# D-lactate-selective amperometric biosensor based on the mitochondrial fraction of *Ogataea polymorpha* recombinant cells

Oleh Smutok[1,*], Maria Karkovska[1], Tetiana Prokopiv[1], Taras Kavetskyy[2,3], Wladimir Sibirnyj[4], Mykhailo Gonchar[1]

[1]Department of Analytical Biothecnology, Institute of Cell Biology NAS of Ukraine, Drahomanov Street 14/16, 79005, Lviv, Ukraine

[2] Drohobych Ivan Franko State Pedagogical University, 82100 Drohobych, Ukraine

[3] The John Paul II Catholic University of Lublin, 20-950 Lublin, Poland

[4]Department of Bioenergy Technologies, Faculty of Biology and Agriculture, University of Rzeszow, Zelwerowicza 4, Rzeszow 35-601, Poland.

## ABSTRACT

During the recent decades, a lot of data about the significance of D-lactate determination in food technology and quality control has been accumulated. Nowadays, the development of new methods for the determination of D-lactate is very relevant, especially with regards to biosensors. To construct a D-lactate-selective biosensor, we suggest using the mitochondria of recombinant yeast cells of *Ogataea (Hansenula) polymorpha* "tr6" (*gcr1 catX/Δcyb2, prAOX_DLDH*) overproducing D-lactate: cytochrome *c*-oxidoreductase (DLDH, EC 1.1.2.4) and lacking an L-lactate-specific enzyme (flavocytochrome $b_2$, E.C. 1.1.2.3). The usage of the pure enzyme is problematic due to the complexity of its isolation and stabilization because of the intramembranous localization of DLDH. The enzyme catalyzes D-lactate oxidation to pyruvate coupled with ferricytochrome *c* reduction to ferrocytochrome *c*.

The constructed biosensor is characterized by high sensitivity (18.5 $A \cdot M^{-1} \cdot m^{-2}$), a low detection limit (3 μM of D-lactate), wide linear ranges, good selectivity and sufficient stability. The real samples analysis of D-lactate in dairy products was performed, and high correlation of the obtained results with the reference approach ($0.7 < r < 1$) and literature data was demonstrated.

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*Corresponding author email: smutok@cellbiol.lviv.ua



## INTRODUCTION

Lactic acid (lactate) is among the most important analytes since it is a universal metabolite of nearly all the living organisms (Thornalley et al., 1990). L- and D-lactic acids are stereoisomers, different in the orientation of the a-hydroxy group. D-lactate is a natural component of many fermented foods like yogurts (de Vrese et al., 1990; Nikolaus & Strehlitz, 2009), sour milk (Scheele, 1931), cheeses (https://secure.megazyme.com/D-Lactic-Acid-Assay-Kit.pdf) and pickled vegetable products (Zhang et al., 2003).

D-lactate can be metabolized slowly in the human body in comparison with the L-lactate isomer and can cause metabolic disorders if ingested in excess. Therefore, the World Health Organization (WHO) recommends a limited daily consumption of D-lactate up to 100 $mg \cdot kg^{-1}$ body weight. Moreover, some countries, *e.g.* Germany, have decided to minimize the D-lactate content of fermented dairy products (Zourari et al., 1992). The high concentration of D-lactate is toxic for children (Stolberg et al., 1982) and people with short bowel syndrome (Hudson et al., 1990; Day & Abbott, 1999; Gurukripa Kowlgi & Chhabra, 2015). Also, D-lactate toxicity depends on the physiological state of renal excretion. Hingorani *et al*. demonstrated that in healthy men continuously infused with a D-lactate solution, excretion rates ranged between 61 and 100 % (Hingorani et al., 1993). In 1993, there was a reported case of chronic D-lactate encephalopathy occurring in a patient with short bowel syndrome and end-stage renal disease (Thurn, et al., 1985).

Nowadays, many physicochemical and chemical methods for the assay of L-lactate have been developed, however, differential determination of lactate enantiomers is rather complicated. It is possible to use ion chromatographic separation or HPLC to determine both forms, but the pretreatment of samples is necessary to distinguish between D- and L-lactate (Rich et al., 1980; Olieman & de Vries, 1988; Okubo et al., 2000).

