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# Effect of yeast pretreatment on the characteristics of yeast-modified electrodes as mediated amperometric biosensors for lactic acid

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ABSTRACT

Carbon paste electrode modified with baker' and wine yeast *Saccharomyces cerevisiae* (a source of flavocytochrome $b_2$) were investigated as amperometric biosensors for L-lactic acid. Before immobilization on the electrode surface, yeast cells were pretreated with various electrolytes, alcohols and weak organic acids. Electrode responses to L-lactic acid were tested in the presence of various mediators (potassium ferricyanide, phenazine methosulfate, 2,6-dichlorophenolindophenol sodium salt hydrate, 1,2-naphthoquinone-4-sulfonic acid sodium salt). The highest (144±7 nA per 0.2 mM L-lactic acid) and the most stable responses were obtained after yeast pretreatment with 30% ethanol using potassium ferricyanide as a mediator. Different electrode sensitivities with mediator phenazine methosulphate probably reflected diverse changes in yeast membrane (and/or cell wall).



## 1. Introduction

The importance of determination of lactic acid in clinical analysis, sports medicine and dairy industry has stimulated the development of amperometric biosensors based on enzymes active for lactic acid such as lactate oxidase [1–4], NAD+-dependent lactate dehydrogenase [5–8] and flavocytochrome $b_2$ [9–12]. All these enzymes catalyze the oxidation of L-lactic acid to pyruvic acid. The advantages of flavocytochrome $b_2$ are that this enzyme is specific for L-lactic acid; it does not require an additional cofactor and is non-specific for mediators. The scheme of mediated electrocatalytic oxidation of lactic acid can be represented as follows:

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

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

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

where Eqs. (1) and (2) are chemical reactions of flavocytochrome $b_2$ with lactic acid and mediator, respectively, Eq. (3) is the electrochemical oxidation of the reduced form of mediator. This reaction is used for amperometric detection of lactic acid. The limitation of flavocytochrome $b_2$ (isolated mostly from yeast *Saccharomyces cerevisiae*, *Hansenula anomala*, *Hansenula polymorpha*) as a biosensing constituent is its poor stability. The possible ways to solve the stability problem are to seek the yeast strains producing stable forms of flavocytochrome $b_2$ [13,14] or to modify electrodes with the whole yeast cells, thus keeping the enzyme in its natural environment. Flavocytochrome $b_2$ is known to be located in the inter-membrane space of yeast mitochondria [15]. The catalytic activity of intracellular enzymes in intact yeast cells may be low due to the impermeability of membrane to the substrate and/or mediator [16]. Relatively thick cell wall (composed mostly from polysaccharides, chitin and proteins) is responsible for the cell's resistance to mechanical stress and presents no real barrier to the diffusion of small molecules and ions [17]. Research of redox processes occurring in the intact yeast cells [18–21] revealed that amperometric measurements using only negatively charged ions (such as ferricyanide) were not possible probably due to electrostatic interaction of mediator with phosphate moieties of membrane phospholipids. The double mediator system containing both lipophilic (such as menadione) and hydrophilic (ferricyanide) was needed to monitor the redox activity inside the yeast cell.

Another way to achieve significantly higher electrode responses compared to those when intact yeast cells were immobilized onto electrode surface is the permeabilization of yeast cells. Common permeabilization methods include cell treatment with solvents, detergents, salts, cell freezing and thawing or electropermeabilization [22,23].

Numerous publications of the effects of various electrolytes, alcohols and weak organic acids on “yeast life” (including possible perturbation of yeast cell membrane integrity) has motivated this investigation of the performance of yeast-modified electrodes

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**Table 1**
Current responses of baker's (BY) and wine (WY) yeast-modified electrodes to 0.2 mM L-lactic acid in phosphate buffer at pH 7.3 containing 0.5 mM $K_3[Fe(CN)_6]$ and 0.5 mM EDTA (operating potential 0.3 V, *vs.* Ag/AgCl, 3 N NaCl))

| Solution used for yeast pretreatment | Current response (BY), nA | Current response (WY), nA |
|---|---|---|
| 0.1 M $KH_2PO_4$+0.1 M KCl (pH 7.3) | 17±4 | 12±6 |
| 0.1 M $KH_2PO_4$ (pH 7.3) | 11±10 | 11±4 |
| 0.1 M KCl | 17±4 | 10±6 |
| 0.1 M $NaH_2PO_4$ (pH 7.3) | 28±8 | 12±8 |
| 0.1 M $NaH_2PO_4$+0.1 M NaCl (pH 7.3) | 42±9 | 12±6 |
| 0.1 M LiCl | 71±10 | 45±5 |
| 0.1 M LiCl+0.1 M $NaH_2PO_4$ (pH 7.3) | 95±7 | 47±8 |
| 0.01 M LiCl+0.1 M $KH_2PO_4$+0.1 M KCl (pH 7.3) | 11±6 | 11±6 |
| 0.025 M LiCl+0.1 M $KH_2PO_4$+0.1 M KCl (pH 7.3) | 58±10 | not tested |
| 0.05 M LiCl+0.1 M $KH_2PO_4$+0.1 M KCl (pH 7.3) | 73±9 | not tested |
| 0.1 M LiCl+0.1 M $NaH_2PO_4$+0.1 M NaCl (pH 7.3) | 93±7 | 40±8 |
| 0.2 M LiCl+0.1 M $NaH_2PO_4$+0.1 M NaCl (pH 7.3) | 99±10 | 45±4 |
| 0.1 M LiCl+0.1 M $KH_2PO_4$+0.1 M KCl (pH 4.6) | 72±14 | 45±8 |
| Water | 11±4 | 12±4 |

containing yeast cells pre-treated with these substances prior to immobilization on the electrode surface. Electrode responses to L-lactic acid in the presence of mediators (potassium ferricyanide, phenazine methosulfate, 2,6-dichlorophenolindophenol sodium salt hydrate, 1,2-naphthoquinone-4-sulfonic acid sodium salt) probably reflected the changes in cell membrane and/or cell wall induced by the cell pre-treatment.

## 2. Experimental

Baker's yeast (BY) *S. cerevisiae* (SEMA, Panevezys, Lithuania) was obtained from local market (shelf life not less than 2 weeks as specified by the producer). Wine yeast (WY) wine strain type K2 killer Rom K-100 HM/HM wt [kill-K2] from the collection of Institute of Botany (Vilnius, Lithuania) was grown on the YEPD medium (1% yeast extract, 2% peptone, 2% glucose and 2.5% agar) until stationary phase. The plates were kept for 3 days at 30 °C. Afterwards the samples of yeasts were stored in the fridge.

