JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Biotechnology and Biological Transformations

Efficient biotransformation of luteolin to isoorientin through adjusting induction strategy, controlling acetic acid and increasing UDP-glucose supply in Escherichia coli

Jianjun Pei, Qing Sun, Linguo Zhao, Hao Shi, Feng Tang, and Fuliang Cao

J. Agric. Food Chem., **Just Accepted Manuscript** • DOI: 10.1021/acs.jafc.8b05958 • Publication Date (Web): 10 Dec 2018

Just Accepted

ACS Publications

is published by the American Chemical Society. 1155 Sixteenth Street N.W., Washington, DC 20036

Efficient biotransformation of luteolin to isoorientin through adjusting induction strategy, controlling acetic acid and increasing UDP-glucose supply in *Escherichia coli*

Jianjun Pei[1, 3#], Qing Sun[1,3#], Linguo Zhao[1, 3*], Hao Shi[2], Feng Tang[4], Fuliang Cao[1, 3]

[1] *College of Chemical Engineering, Nanjing Forestry University, Nanjing, China;*

[2]*Huanyin Institute of Technology, Huanyin, China*

[3] *Jiangsu Key Lab of Biomass-Based Green Fuels and Chemicals, Nanjing, China*

[4] *International Centre for Bamboo and Rattan, Beijing, China*

[*] Corresponding author, Linguo Zhao. College of Chemical Engineering, Nanjing Forestry University, Nanjing 210037, China. Phone: +86-025-85427962. E-mail: njfu2302@163.com, lgzhao@njfu.edu.cn.

[#] These authors contributed equally to this work

Abstract

Isoorientin is a *C*-glycosylated derivative of luteolin and exhibits a number of biological properties. In this study, multiple strategies were adopted to improve isoorientin production from luteolin in *Escherichia coli*. Isoorientin production was improved substantially by adjusting induction strategies and controlling acetic acid accumulation, with maximum isoorientin production reaching 826 mg/L. Additionally, a novel UDP-glucose synthesis pathway was reconstructed in *E. coli* through cellobiose phosphorylase-catalyzed phosphorolysis of cellobiose for the production of glucose 1-phosphate, which serves as a precursor in UDP-glucose formation. The results from two mechanisms of UDP-glucose formation in *E. coli*, cellobiose phosphorolysis and sucrose phosphorolysis, were compared. Increasing the UDP-glucose supply resulted in maximal isoorientin production reaching 1371 mg/L. Finally, isoorientin (1059 mg) was obtained from 1 L of fermentation broth by simple purification steps with a yield of 81.5%. Therefore, this study provides an efficient method for isoorientin production and a novel UDP-glucose synthesis pathway.

Keywords: Isoorientin; *C*-Glucosyltransferase; Cellobiose phosphorylase; Acetic acid; UDP-glucose

Introduction

C-glycosylflavones, which are conjugated with sugar residues through *C*-glycosidic bonds, are present in food and health-care products such as wheat, maize, rice, mung bean, blueberries, lemon juice, acai pulp, rooibos tea and bamboo leave extracts[1-7]. Compared with *O*-glycosylation, *C*-glycosylation is highly stable to acid and resistant to glycosidase hydrolysis[8]. Isoorientin (3',4',5,7-tetrahydroxy-6-*C*-glucopyranosyl flavone), a naturally occurring *C*-glycosylflavone, is found in many plants[7, 9-11]. Isoorientin has been reported to have many pharmacological activities, such as anticancer, antioxidant, hypotensive, anti-inflammatory, antispasmodic, antimicrobial, hepatoprotection, and antiglycation activities[12-19]. Although some *C*-glycosylflavones, including isoorientin, have been extracted from bamboo leaves and *Atractylodes japonica*[7, 20], it is difficult to prepare them because of their low concentration and complex composition in plants. Therefore, large-scale and low-cost *C*-glycosylflavone production has become important.

In plants, *C*-glycosylflavones are synthesized by *C*-glucosyltransferases. *C*-glycosylflavone-related genes from different plants have been reported[21-26]. *C*-glucosyltransferases can be divided into two groups on the basis of their substrate specificity. The *C*-glucosyltransferases in the first group, which catalyze the conversion of only the open-chain form of 2-hydroxyflavanone to *C*-glycosylflavone, were identified in rice, maize, and buckwheat[21-24]. 2-Hydroxyflavanone is generated from flavanone by the cytochrome P450 flavanone 2-hydroxylase in plants[27-28]. Subsequent to this discovery, Brazier-Hicks reconstructed a biosynthetic pathway of *C*-glycosylflavone in yeast by introducing a cytochrome P450 flavanone 2-hydroxylase gene and a *C*-glucosyltransferase gene from *Oryza sativa*. The recombinant yeast biosynthesized 2-hydroxynaringenin-*C*-glucoside from flavanone,

with a maximum titer of 7.2 mg/L[28]. Because it is difficult to express cytochrome P450 flavanone 2-hydroxylase genes in *Escherichia coli* and to prepare the open-chain form of 2-hydroxyflavanone, recombinant *E. coli* was not engineered to produce orientin, vitexin, isoorientin, or isovitexin using the first group of *C*-glucosyltransferases.

The second group of *C*-glucosyltransferases directly *C*-glucosylates the *C*-6 position of flavones, which has been identified only in *Gentiana triflora*[25]. A *C*-glucosyltransferase (Gt6CGT) gene from *G. triflora* was introduced to first construct a recombinant *E. coli* strain to optimize the UDP-glucose synthetic pathway, and the maximum titer of isoorientin in this strain reached 34 mg/L[29]. Thus, it is necessary to improve *C*-glycosylflavone production towards low-cost production.

The soluble expression of key enzymes, enzymatic activity, supply of UDP-glucose, regulation of metabolic processes, and fermentation conditions all affect *C*-glycosylflavone production in *E. coli*. Many recombinant proteins from plants often fail to fold into their native state and accumulate as inclusion bodies when the respective genes expressed in *E. coli*[30]. Thus, expression of genes encoding *C*-glucosyltransferases with high specific activity is essential to improve the production of *C*-glycosylflavone in *E. coli*. On the other hand, the most common byproduct, acetic acid, which is synthesized by phosphotransacetylase/acetate kinase and pyruvate oxidase in *E. coli*, can reduce the level of recombinant enzymes and inhibit the growth of *E. coli*[31-32]. Therefore, avoiding the accumulation of acetic acid in the fermentation process is crucial to the expression of Gt6CGT and *C*-glycosylflavone production.

In addition, a reasonable supply of UDP-glucose *in vivo* is also important to produce *C*-glycosylflavone[33]. Supply of UDP-glucose must meet the catalytic

efficiency of *C*-glucosyltransferase. Two methods, strengthening the endogenous UDP-glucose synthesis pathway and introducing the exogenous UDP-glucose synthesis pathway, have been used to improve the UDP-glucose supply in *E. coli*[30, 34-35]. In this study, a novel UDP-glucose synthetic pathway was reconstructed in *E. coli* by introducing the cellobiose phosphorylase gene (*cep*) from *Saccharophagus degradans* and the UTP-glucose-1-phosphate uridylyltransferase gene (*ugpA*) from *Bifidobacterium bifidum*[33, 36]. Cellobiose phosphorylase can split cellobiose into glucose and glucose 1-phosphate. Subsequently, glucose 1-phosphate will be converted into UDP-glucose by UgpA. Cellobiose phosphorylase-catalyzed phosphorolysis of cellobiose has many advantages such as its circumvention of catabolite repression, better tolerance of common inhibitors under both anaerobic and aerobic conditions, control of favorable energy metabolism and increases in the expression of heterologous genes[36-37]. Thus, introducing *cep* and *ugpA* genes into *E. coli* might improve isoorientin production.

In this paper, the aim was to increase isoorientin biosynthesis in recombinant *E. coli* by optimizing the soluble expression of *C*-glucosyltransferase (Gt6CGT), adjusting induction strategies, controlling acetic acid accumulation and increasing the supply of UDP-glucose, which resulted in successful improvement in isoorientin production on the fermentation level (Fig. 1).

Materials and methods

Strains, plasmids, media, and chemicals

All plasmids and strains used in this research are listed in Table 1. The strains were grown at 37°C in Luria-Bertani (LB) medium that was supplemented with antibiotics when required. Luteolin was purchased from Shaanxi Huike Botanical Development

Co., Ltd (Xian, China). Isoorientin was purchased from MUST Bio-Technology (Chengdu, China). LB medium contained 10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl; TB-Gly medium contained 12 g/L tryptone, 24 g/L yeast extract, 2.32 g/L KH_2PO_4, 12.54 g/L K_2HPO_4, and 10 g/L glycerol; TB-Glc contained 12 g/L tryptone, 24 g/L yeast extract, 2.32 g/L KH_2PO_4, 12.54 g/L K_2HPO_4, and 10 g/L glucose; TB-Dex contained 12 g/L tryptone, 24 g/L yeast extract, 2.32 g/L KH_2PO_4, 12.54 g/L K_2HPO_4, and 10 g/L dextrin; and TB-Mal contained 12 g/L tryptone, 24 g/L yeast extract, 2.32 g/L KH_2PO_4, 12.54 g/L K_2HPO_4, and 10 g/L maltodextrin.

