Int. Agrophysics, 2006, 20, 147-152

INTERNATIONAL
Agrophysics
www.ipan.lublin.pl/int-agrophysics

Optimization of selenium accumulation in *Rhodotorula rubra* cells by treatment of culturing medium with pulse electric field

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Received December 6, 2005; accepted February 14, 2006

A b s t r a c t. Electroporation was used as a method for *Rhodotorula rubra* biomass enrichment in selenium. The highest selenium accumulation in yeast cells was achieved after 10 min pulse electric field (PEF) exposure of 16 h shaken culture. Multiple PEF exposure of yeast culture did not favour selenium accumulation. Optimization of selenium concentration in a medium resulted in an over two-fold increase of its accumulation in cells. About three-fold increase of dead yeast cells was recorded in analysed range of selenium concentrations.

K e y w o r d s: selenium, biomass, *Rhodotorula rubra*, pulse electric field

INTRODUCTION

Selenium is an important microelement that enters anti-oxidation reactions and it is among the regulators of the immune system (Sattar *et al.,* 1997; Hammel *et al.,* 1997; Gazdic *et al.,* 2004). As opposed to other microelements, it is characterized by a narrow range between therapeutic and toxic doses (Graczyk *et al.,* 1994). Selenium concentration of 600-800 mg for person per day is considered as toxic (Food and Nutrition Board, 1980). Incorporation of selenium into yeast cells is one of the ways to diminish the risk of intoxication with the element at supplementing the diet with selenium preparations (Chmielowski *et al*., 1994).

Microbial cells are able to accumulate selenium in the form of organic and inorganic compounds. Two processes influence its accumulation: extracellular binding by active groups of bio-polymers within the cell wall-membrane system and intracellular accumulation combined with selenium ions transport through biological membrane into the cell interior (Danch and Chmielowski, 1985).

In selenium transport, microorganisms may use the sulphur transport mechanisms, which results from similarity and possibility of substituting sulphur by selenium. It may be also incorporated into microbial cells through transport systems of other ions that are physiologically necessary, or during non-specific transport process in complexed form *eg* with sugar substrates (Kabata-Pendias and Pendias, 1994).

Electroporation is one of relatively easy, non-toxic and cheap techniques for incorporating specific macromolecules into the cytoplasm (Prasanna and Panda, 1997; Serpersu and Tsong, 1984). It consists in short-term removal of plasmatic membrane integrity *ie* making such large pores as to facilitate the penetration of studied molecules into the cytoplasm, but small enough to make possible their subsequent closure. When electric field intensity achieves critical value or even higher, a great number of large-size pores is formed and thus irreversible mechanical damage of a cell occurs (Zimmermann, 1986; Fiedurek *et al.,* 2000).

It was plausible to undertake a study utilizing electroporation as a method for the enrichment of *Rhodotorula rubra* biomass in selenium. *Rhodotorula rubra* yeast is usually saprophyte – it does not invoke any disease in humans. Producing the carotenoid pigment is characteristic for that yeast.

MATERIALS AND METHODS

Materials

Rhodotorula rubra biomass used for the study was obtained from Department of Agricultural, Foodstuff and Storage Industry Technology, University of Agriculture in Lublin.

The basic liquid medium for culturing consisted of (in g dm^{-3}): glucose (70), NH_4Cl (7.5), KH_2PO_4 (2.5), $MgCl_2$ 6 H_2O (2), Na_2SO_4 (2), yeast extract (YE) (5), inulin (1) and 40 ml of non-hop medium. The sterilization process was conducted in autoclave under the pressure of 506 hPa 113°C for 20 min.

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Preparation of inoculation material

