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Biotransformation of Flavonoids with *O*-Methyltransferase from *Bacillus cereus*

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Abstract *O*-Methylation is a common modification reaction found in nature, and is mediated by an *O*-methyltransferase (OMT). OMTs have been mainly studied in plants, whereas only a few OMTs have been studied in microbes. When searching the *Bacillus cereus* genome, four putative small molecular OMTs were identified, among which *BcOMT-1* was cloned and expressed in *E. coli* as a his-tag fusion protein. The whole cell expressing *BcOMT-1* was used to methylate several flavonoids. Eriodictyol, luteolin, quercetin, and taxifolin, all of which contain 3' and 4' hydroxyl groups, served as methyl group acceptors for BcOMT-1, whereas naringenin, apigenin, 3,3'-dihydroxyflavone, and 3,4'-dihydroxyflavone did not function as substrates. Analysis of the reaction products using HPLC showed two different peaks, and NMR revealed that the methylation position was at the hydroxyl group of either carbon 3' or 4'. Therefore, this showed that BcOMT-1 used flavonoids containing ortho hydroxyl groups and transferred a methyl group to either of two hydroxyl groups.

Key words: *Bacillus cereus*, biotransformation, flavonoids, *O*-methyltransferase

The biological modification of natural compounds is an attractive area, as it can provide chirality and regioselectivity [17]. Various organisms have been used for biotransformation [11, 12, 18]. However, since the completion of genome projects for certain organisms, it is now feasible to choose a particular gene, analyze its product, and predict a possible substrate, thereby appearing a new tool to manipulate natural products. Flavonoids, a natural product found in nature, are good target molecules for structural modifications, as they include several available active hydroxyl groups [1, 13] that can act as acceptors for methyl or glucose groups.

Methyltransferases are classified into three groups depending on the substrate position: *C*-methyltransferase (CMT), *N*-methyltransferase (NMT), and *O*-methyltransferase (OMT) [8]. OMTs transfer a methyl group to a hydroxyl group in the substrate and produce a methoxy group. Therefore, *O*-methylation is one of the common reactions found in flavonoids. OMTs that use flavonoids as substrates have been studied in plants, since flavonoids are plant-originating compounds [5, 7]. Initially, plant OMTs with molecular weights ranging from 38 to 43 kDa were found to mediate the methylation reaction of flavonoids; yet more recently, smaller molecular weight OMTs ranging from 23 to 27 kDa were also shown to methylate flavonoids [4]. The latter OMTs increased their activity in the presence of Mg^{2+} and thus are called Mg^{2+}-dependent OMT. Meanwhile, only a few OMTs from microorganisms have been reported, and mainly related to the modification of antibiotics as a part of an antibiotic biosynthetic gene cluster [14]. Microorganisms are able to sustain in diverse environments, making them a diverse sources of genetic materials. It is assumed that microorganisms contain enzymes that metabolize flavonoids, and the completion of genome projects from numerous microorganisms makes it possible to clone any gene. Accordingly, based on an interest in modification of flavonoids using genes from diverse sources, including microorganisms, the present study reports on the cloning, heterologous expression, and characterization of an OMT, *BcOMT-1*, from *Bacillus cereus*.

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MATERIALS AND METHODS

Chemicals

The flavonoids and phenolic compounds were purchased from Indofine Chemicals (Somerville, NJ, U.S.A.), and HPLC-grade organic solvents were purchased from Duksan Co. (Ansan, Korea).

Cloning of *BcOMT-1*

Two primers, ATCATATGAAACAAATTAATCGTTATA-TTGAT for forward primer and ATGGATCCTTATTTCT-TCACTTTAGAAACCGTC for reverse primer, were designed based on the sequence of GenBank accession number 30020519. At the 5'-end of each primer, a restriction site (*Nde*I site for forward primer and *Bam*HI site for reverse primer) and two additional bases were attached to facilitate the cloning. The PCR reaction was carried out under the following conditions: 30 cycles of denaturation for 1 min at 95°C, annealing for 1 min at 55°C, and extension for 1 min at 72°C. The PCR product was sequenced and cloned into *Nde*I/*Bam*HI sites of *E. coli* expression vector pET-15b (Novagen, Madison, WI, U.S.A.).

