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Biotransformation of Naringenin to Eriodictyol by *Saccharomyces cerevisiae* Functionally Expressing Flavonoid 3' Hydroxylase

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To increase the biological activities of flavonoids and to enhance their stability and solubility by functionalization reactions (polymerization, esterification, alkylation, glycosylation and acylation), an increase in the number of hydroxyl groups in these molecules is needed. Hydroxylation reactions may be achieved using either chemical or enzymatic methods, the latter being more highly specific than the former. In our study, the flavonoid 3' hydroxylase (F3'H) from *Gerbera hybrid*, functionally expressed in *Saccharomyces cerevisiae*, was used to hydroxylate naringenin (the first flavonoid core synthesized in plants). Furthermore, we studied factors that may affect naringenin hydroxylation by recombinant cell-like yeast growth on selective or rich media and plasmid stability.

The whole recombinant cells hydroxylated naringenin at position 3' to give eriodictyol. In a selective media, the yeast failed to grow to high cell densities (maximum 5 g/L), but the plasmid stability was nearly 90 %, and naringenin hydroxylation reached 100 %. In a rich complex media, the biomass reached 10 g/L, but the yield of naringenin hydroxylation reached only 71 %, and the plasmid stability decreased. When yeast functionally expressing F3'H from *Gerbera hybrid* was used, in a selective media, 200 mg/L of eriodictyol from naringenin was produced.

Keywords: flavonoid, F3'H from *Gerbera hybrid*, hydroxylation, plasmid stability.

Flavonoids are secondary metabolites found in plants [1], in which their roles are as diverse as their structures, contributing to plant protection from environmental aggression [2,3]. In addition, flavonoids have potential benefits to human health [4], but the major hurdles of their formulation are their low solubility and stability. In order to take advantage of their beneficial properties, functionalization reactions (polymerisation, esterification, alkylation, glycosylation and acylation) have 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. Through hydroxylation, the basic flavonoid backbone can serve as useful starting materials for the development of new drugs.

However, the ability to generate either regioselective or stereoselective compounds through chemical hydroxylation presents a major obstacle for the development of new pharmaceuticals. Fortunately, biological hydroxylation using either enzymes or transgenic microbes has the ability to overcome this problem. Enzymatic hydroxylation reactions have been achieved, in particular with either flavonoid hydroxylase (FH) directly extracted from plants or from recombinant *Escherichia coli* or *Saccharomyces cerevisiae* functionally (Table 1) expressing FHs [5,6,12]. In the present study, the 3' hydroxylation of naringenin was evaluated using the microsomal fraction and whole recombinant yeast expressing the F3'H from *Gerbera hybrid*. Also we investigate the effect of media

Table 1: Origin of flavonoid hydroxylation enzyme functionally expressed in recombinant cells.

Flavanone 3 hydroxylase	Flavonoid 3' hydroxylase	Flavonoid 3'5' hydroxylase
<i>Malus domestica</i> cv. M9 [6] <i>Ammi majus</i> , <i>Anethum graveolens</i> , <i>Pimpinella anisum</i> [7] <i>Petunia hybrida</i> [8-10] <i>Petroselinum crispum</i> cv. Italian Giant plants [11]	<i>Brassica napus</i> [12] <i>Callistephus chinensis</i> line '01', <i>Osteospermum</i> hybrid line '082', <i>Gerbera hybrid</i> line 'D1', <i>Hieracium pilosella</i> [5], <i>Sorghum</i> [13] <i>Torenia hybrida</i> [14], <i>Gentian</i> [15] <i>Arabidopsis thaliana</i> [16], <i>Petunia hybrida</i> [17]	<i>Callistephus chinensis</i> line '01', <i>Osteospermum hybrid</i> cv. 'Bamba', <i>Pericallis cruenta</i> cv. 'Blue Bicolor' [5] <i>Verbena temari</i> , <i>Butterfly pea</i> [18] <i>Catharanthus roseus</i> , <i>Petunia hybrida</i> [19]

Table 2: Effect of microsome extraction pressure on naringenin bioconversion yield and protein quantity.

Protein (g/L)	Manual extraction	French press extraction				
		500 bars	750 bars	1000 bars	1500 bars	2000 bars
Naringenin bioconversion yield (%)*	100	66	90	97	83	85

*: Relative to the activity of microsomes extracted manually (100%). The quantity of proteins used for the reaction of naringenin hydroxylation was standardized to 0.25 g/L

culture on naringenin bioconversion, yeast growth and plasmid stability.