Yeast was transferred onto agar scarves several times and cultured for 48 h at 30°C under thermostatic conditions, then inoculum was prepared. Cells from a single scarf served for inoculation of 150 ml of sterile medium in an Erlenmeyer flask. Culture was performed in rotation shaker equipped with water basin, at amplitude 4, 220 r.p.m., for 48 h at 30°C. The after-culture liquid was removed and the remaining cellular biomass from three Erlenmeyer flasks was diluted in sterile water adjusting the final volume to 300 ml. The inoculum prepared in this way was used for grafting the inbreeds that were performed in Erlenmeyer flasks of 500 cm^3 capacity and containing 100 cm^3 of the medium, with subsequent grafting using 10 ml of the inoculum. Conditions at which cultures were performed were identical as during inoculum culturing. After culture completing, the mycelium was centrifuged, washed with distilled water several times and then dried in a lyophilizer (Labconco, Model 64132, Kansas City, MO, USA). Yeast digestion was carried out to determine selenium concentration by application of atomic absorption spectrometry technique (AAS). Aliquots of 100 mg of lyophilized selenium yeast were placed in glass vials, 5 ml mixture of concentrated HNO_3 – $HClO_4$ (3+1, v/v) was added and left till the next day. Then, the sample was heated at 50°C for 1 h, at 70°C for 6 h, and at 125°C for 12 h. After cooling, solutions were transferred into 25 ml capacity measuring flasks, adjusted with distilled water to the mark, and subjected to electrothermal atomic absorption spectrometry (ETAAS) determination.

Objects

The following were the objects in these experiments:

1 – culture to which selenium was added in five doses: the first before culturing, next after 8, 12, 16, 20 h; the sample was treated with pulse electric field (PEF) after 8 h of experiment;

2 – culture to which selenium was added in five doses as previously, but PEF was applied after 12 h;

3 – culture to which selenium was added in five portions as above but treated with PEF after 16 h;

4 – culture with selenium divided into five doses as above but treated with PEF after 20 h.

The control cultures were:

K1 – culture with no selenium and with no PEF;

K2 – culture with the whole selenium amount added into the medium before culturing and with no PEF;

K4 – culture to which selenium was added in five doses and treated with PEF after 8, 12, 16, 20 h.

An additional control sample was:

K3 – to which selenium was added into the medium before culture and treated with PEF after 16 h, was used when setting the optimum culturing time for the PEF treatment.

Methods

Selenium was determined by means of the non-flame atomic absorption spectrometry technique using graphite cell in Spectra AA-880 (Varian) equipment.

Electroporation of *R. rubra* cell membranes was carried out according to the Fig. 1.

To optimize the PEF exposure time for *R. rubra* cells (Fig. 1), a series of cultures - with the whole amount of 4 μg Se ml^{-1} medium added before the experiment – was carried out. Yeast cells were treated with PEF after culturing time estimated earlier.

Finding the optimum selenium concentration in medium (Fig. 1) was made on the basis of cultures performed at culture duration and PEF treatment times determined earlier.

In order to find difference significance between particular groups, t-test was applied to compare independent samples in pairs and variance analysis (ANOVA) for more than two groups. Statistical processing of results was performed using Statistica 6.0 software.

Determination of yeast cells viability

Aliquots of 2 ml of post-culturing liquid were taken, centrifuged and decanted. Yeast biomass remained after centrifuging was washed with distilled water, centrifuged again, decanted and adjusted with water to 2 ml capacity. After thorough stirring, a single drop of the solution was placed in Thom's chamber, one drop of 0.01% solution of methylene blue was added to dye the dead cells. The preparation was covered with microscopic cover glass and living (colourless) and dead (blue) cells were counted at 100x magnitude at the presence of immersion oil. The counting included 16 fields of Thom's chamber. The percentage of dead cells was a mean value from 16 fields calculated according to the formula:

$$\%\ \text{of dead cells} = \frac{\text{number of dead cells}}{\text{sum of dead and living cells}} 100\%\,.$$

RESULTS

To evaluate the optimum culturing time, there were performed cultures exposed to PEF after 8, 12, 16, 20 h of cell reproduction. Maximum selenium accumulation in yeast (about 32 μg g^{-1} d.m.) was recorded after the 16th-hour PEF treatment (Fig. 2).

Another scientific task was aimed at optimising the time of PEF action after 16 h of yeas cells reproduction to elevate the selenium content in cell biomass. The highest selenium accumulation (about 50 μg g^{-1} d.m.) was achieved in cells of culture II after 10 min PEF exposure. It was higher by about 56% in relation to culture I that was performed at optimized time after which cells were treated with PEF. Optimization of PEF action time caused a significant increase of selenium

