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Accumulation of Selenium and Changes in the Activity of Inulinase and Catalase in the Cells of *Kluyveromyces marxianus* on Pulsed Electric Field Treatment

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Pulsed electric field (PEF) of 1 Hz, 1.5 kV, and 1 ms increased the activities of catalase and inulinase over the whole range of applied Se concentrations compared with the non-treated cultures. A significant effect of selenium concentration (in the range of 5–14 μg/ml) on both intra- and extracellular enzyme activities was noted. At a Se concentration of 10 μg/ml, the activities of intra- and extracellular inulinases and extracellular catalase in the PEF-treated cultures reached the maximum of 71 U/g d.m., 46 U/g d.m., and approx. 8 U/ml, respectively. The maximum activity of intracellular catalase of approx. 6 U/ml (with and without PEF) was recorded at 5 μg Se/ml. Further increasing of selenium concentration caused a decrease in the activity of the enzymes.

Keywords: Selenium, *Kluyveromyces marxianus*, pulsed electric field, inulinase

For its antioxidant properties, the vital role of selenium in human and animal metabolism is highly appreciated [1, 4, 9, 13, 20, 21, 31, 32, 37]. However, higher concentrations of selenium in the organism may be harmful as it then acts as a prooxidant, catalyzing the oxidation of thiols and generating superoxide [36, 45]. Se can replace S in sulfhydryl groups of sulfur amino acids, glutathione, and coenzyme A, substantially changing their functions, structure, and properties [15, 16, 35]. Organic Se from yeast grown in selenium-enriched media is a common form of Se for humans and animals, and its antioxidant activity exceeds that of inorganic Se [1, 21, 23].

Supplementation of human food and livestock feed with Se-enriched yeast has become more common. Since the margin between its toxic and essential levels is narrow [8]. accurate Se speciation in nutritional supplements is of great importance.

Se also controls enzyme activity. For instance, it has been shown that the activities of succinate dehydrogenase, cytochrome oxidase, and arginase decrease in the presence of Se. This is probably due to blocking of the SH groups [12]. Any data concerning the effect of Se on changes in inulinase and catalase activities resulting from PEF treatment are lacking.

Several microorganisms are used to produce inulinase, and *Kluyveromyces marxianus* is one of the most effective species. It can be grown on inexpensive media containing inulin such as Jerusalem artichoke, chicory, and dahlia. Inulinases (2,1-β-D-fructan fructanohydrolases) are used in the production of fructose syrups. They hydrolyze inulin (β-2-1-linked fructose polymer with a terminal glucose unit) to fructose in one step [39].

By breaking perhydrol down, catalase protects cells and prevents tissue degradation [10, 33, 34]. It is used for removal of H_2O_2 after cold pasteurization in milk processing. Unfortunately, isolation of the enzyme either from microbial biomass or from animal organisms and its purification are very costly, and the use of isolated catalase is irrational as it is rapidly inactivated by H_2O_2. Therefore, whole cells are used as a source of the enzyme.

For over two decades, PEF has been investigated as a non-thermal technique of food preservation [2, 3, 6, 17, 18]. This food processing technology is mainly used for liquid foods to selectively inactivate microorganisms and enzymes, retaining flavor and keeping nutritive compounds intact [5, 22, 24].

The effect of PEF on microorganism cells is well known. When PEF applied to the cell membranes exceeds a certain critical value, the cells undergo damage [7, 19]. Enzyme inactivation is influenced by field strength, pulse duration

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and shape, number of pulses, and enzyme concentration. Enzymes are generally more resistant to PEF than microorganisms [18, 42, 46]. When PEF of 13–87 kV/cm, 0.5 Hz, 2 μs pulse width, and 30 pulses was applied, lipase, glucose oxidase, and heat-stable α-amylase exhibited activity reduction by 70–85%; peroxidase and polyphenol oxidase showed a moderate 30–40% decrease in their activities, whereas alkaline phosphatase suffered only about 5% reduction of its activity [43].

Recent data suggested that both native and thermally inactivated enzymes could be activated with PEF. A PEF intensity of 12–13 kV/cm with the decaying time of up to 2 ms was applied to six different enzymes, resulting in an up to 20% increase in the activity of peroxidase and β-galactosidase [26]. The authors suggested that peroxidase has heme in its molecular structure, which could be easily transferred depending on electric field. Catalase is also a heme enzyme, and thus its reaction to PEF could be similar. PEF treatment could also cause some conformational change, leading to an increase or decrease in the enzyme activity [16, 46].

The effect of PEF upon enzyme depends on the type of enzyme [18, 26]. Application of PEF to the cultures of *S. cerevisiae* did not affect the activity of catalase. The selenium concentration was the sole parameter that influenced the activity of this enzyme [29].