Potassium ferricyanide, *N*-methylphenazonium methyl sulphate (or phenazine methosulphate, PMS), 2,6-dichlorophenolindophenol sodium salt hydrate (DCPIP) were obtained from Fluka, 1,2-naphthoquinone-4-sulfonic acid sodium salt (NQS) was from Merck. Phosphate buffer was prepared from 0.1 M $KH_2PO_4$ and contained additionally 0.1 M KCl (both from Fluka). The values of pH were adjusted with KOH.

Plain carbon paste was prepared by mixing 100 mg of graphite powder (Merck) with 50 μL of paraffin oil (Fluka). Bulk yeast-modified paste was prepared by mixing 40 mg of yeast with 60 mg graphite and 50 μL of paraffin oil. The pastes were packed into an electrode body consisting of a plastic tube (diameter 2.9 mm) and a copper wire serving as an electrode contact. The layers of the pre-treated yeast cells on the surfaces of a plain carbon paste electrodes were formed by dipping the electrode into the suspensions of yeast prepared from 40 mg yeast in 0.5 mL of solutions of various salts ($KH_2PO_4$, KCl, $NaH_2PO_4$, NaCl, LiCl (all from Fluka) in various combinations, or in solutions of methanol, ethanol or isopropyl alcohol (all from Reakhim, Russia) of various concentrations in phosphate buffer at pH 7.3 or in solutions of acetic acid, sodium and lithium acetate or benzoic acid (all from Reakhim, Russia) at various concentrations. The electrodes were allowed to dry at room temperature for 25–30 min. and then covered with a dialysis membrane (Aldrich-Sigma) pre-soaked in water. To test the effect of time of yeast pretreatment on electrode responses, electrode modifications with pre-treated cells were performed after 1 h and further after every second hour or as indicated (in the cases of methanol or ethanol). All experiments were repeated at least for 3 times. Yeast suspensions were stored at room temperature.

Electrochemical experiments were carried out with a BAS-Epsilon Bioanalytical system (West Lafayette, USA) and a three-electrode cell arranged with a magnetic stirrer. Modified carbon paste electrode served as a working electrode. Platinum wire and Ag/AgCl, 3 N NaCl were, respectively, counter- and reference electrodes. Amperometric measurements were carried out in a stirred solution at an operating potential 0.3 V (vs. Ag/AgCl, 3 N NaCl) in phosphate buffers at pH 7.3 containing 0.5 mM of mediator. In the case of potassium ferricyanide, the solution contained additionally 0.5 mM EDTA (Reakhim, Russia). For some experiments, solutions were deoxygenated by purging argon for 20 min. All measurements were performed at room temperature.

To test the viability of pre-treated cells, 10 mg of the yeasts were suspended with stirring in 0.2 mL of sterile water. Primary culture of the yeast was also suspended for comparison. Both suspensions were seeded on the YEPD medium (1% yeast extract, 2% peptone, 2% glucose and 2.5% agar). The plates were kept for 3 days at 30 °C. The cloning was carried out until separate colonies of the yeast were obtained. The resemblance of the cells was controlled by microscopy.

## 3. Results and discussion

### 3.1. The effect of various electrolytes

Previous our investigation on possibility to use baker's yeast as a cheap source of the enzyme flavocytochrome $b_2$ for the development of amperometic biosensor for lactic acid was focused on the properties of intact yeast-modified carbon paste electrode in combination with various mediators both in the solution or adsorbed on graphite [24,25].

The idea to pre-treat the yeast cells with various electrolytes before immobilization on the electrode surface was inspired by the effect of lithium salts on yeast transformation efficiency and yeast membrane permeability [26–29]. Therefore, yeast suspensions for electrode modification were prepared in solutions of LiCl and other salts (such as $KH_2PO_4$, $NaH_2PO_4$, KCl, and NaCl) commonly employed in bioelectrochemical analysis. Electrode response to 0.2 mM of L-lactic acid in phosphate buffer at pH 7.3 containing 0.5 mM potassium ferricyanide and 0.5 mM EDTA was taken as a measure of the effect of yeast pretreatment since it was determined (as described below) that the highest and the most stable electrode responses were obtained with potassium ferricyanide as a mediator. The results are summarized in Table 1. Current responses of electrodes with yeast (both BY and

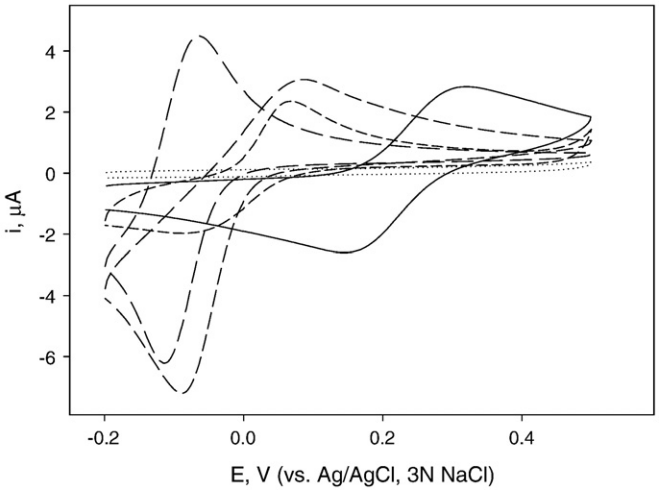


**Fig. 1.** Cyclic voltammograms of electrode modified with LiCl-pretreated BY in phosphate buffer pH 7.3 (dotted line) and in phosphate buffer pH 7.3 containing 1 mM $K_3[Fe(CN)_6]$ (solid line), 1 mM phenazine methosulphate (long-dashed line), 1 mM 2,6-dichlorophenolindophenol sodium salt hydrate (medium-dashed line), 1 mM 1,2-naphthoquinone-4-sulfonic acid sodium salt (short-dashed line). Potential scan rate 50 mV/s.

WY) layer obtained from suspensions containing potassium ions were low (not exceeding 21 nA per 0.2 mM L-lactic). The effect of sodium ions was more expressed for BY-modified electrodes. Both BY- and WY-modified electrodes showed significantly higher responses to L-lactic acid after pretreatment with solutions containing 0.1 M LiCl compared to those after pretreatment with electrolytes without LiCl. The electrode responses probably also indirectly suggested that the ionic strength or pH of solutions were not important factors contributing to the increase of electrode sensitivities. The amount of LiCl in the pretreatment solution was the main factor that predetermined the electrode sensitivities.

