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Effect of Pulsed Electric Fields upon Accumulation of Zinc in *Saccharomyces cerevisiae*

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Cultures of *Saccharomyces cerevisiae* were treated with pulsed electric fields to improve accumulation of zinc in the biomass. Under optimized conditions, that is, on 15 min exposure of the 20 h grown culture to PEFs of 1500 V and 10 µs pulse width, accumulation of zinc in the yeast biomass reached a maximum of 15.57 mg/g d.m. Under optimum zinc concentration (100 µg/ml nutrient medium), its accumulation in the cells was higher by 63% in comparison with the control (without PEFs). That accumulation significantly correlated against zinc concentration in the medium. Neither multiple exposure of the cultures to PEFs nor intermittent supplementation of the cultures with zinc increased the zinc accumulation. The intermittent supplementation of the cultures with zinc and multiple exposures on PEFs could even reduce the accumulation efficiency, respectively, by 57% and 47%.

Keywords: Zinc, biomass, *Saccharomyces cerevisiae*, pulsed electric fields

Zinc is an essential microelement for life. As a structural component of the zinc finger motifs found in many transcription factors and as a catalytic cofactor for RNA polymerase, zinc is required for gene transcription [18, 29]. Zinc, in a biologically relevant form of Zn^{2+} ions, is essential as a catalytic cofactor of many enzymes, including alcohol dehydrogenase, alkaline phosphatase, carbonic anhydrase, and several carboxypeptidases. It also plays a critical structural role in enzymes and many noncatalytic proteins [5, 27, 32]. A deficiency of zinc ions reduces the yeast growth and alcoholic fermentation [9, 10, 12, 17].

Consistent with the importance of zinc to cellular metabolism, zinc deficiency can have severe consequences. In mammals, for example, even moderate zinc deficiency can cause anemia, loss of appetite, immune system defects, developmental problems, and teratogenesis. In the case of humans, most susceptible to zinc deficiency are young children, pregnant and nursing women, and elderly people [8, 18]. Zinc has a narrow margin between therapeutic and toxic doses. One of the ways of reducing the risk of zinc intoxication during supplementation of diet is an incorporation of this element into yeast cells.

Yeasts are known for their ability to accumulate metal ions from aqueous solutions by different physico-chemical interactions (*e.g.,* by adsorption and absorption), or by a metabolism-dependent mechanism [8, 15]. Sorption processes are dependent on disposable functional groups on the cell surface and on the nature of metal ions. Thus, the concentration of free ions, ligand electronegativity, metal cation, ligand charge, and the cavity size have a great influence on the selectivity of metal uptake. Furthermore, the composition of nutritive medium to which the microorganism is exposed affects the amount of metal uptake, because of the cell wall structure and the metabolic state of the cell [6, 15, 20, 27, 30, 32].

Pulsed electric field (PEF) electrotechnology is an emerging technology in the field of food preservation. PEFs have the potential to pasteurize several foods nonthermally *via* exposure to high-voltage short pulses while the material is between the electrodes of a treatment chamber [19, 25, 28]. The electric field affects the cell membranes, and may cause irreversible membrane breakage, alteration in transport of ions, and changes in enzyme structure [4, 11, 13, 16, 23, 33]. It is generally accepted that a PEF exerts its effect by causing the formation of pores in the membrane of treated cells [1, 2, 3, 31]. In a cell subjected to a PEF, induced transmembrane voltage leads to the formation of pores in the membrane and to an increase in its permeability. It may reach a level permiting cell penetration by extracellular molecules. Such a state of membrane permeability for macromolecules following electroporation may persist for a period from several minutes to more than 10 h, depending

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on the type and size of cells, electric field intensity, pulse duration, temperature, and chemical composition of the medium. After cessation of the PEF effect, there may occur a reversible process of self-sealing of the formed pores, with retention of foreign molecules [14, 24, 26].

The aim of this paper was to test whether PEF technology is a suitable technique for the zinc enrichment of *S. cerevisiae*.

Materials and Methods

Culture Maintenance and Inoculum Preparation

Saccharomyces cerevisiae 11 B_1 (industrial strain) from the Yeast Plant (Lublin, Poland) was used.

