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Biotransformation of quercetin by *Gliocladium deliquescens* NRRL 1086

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[ABSTRACT] With an attempt to synthesize high-value isoquercitrin (quercetin-3-*O*-*β*-D-glucopyranoside), we carried out the biotransformation of quercetin (**1**) by *Gliocladium deliquescens* NRRL 1086. Along with the aimed product quercetin 3-*O*-*β*-D- glycoside (**2**), three additional metabolites, 2-protocatechuoyl-phlorogucinol carboxylic acid (**3**), 2,4,6-trihydroxybenzoic acid (**4**), and protocatechuic acid (**5**), were also isolated. The time-course experiments revealed that there were two metabolic routes, regio-selectivity glycosylation and quercetin 2,3-dioxygenation, co-existing in the culture. Both glycosylation and oxidative cleavage rapidly took place after quercetin feeding; about 98% quercetin were consumed within the initial 8 h and the oxdized product (2-protocatechuoyl-phlorogucinol carboxylic acid) was hydrolyzed into two phenolic compounds (2,4,6-trihydroxybenzoic acid and protocatechuic acid). We also investigated the impact of glucose content and metal ions on the two reactions and found that high concentrations of glucose significantly inhibited the oxidative cleavage and improved the yield of isoquercitrin and that Ca^{2+}, Fe^{2+}, Mn^{2+}, Mg^{2+}, and Zn^{2+} inhibited glycosylation. To test the promiscuity of this culture, we selected other four flavonols as substrates; the results demonstrated its high regio-selectivity glycosylation ability towards flavonols at C-3 hydroxyl. In conclusion, our findings indicated that the versatile microbe of *G. deliquescens* NRRL 1086 maitained abundant enzymes, deserving further research.

[KEY WORDS] *Gliocladium deliquescens* NRRL 1086; Quercetin; Biotransformation; Glycosylation; Quercetin-2,3-dioxygenase

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Introduction

Flavonoids are natural polyphenols with diverse biological functions found in herbs, fruits, and vegetables, which can be easily taken by humans [1]. Epidemiological and nutritional studies show that dietary flavonoids have mutiple functions in preventing chronic diseases, such as cancer, diabetes, and cardiovascular diseases [2-8]. The inherent health benefits arouse health-conscious consumers to value products containing flavonoids and attract increasing number of researchers to pay an attention to these compounds. Among the flavonoids family, flavonol quercetin (**1**) is one of the most representative compounds in this sub-class**.** Compared to aglycone-quercetin, isoquercitrin (**2**, quercetin-3-*O*-*β*-D-glucopyranoside) in recent years has been attracting an increasing research attention, due to its better water solubility and multiple pharmacological effects, such as antioxidant, anti-inflammatory, anticarcinogenic, cardioprotective, antidiabetic, anti-allergic, and neuropharmacological activities and prophylaxis of osteoporotic disorders [9-12]. Additionally, isoquercitrin is also an important intermediate in enzymatically modified isoquercitrin (EMIQ) production, which has been approved as a safe food additive in Japan and USA [13]. Although isoquercitrin is widespread in nature, its production is still difficult to meet the high demands in food industry [14]. Though several methods have been developed recently for production of isoquercitrin, including acid hydrolysis [15], microbial transformation [16] and enzymatic transformation [15, 17-18], seeking a more convenient way to synthesize isoquercitrin still has a

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vital significance.

In our previous studies, *G. deliquescens* NRRL 1086 has been proven to be a fruitful glycosylation culture toward a number of natural products [19-22]. Thus we attempted to synthesize isoquercitrin through the microbial culture of *G. deliquescens* NRRL 1086. The preliminary experiments revealed that, after feeding quercetin, the substrate was metabolized quickly and several metabolites could be detected. In the present study, we explored the metabolic pathway of quercetin in *G. deliquescens* NRRL 1086 and investigated several factors impacting the biotransformation. It was hoped that these results would provide valuable insights into the metabolic pattern of this unique microbe.

