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Ilef Limen Ben Amor, Nidhal Salem, Emmanuel Guedon, Jean-Marc Engasser, Leila Chekir-Ghedira, et al.. Preliminary investigation of naringenin hydroxylation with recombinant *E. coli* expressing plant flavonoid hydroxylation gene. *Natural Product Communications*, 2010, 5 (5), pp.777-782. 10.1177/1934578X1000500520 . hal-00799073

HAL Id: hal-00799073

<https://hal.science/hal-00799073v1>

Submitted on 3 Apr 2020

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Preliminary Investigation of Naringenin Hydroxylation with Recombinant *E. coli* Expressing Plant Flavonoid Hydroxylation Gene

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Received: November 11th, 2009; Accepted: March 8th, 2010

Flavonoid hydroxylation is one way to increase the biological activities of these molecules and the number of hydroxyl groups needed for polymerization, esterification, alkylation, glycosylation and acylation reactions. These reactions have been suggested as a promising route to enhance flavonoid solubility and stability. In our preliminary study we hydroxylated naringenin (the first flavonoid core synthesized in plants) with recombinant *E. coli* harboring flavanone 3 hydroxylase (F3H). We demonstrated that recombinant *E. coli* harboring the F3H from *Petroselinum crispum*, can convert naringenin to dihydrokaempferol. The whole cell hydroxylase activity was often influenced by the stability of the plasmid harboring the cloned gene and the biomass yield. When the composition of the growth media became richer the amount of formed product decreased about twofold; the naringenin bioconversion yield in LB media was 70% and decreased to 33% in TB. However, the enrichment of culture media increased the biomass yield nearly threefold in LB media, only 0.5 g/L of bacteria was formed, but in TB there was 1.6 g/L. Thus, LB constitutes the best medium for naringenin bioconversion using the recombinant *E. coli* harboring the F3H; this allows for maximum bioconversion yield and plasmid stability when compared with the fourth tested culture medium. Consequently, *E. coli* harboring F3H from *Petroselinum crispum* can be used to produce flavonoids hydroxylated in position 3 that can serve in additional reactions like polymerization, glycosylation, and acylation,

Keywords: *Escherichia coli*, flavonoid hydroxylation, flavanone 3 hydroxylase.

Flavonoids (aglycon, glycosylated) are widely used in pharmaceutical, cosmetic and food preparations. They have several physico-chemical properties and biological activities, but are characterized by low solubility and stability. In order to take advantage of the beneficial properties, polymerization, esterification, alkylation, glycosylation and acylation of these molecules has been suggested by several authors as a promising route. However, these reactions need free hydroxyl groups in aglycons or in glycosides to react with functional groups (for example, ester, alkyl, glycosyl). In plants, flavonoid hydroxylation can be assured by three major flavonoid hydroxylases: flavonoid 3'5'hydroxylase, flavonoid 3' hydroxylase, and flavanone 3 hydroxylase. To imitate plant hydroxylation, the hydroxylation

reaction can be assured with enzymes purified directly from either plants or from recombinant microorganisms expressing flavonoid hydroxylase genes. This alternative is facilitated by good knowledge of plant genes implicated in flavonoid synthesis.

Due to the important biological properties of 3 and 3'hydroxylated flavonoids [1-3], attempts to produce them have been proposed and investigated. The main works, presented in Table 1, have used purified recombinant flavonoid 3'hydroxylase (F3'H) and flavanone 3 hydroxylase (F3H) [4-6]. These two enzymes are, respectively, a mono and a di-oxygenase, and their use needs cofactor supplementation. F3H, an oxoglutarate dependent dioxygenase, needs also iron, oxygen and ascorbate to be fully active *in vitro*, whereas

Table 1: Different expression systems and purification procedures used to evaluate recombinant F3H activity.

