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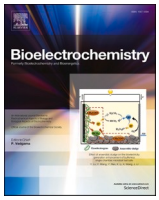

A rapid *in vitro* electrochemical screening of extracellular matrix of *Saccharomyces cerevisiae* by palladium nanoparticles-modified electrodes

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ABSTRACT

Herein, a rapid electrochemical screening of yeasts (*Saccharomyces cerevisiae*) *in vitro* mode depending on their optical density, cultivation time and growth medium used was conducted in 3 min by palladium nanoparticles (Pd-NPs)-modified electrodes. Pd-NPs-modified electrodes operated in cyclic voltammetry mode at low scan rates, *i.e.* 5–20 mV/s supported a low oxidative process in the yeast extracellular matrix. The electrochemical screening relied on an efficient electrooxidation of secondary metabolites, *i.e.* organohydrazines formed in the extracellular medium as a result of microbial activity of yeast cells.

More importantly, during the study the impact of fundamental parameters, *viz.* type of the matrix and pH on electroanalytical response of Pd-NPs-based electrodes in real fermentation medium was investigated in detail. The efficiency of the proposed *in vitro* electrochemical screening of yeast extracellular matrix was not affected by pH of the samples or composition of the multicomponent medium, but more likely exclusively depended on the presence of organohydrazines. The potential of this electroanalytical approach towards profiling of the extracellular matrix of *Saccharomyces cerevisiae* was compared with results obtained by gas chromatography mass-spectrometry (GC–MS) and genetically encoded biosensor (ro-GFP2) assays.

1. Introduction

Yeast cells (*Saccharomyces cerevisiae*) serve as an excellent model tool for addressing various questions in cell biology [1,2]. Although electrochemical properties of yeast cells in the presence of various materials were investigated by scanning electrochemical microscopy [3,4] as well as by electrochemical [5] and physical [6] assays, the composition of the intact extracellular matrix of yeasts mostly remains unknown. Although a wide spectrum of electrodes with different design and analytical merit for individual analysis of physiologically significant small molecular weight bioanalytes, primary and secondary metabolites which can be formed in the extracellular matrix of yeasts was proposed [7–9], almost no attempts were conducted to characterize the extracellular multicomponent matrix of yeast cells. At the same time, this analytical information is important in terms of a rapid biochemical profiling of cells depending on their growth protocols, studying the key parameters of physiological stress or changes induced by the use of specific drugs. More importantly, the discovered primary or secondary metabolites formed in the exogenous matrix of yeasts as a result of their high metabolic activity and change of electric resistance of cell wall [6] could significantly assist novel strategies implemented in Green fuel or fuel cells.

Hitherto, biochemical profiling of the extracellular content of yeasts was conducted for polysaccharides fraction, pyrazines and metabolites containing amino and/or carboxyl groups.[10–12] For this goal gel electrophoresis and gas-chromatography mass spectrometry (GC–MS) were utilized. However, despite the existing libraries GC–MS assay is often limited by the necessity of an extensive sample preparation (*i.e.* extraction, derivatization, etc.), the fragility of the formed metabolites causing their extensive fragmentation or appearance of by-products chemical compounds not present in the original samples. In addition, numerous bioconjugations and interactions [13] between the formed fragments and intact analytes can take place in injectors (~120–200 °C) or occur *in situ* on columns (~up to 250–300 °C) that significantly impacts the obtained results.

Moreover, low molecular weight primary and secondary

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metabolites, *viz.* organic and inorganic peroxides,[14] hydrogen sulfide, mercaptans, hydropolysulfides,[15] endogenous ammonia,[16] organohydrazines [17,14], azides or azines cannot be detected by GC–MS without derivatization and multi-step extraction. In other words, in fact some important signaling bioanalytes remain not discovered due to limitations of conventionally employed bioanalytical approaches. Therefore, the development of alternative strategies which could support extracellular profiling of yeast cells is highly desirable.

Herein, we present an original approach for a rapid *in vitro* electrochemical screening of yeasts performed by palladium-based electrodes operated in a droplet cyclic voltammetry mode. This simple approach relies on the electrochemical detection of organohydrazines formed in yeast extracellular matrix and allows to visualize a minor difference in cultivation conditions of cells or to distinguish wild-type cells from genetically modified variants.

Although several studies devoting to a possible mechanism of hydrazine (as the simplest representative compound of hydrazines) electrooxidation on bulk and nanostructured noble metal-based electrodes were published,[18–21] almost no attempts were conducted to evaluate their electroanalytical performance in complex media. For example, depending on pH and applied polarization mode palladium (Pd)-based electrodes were reported to be electroactive in individual buffered alcohol-containing solutions, aldehydes,[22] ketones, peroxides,[23] and polyphenols [24,25]. However the analytical performance of Pd-modified electrodes in real multicomponent biological matrices and fermentation media, where all these small molecular weight bioanalytes present together, remains unclear.

As a result, starting from the optimization of the electroanalytical protocol for a rapid *in vitro* electrochemical screening of yeasts performed by Pd-modified electrodes (*i*) during the course of investigations we fully validated the proposed approach by alternative advanced bioanalytical assays (*ii*), studied electrochemical activity of Pd-electrode in complex biological matrices (*iii*) and approved its exclusive sensitivity towards electrooxidation of hydrazine-based compounds regardless the content and pH of fermentation media (*iv*).

2. Experimental part

2.1. Chemicals and materials

H_2PdCl_4, $(NH_4)_2HPO_4$, $Na_2HPO_4{\cdot}12H_2O$ with purity of 99.9 % and 25 wt% NH_4OH used to prepare Pd-NPs (basic electrolyte, pH 9.1), urea (99.5 %), adenosine (≥99 %), hydrazine solution (35 wt% in H_2O), pyrrole (98 % in H_2O), dimethylformamide (99 %), ethylamine (97 %), diethylamine (98 %), nicotine acid amide (≥99.5 %), hydrobenzaldehyde azine (99 %), 2,2-azino-bis-ethylbenzo-thiazoline ammonium salt (≥98 %) were received from Merck (Darmstadt, Germany).

Mes-Tris and phosphate buffer, Hartwelĺs HC medium (Hartwelĺs Complete medium), YPD medium were received from Merck (Darmstadt, Germany). Apart from the standard mixture of amino acids, HC medium was supplemented with 10 % of yeast nitrogen base (YNB, 10 %, Merck, Darmstadt, Germany).[14] To compare the impact of growth medium on the content of extracellular matrix of cells grown in HC medium supplemented with YNB, the conventional YPD medium was explored.

For the experiments yeast cells (By4742 line) were used in this study. 96-well plates for ro-GFP-2 were obtained from Merck (Darmstadt, Germany). All pH measurements were made with a digital pH sensor HORIBA LAQUATWIN PH-22 (MMM Tech support GmbH&Co KG, Berlin, Germany). The DRP-110DGPHOX screen printed electrodes (SPE) were obtained from DropSens (Metrohm, Germany).

