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Article

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Biotransformation of chrysin to baicalein: Selective C6-hydroxylation of 5,7-dihydroxyflavone using whole yeast cells stably expressing human CYP1A1 enzyme

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ABSTRACT:

Naturally occurring polyphenolic compounds are of medicinal importance because of their unique antioxidant, anticancer and chemopreventive properties. Baicalein, a naturally occurring polyhydroxy flavonoid possessing a diverse range of pharmacological activities, has been used in traditional medicines for treatment of various ailments. Apart from its isolation from natural sources, its synthesis has been reported via multi-step chemical approaches. Here we report a preparative-scale biotransformation, using whole yeast cells stably expressing human cytochrome P450 1A1 (CYP1A1) enzyme, that allows regioselective C6-hydroxylation of 5,7-dihydroxyflavone (chrysin) to form 5,6,7-trihydroxyflavone (baicalein). Molecular modelling reveals why chrysin undergoes such specific hydroxylation mediated by CYP1A1. More than 92% reaction completion was obtained using a shake-flask based process that mimics fed-batch fermentation. Such highly efficient selective hydroxylation, using recombinant yeast cells, has not been reported earlier. Similar CYP-expressing yeast cell-based systems are likely to have wider applications in the syntheses of medicinally important polyphenolic compounds.

Keywords: Biotransformation, chrysin, baicalein, CYP1A1, yeast cells, hydroxylation

INTRODUCTION

Baicalein (5,6,7-trihydroxyflavone) (**1**) was originally isolated from the roots of *Scutellaria baicalensis* (Chinese skullcap) which is used for treatment of chronic hepatitis, inflammatory diseases, tumors, and diarrhea in China, Korea, Taiwan, and Japan.[1] Its isolation has also been reported from another plant, *Oroxylum indicum* (Indian trumpet flower).[2] Baicalein is known for its anticancer properties in various cancer types[2-22] including pancreatic, gastric and colorectal cancers, multiple myeloma, head and neck cancer, and breast cancer.[23, 24] Baicalein is also considered as an anti-inflammatory[25, 26] that generally protects against oxidative stress,[27] more specifically in cardiac cells,[28] and in cisplatin-induced acute kidney injury.[29] Because of its wide-ranging medicinal applications, an industrial scale protocol for its production would be considered as important.

Baicalein has primarily been produced via five approaches: (a) single-step hydrolysis of the natural product baicalin, a glycoside,[30] (b) 4-step total synthesis from a cinnamic acid derivative,[31] (c) 4-step total synthesis from 3,4,5-trimethoxyphenol,[32] (d) 3-step total synthesis from 2,4,5,6-tetrahydroxyacetophenone,[33] and (e) 6-step synthesis from chrysin[34] involving methylation, bromination, acylation, nucleophilic replacement of Br with OMe, followed by de-acylation and demethylation as key steps.

Selective transformation, using efficient biocatalysts, of a low value phytochemical to high value phytochemical is an exciting area of bioorganic chemistry. Sordon and coworkers[35] have reported biotransformations of natural flavonoids naringenin, hesperetin, chrysin, apigenin, luteolin, quercetin, epicatechin, and biochanin A using the natural yeast, *Rhodotorula glutinis*. This approach produced norwogonin (5,7,8-trihydroxyflavone) from chrysin (5,7-dihydroxyflavone). Here we report a preparative scale process for biotransformation of chrysin to baicalein, via selective C6-hydroxylation using recombinant human cytochrome P450-1A1 (CYP1A1) enzyme expressed within baker's yeast (*Saccharomyces cerevisiae*) cells. This is the first single-step protocol for a high-yield conversion of chrysin to baicalein and, therefore, may serve as a simple and cheap strategy for production of baicalein in an industrial scale.

MATERIALS AND METHODS

General. All chemicals were obtained from Sigma-Aldrich and were used as received. ^{1}H NMR spectra were recorded on Brucker-Avance DPX FT-NMR 400 MHz instrument. Chemical data for protons are reported in parts per million (ppm) downfield from tetramethylsilane and are referenced to the residual proton in the NMR solvent (CD_3OD, 3.31 ppm). ESI-MS were recorded on Waters QTOF mass spectrometer.

HPLC analysis was performed on Shimadzu LC-6AD system connected with C18 column (4.6 x 25 mm, 5 µ). Mobile phase consisted of 0.1% formic acid (A) and methanol (B) using isocratic elution (30: 70 – A: B). Flow rate was 1 mL/min and detection wavelength was 270 nm.

LC-MS analysis was performed on Waters Acquity UPLC system. The column used was C18, 1.7 µ with dimensions of 100 x 2.1 mm (column temp. 30 °C). Binary gradient system was used. Mobile phase A consisted of 5% acetonitrile in water (with 0.1% formic acid). Mobile phase B consisted of acetonitrile with 0.1% formic acid. Gradient details are: Time in min (% B concentration): 0.01 (10), 0.25 (10), 9.00 (100), 10.00 (100), 11.00 (10), 12.00 (10). PDA range: 220 nm to 400 nm; flow rate: 0.3 mL/min.