Cyclic voltammograms of electrodes modified with LiCl-affected yeast recorded in phosphate buffer at pH 7.3 (Fig. 1, dotted line) were constant during prolonged cycling indicating that surface state did not change contrary to the case when bulk yeast-modified carbon paste electrodes [24,25] were used. Constant cyclic voltammograms in solutions of potassium ferricyanide (Fig. 1, solid line), PMS (Fig. 1, long-dashed line), NQS (Fig. 1, medium-dashed line) and DCPIP (Fig. 1, short-dashed line) were recorded after 4 or 5 potential scans and were similar to those of electrodes containing baker's or wine yeast cells treated with water or phosphate buffer at pH 7.3 (not shown). The values of peak separation ($\Delta E_p$) in voltammograms ranged from 0.052 V (for PMS) to 0.17 V (for potassium ferricyanide and NQS) indicating that the highest reversibility of mediator electrochemical processes at yeast-modified electrodes was observed for PMS.

To evaluate the effect of LiCl on permeabilization of yeast cell membranes and/or cell walls, the modes (layered and bulk) of electrode modification and electrode responses in various mediator solutions should be compared. Fig. 2 shows the dependences of electrode (a — with a layer of cells pre-treated with phosphate buffer at pH 7.3, b — with a layer of yeast cells pre-treated with potassium phosphate buffer at pH 7.3 containing 0.1 M LiCl, and c — bulk-paste modified with non-pretreated cells) responses on concentration of L-lactic acid in solutions of potassium ferricyanide, NQS, DCPIP and PMS. The sensitivities of electrodes with a layer of phosphate buffer-pretreated cells (both for BY and WY, shown only for BY) were low with all these mediators and did not exceed 21 nA per 0.2 mM of L-lactic acid (Fig. 2A). The responses of electrodes with a layer of LiCl-affected yeasts (Fig. 2B) were 5 to 7 times higher with potassium ferricyanide compared to those obtained with NQS and DCPIP (both for BY- and WY-modified electrodes). The electrodes were practically insensitive in solution of PMS, whereas the responses of bulk-paste modified electrodes (Fig. 2C) in PMS solution were comparable to those obtained with potassium ferricyanide. These differences of electrode performances probably could be explained by different accessibility of enzyme to mediators and/or substrate. Probably during mixing with graphite, yeast cell walls and/or membranes were damaged, thus, substrate and mediator could reach flavocytochrome $b_2$ (as reflected by electrode sensitivities with all mediators in Fig. 2C), whereas cell treatment with LiCl probably induced permeability changes in the cell membrane and/or cell wall favorable for negatively charged mediators and not favorable for PMS bearing a positive charge on phenazine ring.

The repeatability of the electrode responses to L-lactic acid was tested by running three consecutive calibration curves in the range 0.2 to 1.2 mM. The responses in NQS and DCPIP decayed gradually by 20 to 35% both for BY and WY (and both for bulk yeast-modified paste and for LiCl-pretreated layer) with each subsequent calibration curve. To keep stable responses (95–100% of the initial) in potassium ferricyanide solution, EDTA was necessary as it was observed previously with electrodes modified with dried yeasts [30]. Therefore, in order to study the effects of yeast pre-treatment on electrode sensitivity, the measurements with the same electrode were performed strictly as follows: three calibration plots in the range 0.2 to 1.2 M L-lactate in 0.5 mM potassium ferricyanide solution containing EDTA (to be sure about the stability of the responses), one calibration plot at a time in PMS, then in NQS and DCPIP (all 0.5 mM) solutions. Due to instability

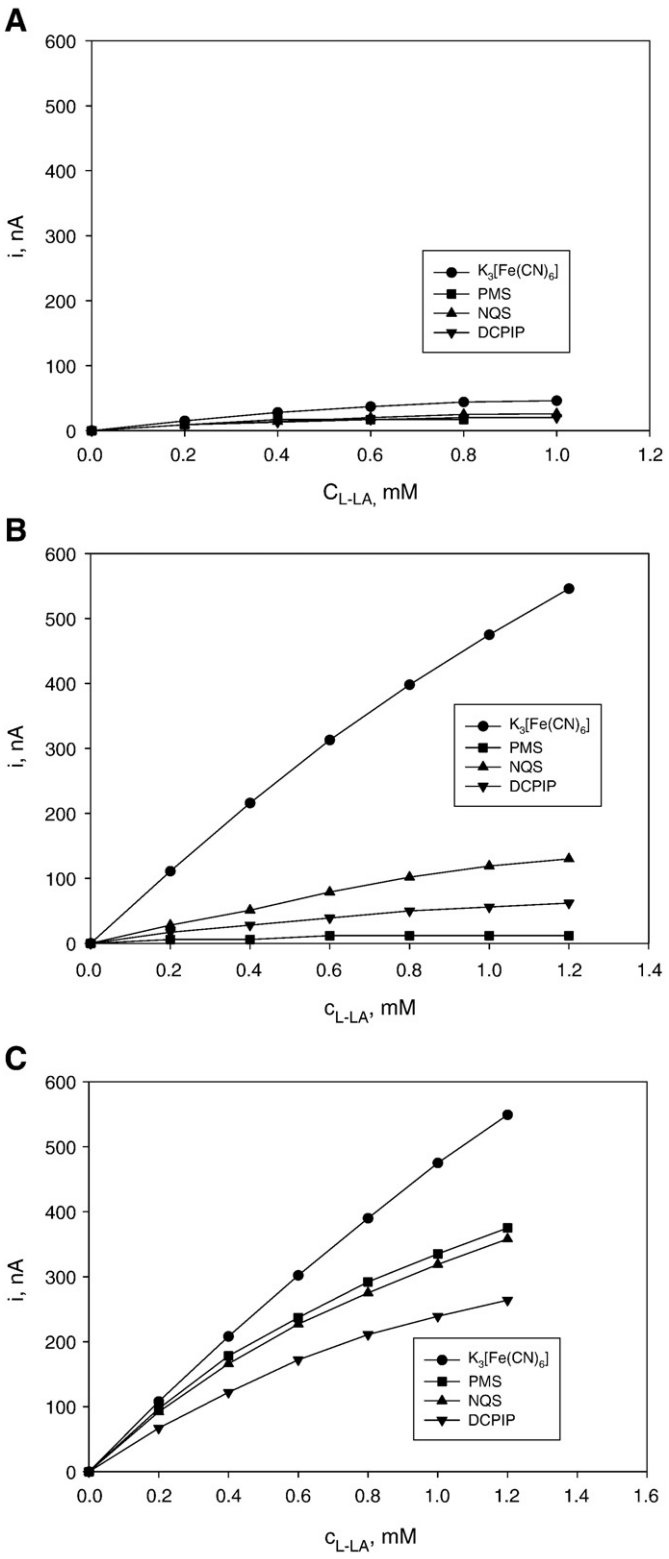


**Fig. 2.** Dependences of current responses of electrodes modified with BY on concentration of L-lactic acid using various mediators (as indicated): A — BY pretreated with phosphate buffer at pH 7.3, B — BY pretreated with 0.1 M LiCl in phosphate buffer at pH 7.3, C — bulk paste electrodes modified with non-pretreated BY. Operating potential 0.3 V (*vs.* Ag/AgCl, 3 N NaCl).