Plasmid construction

Gt6CGT (AB985754.1) was synthesized to incorporate *E. coli* codons. The *Nco*I site was added to the 5′ ends of the gene, the *Bam*HI site was added to the 3′ ends of the gene, and six histidine residues were fused to the *C*-termini of recombinant enzyme. *Gt6CGT* was digested with *Nco*I and *Bam*HI and subcloned into the expression vector pCDFDuet-1 at the *Nco*I and *Bam*HI sites to create pCDFDuet-Gt6CGT.

A cellobiose phosphorylase gene (*cep*, GenBank no. ABD80580.1) was synthesized to incorporate *E. coli* codons. An *Nco*I site was added to the 5’ end of the gene, and an *Eco*RI site was added to the 3’ end of the gene. The synthesized gene (*cep*) was subcloned into pACYCDuet-1 at the *Nco*I and *Eco*RI sites to create pACYCDuet-Cep.

A UTP-glucose-1-phosphate uridylyltransferase gene (*ugpA*, GenBank no. YP_003971086.1) was obtained from pACYCDuet-cscB-Basp-UgpA and digested with *Nde*I and *Kpn*I. Then, *ugpA* was subcloned into pACYCDuet-Cep at the *Nde*I and *Kpn*I sites to produce pACYCDuet-Cep-UgpA.

Expression of Gt6CGT

The plasmid pCDFDuet-Gt6CGT was use for the transformation of *E. coli* BL21(DE3)

to obtain the recombinant strain BL21-CGT-I. The plasmids pCDFDuet-Gt6CGT and pG-KJE8 were used for the co-transformation of *E. coli* BL21 (DE3) cells to obtain the recombinant strain BL21-CGT-KJE8. The plasmids pCDFDuet-Gt6CGT and pGro7 were used for the co-transformation of *E. coli* BL21 (DE3) cells to obtain the recombinant strain BL21-CGT-pGro7. The plasmids pCDFDuet-Gt6CGT and pKJE7 were used for the co-transformation of *E. coli* BL21 (DE3) cells to obtain the recombinant strain BL21-CGT-pKJE7. The plasmids pCDFDuet-Gt6CGT and pG-Tf2 were used for the co-transformation of *E. coli* BL21 (DE3) cells to obtain the recombinant strain BL21-CGT-Tf2. The plasmids pCDFDuet-Gt6CGT and pTf16 were used for the co-transformation of *E. coli* BL21 (DE3) cells to obtain the recombinant strain BL21-CGT-pTf16. Recombinant strains were cultured in 5 mL of LB with appropriate antibiotics overnight. Seed culture (1 mL) was added to 50 mL of fresh LB medium with appropriate antibiotics. Cultures were incubated at 37°C until the OD_{600} reached 0.5-0.6, then induced to express recombinant Gt6CGT by adding isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration between 0.1 and 0.2 mM, and incubated further at 20°C for 24 h, 30°C for 12 h or 37°C for 8 h. Expression of Gt6CGT was examined by SDS-PAGE and the detection of *C*-glucosyltransferase activity.

_C_-glucosyltransferase activity

C-glucosyltransferase activity was measured as described previously [25]. The reaction mixture, which contained 0.5 mM luteolin as a substrate, 1 mM UDP-glucose, 50 mM phosphate buffer (pH 7.5), and various amounts of Gt6CGT in 100 μL, was incubated for 30 min at 50°C. The reaction was stopped by adding 400 μL of methanol, and its products were assayed via high-performance liquid chromatography (HPLC). One unit of enzyme activity was defined as the amount of enzyme necessary to synthesize

1 μmol of isoorientin per min under the assay conditions.

Isoorientin production by recombinant strains

The recombinant strains (BL21-CGT-I) were added to 50 mL of fresh medium in 250-mL shake flasks containing appropriate antibiotics and grown at 37°C until the OD_{600} reached 0.6. A total of 900 mg/L luteolin and 0.1 mM IPTG were added to BL21-CGT-I. Fermentation broths were incubated at 20°C and 180 rpm for 24 h or 48 h. Methanol (five volumes) was added directly to the fermentation broths. The supernatant was harvested by centrifugation at 12,000 x g for 5 min and analyzed using HPLC.

The effects of the induction temperature on isoorientin production were determined by induction at different temperatures (20°C, 30°C, and 37°C) with 0.1 mM or 0.2 mM IPTG. To determine the effects of induction strategies on isoorientin production, BL21-CGT-I was first induced at 20°C with 0.1 mM IPTG for 3 h, 6 h, or 9 h, and then 900 mg/L luteolin was added to produce isoorientin for 48 h. The effects of different carbon sources on isoorientin production were determined by growth in different media (LB, TB-Gly, TB-Glc, TB-Dex, TB-Mal) in 250-mL shake flasks. The samples were measured using a method similar to that described above.

Effects of UDP-glucose supply on isoorientin production

To determine the effects of UDP-glucose supply on isoorientin production in *E. coli*, the plasmids pCDFDuet-Gt6CGT and pACYCDuet-cscB-Basp-UgpA were used for the co-transformation of *E. coli* BL21 (DE3) cells to obtain the recombinant strain BL21-CGT-II. The plasmids pCDFDuet-Gt6CGT and pACYCDuet-Cep-UgpA were used for the co-transformation of *E. coli* BL21(DE3) cells to obtain the recombinant strain BL21-CGT-III. The recombinant strains were cultured in 5 mL of LB with streptomycin (50 μg/mL) and chloramphenicol (40 μg/mL) overnight. Seed culture (1

mL) was added to 50 mL of fresh TB-Gly medium containing streptomycin and chloramphenicol and was grown at 37°C until the OD_{600} reached 0.6. The broth was incubated at 20°C for 6 h after the addition of 0.1 mM IPTG. Then, 900 mg/L luteolin was added to the broth, which was incubated at 20°C for 116 h, and different concentrations of cellobiose were added to the broth at different times after the addition of luteolin.

Preparation and structural identification of isoorientin

BL21-CGT-III was cultured in 50 mL of LB with streptomycin and chloramphenicol overnight. Seed culture (50 mL) was added to 1000 mL of fresh TB-Gly medium in 3000-mL shake flasks containing 50 μg/mL streptomycin and 40 μg/mL chloramphenicol and was grown at 37°C until the OD_{600} reached 0.6. The broth was incubated at 20°C for 6 h after the addition of 0.1 mM IPTG. Then, 900 mg/L luteolin was added to the broth, which was incubated at 20°C for 116 h, and 2% cellobiose was added to the broth at 12 h after adding luteolin. The broth was harvested by centrifugation (20,000 g, 4°C, 10 min) to remove the cells and then applied to an AB-8 column macroporous resin (2.5 x 30 cm, Jianghua, China) equilibrated with distilled water (10 volumes) and eluted with 100 mL of different concentrations of ethanol (5%, 30%, 50% and 80%). The sample was evaporated to dryness and analyzed by LC/MS and HPLC.

Acetic acid quantitation

The acetic acid concentration of the culture was determined by Agilent 7890A gas chromatography, and the sample was prepared as described previously[32]. The column temperature began at an initial temperature of 70°C for 3 min; then, it was raised to 230°C at an incremental rate of 8°C per minute, and it was held for 3 min (30 m x 0.25 mm x 0.25 μm, HP-INNOWAX, Agilent) with N_2 (2 mL/min) as the carrier gas.

The detector temperature was 300°C. The flow rate of the tail gas (N_2) was 25 mL/min, the H_2 flow rate was 30 mL/min, and the air flow rate was 400 mL/min.

Sugar analysis

Maltodextrin and dextrin were determined by the phenol-sulfuric acid method[38]. Glucose and sucrose were determined with an HPLC 1200 system (Agilent, USA) and a Prevail Carbohydrate ES 5 μm column (250 mm×4.6 mm; Grace, USA) according to a previous method[30]. Glycerol was determined using the Glycerol Assay Kit (Jiancheng Bioengineering Institute, Nanjing, China).

HPLC and LC/MS analysis

HPLC analysis of luteolin and isoorientin was performed using an HPLC 1200 system (Agilent, USA) and a C18 (250 × 4.6 mm; i.d., 5 μm) column with methanol (A) and distilled water (B) at an A/B ratio of 55:45 for 15 min. The flow rate was 0.8 mL/min, and detection was performed by monitoring the absorbance at 368 nm. LC/MS analysis for luteolin and isoorientin was performed with an LTQ Orbitrap XL (ThermoFisher Scienticfic, USA) in negative mode with an ion trap analyzer. The ion spray was operated at 25 arbitrary units N_2/min, 3.5 kV, and 300°C.