2.2. Electrochemical electrode preparation and characterization

Palladium nanoparticles (Pd-NPs) were formed on the surface of the working electrode of SPE during galvanostatic deposition performed at the cathodic current of −2.5 mA for 30 s on the one-channel biologic Potentiostat PalmSens4 (PalmSens, Utrecht, Netherlands). The deposition of Pd-NPs was conducted in a droplet mode [23] from Pd-electrolyte of the following composition: H_2PdCl_4, 3.5 g/L; $(NH_4)_2HPO_4$, 20 g/L; $Na_2HPO_4{\cdot}12H_2O$, 100 g/L; and NH_4CI, 25 g/L, pH = 9.1, anode – Pd-wire. The mass of electrodeposited Pd estimated from the charge passed during electrodissolution of Pd in 1 M of HCl in potentiostatic mode was 7 μg.

The morphology of the formed Pd-NPs-modified electrodes was investigated by scanning electron microscopy (SEM) on an FEI Quanta (Hillsboro, OR, USA) 400 FEG system. Secondary and back scattered electron images were acquired at 10 kV accelerating voltage in high vacuum mode.

2.3. Operation of electrochemical electrodes and in vitro yeast cells profiling

SPE electrodes modified by Pd-NPs were explored in cyclic voltammetry (CV) mode at the scan rate of 20 mV/s from −0.4 V to 0.4 V (unless otherwise specified). Before electrochemical studies extracellular matrix was separated from the intact cells via centrifugation at 5000 rpm for 5 min. Afterwards, 150 μL of the obtained solution was dropped on the surface of Pd-NPs-modified electrodes. All the experiments were performed at least in triplicate. For comparison of the recorded CV plots and data acquisition in dependence with cell growth medium, optical density, phase growth, etc. the second scans were taken (unless otherwise specified). The stability of the electrode from run to run was verified in buffered solutions via returning of its baseline to the intact values.

The sensitivity of Pd-NPs-based electrodes towards nitrogen-contained compounds was evaluated based on the following ratio:

$$S = \frac{I}{C}$$

where I – anodic current at 0.3 V, μA;

C – concentration of the tested nitrogen-contained compound, mM.

2.4. Yeast fermentation for electrochemical screening

Yeast samples with a certain optical density (OD) were grouped as follows: cells grown in HC-YNB-peptone + 2 % glucose (**group I**), HC + YNB + 2 % glucose (**group II**); HC-YNB + 1 % peptone + 1 % glucose (**group III**); HC-YNB + 2 % peptone + 2 % glucose (**group IV**) and cells cultivated in a conventional YPD medium + 2 % glucose in the absence of YNB and peptone (**group V**); HC + YNB-uracil + 2 % glucose, mutant cells (**group VI**), see *section 2.5* for the details.

Regardless the selected group, after cultivation and OD measurements cells were removed from the medium by centrifugation performed at 10000 rpm for 5 min. The obtained yeast extracellular matrices were collected for subsequent electrochemical measurements.

2.5. Redox-sensitive green fluorescent protein 2 (roGFP2) assay

Construction of yeast strains and plasmid transformation was performed as previously described.[26] Briefly, a p416TEF plasmid encoding roGFP2-Tsa2ΔC$_R$ was transformed into BY4742 yeast cells. For subtraction of background fluorescence, cells were transformed with an empty p416TEF plasmid. After transformation cells grow under continuous selective pressure to ensure retention of the plasmid (HC medium-uracil). The cells prepared according to this protocol were used for fluorescence measurements as well as for bioelectrochemical profiling (mutant cells representing **group VI**).

For fluorescence measurements, transformed cells were inoculated into liquid selection medium and grown to an OD of ~ 3.5–4.5. Briefly, cells were centrifuged at 1000 *g* for 3 min to remove the growth medium.

Subsequently, the pellet was resuspended in 100 mM Mes/Tris, pH 6 and transferred to a 96-well plate at an OD of 7.5. The plate was then centrifuged at 15 *g* that cells form a loose pellet at the bottom of each well. RoGFP2 has two excitation maxima depending on its redox state. The anionic chromophore, predominantly in reduced roGFP2, is excited at ~ 480 nm. The neutral chromophore, predominantly in oxidized roGFP2, is excited at ~ 400 nm. In both states roGFP2 emits at 510 nm, allowing for ratiometric measurements. Fluorescence of roGFP2 was monitored in a BMG Labtech CLARIOstar fluorescence plate-reader.

For the evaluation of oxidation degree (OxD) the following balance was used:

$$OxD_{roGFP2} = \frac{I390 \bullet I480_{red} - I390_{red} \bullet I480}{I390 \bullet I1480_{red} - I390 \bullet I480_{ox} + I390_{ox} \bullet I480 - I390_{red} \bullet I480}$$

where I_{390} – intensity at 390 nm; I_{390ox} – full oxidized intensity at 390 nm;

I_{390red} – full reduced intensity at 390 nm; I_{480} – intensity at 480 nm;

I_{480ox} – full oxidized intensity at 480 nm; I_{480red} – full reduced intensity at 480 nm.

2.6. Gas-chromatography mass spectrometry (GC–MS) profiling of an intact extracellular matrix of yeast cells

The intact yeasts extracellular matrices were screened by GC–MS on a QP2010 (Shimadzu, Kyoto, Japan) in dependence on the media used, the grow phase of cells and their cultivation time. To this end, 1 μL of the sample/yeast extracellular matrix was injected into the DB-HeavyWAX column (Agilent, California, USA, 30 m × 0.25 mm, film thickness 0.25 μm) at a split ratio 1:10 using the following temperature gradient: starting temperature was 50 °C held for 1 min, raised to 200 °C at 20 K/min for 3 min and then held at 50 K/min at the final temperature of 250 °C for 5 min. MS ion source temperature was set at 200 °C. Mass spectra were recorded at *m/z* 30–300 in TIC and SIM modes. The obtained results were evaluated by means of NIST and WILEY Libraries.

2.7. GC–MS analysis of derivatized yeast extracellular matrix

To control a biochemical route resulting in the formation of hydrazine-based compounds depending on the used growth media and cultivation time, the obtained extracellular matrix was derivatized with acetylacetone for 1 h at 22 ± 1 °C according to previously reported protocol followed by extraction of the formed products (pyrazole in case of hydrazine presence and a mixture of free organohydrazines and azides) with dichloromethane.[27] Next, a chromatographic separation of the derivatized products was conducted on the ZB-5HT Inferno column (Phenomex, CA, USA, 30 m × 0.25 mm × 0.25 μm).

3. Results and discussion

3.1. Exploring the potential of Pd-NPs-modified electrodes for in vitro electrochemical screening of yeast extracellular matrix

The approved mechanical and chemical stability of Pd-NPs-modified electrodes in aqueous solutions [28,29] make them ideal for sensory tasks in biotechnology and applications in cell biology. In a previous study, we reported on the development of *in situ* alginate-based nanoreactors with Pd-NPs in design of the electrocatalytic layer.[14] This *in situ* approach serves as a nanofermenter to provide a friendly environment supporting physiological growth of yeast cells within 24 – 96 h from the one side and as an amperometric detector from the other side.