There are also a number of described photometric and amperometric methods for D-lactate assay, usually based on $NAD^+$-dependent D-lactate dehydrogenase ($NAD^+$-DLDH EC 1.1.1.28) (Noll, 1966; Avramescu et al., 2002; Tap et al., 2002; Pohanka & Zbooil, 2007). A weak point of $NAD^+$-DLDH-based approaches is shifting the equilibrium constant far

towards the D-lactate side, and hence the reaction of the product with toxic compounds, *e.g.* hydrazine, or the use of an additional enzyme such as D-glutamate-pyruvate transaminase becomes necessary to shift the equilibrium towards the product side (Noll, 1966; https://secure.megazyme.com/D-Lactic-Acid-Assay-Kit). On the other hand, they are expensive due to a high price of exogenous cofactors and additional enzymes. Recently there has been described a complex conductometric biosensor for the determination of total lactate, L- and D-lactate in dairy products which combine $NAD^+$-DLDH-based and lactate oxidase (LO) biosensors (Nguyen-Boisse et al., 2013). However, both approaches require additional components: co-enzyme $NAD^+$ (for biosensor based on bacterial DLDH) and horseradish peroxidase (for LO biosensor).

In our opinion, a promising candidate for the construction of D-lactate-selective amperometric biosensor is the D-lactate oxidoreductase of yeast (cytochrome *c*-dependent dehydrogenase, DLDH, EC1.1.2.4). DLDH is a respiratory enzyme involved in electron transfer, located on the cytoplasmic side of the inner membrane of mitochondria (Dym et al., 2000). The enzyme is a FAD- and $Zn^{2+}$-containing protein found in yeast and bacteria with the molecular weight of 63 kDa for DLDH from *Kluyveromyces lactis* and 64 kDa – for DLDH from *Saccharomyces cerevisiae*, respectively (Lodi & Ferrero, 1993; Lodi et al., 1994) DLDH catalyzes D-lactate oxidation to pyruvate coupled with ferricytochrome *c* reduction to ferrocytochrome *c*. The enzyme is selective with respect to the substrate (D-lactate), but nonselective to the nature of electron acceptor. However, its native acceptor (cytochrome *c*), DLDH is able to reduce different low molecular redox mediators (*e.g.* ferricyanide and phenazine methosulfate) (Gregolin & Singer, 1963; Lodi et al., 1999). DLDH is highly selective to D-lactate; however, D-2-hydroxybutyric acid can be also used as an alternative electron donor (Nygaard, 1961). On the other hand, there is a known phenomenon of the activation of yeast mitochondrial DLDH by carboxylic acids like L-malate (Mourier et al., 2008).

The usage of DLDH in the pure enzyme form is problematic due to the complexity of its isolation and stabilization because of the intramembranous localization of DLDH (Tap et al., 2002; Lodi & Ferrero, 1993; Lodi et al., 1999; Rojo et al., 1998). On the other hand, the use of a mitochondrial fraction as a bioselective element opens the possibility of using highly active DLDH in the biorecognition membrane because of native conditions of the enzyme localization.

This paper describes the construction of a new D-lactate-selective amperometric biosensor based on the use of the mitochondrial fraction isolated from recombinant cells of the methylotrophic yeast *Ogataea (Hansenula) polymorpha "*tr6*"* (*gcr1 catX/Δcyb2, prAOX_DLDH*) as a source of DLDH. The specificity of this strain is the over-production of the target enzyme (DLDH) and the lack of the L-lactate specific enzyme (flavocytochrome $b_2$) (Smutok et al., 2014). The developed sensor is characterized by improved operational parameters in comparison with the close analog (Pohanka & Zbooil, 2007).

## MATERIALS AND METHODS

### Materials

Sodium D-lactate, Sodium L-lactate, Sodium Pyruvate, Potassium ferrycianide, Phenylmethylsulfonyl fluoride (PMSF), phenazine methosulfate (PMS), Ethylenediaminetetraacetic acid (EDTA) and 3-(N-morpholino) propanesulfonic acid (MOPS) were obtained from Sigma-Aldrich Chemie GmbH (Taufkirchen, Germany). D(+)-glucose monohydrate was purchased from J.T. Baker (Deventer, The Netherlands); Methanol and ethanol absolute were from Riedel-de Haën (Seelze, Germany); $(NH_4)_2SO_4$, $Na_2HPO_4$, $KH_2PO_4$, $MgSO_4$ and sorbitol were obtained from Merck (Darmstadt, Germany); Cetyl trimethyl ammonium bromide (CTAB) from (Chemapol, Slovakia); 2',7'–dichlorofluorescein diacetate (DCFDA) was obtained from Fluka (Buchs, Switzerland); "Yeast Litin" was obtained from Enzyme (Yaroslavl, Russia). The cathodic electrodeposition paint "GY 83-0270 0005" was from BASF Farben und Lacke (Munster, Germany).