Results and discussion

Effects of soluble expression of Gt6CGT on isoorientin production

Recently, several *C*-glucosyltransferase genes have been cloned and characterized from plants[21-26]. *C*-glucosyltransferase from *Oryza sativa* (rice) has been used for synthesizing natural and novel *C*-glycosylflavones from UDP-glucose *in vitro*[39]. Brazier-Hicks reconstructed the recombinant yeast to produce *C*-glycosylflavones by co-expressing the rice *C*-glucosyltransferase gene with the flavanone 2-hydroxylase gene derived from either licorice, or rice[28]. But most of *C*-glucosyltransferases

catalyze the conversion of only the open-chain form of 2-hydroxyflavanone to *C*-glycosylflavone. *C*-glucosyltransferase (Gt6CGT, AB985754.1) catalyzes the generation of *C*-glycosylflavones (isoorientin and isovitexin) using flavones (luteolin, apigenin) as substrates and has been reported only in *G. triflora*[25]. Luteolin is inexpensive and comparatively easy to prepare. Thus, it is feasible to reconstruct a recombinant strain for producing *C*-glycosylflavone from luteolin. A recombinant *E. coli* strain was successfully constructed by introducing the *Gt6CGT* gene, and the maximum titer of isoorientin was 34 mg/L[29]. Because the expression and activities of key enzymes were closely related to metabolite production in *E. coli*, to increase the expression of Gt6CGT, *Gt6CGT* was optimized by utilizing the codons preferred by *E. coli.*

The recombinant Gt6CGT protein was expressed by adding 0.1 mM IPTG and incubating the culture at 37°C for approximately 6 h. However, the overexpression of Gt6CGT resulted in the production of large amounts of inclusion bodies. The activity of Gt6CGT was only 0.1 nmol/min/mg in the recombinant strain BL21-CGT-I (Fig. 2A, lane 3-4). Molecular chaperones were introduced by plasmids pG-KJE8, pGro7, pKJE7, pG-Tf2, and pTf16 to reduce inclusion body formation by Gt6CGT in BL21-CGT[40-41]. However, compared to those in BL21-CGT, the activity of Gt6CGT and isoorientin production in these recombinant strains did not improve (data not shown). To reduce inclusion body formation by Gt6CGT, we expressed the gene using different strategies such as the use of low-temperature induction and low inducing agent concentrations[30, 42]. Only a small proportion of Gt6CGT was soluble in the cell-free extracts, and most of the enzyme was in inclusion bodies under induction at 30°C with 0.1 mM IPTG (Fig. 2A). Expression at 20°C with 0.1 mM IPTG decreased the inclusion body formation, and the activity of Gt6CGT reached 3.2

nmol/min/mg, which was approximately 32 times higher than that at 37°C with 0.1 mM IPTG (Fig. 2A). But expression at 16°C with 0.05 or 0.1 mM IPTG did not improve the activity of Gt6CGT. These results suggested that it was difficult to further improve the activity of Gt6CGT by reducing the expression of Gt6CGT in *E. coli*. Isoorientin production under different induction conditions indicated the same behavior in BL21-CGT. The highest isoorientin production was 47 mg/L, which occurred when the recombinant strain was induced at 20°C with 0.1 mM IPTG; this production was higher than that at 30°C or 37°C with 0.1 mM IPTG (Fig. 2B). These results indicated that the activity of Gt6CGT was closely related to isoorientin production, and it was difficult to further improve isoorientin production by reducing the amount of Gt6CGT inclusion bodies in *E. coli*. Some fusion tags such as maltose-binding protein (MBP), thioredoxin (Trx), N-utilization substance (NusA) and glutathione-S-transferase (GST) have been used to enhance the solubility of expressed proteins in *E. coli*[43]. Want et al. developed an activity-independent form of SE-PACE (soluble expression phage-assisted continuous evolution) to correct folding-defective variants of maltose-binding protein (MBP) and to improve the solubility of proteins[44]. These methods provide opportunities for further improving the soluble expression of Gt6CGT in *E. coli*.

Effects of induction strategies on isoorientin production

Flavonoids always have antibacterial activity and inhibit the growth of *E. coli*[45-46], so adding luteolin might affect the expression of Gt6CGT in the recombinant strain. The expression of Gt6CGT would be inhibited if luteolin is added too early, and recombinant strains would enter the death phase if luteolin is added too late. Thus, to improve isoorientin production, a reasonable induction and conversion strategy was adopted. The recombinant strain BL21-CGT-I was first induced for a period of time to

express Gt6CGT, after which luteolin was added for the production of isoorientin. As shown in Fig. 3, isoorientin production significantly increased when the recombinant strain was induced according to this strategy. The best strategy was performing the first induction for 6 h, and then adding luteolin. The maximum isoorientin production was 168 mg/L in LB medium for 48 h of bioconversion; this amount was approximately 2 times higher than that of the control (Fig. 3A). The same results were found in TB-Gly medium, and the maximum isoorientin production according to this strategy was 545 mg/L, which was approximately 4.3 times higher than that of the control (Fig. 3B). These results indicated that adjusting the timing of luteolin addition to improve isoorientin production is an effective method despite being very simple.

Effects of carbon sources on the accumulation of acetic acid and isoorientin production

Acetic acid inhibits the expression of enzymes and the growth of *E. coli* [32]. Because the activity of Gt6CGT was closely related to isoorientin production, acetic acid could decrease isoorientin production through inhibiting the expression of Gt6CGT. The main cause of acetic acid production was that the capacity of the tricarboxylic acid cycle or cellular respiration could not keep up with the carbon flooding into the central metabolic system[31].

Different carbon sources could affect acetic acid production through adjusting the carbon metabolism rate in recombinant strains. Thus, the effects of different carbon sources (those in TB-Gly, TB-Glc, TB-Dex, and TB-Mal) on isoorientin production were determined. As shown in Fig. 4, the highest isoorientin production was 826 mg/L, which was found at 80 h in TB-Gly and was approximately 730% of that (113 mg/L) in TB-Glc (Fig. 4C,D). Isoorientin production reached 376 and 358 mg/L at 80 h in TB-Mal and TB-Dex, respectively, which were also higher than that in TB-Glc

(Fig. 4A,B,D). In contrast, the consumption rate of glucose was far higher than those of dextrin and maltodextrin in *E. coli* (Fig. 4A,B), and the concentration of acetic acid in TB-Glc was 1655 mg/L at 32 h, which was higher than those in TB-Dex (371 mg/L) and TB-Mal (317 mg/L) (Fig. 4A,B,D). Glycerol as the carbon source could improve the expression of recombinant enzymes by decreasing specific growth rate and acetic acid accumulation.

The concentration of glycerol could also affect acetic acid accumulation in *E. coli*. As shown in Fig. 5, acetic acid accumulation increased significantly with increasing concentrations of glycerol, which resulted in a reduction in isoorientin production. Isoorientin production was only 265 mg/L in TB-Gly medium containing 4% glycerol, which was 33% of that in TB-Gly medium containing 1% glycerol. However, the concentration of acetic acid in TB-Gly medium containing 4% glycerol was approximately 2.4 times higher than that in medium containing 1% glycerol (Fig. 5).

The flux of additional carbon into the central metabolic system could cause metabolic imbalance, which results in excessive acetic acid production by *E. coli*[32] and acetic acid can inhibit the expression of recombinant enzymes. These results indicated that decreasing the rate of carbon source consumption to inhibit acetic acid accumulation is critical to improving isoorientin production in *E. coli*. However, consuming carbon sources too slowly could also not be conducive to improving isoorientin production in *E. coli*. The consumption rates of dextrin and maltodextrin were slower than those of glycerol, and isoorientin production in TB-Dex and TB-Mal was only 50% of that in TB-Gly, although the concentration of acetic acid in TB-Dex and TB-Mal was lower than that in TB-Gly. Thus, controlling the concentration of acetic acid by adding appropriate carbon sources was crucial to improving isoorientin production in *E. coli*.

Effects of UDP-glucose supply on isoorientin production

The UDP-glucose supply in *E. coli* is another important factor in isoorientin production because Gt6CGT catalyzes the transfer of a glucose residue from its activated form (UDP-glucose) to flavone to form isoorientin[25]. The intracellular UDP-glucose pool can be efficiently increased by introducing sucrose permease (cscB), sucrose phosphorylase (Basp) and uridylyltransferase (UgpA) genes[30, 33-34]. These enzymes use sucrose to synthesize UDP-glucose. The plasmids pCDFDuet-Gt6CGT and pACYCDuet-cscB-Basp-UgpA were used for the co-transformation of *E. coli* to obtain the recombinant strain BL21-CGT-II. However, isoorientin production in BL21-CGT-II was only 445 mg/L in TB-Gly medium without the addition of sucrose, which was far lower than that in BL21-CGT-I (Fig. 6A, 4C). These results indicated that the expression of *cscB, BasP* and *ugpA* could affect the expression of Gt6CGT or the physiological state of *E. coli*. With increasing addition of sucrose, isoorientin production decreased continuously, and the concentration of acetic acid increased continuously (Fig. 6A). The recombinant strains harboring the *cscB*, *Basp* and *ugpA* genes have proven that sucrose could be rapidly transferred and metabolized, resulting in an accumulation of acetic acid and a reduction in isoorientin production[30]. Thus, manipulating the UDP-glucose synthesis pathway in this manner was not suitable for isoorientin production in this study.

