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# D-lactate-selective amperometric biosensor based on the cell debris of the recombinant yeast *Hansenula polymorpha*

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ABSTRACT

A D-lactate-selective biosensor has been developed using cells' debris of recombinant thermotolerant methylotrophic yeast *Hansenula polymorpha*, overproducing D-lactate: cytochrome *c*-oxidoreductase (EC 1.1.2.4, D-lactate dehydrogenase (cytochrome), DlDH). The *H. polymorpha* DlDH-producer was constructed in two steps. First, the gene *CYB2* was deleted on the background of the C-105 (*gcr1 catX*) strain of *H. polymorpha* impaired in glucose repression and devoid of catalase activity to avoid specific L-lactate-cytochrome *c* oxidoreductase activity. Second, the homologous gene *DLD1* coding for DlDH was overexpressed under the control of the strong *H. polymorpha* alcohol oxidase promoter in the frame of a plasmid for multicopy integration in the *Δcyb2* strain. The selected recombinant strain possesses 6-fold increased DlDH activity as compared to the initial strain.

The cells' debris was used as a biorecognition element of a biosensor, since DlDH is strongly bound to mitochondrial membranes. The cells' debris, prepared by mechanic disintegration of recombinant cells, was immobilized on a graphite working electrode in an electrochemically generated layer using an Os-complex modified cathodic electrodeposition polymer. Cytochrome *c* was used as additional native electron mediator to improve electron transfer from reduced DlDH to the working electrode. The constructed D-lactate-selective biosensors are characterized by a high sensitivity (46.3–61.6 A $M^{-1}$ $m^{-2}$), high selectivity and sufficient storage stability.



## 1. Introduction

D-lactate, a product of methylglyoxal pathway in mammalian cells, is a natural enantiomer of L-lactate. In healthy human D-lactate is found in serum and urine in nano- and micromolar concentrations, respectively [1]. Fermented foods like yogurts, pickles and products of microbial fermentation in the human colon are exogenous D-lactate sources [2]. D-lactate in high concentrations is toxic for children and people with short bowel syndrome and provokes encephalopathy [3,4]. Its concentration can be extremely high in human plasma during acidotic episodes as a result of increased production of D-lactate by intestinal bacteria due to malabsorption of carbohydrates [5]. The presence of D-lactate in body fluids indicates traumatic shock or a local bacterial infection like sepsis or meningitis [6]. Moreover, D-lactate is a potential biomarker of diabetic ketoacidosis [7], differential diagnosis of acute appendicitis [8] and predictor of intestinal ischemia and reperfusion injury [9]. All these facts convincingly demonstrate a need for analytical devices for the accurate D-lactate analysis in food industry, as well as clinical diagnostics and medicine. Use of enzyme- and cell-based amperometric biosensors looks very promising due to the favorable coupling of the selectivity of the biological recognition element and the sensitivity of electrochemical transducer.

As a biorecognition element of a D-lactate-selective biosensor we propose to use D-lactate dehydrogenase (cytochrome) (EC 1.1.2.4) (DlDH). DlDH is a FAD- and $Zn^{2+}$-containing membrane-associated protein found in yeast and bacteria [10,11]. The enzyme catalyzes D-lactate oxidation to pyruvate coupled with ferricytochrome *c* reduction to ferrocytochrome *c*. It is characterized as a mitochondrial protein with a molecular weight of 63 kDa for DlDH from *Kluyveromyces lactis* and 64 kDa for DlDH from *Saccharomyces cerevisiae*, respectively [12,13]. The enzyme participates in the lactate/pyruvate metabolism in yeast cells. DlDH is highly selective to D-lactate; however, D,L-α-hydroxybutyric acid can be used as

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alternative electron donor [14]. The enzyme is not selective with respect to electron acceptors and can reduce in addition to its native acceptor (cytochrome *c*) by different low molecular artificial redox mediators (ferricyanide, dichlorphenol indophenols, *etc.* [15]). These properties make it promising for the use as a biorecognition element in amperometric biosensors.