F3H origin, expression host, purification procedures	References
- <i>Malus domestica</i> (Y/TPE) Km=10 μ M, V _{max} =117.5 μ kat/kg - <i>Pyrus communis</i> (Y/TPE)	[17]
<i>Callistephus chinensis</i> (Y/TPE) <i>Daucus carota</i> (Y/TPE) <i>Dendranthema grandiflorum</i> (Y/TPE) <i>Ipomoea batatas</i> (Y/TPE) <i>Matthiola incana</i> (Y/TPE) <i>Petunia hybrida</i> (Y/TPE)	[18]
<i>Petunia hybrida</i> (Y/PE ¹)	[19]
<i>Dianthus caryophyllus</i> (Ec/PE ²) <i>Callistephus chinensis</i> (Ec/PE ²)	[5]
<i>Petroselinum crispum</i> (Y/TPE)	[20, 21]
<i>Petunia hybrida</i> (Ec/CE) (97 mg of proteins from 2 g of cell pellet with 100% of activity recovery) Specific activity 1.7 mkat/kg <i>Petunia hybrida</i> (Ec/PE ²) (3.5 mg of proteins from 2 g of cell pellet with 19.7 % of activity recovery) Specific activity 9.3 mkat/kg <i>Petunia hybrida</i> (Ec/PE ⁴) (0.25 mg of proteins from 2 g of cell pellet with 4.7% of activity recovery) Specific activity 31.8 mkat/kg	[22 a, b, c]

Y: yeast; Ec: *Escherichia coli*; TPE: total protein extract; PE¹: purified enzyme from amylose resin column chromatography; PE²: purified enzyme from Fractogel-EMD DEAE-650 (S), Fractogel-EMD TMAE-650 (S) anionic exchangers and hydroxyapatite columns; PE³: purified enzyme from EMD BioSEC (S) PE⁴: purified enzyme from EMD DEAE 650 (S).

F3H, a cytochrome P450 monooxygenase, requires P450 reductase as a redox partner for proton transfer from NADPH to cytochrome P450. For these reasons the use of mono and di-oxygenase in an *in vivo* system is more attractive compared with the use of isolated enzymes. In fact, in the *in vivo* system, the host strain synthesizes and regenerates cofactors such as NADPH. Therefore, the aim of this work was to develop a single enzymatic step: hydroxylation of naringenin (which constitutes the first and less substituted flavonoid core formed in the phenylpropanoid synthesis pathway of flavonoids). To reach this goal, in a first step, the interest of using whole cells for naringenin hydroxylation with recombinant *Escherichia coli* expressing F3H of *Petroselinum crispum* was demonstrated by comparing the activity of this system with that obtained from the purified enzymes. In a second step, different factors affecting this reaction, such as biomass concentration, medium composition and stability of the plasmid harboring the F3H of *Petroselinum crispum* was investigated.

In general, in an *in vivo* system, it is important that high cell densities are achieved to increase flavonoid production using a recombinant microorganism expressing an heterologous enzyme. However, for recombinant strains, the operating conditions leading to high cell concentration can affect the plasmid stability. So, the effect of different media compositions on growth rate, cell yield, plasmid stability and naringenin hydroxylation were investigated in parallel.

To compare the performances of the *in vivo* and *in vitro* systems, both whole cells and total protein extract (supernatant) were used, in parallel, to hydroxylate naringenin.

F3H belongs to the class of Fe(II)/2-oxoglutarate-dependent dioxygenases, which are a catalytically diverse family of nonheme iron enzymes that oxidize

their primary substrates (naringenin) and at the same time decompose the 2-oxoglutarate co-substrate to form succinate and CO₂.

In this study the hydroxylation activity of the F3H present in the total protein extract (supernatant) obtained from the recombinant *E. coli* harboring either the empty vector (pET-15b) or the recombinant plasmid (pET-15b-F3H of *Petroselinum crispum*) were tested both with and without the Fe(II)/2-oxoglutarate cofactors. Only protein extract prepared from recombinant *E. coli* harboring the F3H from *P. crispum* and tested in the presence of Fe(II) and 2-oxoglutarate, totally converts naringenin to dihydrokaempferol (100%). Thus, it appears that the F3H hydroxylation reaction is strictly dependent on the presence of Fe(II) and 2-oxoglutarate in the reaction medium. Despite the interest in the hydroxylation potential of F3H enzymes in flavonoid transforming processes, their biotechnological use is limited due to their complexity, instability and low recovery yield during purification processes (Table 1).