Indeed, the functional expression of *Gerbera hybrid* F3'H has been previously studied by Seitz *et al.* [5]; the ORF of isolated cDNA is constituted by 1539 base pairs; the encoded protein has a specific activity of 5.63 pkat/mg. The recombinant activity has been explored using the microsomal fraction to convert naringenin to eriodictyol [5]. Generally, the preparation of the microsomal fraction is done manually. In order to facilitate the microsome extraction procedure, we have used a modified extraction method based on the use of a constant disruption system. As can be seen in Table 2, the increase of extraction pressure was accompanied with an increase of protein yield and a decrease in naringenin bioconversion yield. A pressure of 750 and 1000 bars gave nearly the same bioconversion yield as that obtained with manual extraction, but the protein content of the microsomal fraction was more important. Whereas 500, 1500 and 2000 bars gave no amelioration of naringenin bioconversion, this may be attributed to insufficient extraction pressure (500 bars) or to the deleterious effect of pressure on enzyme structure and activity (1500 and 2000 bars). Manual extraction gave the best ratio for bioconversion/ protein quantity, but the use of a constant disruption system is easier and a more comfortable tool, since it permits the characterization of the naringenin bioconversion reaction and enzyme constants.

Whole cells and purified enzymes are both used as biocatalysts, but the former presents the advantages of simplicity, convenient manipulation and low costs, while enzyme purification is tedious and expensive. Moreover, the purification of enzymes could lead to significant loss of activity and cofactors such as NADPH, usually required for enzymes like F3'H [5,12-17]. Consequently, in order to probe the whole cell activity, 1 g/L of recombinant yeast was incubated with naringenin as substrate. In the course of the incubation,

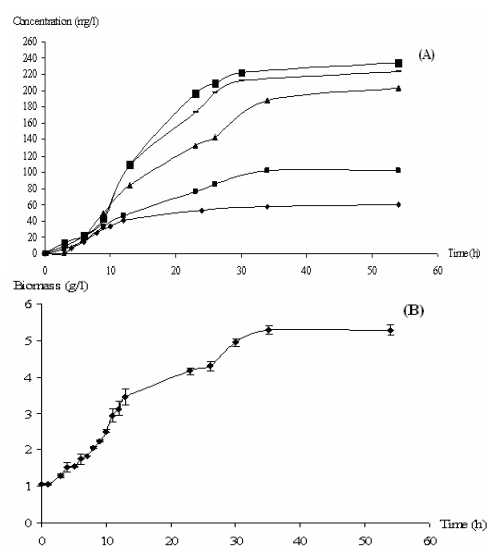


Figure 1 (A): Eriodictyol production by recombinant *S. cerevisiae* expressing the F3'H of *G. hybrid* in the presence of various concentrations of naringenin. (■): 2025 mg/L, (○): 675 mg/L, (▲): 225 mg/L, (■): 100 mg/L, (◆): 50 mg/L. **(B):** Growth curve of the recombinant *S. cerevisiae* expressing the F3'H of *G. hybrid* (means of growth curves of the recombinant yeast strain with different concentrations of naringenin (0, 50, 100, 225, 675 and 2025 mg/L) with standard deviation).

the initial naringenin concentration (100 mg/L) started to decrease and a new product appeared (data not shown). The new product was identified as eriodictyol (LC/MS). Thus, the whole recombinant yeast can be used to hydroxylate naringenin.

In order to investigate the maximum bioconversion ability of *S. cerevisiae* strain harbouring F3'H, the naringenin was added to the culture media at final concentrations of 50, 100, 225, 675 and 2025 mg/L; product formation and residual substrate concentrations against incubation time were plotted (Figure 1). We analysed the yeast growth and the naringenin biotransformation kinetics with *S. cerevisiae* expressing F3'H. In *S. cerevisiae* strain harbouring F3'H, recombinant protein production was induced by galactose in the YPL medium, as described in the experimental section. The growth of the recombinant

strain was not affected by the naringenin concentration (Figure 1). The bioconversion kinetic and growth were slow for the first 10 hours; the biomass was doubled, and 33 to 50 mg/L of naringenin was converted to eriodictyol. Within 10 to 33 hours of the exponential growth phase, the biomass was multiplied by 5 (when compared with the initial biomass) and the growth was associated with the bioconversion kinetic, both of which progress faster than during the first 10 hours after induction. Nearly 200 mg/L of eriodictyol were synthesized from 225, 675 and 2025 mg/L of naringenin. Within 33 to 54 h, the stationary growth phase was attained and only an extra 5 mg/L of eriodictyol, at a maximum, was formed (Figure 1).

