VILNIUS UNIVERSITY

CENTER FOR PHYSICAL SCIENCES AND TECHNOLOGY

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INVESTIGATION OF THE CHEMICAL AND ELECTRICAL EFFECTS ON *SACCHAROMYCES CEREVISIAE* YEAST CELL PROPERTIES

Summary of doctoral dissertation

Physical sciences, chemistry (03 P)

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VILNIAUS UNIVERSITETAS

FIZINIŲ IR TECHNOLOGIJOS MOKSLŲ CENTRAS

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INTRODUCTION

For several decades, the budding yeasts, *Saccharomyces cerevisiae*, have been considered as prototypical eukaryotic cells, ideally suited for use in investigation of many basic phenomena of eukaryotic life and some of their fundamental properties. Yeasts are used not only to produce transgenic proteins, or for fermentation in foods and beverages, but also can be used in microbial fuel cells for the generation of green electricity as well as whole cell sensors.

The *S. cerevisiae* yeast cells are surrounded by cell wall, which provide them not only with protection from osmotic stress, but are also important for their defense against toxic compounds, for self-recognition and for flocculation. The cell wall hydrophobicity and charge are the major determinants of yeast adhesion on abiotic surfaces. The chemical treatment of the cells by adding reducing agents such as dithiothreitol (DTT) or antibiotics are often used in order to change the permeability of the yeast cells or cell wall. Their permeability can also be changed by increasing the external mechanical pressure on them, or by treating them with pulsed electric field.

In order to detect changes of the permeability of the yeast cells, electron and fluorescent microscopy are used. However, the quantitative measurement of the cell permeabilization using these methods is difficult. Moreover, only few methods can be used for the indication of selective cell wall permeabilization without causing lethal influences to the cells.

The atomic force microscopy (AFM) imaging is well suited for the investigation of biological objects as it can be conducted in liquid environment. The AFM techniques are successfully applied for the investigation of mechanical properties of living cells including yeast cells. The AFM is applied for the observation of cell wall oscillations, topological structure of membrane, (visualization of pores, lipids and proteins) and evaluation of cellular interaction on a molecular level.

The lipophilic cations (LC) as a probe to analyze of selective yeast cells wall permeabilization can be applied. Tetraphenylphosphonium salts (for example: TPPBr)

are frequently used for the measurement of the membrane potential of prokaryotic and eukaryotic cells. The use of a potentiometric ion-selective electrode (ISE) is also a convenient method for the quantitative evaluation of the permeability of the yeast cell wall and membrane.

Determination the impact of chemical compounds and physical exposure to the cell surface using AFM and ISE analysis have not been performed. The analysis and regulation of cell surface permeability has great applicability of environmental waste detection using whole cell biosensors. The sensitivity of the whole cell biosensors and the yield of expressed recombinant proteins or products of biocatalysis could be improved and enhanced using chemical compound or pulsed electric field.

The aim of the work:

To investigate the chemical impact on mechanical properties of the yeast cells (*Saccharomyces cerevisiae*) and effect of micro- and nano- second duration high power electrical pulses to the permeability of cells. For these investigations AFM and electrochemical technique for lipophilic ions concentration analysis was used.

The following main tasks of the work are:

- Investigation of the mechanical elasticity of yeast cells (*Saccharomyces cerevisiae*) using AFM and to study a mechanical elasticity changes of yeast cells after chemical treatment by dithiothreitol.
- Determination of TPP^+ absorption rate changes when yeast cells were exposed by high power electric field pulses having duration from 5 to 150 μs and electric field strength up to10 kV/cm using electrochemical method based on measurement of TPP^+ absorption by the yeast.
- Evaluation of the influence of 60 nanosecond duration electrical pulses, which are able to generate electric field strength up to 200 kV/cm, on the rate of TPP^+ absorption by the yeast cells and determination of high power pulsed electric field impact on viability of the yeasts.

Statements to be defended:

1. The dithiothreitol solution has a significant impact on mechanical elasticity of yeast cells. The experimentally obtained dependence between force and indentation of yeast *Saccharomyces cerevisiae* cells can be well described by Sneddon model. The Young module calculated using this model is about 2 times higher for chemically treated yeast cells in comparison to untreated ones.
2. Electrochemical TPP^+ ion concentration measurement method provides the ability to detect yeast cell wall permeability change after the impact by high power electric pulses with duration ranging from ns to μs. Electric pulses generating electric field strength up to 200 kV/cm increase the rate of TPP^+ absorption by the yeast cells.
3. Experimentally obtained TPP^+ ion influx *vs*. electric field strength dependence can be well described using model based on second order kinetic equation, and assuming that absorption coefficient, which characterize the rate of TPP^+ absorption, is the square function of electric field strength. Obtained results show that permeability of the yeast cell wall to the TPP^+ ions depends on energy of the electric pulse.
4. Nanoseconds duration electric pulses generated electric field strength up to 200 kV/cm did not affect viability of the yeasts. However the reduction of the yeast cell viability (up to 60%) is obtained when microseconds duration electric pulses generating electric field strength up to 10 kV/cm are applied.

Novelty of the accomplished research work:

Using the atomic force microscopy it was shown that treatment of yeast cells by chemical compounds, which affect the permeability of the yeast cell, changes also their mechanical elasticity.

A new method based on the measurements of TPP^+ absorption by yeast cells using electrochemical cations selective electrode was suggested to study the permeability

of yeast cell wall. Using this method it was obtained that nanosecond duration strong (up to 200kV/cm) electric field pulses are able to increase the permeability of the cell wall. This increase is not accompanied with decrease of viability of the cells.

Data about strong pulsed electric field impact on permeability of the yeast cell wall and changes of mechanical elasticity by chemical treatment provides the possibility to develop new methods for the modification of yeast cell properties.