Cellobiose phosphorylase-catalyzed cellobiose assimilation has many advantages such as its circumvention of catabolite repression, tolerance of common inhibitors, and ability to increase the expression of recombinant protein[36-37]. Thus, a novel UDP-glucose synthesis pathway was reconstructed in the recombinant strain by introducing the cellobiose phosphorylase gene (*cep*) from *S. degradans* and the uridylyltransferase gene (*ugpA*) from *B. bifidum*[33, 36-37]. As shown in Fig. 6B,

isoorientin production decreased when the UDP-glucose synthesis pathway was introduced into *E. coli*, resulting in 610 mg/L titer in BL21-CGT-III without the addition of cellobiose. However, as cellobiose was added into fermentation broth, isoorientin production increased significantly. Isoorientin production reached 1176 mg/L with the addition of 2% cellobiose, which was 193% of that in samples without added cellobiose (Fig. 6B). Furthermore, the concentration of acetic acid was low, even when the cellobiose concentration reached 40 g/L. These results suggested that the reconstruction of the UDP-glucose synthesis pathway by introducing *cep* and *ugpA* genes could enhance the supply of UDP-glucose and was conducive to increasing isoorientin production in BL21-CGT-III. On the other hand, the merits of cellobiose phosphorylase-catalyzed cellobiose assimilation could also be helpful for the metabolism and growth of bacteria[36-37], which is crucial for metabolite production in *E. coli*. Therefore, the method described herein for the generation of UDP-glucose from cellobiose could be widely used for the glycosylation of flavonoids and other bioactive substances.

Subsequently, the optimal time to add cellobiose was determined in BL21-CGT-III. As shown in Table 2, isoorientin production changed according to the time at which cellobiose was added. The maximum isoorientin production reached 1206 mg/L in BL21-CGT-III when cellobiose was added 12 h after the luteolin had been added. Isoorientin production increased slightly with the addition of cellobiose at the end of fermentation, reaching only 766 mg/L when cellobiose was added 60 h after luteolin had been added.

Time courses for isoorientin production in BL21-CGT-III under the optimal conditions are shown in Fig. 7. The concentration of acetic acid remained moderately low for the whole period of growth in BL21-CGT-III with the addition of cellobiose,

and the consumption rate of cellobiose in BL21-CGT-III was 0.13 g/L/h. These results indicated that the recombinant strain harboring the novel UDP-glucose synthesis pathway could slowly phosphorylate cellobiose to produce UDP-glucose and avoid acetic acid accumulation. At the beginning of bioconversion, the specific productivity was 3.6 mg/L/h during hours 8-32 of bioconversion time. With the addition of cellobiose, the specific productivity gradually increased. The specific productivity was 15.3 mg/L/h during hours 32-116 of bioconversion, which was approximately 4 times higher than that at the beginning of bioconversion. Finally, maximal isoorientin production reached 1371 mg/L at 116 h with a corresponding molar conversion of 97.4%, which was the highest titer of isoorientin reported to date and the first to reach the gram scale in *E. coli*[29]. Thus, this study presented a novel UDP-glucose synthesis pathway with two distinct characteristics: it avoided acetic acid accumulation and slowly and continuously generated UDP-glucose.

In the previous decade, several flavonoid glycosides have already been produced in recombinant strains, which were constructed by introducing UDP-dependent glycosyltransferase genes and the UDP-glucose synthesis pathway[30, 33-34]. De Bruyn et al. developed *in vivo* glycosylation platform in *E. coli* W, resulting in production of hyperoside and quercitrin reaching 0.94 g/L and 1.12 g/L, respectively[33-34]. But most research has focused on *O*-glycosylflavones and the production of *C*-glycosylflavones was also low in recombinant strains. In this study, isoorientin production was the first to reach the gram scale by adjusting induction strategies, controlling acetic acid accumulation and increasing UDP-glucose supply in *E. coli*. The general phenylpropanoid pathway has been clarified in plants and some intermediate metabolites such as pinocembrin (429 mg/L) and naringenin (329 mg/L) have been produced in *E. coli* by constructing the phenylpropanoid biosynthesis pathway[47-49].

426 *C*-glycosylflavones are synthesized from flavones by flavone synthases and
427 *C*-glucosyltransferases. Thus, based on this result, an efficient isoorientin synthesis
428 pathway from glucose could be reconstructed in *E. coli*.

429 **Preparation of isoorientin from fermentation broth**

430 The broth was harvested by centrifugation to remove the cells and residual luteolin.
431 Most of the residual luteolin was removed by centrifugation, and the yield of
432 isoorientin reached 90.1% (Table 3, Fig. 8C) because luteolin had poor water
433 solubility compared with isoorientin. Then, the sample was applied to an AB-8
434 macroporous resin column equilibrated with distilled water, and isoorientin was eluted
435 with 30% ethanol and evaporated to dryness (Fig. 8D). Isoorientin (1059 mg) was
436 obtained by these simple purification steps with a yield of 81.5% (Table 3, Fig. 8).
437 The purity of the product was more than 95% as determined by LC/MS and HPLC
438 (Fig. 8). An analysis of the *m/z* value of the molecular ion $[M-H]^-$ of the
439 bioconversion product (447.0930) showed that its value corresponded to a D-glucose
440 adduct of luteolin (Fig. 8E), and the product had a retention time similar to that of
441 authentic isoorientin (Fig. 8). These results confirmed that the bioconversion product
442 was isoorientin.

443

444 **Acknowledgements**

445 This work was supported by the National Key R&D Program of China
446 (2017YFD0600805), the National Natural Science Foundation of China (31570565),
447 the Open Foundation of Jiangsu Provincial Engineering Laboratory for Biomass
448 Conversion and Process Integration (JPELBCPI2017002), the Qing Lan Project and
449 the Priority Academic Program Development of Jiangsu Higher Education Institutions
450 (PAPD).

References

1. Barreca, D.; Bellocco, E.; Caristi, C.; Leuzzi, U.; Gattuso, G., Flavonoid profile and radical-scavenging activity of Mediterranean sweet lemon (*Citrus limetta* Risso) juice. *Food Chem.* **2011,** *129* (2), 417-422.

2. Gattuso, G.; Caristi, C.; Gargiulli, C.; Bellocco, E.; Toscano, G.; Leuzzi, U., Flavonoid glycosides in bergamot juice (*Citrus bergamia* Risso). *J. Agric. Food Chem.* **2006,** *54* (11), 3929-35.

3. Watanabe, M., An anthocyanin compound in buckwheat sprouts and its contribution to antioxidant capacity. *Biosci. Biotech. Bioch.* **2007,** *71* (2), 579-82.

4. Zielinska, D.; Szawara-Nowak, D.; Ornatowska, A.; Wiczkowski, W., Use of cyclic voltammetry, photochemiluminescence, and spectrophotometric methods for the measurement of the antioxidant capacity of buckwheat sprouts. *J. Agric. Food Chem.* **2007,** *55* (24), 9891-8.

5. Dastmalchi, K.; Flores, G.; Petrova, V.; Pedraza-Penalosa, P.; Kennelly, E. J., Edible neotropical blueberries: antioxidant and compositional fingerprint analysis. *J. Agric. Food Chem.* **2011,** *59* (7), 3020-6.

6. Kang, J.; Li, Z. M.; Tong, W.; Jensen, G. S.; Schauss, A. G.; Wu, X. L., Anti-oxidant capacities of flavonoid compounds isolated from acai pulp (*Euterpe oleracea* Mart.). *Food Chem.* **2010,** *122* (3), 610-617.

7. Zhang, Y.; Bao, B.; Lu, B.; Ren, Y.; Tie, X., Determination of flavone *C*-glucosides in antioxidant of bamboo leaves (AOB) fortified foods by reversed-phase high-performance liquid chromatography with ultraviolet diode array detection. *J. Chromatogr. A.* **2005,** *1065* (2), 177-85.

8. Durr, C.; Hoffmeister, D.; Wohlert, S. E.; Ichinose, K.; Weber, M.; Von Mulert, U.; Thorson, J. S.; Bechthold, A., The glycosyltransferase UrdGT2 catalyzes both *C*- and *O*-glycosidic sugar transfers. *Angew. Chem. Int. Edit* **2004,** *43* (22), 2962-5.

