation 1 was determined to be 7.6 (Fig. 5) which well correlates with the data ob-

tained earlier [14]. Besides, the value of response was shown to be practically independent on the buffer capacity and ion strength of tested solution (Fig. 6a, 6b), which is characteristic for amperometric biosensors, including the LOD-based lactate sensor [16].

Conclusion. The investigation showed that the FC b_2 preparation 1 (40% ammonium sulphate, specific activity 3 U/mg, protein concentration 4.8 mg/ml), immobilized by electrochemical polymerization in PDT polymer, is the most effective for the development of amperometric lactate biosensor based on flavocytochrome b_2 . This biosensor demonstrates better operational and storage stability as compares with the sensor based on lactate oxidase immobilized in PEDT. On the other hand, the latter is advantageous by sensitivity and selectivity, these parameters being decisive to choose a device for the analysis of wine and must - complex mixtures, lactate concentration in which is extremely low. The results of lactate measurement in wine samples by the LOD-based biosensor proved its efficiency while those obtained by the biosensor with immobilized FC b_2 appeared to be unreliable. That is why the LOD-based biosensors are considered as preferable for the application in wine making. The FC b_2 based biosensor due to its high stability can be beneficial for the analysis of other liquids, e.g. blood, in which ethanol is absent while lactate concentration is rather high (0.5 – 2.2 mM [8]) and is within the dynamic range of the sensor under consideration.

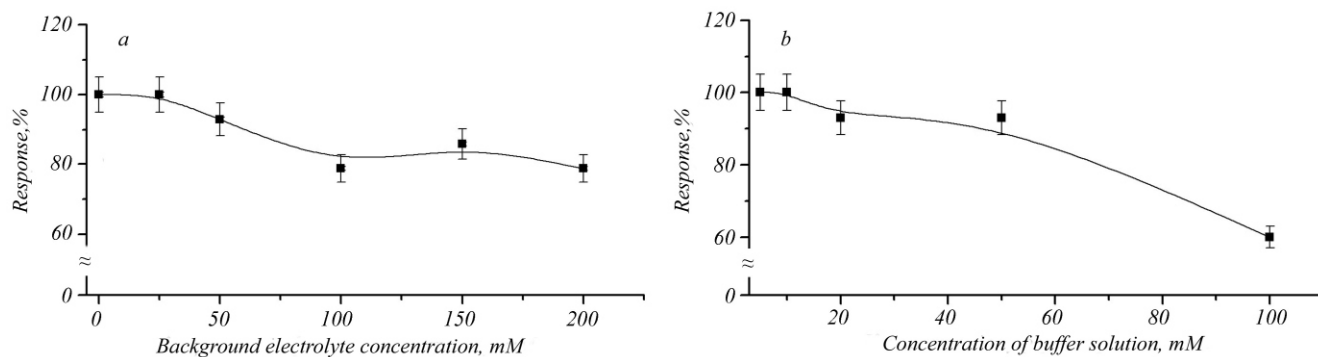


Fig. 6. Dependence of response of amperometric biosensor based on FC b_2 (preparation 1), immobilized in polymer PEDT, on concentration of background electrolyte in buffer (a) and concentration of buffer solution (b). Final concentration of lactate in a measurement cell was 6.4 mM. Measurement in phosphate buffer, pH 7.6, potential of +450 mV vs reference electrode

As it was shown in the previous investigation [13], an essential improvement of the selectivity and sensitivity of FC b_2 -based biosensor could be achieved by applying a lower working potential when mediator with a low redox potential and FC b_2 preparations with a higher specific activity were used. Therefore, we are planning to continue our investigations in this direction.

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Використання L-лактат-цитохром c-оксидоредуктази для створення амперометричного біосенсора при визначенні L-лактату

Резюме

Мета. Розробити амперометричний біосенсор для визначення L-лактату на основі L-лактат-цитохром c-оксидоредуктази (флавоцитохрому b_2 , ФЦ b_2) дріжджів *Hansenula polymorpha*. **Методи.** Експерименти проведено з використанням амперометричного методу вимірювання. Для іммобілізації ФЦ b_2 на поверхню електродів застосовано методи електрохімічної полімеризації та іммобілізації в парах глутарового альдегіду. **Результати.** Обрано препарат ферменту ФЦ b_2 , який після іммобілізації у полі-3,4-етилендіокситіофені демонструє найкращі робочі характеристики. Досліджено селективність, операційну стабільність та стабільність при зберіганні створеного біосенсора, встановлено рН-оптимум його роботи. За

допомогою розробленого високостабільного біосенсора проаналізовано концентрацію L-лактату у модельних розчинах та зразках вина. **Висновки.** Біосенсор на основі ФЦ b_2 , перевагою якого є висока стабільність, можна застосовувати для аналізів крові та інших рідин, які не містять етанолу, а після оптимізації селективності біосенсора – і для аналізу вина.

Ключові слова: амперометричний біосенсор, флавоцитохром b_2 , L-лактат.

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Использование L-лактат-цитохром c-оксидоредуктазы при создании амперометрического биосенсора для определения L-лактата

Резюме

Цель. Разработать амперометрический биосенсор для определения L-лактата на основе L-лактат-цитохром c-оксидоредуктазы (флавоцитохрома b_2 , ФЦ b_2) дрожжей *Hansenula polymorpha*. **Методы.** Все эксперименты проводили с использованием амперометрического метода измерения. Для иммобилизации ФЦ b_2 на поверхность электродов применяли методы электрохимической полимеризации и иммобилизации в парах глутарового альдегида. **Результаты.** Выбран препарат фермента ФЦ b_2 , демонстрирующий после иммобилизации в поли-3,4-этилендиокситиофене наилучшие рабочие характеристики. Исследованы селективность, операционная стабильность и стабильность при хранении созданного биосенсора, установлен рН-оптимум его работы. С помощью разработанного высокостабильного биосенсора проведен анализ концентрации L-лактата в модельных растворах и образцах вина. **Выводы.** Биосенсор на основе ФЦ b_2 , преимуществом которого является высокая стабильность, можно эффективно использовать для анализов крови и других жидкостей, не содержащих этанол, а после оптимизации его селективности – и для анализа вина.

Ключевые слова: амперометрический биосенсор, флавоцитохром b_2 , L-лактат.

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