We describe the development of a D-lactate-selective amperometric biosensor based on cells' debris of the recombinant thermotolerant yeast *H. polymorpha* overproducing DlDH. The specific DlDH activity of the constructed strain is 6-fold increased as compared to the parental strain. The cells' debris of the recombinant strain was immobilized in the matrix of an Os-containing complex modified cathodic electrodeposition paint which plays a double role as a polymeric support and as electron transfer mediator to facilitate efficient electrochemical communication of DlDH with the electrode. The sensor architecture was additionally supplemented with cytochrome *c* as a native mediator of DlDH to improve the sensor characteristics.

## 2. Materials and methods

### 2.1. Materials

D-lactic acid sodium salt and L(+)-lactic acid were obtained from Fluka (Buchs, Switzerland). Phenylmethylsulfonyl fluoride (PMSF) and EDTA were obtained from Sigma (Deisenhofen, Germany). Sodium pyruvate and D(+)-glucose monohydrate were purchased from J.T. Baker (Deventer, The Netherlands). Ethanol absolute was from Riedel-de Haën (Seelze, Germany); $(NH_4)_2SO_4$, $Na_2HPO_4$, $KH_2PO_4$, $MgSO_4$, and $CaCl_2$ were obtained from Merck (Darmstadt, Germany).

The Osmium-complex modified cathodic electrodeposition polymer (*CP-Os*) was synthesized following a previously published procedure [16].

All chemicals were of analytical grade and all solutions were prepared using HPLC-grade water. Solutions of L-lactic acid were prepared in 50 mM phosphate buffer, pH 7.8 followed by neutralization using concentrated NaOH.

### 2.2. Strains, media, cultivation conditions and cells' debris preparation

Yeast strain of *H. polymorpha* NCYC495 leu1-1 [17], C-105 (*gcr1 catX*) [18] and its derivatives were grown in YPS reach medium containing (g $l^{-1}$): yeast extract— 5; peptone—10; sucrose—20 or mineral medium $(NH_4)_2SO_4$—3.5; $KH_2PO_4$—1.0; $MgSO_4 \times 7H_2O$—0.5; $CaCl_2$—0.1; and yeast extract—7.5. A mixture of glucose (10 g $l^{-1}$) and the racemate of L-D-lactate (9 g $l^{-1}$) was used as carbon and energy source. All strains were cultivated at 37 °C unless stated otherwise.

Yeast transformants were selected on the solid YPS medium supplemented with zeocin at 150 mg $l^{-1}$ or geneticin (G418) at 1 g $l^{-1}$.

For cells' debris preparation, the recombinant strain was cultivated in a mineral medium in flasks on a shaker (200 rpm) at 30 °C until the end of the exponential growth phase (~32 h). After washing, the cells were suspended in 50 mM phosphate buffer (pH 7.8) containing 1 mM PMSF and 1 mM EDTA followed by drying. Before experiments, dried yeast cells were re-suspended to 30 mg $ml^{-1}$ in 50 mM phosphate buffer, pH 7.8, containing 1 mM EDTA. The cells were mechanically disrupted with glass beads ($d$=0.8 mm). The cell-free extracts were separated and the cells' debris was re-suspended in a small amount of 50 mM phosphate buffer (pH 7.8) containing 1 mM PMSF and 1 mM EDTA.

The flavocytochrome $b_2$ (FC $b_2$) activity in fresh cell-free extracts was determined spectrophotometrically at 20 °C [19] by monitoring ferricyanide reduction in the absence and presence of L-lactate. The specific activity (SA) of FC $b_2$ (μmol $min^{-1}$ mg $^{-1}$ protein) was calculated as $SA_{FC\ b2} = SA_{+Lact} - SA_{-Lact}$.