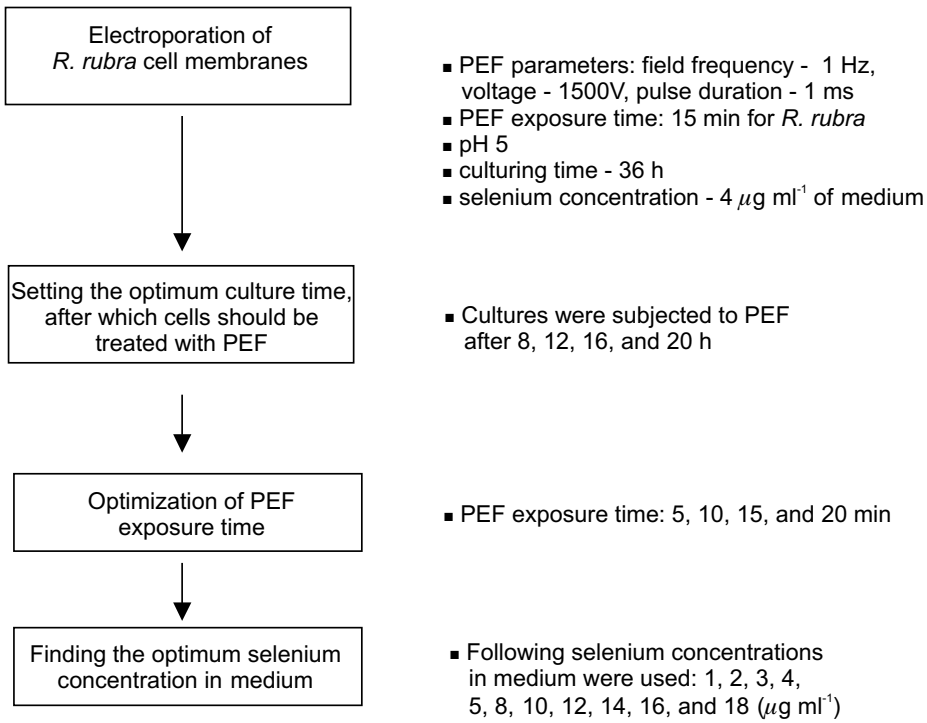

Fig. 1. Electroporation of yeast cell membranes.

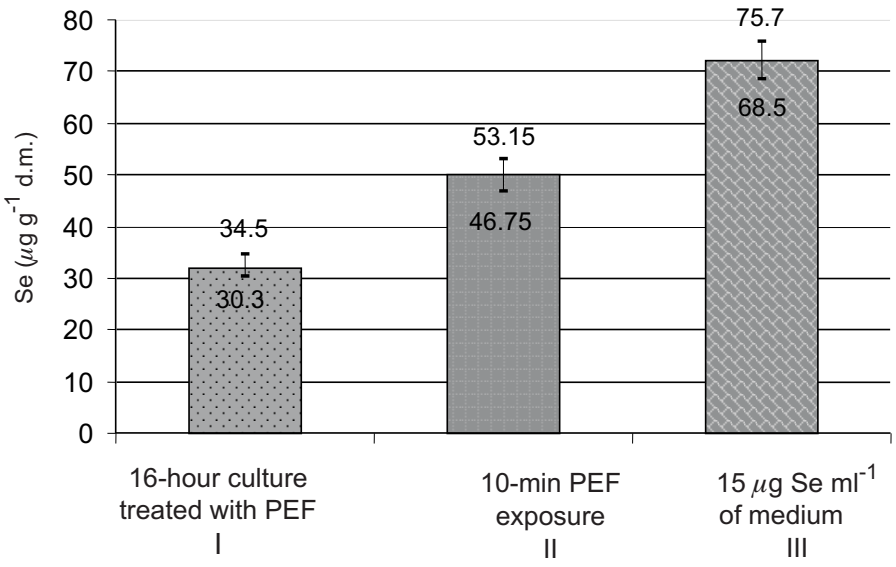

Fig. 2. Influence of culture parameters optimization on selenium accumulation in *R. rubra* cells:
I – optimized culturing time after which cells were treated with PEF; selenium concentration 4 μg ml^{-1} of medium added in five portions; 15-min PEF exposure after 16 h of culturing; II – optimized PEF exposure time; selenium concentration 4 μg ml^{-1} of medium added once before culture beginning; 10-minute PEF exposure after 16 h of culturing; III – optimized selenium concentration 15 μg ml^{-1} of medium added once before culture beginning; 10-min PEF exposure after 16 h of culturing.

content in *R. rubra* cells (Fig. 2).Up-to-date cultures of *R. rubra* were performed at selenium concentration of 4 μg ml^{-1} of medium. Analysis of other experimental results concerning selenium accumulation in yeasts suggested that optimization of selenium concentration in medium is necessary at given parameters of PEF action. Maximum selenium accumulation (about 72 μg g^{-1} d.m.) in *R. rubra* cells was observed at its optimum content amounting to 15 μg ml^{-1}. Optimization of selenium concentration in medium resulted in 44% increase of selenium accumulation in yeast in relation to cultures performed at optimized time, after which cells were exposed to PEF, and at optimum PEF exposure time (Fig. 2).