All chemicals and reagents were of analytical grade and all solutions were prepared using HPLC-grade water. D-lactate standard solution and appropriate dilutions were prepared in 100 mM phosphate buffer, pH 7.8. The DTT-buffer consists of: 100 mM Tris-$H_2SO_4$, pH 9.4 and 10 mM dithiothreitol. Ice-cold buffer for homogenization contained 10 mM Tris-HCl, pH 7.4, 0.6 M sorbitol and 1 mM EDTA. MOPS-KOH buffer consists of: 3-(N-morpholino) propane sulfonic acid in KOH (pH 7.2) and 250 mM sucrose with 1 mM EDTA.

### Cultivation of yeast cells

Recombinant DLDH-overproducing strain *O. polymorpha* "tr6" (*gcr1 catX/Δsyb2, prAOX_DLDH*) is characterized by 6-fold overexpression of DLDH, impaired in glucose repression, devoid of catalase and specific L-lactate-cytochrome *c*: oxidoreductase activities (Smutok et al., 2014).

Cultivation of the *O. polymorpha* "tr6" was performed in flasks on a shaker (200 rpm) at 28 $^{o}$C until the middle of the stationary growth phase (~52 h) in a medium containing (g·l$^{-1}$): $(NH_4)_2SO_4$ - 3.5; $KH_2PO_4$ - 1.0; $MgSO_4$ x $7H_2O$ - 0.5 supplemented with 0.75 % yeast extract. A mixture of glucose (10 g·l$^{-1}$), L,D-lactate racemic mixture (2 g·l$^{-1}$) was used as a carbon and energy source and methanol (1 g·l$^{-1}$), as the inducer for strong constitutive promoter pAOX. After washing, the cells were suspended in 50 mM phosphate buffer, pH 7.8 containing 1 mM PMSF followed by drying. Before experiments, the freshly prepared yeast cells were resuspended to 30 mg·ml$^{-1}$ in 50 mM phosphate buffer, pH 7.8.

**Assay of DLDH activity in mitochondrial fraction**

One unit of the DLDH activity is defined as that amount of the enzyme which forms 1 mol hexacyanoferrate(II) per minute under standard conditions of the assay (20 $^{o}$C, 30 mM phosphate buffer, pH 7.8). Activity was estimated by spectrophotometric monitoring of potassium ferricyanide reduction at λ = 420 nm. During this process, the optical density of the analyzed solution becomes lower. Assay mixture consisted of 30 mM phosphate buffer, pH 8.0; 16 mM sodium D-lactate; 83 mM $K_3Fe(CN)_6$ and 20 µl permeabilized cell suspension (30 mg·ml$^{-1}$).

The specific activity of DLDH was calculated by a formula:

$$A = \frac{\Delta E/\min \cdot V \cdot n}{\varepsilon_{mM} \cdot C \cdot Vm},$$

where ΔE/min – the change of optical density at λ = 420 nm per min; V – total volume of the assay solution, ml; n – dilution of the enzyme before assay; $\varepsilon$mM = 1.04 mM$^{-1}$·cm$^{-1}$, millimolar extinction of reduced hexacyanoferrate(II); V*m* - volume of the added destroyed mitochondrial fraction suspension, ml; C– mitochondrial concentration, mg·ml$^{-1}$.

Specific DLDH activity (SA) in the cells was calculated by a formula, considering a difference between specific DLDH activity (in the presence of D-lactate) and nonspecific ferrireductase activity (without adding D-lactate):

$$SA = A_{+Dact} - A_{-Dact}.$$

**Mitochondria isolation**

The isolation of the mitochondrial fraction from *O. polymorpha* "tr6" cells was performed using modified Gregg's method (Gregg et al., 2009). The procedure was as follows: the precipitated yeast cells were resuspended in DTT buffer (2 ml per 1 g of wet weight cells) and incubated in the shaker at 30 ºC, 220 rpm for 20 min. The cells were

precipitated for 5 min at 3,000 g and resuspended in 20 mM PB, pH 7.4 with 1.2 M sorbitol (7 ml of buffer per 1 g of wet weight cells). The resuspended cells were incubated in the shaker at 30 °C, 220 rpm for 20 min. Then, the cells were precipitated for 5 min at 3,000 g and resuspended in 20 mM PB, pH 7.4 with 1.2 M sorbitol, following the addition of 7 ml of buffer per 1 g of wet weight cells. After repeated centrifugation, the cells were resuspended in the same buffer with the addition of "Yeast Litin" (50 mg of enzyme with the activity of 80 $U \cdot mg^{-1}$ per 1 g of cells) and incubated for 4 hours in the shaker at 220 rpm, 30 °C for the hydrolysis of the yeast cell wall. The formation of the spheroplasts was monitored under the microscope.