It is clear that increasing naringenin concentrations enhanced the production yield and bioconversion kinetic but there is a limit of saturation (of substrate bioconversion). It is not necessary to increase naringenin concentration over 225 mg/L because the bioconversion yield decreases 3 and 9 folds when compared to a total bioconversion (for 675 and 2025 mg/L of naringenin, respectively).

The bioconversion yield is influenced by the initial naringenin concentration and also by the culture media composition. The latter dictates the growth rate of the recombinant yeast, the cell yield, the plasmid stability and naringenin bioconversion. Consequently, we tested, in parallel, the effect of culture conditions on the factors cited above.

Two stage culture modes were used: (i) a growth phase to form the initial biomass and (ii) an induction phase. We tested two culture media; a minimal medium without uracil (to select recombinant yeast harboring plasmid) and a rich medium. We combined these two media, as indicated in Table 3. As can be seen in Table 3, independently of the media used to prepare the inoculums, when the induction was done in YPL rich medium, the biomass production was nearly 10 g/L compared with 5 g/L in SC minimal medium. Despite the use of the same quantity of carbon source and the same initial biomass (adjusted to 1 g/L), biomass production was not the same, indicating that minimal

medium presents a limitation in its saline, vitamin or nitrogen composition.

Within 33 hours of induction, it was notable that SC-SC and YPL-SC two stage culture modes gave the same maximum bioconversion yield (100%), but in SC-YPL and YPL-YPL culture mode the bioconversion yield did not exceed 71% (Table 3). This indicates the importance of induction media in the conversion reaction. Indeed, SC minimal media exert a selection pressure to stabilize the plasmid in cells, and consequently, the F3H expression is maintained to a high level allowing the production of the maximum amount of eriodictyol (100 mg/L of naringenin were totally converted to eriodictyol). As can be shown in Figure 2, after 30 hours of induction, only 58 % of cells in YPL medium contain plasmid, while in SC medium, 90 % of the yeast contains plasmid. We noticed that during the exponential phase, between 6 and 24 hours, the fraction of plasmid free cells increased. This phenomenon may be due to the acceleration of cell division. However, in the stationary phase, this fraction decreased, probably because the media components become limiting and only cells containing the recombinant plasmid can survive uracil limitation.

Over-viewing our work, we have successfully produced eriodictyol, a 3'hydroxylated flavonoid from naringenin, by using a whole cell activity. Recombinant *S. cerevisiae* harboring F3'H from *Gerbera hybrid* was used as a selective single step for high-yield hydroxylation to replace a series of chemical synthetic reactions that suffers from drawbacks of simultaneous formation of diverse by-products and low isolated yield.

Contrary to the works reported in Table 1, we have used another easy method for microsomes (containing the F3'H) extraction, to test enzymatic activity. The use of a cell disruption system at 750 and 1000 bars gave nearly the same naringenin bioconversion yield as that of the classic manual extraction. Moreover, in the present study, we demonstrated that it is not necessary to extract enzyme to achieve the hydroxylation reaction. The whole recombinant *S. cerevisiae* cell expressing flavonoid hydroxylases can hydroxylate naringenin and

Table 3: Effect of culture mode on growth of the recombinant yeast strain and eriodictyol production. SC: selective medium, YPL: rich medium.

Medium used to two stage culture mode		Biomasse (g/L)	Eriodictyol (mg/L)	Biomasse (g/L)	Eriodictyol (mg/L)	Biomasse (g/L)	Eriodictyol (mg/L)	Biomasse (g/L)	Eriodictyol (mg/L)	Biomasse (g/L)	Eriodictyol (mg/L)
Inoculum media	Induction and bioconversion media	Time after induction of recombinant hydroxylase synthesis (h)									
		0		5.5		11		22		33	
SC min	SC min	1	0	1.5	17.5	2.1	47.6	4.4	90.4	5.1	100
		YPL	1	0	1.1	30.7	2.2	85.3	4.7	94	4.8
SC min	YPL	1	0	1.5	12	2.5	36.8	8	71.2	8.3	71
		YPL	1	0	1.7	20	3.7	61.1	8.9	70.4	9.9

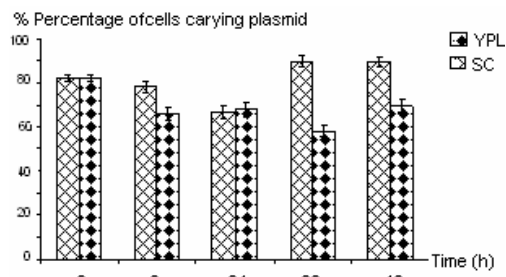


Figure 2: Effect of culture media on the percentage of plasmid containing cells. SC: selective medium, YPL: rich medium.

