

SUMMARY

Bioconversion of toxic substances is the current issue of modern biotechnology, toxicology, microbiology, and practical enzymology. Among these dangerous compounds, there are industrial products of human activity (xenobiotics) and toxic metabolites produced by microorganisms during the biotechnological processes.

Chromate (xenobiotic) and D-lactate (a natural metabolite) are examples of such substances.

In the present study, it was investigated the potential use of unconventional, thermotolerant, methylotrophic yeast *Hansenula polymorpha*, as a source of flavoproteins: flavocytochrome b_2 and D-lactate dehydrogenase, to detoxify D - lactate and chromate. These enzymes are characterized by an absolute stereospecificity toward substrate (L- or D-lactate, respectively), but no selectivity to the electron acceptors *in vitro*.

As a source of the enzyme D-lactate dehydrogenase, yeast *H. polymorpha* can be used to specifically decompose D-lactate, a metabolite, which is potentially harmful to people with increased risk. Recombinant yeast, overexpressing this enzyme, can serve as a cheap biocatalyst for construction of the cell bioreactors to biodegrade D-lactate. Using for this purpose the cells of the recombinant strain of *H. polymorpha* tr6 (live and permeabilized, lyophilized) with increased synthesis of D-lactate dehydrogenase and avoid of L-lactate oxidizing activity, allows oxidative detoxification of D-lactate with a yield of $9 \text{ mM} \cdot \text{h}^{-1}$.

D-lactate is also a precursor used in many chemical syntheses. Synthesis of this enantiomeric lactic acid in pure form is rather expensive. The solution of this problem would be the enzymatic method for stereospecific degradation of L-enantiomer from the racemate. Using purified flavocytochrome b_2 from the yeast *H. polymorpha* allowed obtain D-lactate from the racemate by oxidation of L-lactate, which is a specific substrate for the exploited enzyme.

Because of the intramitochondrial localization of both enzymes, using these enzymes in a pure form is difficult. For this reason, development of alternative overexpression systems, as well as protein engineering approaches to facilitate isolation and purification of the enzymes is still important. To solve this problem, in the work there were used eukaryotic and prokaryotic expression systems for the synthesis of the modified enzymes.

A fusion protein, consisting of protein yEGFP, D-lactate dehydrogenase from the yeast *H. polymorpha* and (His)₆-tag, was expressed in the yeast *S. cerevisiae*. The fusion protein showed fluorescence in the recombinant cells, but no enzymatic activity, characteristic for D - lactate dehydrogenase.

By contrast, eukaryotic secretory system, based on the yeast *Kluyveromyces lactis*, for the expression of D-lactate dehydrogenase derived from *H. polymorpha*, results in the secretion of the truncated and inactive form of the enzyme.

Using a prokaryotic expression system, based on the *E. coli* and plasmid vector of pET32a series for IPTG-induced expression of the yeast ORF *HpCYB2*, encoding flavocytochrome *b*₂, results in the synthesis of the enzyme in the form of inclusion bodies. The fusion protein, consisting of several tags - thioredoxin (Trx), (His)₆-tag, S-tag with flavocytochrome *b*₂ retains enzymatic activity (16 J·mg⁻¹) after solubilization of inclusion bodies in the presence of 0.3% N-lauroylsarcosine.

For microbial bioremediation of chromate, recombinant yeast *H. polymorpha* tr1, overexpressing a mitochondrial flavoprotein, flavocytochrome *b*₂ was used. The enzyme contributes to a higher bioremediation activity of the recombinant yeast cells, compared to the wild type cells. This strain is able for efficient (100%) reductive bioremediation of 1 mM chromate. Using the pure enzyme, it has been shown that Cr(VI) can directly react with a reduced form of flavocytochrome *b*₂, due to the non-specificity *in vitro* toward electron acceptor, resulting in formation of Cr(III) species. Among several tested redox-active dyes, the best mediator activity belongs to dichlorophenolindophenol, which significantly contributes to an increased chromate bioremediation activity of a purified form of the enzyme, as well as of *H. polymorpha* yeast cells (live or in permeabilized, lyophilized form).

It was also shown that the yeast *H. polymorpha* tr1 has the extracellular capacity to reduce toxic chromate and to chelate chromium(III), resulting in the formation of Cr(III) biocomplexes in the culture medium. The physico-chemical analysis shows that isolated biocomplexes Cr(III) consist of at least two components, including protein. The complexes have strong absorbance peaks at 275 and 325 nm, and weaker - at 580 and 975 nm. The isolated Cr(III) biocomplexes are also able for a green fluorescence. They reveal an antioxidant activity (9,9 ÷ 21,9 J · g⁻¹ Cr) of chromium) in the tests based on using ABTS and iron(III).

The isolated Cr(III) bio-complexes show a positive biological activity in physiological studies using rats as the test model: induce a decrease of blood glucose level without any toxic effect (approved by ALT and AST transaminases' assay test).