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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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20

21 **ABSTRACT:**

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

35

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

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39 INTRODUCTION

40 Baicalein (5,6,7-trihydroxyflavone) (**1**) was originally isolated from the roots of *Scutellaria baicalensis*
41 (Chinese skullcap) which is used for treatment of chronic hepatitis, inflammatory diseases, tumors, and
42 diarrhea in China, Korea, Taiwan, and Japan.¹ Its isolation has also been reported from another plant,
43 *Oroxylum indicum* (Indian trumpet flower).² Baicalein is known for its anticancer properties in various
44 cancer types²⁻²² including pancreatic, gastric and colorectal cancers, multiple myeloma, head and neck
45 cancer, and breast cancer.^{23, 24} Baicalein is also considered as an anti-inflammatory^{25, 26} that generally
46 protects against oxidative stress,²⁷ more specifically in cardiac cells,²⁸ and in cisplatin-induced acute
47 kidney injury.²⁹ Because of its wide-ranging medicinal applications, an industrial scale protocol for its
48 production would be considered as important.

49 Baicalein has primarily been produced via five approaches: (a) single-step hydrolysis of the natural
50 product baicalin, a glycoside,³⁰ (b) 4-step total synthesis from a cinnamic acid derivative,³¹ (c) 4-step
51 total synthesis from 3,4,5-trimethoxyphenol,³² (d) 3-step total synthesis from 2,4,5,6-
52 tetrahydroxyacetophenone,³³ and (e) 6-step synthesis from chrysin³⁴ involving methylation, bromination,
53 acylation, nucleophilic replacement of Br with OMe, followed by de-acylation and demethylation as key
54 steps.

55 Selective transformation, using efficient biocatalysts, of a low value phytochemical to high value
56 phytochemical is an exciting area of bioorganic chemistry. Sordon and coworkers³⁵ have reported
57 biotransformations of natural flavonoids naringenin, hesperetin, chrysin, apigenin, luteolin, quercetin,
58 epicatechin, and biochanin A using the natural yeast, *Rhodotorula glutinis*. This approach produced
59 norwogonin (5,7,8-trihydroxyflavone) from chrysin (5,7-dihydroxyflavone). Here we report a
60 preparative scale process for biotransformation of chrysin to baicalein, via selective C6-hydroxylation
61 using recombinant human cytochrome P450-1A1 (CYP1A1) enzyme expressed within baker's yeast
62 (*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. ^1H NMR spectra were recorded on Bruker-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 $^\circ\text{C}$). 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 $^\circ\text{C}$. 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 OD₆₀₀ of ~90.

The harvested cells, ~3.0 mL (OD₆₀₀, ~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 (CD₃OD, 400 MHz, δ ppm): 7.91 (dd, *J* = 4.0, 8.0 Hz,

112 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
113 identical to that reported in the literature³² and TLC matched with reference sample obtained from
114 Sigma-Aldrich (CAS number 491-67-8).

115 **Molecular modeling:** The docking of chrysin with CYP1A1 (PDB ID: 4I8V) was performed using
116 GLIDE module of Schrodinger molecular modeling software, using the protocols as described in our
117 earlier publications.^{36, 37} The docking protocol was validated by docking known ligand α -
118 naphthoflavone (ANF). The interaction pattern of docked ANF and ligand from co-crystallized protein
119 (4I8V) are shown in the supporting information (Section S8).

120 RESULTS AND DISCUSSION

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

133 **Selection of CYPs for biotransformation of chrysin.** In this work, use of recombinant CYP-
134 expressing whole yeast cells has been explored for their ability to be used as biocatalysts for
135 biotransformation reactions. For this purpose, we chose the flavonoid chrysin as a substrate. As the

136 cytochrome P450 family 1 enzymes CYP1A1 and CYP1A2 are reported to oxidize dietary flavonoids,⁴⁰⁻
137 ⁴² herein we chose two yeast-expressed enzymes of the CYP1 sub-family (i.e. CYP1A1 and CYP1A2)
138 as biocatalysts.

139 Two recombinant yeast strains, each containing two chromosomally integrated copies of *CYP1A1* and
140 *CYP1A2* genes under the control of the ethanol-inducible alcohol dehydrogenase 2 (*ADH2*) promoter,
141 were created to enable stable and reproducible biotransformation reactions (plasmid maps shown in
142 Supporting Information, S2). The *ADH2* promoter is repressed in the presence of glucose. The aim was
143 that recombinant yeast cells, containing stably integrated *CYP* expression cassettes, would be grown
144 initially in complete YPD medium (containing 2% glucose) to obtain large optical density, measured at a
145 wavelength of 600 nm (OD₆₀₀) of ~90, in shake flasks. This would occur without any plasmid loss and
146 would be achieved over 3 days through repetitive replenishment of glucose, every 24 h, in the non-
147 selective highly nutritious YPD medium. Cells grown in YPD (Figure 1a) would then be re-suspended
148 in minimal selective SD medium (pH 7.0), supplemented with 1.5% of glucose, for the
149 biotransformation reaction. It was thought that rich full YPD medium may not be appropriate for
150 biotransformation since there is a possibility of substrate binding to its ingredients.