of electrode responses with the latter two mediators, exact comparison of electrode sensitivities was not possible, however, the sensitivities of both BY- and WY-modified electrodes followed the mediator sequence potassium ferricyanide > NQS > DCPIP > PMS.

The effect of time of yeast pre-treatment with phosphate buffer containing LiCl was tested during two days. Faster decrease in electrode sensitivity was observed for BY-modified electrodes: after one day BY and WY-modified electrodes retained, respectively, 64% and 84% of the initial sensitivity to 0.2 mM L-lactic acid with potassium ferricyanide as a mediator. After 2 days of yeast treatment with 0.1 M LiCl, both BY and WY-modified electrodes were insensitive. Viability tests showed that the cells remained viable in phosphate buffer at pH 7.3 both with and without LiCl at least for 2 days.

It should be mentioned that the effect of LiCl on mediated electrode responses to L-lactic acid was investigated for number of wine yeast strains. The strain type K2 killer Rom K-100 HM/HM wt [kill-K2] was chosen as giving the highest electrode responses after pre-treatment with LiCl (although always lower compared to those of BY-modified electrodes). For some of the strains the effect of LiCl was not observed, *i.e.*, electrodes remained practically not sensitive to L-lactic acid with all four mediators used in this research.

### 3.2. The effect of various alcohols

The effects of alcohols on yeast cells include the effects on plasma membrane integrity that induce morphological changes, permeabilize the membrane and facilitate cellular ion leakage [31–36]. To find out the effectiveness of methanol, ethanol or isopropyl alcohol as the permeabilizing agents, yeast cells were treated with different concentrations (10, 20, 30, 40%, v/v) of these alcohols. Electrode response to 0.2 mM of L-lactic acid in phosphate buffer at pH 7.3 containing 0.5 mM potassium ferricyanide and 0.5 mM EDTA again was taken as a measure of the effect of yeast pretreatment. The results showed marked differences in responses of electrodes covered with methanol- or ethanol-pretreated yeast cells (Fig. 3A and B, respectively) and isopropyl alcohol-affected cells (Fig. 3C). First of all, time needed to obtain responses to L-lactic acid comparable (66±8 nA, 20% methanol) or even higher (144±7 nA, 30% ethanol) to those obtained after treatment with LiCl (Table 1) was much longer (at least 20–24 h) compared to that when isopropyl alcohol was used (1 h in 10% isopropyl alcohol resulted in 114±30 nA). In the case of methanol and ethanol, an increase of electrode responses was observed with increasing alcohol concentration up to 30% in the pretreatment solution. Further increase in alcohol concentration resulted in low sensitivity of electrode with methanol-treated cells (Fig. 3A) or complete insensitivity of electrode with ethanol-treated cells (Fig. 3B). The cell suspensions in methanol or ethanol solutions (10 and 20%) remained active during 5 days of monitoring whereas those in 30% of alcohol showed decrease in electrode sensitivity after 2 days (for methanol, Fig. 3A) and 4 days (for ethanol, Fig. 3B). In the case of isopropyl alcohol (Fig. 3C), the highest and the most stable electrode responses were obtained when the lowest concentration (10%) of isopropyl alcohol was used for cell treatment. The responses of electrodes that contained cells treated with 20 or 30% of isopropyl alcohol were lower and decayed markedly after keeping the yeast suspensions overnight. Further increase of isopropyl alcohol up to 40% in the pretreatment solution resulted in complete insensitivity of electrodes. Electrodes with alcohol pretreated WY exhibited very similar properties although were less sensitive (Fig. 3, dashed lines). It should be mentioned again that the strain type K2 killer Rom K-100 HM/HM wt [kill-K2] gave the highest signal from a number of investigated wine yeast strains when immobilized on the electrode surface after treatment with alcohols.

The comparison of the electrode responses using other mediators showed that again the most sensitive electrode responses were obtained with potassium ferricyanide (Fig. 4). The changes in membrane permeability induced by methanol and ethanol were favorable for the same mediators as in the case with LiCl-treated cells.