The obtained spheroplasts were precipitated by centrifugation for 8 min at 2,200 g at 4 °C and resuspended in ice-cold Sorbitol Buffer (10 mM Tris/HCl pH 7.4, 0.6 M sorbitol, 1 mM EDTA) in the proportion of 6.5 ml per 1 g of wet weight cells. All the next steps were performed at 4 °C or on the ice. Spheroplasts were centrifuged for 8 min at 2,200 g at 4 °C and pelleted spheroplasts were resuspended in ice-cold Sorbitol Buffer (6.5 ml of buffer per 1 g of wet weight cells). The centrifugation and washing procedure were repeated again. The final suspension of the spheroplasts was pre-cooled on the ice and homogenized gently using glass Potter-Elvehjem homogenizer. The resulting suspension was added 1 volume (6.5 ml) of ice-cold Sorbitol buffer with 1 mM PMSF. Unbroken cells, nuclei, and large debris were precipitated at 1,500 g, 4 °C for 5 min and, finally, by centrifugation for 5 min at 3,000 g and 4 °C. The resulting supernatant was centrifuged for 15 min at 12,000 g, 4 °C. The supernatant was decanted and the pellet was resuspended in ice-cold Sorbitol buffer (6.5 ml of buffer per 1 g of wet weight cells) and centrifuged for 5 min at 3,000 g, 4 °C. The obtained supernatant was centrifuged for 15 min at 12,000 g, 4 °C, and the precipitate was resuspended in 3 ml ice-cold SEM buffer (10 mM MOPS/KOH pH 7.2, 250 mM sucrose, 1 mM EDTA) and frozen at -25 °C for storage. Before using, the mitochondrial fraction was slowly defrosted at 4 °C.

The activity of DLDH in the isolated mitochondrial fraction was analyzed according to the procedure described in *Section 2.3*.

**Fluorometric microscopy of the mitochondrial fraction**

An inverted fluorescence microscope (Axio Lab. A1., Carl Zeiss, Germany) was used for phase-contrast and fluorescence microscopy of the mitochondrial fraction. The 2',7'-dichlorofluorescein diacetate (DCFDA) was used as an indicator for reactive oxygen species (Ex/Em: ~492–495/517–527 nm). For this experiment, the obtained mitochondria were separated into 3 samples. The first sample was incubated for about 15 min only with 50 μM

DCFDA; to another sample 18 µM D-lactate was added, in addition to DCFDA, to switch in the enzymatic DLDH activity. The last one (control), contained only the mitochondrial fraction, without any reagent. After the incubation for 20 min in the dark at room temperature, the samples were centrifuged at 1,800 g, 4 °C and washed with 50 mM PB, pH 7.8. The obtained precipitate was resuspended in 25 µl 50 mM PB, pH 7.8 and used for fluorometric microscopy analysis.

**Biosensor preparation and evaluation**

Amperometric biosensors were evaluated using constant-potential amperometry in a three-electrode configuration using 4 mm diameter planar gold electrodes DRP-C220AT from "DropSens" (Llanera, Asturias, Spain). Amperometric measurements were carried out using a potentiostat CHI 1200A (IJ Cambria Scientific, Burry Port, UK) connected to a personal computer and performed in a batch mode under continuous stirring in a standard 4 ml mini electrochemical cell at 25 $^{o}$C.

The immobilization procedure was provided using cathode polymer GY83-02700005 as follows: mitochondria suspension in 30 mM PB, pH 7.8 was dropped on the top of the planar gold electrode DRP-C220AT to the final activity of the enzyme up to 5 U·ml$^{-1}$ on the working electrode area. After drying for 2 min at room temperature, the cells' layer was covered with 8 µl 10-fold diluted solution of the cathodic paint GY83-02700005 (pH 5.5). After drying, a well-adhering polymer film was formed. The prepared mitochondria-based electrodes were rinsed by 20 mM PB, pH 7.8 and stored at 4 °C before application.

All the amperometric measurements were carried out in 50 mM PB, pH 7.8. After the background current reached a stable value, increasing concentration of the D-lactate solution was added to the electromagnetic cell at the continuous stirring. Research on the storage stability was carried out using 0.2 mM concentrations of D-lactate.