To fully visualize the PEF influence on selenium accumulation in yeasts, control cultures were performed at the same time *eg* after the 16 h PEF treatment (Fig. 3). Trace selenium amounts (about 1 μg g^{-1} d.m.) were found in control culture K1, to which no selenium was added and no PEF was applied. Significant increase of selenium accumulation in cells was recorded in control culture K2, to which the full selenium dose was added, but no PEF was applied (Fig. 3).

Exposing control culture K3 to PEF resulted in a significant increase of selenium accumulation in reference to K2 culture. Moreover, it was found that selenium accumulation in control culture K3, in which the full selenium amount was added into the medium before culturing and then the 16 h culture was exposed to PEF, did not significantly differ from selenium accumulation in cells from culture I, to which selenium was introduced in five portions and the 16-hour culture was treated with PEF (Fig. 2). Significant decrease of selenium accumulation was found in cells from 36 h control culture K4 that was treated with PEF four times (after 8, 12, 16, and 20 h), as compared to culture K3, exposed to PEF only once (Fig. 3). Selenium content in K4 was about 25 μg g^{-1} d.m. and it was similar to accumulation in cells from culture K2, in which the full selenium dose was added and no PEF was applied (Fig. 3).

Data referring to mean values (M), standard deviations (SD) and statistical significances for selenium accumulation are presented in Table 1.

The above culturing experiments were aimed at finding the influence of selenium concentration in the medium on cell viability (Fig. 4). Selenium content in the range from 1 to 3 μg ml^{-1} had a slight effect on yeast cell atrophy. The percentage of dead cells was about 15%. A significant influence of selenium concentration on cell viability was recorded in the range from 4 to 12 μg ml^{-1}. In this range, the percentage of dead cells was from 30 to 45%.

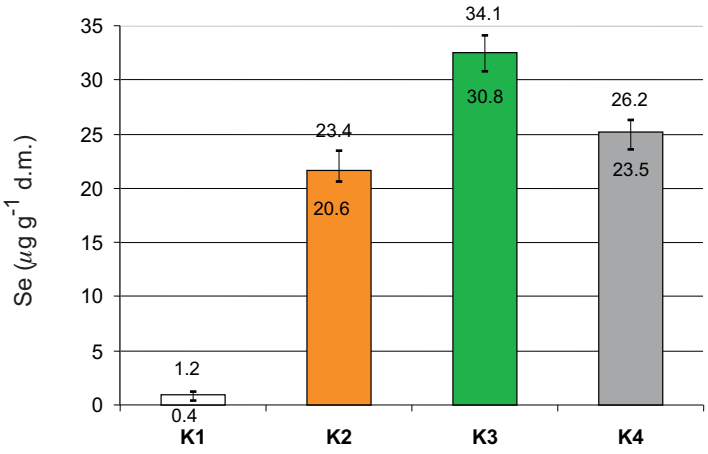

Fig. 3. Selenium concentration in *R. rubra* cells (control cultures: K1 – with no Se and no PEF; K2 – with all Se dose and no PEF; K3 – with all Se dose and PEF after 16 h culturing; K4 – with all Se dose and PEF after 8, 12, 16, and 20 h culturing).

T a b l e 1. Selenium accumulation in *R. rubra* cells in particular groups

Groups	K1	K2	K3	K4	I	II	III
M	0.85a	21.94a.b	32.73b.c.*	24.98c	32.50d.*	50.15d.e	71.62e
SD	0.31	1.10	1.25	1.07	1.70	2.42	2.80

M – mean values, SD – standard deviation; mean values marked with: the same letter (a, b, c, d, e) differ significantly at $P \leq 0.05$, n = 6; asterisk (*) do not differ significantly at $P \leq 0.05$, n = 6.