In addition, the major hurdle to the implementation of dioxygenase (i.e. F3H) catalysis for commercial synthesis is the requirement of stoichiometric amounts of cofactors such as oxoglutarate and Fe(II). However, in whole cells, oxoglutarate can be regenerated *in situ* during biotransformation reactions. This justified our choice to use the hydroxylase activity of cells transformed with the recombinant plasmid (pET-15b-F3H of *Petroselinum crispum*).

The whole cells of *E. coli* containing the F3H gene were incubated with naringenin as substrate. After 5 h of incubation at 37°C, HPLC analysis of the reaction medium showed that 50 mg of naringenin was consumed and a new product, expected to be dihydrokaempferol, was produced. The identification of the naringenin hydroxylation product was realized by a

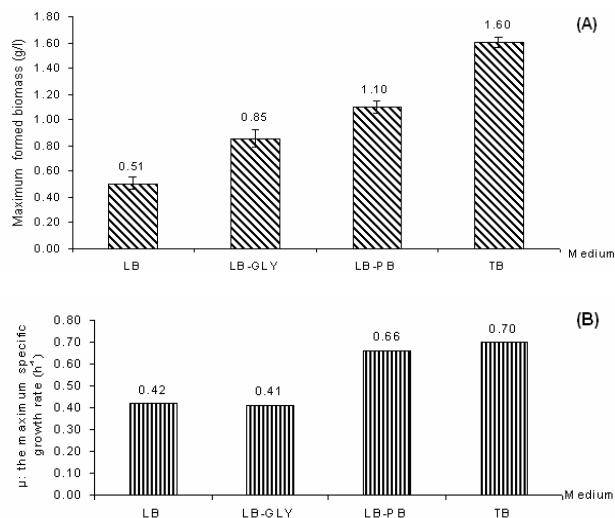


Figure 1: Effect of culture media (A): on maximum biomass formation over a time course of 20 h of culture. (B): on maximum specific growth rate (h^{-1}). LB: Luria Bertani medium, LB-GLY: Luria Bertani medium supplied with glycerol, LB-PB: Luria Bertani medium supplied with phosphate buffer, TB: Terrific Broth medium.

method involving HPLC coupled to mass spectrometry. In order to achieve maximum bioconversion yields, the recombinant *E. coli* should be grown to high cell densities. In a shake flask experiment, all the required nutrients can be either added initially into the basal broth or increased sequentially by adding sources of carbon, phosphorus, sulfur and trace elements [7].

To evaluate the influence of nutrient concentration increases and the nutrient supplementations on growth rate, shake-flask experiments were conducted. In addition to the popular complex LB broth, we also tested other media commonly used for microbial cultures: LB supplied with either glycerol (LB-Gly) or phosphate buffer (LB-PB), and TB (contains glycerol, phosphate buffer and important concentrations of yeast extract and tryptone). As shown in Fig. 1, LB-PB and TB permit increases of cell concentration, respectively of 2 (1.1 g/L) and 3 (1.6 g/L) fold compared with that obtained with LB (0.51 g/L). When, glycerol was added to LB, it did not produce an important increase in cell concentrations (0.85 g/L). Comparison of media composition led to the conclusion that the supplementation of phosphate buffer (LB-PB and TB) and glycerol (LB-Gly and TB) to LB, or the increase of yeast extract and tryptone concentrations (TB) can lead to high cell densities. In addition, it is notable that the supplementation of LB medium with phosphate buffer (LB-PB), and high concentration of yeast extract and tryptone increase the specific growth rate of the recombinant strain. In fact, we observed that TB gave the highest growth rate ($\mu = 0.7 \text{ h}^{-1}$), compared with the other growth media. This was followed by LB-PB, which exhibited a growth rate of 0.66 h^{-1} , and finally,

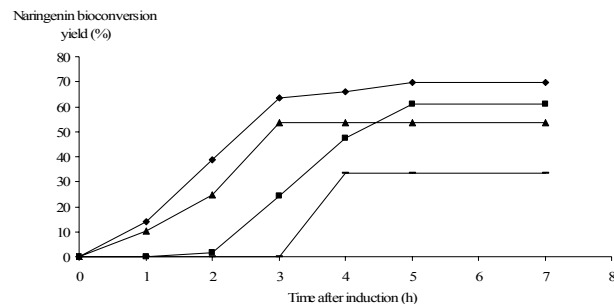


Figure 2: Effect of culture media on the yield of naringenin bioconversion to dihydrokaempferol with recombinant *E. coli* expressing F3H of *P. crispum*. (♦) LB medium, (■) LB-GLY, (▲) LB-PB, (×) TB. LB: Luria Bertani medium, LB-GLY: Luria Bertani medium supplied with glycerol, LB-PB: Luria Bertani medium supplied with phosphate buffer, TB: Terrific Broth medium

LB and LB-Gly, with respectively 0.42 and 0.41 h^{-1} growth rates.