Materials and Methods

Microorganisms and chemicals

G. deliquescens NRRL 1086 was a gift from Prof. J. P. N. Rosazza, University of Iowa, USA. Stock cultures were maintained on Potato Dextrose Agar (PDA) slants and stored at 4 °C prior to use. Quercetin (CAS: 117-39-5), protocatechuic acid (CAS: 99-50-3) and rutin (CAS: 207671-50-9) were purchased from Aladdin Co., Ltd. (Shanghai, China; Product code: quercetin Q111274, protocatechuic acid P104383, and rutin R106911). 2,4,6-Trihydroxybenzoic acid (CAS: 83-30-7) was purchased from Sigma-Aldrich Co. (Shanghai, China; Product code: PH005103). Kaempferol (CAS: 520-18-3) were purchased from Nanjing Spring & Autumn Biological Engineering Co., Ltd. (Nanjing, China) Morin (CAS: 480-16-0), galangin (CAS: 548-83-4) and myricetin (CAS: 529-44-2) were purchased from Nanjing Jingzhu Bio-technology Co., Ltd. (Nanjing,China). Acetonitrile was of HPLC-grade and purchased from Tedia Inc. (Ohio, USA). All the other reagents were analytical grade and commercially available.

Instrumentation and general procedures

The HPLC equipment was an Agilent 1260 Series HPLC system consisting of quaternary pump, autosampler, thermostated column compartment and DAD system (Agilent Technologies, Inc., USA). A reversed phase column (Agilent ZORBAX SB-C_{18}; 5 μm, 4.6 mm × 250 mm) was used for all chromatographic separations. Mobile phase A was formic acid/water (100 : 0.1, *V*/*V*) and mobile phase B was acetonitrile. The gradients used for different substrates and their corresponding biotransformation products were as follows: for quercetin, rutin and morin, 5%−40% B at 0−35 min, 40%−5% B at 35−37 min, 5% B at 37−42 min; for kaempferol, 10%−60% B at 0−35 min, 60%−10% B at 35−38 min, and 10% B at 38−42 min; for galangin, 10%−35% B at 0−15 min, 35%−60% B at 15−35 min, 60%−10% B at 35−38 min, and 10% B at 38−42 min; for myricetin, 5%−20% B at 0−5 min, 20% B at 5−30 min, 20%−35% B at 30−40 min, 35%−5% B 40−42 min, and 5% B at 42−47 min. The column oven was set at 30 °C, and the detection wavelength was set at 254 nm. The flow rate of the mobile phase was 1.0 mL·min^{-1}. ESI-MS experiments were performed on an Agilent 1100 Series MSD Trap mass spectrometer (Agilent Technologies, Inc., USA). ^{1}H nuclear magnetic resonance (NMR) and ^{13}C- NMR spectra were recorded on a Bruker AV-300 or AV-500 spectrometer (Bruker Corporation, Germany) in pyridine-d6 solution with tetramethylsilane as the internal reference, and chemical shifts were expressed in δ (parts per million).

Preparative biotransformation and products isolation

The culture was carried out in potato dextrose (PD) medium prepared as follows: 200 g of peeled potatoes was cut into pieces, boiled in water for 20 min, and filtered; 20 g of glucose, 3 g of KH_2PO_4, and 1.5 g of $MgSO_4·7H_2O$ were added into the filtrate and diluted with distilled water to 1 L before being autoclaved at 121 °C for 15 min and 15 psi. Cultures were grown by a two-stage procedure [23] in 30 mL of potato dextrose (PD) medium held in 150-mL culture flasks. To initiate fermentations, the surface growth from slants of microorganism was suspended in 30 mL of potato dextrose (PD) medium. The resulting suspension was used to initiate stage Ⅰ cultures which were incubated at 28 °C on a rotary shaker at 180 r·min^{-1} for 24 h. One milliliter of *G. deliquescens* NRRL 1086 inoculum derived from the 24 h-hold stage Ⅰ cultures was used to initiate stage Ⅱ cultures, which were incubated for 24 h before receiving 20 mg of quercetin in 1 mL of acetone, and incubations were conducted as above. Using the 24-h stage Ⅱ cultures of *G. deliquescens* NRRL 1086, a total of 300 mg of quercetin were distributed evenly among 15 150-mL flasks. The substrate-containing cultures were incubated for 24 h and then extracted with equal volume of ethyl acetate thrice. The organic solvent layer was collected and evaporated to dryness. The extract was subjected to silica gel column chromatography, gel column chromatography and ODS reverse phase column to afford the products. The structures of transformation products were identified based on mass spectrometry and nuclear magnetic resonance.