**RESULTS AND DISCUSSION**

The usage of the pure D-lactate: cytochrome *c*-oxidoreductase (DLDH, EC 1.1.2.4) in the biosensor construction is problematic due to the complexity of its isolation and stabilization because of the intramembranous localization (Dym et al., 2000; Gregolin & Singer, 1963; Mourier et al., 2008; Smutok et al., 2014). On the other hand, the use of the mitochondrial fraction as a bioselective element opens the possibility of using high activity of DLDH in the biorecognition membrane via native conditions of the enzyme localization. Therefore, to construct a new D-lactate-selective biosensor, we suggest using a mitochondrial

fraction isolated from recombinant cells of methylotrophic yeast *Ogataea polymorpha* "tr6" (*gcr1 catX/Δcyb2, prAOX_DLDH*) overproducing DLDH and avoiding L-lactate-specific activity related to flavocytochrome $b_2$.

For this purpose, the mitochondrial fraction was isolated from the yeast by the modified method of Gregg (Gregg et al., 2009) (*see section 2.4*). The mitochondrial fraction was characterized enzymatically with respect to the specific DLDH activity which was shown to be 4.16 $U \cdot mg^{-1}$ and morphologically using fluorescence microscopy to confirm the native-form of the enzyme inside mitochondria during its isolation (Fig. 1).

Fluorometric analysis using 2',7'-dichlorofluorescein diacetate (DCFDA) was used to confirm the presence of a native DLDH form in the obtained mitochondrial fraction. DCFDA is a specific dye for reactive oxygen species (ROS) (Gomes et al., 2005). Normally, in the yeast mitochondria in the native conditions, ROS are formed in low quantities. In case of using the mitochondria isolated from the cells overproducing DLDH, the increased DLDH-activity would provoke the formation of a high ROS level. The increasing fluorescence after D-lactate pretreatment of the mitochondrial fraction (Fig. 2C) compared with fluorescence without any pretreatment with D-lactate (Fig. 2B) can be regarded as an evidence of DLDH nativity in the isolated mitochondria preparations.

The DLDH is non-selective to the nature of an electron acceptor and besides its native acceptor (cytochrome *c*) this enzyme can reduce phenazine methosulfate (PMS) as a low molecular weight redox mediator (Lodi et al., 1994). So, this mediator (0.2 mM) was selected by us for the construction of a biosensor based on mitochondrial fractions. The optimal working potential of +150 mV *vs* Ag/AgCl for efficient oxidation of PMS on the gold electrode was applied. The mitochondria were held on the working electrode using commercial cathodic paint GY 83-0270 005 according to Karkovska et al., 2015.

The operational parameters of a mitochondria-based biosensor were evaluated using chronoamperometry (Fig. 2).

The sensor demonstrated the maximal response ($I_{max}$) for D-lactate of about 183.9±6.4 nA. The calculated value of $K_M^{app}$ for D-lactate derived from the calibration plot for the sensor was estimated as 0.17±0.02 mM (Fig. 2). The limit of detection (LOD) of the developed biosensor was calculated as 3 μM of D-lactate with a response time (90 % response to the analyte) less than 10 s.

The linearity range for the amperometric biosensor was shown to be from 30 up to 300 μM D-lactate (Fig. 3).

Some substances, usually present in dairy products, were studied with the purpose of testing their possible interfering effect on D-lactate analysis. The levels of common blood metabolites (glucose, pyruvic acid and especially, L-stereoisomer of lactic acid) in biological samples are an order higher compared with the concentration of D-lactate (Silanikove et al., 2014; Shapiro & Silanikove, 2011; Ahmed et al., 2013). It was shown that the mentioned above analytes (as well as ethanol) had no interfering effect on the biosensor analysis of D-lactate (*See Supplementary Materials Fig. S1*). Complete absence of response toward L-lactate is explained by the impaired activity of L-lactate cytochrome *c*-oxidoreductase due to the deletion of the *CYB2* gene coding for this enzyme synthesis in the recombinant strain.

The stability of D-lactate-selective bioelectrode based on the mitochondrial fraction was characterized by the response toward 0.2 mM D-lactate during the storage at +4 °C (*See Supplementary Materials Table S1*). The storage stability of the biosensor was characterized as the residual response of the biosensor (%) relative to the initial value of the signal (the first day of analysis). The half-life of the sensor (50 % response of initial response) was estimated as 11 days of storing at +4 °C (*Table S1*). It should be noted that the D-lactate-selective biosensor based on the mitochondria from *O. polymorpha* "tr6" recombinant cells demonstrated sufficient stability for the organelle-based sensor and can be used for determination of D-lactate for several days, in case of being recalibrated with the D-lactate standard solution.