LITERATURE REVIEW

In this chapter the literature of common yeast cells and their wall properties describing the composition of cell membrane and cell wall structures is reviewed. This chapter also describes the impact of chemical treatment and pulsed electric field exposure to the yeast cell plasma membrane and their wall. The methods, which were commonly used to describe the mechanical properties and permeability of the yeast cell, are reviewed. The yeast cell mechanical properties and mammalian cell permeability changes after the exposure to the pulsed electric field are discussed.

EXPERIMENTAL

Application of AFM for the visualization of cells and investigation of their mechanical properties

In this study we demonstrate the applicability of AFM for the visualization of cell wall lesions which appears after transformation procedure. The baker's yeast cells were treated by the chemical compounds (e.g.: 1,4-dithiothreitol and lithium acetate), which are mainly used to increase the permeability of the cell wall.

After the deposition on the glass plate or after the trapping into the pores of polycarbonate membrane the yeast cells were investigated by AFM. Here presented results show the ability of AFM technique to be routinely used for fast visualization of yeast cells surface in order to observe the success of cell transformation procedure.

The measurements were performed in deionized water (DI) utilizing BioScope II atomic force microscope models from Bruker. The silicon nitride probes with the triangular cantilevers with the spring constants below 0.1 N/m containing four-sided pyramidal tips of 20 – 60 nm radiuses were used. The 175×175 μm^2 area of a membrane was scanned to localize the position of pores with mechanically trapped yeast cells. After treatment by DTT and lithium acetate the morphological changes of yeast cell wall are clearly observed.

When a soft sample is probed, the force induced by elastic cantilever indents the sample, and deflection of the cantilever is reduced. The indentation depth δ is determined by the following equation: $\delta = (z - z_0) - (d - d_0)$, where z is the position of the probe, z_0 – the offset, d – the deflection of the cantilever and d_0 – the initial contact point. The Hertz–Sneddon indentation model is applied in order to describe the mechanical interaction and to find Young's modulus (E) by the equation:

$$F = \frac{2}{\pi} \cdot \frac{E}{1-\nu^2} \cdot \frac{1}{\tan(\alpha)} \cdot \delta^2 \qquad (1)$$

where ν is Poisson's ratio and α is the half opening cone angle of the tip of the AFM probe.

Equipment for the generation of pulsed electric field

The experimental setup for the electroporation of the yeast is presented in Figure 1. It was made in the scope of this dissertation and it consists of high voltage DC power supply, square-wave pulse generator, a real time oscilloscope, an isolated gate bipolar transistor (IGBT) and commercial cuvette of 120 μL volume with plate-shape electrodes of 2 cm^2 and gap between electrodes of 1 mm.

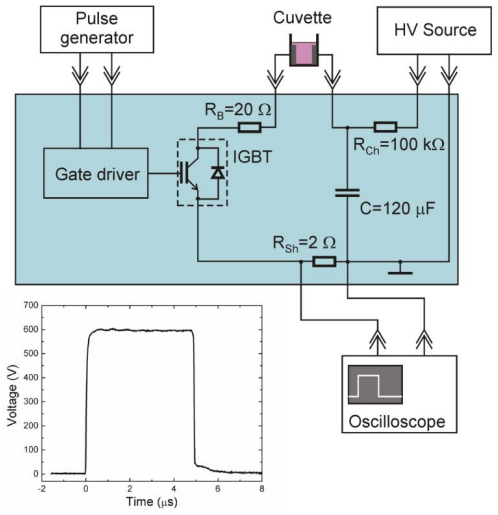

Fig. 1. The experimental setup used for the yeast cell electroporation in microsecond pulse duration range. The picture below is the typical pulse shape at the 600 V amplitude and 5 μs pulse duration.

In the case of impact of nanosecond pulse duration to the yeast cells the used experimental setup is presented in Figure 2. The apparatus consisted of the following parts: an optically-triggered spark-gap switch, a Nd:LSB laser (1062 nm wavelength, 450 ps duration, 1 mJ pulse energy), a laser power supply, an electrical pulse generator with an amplitude of 25 V and variable pulse duration from 1 μs to 1 ms, a pulse-forming line (coaxial cables of different length for discrete pulse duration of 10, 40, 60, and 92 ns), a variable (from 2.5 to 25 kV) high voltage (HV) DC power supply, a resistive power divider (1:86), a 75 Ω impedance transmission line, a cuvette for biological cell electroporation, a transformer with impedance from 75 Ω to 50 Ω, an attenuator (40 dB) and a 6 GHz frequency band DPO 70604 Tektronix real-time oscilloscope.

Preparation of the yeast cells suspension

The yeast cells used for the electroporation procedure were grown in YPD growth medium to their exponential growth phase (OD_{600nm} 0.8-1.0), then washed with distilled water. In the case of mechanical measurement the cells were suspended in the buffer with 0.1 M lithium acetate and incubated for 45 min at 30 ºC. Then the 25 μL of 1 M DTT solution was added and the mixture was incubated for 15 min at the same temperature. After this treatment, yeast cells were washed twice in 1 mL of chill water to

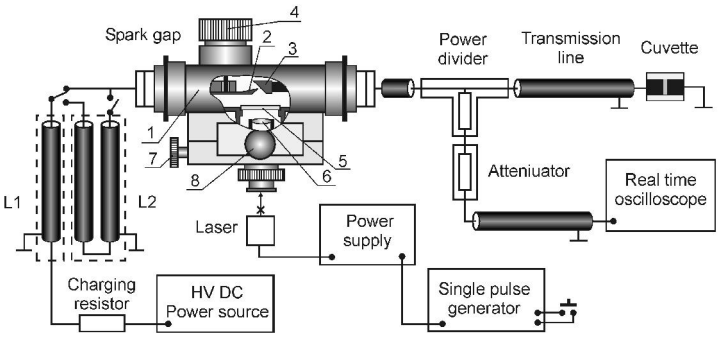

Fig. 2. Schematic diagram of the apparatus used for the nanoporation of biological cells. Spark-gap components: 1-casing, 2 - anode, 3 - cathode, 4 - knob for control the distance between electrodes, 5 - optical window, 6 - lens, 7, 8- knobs for horizontal and vertical adjustment of the laser beam.