Determination of the influences of incubation time and initial substrate concentration on the microbial biotransformation

This part of experiments focused on the effects of incubation time and initial concentration of Substrate **1** on the yields of main products. For the incubation time test, the samples were taken at defined intervals over a period of 72 h after adding 20 mg of **1** into each flask. For the substrate concentration test, the amount of **1** varied from 5 to 25 mg in each flask, and the incubation time was set at 12 h. When sampling at defined times, 1 mL of the culture media were removed. All the samples were taken in triplicate, freeze-dried, dissolved in methanol, and then analyzed by HPLC. The yields of substrates and corresponding products were calculated in molar concentration, according to the standard curves for each compound.

Determination of the influences of metal ion on the microbial biotransformation

Five kinds of metal salts of $MgSO_4$, $ZnSO_4·7H_2O$, $FeSO_4·7H_2O$, $MnSO_4$, and $CaCl_2$ were separately added into the PD medium at a final concentration of 1 mmol·L^{-1}, aim-

ing to investigate the impact of different metal ions on the glycosylation and quercetin 2,3-dioxygenation. The initial content of substrate **1** was 20 mg and the incubation time was set at 12 h. All the samples were analyzed in triplicate and treated the same as that in aforementioned time-course experiments.

Analysis of substrate promiscuity

Glycoside rutin (**6**) and four aglycons of kaempferol (**7**), morin (**8**), galangin (**9**) and myricetin (**10**) were employed for the biotransformation. The structural differences between **1** and **7**−**10** were of the hydroxyl groups linked on different carbon positions of ring B. The general preparation procedures of the glycosylated products kaempferol-3-*O*-*β*-D- glucoside (**11**), morin-3-*O*-*β*-D-glucoside (**12**), galangin-3-*O*-*β*-D-glucoside (**13**) and myricetin-3-*O*-*β*-D-glucoside (**14**) were the same as quercetin. The yields of their products were tested by HPLC. The initial content of each substrate was 20 mg and the incubation time was set at 24 h. All the samples were analyzed in triplicate, following the same treatments as in the aforementioned time-course experiments.

Results

Identification of biotransformation products

The HPLC spectra of the extracted culture revealed that there were four metabolites appeared in the biotransformation. Compounds **2** and **3** were rapidly formed in early hours (Fig. 1A), while Compound **4** and **5** were gradually generated at late stage along with the disappearance of Substrate **1** (Fig. 1B).

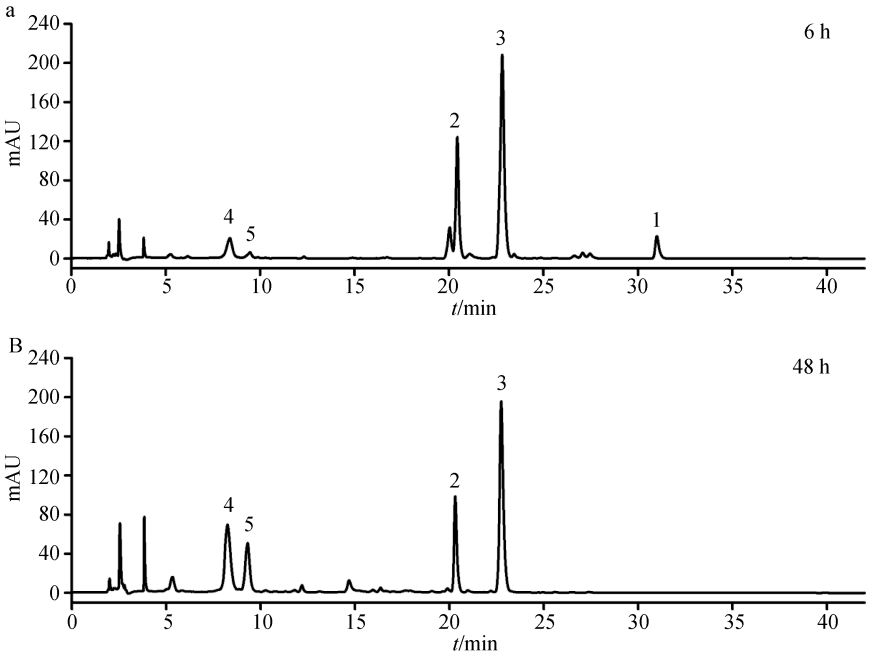


Fig. 1 HPLC spectra of the biotransformation of quercetin (1) by *G. deliquescens* NRRL 1086 at 6 h (A) and 48 h (B)