Biotransformation experiment. Yeast strains, each of which contain two copies of human *CYP1A1* or *CYP1A2* genes, downstream of the *ADH2* promoter, integrated into chromosomal loci of the genome of the yeast strain W303-1a (ATCC 208352), were used for biotransformation (Section S2 of supporting information). Expressed CYP1A1 and CYP1A2 proteins were confirmed by Western blotting (supporting information-S4). The strains, from frozen stocks, were revived in 250 mL Erlenmeyer baffled flasks containing 50 mL YPD (Yeast, Peptone, Dextrose) medium with composition (g/L): peptone 20; yeast extract 10; glucose 15.0, pH 6.0. The flasks were shaken at 200 rpm, at 28 °C. Three consecutive YPD pre-cultures were grown for high biomass production, before addition of the substrate to cells grown in SD (Synthetic Defined) medium. Typically, loopful of CYP-containing freshly grown yeast cells was inoculated in a 500 mL Erlenmeyer baffled flask separately containing 100 mL YPD medium (pre-culture -1) at 28 °C for 24 h. The cells were harvested after 24 h and inoculated into a new 500 mL baffled flask containing 100 mL YPD medium (pre-culture -2) at 30 °C for 18 h. The process was repeated three times for the cells to reach an OD_{600}, of ~90.

The harvested cells, ~3.0 mL (OD_{600}, ~90), were inoculated in 50 mL of minimal SD medium contained in a 1 L baffled flask. Composition of SD medium (g/L): dextrose 1.0; dipotassium phosphate 7.0; monopotassium phosphate 2.0; sodium citrate 0.50; magnesium sulphate 0.10; ammonium sulphate 1.0, pH 7.0 ± 0.2 at 28 °C. Initially, reaction was carried out with 0.2 mg/mL of chrysin. Later, the chrysin at different concentrations *viz*. 0.2, 1, 2, 5 and 10 mg/mL were incubated in 50 mL of SD medium (DMSO was used for initial dissolution of the compound, and keeping the DMSO concentration < 0.5% in final reaction medium) for 72 h at 28 °C, 200 rpm. After every 24 h, the medium was replenished with 1.5% w/v of glucose. For optimization of incubation time, the SD cell culture media were harvested after 24, 48, 72, 96, 120, 144 and 160 h, and were then analysed on TLC and HPLC/ LC-MS to monitor the yields of biotransformation at each time point.

Isolation and characterization of baicalein. The reaction media was extracted with ethyl acetate (3 times). The combined ethyl acetate layer was concentrated on vacuo-rotavapor to obtain crude extracts that contained the biotransformation product. The crude residue was loaded on a reverse phase (C18) silica gel column packed in water. The crude extract was loaded on the column by making a slurry with C18 silica gel mesh 200-400 (Sigma-Aldrich, product no. 377635). The column was then eluted with increasing concentrations of methanol in water. The desired product was collected at 50% methanol in water. Evaporation of the solvent gave a yellow solid which was characterized as baicalein (**1**). Yellow powder; m.p. 262-265 °C (Lit. 264-265 °C); TLC: R_f = 0.5 (3% methanol in DCM) and 0.8 (40% EtOAc: hexane with 0.1% acetic acid); ^1H NMR (CD_3OD, 400 MHz, δ ppm): 7.91 (dd, J = 4.0, 8.0 Hz, 2H), 7.48 (m, 3H), 6.66 (s, 1H), 6.55 (s, 1H); ESI-MS: *m/z* 271.10 $[M+H]^+$. The spectral data was identical to that reported in the literature[32] and TLC matched with reference sample obtained from Sigma-Aldrich (CAS number 491-67-8).

Molecular modeling: The docking of chrysin with CYP1A1 (PDB ID: 4I8V) was performed using GLIDE module of Schrodinger molecular modeling software, using the protocols as described in our earlier publications.[36, 37] The docking protocol was validated by docking known ligand α-naphthoflavone (ANF). The interaction pattern of docked ANF and ligand from co-crystallized protein (4I8V) are shown in the supporting information (Section S8).

RESULTS AND DISCUSSION

CYP enzymes are known for their exceptional ability to carry out hydroxylation, epoxidation or demethylation reactions in a regioselective fashion, both in plants and humans. It is essential for human CYP450 enzymes to be integrated on the endoplasmic reticular (ER) membranes to manifest its native activity. Baker's yeast cells possess ER membranes which are not present in prokaryotic *E. coli* cells. Eukaryotic baker's yeast cells were chosen because they can grow as rapidly as bacterial cells and they are as amenable as bacteria for scale-up in fermentors. Using Baker's yeast cells, we have developed an efficient technology for stable expression of human CYP enzymes within these cells. Using this platform, recombinant baker's yeast cells can continue to express CYPs in shake flasks, over a week or more, with increasing activities, under conditions that mimic fed-batch growth (Supporting Information; S1). Microsomal CYP enzymes, isolated from these same recombinant yeast cells, have successfully been used by us earlier as drug discovery tools for screening synthetic compounds and natural product repositories to identify possible cancer chemopreventive agents.[37-39]

Selection of CYPs for biotransformation of chrysin. In this work, use of recombinant CYP-expressing whole yeast cells has been explored for their ability to be used as biocatalysts for biotransformation reactions. For this purpose, we chose the flavonoid chrysin as a substrate. As the cytochrome P450 family 1 enzymes CYP1A1 and CYP1A2 are reported to oxidize dietary flavonoids,[40-42] herein we chose two yeast-expressed enzymes of the CYP1 sub-family (i.e. CYP1A1 and CYP1A2) as biocatalysts.