Methyltransferase Reaction with Whole Cells of *E. coli* Expressing *BcOMT-1*

E. coli BL21 transformant harboring *BcOMT-1* was grown in LB medium containing 50 μg/ml ampicillin until absorbance at 600 nm reached 0.8. At this point, IPTG was added to a final concentration of 1 mM and the culture was grown for 5 more hours at 30°C. The cells were harvested and resuspended in the LB medium containing 50 μg/ml ampicillin. Substrates were added in the final concentrations at 70 μM and the mixture was incubated at 30°C for 18 h. The supernatant was then collected and extracted using ethyl acetate. Finally, the ethyl acetate extract was evaporated in a speed vacuum and dissolved in methanol.

Analysis of Metabolites Produced by BcOMT-1

The sample was analyzed by thin layer chromatography on TLC plates (Silicagel 60 F254, Merck, Germany) with a solvent system of benzene/ethyl acetate (2/1). The plate was visualized by ultraviolet light or spraying with 1% $FeCl_3$ solution, and the analytical HPLC performed using a Varian HPLC (Walnut Creek, CA, U.S.A.) equipped with a photodiode array detector. To analyze the flavonoids, a Waters Symmetry C18 column (3.5 μm particle size, 4.6× 250 mm, Milford, MA, U.S.A.) was used with a mobile phase, consisting of 0.1% formic acid (pH 3.0). The elution program for the mobile phase, which was composed of phosphate buffer-acetonitrile solution containing 0.1% formic acid, was as follows: 10% acetonitrile at 0 min, 30% acetonitrile at 10 min, 60% acetonitrile at 40 min, 90% acetonitrile at 45 min, 10% acetonitrile at 50 min. The flow rate was 1 ml/min and UV detection was performed at 340 nm.

Determination of Methylation Position Using Nuclear Magnetic Resonance Spectroscopy

The reaction products were separated using HPLC, as described above. For identification by nuclear magnetic resonance (NMR) spectroscopy, each eluent containing the reaction products was collected twenty times and evaporated under reduced pressure. The dried remnant was dissolved in ethyl a cetate, and the supernatant separated by centrifugation and evaporated again under reduced pressure. The final remnant was dissolved in dimethylsulfoxide-d6 for the NMR experiments. NMR spectroscopy was carried out as described by Kim *et al.* [10].

RESULTS AND DISCUSSION

Isolation and Expression of *BcOMT-1*

The *B. cereus* genome has already been completed and most genes were annotated [6]. Therefore, when searching for the *B. cereus* protein coding genes, four *O*-methyltransferase genes were found: *BcOMT-1*, *BcOMT-2* (GenBank accession number 30022459), *BcOMT-3* (GenBank accession number 30019359), and *BcOMT-4* (GenBank accession number 30020293). All the OMTs found in *B. cereus* have a lower molecular weight and contain catechol *O*-methyltransferase (*COMT*), caffeoyl-CoA *O*-methyltransferase (*CCoAOMT*), and a family of bacterial *O*-methyltransferases that may be involved in antibiotic production. A BLAST analysis of the four BcOMTs revealed that BcOMT-1 and -2 were likely to be involved in the methylation of small compounds, such as flavonoids, whereas BcOMT-3 is predicted to be involved in antibiotic modification and BcOMT-4 is predicted to transfer a methyl group to protein. A primer set was designed for *BcOMT-1* and the gene cloned by a PCR. *BcOMT-1* consists of a 636-bp open reading frame that encodes a 22.8-kDa protein, and showed a 55–58%

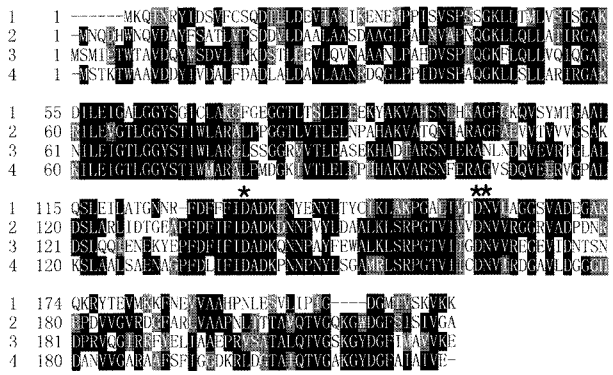


Fig. 1. Alignments of BCOMT1 with other OMTs from various OMTs.