Compound **2** (32 mg) was obtained as yellow powder (methanol). It showed a positive color reaction of hydrochloric acid−magnesium. The ESI-MS of Compound **2** revealed the molecular ion at *m/z* 465.1 $[M + H]^+$, indicating that a 162 amu mass of hexose group might be introduced to compound **1** ($C_{15}H_{10}O_7$, MW 302). The ^{13}C NMR spectra showed the presence of six new signals at δ 100.85, 74.02, 76.45, 69.89, 77.46 and 60.92 (Table 1), which are characteristic of D-glucose. The ^1H NMR spectra (500 MHz, DMSO-d_6) showed the presence of one *β*-glucopyranolsyl unit of signals of δ 5.46 (1H, d, *J* = 7.0 Hz, H-1″) and δ 3.09−3.59 (5H, m, remaining sugar protons). A coupling constant of 7.0 Hz for H-1″ indicated that the stereochemistry of the glycosidic linkage at C-1″ of D-glucose is *β*. Compound **2** was therefore identified as quercetin-3-*O*-*β*-D-glucopyranoside.

Compound **3** (78 mg) was obtained as white powder (methanol). It showed a negative color reaction of hydrochloric acid−magnesium. The molecular formula of compound **3**, $C_{14}H_{10}O_8$, was established by the $[M - H]^-$ ion at *m/z* 304.9 in negative ion mode of ESI-MS as well as from its ^{13}C NMR data, indicating that compound **3** lose a carbon. The ^{13}C NMR spectrum showed the disappearance of signals at C-2 and C-3, and the presence of a new carbon signal at δ 164.28. Compared to the ^{13}C NMR data of compound **1**, the cleavage of carbon-carbon double bonds between C-2 and C-3 occurred, forming a carbonyl group at C-2. The ^1H NMR spectra (500 MHz, DMSO-d_6) showed the presence of 5H signals of δ 6.12 (1H, d, *J* = 2.5 Hz, H-6), δ 6.23 (1H, d, *J* = 2.0 Hz, H-8), δ 6.86 (1H, d, *J* = 8.0 Hz, H-5′), δ 7.45 (1H, d, *J* = 8.5 Hz, H-6′), δ 7.43 (1H, d, *J* = 2.0 Hz, H-2′) at δ 7.12−7.45, closing to the signals of A-ring and B-ring of **1**. It suggested that **3** maintained the structure of A-ring and B-ring. The ^1H NMR data of **3** showed the disappearance of the signal of hydrogen of hydroxyl at C-3 at δ 9.40−13.38 and exhibited a new signal at

δ 13.38, indicating a carboxyl group at C-4. Compound **3** was identified as 2-protocatechuoyl-phlorogucinol carboxylic acid.

Table 1 ^{13}C NMR spectroscopic data of compounds 2, 11, 12, 13, and 14

Carbon	2	11	12	13	14
2	156.2	156.3	156.3	159.2	156.1
3	133.3	133.2	133.3	136.9	133.5
4	177.4	177.4	177.4	180.2	177.3
5	161.2	161.2	161.2	163.7	161.2
6	98.6	98.6	98.6	100.5	98.6
7	164.0	164.1	164.1	166.7	164.1
8	93.4	93.6	93.5	95.4	93.3
9	156.1	156.2	156.1	159.2	156.2
10	103.9	103.9	104.0	106.5	103.9
1′	121.1	120.8	121.1	132.5	120.0
2′	115.1	130.8	115.2	129.7	108.5
3′	144.7	115.0	144.8	130.8	145.3
4′	148.4	159.9	148.4	132.4	136.7
5′	116.1	115.0	116.2	130.8	145.3
6′	121.5	130.8	121.6	129.7	108.5
1″	100.8	100.9	100.9	104.3	100.9
2″	74.0	74.1	74.1	76.2	73.9
3″	76.4	77.4	77.5	78.9	77.6
4″	69.9	69.9	69.9	71.9	69.9
5″	77.5	76.4	76.5	78.5	76.6
6″	60.9	60.8	61.0	63.2	61.1

Compounds **4** (12 mg) and **5** (4 mg) were obtained as white powder. Based on the structure of **3** and literature [24], we speculated they were generated from **3** by esterase hydrolysis. Compounds **4** and **5** were identified by comparison with authentic standard of 2,4,6-trihydroxybenzoic acid (**4**) and protocatechuic acid (**5**) by TLC and HPLC-DAD spectra.