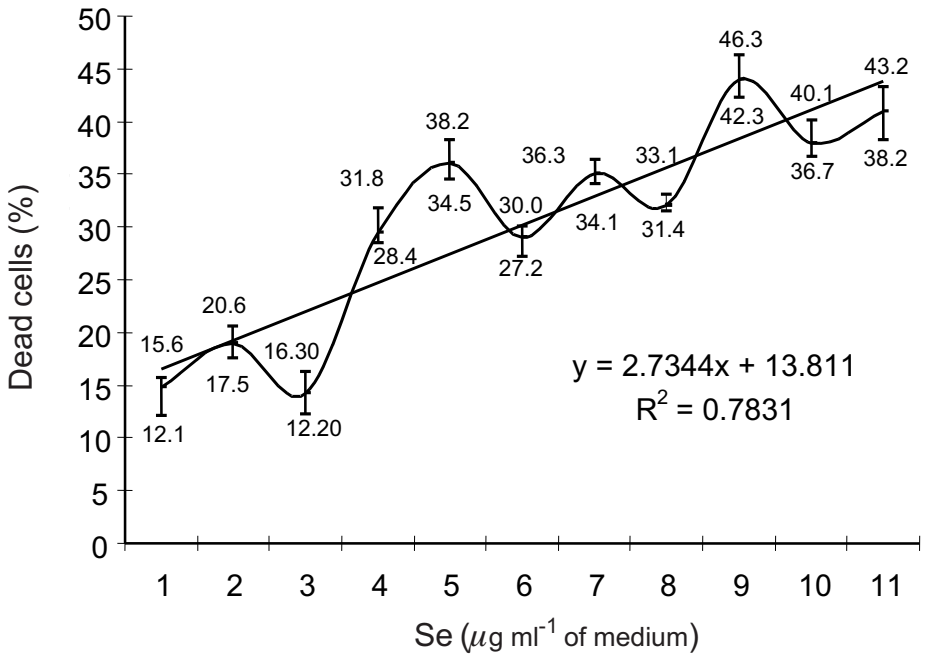

Fig. 4. Influence of selenium concentration on *R. rubra* cell viability.

DISCUSSION

On the basis of presented results it can be concluded that finding the optimum selenium concentration and its dosage was necessary, because probably different interactions of selenium with cells of different yeast types were the main reason for its differentiated accumulation.

Podgórska and Bujak (1985) studied the selenium influence on growth of selenium yeast *S. cerevisiae* and *C. tropicalis*. They proved inhibition of selenium at 1 μg Se ml^{-1} of medium dose. *E. coli* bacteria manifested differentiated resistance towards the increase of selenium concentration in medium. According to Opieńska-Blauth and Iwanowski (1952), concentration of 7.9 μg Se ml^{-1} of medium obviously inhibited growth of *E. coli*, whereas Tuve and Wiliams (1961) found that the concentration below 10 μg Se ml^{-1} of medium did not influence *E. coli* growth. The divergence in results is probably caused by other interactions of selenium with different microorganisms cells or differentiated conditions during the culture.

Finding the PEF exposure time was a significant factor in *R. rubra* culture. Exposure of selected culture to pulse electric field had a significant influence on selenium accumulation in cells. Higher selenium accumulation was found in cultures treated with PEF as compared to those not exposed to PEF. Results of an unpublished study revealed different resistance of different yeast types towards PEF treatment and to selenium accumulation. Cells of *Kluyveromyces marxianus*, for which optimum PEF exposure time was the shortest, appeared to be the most vulnerable to electric field action. Cells of *S. cerevisiae* were the most resistant – the optimum PEF treatment time was five times longer (own study, unpublished data).

CONCLUSIONS

1. The dependence between selenium accumulation in yeast cells and its concentration in culturing medium has been proved. Achieved results indicate yeast resistance to selenium accumulation.

2. Multiple PEF exposure of yeast cultures did not favour selenium accumulation in cells, nor did the addition of the whole selenium amount into the medium in five doses during the culture.

3. Selenium accumulation in cells was found to be dependent on culturing time after which they were treated with PEF. Optimization of culturing time after which *R. rubra* cells were exposed to PEF as well as optimization of PEF exposure time resulted in over two-fold increase of selenium accumulation in relation to the control culture with no PEF application.

4. Increase of selenium concentration in the medium caused yeast cell atrophy. About three-fold increase of dead yeast cells was recorded within the range of applied selenium concentrations.

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