Two recombinant yeast strains, each containing two chromosomally integrated copies of *CYP1A1* and *CYP1A2* genes under the control of the ethanol-inducible alcohol dehydrogenase 2 (*ADH2*) promoter, were created to enable stable and reproducible biotransformation reactions (plasmid maps shown in Supporting Information, S2). The *ADH2* promoter is repressed in the presence of glucose. The aim was that recombinant yeast cells, containing stably integrated *CYP* expression cassettes, would be grown initially in complete YPD medium (containing 2% glucose) to obtain large optical density, measured at a wavelength of 600 nm (OD_{600}) of ~90, in shake flasks. This would occur without any plasmid loss and would be achieved over 3 days through repetitive replenishment of glucose, every 24 h, in the non-selective highly nutritious YPD medium. Cells grown in YPD (Figure 1a) would then be re-suspended in minimal selective SD medium (pH 7.0), supplemented with 1.5% of glucose, for the biotransformation reaction. It was thought that rich full YPD medium may not be appropriate for biotransformation since there is a possibility of substrate binding to its ingredients.

For initial optimization, reactions were performed in SD with 10 mg of chrysin in baffled flasks shaking at 200 rpm for 72 h, at 28 °C (Figure 1b). After every 24 h, the medium was replenished with fresh glucose to a final concentration of 1.5%. Glucose was exhausted after 12 h of growth of yeast cells when it was converted to ethanol. Hence, the ethanol-inducible *ADH2* promoter was fully induced every 12 h before more glucose was added to the medium for further rounds of expression.

Figure 1.

The reaction media at each time point was extracted with ethyl acetate. The extracts were analyzed by TLC followed by HPLC/ LC-MS. The formation of new product on TLC with lower R_f value than the substrate gave us an indication of a hydroxylation reaction (Supporting Information, S3). LC-MS analysis of the reaction mixture confirmed the product as mono-hydroxy chrysin with mass of *m/z* 270. The same product was formed using both the enzymes, CYP1A1 and CYP1A2, expressed within yeast cells. However, conversion of substrate to product was better with CYP1A1. Therefore, for subsequent scale-up experiments, only CYP1A1-expressing yeast cells were used for biotransformation.

Scale up and optimization of biotransformation reaction. Scale-up was performed as above; cells were at first cultivated in non-selective YPD media for 72 h, with fresh glucose (2%) being added every 24 h. Cells were re-suspended in selective SD minimal medium. Chrysin at different concentrations *viz*. 0.2, 1, 2, 5 and 10 mg/mL were dissolved in DMSO and further incubated in 50 mL of SD medium (keeping the DMSO concentration < 0.5%) for 72 h at 28 °C, 200 rpm. The chrysin-containing cell culture media were replenished with glucose every 24 h to a final concentration of 1.5%. The reaction using chrysin at concentration of 2 mg/mL (100 mg of chrysin) was observed to be the most efficient. With 5 and 10 mg/mL concentrations, incomplete biotransformation occurred, probably because the small number of cells, used for growth in these specific experiments, was inhibited by the substrate.

Based on these results, it was decided to optimize the time period for biotransformation. For this, we chose 2 mg/mL concentration of substrate with reaction time points of 24, 48, 72, 96, 120, 144 and 160 h. HPLC analyses of the reaction mixture after 24, 48, 72, 96, 120, 144 and 160 h incubations indicated that, at 144 h, there was nearly complete conversion (>92%) of chrysin to the product. The HPLC chromatograms of reaction mixtures at representative four time intervals are shown in Figure 2. In HPLC analysis, the concentration of sample injected at each time interval was kept constant. The increase in the AUC of baicalein with increase in the time, from 24 h to 144 h, is indicative of the fact that the highest amount of baicalein is formed at the 144 h time point.

Figure 2

The relative percentages (AUC) of chrysin and baicalein at different time intervals are depicted in Figure 3. As shown in Figure 3, the percentage conversion of chrysin to baicalein increased with time.

Similarly, Figure 3b shows increased peak height (mAU) of baicalein with increase in reaction time. Figures 3a and 3b, in combination, indicate that 144 h is the optimal reaction time for this transformation. Further details of HPLC analysis are provided in supporting information, section S5.

Figure 3.