Compound **11** (57 mg) was obtained as yellow powder (methanol). The ESI-MS of compound **11** revealed the molecular ion at *m/z* 449.2 $[M + H]^+$ indicating a 162 amu mass of glucosyl group might be introduced to kaempferol ($C_{15}H_{10}O_6$, MW 286.2). The ^{13}C NMR spectrum showed the presence of six new signals (Table 1). A coupling constant of 7.35 Hz for H-1″ indicated that the stereochemistry of the glycosidic linkage at C-1″ of D-glucose was *β*. ^{1}H NMR (500 MHz, DMSO-d_6) δ: 8.03 (2H, d, *J* = 8.75 Hz, H-2′, H-6′), 6.88 (2H, d, *J* = 8.7 Hz, H-3′, H-5′), 6.42 (1H, d, *J* = 1.5 Hz, H-8), 6.20 (1H, d, *J* = 1.5 Hz, H-6), 5.45 (1H, d, *J* = 7.35 Hz, H-1″), and 3.17−3.57 (5H, m, H-2″, H-3″, H-4″, H-5″, H-6″). Thus, compound **11** was established as kaempferol-3-*O*-*β*-D-glucoside.

Compound **12** (15 mg) was obtained as yellow powder (methanol). The ESI-MS of compound **12** revealed the molecular ion at *m/z* 487.1 $[M + Na]^+$ indicating a 162 amu mass of glucosyl group might be introduced to morin ($C_{15}H_{10}O_7$, MW 302.2). The ^{13}C NMR spectrum showed the presence of six new signals (Table 1). A coupling constant of 6.6 Hz for H-1″ indicated that the stereochemistry of the glycosidic linkage at C-1″ of D-glucose is *β*. ^{1}H NMR (300 MHz, DMSO-d_6) δ: 7.57 (1H, s, H-6′), 6.84 (1H, d, *J* = 8.9 Hz, H-5′), 6.40 (1H, s, H-8), 6.20 (1H, s, H-6), 6.43 (1H, s, H-3′), 5.46 (1H, d, *J* = 6.6 Hz, H-1″), 3.17 (1H, m, H-2″), 3.22 (2H, m, H-3″, H-5″), 3.56 (1H, m, H-4″), and 4.21 (1H, d, *J* = 11.5 Hz, H-6″). Compound **12** was established as morin-3-*O*-*β*-D-glucoside.

Compound **13** (27 mg) was obtained as yellow powder (methanol). The ESI-MS of Compound **13** revealed the molecular ion at *m/z* 455.1 $[M + Na]^+$, indicating that a 162 amu mass of glucosyl group might be introduced to galangin ($C_{15}H_{10}O_5$, MW 270.2). The ^{13}C NMR spectrum showed the presence of six new signals (Table 1). A coupling constant of 6.6 Hz for H-1″ indicated that the stereochemistry of the glycosidic linkage at C-1″ of D-glucose is *β*. ^{1}H NMR (300 MHz, CH_3OD) δ: 6.22 (1H, s, H-6), 6.40 (1H, s, H-8), 7.50 (3H, m, H-3′/H-4′/H-5′), 8.11 (2H, m, H-2′/H-6′), 3.17−3.27 (1H, m, H-2″), 3.34−3.41 (2H, m, H-3″/H-5″), 3.46−3.52 (1H, m, H-4″), 3.65−3.69 (1H, d, *J* = 11.5Hz, H-6″), and 5.31 (1H, d, *J* = 6.6Hz, H-1″). Compound **13** was established as galangin-3-*O*-*β*-D-glucoside.

Compound **14** (24 mg) was obtained as yellow powder (methanol). The ESI-MS of Compound **14** revealed the mo-

lecular ion at *m/z* 503.1 $[M + Na]^+$ indicating that a 162 amu mass of glucosyl group might be introduced to myricetin ($C_{15}H_{10}O_8$, MW 318.2). The ^{13}C NMR spectrum showed the presence of six new signals (Table 1). A coupling constant of 7.5 Hz for H-1″ indicated that the stereochemistry of the glycosidic linkage at C-1″ of D-glucose was *β*. ^1H NMR (300 MHz, DMSO-d_6) δ: 7.19 (2H, s, H-2′, H-6′), 6.19 (1H, s, H-6), 6.37 (1H, s, H-8), 12.64 (1H, s, 5-OH), 5.45 (1H, d, *J* = 7.5 Hz, H-1″), 3.17−3.19 (1H, m, H-2″), 3.22−3.24 (2H, m, H-3″, H-5′), 3.46−3.52 (1H, m, H-4″), and 3.65−3.69 (1H, d, *J* = 11.5 Hz, H-6″). Compound **14** was established as myricetin-3-*O*-*β*-D-glucoside.

