

Bioconversion of Daidzein to 3'-ODI by *Bacillus Subtilis* Spore Displayed Tyrosinase

Afrouzossadat Hosseini Abari *

Assistant Professor, Department of Biology, Faculty of Sciences, University of Isfahan, Isfahan, Iran, af.hosseini@sci.ui.ac.ir

Abstract

Introduction: *Bacillus subtilis* spore surface display technique has long been used to display antigens and enzymes for medical and industrial proposes. In this technique, the enzyme is genetically immobilized on the spore surface and one of the capabilities of spore displayed enzyme is the reusability. In this study, spore displayed tyrosinase was used for the bioconversion of soybean extracted daidzein to more hydroxylated, anticancer compound, 3'-ODI.

Materials and methods: *Bacillus subtilis* DB104 (pSDJH-cotE-tyr) which was constructed in our previous study was used as an enzyme source. The reaction was done in 37°C for 1 hour. To detect the product of the reaction, *high-performance liquid chromatography* was used.

Results: The results revealed that 1mM of daidzein was converted to about 1mM 3'-ODI during 60 min by 4×10^8 spores. The retained activity of the spore displayed tyrosinase was also detected about 58% after three times usage.

Discussion and conclusion: Spore surface displayed enzymes have created a new way to improve enzyme stability and reusability. Our results showed that active tyrosinase on the surface of the spores has the potential to be used in industrial conditions to produce more hydroxylated isoflavones.

Key words: Bioconversion, Tyrosinase, Spore Surface Display, Daidzein, 3'-ODI

*Corresponding Author

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Introduction

Daidzein (4', 7-dihydroxyisoflavone) is a natural isoflavone and a bioactive compound isolated from soybean (1). The protective effects of daidzein against various diseases such as breast and prostate cancer, diabetes and cardiovascular diseases have been investigated. The pharmaceutical mechanism of daidzein is because of the structural similarities to mammalian estrogens. Daidzein has antiproliferative effects and it is an antiangiogenesis compound which plays a remarkable role in the inhibition of the migration of cancer cells to prevent metastasis (2). Phenolic compounds, especially ortho-dihydroxylated phenolic compounds, have the potential to prevent cancer (3). 3'-ODI (7, 3', 4'-trihydroxyisoflavone) is one of the ortho-dihydroxylated isoflavones which is not present naturally (4). Anti-oxidative and anti-cancer effects of 3'-ODI have been investigated in several researches (5, 6). CytP450 and tyrosinase were used for the bioconversion of daidzein, to 7, 3', 4'-trihydroxyisoflavone (3'-ODI) to increase the antioxidant effects (7). Cyt P450 can selectively introduce atomic oxygen into non-activated carbon-hydrogen bonds; however, the utilization of Cyt P450 in industry, because of their low efficiencies, low yields, and necessity for cofactor has several problems (8). Tyrosinase, a type III copper containing monooxygenase, is an applicable enzyme in cosmetic, drug development and pharmaceutical industries and also it has been used in variety of biotechnological applications such as bioremediation, biosensor, biomedicine, and biocatalysis (9, 10).

The most important reaction organized by tyrosinase is the bioconversion of L-tyrosine to L-DOPA (3,4-di hydroxy phenylalanine) which is a drug for Parkinson disease (11). Unlike the Cyt P450, tyrosinase is simply used in industry

with high efficiencies and outputs (8). Due to the high utilization of the enzyme, it is necessary to immobilize the enzyme on appropriate support for the efficient usage (12). Spore surface display technique is a developmental genetic engineering method to express and immobilize proteins on the surface of *Bacillus subtilis* spores (13). According to the spore resistance to heat shocks, pH changes and UV radiation, genetically immobilized protein can be used in such extreme conditions. The spore surface display technique has been used to develop vaccines and biosensors successfully (14, 15).

In this study, we used the spore displayed tyrosinase as a stable and reusable enzyme for synthesis of 3'-ODI from daidzein.

Materials and Methods

Bacterial Strain and Culture Condition: *Bacillus subtilis* DB104 (pSDJH-cotE-tyr) which was constructed in our previous study was used as an enzyme source. To collect the spores, vegetative cells were inoculated into Difco sporulation medium (DSM) and cultivated for 24h at 37°C in a shaker incubator (200rpm). This medium contained 0.8% w/v nutrient broth, 0.1% KCl, 0.025% MgSO₄·7H₂O, 1mM Ca(NO₃)₂, 0.01mM MnCl₂, and 0.01mM FeSO₄ in 1L distilled water, pH 7. The spores were then purified using renografin (sodium diatrizoate, S-4506, Sigma) gradient method (16).

Bioconversion Assay of Daidzein to 3'-ODI: The purified spores (4×10^8) were used as an enzyme source for the bioconversion of daidzein to 3'-ODI. The reaction of 1mM daidzein (**D7802** Sigma) was done in 50mM tris- HCl buffer pH 8 with 5mM ascorbic acid and 10µM CuSO₄ in 37°C for 1 hour. HPLC (YL9100, Young Lin instrument) was used to monitor the concentrations of substrates and products. The experiments were repeated in triplicate.

Reusability of the Enzyme: To assay the enzyme reusability, the spores were washed three times with Tris-HCl buffer (pH 8), and the formation of the product was monitored by HPLC again. The HPLC graphs were analyzed by ChromNAV 2.0 HPLC software.

Results

Bioconversion of Daidzein to 3'-ODI by Spore Displayed Tyrosinase: Bioconversion of daidzein to 3'-ODI by spore displayed tyrosinase was monitored by HPLC. As it is

shown in figure 1, after 60 min, almost all content of daidzein were converted to 3'-ODI. Tyrosinase transferred one hydroxyl group on daidzein to create 3'-ODI (Fig. 1).