The comparative analysis of the operational parameters of the developed D-lactate amperometric biosensor and its previous analog is shown in Table 1.

The current sensor demonstrated a lower limit of detection (about 3 μM) in comparison with the closest analog (56 μM), described by Pohanka & Zbooil (2007),

essentially higher sensitivity (53-fold) with the same selectivity and sufficient storage stability.

The developed biosensor was tested on the model samples of fermented yogurt and milk. The commercial yogurt and milk samples were additionally fermented at +34 °C for 24 hours (to increase D-lactate content) and filtrated through 0.2 μm pores Minisart® NML Syringe Filter (Sartorius GmbH, Gottingen, Germany) prior to D-lactate analysis.

The sensitivity value for D-lactate-selective mitochondria-based bioelectrode calculated from the calibration plot was about 18.5 $A \cdot M^{-1} \cdot m^{-2}$ (Fig. 3 (right)).

As shown in Fig. 4, D-lactate content in tested drinking yogurt and milk was estimated as 63.4 ± 3.1 mM and 3.25 ± 0.6 mM D-lactate, respectively. These results are in good agreement with the published data (Olieman & de Vries, 1988; Marrazza et al., 1994; Shapiro & Silanikove, 2010) and tightly correlate ($0.7 < r < 1$) with the results, obtained using an adapted enzymatic method for the analysis of D-lactate (Gonchar et al., 2009): 60.6 ± 1.3 mM for yogurt and 3.8 ± 0.3 mM D-lactate for milk (Table 2).

Good correlation between the obtained results on the D-lactate analysis, conducted using the developed prototype of D-lactate-selective biosensor, and literature data can be regarded as a good platform for the production of a commercial biosensor for inexpensive and rapid quantitative determination of D-lactate in dairy products. This is especially relevant in view of the recently reported work concerning a construction of the laboratory prototype of a bioreactor for the oxidation of toxic D-lactate from dairy products (Karkovska et al., 2016), because this technology requires automatic control of the efficacy of D-lactate conversion.

## CONCLUSIONS

The mitochondrial fraction isolated from recombinant cells of the methylotrophic yeast *O. polymorpha* "tr 6" (*gcr1 catX/Δcyb2, prAOX_DLDH*) with a highly specific DLDH activity and complete absence of an L-lactate-specific enzyme has been obtained and used for the construction of the new D-lactate-selective amperometric biosensor.

It was shown that mitochondria-based bioelectrode is suitable for the determination of D-lactate in the concentration range from 30 up to 300 μM with the sensitivity of about 18.5

$A \cdot M^{-1} \cdot m^{-2}$, high selectivity, and sufficient stability. The biosensor can be used for several days, in case of recalibrating the bioelectrode. In comparison with the described DLDH-based D-lactate-selective biosensor, the mitochondria-based sensor demonstrated 18.6-fold lower limit of detection with essentially higher sensitivity (53 fold).

The D-lactate-selective amperometric biosensor was tested on the real samples of fermented milk and drinking yogurt. The obtained D-lactate values are in good correlation with the published data, so this biosensor is suggested for successful application in the determination of D-lactate for quality control of food products.

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**Conflict of Interest:** The authors declare no conflict of interest.

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**Table 1.** Comparison of the current D-lactate-selective mitochondria-based biosensor with described biosensor, based on DLDH from *S. cerevisiae* (Pohanka & Zbooil, 2007)

| | Limit of detection, μM | Sensitivity, $A \cdot M^{-1} \cdot m^{-2}$ | Linearity range, mM | Time of response, s |
|---|---|---|---|---|
| Current biosensor based on intact mitochondria | 3 | 18.5 | 0.03-0.30 | less than 10 |
| DLDH-based biosensor (Pohanka & Zbooil, 2007) | 56 | 0.35 | 0.06-100 | none indicated |

**Table 2.** The analysis of the fermented samples of milk and yogurt by the mitochondria-based D-lactate-selective biosensor (spectrophotometric method was used as a reference one)

| | The biosensor | Spectrophotometric method |
|---|---|---|
| | [D-lactate], mM | |
| Milk | 3.25 ± 0.57 | 3.8 ± 0.25 |
| Yogurt | 63.4 ± 3.1 | 58.6 ± 0.34 |