other flavonoids (like apigenin ;data not shown)). As substrate concentration increased, the naringenin bioconversion yield decreased and goes much slower. This may be ascribed to a substrate saturation phenomenon, low substrate solubility in aqueous medium, low substrate penetration to cells, and limitation in medium composition... Special techniques, such as two-phase biocatalysis and cell immobilization and/or permeabilization could be used to increase the concentration tolerance of this reaction [20].

Naringenin bioconversion to eriodictyol depends on the production of the recombinant F3'H enzyme. Synthesis of the cloned gene product is influenced by genetic and environmental factors, including plasmid stability, cell growth rate and medium composition. We have tested four two stage culture modes using two culture media: the SC minimal and YPL rich medium. We found that the naringenin bioconversion yield depends on the media used to induce the recombinant F3'H production, independently of the medium used to form the initial yeast biomass. SC minimal medium used as an inductor gave the best bioconversion/biomass ratio; 20 mg of eriodictyol per g of recombinant yeast (dry weight), when compared with the YPL rich complex medium. The induction in this latter case gave a ratio of 7.1 mg of eriodictyol per g of recombinant yeast. In addition, culture in the minimal medium offers the advantage of maintaining high plasmid stability. Thus, the decrease of naringenin bioconversion, in rich media, may be correlated to plasmid instability. It is notable (Figure 2) that the fraction of plasmid-carrying cells decreased during the exponential growth phase, but increased during the stationary phase. This phenomenon may be ascribed to the death rate difference between the plasmid-free and plasmid carrying cells caused by starvation in the stationary phase. Indeed, the high segregational instability of recombinant *S. cerevisiae* is primarily caused by asymmetric cell division, which can lead to the formation of 9 to 43 buds per cell [21]. In contrast to binary cell division in *E. coli*, budding results in an unequal distribution of plasmids among daughter cells, leading to an increased probability of plasmid-free cell emergence, especially in

the exponential growth phase. In theory, plasmid-free cells respond to environmental changes much quicker than plasmid carrying cells. Plasmid-free cells may grow faster than plasmid carrying cells when there is plenty of growth substrate, but they also may lyse or die faster upon depletion of growth substrate (uracil). Thus, pulse addition of growth substrate at an appropriate time interval, with a significant starvation period between two consecutive feedings during fedbatch fermentation may have positive effects on stabilizing plasmid and enhancing protein production [22]. Other various operating strategies have been proposed to address the problem of competitive plasmid instability in recombinant yeast. These include cycling growth rate changes, cycling of substrate levels, cycling dissolved oxygen tension, dilution rate cycling, and autoselection systems [23].

Our work represents the first report of using recombinant yeast whole cell activity to achieve a single flavonoid hydroxylation step. Yan *et al* [24] have reported the use of whole cells expressing multiple enzymes to produce eriodictyol from caffeic acid. Their recombinant system produced 6.5 mg/L of eriodictyol from 180 mg/L of caffeic acid. In the present work, we produced 200 mg/L of eriodictyol from 225 mg/L of naringenin. Consequently, eriodictyol formation is 24 orders of magnitude higher than that reported [24]. This work opens up alternative routes for the production of additional hydroxylated flavonoid structures from other non-hydroxylated flavonoid precursors, using recombinant *S. cerevisiae* cells and by engineering substrate specificity of the F3'H by using site directed mutagenesis, laboratory evolution or chimeragenesis and engineering improved redox partners [25]. Generally, the relatively poor stability of P450 monooxygenases, such as F3'H, still impede the technical application of these enzymes. Immobilization of enzyme or recombinant permeabilized yeast has proven useful for *in vitro* biotransformations [26]. A combination of chemical, physical and biological approaches will contribute to the production of polyhydroxylated flavonoid structures needed for functionalization reactions, like glycosylation [4,27], acylation [4], methylation [4,28], and sulfation [4,29].

Experimental

Plasmid and cell transformation: The F3'H from *Gerbera hybrid* was generously given by Pr. Christian Seitz (Technical University Munich, Germany) in yeast plasmid pYES2.1/V5-His-TOPOZ® [5]. The *Saccharomyces cerevisiae* INVSc1 strain was purchased from Invitrogen, (Paisley, UK). The yeast transformation was carried out according to the manufacture's prescription. Chemicals used in this study were purchased from Sigma Chemicals (St. Louis, (USA)).