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

156 **Figure 1.**

157 The reaction media at each time point was extracted with ethyl acetate. The extracts were analyzed by
158 TLC followed by HPLC/ LC-MS. The formation of new product on TLC with lower R_f value than the
159 substrate gave us an indication of a hydroxylation reaction (Supporting Information, S3). LC-MS

160 analysis of the reaction mixture confirmed the product as mono-hydroxy chrysin with mass of m/z 270.
161 The same product was formed using both the enzymes, CYP1A1 and CYP1A2, expressed within yeast
162 cells. However, conversion of substrate to product was better with CYP1A1. Therefore, for subsequent
163 scale-up experiments, only CYP1A1-expressing yeast cells were used for biotransformation.

164 **Scale up and optimization of biotransformation reaction.** Scale-up was performed as above; cells
165 were at first cultivated in non-selective YPD media for 72 h, with fresh glucose (2%) being added every
166 24 h. Cells were re-suspended in selective SD minimal medium. Chrysin at different concentrations *viz.*
167 0.2, 1, 2, 5 and 10 mg/mL were dissolved in DMSO and further incubated in 50 mL of SD medium
168 (keeping the DMSO concentration < 0.5%) for 72 h at 28 °C, 200 rpm. The chrysin-containing cell
169 culture media were replenished with glucose every 24 h to a final concentration of 1.5%. The reaction
170 using chrysin at concentration of 2 mg/mL (100 mg of chrysin) was observed to be the most efficient.
171 With 5 and 10 mg/mL concentrations, incomplete biotransformation occurred, probably because the
172 small number of cells, used for growth in these specific experiments, was inhibited by the substrate.

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

181 **Figure 2**

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

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

187 **Figure 3.**

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

193 The ^1H NMR of chrysin contains a typical bunch of three singlets at chemical shift values in the range of
194 6 to 7 ppm. These three singlets at δ 6.24, 6.49 and 6.74 ppm correspond to the protons present at C6,
195 C8 and C3 positions. The C6-proton appears with an up-field shift in comparison to two other aromatic
196 protons at C3 and C-6, because of the shielding effect from two adjacent C5 and C7 hydroxyls. It is
197 obvious that the hydroxylation reaction is possible, either on A or C ring. Since there was no change in
198 the chemical shift value pattern of C ring (δ 7.91, dd, 2H and δ 7.48, m, 3H), it would indicate that the
199 C-ring is intact and no hydroxylation had taken place on this ring. The hydroxylation on A ring has two
200 possibilities, either C6- or C8 hydroxylation. Hydroxylation at C6-position will form baicalein (5,6,7-
201 trihydroxyflavone) whereas hydroxylation at C8-position will form norwogonin (5,7,8-
202 trihydroxyflavone) as a product. It was interesting to see that, in the ^1H NMR of the obtained product,
203 the up-field singlet (δ 6.24 ppm) disappeared. This singlet peak in chrysin corresponds to the proton
204 present at C6-position. This gave us a clear indication that hydroxylation occurred at the C6-position,
205 which means that the product is 5,6,7-trihydroxyflavone. This is the naturally occurring flavone,
206 commonly named as 'baicalein'. Furthermore, on comparison of the ^1H NMR of the obtained product
207 with norwogonin,³⁵ the possibility of norwogonin as the product was ruled out. In literature, the
208 biotransformation of chrysin using natural yeast, *Rhodotorula glutinis* yielded C-8 hydroxylated product

209 norwogonin,³⁵ however in the present study, C-6 hydroxylated product (baicalein) was formed, which
210 may be possibly because of the regio-specificity of the CYP1A1 enzyme.

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

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

217 **Figure 4.**

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

227 **Figure 5.**

228 Our efforts using yeast whole cells have resulted in the development of a reproducible preparative-scale
229 biotransformation process for the conversion of chrysin to baicalein (5,6,7-trihydroxyflavone).
230 According to the literature, the medicinal effects of baicalein are more profound than that of chrysin.
231 Furthermore, commercially available baicalein is at least 60-times more expensive than chrysin. Thus,

232 this protocol described here can be utilized for production of a high value phytochemical from a low
233 value one, using a simple, low-cost, one-step biotransformation reaction.

