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The use of biotransformation for antioxidants synthesis

Summary

Biotransformation processes based on the use of microorganisms as biocatalysts are an alternative to chemical synthesis of the useful compounds. Methods with whole-cell biocatalysts, due to the low acquisition cost and greater enzymatic stability, in contrast to isolated enzymes, are commonly used both in the process of obtaining goods by fermentation and for biotransformation. Catalytic potential of microorganisms used in bioconversion of xenobiotics, makes biocatalysis a competitive tool in the synthesis of practical compounds.

Knowledge about the adverse effects of free radicals on a living organism, requires a constant search for substances that are able to inhibit their harmful effects. Such compounds are antioxidants, used in the treatment and prevention of many diseases. That leads to the constant search for new compounds with this activity and new methods of obtaining them - alternative to the processes that are harmful to the environment. The aim of this work was to use fungi for the synthesis of polyphenolic compounds with antioxidant activity by hydroxylation of the cheap substrate - phenylethanol.

The following filamentous fungi were used in presented work: *Aspergillus niger*, *Rhizopus oryzae*, *Beauveria brongniartii* and *Beauveria bassiana*. Biotransformation of 2-phenylethanol with participation of *Aspergillus niger* KKP 2301, *Rhizopus oryzae* DSM 1185, *Beauveria bassiana* DSM 875, led to the synthesis of the antioxidants: hydroxytyrosol, tyrosol and 4-hydroxyphenylacetic acid and a valuable chiral product of pharmaceutical significance: 1-phenylethane-1,2-diol (in the case of *A. niger* as pure isomer (*S*)). The use of *Beauveria brongniartii* (DSM 6651) resulted in degradation of xenobiotic (2-phenylethanol), which indicates that this microorganism, after further confirmatory studies, could be used in

bioremediation processes. Experiments differed in the type and form of the biocatalyst (free and immobilized mycelium, spore suspension), process conditions and the type of reactor (250 mL flask, flow bioreactor, batch reactor- New Brunswick Scientific BioFlo). Depending on the approach, different efficiency and products (mixture or single product) were obtained.

Studies with *A. niger* began with the application of its resting form - spore suspension. Results indicated the synthesis of one of the antioxidants- hydroxytyrosol. In order to find conditions for the effective bioconversion, the biotransformation medium, substrate concentration, and time of process were manipulated, as well as spores activity was modified (temperature shock, preincubation without substrate). Procedure with biotransformation medium supplemented with glucose (13 mM) proved to be the most effective in the synthesis of hydroxytyrosol (1.4 mg/50 mL starting from 15 mg/50 mL of substrate, 7.4% efficiency), compared to the biotransformation carried out only in the water (0.27 mg/50 mL starting from 30 mg/50 mL of substrate, 0.7%) or in the medium with lower glucose concentration (4 mM) (0.9 mg/50 mL starting from 15 mg/50 mL of substrate, 4.7%) or in Czapek-Dox medium (0.7 mg/50 mL starting from 30 mg/50 mL of substrate, 1.8%). Modifications of the spores, in order to initiate the germination process and stimulate their enzymatic activity, relying on spores incubation under thermal shock conditions (0.5 mg/50 mL hydroxytyrosol starting from 30 mg/50 mL of substrate, 1.3% efficiency), or preincubation in medium without substrate (1.2 mg/50 mL hydroxytyrosol starting from 15 mg/50 mL of substrate, 6.2%) did not improve bioconversion.

In next experiments, the catalytic activity of vegetative form of *A. niger* (mycelium) was studied. Experiments with mycelium of *A. niger* allowed to obtain a mixture of two antioxidants: 4-hydroxyphenylacetic acid and hydroxytyrosol. Initial studies using free mycelium of *A. niger* resulted in 0.7 mg/50 mL of a mixture of 4-hydroxyphenylacetic acid and hydroxytyrosol starting from 15 mg/50 mL of substrate (3.7% efficiency). Cell sensitivity