Medium for agar slants and inoculum growth (g/l): sucrose (20), NH_4Cl (3.2), KH_2PO_4 (2.5), Na_2SO_4 (2.0) (POCH, Gliwice, Poland), yeast extract (YE) (5.0), agar (15) (DIFCO, Detroit, MI, USA), and unhoped wort (40.0 ml) (Lublin Breweries S.A., Lublin, Poland) at pH 5. Experimental medium for *S. cerevisiae* contained (g/l), peptone (10) (Sigma-Aldrich Co, St. Louis, MI, USA), YE (5), and glucose (10) (POCH, Gliwice, Poland) [7].

Biomass Cultivation

Yeast was passaged three times for agar slants, grown for 48 h in a thermostat at 30°C, and finally an inoculum was prepared. Cells from a single slant were used to inoculate 150 ml of sterile medium in an Erlenmeyer flask. Cultures were grown on a rotary shaker with water bath, at amplitude 4 and at 220 rpm for 48 h at 30°C. The fermented medium was centrifuged, washed three times with sterile water, and the remaining washed biomass of cells from three Erlenmeyer flasks was diluted with sterile water to a final volume of 300 ml. The inoculum was made of 48 h yeast cultures with OD_{400} of 2.2 (Spekol 11; Carl Zeiss, Jena, Germany). Biomass inoculum with washing was 12 g d.m/l. The inoculum prepared in this manner was used for inoculation of submerged culture grown in 500 ml Erlenmeyer flasks, each containing 100 ml of medium inoculated with 10 ml of inoculum. The culturing conditions were identical to those applied for the inoculum preparation. After 42 h culturing, the mycelium was centrifuged, washed several times with distilled water, and then lyophilized in a Labconco freeze dryer (Model 64132; Kansas City, MO, USA).

PEF Treatment and Enrichment with Zinc

S. cerevisiae cultures grown in flasks were agitated for 20 h and then treated with PEF for 15 min (electroporator ECM 830; BTX Harvard Apparatus, USA) at the field frequency of 1 Hz and 20 µs pulse width. Simultaneously, voltage was optimized on the field exposures of 50, 100, 150, 200, 250, 500, 1,000, 1,500, 2,000, 2,500, and 3,000 V; respectively 0.1, 0.2, 0.3, 0.4, 0.5, 1.0, 2.0, 3.0, 4.0, 5.0, and 6.0 kV/cm.

The concentration of zinc in the culture medium was set at the optimum voltage of 1500 V. Subsequent culturing of *S. cerevisiae* was carried out in the medium containing 10, 100, 200, 400, 500, 750, and 1,000 µg/ml zinc from solutions of the $ZnSO_4$.

Optimization of the PEF exposure time for the yeast cultured for 20 h was performed for 5, 10, 15, and 20 min, at optimum voltage of 1,500 V and optimum zinc concentration in the culture medium (100 µg/ml). The PEF pulse widths of 10, 20, 50, 75, 100, 125, and 150 µs were selected at 1,500 V on the 15 min exposure.

Optimization of the time after which yeast cells were treated with PEF was performed after 8, 12, 16, 20, and 24 h culturing at 1,500 V, 10 µs pulse width, and 15 min of exposure. At optimum PEF parameters and 100 µg/ml zinc concentration, the medium was additionally enriched with subsequent 25 µg/ml doses of zinc after 8, 12, 16, and 20 h of culturing. Subsequent culturing was performed under optimized conditions and these cultures were PEF treated several times after 8, 12, 16, and 20 h. The results allowed to check the role of enrichment with zinc and number of exposures to PEF upon the zinc accumulation in the yeast cells. Samples not treated with PEF and without zinc in the medium (K1) or with 100 µg Zn/ml (K2) served as controls.

Determination of the Zinc Concentration

Mineralization of yeast for the determination of the zinc concentration using the method of flame atomic absorption spectrophotometry (FAAS, VARIAN AA 280 FS) was conducted as follows: 250 mg samples of lyophilized zinc-enriched yeast were weighed into glass thimbles, and 3 ml of HNO_3 was added. Determination by the FAAS method was carried out according to the Norm PN-EN 14082:2004. A standard curve was plotted with the Zn concentrations of 0.5, 1, 2, 5, 190, 15, and 20 mg/l, and the determination coefficient was equal to 1. Samples were digested in a MARS5 (CEM Corporation) microwave oven at 200°C for 30 min. After cooling, solutions were transferred to 50 ml measuring flasks and topped up with deionized water.