One unit of DlDH activity is defined as the amount of the enzyme which oxidizes 1 μmol of D-lactate in 1 min under standard conditions of the assay (20 °C; 30 mM phosphate buffer, pH 7.5; 0.33 M D-lactate, 0.83 mM $K_3[Fe(CN)_6]$, 1 mM EDTA, 1 mM PMSF).

The *Escherichia coli* DH5α strain (Φ80d*lacZ*ΔM15, *recA*1, *endA*1, *gyrA*96, *thi*-1, *hsdR*17($r_K^-$, $m_K^+$), *supE*44, *relA*1, *deoR*, Δ(*lacZYA-argF*) U169) was used as a host for plasmid propagation. Strain DH5α was grown at 37 °C in an LB medium as described previously [20]. Transformed *E. coli* cells were maintained on a medium containing 100 mg $l^{-1}$ ampicillin.

### 2.3. Molecular techniques and plasmids construction

Standard cloning techniques were applied [20]. PCR-amplification of the fragments of interest was done with Platinum® *Taq* DNA Polymerase High Fidelity (Invitrogen) according to the manufacturer specification. PCR was performed in a GeneAmp® PCR System 9700 thermocycler (Applied Biosystems). Transformation of the yeast *H. polymorpha* by electroporation was carried out as described previously [21]. Preparation of the total DNA from yeast was carried out by using the DNeasy® Tissue Kit (Qiagen). Plasmid DNA isolations from *E. coli* were performed by using NucleoSpin® Plasmid (Macherey-Nagel).

To construct the deletion cassette for *H. polymorpha* gene *CYB2* coding for L-lactate-cytochrome *c* oxidoreductase the 5'-uncoding region (~1.3 kb) of *CYB2* gene was amplified *via* PCR using the pair of primers Ko258 / Ko259 and the total DNA of *H. polymorpha NCYC 495 leu1-1* strain as template. Amplification of the 3'-uncoding region (~1.0 kb) of *CYB2* gene was performed using the pair of primers Ko260 / Ko261. Both fragments were fused by overlap PCR using the primers Ko258 / Ko261 double digested with *Kpn*I and *Hind*III and cloned to the corresponding sites of the basic plasmid pUC57 (Fermentas, Vilnius, Lithuania). *Kpn*I and *Hind*III restriction sites were introduced into the primers to simplify cloning (sequences for primers used through this work and corresponding restriction sites are represented in Table SD.1 (see Supplementary data). Finally the gene ZeoR conferring resistance to the antibiotic zeocin was amplified with the pair of primers VA158/VA159 and the plasmid pPICZ-B (Invitrogen, Carlsbad, CA, USA) as a template. ~1.2 kb fragment was cloned as *Pst*I-fragment between 5′- and 3′-uncoding regions of *CYB2* gene to create plasmid pLR_cyb2_Z (~6.2 kb). The accuracy of constructed plasmid pLR_cyb2_Z (Fig. 1A) was verified by sequencing.

The *H. polymorpha DLD1* gene with a terminator region (scaffold_7:338354-340365 in the *H. polymorpha* genome database [22]) and the alcohol oxidase gene promoter were amplified from the genomic DNA of *H. polymorpha* strain NCYC 495 *leu1-1* using the corresponding pairs of primers: Ko248 / Ko249 and Ko184/Ko247. The primers Ko184 and Ko249 were used to obtain a ~3.0 kb fragment containing the *H. polymorpha DLD1* gene with a terminator region driven by the alcohol oxidase gene promoter by overlap PCR. 6His-tag sequences were introduced before the ATG start codon of *DLD1* ORF to facilitate DlDH single step purification. The fused fragment was treated with restriction endonuclease *Bam*HI and cloned into the *Bam*HI-linearized and dephosphorylated plasmid pGLG61 [23], resulting in the recombinant constructs pGLG61_DLD1_Hp (Fig. 1B).