9. Yong-Chun, J.; Lin, Y.; Yuan, K., A novel high-performance liquid chromatography fingerprint approach to discriminate *Phyllostachys pubescens* from China. *Pharmacogn Mag* **2012,** *8* (29), 42-8.

10. Peng, J.; Fan, G.; Hong, Z.; Chai, Y.; Wu, Y., Preparative separation of isovitexin and isoorientin from *Patrinia villosa* Juss by high-speed counter-current chromatography. *J. Chromatogr. A.* **2005,** *1074* (1-2), 111-5.

11. Budzianowski, J.; Budzianowska, A.; Kromer, K., Naphthalene glucoside and other phenolics from the shoot and callus cultures of *Drosophyllum lusitanicum*. *Phytochemistry* **2002,** *61* (4), 421-5.

12. Pacifico, S.; Scognamiglio, M.; D'Abrosca, B.; Piccolella, S.; Tsafantakis, N.; Gallicchio, M.; Ricci, A.; Fiorentino, A., Spectroscopic characterization and antiproliferative activity on HepG2 human hepatoblastoma cells of flavonoid *C*-glycosides from *Petrorhagia velutina*. *J. Nat Prod.* **2010,** *73* (12), 1973-8.

13. Yuan, L.; Wang, J.; Xiao, H.; Wu, W.; Wang, Y.; Liu, X., MAPK signaling pathways regulate mitochondrial-mediated apoptosis induced by isoorientin in human hepatoblastoma cancer cells. *Food Chem. Toxicol.* **2013,** *53* (3), 62-68.

14. Yuan, L.; Wei, S.; Wang, J.; Liu, X., Isoorientin induces apoptosis and autophagy simultaneously by reactive oxygen species (ROS)-related p53, PI3K/Akt, JNK, and p38 signaling pathways in HepG2 cancer cells. *J. Agric. Food Chem.* **2014,** *62* (23), 5390-400.

15. Zhang, Y.; Jiao, J.; Liu, C.; Wu, X.; Zhang, Y., Isolation and purification of four flavone C -glycosides from antioxidant of bamboo leaves by macroporous resin column chromatography and preparative high-performance liquid chromatography. *Food Chem.* **2007,** *107* (3), 1326-1336.

16. Hu, C.; Zhang, Y.; Kitts, D. D., Evaluation of antioxidant and prooxidant activities of bamboo *Phyllostachys nigra* var. *Henonis* leaf extract in vitro. *J. Agric. Food Chem.* **2000,** *48* (8), 3170-6.

17. Yuan, L.; Han, X.; Li, W.; Ren, D.; Yang, X., Isoorientin prevents hyperlipidemia and liver Injury by regulating lipid metabolism, antioxidant capability, and inflammatory cytokine release in high-fructose-fed Mice. *J. Agric. Food Chem.* **2016,** *64* (13), 2682-9.

18. Luan, G.; Wang, Y.; Wang, Z.; Zhou, W.; Hu, N.; Li, G.; Wang, H., Flavonoid glycosides from fenugreek seeds regulate glycolipid metabolism by improving mitochondrial function in 3T3-L1 adipocytes in vitro. *J. Agric. Food Chem.* **2018,** *66* (12), 3169-3178.

19. Yuan, L.; Li, X.; He, S.; Gao, C.; Wang, C.; Shao, Y., Effects of natural flavonoid isoorientin on growth performance and gut microbiota of Mice. *J. Agric. Food Chem.* **2018,** *66* (37), 9777-9784.

20. Kim, Y. C.; Jun, M.; Jeong, W. S.; Chung, S. K., Antioxidant properties of flavone *C*-glycosides from *Atractylodes japonica* leaves in human low-density lipoprotein oxidation. *J. Food Sci.* **2010,** *70* (9), S575-S580.

21. Brazier-Hicks, M.; Evans, K. M.; Gershater, M. C.; Puschmann, H.; Steel, P. G.; Edwards, R., The *C*-glycosylation of flavonoids in cereals. *J . Biol. Chem.* **2009,** *284* (27), 17926-34.

22. Falcone Ferreyra, M. L.; Rodriguez, E.; Casas, M. I.; Labadie, G.; Grotewold, E.; Casati, P., Identification of a bifunctional maize *C*- and *O*-glucosyltransferase. *J . Biol. Chem.* **2013,** *288* (44),

31678-88.

23. Nagatomo, Y.; Usui, S.; Ito, T.; Kato, A.; Shimosaka, M.; Taguchi, G., Purification, molecular cloning and functional characterization of flavonoid *C*-glucosyltransferases from *Fagopyrum esculentum* M. (buckwheat) cotyledon. *Plant J.* **2014,** *80* (3), 437-48.

24. Hirade, Y.; Kotoku, N.; Terasaka, K.; Saijo-Hamano, Y.; Fukumoto, A.; Mizukami, H., Identification and functional analysis of 2-hydroxyflavanone *C*-glucosyltransferase in soybean (*Glycine max*). *FEBS Lett.* **2015,** *589* (15), 1778-86.

25. Sasaki, N.; Nishizaki, Y.; Yamada, E.; Tatsuzawa, F.; Nakatsuka, T.; Takahashi, H.; Nishihara, M., Identification of the glucosyltransferase that mediates direct flavone *C*-glucosylation in *Gentiana triflora*. *FEBS Lett.* **2015,** *589* (1), 182-7.

26. Zhang, Z.; Liang, Z.; Yin, L.; Li, Q. X.; Wu, Z., Distribution of four bioactive flavonoids in maize tissues of five varieties and correlation with expression of the biosynthetic genes. *J. Agric. Food Chem.* **2018,** *66* (40), 10431-10437.

27. Du, Y.; Chu, H.; Chu, I. K.; Lo, C., CYP93G2 is a flavanone 2-hydroxylase required for *C*-glycosylflavone biosynthesis in rice. *Plant physiol.* **2010,** *154* (1), 324-33.

28. Brazier-Hicks, M.; Edwards, R., Metabolic engineering of the flavone-*C*-glycoside pathway using polyprotein technology. *Metab. Eng.* **2013,** *16*, 11-20.

29. Shrestha, A.; Pandey, R. P.; Dhakal, D.; Parajuli, P.; Sohng, J. K., Biosynthesis of flavone *C*-glucosides in engineered *Escherichia coli*. *Appl. Microbiol. Biot.* **2018,** *102* (3), 1251-1267.

30. Pei, J.; Dong, P.; Wu, T.; Zhao, L.; Fang, X.; Cao, F.; Tang, F.; Yue, Y., Metabolic engineering of *Escherichia coli* for astragalin biosynthesis. *J. Agric. Food Chem.* **2016,** *64* (42), 7966-7972.

31. Valgepea, K.; Adamberg, K.; Nahku, R.; Lahtvee, P. J.; Arike, L.; Vilu, R., Systems biology approach reveals that overflow metabolism of acetate in *Escherichia coli* is triggered by carbon catabolite repression of acetyl-CoA synthetase. *BMC Syst. Biol.* **2010,** *4*, 166.

32. Shi, X.; Xie, J.; Liao, S.; Wu, T.; Zhao, L. G.; Ding, G.; Wang, Z.; Xiao, W., High-level expression of recombinant thermostable β-glucosidase in *Escherichia coli* by regulating acetic acid. *Bioresource technol.* **2017,** *241*, 795-801.

33. De Bruyn, F.; De Paepe, B.; Maertens, J.; Beauprez, J.; De Cocker, P.; Mincke, S.; Stevens, C.; De Mey, M., Development of an in vivo glucosylation platform by coupling production to growth: production of phenolic glucosides by a glycosyltransferase of *Vitis vinifera*. *Biotechnol. Bioeng.* **2015,**

112 (8), 1594-603.

34. De Bruyn, F.; Van Brempt, M.; Maertens, J.; Van Bellegem, W.; Duchi, D.; De Mey, M., Metabolic engineering of *Escherichia coli* into a versatile glycosylation platform: production of bio-active quercetin glycosides. *Microb. Cell Fact.* **2015,** *14*, 138.

35. Mao, Z.; Shin, H. D.; Chen, R. R., Engineering the *E. coli* UDP-glucose synthesis pathway for oligosaccharide synthesis. *Biotechnol. Prog.* **2006,** *22* (2), 369-74.

36. Sekar, R.; Shin, H. D.; Chen, R., Engineering *Escherichia coli* cells for cellobiose assimilation through a phosphorolytic mechanism. *Appl. Environ. Microb.* **2012,** *78* (5), 1611-4.

37. Shin, H. D.; Wu, J.; Chen, R., Comparative engineering of *Escherichia coli* for cellobiose utilization: Hydrolysis versus phosphorolysis. *Metab. Eng.* **2014,** *24*, 9-17.

38. Masuko, T.; Minami, A.; Iwasaki, N.; Majima, T.; Nishimura, S.; Lee, Y. C., Carbohydrate analysis by a phenol-sulfuric acid method in microplate format. *Anal. Biochem.* **2005,** *339* (1), 69-72.

