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| Title: | Combinatorial approaches for *de novo* production of flavonoids in *Escherichia coli* |


Abstract:

Polyphenols are plant secondary metabolites that are produced in response to biotic and abiotic stress. Flavonoids, one of the most representative group of these metabolites, includes at least 9000 compounds. Among them, naringenin has been widely studied due to its interesting biological activities, namely anticancer, anti-inflammatory, and antioxidant. Due to its potential applications and to the attempt to satisfy the industrial demand, there has been an increased interest in the heterologous production of this compound in a microbial chassis. The production of naringenin by a microorganism involves a first step of conversion of tyrosine into coumaric acid by tyrosine ammonia-lyase (TAL). Afterwards, coumaric acid is converted into coumaroyl-CoA by 4-coumarate-CoA ligase (4CL). Coumaroyl-CoA is further converted into naringenin chalcone by chalcone synthase (CHS) and then into naringenin by chalcone isomerase (CHI). In this work, we aimed to design, construct and validate an efficient biosynthetic pathway to produce naringenin in *Escherichia coli* by performing a step-by-step optimization. To construct the biosynthetic pathway to produce naringenin, TAL, 4CL, CHS, and CHI genes from different organisms were selected. Initially, TAL from *Rhodotorula glutanis* (*Rg*TAL) and TAL from *Flavobacterium johnsoniae* (*Fj*TAL) were cloned into the pRSFDuet-1 vector and were further expressed in three different *E. coli* strains (*E. coli* BL21, K12 MG1655 and M-PAR-121) to select the best enzyme and strain to produce coumaric acid. The highest production was obtained in the *E. coli* M-PAR-121 strain expressing *Fj*TAL (2.54 g/L of coumaric acid from 40 g/L glucose). *E. coli* M-PAR-121 is a tyrosine-overproducing strain and it can produce high amounts of tyrosine from glucose that can be converted into coumaric acid. Thus, this strain and enzyme were chosen to construct the complete biosynthetic pathway. Afterwards, 4CL and CHS steps were constructed and validated. Specifically, 4CL from *Arabidopsis thaliana* (*At*4CL), 4CL from *Vitis Vinifera* (*Vv*4CL), and 4CL from *Petroselinum crispum* (*Pc*4CL) were cloned into the pACYCDuet-1 vector. Moreover, CHS from *A. thaliana* (*At*CHS), CHS from *Petunia hybrida* (*Ph*CHS), and CHS from *Curcubita maxima* (*Cm*CHS) were cloned into pCDFDuet-1 vector. Twelve different combinations of the 4CL and CHS genes were expressed in the best strain able to produce coumaric acid and the naringenin chalcone production from glucose was evaluated. The best naringenin chalcone production was obtained in the *E. coli* M-PAR-121 strain expressing pRSFDuet_*Fj*TAL,

pACYCDuet_*At*4CL and pCDFDuet_*Cm*CHS (311.0 mg/L). Aiming to increase the productivity of the engineered strain, the metabolic burden of the cells was reduced by cloning the three genes in only two plasmids. From the different combinations tested, *E. coli* M-PAR-121 strain holding pRSFDuet_*Fj*TAL_*Cm*CHS and pACYCDuet_*At*4CL has reached the highest production of naringenin chalcone (560.2 mg/L). Afterwards, the last step of the biosynthetic pathway was validated. At this point, CHI from *A. thaliana*, CHI from *M. sativa* and CHI from *C. maxima* were tested. The maximum production was achieved in the *E. coli* M-PAR-121 strain expressing pRSFDuet_*Fj*TAL_*Cm*CHS and pACYCDuet_*At*4CL_*At*CHI (366.6 mg/L). This production was one of the highest reported so far in shake-flask experiments using glucose as substrate.