remove residual DTT solution. After that cells were re-suspended in 1 mL of 1 M sorbitol, 20 mM TRIS, pH 7,4 (EPB) and kept at 4 °C). The final concentration of the yeasts was kept at (4-6)×10^9 colony forming unit (CFU)/ml. The electroporation procedures were carried out on 120 µL of EPB. TPPBr to a final concentration of 5×10^{-6} M was added before the electroporation. After electroporation and incubation them for an additional 3 min, the yeast cells were precipitated and the 50 µL of resultant supernatant was mixed with 200 µL of electrode calibration solution (2×10^{-6} M). It means that initial TPP^+ concentration N_s was $[TPP^+]_{initial}$ = 2.6×10^{-6} M. Such solution was used for the measurement of the residual TPP^+ ion concentration in supernatant ($[TPP^+]_{supernatant}$). The quantity (N) of the TPP^+ absorbed by the yeast cells was calculated as follows: $N = [TPP^+]_{initial} - [TPP^+]_{supernatant}$, i.e., $N = N_s - N_{supernatant}$. A combined tetraphenylphosphonium selective electrode was used for this purpose. In order to investigate the TPP^+ accumulation level, these measurements were conducted at different time intervals.

Methods and equipment for yeast cell permeabilization and viability studies

Evaluation of the proliferation of yeast cells after the action of pulsed electric field have been performed using yeast proliferation assay based on WST conversion. After incubation of the yeast cells for 4 h at 30 °C, measurements of the absorption at a 460 nm wavelength were conducted with a micro-plate reader. The proliferation assay was chosen instead of the plating method because yeast viability experiments using the plating method have shown a very wide distribution of the results and this distribution can also be explained by the yeasts' ability to form flocks.

In order to investigate the changes of the viability of the yeast cells after the exposure of nanosecond high power pulsed electric field (HPEF) the plate count method was used. Viability of the yeast cells was calculated by plating aliquots of a suitable dilution (10^{-6} cells/mL) of the cell suspension on YPD agar plates. Plates were incubated during 4 days at 30 °C temperature. Survived cells able to form a colony were counted and expressed in decimal reduction ratio: $n = (\log N_0 - \log N_f)$, where N_0 is the count of yeast colonies not affected by HPEF; N_f is count of surviving population after an exposure by HPEF.

To determine the effectiveness of the plasma membrane electroporation, a calcein blue release assay was performed. The yeast cells were concentrated to 1.5×10^8 CFU/ml in 100 µL of the EPB buffer and then 400 µM of acetoxymethyl (AM) esterified calcein blue AM, which is a cell permeable derivate of calcein blue, was added. The cells were observed using a Olympus BX51TF fluorescence microscope. Calculating the amount of fluorescent and non-fluorescent yeast cells in the cuvette using the dye release test the electroporation efficiency of the pulsed electric field was determined. This test is based on the fact that the electroporated cells are practically permeable to such dyes as calcein. At least one hundred yeast cells were counted in each experiment.

RESULTS AND DICCUSSION

AFM imaging and the changes of the yeast cell elasticity induced by the action of chemical compounds

The baker's yeast cells deposited on glass were imaged by AFM in air before and after the electroporation procedure. Morphological changes of the cell walls were

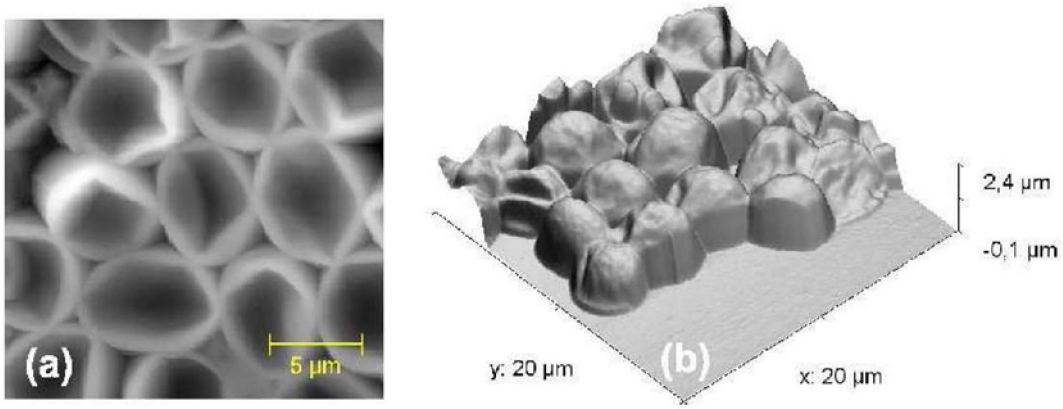

Fig. 3. The AFM image (a) of over dried baker's yeast cells, which are deposited on the glass substrate. As it is shown cell wall is fall down inside of cell, and these cells are dead and cannot be recovered. AFM amplitude error corresponding 3D-topography image (b) of baker's yeast cell deposited on glass.

revealed. Here presented results shows that the AFM technique could be routinely used for quick visualization of yeast cells surface changes to observe the success of cell electroporation procedure (Fig. 3).

The changes of cell wall morphology for baker's yeast cells after the deposition on a substrate and imaged in air considerable difference was observed. To avoid this morphological changes other method of immobilization of the yeast cells was used.