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

240 ASSOCIATED CONTENT

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

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252 Author Contributions

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

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

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261 **Notes**

262 The authors declare no competing financial interest.

263

264 **ABBREVIATIONS**

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

270

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FIGURE LEGENDS

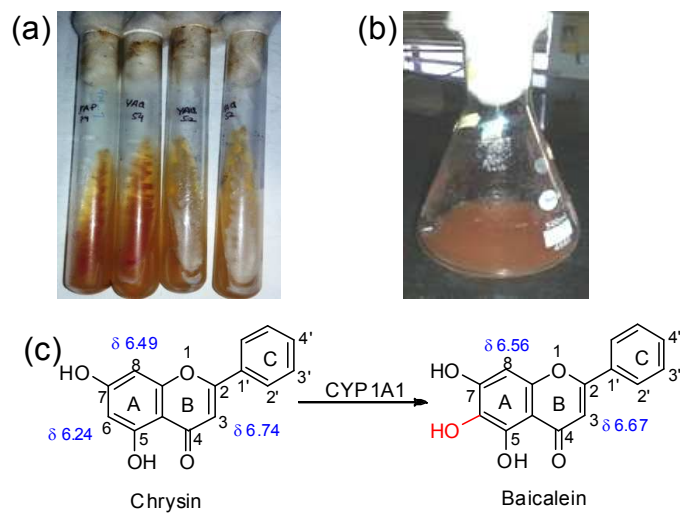
Figure 1. (a) CYP1A1-expressing cells grown in YPD Medium in test-tubes; (b) Biotransformation reaction using CYP1A1 in SD medium in shake-flasks; (c) scheme showing conversion of chrysin to baicalein, and their key ^1H NMR chemical shift values

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 (\pm 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 (\pm 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.