Isolation and characterization of baicalein. Initial isolation attempts using normal phase silica gel column chromatography showed significant loss in the product yield. Therefore, we attempted reverse phase C18 silica gel column chromatography where water-methanol was used as the mobile phase. The product was isolated at 50% methanol in water and was characterized by spectral analysis and by comparison of its TLC with reference sample.

The ^{1}H NMR of chrysin contains a typical bunch of three singlets at chemical shift values in the range of 6 to 7 ppm. These three singlets at δ 6.24, 6.49 and 6.74 ppm correspond to the protons present at C6, C8 and C3 positions. The C6-proton appears with an up-field shift in comparison to two other aromatic protons at C3 and C-6, because of the shielding effect from two adjacent C5 and C7 hydroxyls. It is obvious that the hydroxylation reaction is possible, either on A or C ring. Since there was no change in the chemical shift value pattern of C ring (δ 7.91, dd, 2H and δ 7.48, m, 3H), it would indicate that the C-ring is intact and no hydroxylation had taken place on this ring. The hydroxylation on A ring has two possibilities, either C6- or C8 hydroxylation. Hydroxylation at C6-position will form baicalein (5,6,7-trihydroxyflavone) whereas hydroxylation at C8-position will form norwogonin (5,7,8-trihydroxyflavone) as a product. It was interesting to see that, in the ^{1}H NMR of the obtained product, the up-field singlet (δ 6.24 ppm) disappeared. This singlet peak in chrysin corresponds to the proton present at C6-position. This gave us a clear indication that hydroxylation occurred at the C6-position, which means that the product is 5,6,7-trihydroxyflavone. This is the naturally occurring flavone, commonly named as 'baicalein'. Furthermore, on comparison of the ^{1}H NMR of the obtained product with norwogonin,[35] the possibility of norwogonin as the product was ruled out. In literature, the biotransformation of chrysin using natural yeast, *Rhodotorula glutinis* yielded C-8 hydroxylated product norwogonin,[35] however in the present study, C-6 hydroxylated product (baicalein) was formed, which may be possibly because of the regio-specificity of the CYP1A1 enzyme.

Mass analysis of the isolated product showed *m/z* peak at 271 in ES+ve mode, which matched with the predicted product. The final confirmation of the assigned product was done by co-TLC and HPLC analysis with the reference standard of baicalein (CAS number: 491-67-8)' purchased from Sigma-Aldrich. TLC images as well as HPLC analysis clearly matched the reference standard (TLC images are shown in Supporting Information -S3).

The LC-MS analysis was also performed for the extract as well as isolated baicalein (Figure 4).

Figure 4.

Docking of chrysin with CYP1A1. In order to decipher the rationale for regioselective hydroxylation, chrysin was docked with the substrate binding site of CYP1A1 enzyme (PDB ID: 4I8V). The interaction pattern of chrysin with CYP1A1 is depicted in Figure 5. It is interesting to note that the A-ring of chrysin orients towards the heme. Furthermore, the C-6 carbon of A-ring is present in close-proximity with heme protein, suggesting that the reactive heme-oxo intermediate should possibly form at this position. Other key interactions which help in stabilizing this orientation of chrysin includes: (a) hydrophobic π-π interactions of Phe-224 of I-helix with B and C rings; (b) π-π interactions of Phe-123 with A ring; and (c) polar H-bonding of C-7 hydroxyl group with Ser-122. This observed orientation, excludes the possibility of hydroxylation at C-8 and at aromatic CH of B and C rings.

Figure 5.

Our efforts using yeast whole cells have resulted in the development of a reproducible preparative-scale biotransformation process for the conversion of chrysin to baicalein (5,6,7-trihydroxyflavone). According to the literature, the medicinal effects of baicalein are more profound than that of chrysin. Furthermore, commercially available baicalein is at least 60-times more expensive than chrysin. Thus, this protocol described here can be utilized for production of a high value phytochemical from a low value one, using a simple, low-cost, one-step biotransformation reaction.

In conclusion, we have demonstrated the ability of whole yeast cells, that overexpress the human CYP1A1 enzyme, to catalyse biotransformation of >92% of the natural flavonoid chrysin to baicalein. Optimal aeration, neutral pH and maintenance of glucose concentration, throughout the reaction, played very important roles in the biotransformation reaction. The example demonstrated in this paper, provides an opportunity for further exploring the utility of stable recombinant CYP enzyme-expressing yeast cells for industrial production of medicinally important polyphenolic compounds.