The immobilization was performed by extrusion of the prepared suspension of yeast cells in DI water through a polycarbonate membrane with pore dimensions comparable to the diameter of baker's yeast cells (5 μm). The membrane was rinsed in DI water to remove non-trapped cells. The small piece of excised membrane was fixed on a smooth substrate and transferred to AFM scan stage for topography and force imaging. To improve measurement accuracy, the study of intact and chemically treated cells was performed under the same conditions with the same probe. Deflection-distance curves were measured at least five times at one point and in many points around the center of a selected cell. The position (localization depth) of the cell in the hole was controlled by comparing AFM images acquired before and after force measurements. Living cells with similar localization in the hole and without any features such as a bud scar were probed.

The indentation depth δ can be calculated by analyzing difference in deflection–height (*d*-*z*) curves probed by AFM for both hard and soft samples. Young's modulus is calculated by the least-squares fitting of the experimental data can be directly fitted without conversion to force–indentation curves. Young's modulus *E*, the initial contact

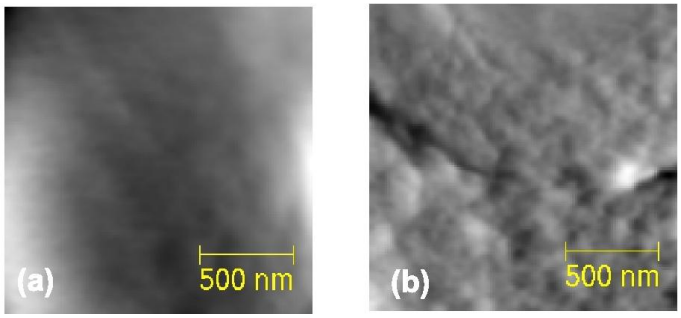

Fig. 4. The AFM topography images of intact (a) and treated (b) cells. The chemically treated yeast cells show considerably increased roughness of the surface compared to almost smooth surface of the intact cells.

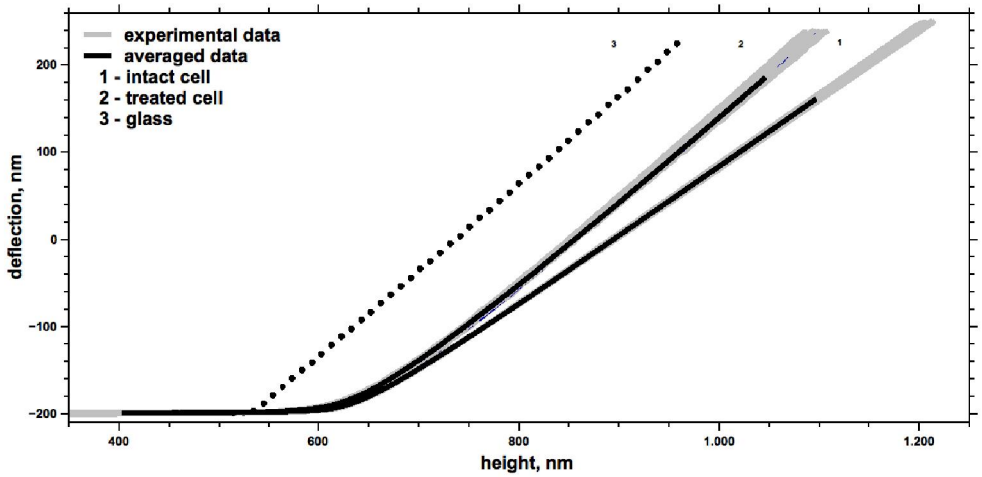

Fig. 5. The AFM *d-z* curves for two types of yeast – intact cells (1) and treated cells (2) affected by Li^+ cations and DTT treatment. The dotted line corresponds to glass; grey lines present experimental curves measured for intact and treated cells in different points around the centre; thick brushed and smooth lines are averages of the experimental data.

point z_0 and the deflection offset d_0 were estimated. Poisson's ratio of a living cell is assumed to be 0.5. In order to investigate the impact of DTT to the cell wall roughness the AFM topography images of an intact yeast cell (Fig. 4 a) and cell treated by DTT (Fig. 4 b) were performed.

The morphological changes in the cell wall roughness of treated yeast cells are clearly observed. The wall of the intact cell is almost smooth, while the wall of the treated cell became rough with holes and many protrusions.

Investing the elasticity of the yeast *d-z* curves was obtained (Fig. 5). Two sets of grey lines correspond to the experimental *d-z* curves measured for the intact cell (1) and for the chemically treated cell (2). The dotted curve (3) shows the *d-z* data of the 'hard material' (a glass plate). All data are aligned relatively to the data of the glass plate. Two smooth black lines are averages of the experimental data. The distance between *d-z* curves for the glass plate and for the yeast cell corresponds to the indentation depth δ and, as seen in Figure 5, the treated cells show higher mechanical resistance characterized by smaller indentation. It was assumed that such chemical treatment softens a cell wall because it breaks chemical bonds and reduces interactions between different macromolecules involved into the cell wall composition. The presented

experimental data show that stiffness of treated cells is increasing compared with that of intact ones.

Figure 6 presents the least-squares fitting of the experimental *d-z* curves shown in Figure 5 utilizing the Sneddon model Eq. (1). Thick gray lines correspond to the averaged experimental data and thin black lines are the data calculated according to the model. According to the applied model, Young's modulus is not constant over the whole range of indentation. In Figure 6, for the 'height' scale up to about 600 nm, the deflection of the cantilever rises very slowly, and then it quickly increases. The applied Sneddon model for a full curve cannot fit the experimental data well in the region near the point where contact is established as it is shown in Figure 6a. Table 1 lists two sets of Young's modulus estimated for both intact and treated cells. Young's modulus was separately estimated for three different regions: (i) for small cantilever deflection (500–600 nm), (ii) for high cantilever deflection (620–800 nm) and (iii) for the full range of deflection (550 –800 nm), which includes both parts.