Determination of the Yeast Cell Viability

Cell viability was determined in the Thoma chamber, dyeing necrotic yeast cells with the 0.01% methylene blue solution. The percentage of dead cells was the mean of 16 fields calculated according to the formula

$$\% \text{ dead cells} = (\text{number of dead cells}/\text{sum of dead and living cells}) \times 100\%$$

Determination of the Crop of Yeast Biomass

Biomass was estimated from optical density at 400 nm. Dry mass was calculated by referring to a standard curve of cell mass versus absorbance. The fermented medium from culturing (2 ml) was centrifuged (3,000 rpm), supernatant was discarded, and cells were rinsed with deionized water and brought to the original volume of 2 ml. Turbidimetric measurements were run against pure water at λ=400 nm, in a 2 mm measurement cell. The amount of dry residue was calculated using the equation for the standard curve: $A_p=0.4476c$, where A_p and c are apparent absorbance and concentration (mg/ml), respectively.

Data Analysis

Significant differences between particular groups were found by applying the Student t-test to compare independent samples in pairs, and variance analysis (ANOVA) was used for more than two groups. Statistical processing of results was performed using the Statistica 6.0 software.

Results and Discussion

The conducted research proved that low voltage of 50 to 500 V did not influence zinc accumulation in the cells

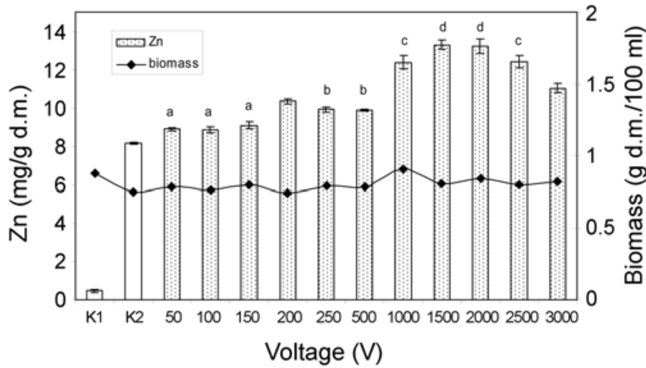

Fig. 1. Effect of voltage PEF treatment on zinc accumulation in *S. cerevisiae.*
Means with the same letters are not significantly different ($P \geq 0.05$; n=6).

which was at a level ca. 9–10 mg/g d.m. A similar value was noted for the control medium K2 which was not treated with PEF (Fig. 1).

In the control culture K1, zinc accumulation came to 0.5 mg/g d.m. Statistically significant changes of accumulation were noted in the range of high voltages. The maximum accumulation of zinc in *S. cerevisiae* (13.29 mg/g d.m) was observed at the optimum voltage of 1,500 V, and it was 63% higher than that observed for the culture K2. An increase of the voltage over 2,000 V caused a statistically significant decrease of zinc accumulation in the cells (Fig. 1).

What is more, the viability of cells was also significantly influenced by high voltage. In the range of 500–3,000 V, the number of dead cells was 3.5 times higher. At the voltage of 1,500 V, dead cells constituted 14% of the culture. The voltages used did not cause any changes in biomass, which reached the range 7.5–9.2 g/l at the voltages, respectively, of 200 and 1,000 V (Fig. 2).

The effect of zinc concentration in the medium on its accumulation in the cells treated with PEF was also significant. Initially, accumulation of this element increased together with increasing concentration. The maximum level of accumulation (13.29 mg/g d.m) was noted at the optimum Zn concentration

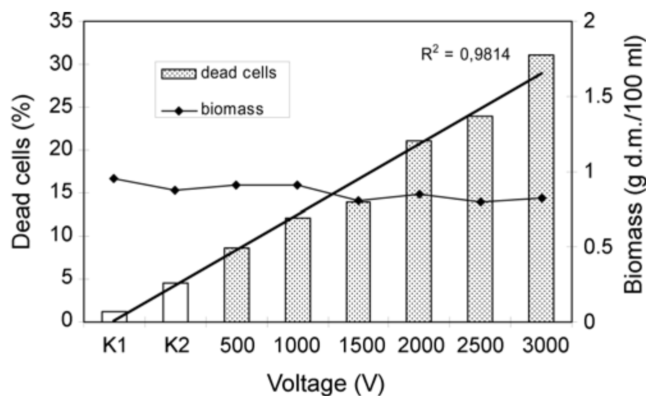

Fig. 2. Effect of PEF voltage on the viability of *S. cerevisiae* cells.