### 2.4. Biosensor preparation and evaluation

#### 2.4.1. Apparatus and techniques

Amperometric cells' debris-based biosensors were evaluated using constant-potential amperometry in a three-electrode configuration with an Ag/AgCl/KCl (3 M) reference electrode and a

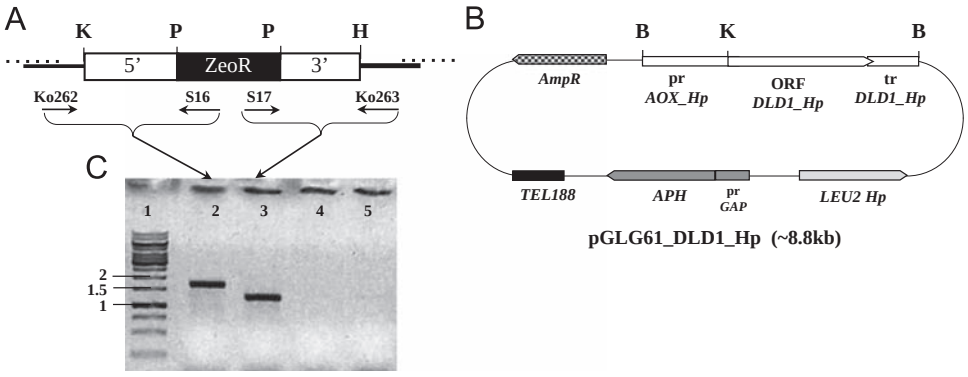


**Fig. 1.** (A) Scheme of the *CYB2* deletion cassette. Primers Ko262, S16, S17, Ko263 (see Table SD.1, Supplementary data) indicated as arrows, (B) Scheme of the plasmid pGLG61_DLD1_Hp (~8.8 kb). The *HpAOX* promoter and *DLD1* ORF with terminator region are shown as open boxes. The *LEU2* gene *H. polymorpha* is shown as lite gray box. The gene *AmpR* conferring resistance to ampicillin is shown as chequered boxes. The *H. polymorpha* truncated glyceraldehyde-3-phosphate dehydrogenase (*GAP*) promoter and the geneticin resistance gene (*APH*) are shown as gray boxes, and the tellomeric region (*TEL188*) as black box. Restriction sites: B, *Bam*HI; K, *Kpn*I; P, PstI; (C) Representative electroforegram of the PCR analyses of the *Δcyb2* strain. The fragment lengths of the molecular weight marker are given in kb (line 1), lines 2 and 3—PCR fragments amplified with pairs of primers Ko262/S16 and S17/Ko263 from the genomic DNA of the strain *Δcyb2*, lines 4 and 5—amplification with the same pairs of primers using the total DNA of strain C-105 as a control.

Pt-wire counter-electrode. Amperometric measurements were carried out using a bipotentiostat (EP 30, Biometra, Göttingen, Germany) connected to a personal computer *via* a RS232 port for data acquisition.

Graphite rods (type RW001, 3.05 mm diameter, Ringsdorff Werke, Bonn, Germany) were used as working electrodes. They were sealed in glass tubes using epoxy glue, thus forming disk electrodes. Before sensor preparation, the graphite electrodes were polished with emery paper and cleaned with water in an ultrasonic bath.

#### *2.4.2. Immobilization of cells' debris by entrapment within a cathodic electrodeposition paint*

2 μl of cells' debris suspension with a DlDH specific activity of 10 U $ml^{-1}$, 2 μl of cytochrome *c* (30 U $ml^{-1}$) and 2 μl of cathodic paint (*CP-Os*) were mixed and dropped onto the surface of a graphite electrode. In a miniaturized electrochemical cell the cathodic paint was finally precipitated using a potentiostatic pulse sequence with pulses to a potential of −1200 mV for 0.2 s and a resting phase at a potential of 0 mV for 5 s. At the applied cathodic potential, water is reduced at the electrode surface leading to an increase of the pH-value in the diffusion zone in front of the working electrode surface [24]. By this, the cathodic paint was deprotonated causing a significant change in its solubility which leads to the precipitation of the polymer on the electrode surface simultaneously entrapping the cells' debris and cytochrome *c*. Prior to use, the electrode was rinsed with 50 mM phosphate buffer, pH 7.8. Between experiments, the modified electrodes were stored in 50 mM phosphate buffer, pH 7.8, at 4 °C.