Hydroxylation reaction by microsomes extracted from *S. cerevisiae* expressing *Gerbera hybrid* F3'H:

Microsomes were prepared as described by Pompon *et al.* [30]. Ten mL of the minimal growth medium (SC minimal composed of 20 g glucose, 1.92 g yeast synthetic drop-out media without uracil, and 6.7 g yeast nitrogen base without amino acids in 1L of distilled water) were inoculated with the recombinant yeast and grown to stationary phase. Cells were harvested by centrifugation (3000 rpm for 5 min), then used to inoculate 250 mL of rich induction medium composed of 20 g/L galactose, 10 g/L peptone and 10 g/L yeast extract (YPL) and incubated overnight at 28°C, with shaking at 200 rpm. Cells were harvested by centrifugation at 7000 g for 10 min at 4°C. The cell pellet was washed with TEK buffer (10 mM Tris-HCL (pH=7.5), 1 mM EDTA and 100 mM KCl). After centrifugation, cells were suspended in 50 mL TESB buffer (50 mM Tris-HCl (pH=7.5), 1 mM EDTA, 0.6 mM sorbitol, 1% BSA and 20 mM β -mercaptoethanol). The cell suspension was passed through either a constant cell disruption system or disrupted manually, as described [30]. Five extraction pressures were applied: 500, 750, 1000, 1500 and 2000 bars. The suspension was centrifuged at 10000 g for 15 min at 4°C. The supernatant was recovered and the microsomes precipitated with $MgCl_2$ (125 mM) for 30 min at 4°C. Microsomes were then recovered by centrifugation for 20 min at 15000 rpm at 4°C and suspended in 3 mL cold TEG (50 mM Tris-HCL (pH=7.5), 1 mM EDTA, 30% glycerol). Protein quantification was determined by the bicinchoninic acid method [31].

Determination of naringenin hydroxylation product by whole cells of *S. cerevisiae* expressing F3'H:

Ten mL of culture grown to stationary phase in SC minimal medium was used to inoculate 100 mL of a rich induction medium. Naringenin was added at a concentration of 100 mg/L and the mixture was incubated on a shaker at 28°C for 55 h. 750 μ L of supernatant was collected and mixed with 250 μ L of acetonitrile. Identification of the hydroxylation products was monitored using HPLC and LC/MS analysis.

Effect of substrate concentration on bioconversion kinetics with *S. cerevisiae* expressing F3'H:

Thirty mL of culture grown to stationary phase in SC minimal medium was used to inoculate 250 mL of SC minimal medium. Cells were harvested by centrifugation at 3000 rpm for 5 min before their transfer to SC medium, with different concentrations (50, 100, 225, 675 and 2025 mg/L) of the substrate. The mixture was incubated on a shaker at 28°C for 55 h. 750 μ L of the supernatant was collected and mixed with 250 μ L of acetonitrile. Quantification of the metabolites and parent material was monitored using HPLC.

Determination of the appropriate culture modes for growth of the recombinant *Saccharomyces cerevisiae* and naringenin bioconversion:

A single colony of recombinant yeast strain was placed in 30 mL of (20%) glucose supplemented SC minimal media or in YPL rich medium and incubated at 28°C till stationary phase. The culture was centrifuged for 3 min at 5000 rpm and the pellet suspended in (20%) galactose supplemented fresh minimal (SC) or complex media (YPL). This gives four types of culture, as seen in Table 3. Naringenin was added to a final concentration of 100 mg/L and its transformation was assessed for 34 h.

Analytical method: Cell density was measured by the dry cell weight method. For determining dry cell weight, 10 mL of 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 0.45 g/L dried cell weight. 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 (SC min) to the total number of cells growing on non selective YPL medium (including plasmid-free and plasmid-containing cells). Cell samples were diluted to make the colony counts within the range of 30-300. All plate counts were taken from the average of at least 3 replicates.

HPLC analysis: A Thermo Finnigan (San Jose, CA, USA) HPLC apparatus equipped with an U.V. 6000 LP detector was used. For analytical scale, the mobile phase consisted of water and acetonitrile and was programmed as follows; 10% acetonitrile from 0 min to 12 min, then a linear gradient was used starting from 40% acetonitrile to 60% at 17 min, followed by 8 min of a constant eluant of 10% acetonitrile. The flow rate was 1 mL/min; detection wavelength 296 nm; column temperature 28°C.

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