Metabolic pattern of quercetin in G. deliquescens *NRRL 1086*

When compounds **2** and **3** were used as substrates individually, the HPLC analysis revealed that there was a small peak with the retention time of 30 min (Fig. 2A), indicating that a small amount of Compound **1** was generated in the initial 6 h. As fermentation time prolonged to 12 h, **1** was completely degraded into **3** (Fig. 2B). It suggested that *G. deliquescens* NRRL 1086 could also hydrolyze the glycosidic bond besides glycosylation. When the fermentation process prolonged to 48 h, the amount of Compound **2** was reduced and **3** was increased continually and the peak of **1** could not be detected (Figs. 2C and 2D). All these results suggested that **3** could also be derived from **2** by glycosidic hydrolysis first and then oxidative cleavage of ring-C. Figs. 2E and 2f showed the biotransformation of Compound **3**. The results demonstrated that **4** and **5** were derived from **3**. The change of production level of Compounds **4** and **5** at 24 h and 48 h implied that **5** could be further degraded. Based on these biotransformation products and process studies, the metabolic pathway of **1** was proposed (Fig. 3).

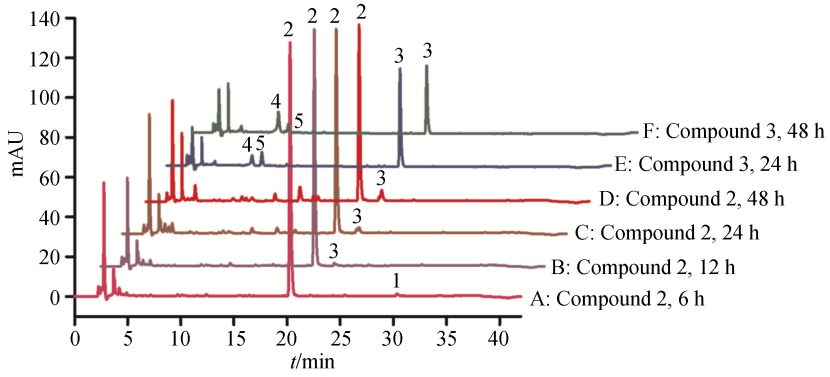


Fig. 2 HPLC spectrum of biotransformation of compounds 2 and 3 by *G. deliquescens* NRRL 1086. A−D: 5 mg of compound 2 per flask was added and sampled at 6, 12, 24 and 48 h. E−F: 5mg of compound 3 per flask was added and sampled at 24 and 48 h

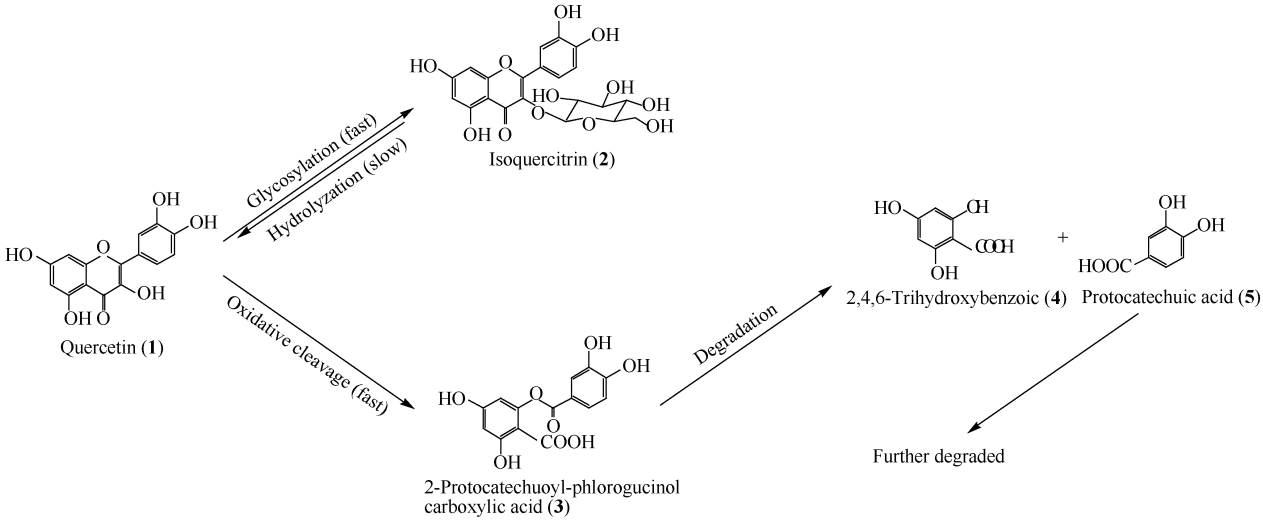


Fig. 3 Metabolic pathways of quercetin (1) in *G. deliquescens* NRRL 1086 culture