1, *B. cereus* BcOMT-1; 2, *Burkholderia cepacia*; 3, *Bacillus clausii*; 4, *Mesorhizobium loti*. Asterisk (*) above the amino acid indicates Mg^{+2} binding amino acids.

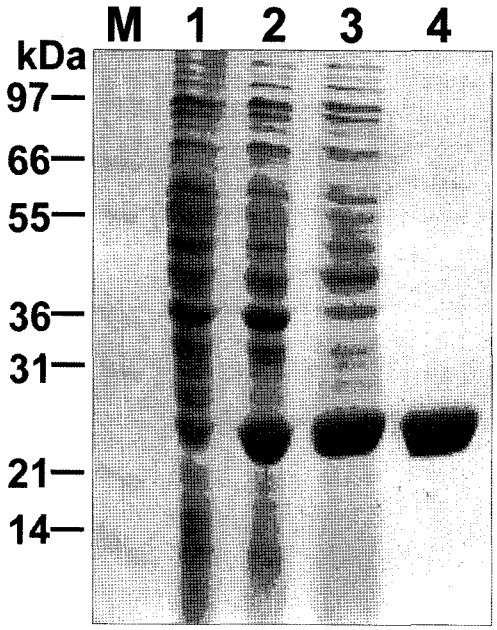


Fig. 2. Expression and purification of recombinant BcOMT-1. M, molecular weight marker; 1, uninduced *E. coli* lysate; 2, induced *E. coli* total lysate; 3, soluble fraction of induced *E. coli* lysate; 4, purified recombinant BcOMT-1.

similarity with OMTs from *Burkholderia cepacia*, *Bacillus clausii*, and *Mesorhizobium loti*, none of which have not been functionally characterized (Fig. 1). In addition, it displayed a similarity (30–40%) with other lower molecular weight OMTs, such as COMT from mice and CCoAOMTs from plants. Furthermore, a comparison of BcOMT-1 with rat catechol OMT, for which the three-dimensional structure has already been determined [18], revealed that the Mg^{+2} binding sites in BcOMT-1 were the same as those in rat catechol OMT.

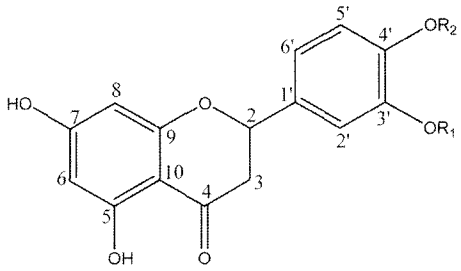
To express *BcOMT-1* in *E. coli*, the open reading frame of *BcOMT-1* was cloned into a pET15b vector and expressed as a His-tag fusion to facilitate purification. As shown in Fig. 2, BcOMT-1 was successfully expressed and purified, and the BcOMT-1 was about 24 kDa based on SDS-PAGE, which agreed with the sum of the predicted molecular weight of BcOMT-1 and the few extra amino acids for purification.

Characterization of *BcOMT-1*

The amino acid sequence of *BcOMT-1* revealed a potential involvement in the modification of caffeoyl-CoA, which provides the building blocks for lignin biosynthesis. However, the recent elucidation of additional functions in this class of OMTs [4] prompted an investigation of BcOMT-1 as regards the modification of flavonoids. Instead of using the expressed BcOMT-1 for the substrate study, an *E. coli* transformant expressing BcOMT-1 was used.