All the experiments were carried out independently in biological triplicates, and the reported results were the average of three replicate experiments.

## 3. Results and discussion

### *3.1. Construction of the recombinant H. polymorpha strain overproducing DlDH*

The *H. polymorpha* mutant *Δcyb2* was constructed by the deletion of the internal part of the *CYB2* gene ORF. For that the HindIII-linearized plasmid pLR_cyb2_Z was transformed to the recipient strain *H. polymorpha* C-105 (*gcr1 cat*X), impaired in glucose repression and devoid of catalase activity. Selected zeocin resistance transformants were subjected to PCR genotyping.

**Table 1**
Specific activities of FC $b_2$ and DlDH in initial and constructed strains.

| Strain | Specific activity (U $mg^{-1}$) | |
|---|---|---|
| | FC $b_2$ | DlDH |
| C-105 | 0.27 ± 0.01 | 0.30 ± 0.02 |
| *Δcyb2* | ND | 0.29 ± 0.01 |
| *Δcyb2/DLD1* | ND | 1.82 ± 0.09 |

ND—not detectable.

Among several tens of transformants, a few strains possessed the correct substitution of the internal part of the *CYB2* gene by a fragment harboring gene conferring resistance to zeocin. Confirmation of the proper *CYB2* deletion was performed by PCR with primers Ko262 and Ko263 homologous to the 5'- and 3'-uncoding regions outside of the DNA sequences presented on the deletion cassette. DNA fragments with an expected length 1.56 kb and 1.26 kb were amplified from the genome of the *Δcyb2* strain using pairs of the primers Ko262/S16 and S17/Ko263, respectively (Fig. 1A and C). The primers S16 and S17 are homologous to the sequence of the ZeoR gene. The obtained results provided a clear evidence of the correct replacement of *CYB2* by the ZeoR gene. The constructed *Δcyb2* strain was used for further experiments.

The recombinant plasmid pGLG61_DLD1_Hp (Fig. 1B) derived from pGLG61 was transformed to the recipient strain of *H. polymorpha* (*Δcyb2 gcr1 cat*X). The transformants were grown on an YPS medium in the presence of increasing concentrations of G418. The highest concentration of G418 which allowed the transformants to grow was 1 mg $ml^{-1}$. The transformants were stabilized by cultivation in non-selective media for 12–14 generations with further shifting to the selective media with G418. The presence of the expression cassette in the stable transformants was examined by diagnostic PCR using the primers Ko184/Ko249 and the genomic DNA of stable transformants as a template. Fragments of predictable size (~3.0 kb) were obtained (data not shown).

The constructed recombinant strains expressing *H. polymorpha DLD1* and the parental strains *Δcyb2* and C-105 were subjected to biochemical analyses. Specific activities of FC $b_2$ and DlDH were assayed. The strains *Δcyb2* and *Δcyb2/DLD1* did not possessed detectable level of FC $b_2$ conferring deletion of the *CYB2* gene. Specific activities of DlDH in the strain *Δcyb2/DLD1* were 6-fold increased as compared to the parental strain *Δcyb2* and the initial strain C-105, reaching 1.82 U $mg^{-1}$ (Table 1). To summarize, the strain *Δcyb2/DLD1* of *H. polymorpha* is characterized by increased