Further work in our laboratory led us to realize the potential of individual Pd-NPs for a rapid *in vitro* electrochemical screening of the extracellular matrix of yeasts depending on the used growth media. The absence of a polymer matrix (*viz.* alginate, poly-lysine, etc.) in the construction of electrodes allows for a marked difference in bioelectrochemical profile of yeasts in CV mode.

The electrode surface formed under the used experimental conditions was represented by nanoparticles of palladium with a diameter ranged from 60 to 80 nm, see **ESI,** Fig. S1. Interestingly, that the response of these Pd-NPs-modified electrodes at the anodic range of potential towards extracellular matrices from the **groups I – IV** was very different, Fig. 1. A clear formation of an oxidation peak between 0.18 and 0.35 V for the samples representing **groups II, III and IV** obtained for the cells cultivated for 24 h was established. At the same time, for the sample from the **group I** (prepared in the reduced amount of amino source, cultivation time of cells was 24 h) with a lack of growth (OD = 0.2) this oxidation peak was not observed. However, compared to a pure HC medium that has never been in contact with cells, an increase in current was recorded for this sample over the entire potential range.

The intensity of the characteristic peak at ~ 0.30 – 0.35 V for the samples representing **groups I-III** had a linear dependency on the cell OD, Fig. 1**A** (shown for the **group II** as a case study). CV plots and the position of peak maximum were also different in dependence with media used to grow yeasts: electrochemical peak recorded at 0.30 – 0.35 V for **group II** samples (HC medium supplemented with YNB) was shifted to 0.2 V for the **group IV** (HC medium supplemented with 2 % peptone was

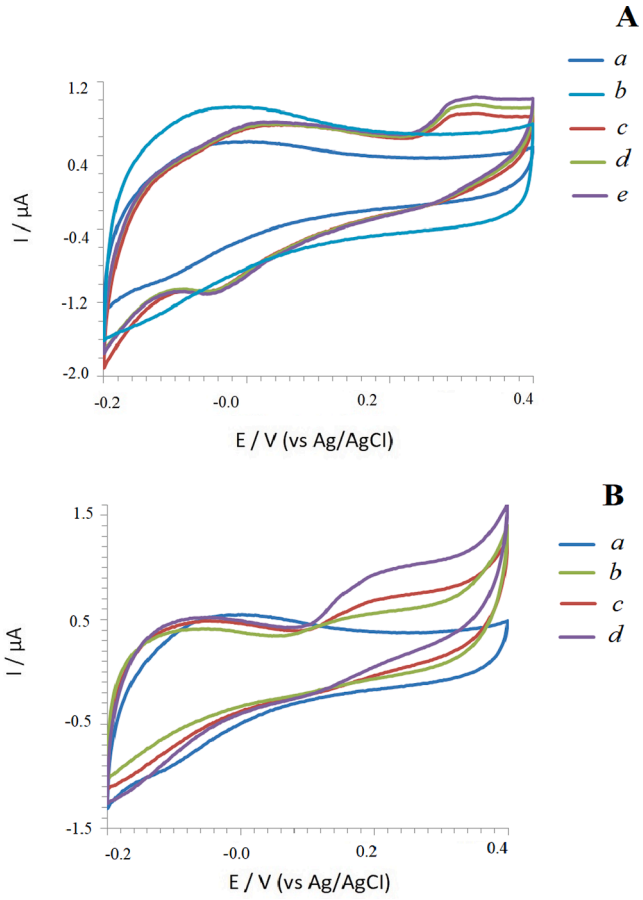

Fig. 1. CV plots (second scans shown) recorded at 20 mV/s from Pd-NPs-modified electrode in 150 μL droplet of **(A)**: *a* – HC medium that has never been in contact with cells, pH = 4.1, OD = 0; *b* – **group I** extracellular matrix, OD = 0.2 (no cell growth was observed), pH = 3.55; *c* – **group II** extracellular matrix, OD = 3.6, pH = 3.48; *d* – **group II**, OD = 4.2, pH = 3.39; *e* – **group II**, OD = 5.5, pH = 3.3. **(B)**: *a* – HC medium, OD = 0; *b* – **group III** extracellular matrix, OD = 6.1, pH = 5.30; *c* – **group IV** extracellular matrix, OD = 6.7, pH = 5.5; *d* – **group IV extracellular matrix**, OD = 7.7, pH = 5.2. *Note*: regardless the medium type cells were cultivated for 24 h followed by their removal and subsequent analysis of the extracellular matrix. CV measurements were repeated in triplicate with the same results.

used to grow cells of this group), Fig. 1**B**. Moreover, for the same peptone-containing medium the signal intensity at the anodic range depended on glucose and peptone concentration: the anodic current for **group III** cells grown in 1 % glucose and 1 % peptone was lower compared to the current recorded for the cells grown in 2 % glucose and 2 % peptone (**group IV**), see Fig. 1**B**. This means that utilizing Pd-NPs-modified electrodes it is readily possible to monitor minor changes in cultivation conditions of yeasts and analyze primary factors affecting their growth.

In contrast to diverse electrochemical profiles recorded for yeast extracellular matrices representing **groups I – IV**, CV plots obtained for the endogenous extracts of cells grown in YPD medium (**group V**) coincided with CV of a pure YPD medium (reference sample) which was not in contact with cells, see **ESI, Fig. S2**. Hence, electrochemical screening of cells from the **group V** by Pd-NPs-modified electrodes in CV mode appears to be challenging. We assume that it is more likely due to the absence of electroactive compounds that are formed in the extracellular matrix of yeasts that could be oxidized on Pd-NPs at the anodic range of potentials, see *section 3.3* for the details.

Next, we explored the performance of Pd-NPs for a rapid electrochemical screening of yeast extracellular matrices in time dependent experiments. From Fig. 2 it can be seen that the increase of the cultivation time from 24 h to 72 h for cells grown in the same medium (shown for cells representing **group II** prepared in HC medium with the added YNB) was accompanied by a significant increase of the anodic current recorded in the range of 0.30 – 0.35 V. At the same time, the changes in OD and pH of these samples were not informative enough to distinguish them based on cultivation time: *i.e.* OD = 6.8 ± 0.3 (24 h) *versus* OD = 7.2 ± 0.2 (72 h).

In other words, from Fig. 1 and Fig. 2 it is obvious, that not only the type of medium used (*i*), density (OD) of cells (*ii*) and the content of glucose (*iii*) in the growth media can affect the characteristic signal recorded from Pd-NPs-modified electrodes for the extracellular matrix in CV mode but also dynamic cultivation conditions of cells, *viz.* cultivation time (*iv*). This altogether highlights the advantage of this electrochemical approach for a rapid electrochemical screening of yeast extracellular matrix.