The culture media have an impact on growth, biomass yield and also on bioconversion kinetics. As shown in Fig. 2, LB, LB-Gly and LB-PB media permit the production of the highest concentration of dihydrokaempferol from 50 mg/L of naringenin. Five hours after induction of the recombinant protein synthesis with IPTG, naringenin bioconversion yields were, respectively 70, 61 and 53 %. However, in TB, the conversion of naringenin was reduced by half (33%) when compared with the bioconversion in LB (70%).

It is notable that the four tested media did not give the same naringenin bioconversion profiles. Three hours after induction with IPTG, respectively 64, 53, and 24 % of naringenin present in LB, LB-PB and LB-Gly were converted to dihydrokaempferol. However, there was no naringenin bioconversion during the first three hours after induction of protein expression in TB; dihydrokaempferol was detected only 4 hours after induction with IPTG.

Surprisingly, in TB the bioconversion kinetic is speedier than in all the other media (33 mg h^{-1}). In LB, LB-Gly and LB-PB, the bioconversion speeds were about 20, 20.1 and 15.7 mg h^{-1} .

The impact of media composition on recombinant cell stability revealed that LB medium permitted the highest plasmid stability during the conversion reaction, as seen in Table 2 (97 % of cells contain the recombinant plasmid). During culture, the TB medium maintained plasmid stability at nearly 79 %. However, LB-PB increased the probability of plasmid free cell emergence with about 46 % of the cells in the culture not harboring the plasmid, and thus not expressing the recombinant F3H activity. Addition of glycerol to LB, as a supplementary carbon source, did not modify cell

Table 2: Effect of culture media on plasmid containing cell %.

Medium	T = 0 h	T = 6 h	T = 12 h	T = 18 h	T = 24h
LB	96.12	93.75	94.57	94.79	96.77
LB-PB	96.12	72.53	64.63	59.45	54.41
LB-Glycerol	96.12	81.34	67.59	64.87	63.21
TB	96.12	81.72	80.35	79.65	78.89

growth ($\mu = 0.41 \text{ h}^{-1}$), whereas it decreased the plasmid stability to 63 % compared with 97% in LB medium.

Selective hydroxylation is an important step for flavonoid functionalization and, therefore, in industrial manufacturing of valuable flavonoids. Chemical synthesis is usually used towards this end, but suffers from drawbacks of simultaneous formation of diverse by-products and low production yield. Biotransformation, however, is now showing an increasing significance in this field because of high regio- and stereo-specificity. In some cases, a series of chemical synthetic reactions could be replaced with one single step of high-yield biocatalysis. These kinds of processes are today run at a scale of thousands of tons per year [8, 9]. Whole cells and purified enzymes are both used as biocatalysts, but the former presents the advantages of simple and convenient manipulation and low costs, while enzyme purification is tedious and expensive. Moreover, the purification of enzymes could lead to significant loss of activity, since cofactors are usually required for enzymes like hydroxylases. Previous works have reported the possibility of naringenin hydroxylation with purified recombinant flavonoid hydroxylase (Table 1). In the present study, we demonstrated that recombinant *E. coli* expressing F3H of *P. crispum* can hydroxylate carbon 3 of naringenin to produce dihydrokaempferol.

In order to achieve maximum productivity, it is important to explore the bacterial culture density limits. The only obstacle to unlimited growth of bacteria in liquid culture is based on the assumption that bacteria continue to divide as long as their nutritional requirements are met and no toxic or inhibitory factors are excreted into the medium by the bacterial community throughout the course of growth. In shake-flask experiments, we evaluated the influence of media culture enrichment on growth rate, biomass and bioconversion yields and plasmid stability. We have found that, in TB medium, the maximum biomass and growth rate are achieved. This is in accordance with result described previously [10, 11].