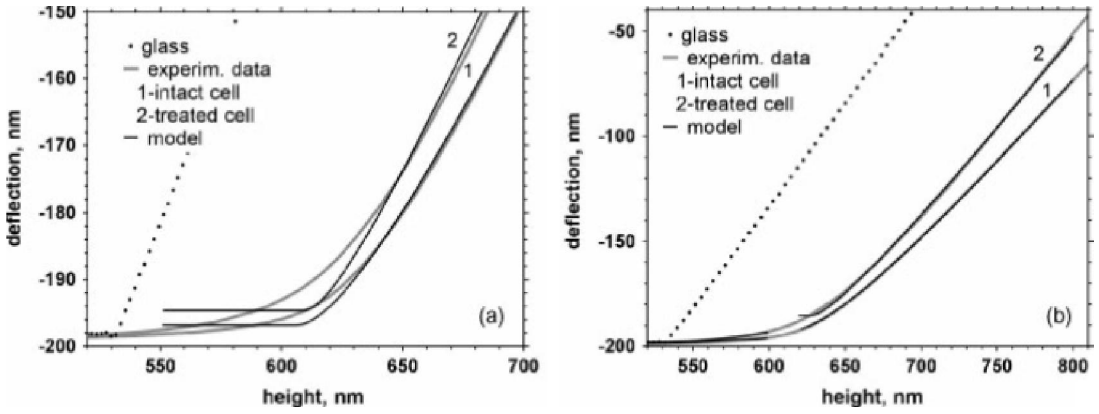

Fig. 6. The least-squares method based approximation of the experimental data (represented by thick grey lines) by the Sneddon model (represented by thin black lines) for intact cells (1) and chemically treated cells (2). Dots show experimental data for the hard material (glass). (a) The plot presents the deflection of model data and errors in determination of the initial contact point when the fit was applied for almost full range of experimental data. (b) The experimental data were fitted twice, for small and high deflections, as shown by two sets of black lines for each curve.

Table 1. The Young's modulus calculated for different regions of cantilever deflection.

Fitting range, nm	500 – 600	620 – 800	550 – 800
E, MPa (intact cell)	0.12 ± 0.82	1.00 ± 0.04	0.84 ± 0.17
E, MPa (treated cell)	0.32 ± 0.12	2.14 ± 0.10	1.93 ± 0.48

Though ignoring the low part of the experimental data, as it was done in the study by other authors, the obtained Young's modulus of the intact cell remains similar at about 1 MPa. For the treated cell, the approximation is still badly suitable as it is shown in Fig. 6b. Enlarging the range, which is fitted for the upper deflections region, Young's modulus is slowly increasing.

The DTT solution has a significant impact on yeast *Saccharomyces cerevisiae* cells mechanical elasticity. The dependence of force to indentation can be described using Sneddon model. According it the Young modules of the yeast cells could be calculated. After the chemical treatment of the yeast cells the increase of Young modules of 2 times could be observed.

Pulsed electric field induced effects on yeast cell memebrane and cell wall permeabilization and viability

The quantity of the TPP^+ absorbed by the yeast cells was expressed as accumulation level and was given by the ratio $(N/N_m)\times 100\%$. Here N_m is the maximal concentration of the molecules that can be accumulated inside the yeasts (steady-state accumulation level). Initial concentration of TPP^+ (N_s) was always kept higher than N_m. The rate of the lipophilic cation absorption of the untreated yeast cells is lower in comparison to the treated by the pulsed electric field (PEF). A TPP^+ accumulation level of 90 % was reached after 100 min for the untreated cells and after 40 min for the yeast cells treated with a 4.8 kV/cm electric field strength and 150 μs duration pulse (Fig. 7). It has to be noted that the steady-state accumulation level is not changed by the PEF action

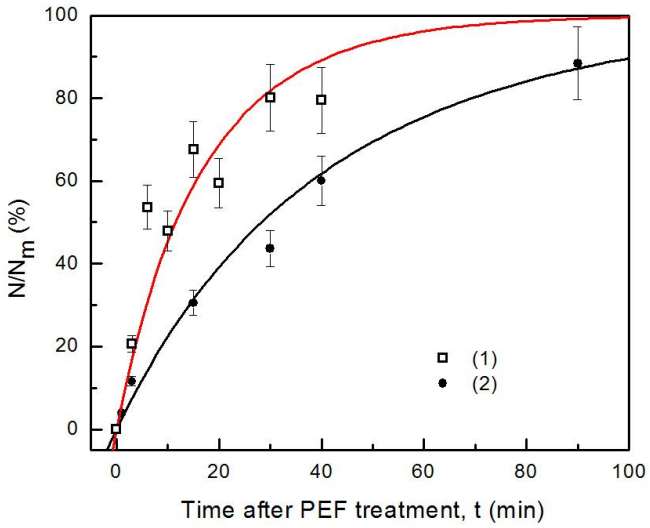

Fig. 7. TPP^+ absorption kinetics of: (1) yeast cells treated with 150 μs duration and 4.8 kV/cm strength PEF, (adjusted R^2 = 0.863) and (2) untreated yeast cells, (adjusted R^2 = 0.989). The duration of the pause from the PEF exposure until the measurements were taken is indicated. The curves were obtained using Equation (3).

on the yeast cells. The results of the TPP^+ accumulation by the yeast cells that were obtained after treating them with a single square shape pulse of 5, 10, 50, 100 and 150 μs