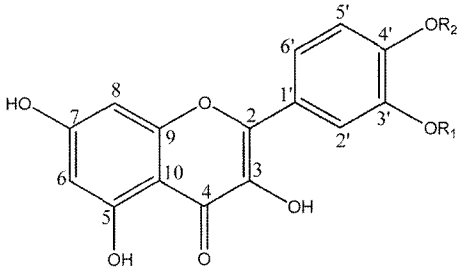
The whole cells containing *BcOMT-1* were incubated with apigenin, caffeic acid, catechol, esculetin, genistein, luteolin, naringenin, or quercetin as substrates to determine whether BcOMT-1 could modify any of these compounds. As a negative control, an *E. coli* transformant containing the empty vector was used. Analysis of the reaction products from BcOMT-1 using thin layer chromatography showed that only esculetin, luteolin, and quercetin gave a new product with a different R_f value, whereas no new reaction product was detected in the negative control. The reaction products from the three substrates were analyzed further using HPLC. Esculetin that contains 6' and 7' hydroxyl groups produced two products, corresponding to scopoletin (7-hydroxy-6-methoxy esculetin) and isoscopoletin (6-hydroxyl-7-methoxy esculetin). However, the conversion rate of esculetin was only about 30% compared with that of eriodictyol (see below). Analysis of the luteolin reaction product by HPLC revealed one peak, whereas the quercetin reaction product(s) revealed two peaks. Among the flavonoids tested, only luteolin and quercetin contained a 3'-hydroxyl group. Thus, it would appear that BcOMT1 transferred a methyl group to the 3'-hydroxy group in the flavonoids. Therefore, using flavonoids containing a 3'-hydroxy group, such as eriodictyol and taxifolin, as the potential substrates of BcOMT-1, the reaction products were analyzed by HPLC. The reaction products from eriodictyol and taxifolin generated two new peaks that exhibited different retention times from those for substrates (Table 2). In addition, an LC/MS analysis of the reaction products from the above four substrates showed an increase of 14 Da in the determined masses, corresponding to the addition of a methyl group to the products formed. The HPLC profiles for the eriodictyol reaction products were compared with those of the authentic 3'-methoxy eriodictyol (homoeriodictyol) and 4'-methoxy eriodictyol (hesperetin). The reaction product eluted at 14.57 min with eriodictyol had identical retention times and UV spectra to the authentic homoeriodictyol, while the product at 14.94 min had the same retention times and UV spectra as the authentic hesperetin (Fig. 3), indicating that BcOMT-1 transferred a methyl group to a 3' or 4' hydroxyl group in eriodictyol. No 3', 4'-dimethoxy eriodictyol was observed. Furthermore, the retention time and UV spectra for the reaction products from luteolin, quercetin, and taxifolin were also compared with those for the corresponding authentic methylated compounds, if available, including chrysoeriol (3'-methylated luteolin), diosmetin (4'-methylated luteolin), isorhamnetin (3'-methylated quercetin), tamarixetin (4'-methylated quercetin), and hesperetin (4'-methylated taxifolin). The retention time and UV spectra for the reaction products from each substrate were identical to those for the corresponding authentic compounds, thereby implying that BcOMT-1 transferred a methyl group to either a 3' or 4' hydroxyl group in the above compounds. To confirm these results,

Table 1. Assignment of ^{1}H NMR data of metabolites I and II from eriodictyol and quercetin produced by BcOMT1.