In addition, a clear difference in the electrochemical response of Pd-NPs was observed between mutant (**group VI**) and wild-type yeast cells (*v*). It should be noted that the mutant and wild-type yeasts had a very

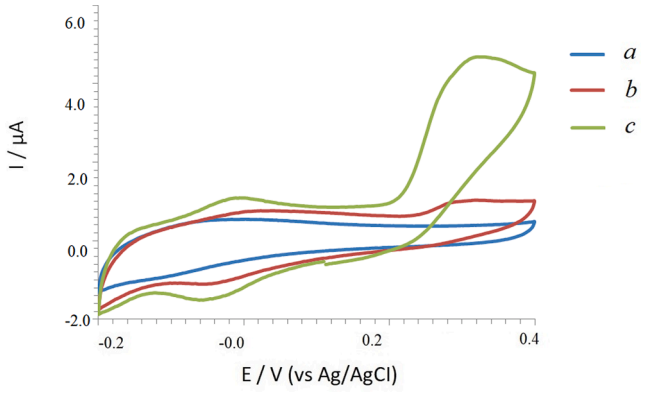

Fig. 2. CV plots (second scans shown) recorded from Pd-NPs-modified electrode at 20 mV/s in 150 μL droplet of: *a* – HC medium; *b* – extracellular matrix of **group II** cells, cells were cultivated for 24 h, OD = 6.8; *c* – the same extracellular matrix as shown in *b*, cell cultivation time 72 h, OD = 7.2. *Note:* pH of the samples was in the range of 3.5 ± 0.2. Electrochemical measurements were repeated at least in triplicate with the same results. The reproducibility of the peak maximum at the characteristic anodic range for the extracellular matrix obtained from yeast cells with OD = 7.2 and cultivation time 72 h at Pd-NPs-modified electrodes taken from three different batches was in the range of 5.8 ± 0.3 μA.

similar optical density (OD) and pH. In contrast to almost equal OD and pH values, these samples gave a very different electrochemical response at the anodic range of potentials on Pd-NPs-modified electrode that allowed us to distinguish them, **ESI, Fig. S3**. Thus, the current at 0.3 V for the mutant cells with OD = 4.9 ± 0.5 did not exceed 2.2 ± 0.2 μA (**group VI**). At the same time the current for the wild-type cells with the same OD and pH reached the level of 3.1 ± 0.3 μA (**group II**). The reason for this phenomenon will be discussed in *section 3.3 "Validation of signaling electroactive metabolites"*.

3.2. Some features of the electrochemical screening of yeast extracellular matrix and validation of electrochemical dependencies

Further, to study the reason of the current increase observed for the **group I** samples (no cell growth was observed, see Fig. 1**A**, curve *b*) in the absence of a clear oxidative peak or wave, we screened it in CV mode at low scan rates, *viz.* 5 mV/s, 10 mV/s and 20 mV/s in the reduced potential range (0 – 0.4 V). For comparison, **group II** and **group IV** samples were tested at the same modified electrochemical conditions. With this electrochemical approach we aimed to eliminate the impact of cathodic processes on the obtained dependencies in the anodic range.

Remarkably, the CV plots obtained at low scan rates were also very different depending on the growth conditions used (Fig. 3**A,D,G**), that was in line with the results obtained previously, see Fig. 1. However, due to significant capacitive currents recorded for Pd-NPs-based electrodes explored in the presence of yeast extracellular matrix, the faradaic currents corresponding to electron transfer (faradaic) reactions could be simply invisible or masked.

To verify the faradaic response of Pd-NPs in different multicomponent media, several data processing approaches were applied, *e.g.* the capacitive currents divided by scan rate, I/v and CV current response divided by square root of potential scan rate, $I/v^{0.5}$. The first approach allows to distinguish the area on CVs recorded from Pd-NPs-based electrodes where the current responses determine only by charging of double electric layer (capacitance). The second approach relates to faradaic responses described by diffusion kinetics (*i.e.* there is a stage of diffusion limitation of reaction rate) and allows to verify the kinetic nature of the observed signals.

As it is seen from Fig. 3**B** capacitive currents divided by scan rate, I/v, were the same for samples representing **group I**. In contrast, a difference between I/v value for various scan rates for samples from the **group II** and **group IV** (Fig. 3 **E,H**) can readily be attributed to faradaic responses. In case of faradaic responses the CV currents divided by the square root of potential scan rate, $I/v^{0.5}$, should be the same for various scan rates in the target potential window (*i.e.* 0.2 – 0.4 V). As it is seen in Fig. 3**C** regardless of the scan rates there are no parts where the current responses are equal for the sample from the **group I**. In other words, the faradaic processes in the anodic range of potentials can be observed exclusively for samples from of the **group II** and **group IV** (Fig. 3**F,I**).

To sum it up, during this set of experiments we managed to (*i*) validate the influence of low-rate process of electrochemical nature on current responses in CV mode recorded from Pd-NPs-modified electrodes (in order to record the electrooxidation of the target compound in the multicomponent media the scan rates should not exceed the level of 20 mV/s); and (*ii*) confirmed faradaic responses for the extracellular medium representing **group II** and **group IV**, Fig. 3 **D,G.** Finally, it was established that in order to make the difference in electrochemical profile between the samples more pronounced in dependence with the used growth medium, Pd-NPs-modified electrode supporting low oxidative process in the yeast extracellular matrix should be operated at very low scan rate, *viz.* 5 mV/s, see Fig. 4**.**

The dependencies recorded in CV mode (Fig. 4) for the samples from these three groups were in a line with results obtained by GC–MS. Hitherto, GC–MS profile for the extracellular matrix of cells grown in HC medium supplemented with YNB (**group II**) was different *vs* GC–MS profile of the extracellular matrix of cells grown in HC medium with the