The enrichment of culture media with glycerol (LB-Gly), phosphate (LB-PB), or glycerol, phosphate and nitrogen (TB), is correlated with an increase of biomass yields and growth rates, but with a decrease of plasmid

stability, as well as a decrease in dihydrokaempferol production. In our investigation, we demonstrated that LB can be used as a complex medium, in shake flask fermentation, as long as plasmid stability is maintained during the conversion reaction and thus F3H activity expressed as dihydrokaempferol formation is preserved. However, the other culture media can be used if the culture process is optimized, for example by gradual addition of nutrients (feed batch culture).

Our work represents the first report of using whole cell activity to achieve a single flavonoid hydroxylation step. In the present preliminary work, we produced 35 mg/L of dihydrokaempferol from 50 mg/L of naringenin. This work open up alternative routes for the production of additional hydroxylated flavonoid structures from other non-hydroxylated flavonoid precursors using recombinant *E. coli* cells. Indeed, flavonoid hydroxylation at position 3 enhances the antioxidant, antiradical, anticancer and antitumor properties [12]. This result opens the door to further extension of the flavonoid pathway in *E. coli* with the many known biosynthetic genes to reproduce pathways for the synthesis of significant quantities of other important plant-derived flavonoids, such as flavonols, flavones, isoflavonoids, condensed tannins, and anthocyanin pigments. *In vivo* feeding with exogenous non-costly precursor compounds will provide an elegant method to produce valuable flavonoids when the actual extraction yields from plants do not exceed 1 %. This technique will provide additional hydroxyl groups in flavonoid structures and sufficient quantities of hydroxylated flavonoids needed for functionalization reactions like flavonoid glycosylation [13,14], acylation [13], methylation [13,15], and sulfation [13,16].

Experimental

Chemicals: Yeast extract, tryptone, peptone, agar, KH_2PO_4 , K_2HPO_4 , isopropyl- β -D-thiogalactopyranoside (IPTG), NaCl, naringenin, ampicillin, chloramphenicol and glycerol were purchased from Sigma-Aldrich [Sigma-Chemical, Co., St. Louis, (USA)]. Acetonitrile and ethanol were purchased from Fisher, (Loughborough, UK).

Plasmids and cell transformation: The *f3h* gene from *Petroselinum crispum* was generously given by Professor Stefan Martens (Institute of Pharmaceutical Biology, Philips University Marburg, Germany) in *E. coli* plasmid pET15b. *E. coli* BL21 DE3 Rosetta 2 (Laboratory collection) was used as host strain for the expression of the recombinant F3H. The bacterial cells were transformed by electroporation (Bio-Rad Gene Pulser electroporator, California, USA).

Media composition: Luria Bertani (LB) was constituted of 5 g/L yeast extract, 10 g/L tryptone and 10 g/L NaCl; the pH was adjusted to 7 with NaOH (10 N). The LB-phosphate buffer (LB-P) was composed of LB supplied with 0.1 % phosphate buffer (KH₂PO₄ 23.15 g/L and K₂HPO₄ 125 g/L), and LB-glycerol (LB-Gly) contained LB supplied with 10 g/L of glycerol. Terrific Broth (TB) was the mix of 12 g/L tryptone, 24 g/L yeast extract, 0.1 % phosphate buffer and 10 g/L of glycerol.

Heterologous expression in E. coli and enzyme assay: F3H-pET15-b was used to transform *E. coli* Rosetta 2. The expression of *Petroselinum crispum* F3H was evaluated *in vitro*. For that purpose, the recombinant strain was cultured overnight at 37°C, with shaking, in 3 mL of LB medium supplemented with 100 µg/mL of ampicillin and 34 µg/mL of chloramphenicol (preinoculum). The following day, the preinoculum culture was used to seed 50 mL LB; the initial cell density was 25 10⁻³ g/L. The culture was grown at 37°C, until it reached a cell density of 0.2 g/L. Then, the inducer IPTG (final concentration: 0.1 mM) was added to the culture medium and incubation continued to allow recombinant protein synthesis. After 3 h of induction, the recombinant cells were harvested by centrifugation at 3500 x g, for 10 min at 4°C. The cell pellet was washed with Tris HCl buffer (0.1 M, pH 7.5) and treated with 1 mg/mL of lysosyme for 30 min at 4°C. After 2 min of sonication, the mixture was centrifuged at 3500 x g, for 30 min at 4°C [4]. The supernatant, containing the soluble recombinant F3H, was recovered to evaluate the recombinant enzyme ability to hydroxylate naringenin.