In this study, the effects of low-intensity PEF upon Se accumulation in *Kluyveromyces marxianus* cultures and on changes of the inulinase and catalase activities were checked.

Materials and Methods

A strain of *Kluyveromyces marxianus* (K-1) obtained from the Department of Biotechnology, Human Nutrition and Science of Food Commodities, University of Life Sciences, Lublin, Poland was used for the experiments.

The strain was subcultured in an optimized liquid medium composed of (g/l) glucose (70.0), NH_4Cl (7.5), KH_2PO_4 (2.5), $MgCl_2 \cdot 6H_2O$ (2.0), Na_2SO_4 (2.0), inulin (1.0), and yeast extract (YE) (5.0), and 40 ml of unhopped wort at pH 5. The selenium concentration in the culture medium was 4 μg/ml. In order to determine the optimum concentration of this microelement, the following doses of Se were added: 1, 2, 3, 5, 8, 10, 12, 14, 16, and 18 μg Se/ml culture medium. Electroporation of cell membranes was conducted at optimized parameters: the field frequency of 1 Hz, voltage of 1.5 kV, and time of pulse length of 1 ms. The yeast culture was treated with PEF for 3 min after culturing for 16 h [28]. After 36 h of culturing, the yeast biomass was centrifuged, washed with distilled water, and dried in a freeze-drier (Model 64132; Labconco, Kansas City, MO, U.S.A.).

Determination of Selenium Concentration

Selenium was determined with the use of atomic absorption spectroscopy (a flameless technique employing a graphite cuvette) in the Varian Spectra AA-880 apparatus.

Determination of Extracellular Activity of Inulinase in Post-Culture Media

The reaction mixtures, composed of 0.2 ml of post-culture medium from the cultivation of *K. marxianus* and 0.8 ml of McIlvaine buffer (pH 5.0) containing 1% inulin, were incubated in a thermostat at 50°C for 30 min. Then, 3 ml of 3.5-dinitrosalicylic acid (DNS) was added to the mixtures, and the whole preparations were incubated for 5 min in a boiling water bath. In the next step, the samples were cooled to 20°C and diluted by adding 11 ml of distilled water. Finally, the absorbance of the samples was measured at λ=520 nm against a reference sample containing pure reagents. The amounts of liberated reducing sugars were calculated on the basis of a standard curve plotted for fructose.

One unit of inulinase activity (U) was defined as the amount of enzyme that liberated, in the experiment conditions, 1 μmol fructose equivalent from the substrate in 1 ml of reaction mixture per minute.

Activity of inulinase was calculated according to the formula:

$$A = c \cdot R / 0.18 \cdot t$$

where A=activity of enzyme (mmol/ml/30 min)
c=amount of sugars (mg) in 1 ml of mixture read from a standard curve
R=multiplicity of dilution
t=time of sample incubation in minutes
0.18=mass of 1 μmol fructose/mg

Results of the determination of inulinase activity were presented in units of activity/g dry mass (U/g d.m.).

Extraction of Intracellular Inulinase and Determination of Its Activity

A 5-ml volume of post-culture medium was centrifuged, and the obtained yeast biomass was suspended in 5 ml of 0.05 M acetate buffer (pH 4.8). Then, the suspension was placed in a closed test tube and incubated in a thermostat at 50°C for 18 h in order to enhance the autolysis of the cells and secretion of the enzyme. Samples were centrifuged at a relative centrifugal force (RCF) of 1,000 ×*g* at 4°C for 15 min. The supernatant was a source of enzymes connected with the cell.

For determination of intracellular inulinase, 0.8 ml of McIlvaine buffer (pH 5) containing 1% inulin was added to 0.2 ml of extract. The further procedure was the same as in the case of determination of extracellular inulinase.

Determination of Intra- and Extracellular Activities of Catalase

Catalase activity was determined according to the procedure of Fiedurek and Gromada [14]. Intracellular catalase was extracted several times from homogenized disintegrated cells using 0.1 M McIlvane buffer at pH 7.0. A unit of catalase activity (U) was defined as the number of μmoles of hydrogen peroxide decomposed by 1 ml of post-culture medium in 30 min.