39. Bungaruang L.; Gutmann A.; Nidetzky B, Leloir glycosyltransferases and natural product glycosylation: biocatalytic synthesis of the *C*-glucoside nothofagin, a major antioxidant of Redbush herbal tea. *Adv Synth Catal.* 2013, 355, 2757-63.

40. Nishihara, K.; Kanemori, M.; Kitagawa, M.; Yanagi, H.; Yura, T., Chaperone coexpression plasmids: differential and synergistic roles of DnaK-DnaJ-GrpE and GroEL-GroES in assisting folding of an allergen of Japanese cedar pollen, Cryj2, in Escherichia coli. *Appl. Environ. Microb.* **1998,** *64* (5), 1694-9.

41. Nishihara, K.; Kanemori, M.; Yanagi, H.; Yura, T., Overexpression of trigger factor prevents aggregation of recombinant proteins in *Escherichia coli*. *Appl. Environ. Microb.* **2000,** *66* (3), 884-9.

42. Zhang, Y.; Gu, H.; Shi, H.; Wang, F.; Li, X., Green synthesis of conjugated linoleic acids from plant oils using a novel synergistic catalytic system. *J. Agric. Food Chem.* **2017,** *65* (26), 5322-5329.

43. Esposito D.; Chatterjee DK, Enhancement of soluble protein expression through the use of fusion tags. *Curr. Opin. Biotechnol.* **2006**, 17(4): 353-358.

44. Wang T.; Badran AH.; Huang TP.; Liu DR, Continuous directed evolution of proteins with improved soluble expression. *Nat. Chem. Biol.* **2018**, 14(10): 972-980

45. Plaper, A.; Golob, M.; Hafner, I.; Oblak, M.; Solmajer, T.; Jerala, R., Characterization of quercetin binding site on DNA gyrase. *Biochem. Bioph. Res. Co.* **2003,** *306* (2), 530-6.

46. Bernard, F. X.; Sable, S.; Cameron, B.; Provost, J.; Desnottes, J. F.; Crouzet, J.; Blanche, F.,

571 Glycosylated flavones as selective inhibitors of topoisomerase IV. *Antimicrob. Agents Ch.* **1997,** *41* (5),
572 992-8.
573 47. Fowler ZL.; Gikandi WW.; Koffas MA, Increased malonyl coenzyme A biosynthesis by tuning the
574 *Escherichia coli* metabolic network and its application to flavanone production. *Appl. Environ.*
575 *Microbiol.* 2009, 75(18):5831-5839.
576 48. Koopman F.; Beekwilder J.; Crimi B.; van Houwelingen A.; Hall RD.; Bosch D.; van Maris AJ.;
577 Pronk JT.; Daran JM, De novo production of the flavonoid naringenin in engineered *Saccharomyces*
578 *cerevisiae*. *Microb. Cell Fact.* 2012, 11:155.
579 49. Wu J.; Yu O.; Du G.; Zhou J.; Chen J, Fine-tuning of the fatty acid pathway by synthetic antisense
580 RNA for enhanced (2S)-naringenin production from L-tyrosine in *Escherichia coli*. *Appl. Environ.*
581 *Microbiol.* 2014, 80(23):7283-7292.
582
583
584
585
586
587
588
589
590
591
592
593
594
595
596
597

598

599 **Figure legends**

600 **Fig. 1** The schematic diagram for improvement of isoorientin production from
601 luteolin in *E. coli*. Abbreviations for enzymes: LacY, lactose permease; Cep,
602 cellobiose phosphorylase; UgpA, uridylyltransferase.

603 **Fig. 2** Effects of soluble expression of Gt6CGT on isoorientin production. (A)
604 SDS-PAGE analysis of recombinant Gt6CGT in BL21-CGT-I. Lane M: protein
605 marker, lane 1, 3, 5, 7, 9: total protein in BL21-CGT-I; lane 2, 4, 6, 8, 10: soluble
606 protein in BL21-CGT-I. (B) Isoorientin production in BL21-CGT-I. Arrow
607 represented recombinant Gt6CGT.

608 **Fig. 3** Effects of induction strategies on isoorientin production in LB medium (A) and
609 TB-Gly medium (B).

610 **Fig. 4** Effects of carbon sources on accumulation of acetic acid and isoorientin
611 production. (A) Accumulation of acetic acid and isoorientin production in TB-Mal. (B)
612 Accumulation of acetic acid and isoorientin production in TB-Dex. (C) Accumulation
613 of acetic acid and isoorientin production in TB-Gly. (D) Accumulation of acetic acid
614 and isoorientin production in TB-Glc.

615 **Fig. 5** Effects of different concentrations of glycerol on accumulation of acetic acid
616 and isoorientin production.

617 **Fig. 6** Effects of different concentrations of sucrose and cellobiose on accumulation of
618 acetic acid and isoorientin production. (A) Effects of sucrose on accumulation of
619 acetic acid and isoorientin production in BL21-CGT-II at 104 h. (B) Effects of
620 cellobiose on accumulation of acetic acid and isoorientin production in BL21-CGT-III
621 at 104 h.

622 **Fig. 7** Time course for isoorientin production under optimal conditions.

623 **Fig. 8** HPLC and LC/MS analysis of isoorientin production. (A) Authentic isoorientin
624 and luteolin. (B) HPLC analysis of fermentation broth. (C) HPLC analysis of the
625 sample by centrifugation. (D) HPLC analysis of the sample by AB-8 column. (E)
626 LC/MS analysis of isoorientin production.

Table 1 Plasmids and strains used in this study

Plasmids/strains	Descriptions	References
Plasmids		
pCDFDuet-1	CDF ori; Strep^r	Novagen
pACYCDuet-1	P15A ori; Cm^r	Novagen
pCDFDuet-Gt6CGT	pCDFDuet-1 carrying *Gt6CGT* from *Gentiana triflora,* T7 promoter.	This study
pACYCDuet-Cep	pACYCDuet-1 carrying cellobiose phosphorylase gene (cep) from *Saccharophagus degradans,* T7 promoter.	This study
pACYCDuet-Cep-UgpA	pACYCDuet-1 carrying *cep* from *Saccharophagus degradans* and UTP-glucose-1-phosphate uridylyltransferase gene (*ugpA*) from *Bifidobacterium bifidum,* T7 promoter.	This study
pACYCDuet-cscB-Basp-UgpA	pACYCDuet carrying sucrose permease gene (*cscB*) from *E. coli* W, sucrose phosphorylase gene (*Basp*) from *B. adolescentis*, and *ugpA* from *B. bifidum,* T7 promoter.	[30]
pG-KJE8	Carrying chaperone protein DnaK gene (*dnaK*), chaperone protein DnaJ gene (*dnaJ*), chaperone protein GrpE gene (*grpE*), chaperone protein GroES gene (*groES*) and chaperone protein GroEL gene (*groEL*); *dnaK, dnaJ* and *grpE* were promoted by araB promoter. *grpES* and	TaKaRa

	groEL were promoted by tetR promoter.	
pGro7	Carrying *groES* and *groEL genes*; *groES* and *groEL* were promoted by araB promoter.	TaKaRa
pKJE7	Carrying *dnaK*, *dnaJ* and *grpE genes*; *dnaK*, *dnaJ* and *grpE* were promoted by araB promoter.	TaKaRa
pG-Tf2	Carrying *groES* and *groEL genes,* and trigger factor gene (*tig*); *groES, groEL* and *tig* were promoted by Pzt-1 promoter.	TaKaRa
pTf16	Carrying *tig*; *tig* was promoted by araB promoter.	TaKaRa
Strains		
BL21 (DE3)	*Escherichia coli* BL21 (DE3)	Novagen
BL21-CGT-KJE8	BL21(DE3) harboring pCDFDuet-Gt6CGT and pG-KJE8	This study
BL21-CGT-pGro7	BL21(DE3) harboring pCDFDuet-Gt6CGT and pGro7	This study
BL21-CGT-pKJE7	BL21(DE3) harboring pCDFDuet-Gt6CGT and pKJE7	This study
BL21-CGT-Tf2	BL21(DE3) harboring pCDFDuet-Gt6CGT and pG-Tf2	This study
BL21-CGT-pTf16	BL21(DE3) harboring pCDFDuet-Gt6CGT and pTf16	This study
BL21-CGT-I	BL21(DE3) harboring pCDFDuet-Gt6CGT	This study
BL21-CGT-II	BL21(DE3) harboring pCDFDuet-Gt6CGT and	This study

	pACYCDuet-cscB-Basp-UgpA	
BL21-CGT-III	BL21(DE3) harboring pCDFDuet-Gt6CGT and pACYCDuet-Cep-UgpA	This study

Table 2 Effects of cellobiose addition on isoorientin production in BL21-CGT-III

Strains	Cellobiose (g/L)	Time of cellobiose addition	Isoorientin (mg/L) [a]
BL21-CGT-III	10	0 h after adding luteolin	1003 ± 19
	10	12 h after adding luteolin	1206 ± 21
	10	24 h after adding luteolin	998 ± 20
	10	36 h after adding luteolin	934 ± 17
	10	48 h after adding luteolin	831 ± 16
	10	60 h after adding luteolin	766 ± 18

[a] The concentration of isoorientin was determined at 104 h.