Metabolite I; R_1=CH_3, R_2=H

Metabolite II; R_1=H, R_2=CH_3



Metabolite I; R_1=CH_3, R_2=H

Metabolite II; R_1=H, R_2=CH_3

Position	Eriodictyol				Position	Quercetin			
	Homoeriodictyol	Metabolite I	Hesperetin	Metabolite II		Isorhamnetin	Metabolite I	Tamarixetin	Metabolite II
	^{1}H/ppm(Hz)	^{1}H/ppm(Hz)	^{1}H/ppm(Hz)	^{1}H/ppm(Hz)		^{1}H/ppm(Hz)	^{1}H/ppm(Hz)	^{1}H/ppm(Hz)	^{1}H/ppm(Hz)
2	5.42(dd;2.9,12.9)	5.43(dd;2.8,12.9)	5.42(dd;3.1,12.3)	5.43(dd;3.1,12.3)	6	6.19(d;2.1)	6.20(d;1.8)	6.19(d;2.0)	6.19(d;1.9)
3	2.68(dd;3.0,17.2)	2.68(dd;3.0,17.2)	2.71(dd;3.2,17.1)	2.71(dd;3.2,17.1)	8	6.47(d;2.1)	6.48(d;1.9)	6.42(d;2.0)	6.42(d;2.0)
	3.31(m)	3.31(m)	3.18(dd;12.3,17.1)	3.19(dd;12.4,17.1)	2'	7.75(d2.1)	7.75(d;1.8)	7.66(d;1.9)	7.67(d;2.6)
6	5.88(d;2.2)	5.88(d;2.2)	5.88(d;2.0)	5.87(d;2.1)	5'	6.94(d;8.5)	6.94(d;8.5)	7.08(d;8.4)	7.08(d;8.5)
8	5.89(d;2.2)	5.89(d;2.1)	5.90(d;2.1)	5.89(d;2.1)	6'	7.68(dd;2.1,8.5)	7.69(dd;2.0,8.4)	7.65(dd;2.3,8.4)	7.65(dd;2.3,8.8)
2'	7.09(d;2.0)	7.09(d;2.0)	6.93(d;2.0)	6.93(d;2.1)	3'-OMe	3.84(s)	3.84(s)	-	-
5'	6.79(d;8.1)	6.79(d;8.1)	6.92(d;8.3)	6.92(d;8.4)	4'-OMe	-	-	3.84(s)	3.84(s)
6'	6.90(dd;2.0,8.2)	6.90(dd;2.1,8.3)	6.86(dd;2.0,8.3)	6.87(dd;2.1,8.2)					
3'-OMe	3.78(s)	3.78(s)	-	-					
4'-OMe	-	-	3.77 (s)	3.77 (s)					

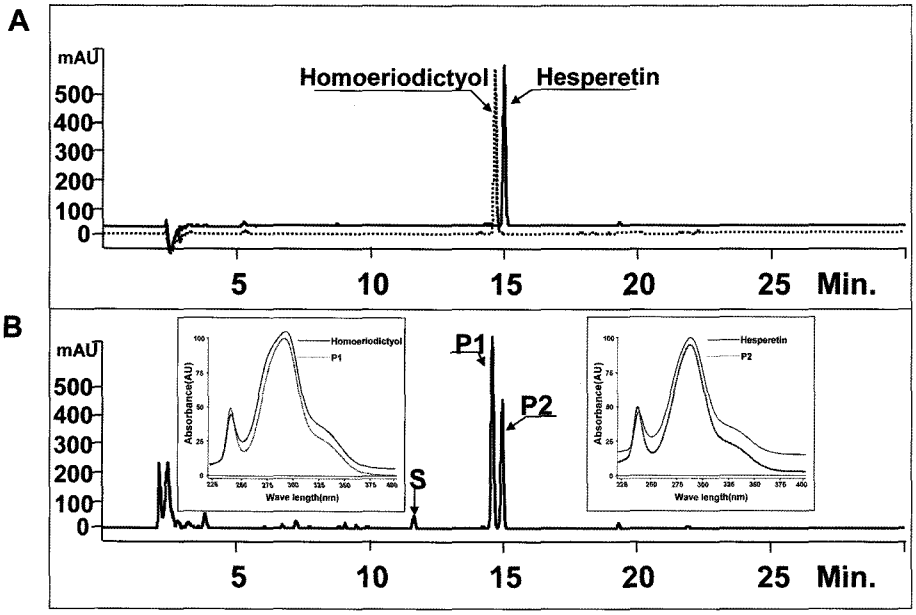


Fig. 3. HPLC profile of eriodictyol reaction product.
A. Authentic 3'-*O*-methylated eriodictyol (homoeriodictyol) and 4'-*O*-methylated eriodictyol (hesperetin). **B.** S, substrate (eriodictyol); P1, P2, reaction products of eriodictyol. Left inset is UV spectra for P1 (dotted line) and homoeriodictyol (straight line). Right inset is UV spectra for P2 (dotted line) and hesperetin (straight line).

the methylation positions of the eriodictyol and quercetin reaction products were determined using NMR. As the retention times for metabolites I and II from eriodictyol produced by BcOMT-1 were matched to homoeriodictyol and hesperetin, respectively, in the HPLC chromatogram, the ^{1}H NMR data for metabolites I and II were compared with that for homoeriodictyol and hesperetin, respectively. As listed in Table 1, the chemical shifts for each reaction product were identical to those for the corresponding authentic compounds. Likewise, the ^{1}H NMR data for the metabolites from quercetin were the same as that for isorhamnetin and tamarixetin, respectively.