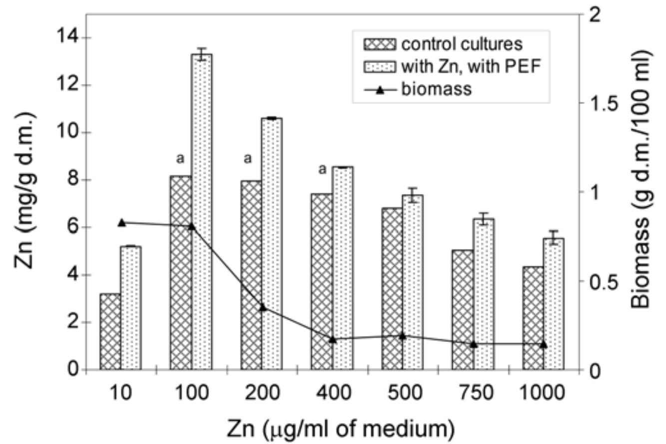

Fig. 3. Zinc accumulation in *S. cerevisiae* depending on its supplementation level in the medium.
Control cultures: no PEF, supplemented with Zn. Means with the same letters are not significantly different ($P \geq 0.05$; n=6).

of 100 µg/ml medium, and this value was 2.5 times higher in comparison with the culture where Zn concentration was 10 µg/ml medium. Further increase of this parameter led to significant decrease of zinc accumulation in the cells, where at Zn concentration 1,000 µg/ml medium it was equal to 5.57 mg Zn/g d.m, and this value was 2.5 times lower than for the optimum concentration. In the control cultures not treated with PEF, in the whole range of Zn concentrations used, accumulation of zinc was significantly lower than in the cultures treated with PEF. Zinc accumulation in the cells was equal to 3.19 mg/g d.m and 8.16 mg/g d.m for the concentrations 10 and 100 µg Zn/ml medium, respectively (Fig. 3). The greatest influence of PEF on zinc accumulation was observed for Zn concentration 100 µg/ml medium and it was 63% higher than in the control medium (without PEF treatment).

The results demonstrated that biomass and cells viability were significantly affected by Zn concentration in the

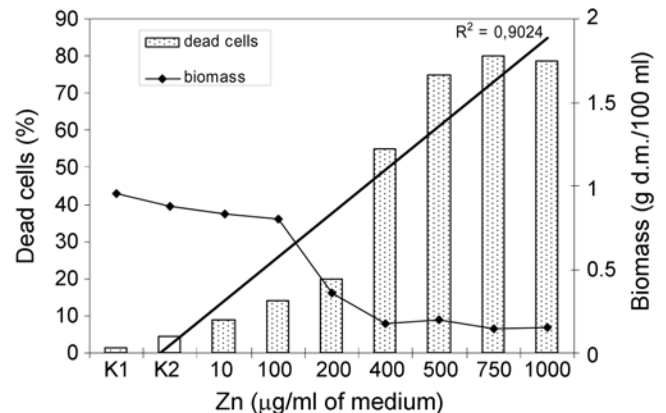

Fig. 4. Effect of zinc concentration on the viability of *S. cerevisiae* cells.

medium. Concentration below 100 μg Zn/ml had no effect on biomass, which was equal 0.8–0.9 g d.m/100 ml. Very similar results were obtained for the control cultures K1 and K2. Significant decrease of biomass, from 0.35 to 0.15 g d.m/100 ml, was observed for the Zn concentration range 200–1,000 μg/ml. Moreover, it was also observed that together with increasing Zn concentration in the medium, the number of dead cells increased as well. At the optimum concentration of 100 μg Zn/ml, 14% of cells in the medium were dead. For the Zn concentration range 400–1,000 μg/ml, the number of dead cells was 5 times higher and was equal, respectively, to 55% and 78%. The correlation between biomass decline and cell viability was significant (Fig. 4).