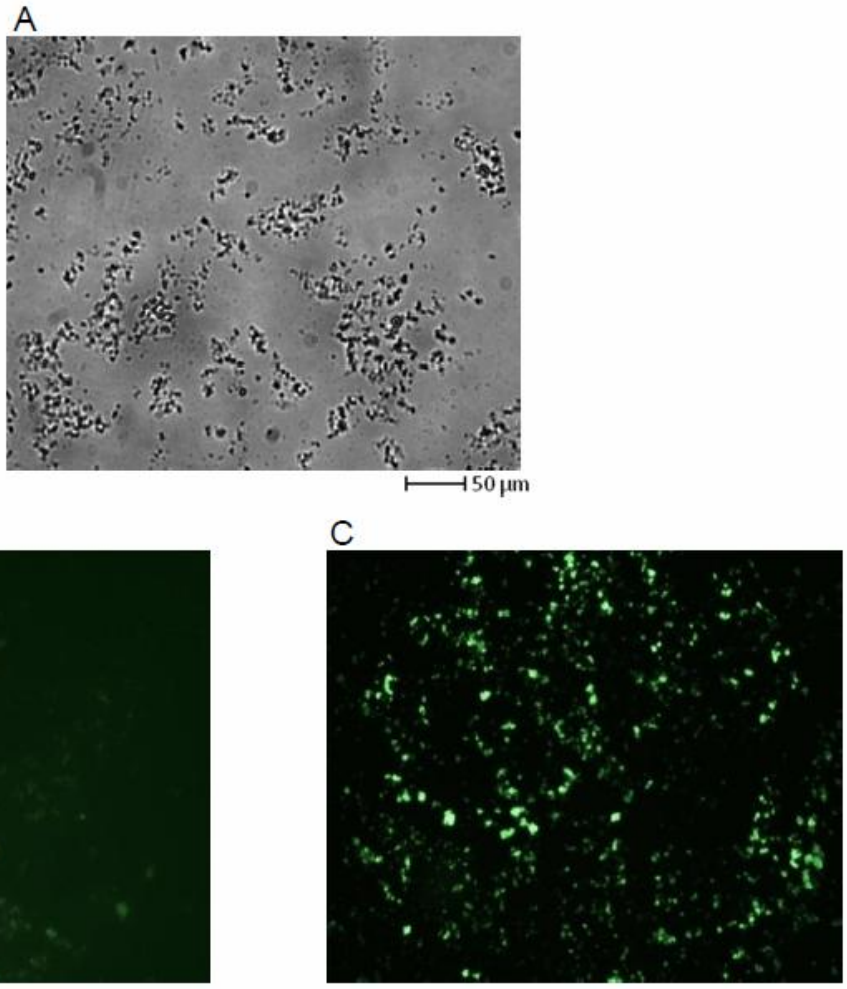


**Fig.1.** Phase-contrast and fluorescence microscopy the mitochondrial fraction isolated from the recombinant *O. polymorpha* cells. A) phase-contrast image of mitochondrial fraction, magnifications 40 x 20; B) fluorescence microscopy of mitochondrial fraction treated with 2',7'-dichlorofluorescein diacetate (DCFDA) without addition of D-lactate; C) fluorescence microscopy in the presence of DCFDA and 18 µM D-lactate as a substrate of DLDH.

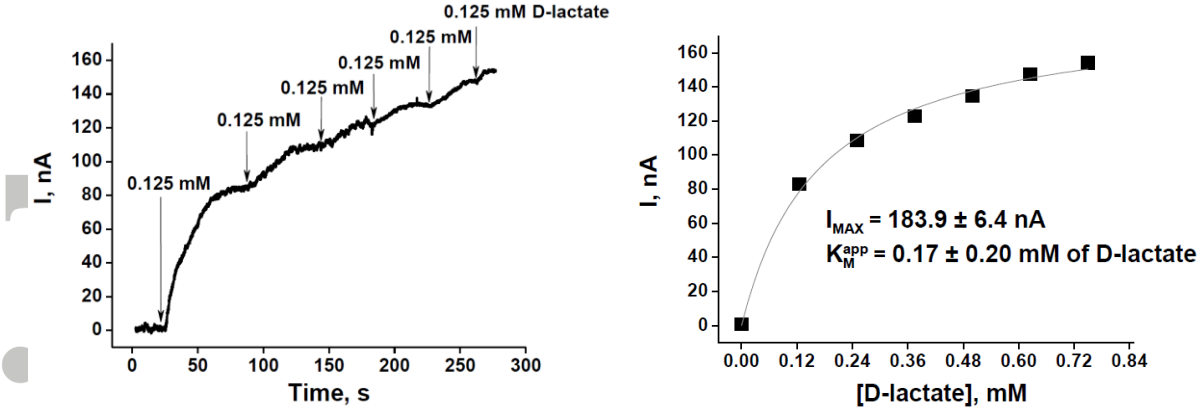


**Fig. 2.** Chronoamperogram (left) and calibration current response upon subsequent additions of an increasing concentration of D-lactate (right) for 4 mm gold planar electrode «DropSens» *C220*, modified by 20 µl of intact mitochondrial fraction with specific DLDH activity of 3 U·ml$^{-1}$, covered by commercial cathodic paint GY 83-0270 005. Conditions: working potential +150 mV *vs* Ag/AgCl reference electrode, 0.2 mM PMS, 60 mM phosphate buffer, pH 7.8.