**Figure 1.**

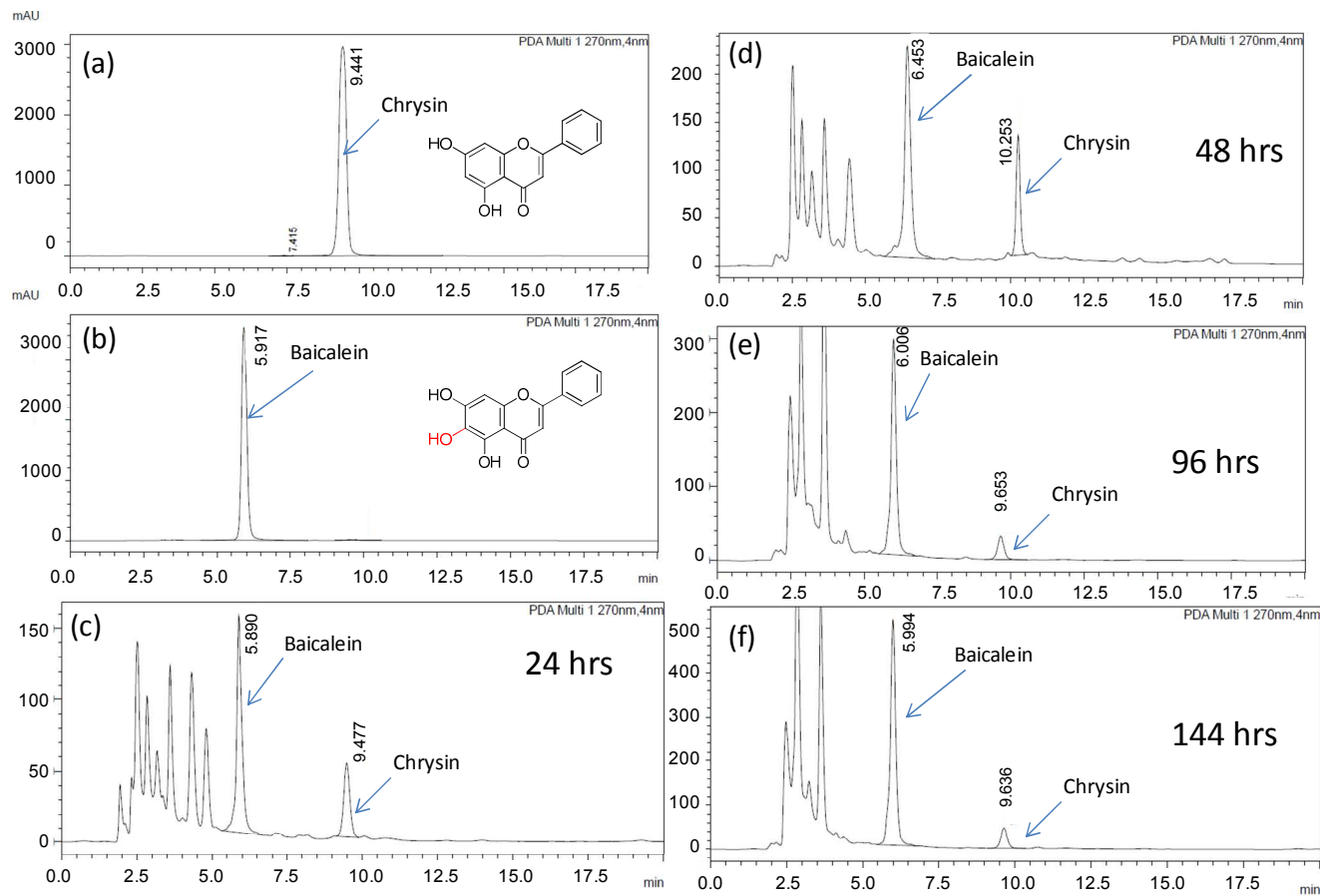
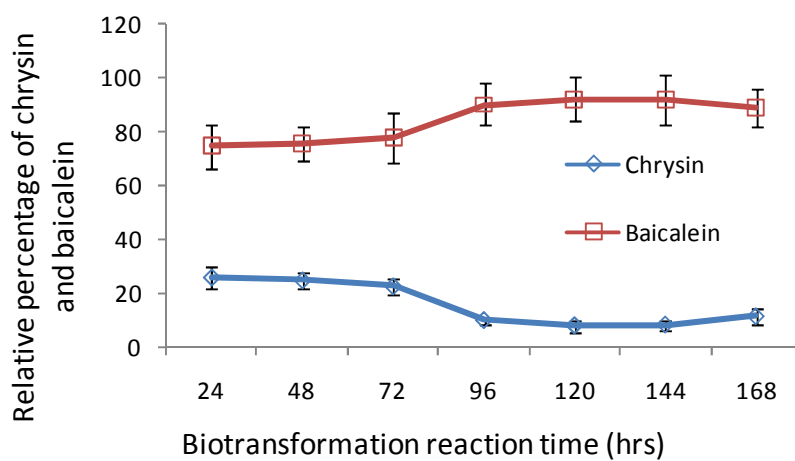
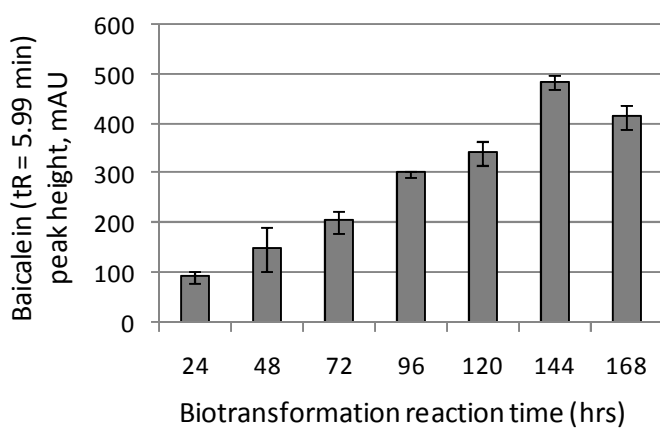


Figure 2.



(a)



(b)

Figure 3.