Table 3 Purification of isoorientin from fermentation broth

Step	Isoorientin (mg)	Yield (%)	Purity
Fermentation broth	1300 ± 28	100%	ND
Centrifugation	1171 ± 16	90.1 ± 1.2%	ND
AB-8 column	1059 ± 15	81.5 ± 1.2%	95 ± 0.8%

ND: not determined.

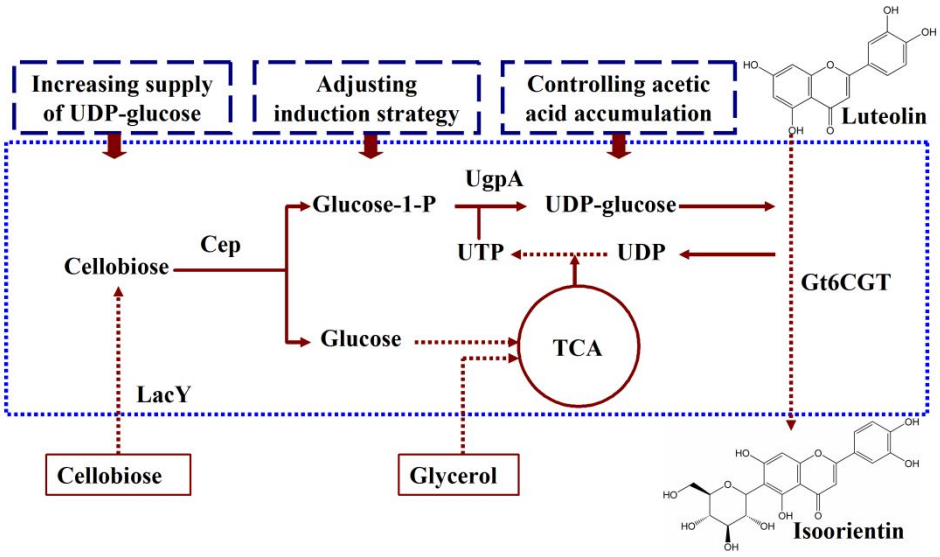


Figure 1

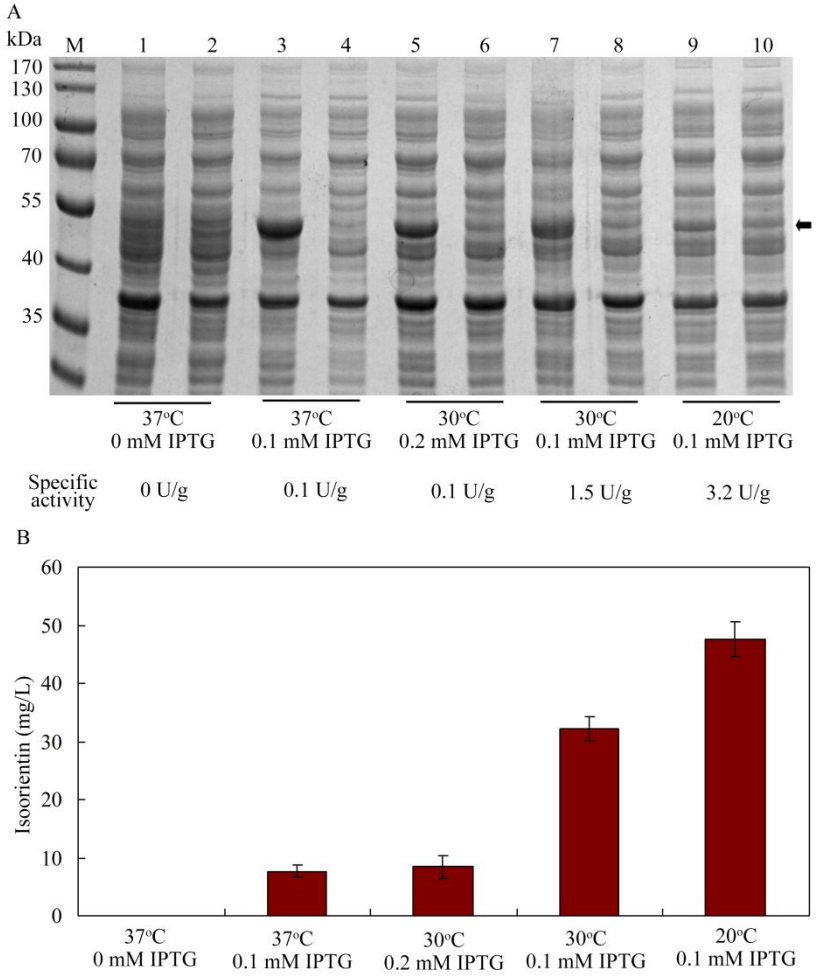


Figure 2