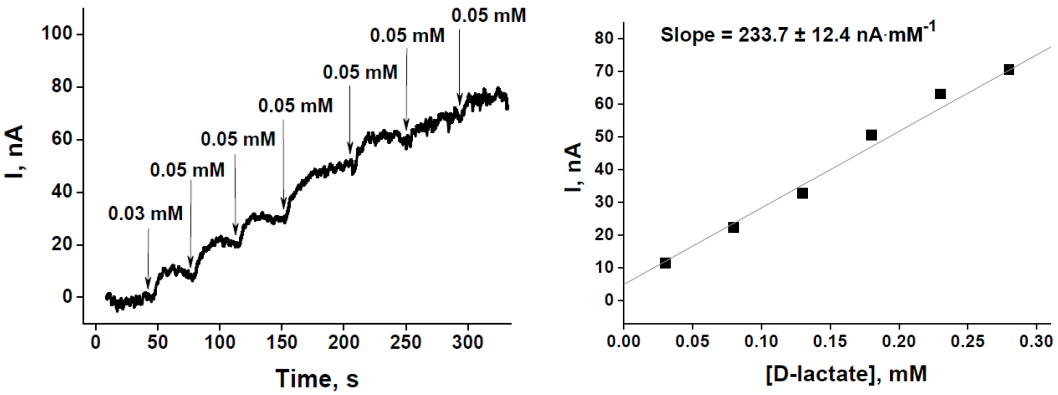


**Fig. 3.** Chronoamperogram (left) and calibration current response upon subsequent additions of D-lactate (right) for linearity test for 4 mm gold planar electrode «Dropsens» *C220*, (working area of 12.6 $mm^2$), modified by 20 µl of intact mitochondrial fraction with specific DLDH activity of 3 $U \cdot ml^{-1}$ and covered by GY 83-0270 005. Conditions: working potential +150 mV *vs* Ag/AgCl reference electrode, 0.2 mM PMS, 60 mM phosphate buffer, pH 7.8.

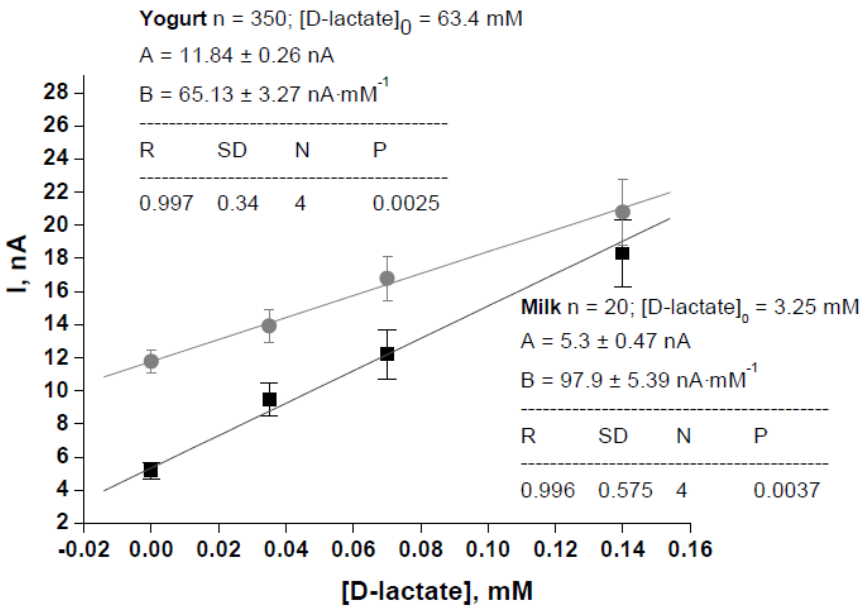


**Fig. 4.** The calibration curves for determination of D-lactate by a "*standard addition mode*" using mitochondria-based D-lactate-selective biosensor in the samples of fermented milk and yogurt analyzed without additional pretreatment.