Results and Discussion

The study showed that PEF affected the accumulation of selenium in *K. marxianus* cells. In 16-h cultures treated with PEF for 3 min, the concentration of this element was about 167 μg/g d.m. The application of PEF caused an

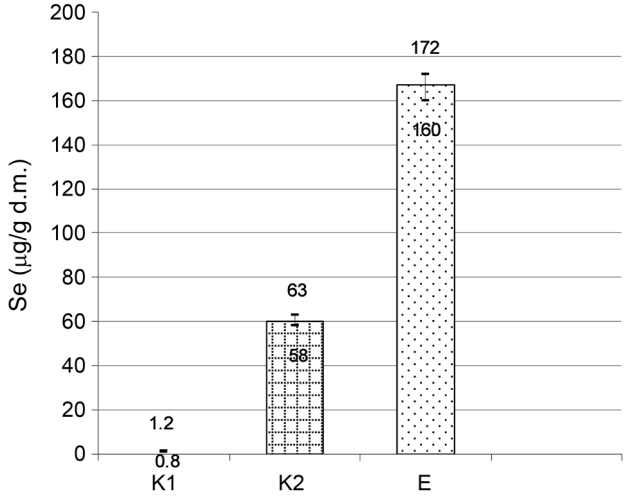

Fig. 1. Effect of pulsed electric field (PEF) on selenium accumulation in the cells of *K. marxianus* (control cultures: K1, without Se and PEF treatment; K2, 4 μg Se/ml medium and without the PEF treatment; E, 4 μg Se/ml medium and 3-min exposure to PEF after 16-h of cultivation).

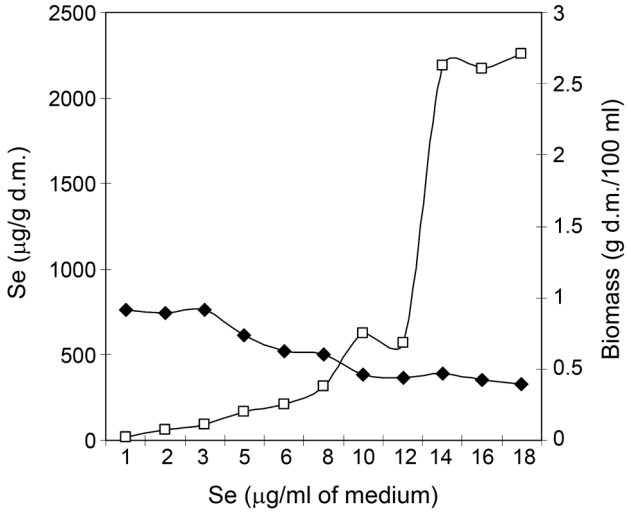

Fig. 2. Effect of selenium concentration in culture medium on its accumulation in cells.

almost triple increase in selenium concentration in comparison with the control culture (Fig. 1).

The maximum accumulation of selenium in the cells of approx. 2,250 μg/g d.m. was recorded at 14 μg Se/ml of the culture medium. A further increase in selenium concentration only insignificantly affected its accumulation level. Optimalization of this parameter resulted in an approx. 13 times higher accumulation of selenium in the yeast cells (Fig. 2).

Simultaneously, together with increasing concentration of selenium in the medium, decay of the cells was observed. At selenium concentration in the range of 1–6 μg/ml, the number of the dead cells was over 3.5 times higher than in the control. Approx. 60% dead cells was observed at concentrations between 8 and 18 μg Se/ml [28].

Former studies showed that PEF influenced the accumulation of selenium in the cells of various yeasts [27]. In the present experiment, changes of the inulinase and catalase activities in *K. marxianus* cells after treatment of the cultures with PEF at different Se concentrations were demonstrated.

In order to determine the effect of selenium concentration in *K. marxianus* cells on the enzyme activity, shaken-flask cultivations were carried out at optimum composition of the culture medium and PEF parameters.

Many authors have indicated a strong influence of Se on the activities of oxidoreductase enzymes, such as catalase, glutathione peroxidase, and superoxide dismutase [13, 25]. The activity of certain enzymes of the glycolytic pathway and the respiratory duct decreased in the presence of selenium probably because of blocking of the SH groups [30].

The applied PEF parameters affected the catalase and inulinase activities. In the cultures treated with PEF and in the control cultures, the activities of intra- and extracellular catalase and inulinase were higher than in the non-PEF-treated cultures, regardless of the selenium concentration (Tables 1 and 2). Selenium concentration in the medium from 1 μg/ml to 3 μg/ml caused a slight increase in the

Table 1. Effects of PEF and selenium concentration in culture medium on extra- and intracellular catalase activities in *K. marxianus.*