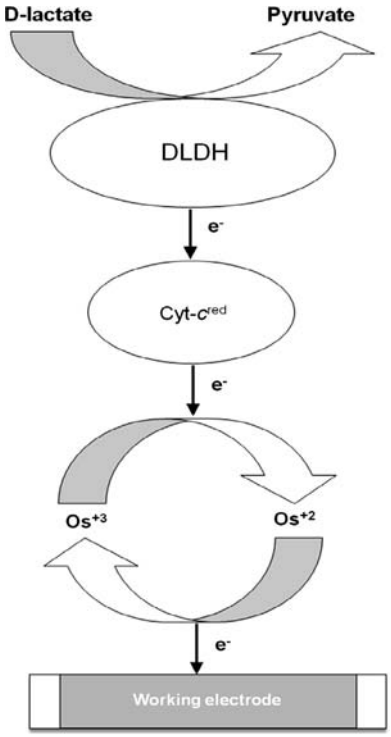


**Fig. 2.** Scheme of a D-lactate selective amperometric biosensor.

DlDH activity and abolished activity of FC $b_2$. This property defines the strain *Δcyb2/DLD1* as a suitable biorecognition element for the construction of biosensors selective for D-lactate.

### 3.2. Evaluation and optimization of a biosensor

DlDH is a mitochondrial membrane-associated protein. Yeast membranes in intact cell complicate communication between DlDH, its substrate and electron acceptors. Hence, the DlDH enriched cells' debris from the recombinant *H. polymorpha strain Δcyb2/DLD1* overexpressing the *DLD1* gene was chosen for construction of D-lactate-selective biosensors. DlDH catalyzes conversion of D-lactate to pyruvate and simultaneous electron transfer to cytochrome *c*, a natural electron acceptor for DlDH in living yeast cells. Due to this, additional integration of exogenous cytochrome *c* into the sensor architecture was supposed to improve the sensor characteristics. The construction of sensors with a bienzyme architecture requires simultaneous co-immobilization of cells' debris and cytochrome *c*. For this purpose, the Osmium complex modified electrodeposition cathodic paint (*CP-Os*) was used. *CP-Os* combines two functions: electron transfer and retention of the biorecognition element on the electrode surface.

The principal scheme of the electron transfer in the proposed bienzyme D-lactate-selective sensor is presented in Fig. 2.

To estimate the optimal working potential for the bienzyme-*CP-Os* sensor architecture, differential pulse voltammetry was used (Fig. 3).

The differential pulse voltammogram shows three peaks which represent the different electroactive compounds immobilized at

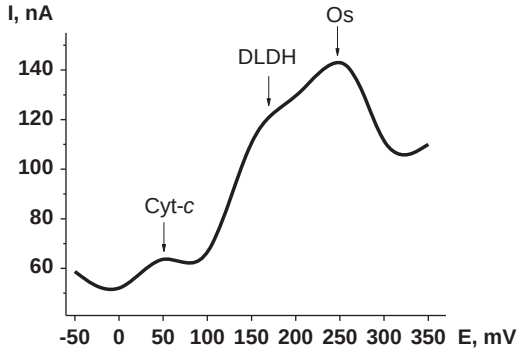


**Fig. 3.** Differential pulse voltammogram of a *bienzyme-CP-Os*-modified electrode (conditions: scan rate 7 mV $s^{-1}$, modulation time 40 ms, modulation amplitude 50 mV *vs* Ag/AgCl/3 M KCl in 50 mM phosphate buffer, pH 7.8).

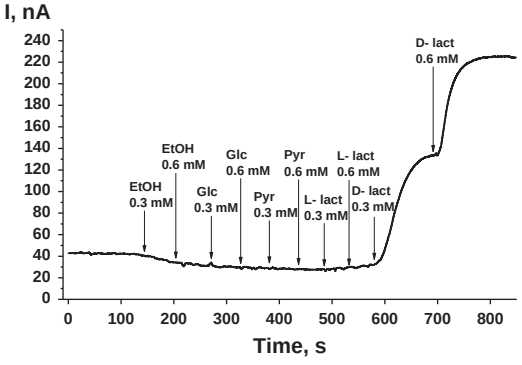


**Fig. 5.** Chronoamperometric analysis of the selectivity of the developed D-lactate biosensor. Abbreviation: EtOH—ethanol, Glc—glucose, Pyr—pyruvate, L-lact—L-lactate, D-lact—D-lactate. Conditions: +250 mV *vs* Ag/AgCl/3 M KCl in 50 mM phosphate buffer, pH 7.8.