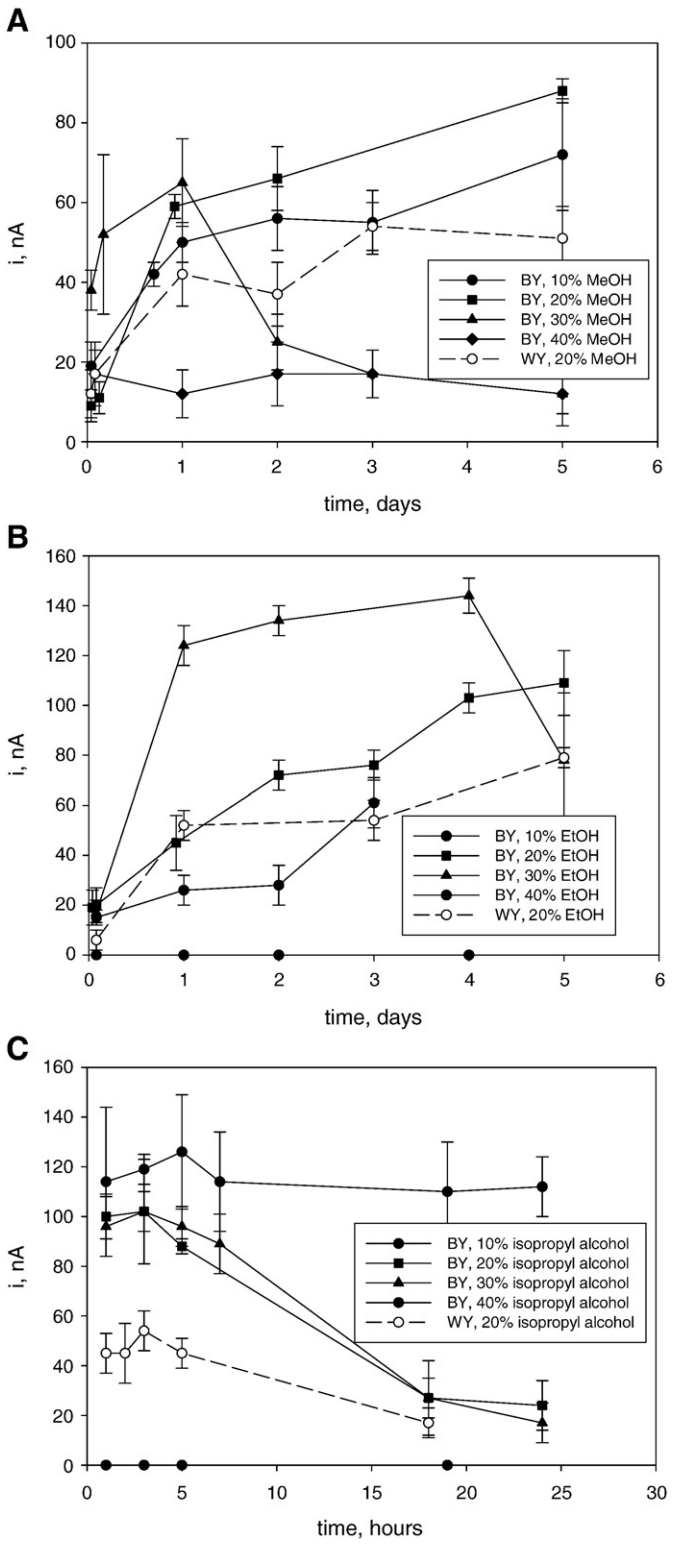


**Fig. 3.** Dependences of current responses of electrodes modified with a layer of alcohol pretreated BY (solid lines) or WY (dashed lines) on time of yeast pretreatment in: A — methanol at various concentrations (as indicated), B — ethanol at various concentrations (as indicated), C — isopropyl alcohol at various concentrations (as indicated). Electrode operating conditions: potential 0.3 V (vs. Ag/AgCl, 3 N NaCl), mediator $K_3[Fe(CN)_6]$, pH 7.3, concentration of L-lactic acid: 0.2 mM.

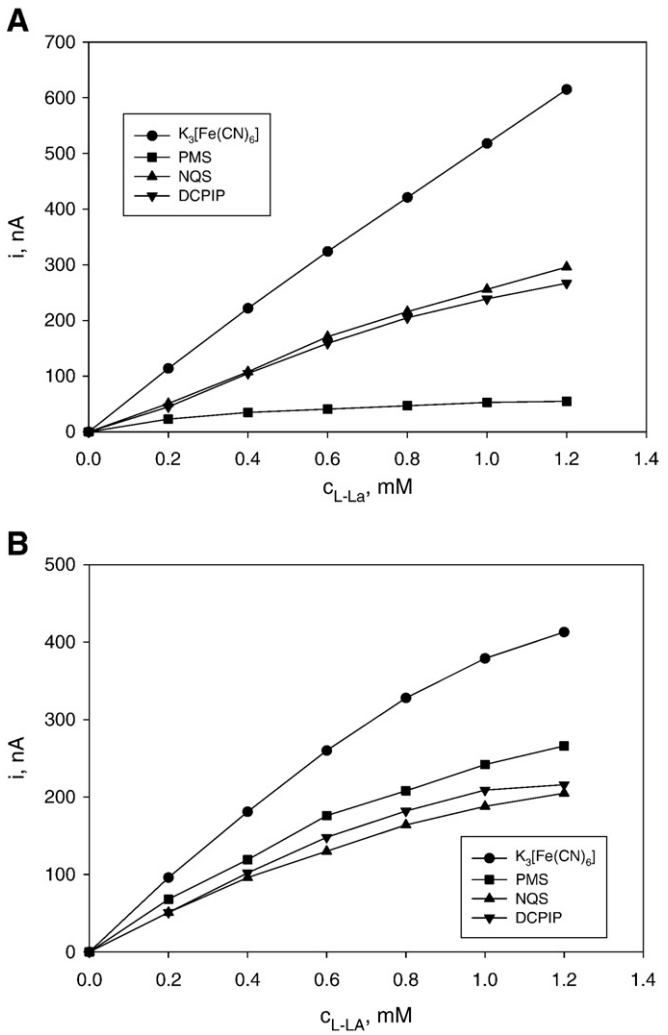


**Fig. 4.** Dependences of current responses of electrodes modified with a layer BY on concentration of L-lactic acid using various mediators (as indicated): A — BY pretreated with 30% ethanol (for 2 days), B — BY pretreated with 20% isopropyl alcohol (for 1 h). Operating potential 0.3 V (*vs.* Ag/AgCl, 3 N NaCl), pH 7.3.

After pre-treatment with methanol or ethanol, the sensitivities of both BY- and WY-modified electrodes followed the same mediator sequence: potassium ferricyanide>NQS>DCPIP>PMS (Fig. 4A, shown only for electrode containing BY treated with 30% ethanol for 2 days).

However, after pre-treatment with isopropyl alcohol, electrode responses (both with BY and WY) in PMS solution were almost comparable to those in potassium ferricyanide (Fig. 4B, shown for BY pre-treated with 10% isopropyl alcohol for 1 h). This probably suggested that the integrity of cell membrane was affected to higher extent (probably even disrupted) compared to that obtained with methanol or ethanol; thus, all mediators could reach the enzyme. Besides, after treatment with isopropyl alcohol, a deviation in the linearity in the dependence of current responses on concentration of lactic acid for BY-modified electrodes was noticed with PMS as a mediator. Fig. 5 shows the recordings of current responses of electrode modified with a layer of BY pretreated with 10% isopropyl alcohol upon addition of aliquots of lactic acid to PMS (dashed line) and, for comparison, to potassium ferricyanide (solid line) solutions. In the case of PMS, the first and the second increments of current responses were obviously lower compared to those obtained with subsequent additions. If this is related to the consumption of reduced PMS due to reaction with oxygen dissolved in the solution [37], one should expect that the linearity in the dependence of current responses on concentration of lactic acid should be obtained after removal of dissolved oxygen. However, experiments showed that the linearity was not improved after purging the solutions with argon. As this non-linearity with PMS as a mediator was not observed for bulk yeast-modified electrodes or with electrodes containing WY treated with isopropyl alcohol, the reason remains unclear and needs further investigation.