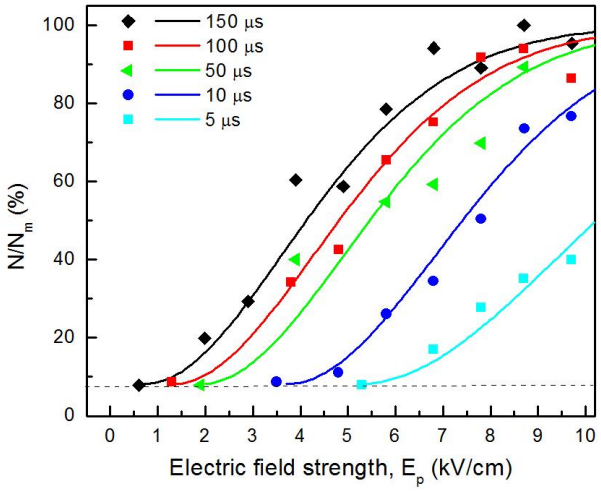

Fig. 8. The effect of PEF strength on TPP^+ accumulation in the cells. The experimental data (symbols) show how the TPP^+ accumulation depends on the field strength after τ = 3 min since the PEF treatment at different square shaped electric pulse duration. The curves in the solid lines were obtained using equation (5). The dashed line represents the amount of TPP^+ which was absorbed by yeast cells up to 3 min after treatment by PEF. Error margins are 10 %. Adjusted R^2 was obtained in the range of 0.899 - 0.973.

duration and different electric field strengths are presented in Figure 8. The measurements of residual TPP^+ concentration in the buffer were performed after 3 min of incubation. Therefore, the dependences of TPP^+ absorption amount on electric field strength are represented in Figure 8 above 9 % level (dashed line) which corresponds to the TPP^+ absorption during these 3 min. As can be seen in Figure 8, the TPP^+ absorption (symbols) definitely depends on the strength and pulse duration of the applied electric field. By increasing the PEF strength at constant pulse duration, the TPP^+ absorption by the yeast is increased and tends to saturation when the applied PEF exceeds 9 kV/cm. It is evident that for different pulse durations these dependences become observable at a certain critical electric field strength called threshold electric field (E_{th}). The E_{th} is a function of the electric pulse duration (t_p) and decreases with an increase of t_p (see Fig.9). The $E_{th\,p}$ at which the TPP^+ absorption starts to increase is inversely proportional to the square root of the electric pulse duration t_p. The inset confirms such relation. It is known, that the TPP^+ accumulation process by the yeasts is very slow, and the equilibrium is reached in 120 min. Therefore, the influence of expulsion from the yeast can be omitted from the accumulation process because all the measurements were performed 3 min after the PEF action.

For this reason TPP^+ accumulation process can be described by the following

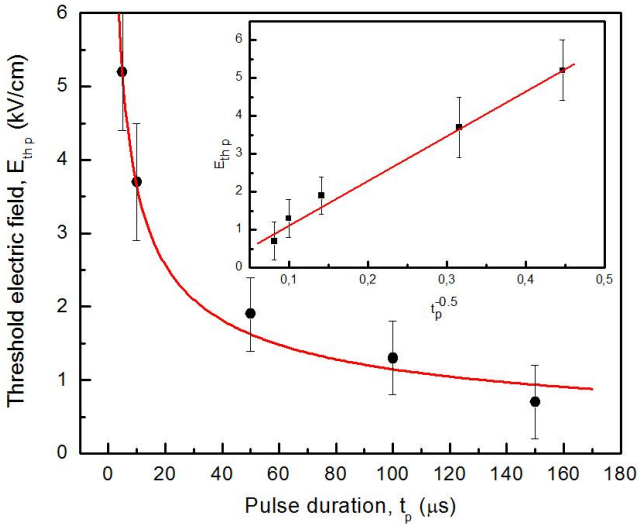

Fig. 9. The dependence of the critical electrical field (E_{thp}) and the pulse duration t_p on the TPP^+ absorption. The calculation of the critical electrical field needed to start the TPP^+ absorption process was done using equation (5). The inset presents the same $E_{th\,p}$ values as a function of $(t_p)^{-0.5}$.

equation:

$$\frac{dN}{dt} = k_a \left(N_m - N\right)\left(N_s - N\right) \tag{2}$$

here: N is the number of the TPP^+ molecules per volume unit (concentration) absorbed by the yeasts; $(N_m - N)$ is a concentration of free absorption sites in the yeasts; $(N_s - N)$ is a concentration of available TPP^+ molecules within the buffer solution; k_a is a coefficient, which quantifies the rate of the absorption process.

The solution of Equation (2) in the case when $N_m/N_s < 1$ is the following:

$$N = N_m \frac{1 - \exp[N_s(R-1)k_a t]}{1 - R \cdot \exp[N_s(R-1)k_a t]} \tag{3}$$

Here: $R = N_m/N_s$; while t is the duration of absorption process.

The absorption rate coefficient k_a depends on the strength of the electric field as follows:

$$k_a = k_{a0} + b \cdot \Delta E^2 \tag{4}$$

Here k_{a0} is the absorption rate at zero electric field; b is the constant, which indicates the electric field influence on the absorption rate of the TPP^+ ions and which increases due to the action of the electric field on the yeast cells.

At fixed time instant (τ), the dependence of $N = f(E)$ can be obtained from (3) and (4) equations:

$$\frac{N}{N_m} \cdot 100\% = \frac{1 - A \exp B \cdot \left(E_p - E_{thp}\right)^2}{1 - R \cdot A \exp B \cdot \left(E_p - E_{thp}\right)^2} \tag{5}$$

here: $A = \exp[N_s \cdot (R-1) \cdot k_{a0}\tau]$, (6)

$$B = B_0 \cdot [1 - \exp(\frac{-t_p}{t_{ch}})]^2, B_0 = N_s \cdot (R-1) \cdot b\tau \tag{7}$$