ASSOCIATED CONTENT

Supporting Information. Additional experimental details. This material is available free of charge via the Internet at http://pubs.acs.org

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Author Contributions

The enzyme expression in yeast cells was executed by I. S. Williams, L. Gatchie and B. Chaudhuri. Pre-culturing of yeast cells and their growth in SD medium followed by set-up of biotransformation experiment was performed by S. Chib and S. Saran. Product isolation and characterization was done by

V. Nuthakki, R.A. Vishwakarma and S. B. Bharate. LS-MS analysis was done by N.A. Narkhede and molecular modeling by P. Joshi and S.B. Bharate.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS

ADH2, alcohol dehydrogenase 2; ANF, alpha-naphthoflavone; CYP1A1, cytochrome P450 group enzyme 1A1; CYP1A2, cytochrome P450 group enzyme 1A2; DMSO, dimethyl sulfoxide; ER, endoplasmic reticulum; HPLC, high performance liquid chromatography; LC-MS, liquid chromatography mass spectrometry; OD, optical density; PDB, protein data bank; SD, Synthetic Defined; TLC, thin-layer chromatography; YPD, Yeast, Peptone, Dextrose;

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FIGURE LEGENDS

Figure 1. (a) CYP1A1-expressing cells grown in YPD Medium in test-tubes; (b) Biotransformation reaction using CYP1A1 in SD medium in shake-flasks; (c) scheme showing conversion of chrysin to baicalein, and their key ^{1}H NMR chemical shift values

Figure 2. HPLC analysis of biotransformation reaction at different time intervals. (a) HPLC chromatogram of chrysin reference standard; (b) HPLC chromatogram of baicalein reference standard; (c) HPLC chromatogram of biotransformation reaction after 24 hrs of incubation time; (d) HPLC chromatogram of biotransformation reaction after 48 hrs of incubation time; (e) HPLC chromatogram of biotransformation reaction after 96 hrs of incubation time; (f) HPLC chromatogram of biotransformation reaction after 144 hrs of incubation time. The concentration of sample injected is kept constant at each time interval; therefore an increase in AUC (and peak height) with the increase in incubation time indicates progress of the reaction.

Figure 3. (a) Relative percentage (±SD) of chrysin and baicalein at different time intervals during a typical biotransformation reaction (the percentages are based on the AUC of the peaks in HPLC analysis at 270 nm). (b) Baicalein (t_R = 5.99 min) peak height (±SD) at different time intervals during biotransformation reaction.

Figure 4. LC-MS analysis of reaction mixture at 144 h. (a) LC chromatogram of reaction mixture after 144 h of incubation. (B) LC chromatogram of isolated product; (c) Mass spectra of peak at t_R 4.69 min.

Figure 5. Molecular docking of chrysin with CYP1A1 (PDB: 4I8V), showing the predicted site of hydroxylation as C6.

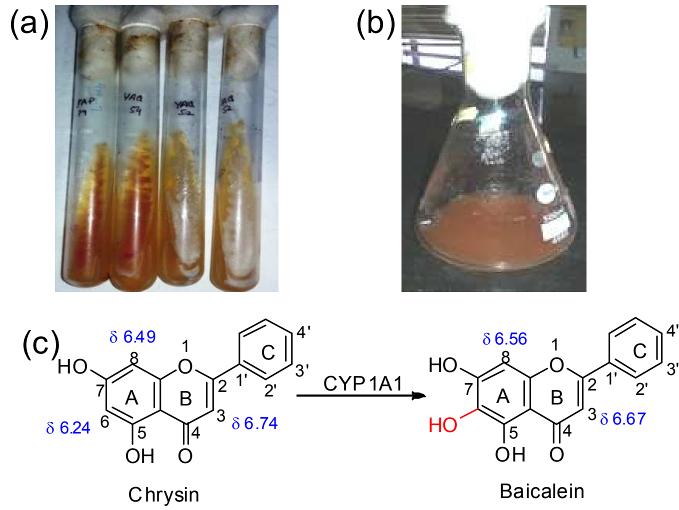

(a)
(b)
(c)
δ 6.49
HO
8
1
O
C
4'
3'
2'
1'
2
3
4
5
6
7
A
B
δ 6.24
δ 6.74
OH
O
Chrysin
CYP1A1
δ 6.56
HO
HO
δ 6.67
OH
O
Baicalein

Figure 1.