Naringenin hydroxylation with total cell extract: In a total volume of 750 µL, 1 mM naringenin, 250 µM 2-oxoglutarate, 100 µM FeSO₄, 5 mM Na-ascorbate and 100 µL of protein extract (supernatant) were incubated with shaking for 30 min at 37°C in open vials. The reaction was stopped with 250 µL of acetonitrile, and the presence of naringenin hydroxylation product was analyzed by HPLC.

Cultivation method: Bioconversion using whole cell *E. coli* activity was performed in shake flasks. Thirteen mL of each tested growth medium was inoculated with 1 mL of *E. coli* pre-culture. Antibiotics were added to a final concentration of 100 µg/mL of ampicillin and 34 µg/mL of chloramphenicol. Each culture was incubated at 37°C with shaking at 150 rpm. When the culture reached an exponentially growing phase (OD_{600nm} = 0.4), the inductor IPTG was added to a final concentration of 0.1 mM. One h later, naringenin was added to a final concentration of 50 mg/L. The naringenin conversion reaction was followed during 15 h. Every hour, 750 µL of the growing culture medium

was taken and mixed with 250 µL of acetonitrile to stop the hydroxylation reaction.

Analytical method: Cell density was measured by the dry cell weight method. For that, 10 mL of the sample was spun in a pre-weighed centrifuge tube, at 5000 rpm for 5 min. The cell pellet was washed with deionized water and dried at 100°C for 24 h. The tube was weighed along with the dried pellet and used to calculate the dry weight of the sample. Total cell concentration was determined by measuring the optical density at 600 nm (OD_{600nm}). One unit of OD_{600nm} was found to be equivalent to about 0.5 g/L dried cell weight.

Plasmid stability, as well as the percentage of plasmid-containing cells, was determined by the replica-plating technique. The percentage of plasmid stability was calculated as the ratio of the number of plasmid-containing cells forming the clones in selective plates (LB with ampicillin) to the total number of cells growing on non selective LB medium (including plasmid-free and plasmid-containing cells). Cell samples were diluted to obtain colony counts within the range 30-300 colony/plate. All plate counts were taken from the average of at least 3 replicates.

For HPLC analysis, a ThermoFinnigan (San Jose, CA, USA) HPLC apparatus equipped with a UV 6000 LP detector was used. The column was an Appolo C₁₈ (5µm, Ø 4.6 mm × 250 mm). For analytical scale, the mobile phase consisted of water and acetonitrile and was programmed as follows; 10% acetonitrile from 0 min to 12 min; from 12 min, a linear gradient was used starting from 40% acetonitrile rising to 60% at 17 min, followed by 8 min of 10% acetonitrile. The flow rate was 1 mL/min; detection wavelength was 296 nm; column temperature was 28°C.

Structure elucidation of the hydroxylation reaction product was performed by MS analysis. Liquid chromatography was performed with the same parameters as described for HPLC analysis. The MS was obtained using a ThermoElectron (ThermoFisher scientific, Waltham, MA) mass spectrometer in ESI mode. Scans were acquired in the negative ion mode between 50 and 300 *m/z*. The nebulizer and desolvation gas were ultrapure nitrogen. The MS parameters were as follows: capillary temperature, 300°C; sheath gas, 50 arbitrary units; auxiliary and sweep gas, 10 units.

Acknowledgments - The authors would like to thank the 'Ministère Tunisien de l'Enseignement Supérieur et de la Recherche Scientifique' and the 'Institut Français de Coopération' for the financial support of this study. Furthermore, authors would like to thank Professor

Stefan Martens (Institute of Pharmaceutical Biology, Philips University Marburg, Germany) for giving them

the plasmid pET15b harbouring *f3h* gene from *Petroselinum crispum*.

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