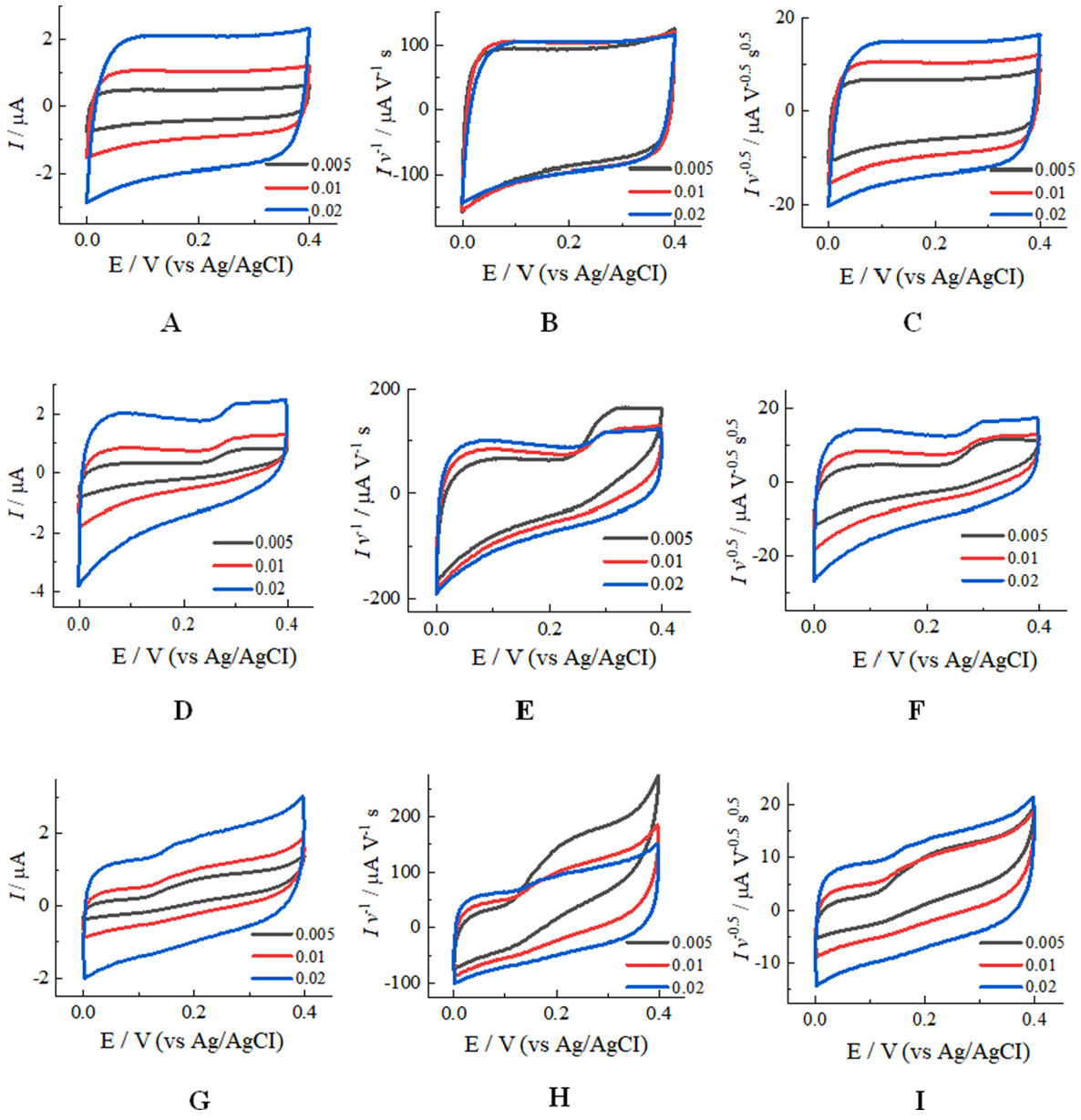

Fig. 3. Validation of the electrochemical screening of yeasts by Pd-NPs-based electrodes operated at the reduced cathodic range. **A,D,G** – CV plots recorded at 5, 10 and 20 mV/s for **group I** yeast extracellular matrices (**A**), OD = 0.2, pH = 3.53, **group II** (**D**), OD = 5.5, pH = 3.3 and **group IV** (**G**) OD = 7.7, pH = 5.2. **B,E,H** – CV plots in coordinates: current divided by scan rate as a function of potential, **C,F,I** – CV plots in coordinates: current divided by square root of scan rate as a function of potential. *Note:* cells were cultivated for 24 h regardless the selected group.

added 2 % peptone (**group IV**), Fig. 5. In general, a high level of ethanol (*i.e.* 180 ± 20 mM for the cells cultivated for 24 h) and acetic acid was detected in **group II** and **IV samples**. However, Pd-NPs-modified electrodes under the used electrochemical conditions, pH (3–5.5) and matrix (HC medium, peptone or YPD) did not show any sensitivity to alcohols and acetic acid up to 750 mM and 2 M, respectively.

Notably, numerous carbonyl-compounds known as cyto-toxicants, *e.g.* aldehydes and ketones were detected by GC–MS in the samples representing **group I** that explains the absence of an intensive growth of cells in this medium (see Fig. 1**A**, *line b,* OD = 0.2). At the same time, ethanol and acetic acid as direct products of successful glucose transformation by yeasts during their physiological growth were also not verified in the extracellular matrix of this sample highlighting non optimal conditions used for their fermentation, Fig. 5**a**. Hence, it can be assumed that the presence of a high amount of aldehydes and ketones in **group I** samples and not electrooxidized on Pd-NPs-based electrode in the presence of multicomponent medium can be a reason of the raised capacities currents (see Fig. 4**a**) over the entire range of potentials. At the same time, in this sample no electroactive compound responsible for the appearance of an oxidative peak at 0.20 – 0.35 V similar to **group II** and **group IV** can be assumed (no electrooxidation peak or wave formation in CV, see also Fig. 1**A**, *line b*).

The trends obtained by Pd-NPs-modified electrodes in CV mode and verified by GC–MS were also in line with results of ro-GFP 2-based assay, see **ESI,** Fig. S4. Thus, ro-GFP 2 sensor response recorded during 180 min toward H_2O_2-induced oxidative stress for the cells grown in HC medium supplemented with YNB (**group II**) was different *vs* cells grown in HC medium with the added 2 % peptone (**group IV**). The change of the ro-GFP 2 signal intensity indicates different RedOx activity and metabolism of yeasts depending on the growth medium used. It should be mentioned that these data were obtained after resuspension of cells taken from the medium in Mes-Tris buffer. Due to a raised strong

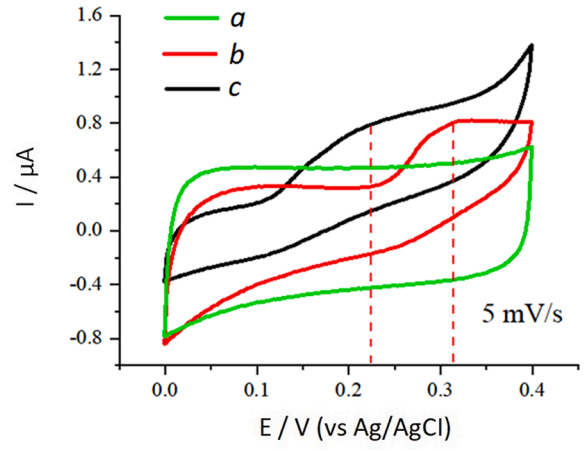

Fig. 4. Summary of CV plots (second scans shown) obtained from Pd-NPs-modified electrode in the reduced cathodic limit at 5 mV/s for the yeast extracellular matrices representing **group I** (*a, no growth, OD = 0.2*), **group II,** OD = 5.5, pH = 3.3 (*b*), **group IV**, OD = 7.7, pH = 5.2 (*c*). *Note:* cultivation time for all tested cells (prior to their removal from the growth media) was 24 h.

fluorescence signal, a direct measurement in a target growth medium utilizing ro-GFP 2 appears to be challenging, **ESI**, Fig. S5.Thus, 60 min after starting of an assay the oxidation signal obtained in HC medium supplemented with YNB continuously increased even in the absence of external oxidants (H_2O_2) that significantly limits a possibility for biochemical profiling of yeasts at physiologically relevant conditions.

In this regard, a rapid electrochemical screening of samples under physiological conditions in dependence with the medium used and growth phase based on the proposed electroanalytical approach via Pd-NPs-modified electrodes can be advantageous at least in several aspects, see Table 1.

The comparison of the present electrochemical approach with results observed by some other authors who have performed electrochemical yeasts characterization also indicates several advantages. Thus, in the majority the characterization of yeasts is based on the usage of RedOx mediators for charge transfer of yeast cells.[3–6] After such a pre-treatment procedure the change of physical properties of yeast cell membrane can be observed by SEM, AFM, optical microscopy or electrochemical studies. Other invasive amperometric approaches utilizing Pt-wire and glassy electrode, respectively, were based on the usage of a tetrazolium salt and hydroquinones (as metabolites of yeast cells microbial activity) reduction in alkaline media.[30,31] The promotion of the metabolism of quinones and hydroquinones was induced by the increase of plasma membrane permeability by ethanol molecules.