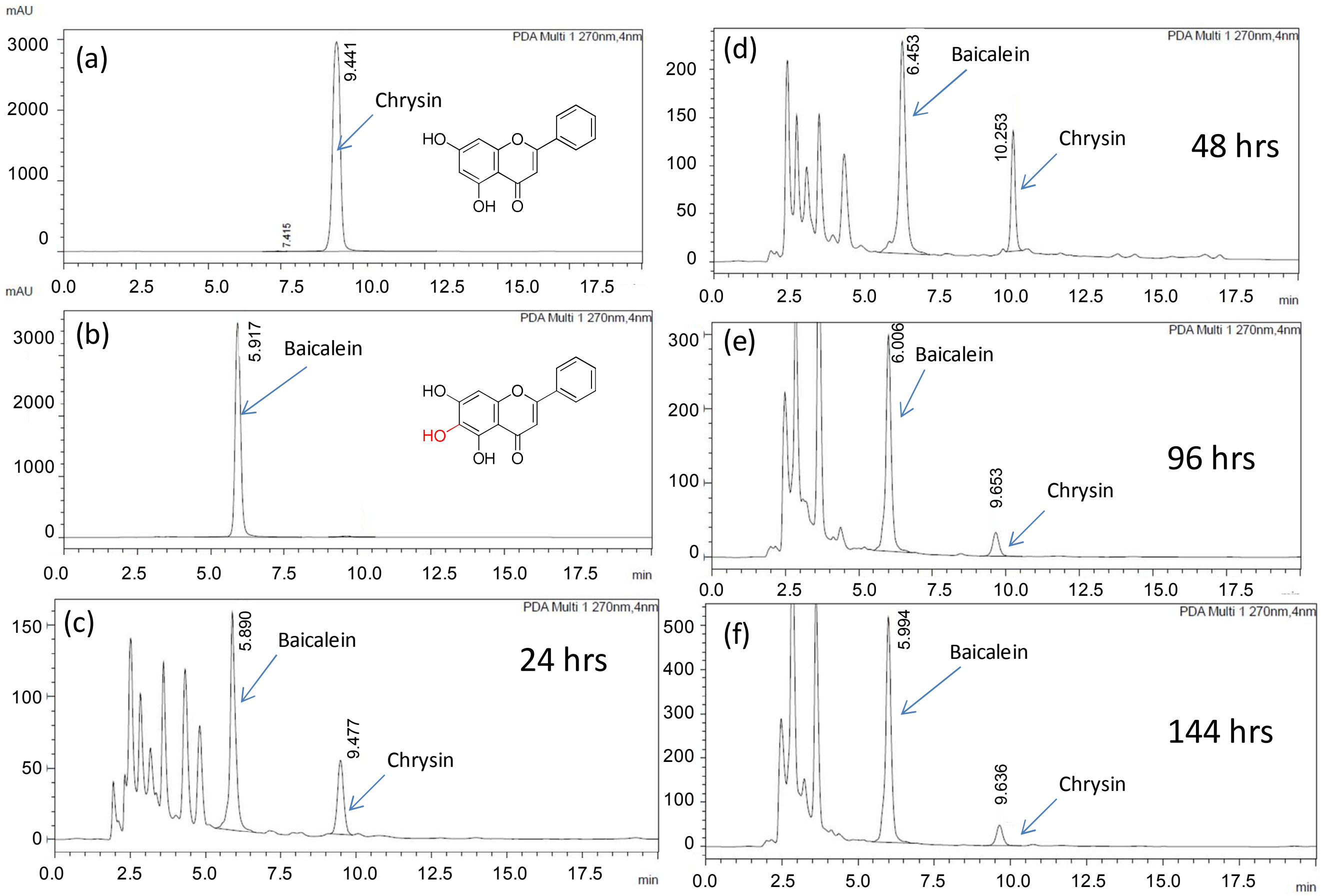
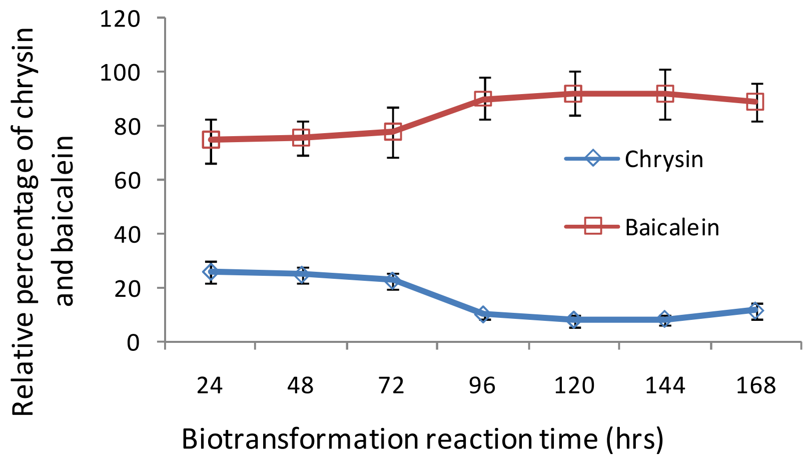
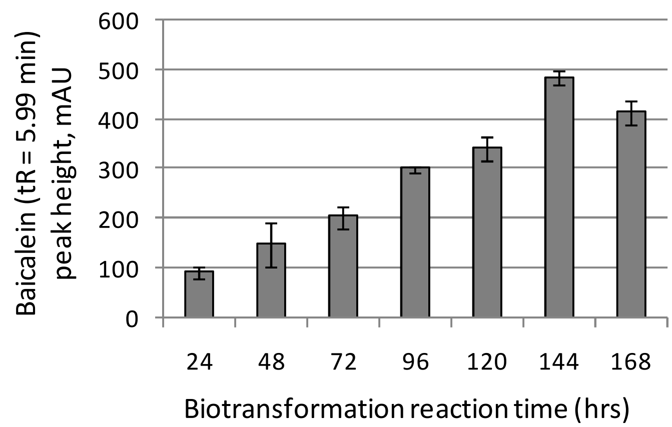

(a)
Chrysin
9.441
HO
OH O
(b)
Baicalein
5.917
(c)
Baicalein
Chrysin
24 hrs
(d)
Baicalein
Chrysin
48 hrs
(e)
Baicalein
Chrysin
96 hrs
(f)
Baicalein
Chrysin
144 hrs

Figure 2.