BcOMT-1 did not methylate apigenin or naringenin, both of which contain a 4' hydroxyl group, yet not a 3' hydroxyl group. Furthermore, when 3,3'-dihydroxyflavone and 3,4'-dihydroxyflavone were used as substrates, BcOMT-1 did not provide any reaction products. Thus, when taken together, BcOMT-1 only methylated flavonoids containing 3' and 4' hydroxyl groups, and transferred a methyl group to either a 3' or 4' hydroxyl group in the flavonoids.

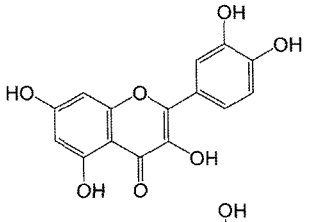
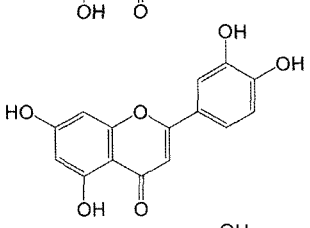
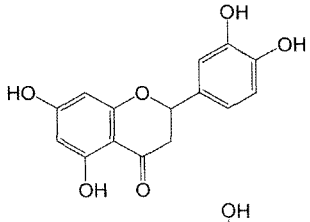
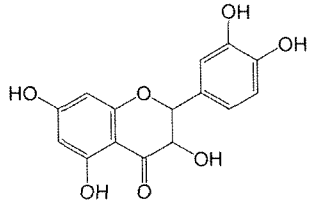
OMTs that use the flavonoids containing 3' and 4' hydroxyl as substrates are usually Mg^{2+}-dependent. The Mg^{2+} ion plays a role in binding ortho hydroxyl groups at the active sites with two possible configurations [3]. Once a hydroxyl group is methylated, the binding force between the Mg^{2+} and the substrate becomes weak and the substrate is released, resulting in just a monomethylated product. Thus, to examine whether BcOMT-1 was Mg^{2+}-dependent, 100 μM Mg^{2+} was added to the purized enzyme dialyzed in a 10 mM sodium phosphate buffer (pH 7.0). Analysis of the reaction product using HPLC showed that Mg^{2+} enhanced the enzyme activity by 20% (data not shown). Therefore, BcOMT-1 would appear to be a typical Mg^{2+}-dependent *O*-methyltransferase that transfers a methyl group to either ortho methyl group position with a different ratio.

The substrate preference of BcOMT-1 was investigated using flavanones, taxifolin and eriodictyol, and flavones, quercetin and luteolin. Eriodictyol was the best substrate tested, followed by taxifolin, luteolin, and quercetin. In addition, the flavanones (taxifolin and eriodictyol) turned out to be better substrates for BcOMT-1 than the flavones (quercetin and luteolin), indicating that the double bond in C-ring has a negative influence on BcOMT-1 reactivity. The presence of a 3 hydroxy group also decreased the methyl acceptor ability based on comparison between quercetin and luteolin and between taxifolin and eriodictyol (Table 2).

The *in vivo* function of BcOMT-1 is still unclear. It is known that some *Bacillus* sp. are able to degrade flavonoids [2]. Although several attempts were made to monitor the fate of flavonoids in *B. cereus*, the flavonoids were likely degraded after incubation with *B. cereus*. In *Streptomyces* sp., the *O*-methylation of flavonoids is the initial step for their degradation [15]. As microorganisms invariably confront exogenous compounds, including several phenolic compounds, they may have developed an ability to metabolize or utilize them.