Reusability of Spore Displayed Tyrosinase: The reusability of tyrosinase-displaying spores was also investigated by washing them repeatedly with a buffer. Retained activity of immobilized tyrosinase was about 58% after three times washing with Tris-HCl buffer, pH 8 (Fig. 2).

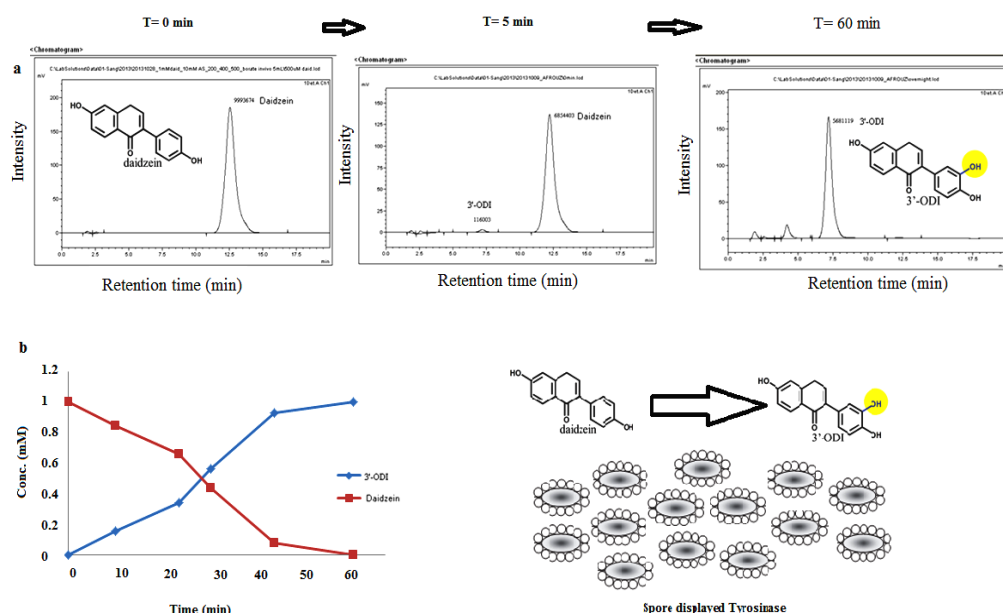


Fig. 1- HPLC result of the spore displayed tyrosinase reaction for producing 3'-ODI. (a) The peaks at retention time 12.5 min and 7.5 min are related to daidzein and 3'-ODI, respectively. (b) Time profile of daidzein consumption and 3'-ODI production.

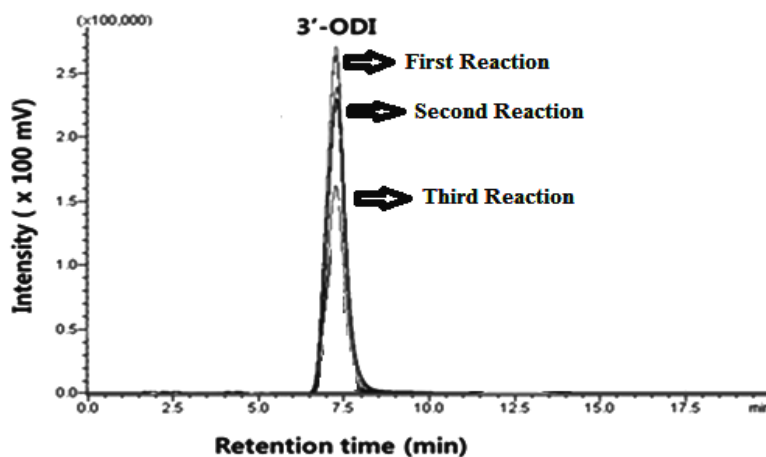


Fig. 2- HPLC result of the reusability of spore displayed tyrosinase after three times washing

Discussion and Conclusion

Daidzein, a natural bioactive compound in soybean was easily converted to 3'-ODI by spore displayed tyrosinase during 1 hour. 3'-ODI is not present naturally and because of the extra hydroxyl group, it is more active than daidzein (4). Due to the spore resistance to harsh conditions such as low or high temperatures, acidic or alkali pH, the spore displayed enzymes also are very active in various industrial conditions (15). It shows the merit of the spore displayed tyrosinase than the pure tyrosinase. Recently, enzyme immobilization has been considered as an essential tool to improve stability and reusability of industrial enzymes (17). Our results also demonstrated 58% retained activity after three times usage. In our previous work, tyrosinase from *Bacillus megaterium* was displayed on the surface of *Bacillus subtilis* spores by CotE as an anchor protein. Our results revealed that the spore displayed tyrosinase can be active during 15 days maintenance in room temperature; however, the free enzyme was active for a few hours. The spore displayed tyrosinase was also active during 6 times washing steps (12). All the results showed that the spore displayed tyrosinase is a good candidate for being used in industry and bioconversion of natural useful compounds. Our previous research also showed 62% retained activity when spore displayed tyrosinase was used to produce dopachrome from L- Tyrosine after six times washing (12). In another research, Tsai and Meyer observed 83% retained activity of spore displayed esterase (18). The need to produce strong enzymes have been made the spore display technique as a suitable novel technique for immobilization.

In conclusion, the spore displayed tyrosinase was used as an efficient tool for bioconversion of daidzein to 3'-ODI. The genetically immobilized enzyme showed reusability potential for several times. It

suggests that the spore displayed tyrosinase has an interested potential for industrial usage.

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