Relative percentage of chrysin and baicalein
120
100
80
60
40
20
0
24
48
72
96
120
144
168
Biotransformation reaction time (hrs)
Chrysin
Baicalein

(a)


Baicalein (tR = 5.99 min) peak height, mAU
600
500
400
300
200
100
0
24
48
72
96
120
144
168
Biotransformation reaction time (hrs)

(b)

Figure 3.

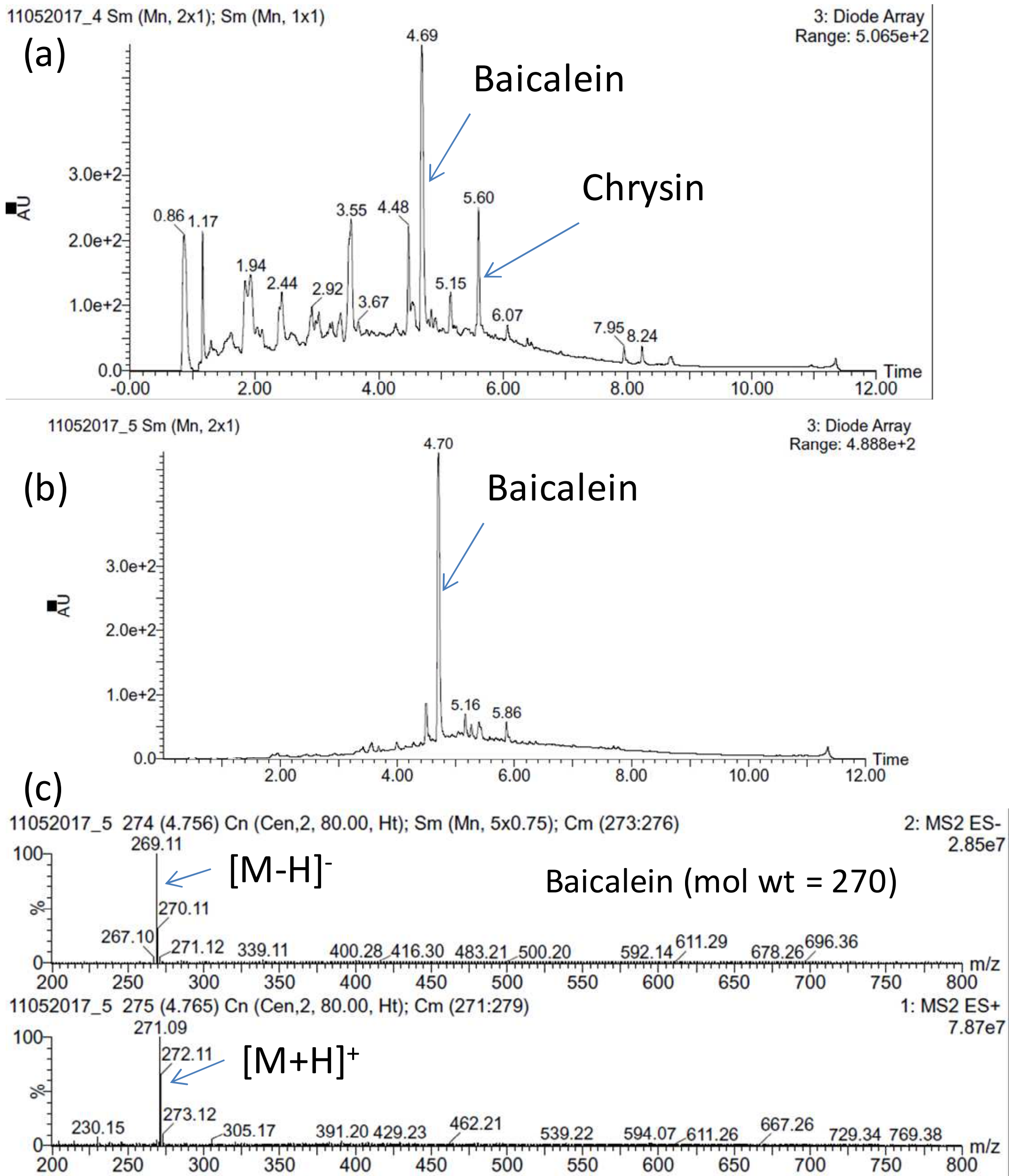

11052017_4 Sm (Mn, 2x1); Sm (Mn, 1x1)
3: Diode Array
Range: 5.065e+2
(a)
Baicalein
Chrysin
AU
Time
11052017_5 Sm (Mn, 2x1)
3: Diode Array
Range: 4.888e+2
(b)
Baicalein
AU
Time
(c)
11052017_5 274 (4.756) Cn (Cen,2, 80.00, Ht); Sm (Mn, 5x0.75); Cm (273:276)
2: MS2 ES-
2.85e7
[M-H]-
Baicalein (mol wt = 270)
m/z
11052017_5 275 (4.765) Cn (Cen,2, 80.00, Ht); Cm (271:279)
1: MS2 ES+
7.87e7
[M+H]+
m/z

Figure 4.