The electrodes with yeast cells pre-treated with 20% methanol or 30% ethanol possessed the characteristics suitable for determination of L-lactic acid, however, only with potassium ferricyanide as a mediator. The sensitivities to 0.2 mM L-lactic acid were 88±3 and 144±7 nA (these are the mean values of three different electrode preparations), respectively, for methanol and ethanol-treated samples. The linearity (with $R^2$ value not lower than 0.998) in the current dependences on concentration of L-lactic acid was up to 1.1±0.1 mM for different electrode preparations. The repeatability and operational stabilities of the electrode responses were tested by running six to eight consecutive calibration curves in the range 0.2 to 1.2 mM, then keeping the electrodes in the solution of potassium ferricyanide or phosphate buffer at pH 7.3 for 1 h, and again measuring the electrode responses. The loss of sensitivity after this test practically was not observed. However, after keeping the electrodes in phosphate buffer overnight, only 30 to 45% of initial sensitivity was obtained. The apparent Michaelis constant ($K_M$') and the maximum values of the currents were obtained from Lineweaver–Burke plot ($i^{-1}$ vs. [L-lactic acid]$^{-1}$) and were, respectively, 4.1±0.4 mM and 2.5±0.3 μA (for 30% ethanol-treated BY).

Detection limits (calculated from the response ratio to noise 3:1) were 3 to 6 μM, *i.e.*, lower compared to those when the yeasts were admixed to carbon paste (16–21 μM [26]).

Viability measurements showed that, after pretreatment with methanol and ethanol at concentrations higher than 20% and isopropyl alcohol at all tested concentrations, cells were non viable.

### 3.3. The effect of weak organic acids

Weak organic acids such as benzoic, acetic or sorbic acids have been widely used to prevent microbial spoilage of food and beverages by inhibiting the growth of microorganisms [38–43]. Growth inhibition can be caused by interference with cell membrane and/cell wall, metabolic activity, protein synthesis system, *etc*. It is widely accepted that the molecules of undissociated weak organic acids pass through the cell membrane, dissociate in the cytoplasm, release protons and inhibit the growth of yeasts due to acidification of the cytoplasm.

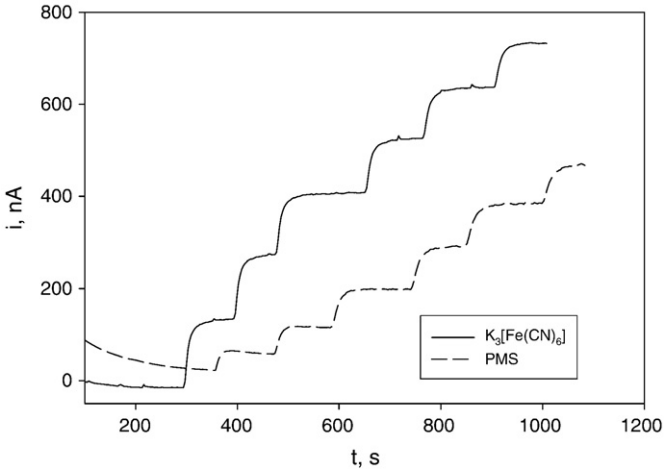


**Fig. 5.** Current responses of electrodes modified with a layer of isopropyl alcohol pre-treated BY upon subsequent additions of aliquots of L-lactic acid in phosphate buffer at pH 7.3 containing 0.5 mM $K_3[Fe(CN)_6]$ and 0.5 mM EDTA (solid line) and 0.5 mM PMS (dashed line). Operating potential 0.3 V (*vs.* Ag/AgCl, 3 N NaCl ).

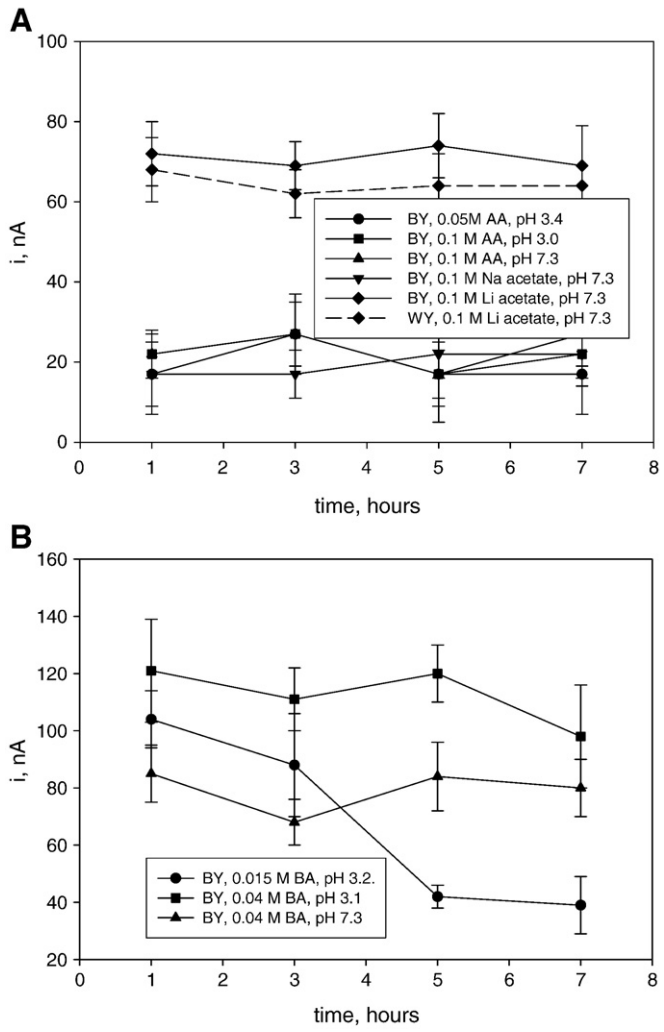


**Fig. 6.** Dependence of current responses of electrodes modified with a layer of BY (solid line) and WY (dashed line) on time of yeast pretreatment: A — BY and WY pretreated with acetic acid at various concentrations and solution pH (as indicated), 0.1 M Na and Li acetate, B — BY pretreated with various concentrations of benzoic acid (as indicated). Electrode operating conditions: operating potential 0.3 V (*vs.* Ag/AgCl, 3 N NaCl), mediator $K_3[Fe(CN)_6]$, pH 7.3, concentration of L-lactic acid: 0.2 mM.