All the above-mentioned assays required a preliminary treatment of yeasts solution by RedOx mediators prior to the subsequent electrochemical studies. Apart from determination of yeast cell density and viability the above mentioned assays did not allow to screen the cells in dependence with the type of used growth medium and growth time or to distinguish wild-type cells from the mutants.

To sum it up, the advantageous of the proposed electrochemical approach toward screening of yeast extracellular matrices based on Pd-NPs-modified electrodes can be summarized as follows:

~ no additional sample preparation, pretreatment by RedOx mediators and derivatization is required;

~ the use of a droplet mode (a droplet of the sample can be taken from the fermenter followed by centrifugation for 5 min to obtain extracellular matrix for the subsequent *in vitro* analysis);

~ the advanced bioanalytical information: samples can have the same OD and pH, but different amounts of electroactive component (due to a different life cycle) followed by its electrooxidation at Pd-NPs that opens up new possibilities for their profiling, monitoring and control;

~ possibility to visually distinguish mutants from wild-type cells;

~ no need to induce, change or to study cell permeability depending on the type and amount of external RedOx mediators;

~ rapid optimization of cultivation conditions for wild-type yeasts and their various mutants.

3.3. Validation of signaling electroactive metabolites

In our previous work we reported on a formation of secondary metabolites, *viz.* organohydrazines in yeast extracts as a result of bioconjugation process.[14] Returning to Fig. 5 it should be mentioned that no hydrazine-derivatives were directly seen on the TIC-chromatograms in EtOH/H_2O containing samples without implementation of preliminary derivatization or product extraction steps.

To clarify the nature of the observed characteristic signal (Fig. 1,2) on Pd-NPs-modified electrodes and to confirm a possible formation of hydrazine-based products in the extracellular matrix of tested yeasts, we derivatized the samples with acetylacetone according to a protocol reported in [27], *see Experiment*.

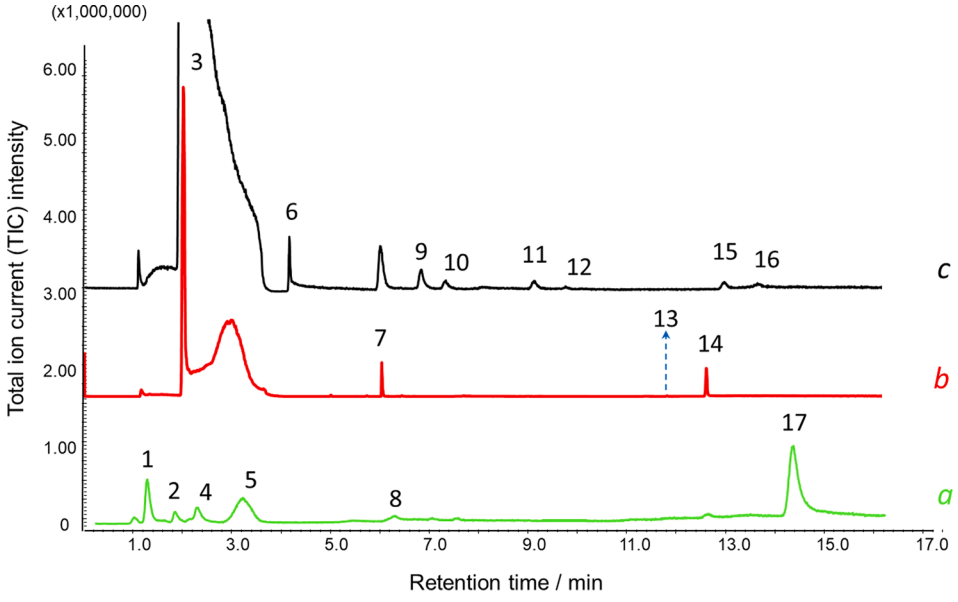

Fig. 5. GC–MS TIC-chromatograms obtained from the ZB-WAX plus column indicating the impact of the used fermentation medium on the microbial activity of yeasts of **group I** (*a*), **group II** (*b*) and **group IV** (*c*). Peak assignments: 1 – oxygen; 2 – 2-methyl-propanal; 3 – ethanol; 4 – 2-methyl-butanal; 5 – carbamic acid methyl ester; 6 – 5-methyl-2-heptanamine; 7 – acetic acid; 8 – furfuralcohol; 9 – 5-methyl-2-furancarboxylaldehyde (5-methyl-2-furfural); 10 – phenylacetaldehyde; 11 – 2-phenylethanol; 12 – 2-formy-1-methylpyrrole; 13 – 2-ethyl-crotonaldehyde; 14 – 2-methyl-propanoate butanol; 15 – 2-butene 1,4-diol; 16 – 4H-Pyran-4-one (4-Pyrone); 17 – 5-hydroxymethyl-2-furancarboxaldehyde. *Note*: extracellular matrices were used in their intact form without any pretreatment and preliminary derivatization steps.

Table 1
A comparative table of bioanalytical assays employed for biochemical screening of extracellular matrix of yeasts.

Parameter	GC–MS	Electroanalytical assay based on Pd-NPs-modified electrode	Genetically encoded biosensors, ro-GFP2
Invasive/non-invasive*	invasive	non-invasive	non-invasive
Duration of analysis, including sample pre-treatment	from 20 to 60 min	3 min	8 days
Type of matrix	allows mearsurments directly in extracellular medium after centrifugation of cells	allows mearsurments in cell medium as well as in a suspension of cells + medium	allows exclusivly mearsurments in cells resuspended in buffer solution
Possibility to conduct cell profiling at physiologically relevant conditions	no	yes	no
Simplycity of analysis	requires expensive instruments and experienced personal	simple	requires essential genetical manipulations
Degree of portativity	non portative	portative, allows a rapid profiling of cells *in vitro* mode	non-portative
Phase of bioanalyte control mode	extracellular	extracellular	intracellular

* sample destructive or not.

GC–MS profiles of the samples after derivatization followed by subsequent extraction with dichloromethane were very different and quite complex as compared to GC–MS profiles obtained for non-derivatized analogues (see **ESI, Fig. S6** in comparison with Fig. 5). Apart from pyrazoles found as direct derivatization products of hydrazine standard solution prepared in HC medium, a wide range of alkanes, un-derivatized azines, pyrroles, azides, N-[3-*t*-butylimino-1,2-dimethyl-propyl]aziridine, benzotriazole and organohydrazines (*i.e.* methylhydrazine hydrochloride) was detected. More importantly, GC–MS profile recorded for the standard of derivatized N_2H_4 was almost identical with a chromatogram and mass spectra obtained for the derivatized sample of **group II**. On the other hand, the conducted GC–MS experiments of the derivatizied samples allows to exclude possible *in situ* formation of organohydrazines from amino acids contained in medium and aldehydes formed in the extracellular matrix during cultivation of cells directly on the electrode due to electrocatalytic ability of Pd-NPs [32].