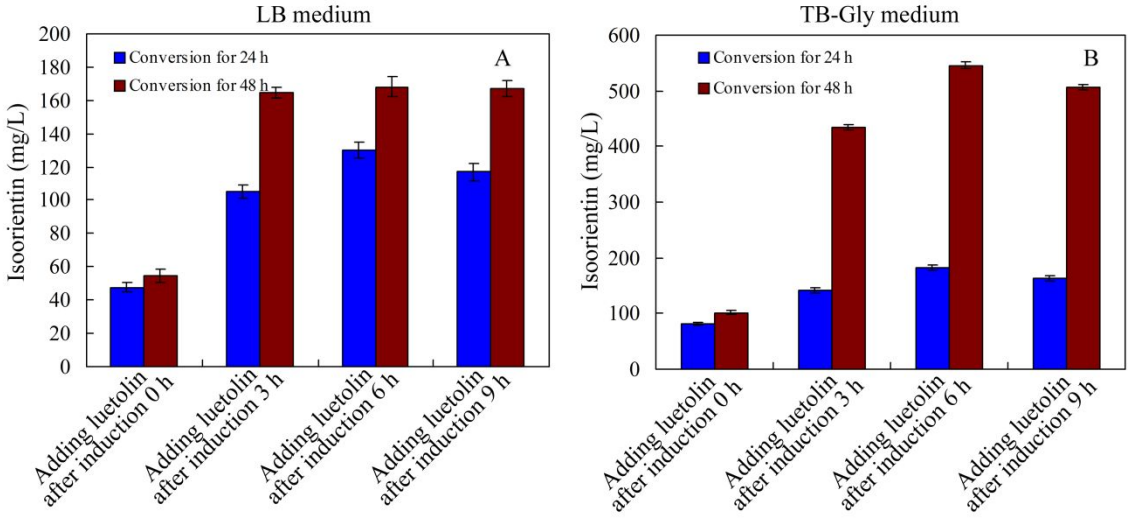


Figure 3

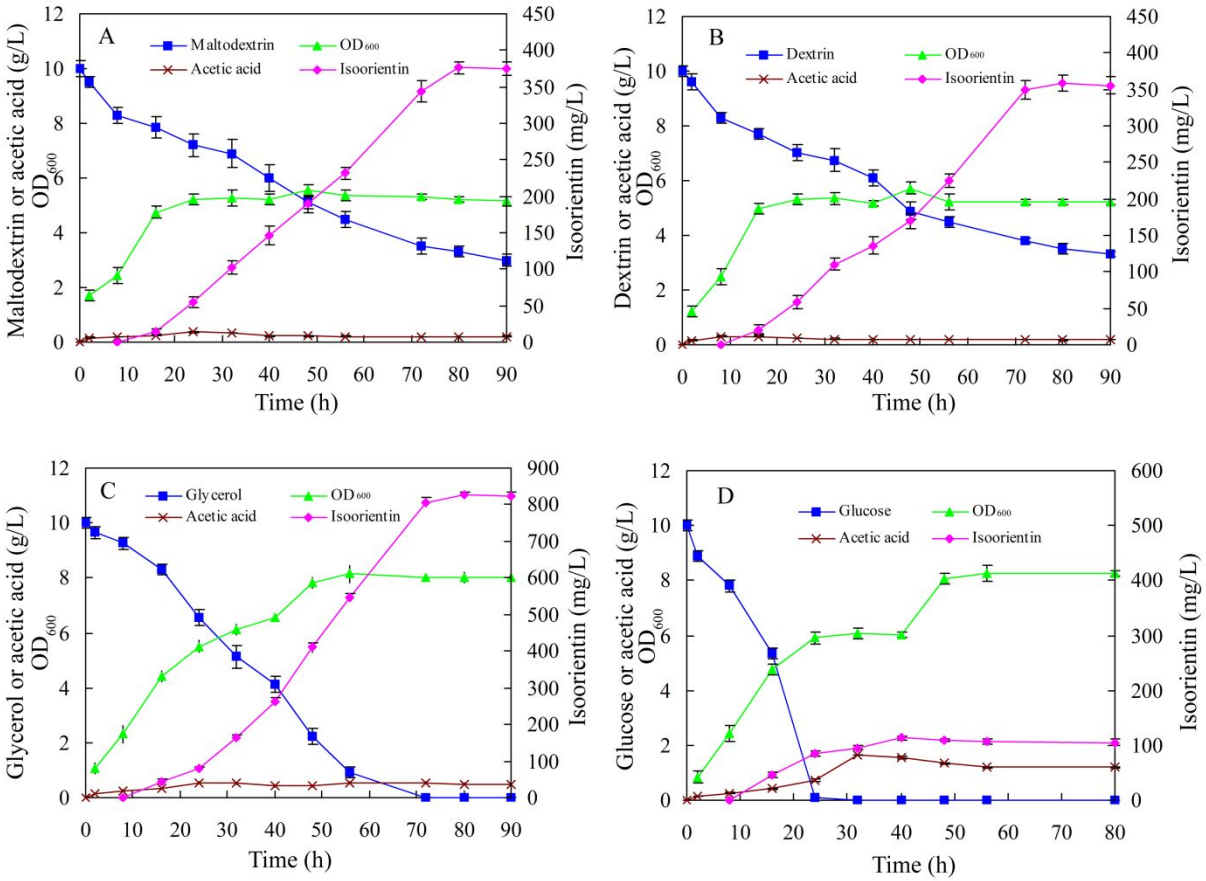


Figure 4

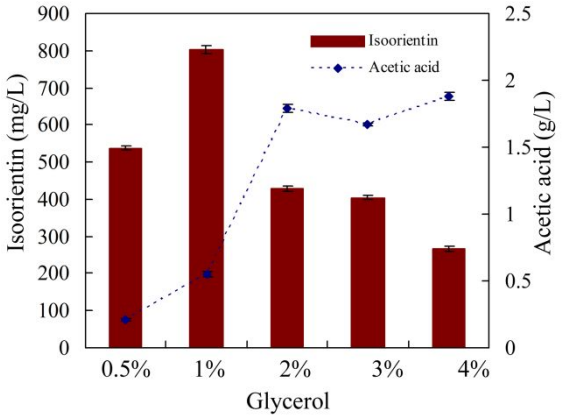


Figure 5

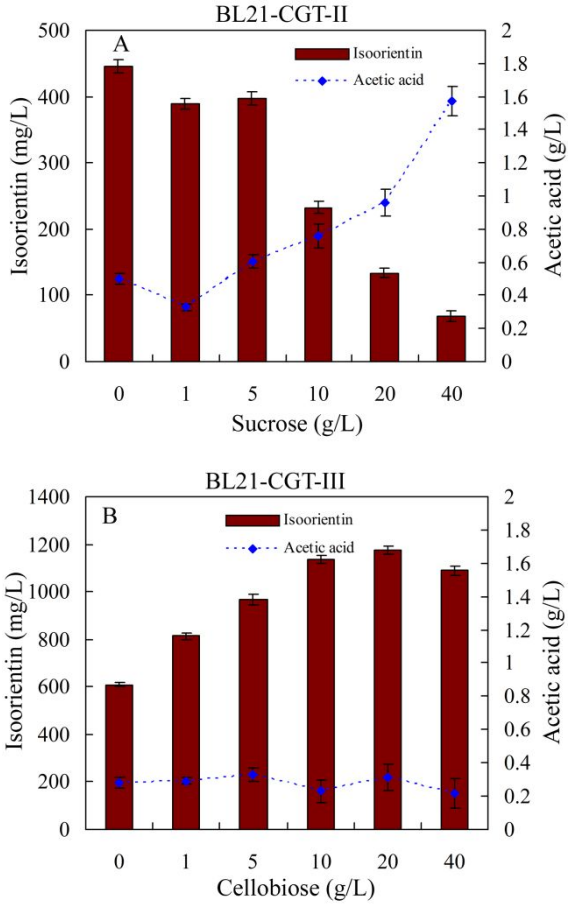


Figure 6

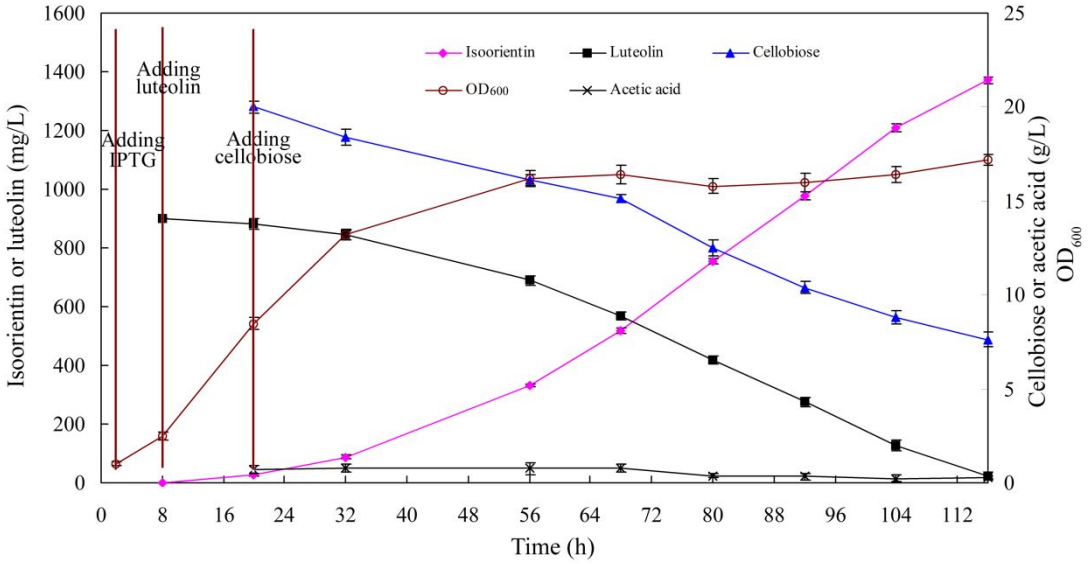


Figure 7

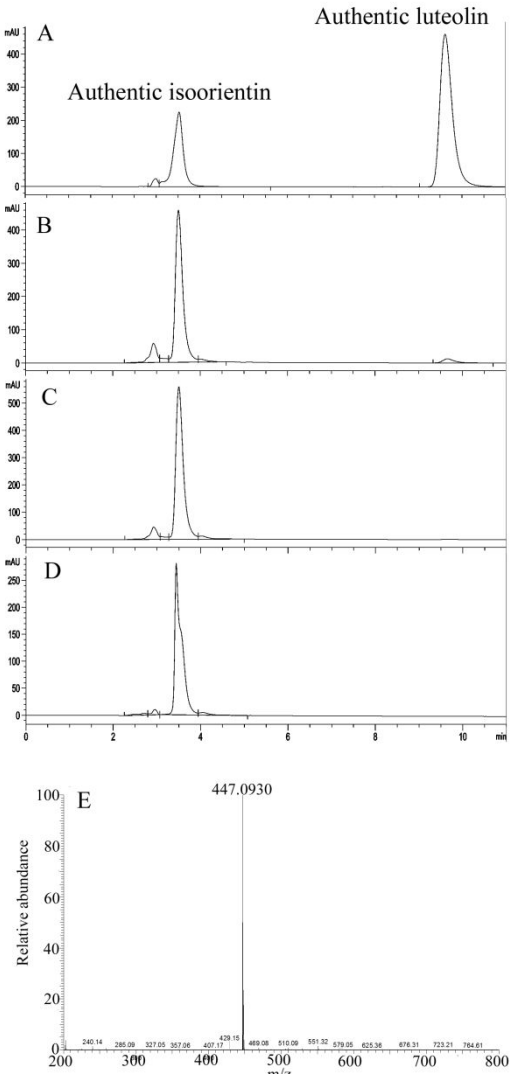
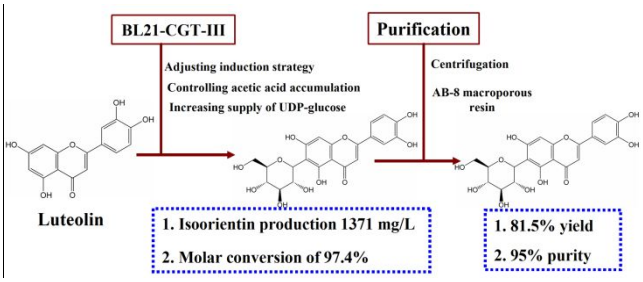


Figure 8



Abstract graphic