The electrode sensitivities to lactic acid after treatment with acetic acid (AA) and benzoic acid (BA) showed marked differences (Fig. 6). After treatment with AA or Na acetate at various concentrations and pH values, the sensitivities of both BY and WY-modified electrodes did not exceed 40 nA per 0.2 mM L-lactic acid. Higher sensitivity (74±8 nA and 68±8 nA, for BY and WY, respectively) was observed only after treatment with Li acetate, probably due to the effect of Li ions rather than acetate (Fig. 5A). The sensitivities of both BY- and WY-modified electrodes again followed the mediator sequence potassium ferricyanide>NQS>DCPIP>PMS. The effect of benzoic acid was more obvious, probably due to higher lipophilic nature of this acid. Electrode sensitivities to 0.2 mM of L-lactic acid in the presence of potassium ferricyanide as a mediator increased with the increase of benzoic acid (dissolved in water) in the pretreatment solution, *i. e*., 104±10 nA and 121±18 nA, respectively, for 0.015 M and 0.04 M of benzoic acid. Electrodes containing BY pretreated with benzoic acid in phosphate buffer at pH 7.3 (where benzoic acid was in more dissociated form) also exhibited rather high sensitivities (85±10 nA), *i.e*., the cell membrane (and/or cell wall) permeability was affected both by undissociated and dissociated forms of BA. Faster decrease in electrode sensitivity was observed when yeast cells were affected with lower concentration (0.015 M) of BA. After keeping the suspensions overnight, the sensitivities in all cases did not exceed 45 nA per 0.2 mM of lactic acid. WY-modified electrodes also exhibited sensitivity to lactic acid, however, with low reproducibility. The loss of sensitivities (from 62±14 nA to 22±12 nA) of WY-modified electrodes varied from 3 to 6 h.

Again the same mediator sequence (potassium ferricyanide>NQS>DCPIP>PMS) for both BY and WY was observed.

## 4. Conclusions

The pretreatment of cells of commercially available baker's yeast *S. cerevisiae* with 20% methanol or 30% ethanol before immobilization on the electrode surface resulted in effective method to gain relatively high amperometric responses to L-lactic acid. However, stable responses could be obtained only with potassium ferricyanide as a mediator and only in the presence of EDTA. Amperometric measurements suggested that the yeast treatment with Li salts, methanol, ethanol or benzoic acid caused similar "mild" permeabilization of yeast cell membrane and/or cell wall that resulted in electrode sensitivity to lactic acid when negatively charged mediators were used. Yeast treatment with isopropyl alcohol probably resulted in more severe effects on the cell membrane and/or cell wall as amperometric responses to lactic acid were obtained with all mediators. Preliminary studies of the responses of electrodes modified with "aged "cells (under various conditions) also revealed "mild" permeabilization. This kind of amperometric investigation probably could indirectly reflect the properties of yeast cell membrane and/or cell wall and the velocity of changes of cell membrane and/or cell wall caused by various substances (e.g., relatively fast changes after treatment with LiCl or isopropyl alcohol and relatively slow ones after treatment with methanol or ethanol).

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## References

[1] J. Kulys, W. Schuhmann, H.L. Schmidt, Carbon paste electrodes with incorporated lactate oxidase and mediators, Anal. Lett. 25 (1992) 1011–1024.
[2] U. Spoh, D. Narasaiah, L. Gorton, The influence of the carbon paste composition on the performance of an amperometric bienzyme sensor for L-lactate, Electroanalaysis 8 (1996) 507–514.
[3] R. Garjonyte, Y. Yigzaw, R. Meskys, A. Malinauskas, L. Gorton, Prussian Blue- and lactate oxidase-based biosensor for lactic acid, Sens. Actuators, B 79 (2001) 33–38.
[4] S. Suman, R. Singhal, A.L. Sharma, B.D. Malhotra, C.S. Pundir, Development of a lactate biosensor based on conducting copolymer bound lactate oxidase, Sens. Actuators, B 107 (2005) 768–772.
[5] M. Gerard, K. Ramanathan, A. Chaubey, B.D. Malhotra, Immobilization of lactate dehydrogenase on electrochemically prepared polyaniline films, Electroanalysis 11 (1996) 450–452.
[6] A. Chaubey, M. Gerard, R. Singhal, V.S. Singh, B.D. Malhotra, Immobilization of lactate dehydrogenase on electrochemically prepared polypyrrole-polyvinylsulfonate composite films for application to lactate biosensors, Electrochim. Acta 46 (2000) 671–680.
[7] A.C. Pereira, D.V. Macedo, A.S. Santos, L.T. Kubota, Amperometric biosensor for lactate based on Meldola's Blue adsorbed on silica gel modified with niobium oxide, Electroanalysis 18 (2006) 1208–1214.
[8] C.L. Lin, C.L. Shih, L.K. Chau, Amperometric L-lactate sensor based on sol–gel processing of an enzyme linked silicon alkoxide, Anal. Chem. 79 (2007) 3757–3763.
[9] J.J. Kulys, G.J. Svirmickas, Reagentless lactate sensor based on cytochrome $b_2$, Anal. Chim. Acta 117 (1980) 115–120.
[10] S.L. Staskeviciene, N.K. Cenas, J.J. Kulys, Reagentless lactate electrodes based on electrocatalytic oxidation of flavocytochrome $b_2$, Anal. Chim. Acta 243 (1991) 167–171.
[11] A. Amine, J. Deni, J.M. Kaufmann, Amperometric biosensor based on carbon paste mixed with enzyme, Bioelectrochem. Bioenerg. 34 (1994) 123–128.
[12] O. Smutok, G. Gayda, M. Gonchar, W. Schuhmann, A novel L-lactate-selective biosensor based on flavocytochrome $b_2$ from methylotropic yeast *Hansenula polymorpha*, Biosens. Bioelectron. 20 (2005) 1285–1290.
[13] O.V. Smutok, G.S. Os'mak, G.Z. Gaida, M.V. Gonchar, Screening of yeasts producing stable L-lactate cytochrome *c* oxidoreductase and study of the regulation of enzyme synthesis, Microbiology 75 (2006) 20–24.