Apart from pyrazoles, azines and azides GC–MS analysis of the samples from the **group IV** revealed the formation of the significant amount of un-derivatized 1-methyl-1-phenylhydrazine (similarity degree with the database was 88 %). It means that free un-derivatized organohydrazines with different chemical nature were formed in the extracellular matrix of yeasts (see Fig. 1**A,B**) during their physiological growth in HC medium supplemented with YNB (**group II**, *viz.* methylhydrazine hydrochloride) and HC medium with added 2 % peptone (**group IV**, 1-methyl-1-phenylhydrazine**).** This fact can also explain difference in forms of CV plots and position of peak maximum recorded for the samples from these two groups, see Fig. 4**b,c** (0.32 V found for the **group II** *vs* 0.24 V for the **group IV**).

However, not only a different chemical nature of the formed organohydrazines, but also pH value of the samples within **group II** and **group IV** could be responsible for a different position of the peak maximum, see Fig. 4. The subsequent model experiment performed for the **group II** extracellular matrix by adjusting pH from the intact 3.3 to 5.2 revealed a shifting of the peak max on the CV curve to the same position what was obtained for the **group IV** with pH = 5.2, Fig. 6. Meanwhile, altering the pH value from 3.3. to 5.2 for the medium which has never been in contact with cells (reference sample) was not accompanied by above-mentioned effect. The pH change for a pure medium only slightly affected base line of the electrode, **ESI**, **Fig. S7**.

Notably, that after derivatization of growth-deficient **group I** samples no pyrazoles, free/un-derivatized hydrazine- or hydrazone-related compounds were detected by GC–MS that was in line with results of yeasts profiling by Pd-NPs-modified electrodes (see Fig. 1**Ab**, Fig. 3**a**, no faradaic current increase in the anodic range).

Speaking about possible source of hydrazine derivatives appearance in the extracellular matrix of yeasts representing **groups II-IV**, several routes could be assumed: *e.g.* pathway based on interactions between ammonia or amines and aliphatic ketones [33], multi-step interactions involved urea [34] or by reacting ammonia (*S. cerevisiae* can produce ammonia in the range of 0.69 ± 0.3 ppm/h per cm^2 as a result of amino acids cleavage [16]) with chloramine [35]. Alternatively, hydrazones and reduced hydrazines can be produced by peroxides-induced routes in the presence of ketones and ammonia [14] or as a result of bio-conjugation between alkoxy-amines and aldehydes resulting in the formation of oximes and hydrazones followed by hydrolysis to corresponding organohydrazines.[36] Regardless of the employed route formed oximes, hydrazones, hydrazines and organohydrazines in HC or peptone-containing media can readily be assigned to the formed secondary metabolites of yeasts.

The lack of amino source in growth media and nitrogen deficiency (yeast-assimilable nitrogen, YAN) leads to adjusting of yeasts metabolism so that they begin to utilize their own amino acids.[37] In other words, the lack of amino source in yeast growth media cannot support the above mentioned pathways underlying the formation of hydrazines and organohydrazines in the extracellular matrix.

This assumption was in line with results of GC–MS analysis obtained for the derivatized extracellular matrix of cells representing **group V**. The cells from this group were grown in YPD medium which didn't contain extra ammonium source (YPD-YNB-peptone). Neither pyrazoles as direct products of chemical derivatization of hydrazine nor un-derivatized free organohydrazines were detected in the **group V** extracellular matrix. The absence of hydrazine and organohydrazine-based substances in these samples was also confirmed by CV studies utilizing Pd-NPs-modified electrodes: no characteristic electrochemical peak at

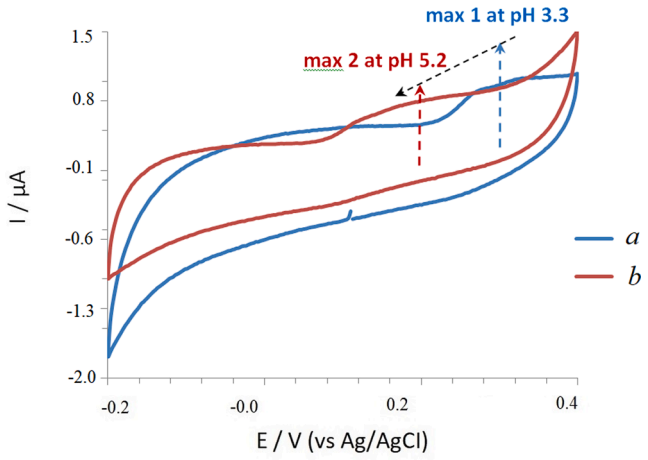

Fig. 6. CV plots recorded from Pd-NPs-modified electrode at 20 mV/s in the yeast extracellular matrix: *a* – **group II**, OD = 4.4, pH = 3.3; *b* – sample *a* with an adjusted pH of 5.2. *Note:* The peak max shift from 3.2 V to 0.20 V was induced by pH change of sample from 3.3 to 5.2.

the anodic range of potential was seen on CV plot for **group V**, see **ESI**, **Fig. S2**.

Moreover, the lack of specific amino source in growth media (HC-YNB-uracil) could also explain the reason of the decreased intensity in the characteristic signal obtained for the extracellular matrix of mutant cells, **group VI** (**ESI,** Fig. 3) as compared to **group II** wild-type samples. In this regard, maintaining the amino source in growth media at a constant level seems to be an important factor for conducting an efficient electrochemical screening of yeast extracellular matrix based on hydrazines electroactive products.

The spiking experiment of **group II** yeast matrices by standard solution of hydrazine approved the nature of chemical class of compounds (hydrazine-based) that caused the appearance of the characteristic signal on Pd-NPs-based electrodes, Fig. 7, *lines a,b*. Testing of hydrazine solution prepared in the same growth medium as an external standard (Fig. 7**c**) revealed the same form of CV plot and the same peak maximum. Moreover, the subsequent CV analysis of derivatized sample of **group II** assuming the formation of pyrazole-based compounds indicated a dramatic decrease of the characteristic signal at the range of 0.30 – 0.35 V (almost until the base line of the electrode), Fig. 7**d** (see in comparison with Fig. 7**a**). Obviously, Pd-NPs-modified electrode is not sensitive enough to pyrazoles formed upon derivatization (*see the next section*). In other words, the tandem of model GC–MS and CV experiments confirmed the formation of organohydrazine derivatives in the extracellular matrix of yeast cells representing **groups II-IV**.

3.4. *Electrooxidation of hydrazine at Pd-NPs-modified electrodes in multicomponent media: Superior sensitivity in the presence of interfering species*

Apart from ethanol and acetic acid, a wide range of amines, pyrroles and aldehydes was detected in the extracellular intact (un-derivatized) matrices of yeasts, see Fig. 5. Therefore, the focus next was tuned to the comparative study of electrooxidation of various nitrogen-containing compounds in HC medium supplemented with the additional amount of the amino source YNB (modeling **group II** samples) on Pd-NPs-modified electrodes.

The sensitivity of electrooxidation of nitrogen-containing compounds at 0.3 V increased from 0.027 ± 2 μA/mmol to 4.2 μA/mmol in the following sequence: adenosine < pyrazole < formamide < urea ≤ nicotinamide < pyrrole < ammonia < ethanolamine < diethylamine < hydrobenzaldehyde azine < 2,2-azino-bis-ethylbenzothiazoline ammonium salt.