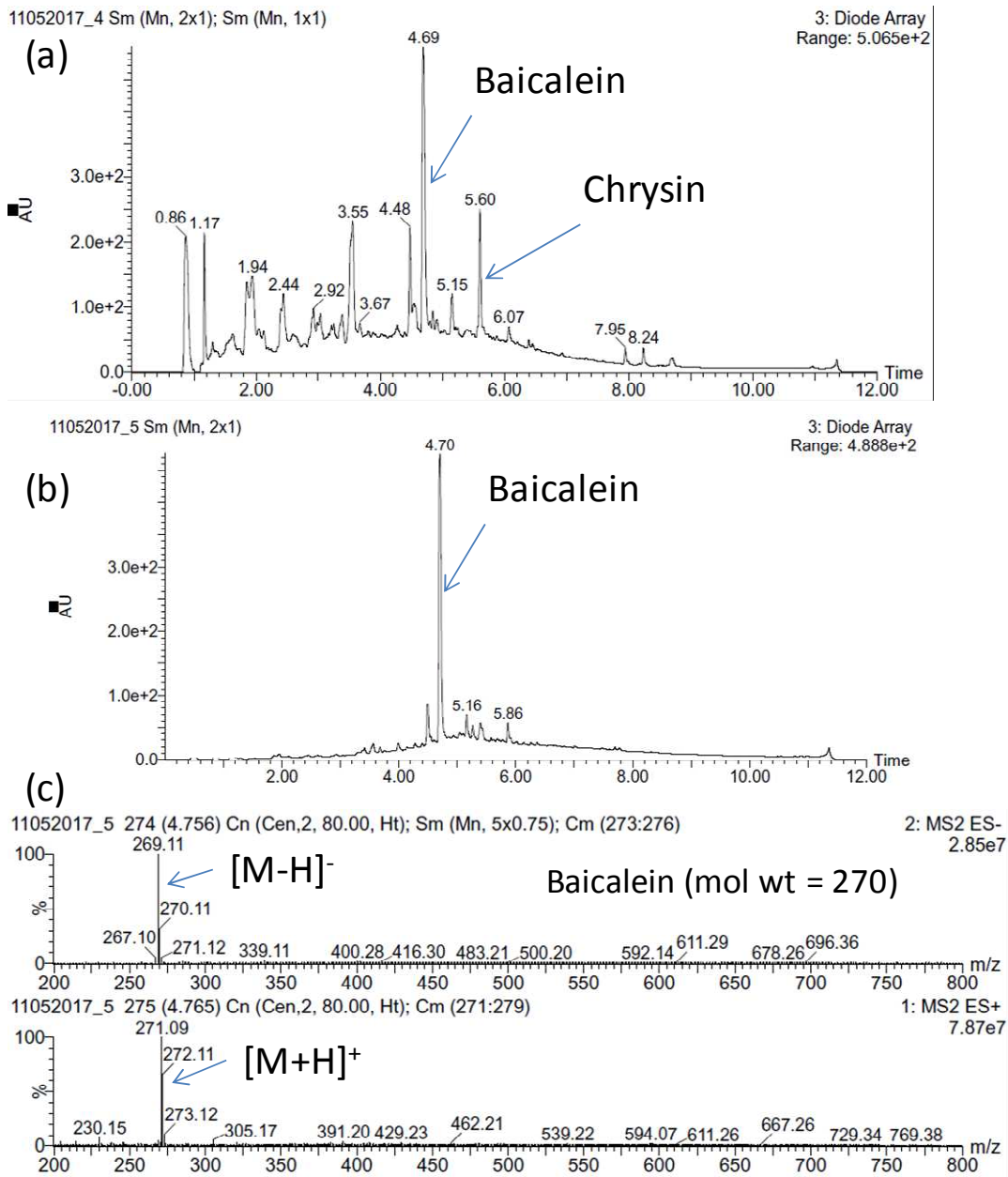


Figure 4.

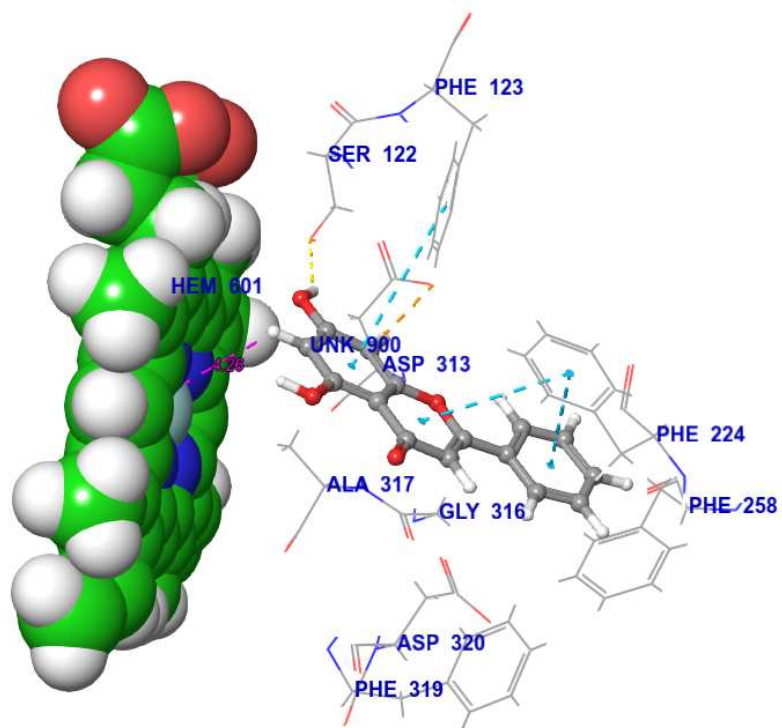


Figure 5.

TOC graphic