Selenium concentration (μg/ml)	Extracellular catalase activity (U/ml)		Intracellular catalase activity (U/ml)	
	with PEF	no PEF	with PEF	no PEF
0	1.09±0.15	0.75±0.13	1.99±0.09	1.08±0.13
1	0.95±0.18	0.68±0.18	1.85±0.12	0.98±0.06
2	0.90±0.14	1.16±0.11	2.14±0.09	1.24±0.09
3	1.35±0.15	0.88±0.16	3.19±0.08	1.30±0.11
5	3.32±0.13	2.99±0.14	6.12±0.11	5.88±0.06
8	7.99±0.11	5.03±0.11	4.51±0.11	3.37±0.12
10	8.08±0.12	5.09±0.15	3.18±0.07	1.03±0.09
12	7.41±0.14	4.27±0.14	3.14±0.1	1.91±0.13
14	6.12±0.13	3.78±0.16	2.97±0.13	1.85±0.07

Table 2. Effects of PEF and selenium concentration in culture medium on extra- and intracellular inulinase activities in *K. marxianus.*

Selenium concentration (μg/ml)	Extracellular inulinase activity (U/g d.m.)		Intracellular inulinase activity (U/g d.m.)	
	with PEF	no PEF	with PEF	no PEF
0	12.65±0.15	5.81±0.13	30.25±0.08	2.12±0.11
1	15.28±0.19	6.14±0.18	33.99±0.12	2.67±0.07
2	16.83±0.16	6.47±0.21	41.14±0.2	4.04±0.19
3	13.08±0.18	6.53±0.26	39.38±0.19	5.17±0.15
5	19.59±0.14	6.94±0.19	43.95±0.25	6.24±0.23
8	30.22±0.10	7.65±0.17	54.77±0.23	6.25±0.14
10	46.12±0.13	9.46±0.25	71.50±0.17	2.71±0.17
12	28.62±0.14	3.54±0.16	69.87±0.16	1.88±0.18
14	27.89±0.13	2.74±0.13	66.17±0.18	1.74±0.16

catalase and inulinase activities, in the PEF-treated and non-treated cultures. A significant effect of selenium concentration in the range of 5–14 μg/ml in the culture medium on the activities of both enzymes was noted. An increase in the Se concentration up to 10 μg/ml was followed by an initial rise in their activities. However, further increase in the Se concentration provided an opposite effect. Similar results were obtained for the cultures left without treatment with PEF.

In the latter cultures, the activity of extracellular inulinase was higher than in the PEF-treated cultures.

The applied PEF parameters affected catalase and inulinase activities. The activities of intra- and extracellular catalase and inulinase were higher than in the non-treated cultures (Tables 1 and 2). Selenium concentration in the medium from 1 μg/ml to 3 μg/ml caused a slight increase in catalase and inulinase activities in the PEF-treated and untreated cultures. A significant effect of the selenium concentration in the medium on the activities of both enzymes was noted in the range of 5–14 μg/ml. An increase in the Se concentration to 10 μg/ml was followed by a rise in their activities. However, further increase in the Se concentration had an opposite effect. Similar results were obtained for the cultures untreated with PEF.

In the PEF-treated cultures, irrespective of the selenium concentration, the activity of intracellular inulinase was approximately twice as high as that of the extracellular inulinase. Maximum activities of extra- and intracellular inulinases in the cultures, 46 U/g d.m. and 71 U/g d.m., respectively, were noted at the selenium concentration of 10 μg/ml, at which the activity of extracellular catalase was approx. 8 U/ml. A further increase in the selenium concentration caused a decrease in the activities of the enzymes (Tables 1 and 2). The highest activity of intracellular catalase in the biomass of about 6 U/ml was recorded at the selenium concentration of 5 μg/ml, (Table 1).

The electric field may affect the activities of some enzymes. Usually enzymes can be partly or completely inactivated by applying a high-intensity electric field [43, 44, 47]. The activity of plasmin, an enzyme originating from cow milk, suspended in simulated milk ultrafiltrate was reduced by 90% after the application of 50 pulses at an intensity of 45 kV/cm [41]. Castro *et al*. [11] reported that 70 pulses of 0.7 ms at 22 kV/cm reduced by 65% the alkaline phosphatase activity in milk. The activity of the protease from *Pseudomonas fluorescens* was reduced by 30% after exposure to 20 pulses of 0.7 ms with the field intensity of 6.2 kV/cm [40]. On the other hand, a weak electric field may enhance the activities of enzymes. A field of 20 V/cm and 300–1,000 Hz stimulated Na^+, K^+-ATPase isolated from human erythrocytes [38].

Zhao and Yang [46] reported that the activity of lysozyme was a function of the applied PEF strength and treatment time. The results indicated that the unfolding of the tertiary structure of lysozyme was induced by PEF treatment at 35 kV/cm for 1,200 μs.

The present studies demonstrated that treatment of *K. marxianus* cultures with low-intensity PEF caused an increase in selenium accumulation in the cells and enhanced catalase and inulinase activities. Selenium accumulation and changes of the enzyme activity depended also on the selenium concentration in the medium.

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