to xenobiotic (2-phenylethanol) was also studied and the increasing of its concentration to 30 mg/50 mL resulted in lower efficiency (0.35 mg/50 mL, 0.9% efficiency). In the next tests, cultivation medium was supplemented with the substrate (2-phenylethanol), in order to increase the synthesis / activity of enzymes involved in oxidation processes and that resulted in formation of a single compound: 4-hydroxyphenylacetic acid (7 mg/50 mL starting from 30 mg/50 mL of substrate, 18% efficiency). To increase the stability of the biocatalyst and efficiency of the bioconversion process, in further studies the immobilized (polyurethane foams) mycelium of *A. niger* was used. This approach increased antioxidant synthesis compared to free cells (0.35 mg/50 mL, 0.9% efficiency) under the same reaction conditions (30 mg/50 mL of substrate): 1.3 mg/50 mL of a mixture of 4-hydroxyphenylacetic acid and hydroxytyrosol (3.5% efficiency). Results showed that the immobilized biocatalyst is more stable to external factors i.e. process conditions, xenobiotics, which can cause loss of biocatalyst activity, and that is the value of performed experiments. To increase the scale of the process, it was decided to use two reactors: designed flow bioreactor and batch bioreactor (New Brunswick Scientific, BioFlo Model C32, 1.3 L). Experiments conducted in a flow bioreactor packed with immobilized cells of *A. niger* (polyurethane foams 1060-1600 μm) allowed to increase the synthesis of products and obtained 11.4 mg/150 mL of a mixture of antioxidants (4-hydroxyphenylacetic acid and hydroxytyrosol) starting from 90 mg/150 mL of substrate (10% efficiency). Another bioconversion of 2-phenylethanol carried out by free mycelium of *A. niger* in an aerated batch bioreactor (New Brunswick Scientific, BioFlo Model C32) led to the synthesis of a completely different product than in other experiments - (*S*)-1-phenylethane-1,2-diol (335 mg/750 mL starting from 458 mg/750 mL of substrate, 65% efficiency) - a compound used in the synthesis of drugs (fluoxetine and norfluoxetine). A semi-preparative procedure of the preparation of this compound was covered by the patent application: *„Method of obtaining (S)-1-phenylethane-1,2-diol"* no. P.429923 of 15.05.2019.

Rhizopus oryzae was another biocatalyst used to transform 2-phenylethanol. Conducted biotransformations led to the synthesis of tyrosol and mainly chiral (unspecified configuration) 1-phenylethane-1,2-diol. According to the process conditions, a mixture of products with different concentration was obtained. First, the impact of substrate concentration on the process effectiveness was investigated. In experiments with free mycelium of *R. oryzae*, after increased the substrate to 30 mg/50 mL, a greater amount of a mixture of products was obtained (1.2 mg/50 mL of tyrosol and 1-phenylethane-1,2-diol, 3.5 % efficiency), maintaining the efficiency at a slightly lower level, as when using less amount of substrate (15 mg/50 mL) (0.7 mg/50 mL products, 4% efficiency). Further increasing of the substrate concentration to 60 mg/50 mL resulted in formation much more products, but reduced the process efficiency (1.8 mg/50 mL of products, 2.6% efficiency), which could be due to inhibition of enzymatic activity by too high concentration of the substrate. Modification relying on preincubation of *R. oryzae* cells under nutrient deficit conditions for 24 hours in order to direct metabolism on the use of substrate when the availability of nutrients is limited, indicated the formation only trace amounts of products and a large amount of unreacted substrate was observed. Also for this biocatalyst, in order to increase its stability and effectiveness of the bioconversion process, in further experiments, its immobilized (polyurethane foams) mycelium was used. This approach allowed to more effective bioconversion of the substrate to tyrosol and 1-phenylethane-1,2-diol (1.9 mg/50 mL of a mixture of products starting from 30 mg/50 mL of substrate, 5.5% efficiency), compared to free cells of this microorganism. Also in this case, in order to increase the scale of the process, it was decided to use a bioreactor with a flow system, unfortunately this attempt was unsuccessful and further research with this biocatalyst was not continued.

The biotransformation of 2-phenylethanol catalyzed by another biocatalyst - *Beauveria bassiana*, led to the synthesis of only trace amounts of tyrosol and 1-phenylethane-1,2-diol, therefore after initial testing, further experiments were not continued.

The use of *Beauveria brongniartii* as a biocatalyst led to the substrate degradation (2-phenylethanol). Substrate incubated with that microorganism was progressive degraded. The total mineralization of the compound was obtained after only one day of bioconversion. However, after an hour, almost half of 2-phenylethanol was lost, and after 5 hours only 16.5% of the compound remained.