The TPP^+ absorption kinetic result was fitted by Equation (3) (Fig. 7). The characteristic time constants of the kinetics processes: $1/[N_s(R-1)k_a$ (see equation (3)) obtained by the fitting procedure were 48.6 min for yeasts untreated by PEF and 19.8 min for those affected by PEF. Moreover, evaluation of the TPP^+ kinetics demonstrates that the PEF affects the rate of TPP^+ uptake, but not the TPP^+ accumulation level. The influx of TPP^+ into the yeast cells after their treatment with PEF of different durations and amplitudes was determined. It can be concluded that the experimental data could be well explained by the proposed model. The fitting of these data by the Equation (5) is represented by the solid lines in Figure 8. As a result of fitting procedure, the parameter B values as a function of pulse duration t_p were obtained (Fig. 9). The obtained value of characteristic time t_{ch} required to charge the biological object shows that the treated objects are larger than one yeast cell. Taking into account t_{ch} = 4 μs, the evaluation of average diameter (D) of biological objects exposed by PEF using formula $D = 2\ t_{ch}/\rho_{ef}C_m$, here ρ_{ef} is the resistivity of suspension (≈ 3 kΩ·cm), C_m is the capacitance of the membrane per unit area (1 μF/cm2) reveal, what D ≈ 26 μm. It confirms that we are dealing with yeast cell aggregates. Our experiments shows that the

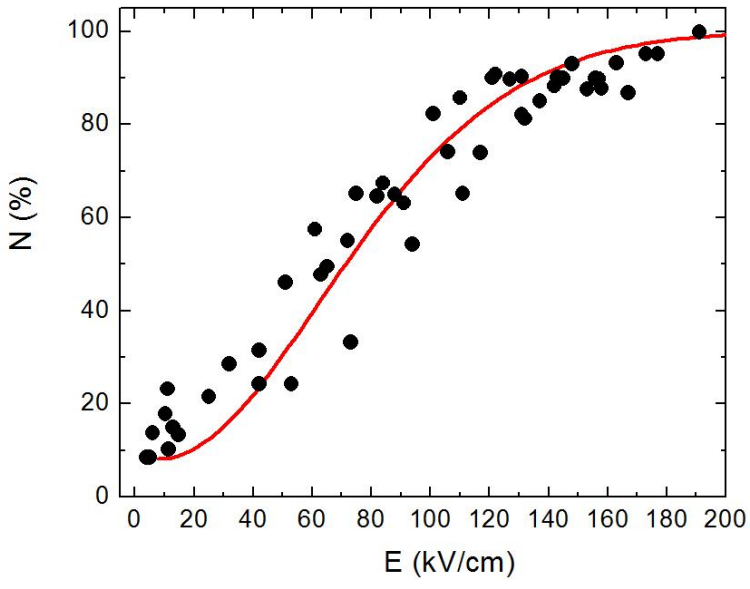

Fig. 10. The dependence of the extracellular TPP^+ ions concentration in the suspension of yeasts after the treatment on the electric field strength of HPEF. Data from 3 independent experiments (symbols) show the dependence of how TPP^+ accumulation on pulsed electric field strength. Measurements were performed 3 min after square-shaped 60 ns duration pulse action. Curve in solid line was obtained using Equation (5).

pulsed electric field focuses mainly on the larger aggregates (equivalent cell) consisting of several cells and diameter of the aggregates varies from 20 to 30 μm.

It is known, that very short electric pulses, shorter than charging time of the biological object (cell or equivalent cell), cannot induce permeabilization changes of the plasma membrane. The analysis of single 60 ns pulse duration high power electric field shows that effectiveness of electrically induced increment of TPP^+ absorption by yeast depends on $t_p \times (E\text{-}E_{th})^2$, i.e. proportional to the energy of electric field pulse (Fig. 10). The same dependence has been shown for microsecond pulse duration exposure. In order to find out the impact of HPEF on the yeast vital functions two different quantitative methods have been used. Plating efficiency was determined using decimal reduction ratio, which gave quantitative results about inactivation rate. Increasing the strength of pulsed electric field survivor rate decreasing only till 0.6 log cycles (Fig. 11a). HPEF impact on yeast viability using microbial cell proliferation assay was done in order to investigate instant effect. Figure 11 b shows survival curves obtained after 4 h when yeasts were treated by 60 ns duration and 150 μs duration electrical pulses. However, no significant impact of nanosecond duration HPEF on yeast proliferation rate was found. To investigate the damage of yeasts cells plasma membrane after nanosecond HPEF action, the fluorescence probe calcein blue AM (molecular weight 321.28) was used. This observation demonstrates that HPEF induced electroporation of cells plasma

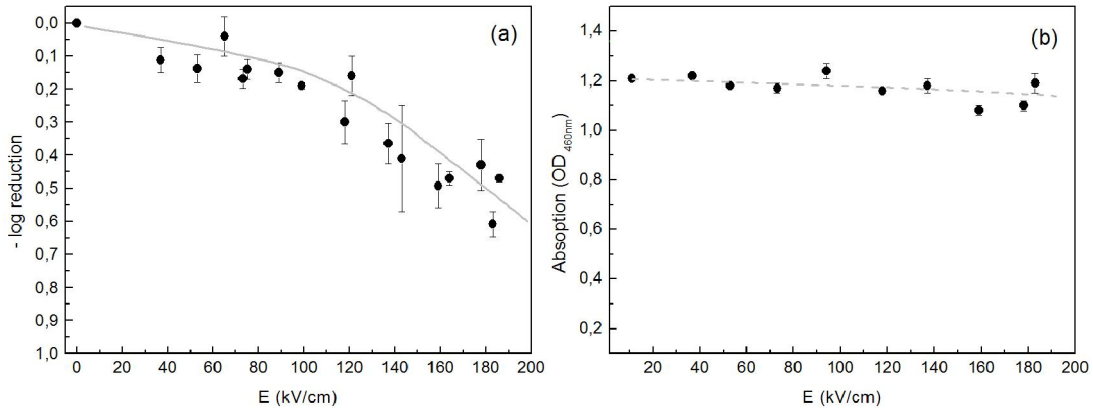

Fig. 11. The effect on the inactivation of yeasts by 60 nanosecond HPEF exposure. (**a**) The reduction rate of yeast growth after 4 days in YPD plates. (**b**) the proliferation of yeast after 4 h of incubation in synthetic medium after nanosecond HPEF exposure. The data are presented as means ± standard deviation of 3 experiments.

membrane is not sufficient to release the calcein blue probe.