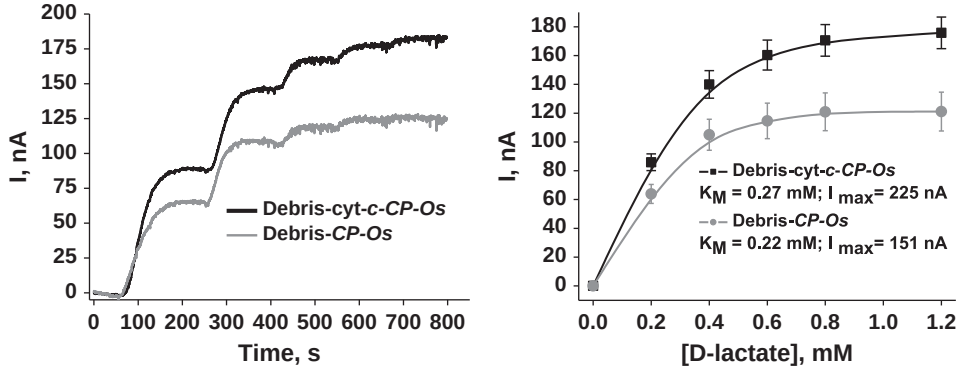


**Fig. 4.** Chronoamperometric responses (left) and calibration curves as average of 3th independent measurements (right) obtained by using a bienzyme-*CP-Os*-modified electrode (black line) and a similar bioelectrode obtained without addition of exogenous cytochrome *c* (gray line) upon consecutive addition of D-lactate (conditions: +250 mV *vs* Ag/AgCl/3 M KCl in 50 mM phosphate buffer, pH 7.8).

**Table 2**
Storage stability of the biosensors based on the cell debris of the recombinant yeast *H. polymorpha*.

| Remaining response, % from the initial output | Time of storage (days) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| *Debris-CP-Os* | 95.6 | 89.8 | 83.7 | 80.2 | 78.3 | 74.8 | 67.4 | 63.6 | 59.8 | 53.7 |
| *Debris-cyt-c-CP-Os* | 98.8 | 97 | 94.4 | 90.5 | 89.3 | 85.1 | 80.2 | 78.8 | 75.3 | 71.4 |

the electrode surface. The peak at +50 mV corresponds to direct electron transfer from cytochrome *c* to the electrode surface [25]. The peak at 160 mV could support the hypothesis of DLDH redox activity. We suppose that even cellular DlDH as a FAD- and $Zn^{2+}$-containing protein is able to direct electron transfer. The negligible peak at +150 mV could be a result of electron transfer from DLDH to the electrode surface. The highest peak at +250 mV corresponds to the redox reaction of the polymer-tethered Os-complex (see Fig. SD 2, Supplementary data). The latter potential is supposed to be optimal for effective electron transfer for bienzyme-*CP-Os* sensor architecture. Thus, further experiments were performed at a working potential +250 mV *vs* the Ag/AgCl/3 M KCl reference electrode.

The comparison of the chronoamperometric responses and calibration curves obtained for a bienzyme-*CP-Os* sensor architecture and sensor without exogenous cytochrome *c* is shown in Fig. 4.

A typical sensor response for both sensor architectures demonstrated a maximal current $I_{max}$ at substrate saturation for the bienzyme-*CP-Os*-modified electrode of 225 ± 14 nA, while the bioelectrode without exogenous cytochrome *c* possessed an $I_{max}$ of 150 ± 11 nA under the same conditions.