Optimization of the PEF exposure time showed that an initial extending of time caused an increase of Zn concentration in the cell biomass. The maximum accumulation of this element in the cells (13.29 mg/g d.m) was obtained after 15 min of exposure to PEF. It was higher by ca. 36% or 50% than that noted after 5 and 20 min of PEF treatment (Fig. 5).

The study revealed that pulse width had also a significant effect on zinc accumulation in the cells. The maximum accumulation of zinc in the yeast biomass (15 mg/g d.m) was reached at the pulse width of 10 μs. This value was higher by 84% than in the case of the medium treated with pulse width of 150 μs. Statistically significant decrease of zinc accumulation was noted when the pulse width was higher than 10 μs (Fig. 6).

This parameter also had an influence on the cell viability (Fig. 7). At the optimum pulse width of 10 μs, the percentage of dead cells (4,58%) was similar to that observed for the control medium K2 that was not treated with PEF. Further increase of the pulse width to 50 μs led to an almost 10-times higher number of dead cells (48%). In the range 75–125 μs, the changes in viability were statistically significant.

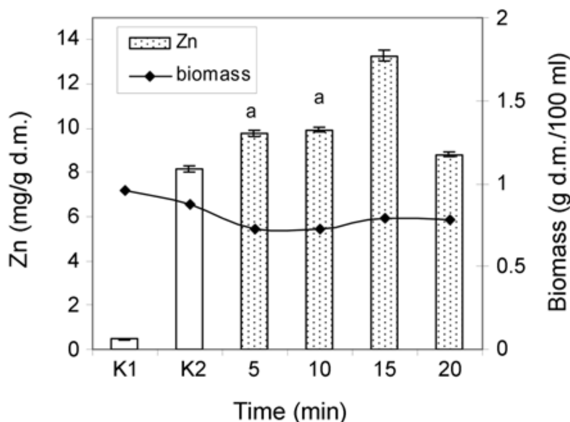

Fig. 5. Accumulation of zinc in the cell biomass dependent on the time of exposure to PEF.
Means with the same letters are not significantly different (P≥0.05; n=6).

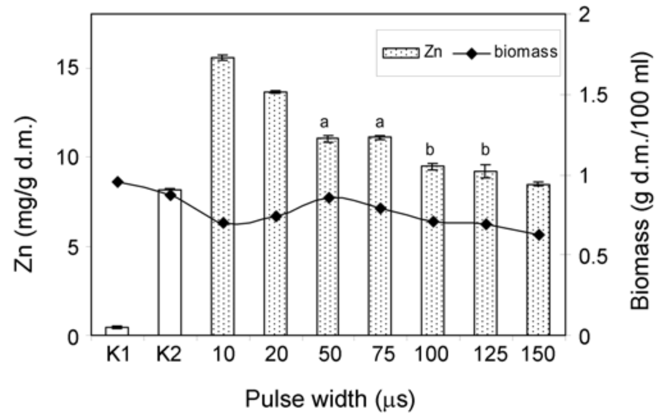

Fig. 6. Effect of PEF pulse width on accumulation of zinc in *S. cerevisiae.*
Means with the same letters are not significantly different (P≥0.05; n=6).

At the pulse width of 125 μs, the percentage of dead cells was the highest (ca. 60%).

For the complete representation of the effect of PEF treatment on zinc accumulation in the cells, the control media were conducted at the same time. In the control K1, which was not supplemented with zinc and not treated with PEF, zinc accumulation was on the level of 0.5 mg/g d.m. Statistically significant increase of zinc accumulation was observed in the case of the control K2, which was supplemented with the total dose of zinc but was not treated with PEF. Simultaneously, the level of accumulation (8.16 mg/g s.s) was lower than in the case of the media treated with PEF.

The study showed that the time of culturing after which cells were treated with PEF significantly affected accumulation of zinc. At the conditions used, the maximum zinc accumulation in the cells (ca. 15.5 mg/g d.m) was obtained after PEF treatment of 20 h culturing. Zinc concentration was higher by ca. 72% or 57% than in the case of the media treated with PEF after 8 and 24 h of cells multiplication (Fig. 8).