It was concluded, that increase of TPP^+ influx into the cell is a result of PEF action on the plasma membrane causing electroporation in the range from nanosecond to microsecond pulse duration. Changing amplitude of pulsed electric field the rate of TPP^+ absorption process can be control. The obtained 65 time increase of yeast cell wall permeability to TPP^+ molecules after the exposure of 60 ns PEF, which do not, has an impact on cell viability, comparing with the intact yeast cells. Such permeability changes were 3.5 times higher in comparison to the yeast cells exposured to 150 μs duration PEF. Such finding can be useful for the enhancement the selectivity and specificity of biosensors or expand the yield of products in biocalysis process.

CONCLUSIONS

1. Mechanical tension of cell wall increased after the treatment of yeast (*Saccharomyces cerevisiae*) cells by DTT. This phenomenon is based on the decrease of the elasticity of the multilayered cell wall structure, after the DTT treatment affecting cell wall protein disulphide bonds.
2. Sneddon model could be applied to describe the mechanical properties of the yeast cell wall. Young's modules were obtained using this model: at the low deformations modules were: 0.12 ± 0.82 MPa for intact cells and 0.32 ± 0.12 MPa for treated cells. For the large deformation Young's modules of the intact yeast cells was 1.00 ± 0.04 MPa and for the treated ones it was 2.14 ± 0.1 MPa.
3. The increase of the permeability of yeast cell wall to TPP^+ ions was obtained after the impact by strong microsecond duration pulsed electric field. The TPP^+ accumulation process can be described by the second order kinetic equations, where the association coefficient is a parabolic function of the electric field strength. This indicates the dependence of permeability effectiveness on the pulse energy, when the amplitude of electrical field exceeds the threshold electric field.

4. Electrochemical TPP^+ ion absorption detection method allows indicating the cell wall permeability after the exposure by high power electric field with duration of nanoseconds, which does not affect a viability of the yeast. Electroporation efficiency depends on the energy of the pulse. The obtained value of characteristic time required to charge the biological object is 4 μs. Indicating that, the electrical pulse charge aggregates consist of several yeast cells in diameter of 26 μm. In the case of nanosecond duration pulses the charging time was 0.9 μs suggesting, that such electric field pulse can charge individual yeast cell.
5. No significant impact of nanosecond duration high power electric pulses to viability of yeast cells was found. Meanwhile, electric pulses of microseconds creating up to 6 kV/cm electric field strength decrease the viability up to 60%.
6. Microsecond and nanosecond duration electric pulses generating electric field strength up to 200 kV/cm enhances yeast wall permeability to the charged molecule such as TPP^+. Using 150 μs duration pulsed electric field is possible to enhance the TPP^+ absorption rate till 18.4 times in comparison with the intact cells without the loss of viability. However, using 60 ns duration pulsed electric field the TPP^+ absorption rate increase up to 65 times comparing with the untreated yeast cells. Such PEF-based procedure could be applicable to enhance the rate of biosensors.

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3. **A. Stirke,** A. Zimkus, S. Balevicius, A. Ramanaviciene, A. Ramanavicius, V. Stankevic, N. Zurauskiene, L. Chaustova. (2012). Effects of nanosecond high power pulsed electric field on yeast Saccharomyces cerevisiae cell wall permeability. *IEEE Proceedings on 4th Euro-Asian Pulsed Power Conference 19th International Conference of High-Power Particle Beams*: 161-164.
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Curriculum Vitae

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Cheminio ir elektrinio poveikių *Saccharomyces cerevisiae* mielių ląstelių savybėms tyrimas

Santrauka

Mielių (*Saccharomyces cerevisiae*) taikymas biologiniuose jutikliuose ar biokatalizėje ribojamas jų ląstelės sienelės struktūros, kuri apsunkina reikiamų medžiagų transportą. Todėl darbo tikslas – ištirti mielių ląstelių mechaninių savybių ir plazminės membranos bei sienelės pralaidumo pakitimus, veikiant mielių ląsteles cheminėmis medžiagomis bei mikro- ir nanosekundžių trukmės didelės galios impulsais naudojant atominių jėgų mikroskopą bei tetrafenilfosfonio katijonų elektrocheminę analizę. Nustatyta, kad ditiotreitolis (DTT) keisdamas mielių sienelių pralaidumą, pakeičia mielių ląstelių mechanines savybes. Esant didelėms deformacijoms mielių ląstelių tamprumo modulis (Young‘o) padidėjo nuo 1,00 ± 0,04 MPa nepaveiktoms iki 2,14 ± 0,1 MPa DTT paveiktoms mielėms. Tiriant impulsinio elektrinio lauko (IEL) poveikį mielių sienelės pralaidumui, impulso trukmių nuo mikrosekundžių iki nanosekundžių diapazone stebima tiesinė TPP^+ absorbcijos greičio priklausomybė nuo impulso energijos. Nustatyta, kad veikiat mieles 60 ns trukmės impulsais ląstelių pralaidumas TPP^+ molekulėms, nepažeidžiant mielų gyvybingumo, padidėjo iki 65 kartų lyginant su nepaveiktomis elektriniu lauku mielių ląstelėmis, t.y. 3,5 karto daugiau negu mieles paveikus 150 μs impulsais. Tyrimų rezultatai rodo, kad IEL galima didinti visos ląstelės biologinių jutiklių spartą ir atrankumą.