In addition, the calculated values of $K_M^{app}$ for D-lactate derived from the calibration plots for sensors based on combination of cells' debris with cytochrome *c* exhibit a higher value: 0.27 mM as compared to 0.22 mM for the bioelectrode without exogenous cytochrome *c*. The increased linear detection range is more suitably adapted to the typical concentration range of D-lactate in real biological samples, thus avoiding potentially necessary dilution steps. The sensitivity of the sensors to D-lactate was 61.6 A $M^{-1}$ $m^{-2}$ (with cytochrome *c*) and 46.3 A $M^{-1}$ $m^{-2}$ (without exogenous cytochrome *c*). The detection limit for both sensor architectures was determined to be 3.1 μM (for a probability $\alpha$=0.995).

For the application of the D-lactate biosensor in the real samples, the selectivity with respect to potential interferences such as L-lactate, pyruvate, D-glucose and ethanol is of great importance. Hence, the amperometric current response of the proposed D-lactate sensor was evaluated with respect to the mentioned compounds (Fig. 5).

The developed biosensor exhibits a high selectivity towards D-lactate. No current increase was observed for all tested potential interfering compounds (Fig. 5).

The storage stability of the both types of D-lactate sensor was investigated. Biosensors were prepared following the optimal preparation protocol. The stability tests were performed at a constant temperature of 24 °C using 1 mM solutions of D-lactate in 50 mM phosphate buffer, pH 7.8, containing 0.2 mM PMSF. Between amperometric D-lactate determinations the sensors were stored at +4 °C (Table 2).

The sensors based on entrapment of the DlDH enriched cells' debris within an Os-containing cathodic electrodeposition polymer could be used for more than 10 days with intermittent storage in buffer in the refrigerator. The significantly improved stability of the sensor containing exogenous cytochrome *c* could be explained by protein–protein interactions. In our case, one protein (cytochrome *c*) is a natural substrate of D-lactate-oxidizing enzyme. It is generally recognized that enzymes are stabilized in the presence of their substrates. Moreover, an application of *H. polymorpha* recombinant cells with subsequent removing of low-molecular proteinases during cell disruption and the following washing steps could also preserve the DlDH activity.

The main characteristics of the developed biosensor were compared to enzyme-based sensor with carbon paste electrode modified by DlDH from the baker's yeast [26]. The proposed sensors are more sensitive (170–230 fold) than biosensor based on DlDH from the baker's yeast: their sensitivities were estimated as 61.6 A $M^{-1}$ $m^{-2}$ and 46.3 A $M^{-1}$ $m^{-2}$ as compared to 0.27 A $M^{-1}$ $m^{-2}$ (1.91 nA $M^{-1}$) for biosensor based on DlDH-modified carbon paste electrodes. The developed cells' debris-based biosensor is also characterized by a lower detection limit (3.1 μM D-lactate) as compared to 56 μM for the DlDH-modified carbon paste electrode. Both sensors demonstrate a high selectivity and satisfying storage stability.

## 4. Conclusions

D-lactate-selective biosensors based on cells' debris of recombinant yeast cells of *H. polymorpha* lacking L-lactate cytochrome *c* oxidoreductase activity and at the same time overproducing D-lactate cytochrome *c* reductase have been proposed. The polymer-bound osmium complex supports the communication between the biorecognition elements and the electrode surface in the absence of additional free-defusing redox mediators. The characteristics of the sensors in the absence and presence of cytochrome *c* have been compared. The sensor with the bi-enzyme architecture possesses improved properties, such as sensitivity and stability as compared with sensors without addition of exogenous cytochrome *c*. Introduction of the *Δcyb2* mutation to the target strain provides an excellent selectivity of the developed microbial sensors. The developed microbial sensor is supposed to be successfully applied for the determination of D-lactate in food technology.

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## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2014.02.041.

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