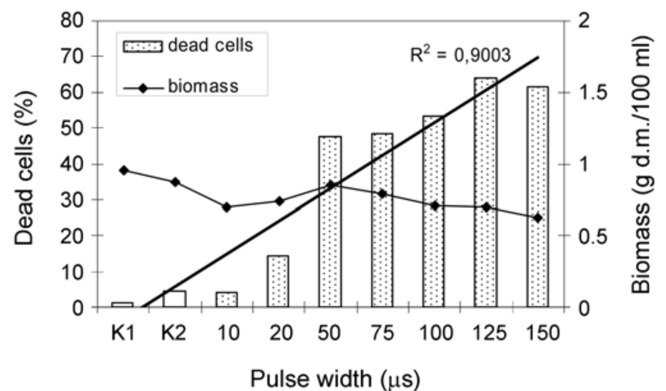

Fig. 7. Effect of PEF pulse width on the viability of *S. cerevisiae* cells.

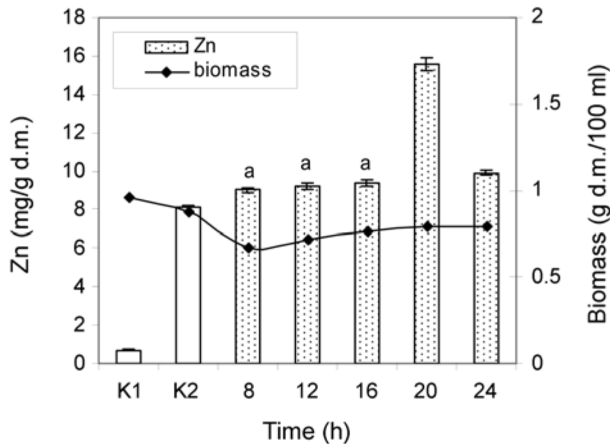

Fig. 8. Effect of post PEF treatment culturing duration on the zinc concentration in *S. cerevisiae* cells.
Means with the same letters are not significantly different ($P \geq 0.05$; $n=6$).

It was proved that multiple exposure of the yeast medium to PEF did not affect significantly the increase of zinc accumulation in the cells. For instance, Zn accumulation after 4-fold 15 min exposure to PEF after 8, 12, 16, and 20 h culturing was on the level of 8.3 mg/g d.m ± 0.23 and was 47% lower than the maximum value of 15.57 mg/g d.m noted after a single PEF treatment.

It was also revealed that addition of a total zinc to the medium in four doses after 8, 12, 16, and 20 h of culturing did not increase its accumulation in the cells. This kind of supplementation resulted in a level of accumulation equal 6.70 mg/g d.m ±0.21. It was 57% lower in comparison with the medium where zinc was added in one dose at the beginning of the culturing.

Pankiewicz and Jamroz [21] investigated the influence of PEF on accumulation of magnesium in *S. cerevisiae.* Under optimized conditions, that is, on 15 min exposure of the 20 h grown culture to PEF of 2,000 V and 20 µs pulse width, accumulation of magnesium in the yeast biomass reached the maximum of 3.98 mg/g dm. It constituted 40% of total magnesium in the medium.

Pasternakiewicz and Tuszyński [22] studied the effect of zinc ions on growth of *S. cerevisiae.* They used two strains: Frencz Mautner and YT_{411x5p} hybrid. In the concentration range of 10−100 µmol/l, zinc ions enhanced the dynamics of yeast cell multiplication and the production of biomass, but the optimal zinc concentrations were different for both strains.

Stehlik-Tomas *et al.* [27] enriched *S. cerevisiae* cells with zinc, copper, and manganese. They investigated the effect of medium supplementation with salts of these metals on biomass and ethanol production by the yeast. Addition of the Zn^{2+}, Cu^{2+}, and Mn^{2+} caused an increase of yeast biomass and it was the highest at medium pH 5. The highest concentration of zinc in dry matter (700 µg/g) was achieved in anaerobic conditions at medium pH 4.

The moment of zinc supplementation also affects its accumulation. According to the literature data [27], metal ions binding by yeast is connected to their life activity, and consequently to the phase of cell growth.

In conclusion, optimized voltage (1,500 V), exposure time (15 min), pulse width (10 µs), and point of treatment in the course of the growth of cultures (20 h) provide the highest level of the zinc accumulation in *S. cerevisiae*. At 100 mg/ml zinc concentration in the medium, 63% accumulation of that element was achieved. That accumulation significantly correlated with the zinc concentration in the medium.

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