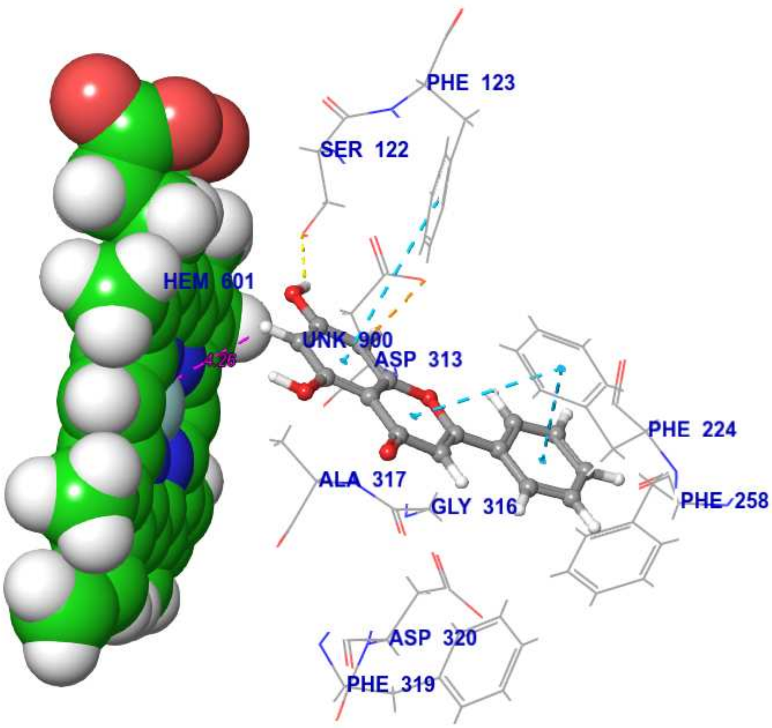
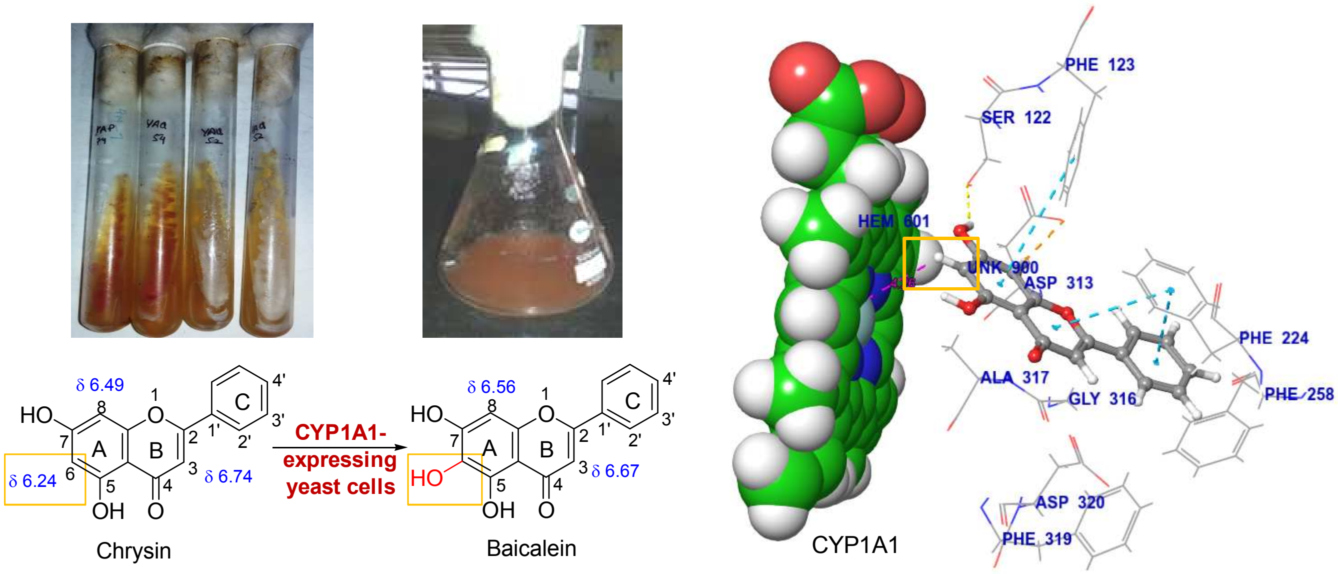

PHE 123
SER 122
HEM 601
UNK 900
ASP 313
PHE 224
PHE 258
ALA 317
GLY 316
ASP 320
PHE 319

Figure 5.

TOC graphic


δ 6.49
HO
δ 6.24
OH
O
Chrysin
δ 6.74
CYP1A1-
expressing
yeast cells
δ 6.56
HO
HO
OH
O
Baicalein
δ 6.67
A
B
C
HEM 601
UNK 900
PHE 123
SER 122
ASP 313
PHE 224
ALA 317
GLY 316
PHE 258
ASP 320
PHE 319
CYP1A1