Notably, the sensitivity of detection of almost all tested nitrogen-containing compounds (except azines) did not exceed the level of 0.2 μA/mmol. The absence of Pd-NPs sensitivity to pyrazole (this is a direct derivatization product of hydrazine) oxidation can readily explain dropping of the characteristic signal shown in Fig. 7**d** *(section 3.3)*. This model experiment utilizing derivatization of hydrazine to pyrazole also highlights an exclusive selectivity of Pd-NPs-modified electrode to hydrazines.

The sensitivity of Pd-NPs-modified electrodes to the electrooxidation of alcohols, aldehydes and ketones in real yeasts medium was almost zero over the entire potential range of regardless of their concentration and pH, Fig. 8**a** (shown for 100 mM of EtOH as a case study). Hence, it appears to be impossible to extend the potential of this electroanalytical approach towards electrochemical detection of alcohols and aldehydes in complex media by tuning of the pH of samples or polarization mode of the electrode. However, earlier it was reported that Pd-NPs-based electrodes explored in buffered standard solutions at basic pH had a moderate activity towards alcohols and aldehydes electrooxidation.[22] The observed suppression phenomenon in our case impacting alcohols and aldehydes electrooxidation on Pd-NPs-based amperomeric electrodes is more likely related with a raised matrix effect.

In contrast, the sensitivity to azines in the same matrix/medium at a potential of 0.3 V was found at the level of 2 ± 0.3 μA/mmol for hydrobenzaldehyde azine, 4 ± 0.4 μA/mmol for 2,2-azino-bis-ethylbenzothiazoline ammonium salt and 15 ± 0.5 μA/mmol for hydrazine as the simplest representative compound of hydrazinés class. Therefore, we assume that in the multicomponent media (yeast extracellular matrix) exclusively azine- and hydrazine-related compounds can be detected by Pd-NPs-modified electrodes at the anodic potentials regardless of pH, Fig. 8**b,c**.

This assumption agreed well with the previously reported dependency of hydrazine electrooxidation on palladium-modified electrodes.[18] Thus, in both media at pH 2 and 12 a high sensitivity of palladium to hydrazine electrooxidation was observed due to a formation of protonated ($N_2H_5^+$) and unprotonated (N_2H_4) hydrazine forms at acidic and basic pH, respectively. By pH altering from 2 to 12 the peak corresponding to hydrazine electrooxidation was shifted from ~ 0.25 – 0.30 V to ~ 0 V. The sensitivity of N_2H_4 electrooxidation at pH 2 in HC medium was an order of magnitude higher than in the same medium at pH 12. In other words, the characteristic signals observed on Pd-NPs-modified electrodes for the yeast extracellular extracts/matrices can readily be attributed to the oxidation of hydrazine-based compounds if their formation took place.

Having evidenced the exclusive electrocatalytic ability of Pd-NPs-modified electrodes to hydrazines regardless of pH and medium content, a reliable electrochemical screening of yeast samples/extracellular matrix based on these types of compounds followed by comparison of CV profiles can be conducted. The exception to the use of this electrochemical approach is the extracellular matrix of cells grown in YPD medium in reduced amount/lack of amino source (**group V**). At these

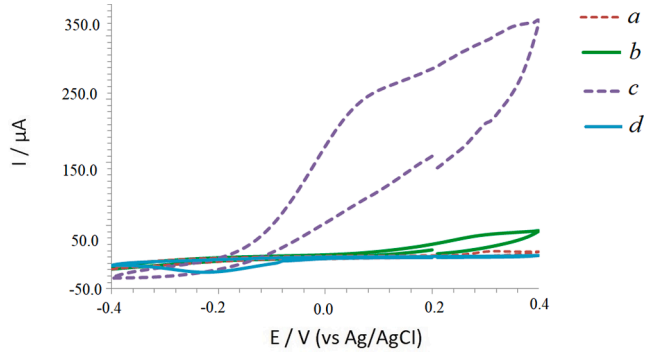

Fig. 7. CV plots (second scans shown) obtained during screening of samples at the scan rate of 20 mV/s: *a* – yeast extracellular matrix of **group II**, (cells were grown in HC medium supplemented with YNB), prior to centrifugation cells had OD = 7.1; *b* – sample *(a)* + 10 mM of N_2H_4; *c* – 100 mM N_2H_4 in HC medium supplemented with YNB; *d* – sample (*a*) after derivatization.

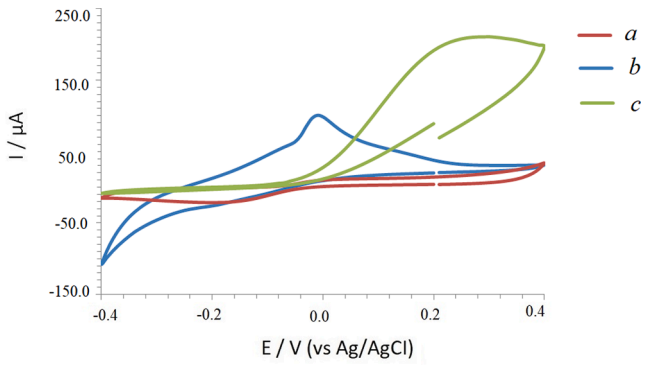

Fig. 8. CV plots (second scans shown) recorded from the surface of Pd-NPs-modified electrode at 20 mV/s in a droplet of: 100 mM of EtOH, pH 12 (*a*), 1 mM N_2H_4, pH 12 (*b*) and 1 mM N_2H_4, pH 2 (*c*). *Note*: HC medium supplemented with YNB was used for sample preparation in all experiments.

conditions the formation of electroactive signaling compounds, *viz.* organohydrazines cannot be expected (see **ESI,** Fig. S2 and discussion *in section 3.1*).

4. Conclusions

In this study a rapid electrochemical approach for *in vitro* screening of yeast extracellular matrix utilizing Pd-NPs-modified electrodes and organohydrazines as signaling secondary metabolites was proposed. This approach allows to visualize a minor difference in cultivation conditions of cells (glucose content and type of the used fermentation media, cultivation time) as well as to distinguish wild-type cells from genetically modified variants. The performance of the fabricated electrodes was fully validated by advanced bioanalytical techniques.

The next step of the proposed electrochemical approach will be the development of biological protocols for treating yeasts to study the impact of various supplements, *viz.* loading of drugs/chemicals or antioxidants on cell response evaluated by Pd-NPs-modified electrodes. This step should be implemented in a close collaboration with biologists. Finally, by summarizing the data in a form of database, including CV plots of standard (STDs) samples and development of qualitative and quantitative comparative criteria between corresponding STDs and real samples, this rapid approach can be realized as a standard tool for electrochemical screening of cells on a daily basis. In addition, given the formation of organohydrazines as secondary metabolites in the extracellular matrix of yeast cells under certain cultivation conditions could open up the new era in hydrazine-based fuel cells and development of sustainable energy supply.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioelechem.2022.108283.

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