[14] O. Smutok, K. Dmytruk, M. Gonchar, A. Sibirny, W. Schuhmann, Permeabilized cells of flavocytochrome $b_2$ over-producing recombinant yeast *Hansenula polymorpha* as biological recognition element in amperometric lactate biosensors, Biosens. Bioelectron. (2007).
[15] S. Daff, J. Ingledew, G.A. Reid, S.K. Chapman, New insights into the catalytic cycle of flavocytochrome $b_2$, Biochemistry 35 (1996) 6345–6350.
[16] M.E. van der Rest, A.H. Kammings, A. Nakano, Y. Anraku, B. Poolman, W.N. Konings, The plasma membrane of *Saccharomyces cerevisiae*: structure, function, and biogenesis, Microbiol. Rev. 59 (1995) 304–322.
[17] F.M. Klis, A. Boorsma, P.W.J. De Grot, Cell wall construction in *Saccharomyces cerevisiae*, Yeast 23 (2006) 185–202.
[18] K.H.R. Baronian, A.J. Downard, R.K. Lowen, N. Pasco, Detection of two distinct substrate-dependent catabolic responses in yeast cells using a mediated electrochemical method, Appl. Microbiol. Biotechnol. 60 (2002) 108–113.
[19] A. Heiskanen, J. Yakovleva, C. Spegel, R. Taboryski, M. Koudelka-Hep, J. Emneus, T. Ruzgas, Amperometric monitoring of redox activity in living yeast cells: comparison of menadione and menadione sodium bisulfite as electron transfer mediators, Electrochem. Commun. 6 (2004) 219–226.
[20] J. Zhao, M. Wang, Z. Yang, Q. Gong, Y. Lu, Z. Yang, Mediated electrochemical measurement of inhibitory effects of furfural and acetic acid on *Saccharomyces cerevisiae* and Candida shehatae, Biotechnol. Lett. 27 (2005) 207–211.
[21] M. Wang, J. Zhao, Z. Yang, Z. Du, Z. Yang, Electrochemical insights into the ethanol tolerance of *Saccharomyces cerevisiae*, Bioelectrochemistry 71 (2007) 107–112.
[22] S.F. D'Souza, Microbial biosensors, Biosens. Bioelectron. 16 (2001) 337–353.
[23] R.R. Chen, Permeability issues in whole-cell bioprocesses and cellular membrane engineering, Appl. Microbiol. Biotechnol. 74 (2007) 730–738.
[24] H. Ito, Y. Fukuda, K. Murata, A. Kimura, Transformation of intact yeast cells treated with alkali cations, J. Bacteriol. 153 (1983) 163–168.
[25] R. Garjonyte, A. Malinauskas, Investigation of baker's yeast *Saccharomyces cerevisiae*- and mediator-based carbon paste electrodes as amperometric biosensors for lactic acid, Sens. Actuators, B 96 (2003) 509–515.
[26] R. Garjonyte, V. Melvydas, A. Malinauskas, Mediated amperometric biosensors for lactic acid based on carbon paste electrodes modified with baker's yeast *Saccharomyces cerevisiae*, Bioelectrochemistry 68 (2006) 191–196.
[27] A. Venancio, L. Dominguez, N. Lima, Transformation of a flocculating *Saccharomyces cerevisiae* using lithium acetate and pYAC4, J. Basic Microbiol. 39 (1999) 37–41.
[28] T. Morita, K. Takayama, A simple and efficient procedure for transformation of Schizosaccharomyces pombe, Yeast 21 (2004) 613–617.
[29] A. Zimkus, L. Chaustova, V. Razumas, Effect of lithium and sodium cations on the permeability of yeast *Saccharomyces cerevisiae* cells to tetraphenylphosponium ions, Biologija 2 (2006) 47–49.
[30] R. Garjonyte, V. Melvydas, A. Malinauskas, Investigation of amperometric biosensors for lactic acid based on baker's and wine yeast *Saccharomyces cerevisiae*, (accepted for publication in Microchimica Acta, doi:10.1007/s00604-008-0055-9).
[31] Y. Liu, H. Hama, Y. Fujita, A. Kondo, Y. Inoue, A. Kamura, H. Fukuda, Production of *S*-lactoylglutathione by high activity whole cell biocatalysts prepared by permeabilization of recombinant *Saccharomyces cerevisiae* with alcohols, Biotechnol. Bioeng. 64 (1999) 54–60.
[32] A. Kondo, Y. Liu, M. Furuta, Y. Fujita, T. Matsumoto, H. Fukuda, Preparation of high activity whole cell biocatalyst by permeabilization of recombinant flocculent yeast with alcohol, Enzyme Microb. Biotechnol. 27 (2000) 806–811.
[33] R.M. Birch, G.M. Walker, Influence of magnesium ions on heat shock and ethanol stress responses of *Saccharomyces cerevisiae*, Enzyme Microb. Technol. 26 (2000) 678–687.
[34] C. Quintas, E. Lima-Costa, M.C. Loureiro-Dias, The effect of ethanol on the plasma membrane permeability of spoilage yeasts, Food Technol. Biotechnol. 38 (2000) 47–51.
[35] E. Canetta, A.K. Adya, G.M. Walker, Atomic force microscopic study of the effects of ethanol on yeast cell surface morphology, FEMS Microbiol. Lett. 255 (2006) 308–315.
[36] E. Marza, N. Camougrand, S. Manon, Bax expression protects yeast plasma membrane against ethanol-induced permeabilization, FEBS Lett. 521 (2002) 47–52.
[37] F. Ricci, A. Amine, D. Moscone, G. Palleschi, A probe for NADH and $H_2O_2$ amperometric detection at low applied potential for oxidase and dehydrogenase based biosensor applications, Biosens. Bioelectron. 22 (2007) 854–862.
[38] A.D. Warth, Effect of benzoic acid on growth yield of yeasts differing in their resistance to preservatives, Appl. Environ. Microbiol. 54 (1988) 2091–2095.
[39] M. Stratford, P.A. Anslow, Evidence that sorbic acid does not inhibit yeast as a classic "weak acid preservative", Lett. Appl. Microbiol. 27 (1998) 203–206.
[40] P. Piper, C.O. Calderon, K. Hatzixantis, M. Mollapour, Weak acid adaptation: the stress response that confers yeasts with resistance to organic acid food preservatives, Microbiology 147 (2001) 2635–2642.
[41] N. Arneborg, L. Jespersen, M. Jakobsen, Individual cells of *Saccharomyces cerevisiae* and Zygosaccharomyces bailii exhibit different short-term intracellular pH responses to acetic acid, Arch. Microbiol. 174 (2000) 125–128.
[42] K.C. Thomas, S.H. Hynes, W.M. Ingledew, Influence of medium buffering capacity on inhibition of *Saccharomyces cerevisiae* growth by acetic and lactic acids, Appl. Environ. Microbiol. 68 (2002) 1616–1623.
[43] R. Hazan, A. Levine, H. Abeliovich, Benzoic acid, a weak organic acid food preservative, exerts specific effects on intracellular membrane trafficking pathways in *Saccharomyces cerevisiae*, Appl. Environ. Microbiol. 70 (2004) 4449–4457.