The biotransformation kinetics of eriodictyol with *E. coli* expressing BcOMT-1 was analyzed as described in Kim *et*

Table 2. Relative activity of BcOMT-1 towards several substrates.

Substrate	Structure	Retention times (S/P1/P2; min)*	Conversion yields (μM)	Relative conversion rate (%)
Quercetin		11.78/14.85/14.99	86	90.9
Luteolin		11.52/14.38	76	80.0
Eriodictyol		11.74/14.59/14.96	94	100
Taxifolin		7.61/9.90/10.42	81	86.2

*S, Substrate; P1, Reaction product 1; P2, Reaction product 2 (100 μM of each substrate was used).
*The *E. coli* transformant expressing BcOMT-1 was used and reactions were carried out for 16 h.
*Relative conversion rate was calculated with activity against eriodictyol as 100%.

al. [10]. Eriodictyol was added at a final concentration of 70 μM and the supernatant was harvested at 0.5, 1, 1.5, 2, 2.5, 3, 4, 6, 8, 10, 12, and 24 h. The amount of eriodictyol continued to decrease with a corresponding increase in the amount of homoeriodictyol and hesperetin (Fig. 4). After 24 h of incubation at 30°C, the concentrations of eriodictyol (substrate), homoeriodictyol (reaction product), and hesperetin (reaction product) were 10.6, 25.1, and 16.5 μM, respectively. Thus, the sum of the substrate and the reaction products was 52.2 μM, which left about 18 μM unaccounted for when compared with the initial concentration of eriodictyol. However, the concentration of eriodictyol after 24 h incubation with *E. coli* transformant containing pET15b vector was reduced to 51.7 μM, indicating that about 18 μM of eriodictyol was metabolized.

The biotransformation of natural products, including flavonoids and alkaloids, has already been carried out by other research groups [16, 20]. As such, a methylation reaction is known to require a *S*-adenosyl-L-methionine (AdoMet) as a cofactor. However, this expensive cofactor is not needed when using a transgenic microbe, which is

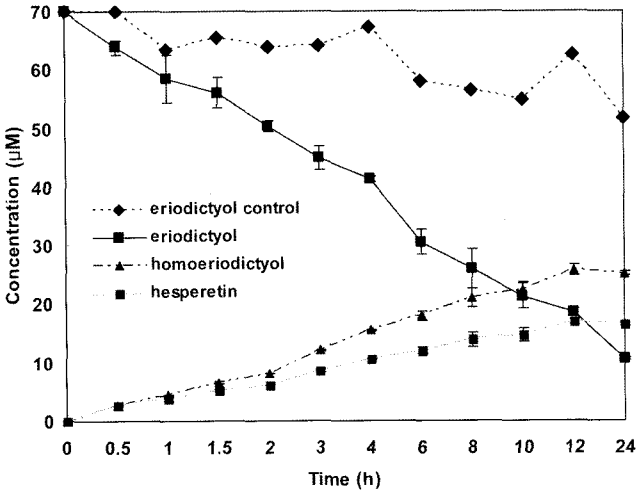


Fig. 4. Biotransformation of eriodictyol with *E. coli* expressing BcOMT-1.
The *E. coli* transformant containing *BcOMT-1* was grown in LB containing 50 μg/ml ampicillin and BcOMT-1 was induced. After addition of eriodictyol at 70 μM, the mixture was shaken at 30°C. Samples were collected at the indicated time and the amounts of reactant and product quantified using HPLC.

one of the advantages to using a biotransformation for the modification of natural compounds.

Flavonoids are mainly produced in plants. Even though several plant flavonoid OMTs have already been cloned and characterized, most of the characterized OMTs from plants have larger molecular weights than BcOMT-1 [9]. The only plant OMT with the similar molecular weight as BcOMT-1 was PFOMT from ice plant [4]. It also produced two reaction products with quercetin, and the ratio of 3'-*O*-methylated quercetin to 4'-*O*-methylated one is about 3:2. Thus, they have similar enzymatic characteristics, despite only 46% similarity at the amino acid level.

Acknowledgments

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