Time-course analysis of biotransformation of quercetin by G. deliquescens *NRRL 1086*

As shown in Fig. 4, at different incubation periods after the feeding of **1** the accumulation of the products changed notably in *G. deliquescens* NRRL 1086; HPLC analysis revealed that more than 70% of **1** could be rapidly consumed in the initial 4 h. Compound **2** accumulated to its maximum conversion rate of about 25% while the value of Compound **3**

was about 46% at 12 h. During this stage, the biotransformation mainly underwent glycosylation and oxidative cleavage reaction. This indicated that the optimal harvest time for Compound **2** was within 8−12 h. After 12 h, Compounds **4** and **5** rapidly accumulated. The results showed that degradation reactions played the chief role at the later stage of fermentation. Theoretically, the generation of **4** and **5** were supposed to be equimolar ratio. However, the yield of Compound **4** reached to 36%, nearly two times higher than that of Compound **5**.

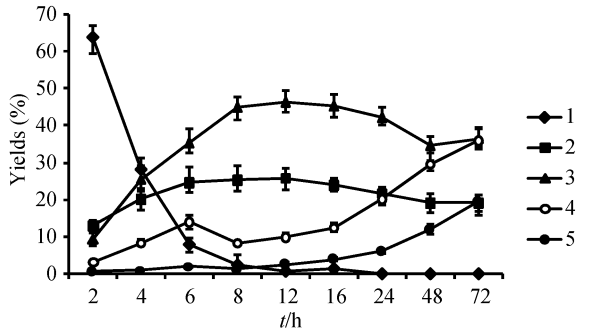


Fig. 4 Time–course curve of substrate 1 and products 2, 3, 4, and 5

Substrate feeding concentration investigation of the biotransformation

As shown in Fig. 5, under the same conditions of fermentation, different amounts of substrate **1** were distinguished on the yields of products. When the initial content of **1** was 5 mg per flask, the yields of Compounds **4** and **5** reached up to 34% and 8%, respectively. This suggested that the biotransformation in 5 mg per flask entered the later stage and a large amount of **3** was degraded. The ratio between the yield of **2** and **3** was significantly different from the 10 mg and above per flask. It seemed that most of the biotransformation went towards the oxidative cleavage reaction and the intial substrate concentration could impact the level of the two types of reaction. The biotransformation in groups of 15, 20, and 25 mg per flask remained at preliminary equilibrium stage. When the feeding amount of **1** was 20 mg per flask, the yield of **2** accumulated to 25%. With the adding amount being increased to 25 mg per flask, the conversion rate of **2** decreased to 18% and the yield of **3** could reach as high as 60%. The respective substrate feeding concentration corresponding to the maximum yields of products **2** and **3** also showed the obvious differences in optimal substrate concentration and substrate inhibition between two types of enzymes.

Influences of different amounts of glucose in the culture medium on the microbial glycosylation

Glycosylation is one of the most important reactions in organism. External glucose would affect the energy metabolism and sugar activation of this microbe. As shown in Fig. 6, different amounts of glucose in the culture medium had significant impact on these two reactions. With the increasing

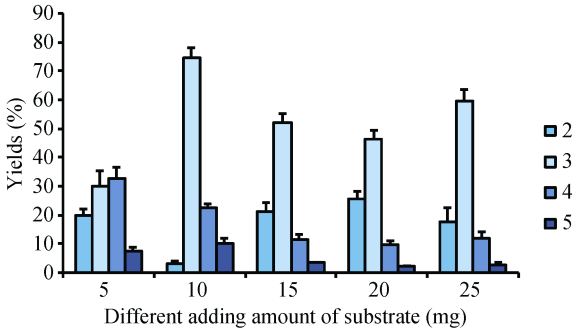


Fig. 5 The yields of substrate and each product with different adding amount of 1. The incubation time was set at 12 h

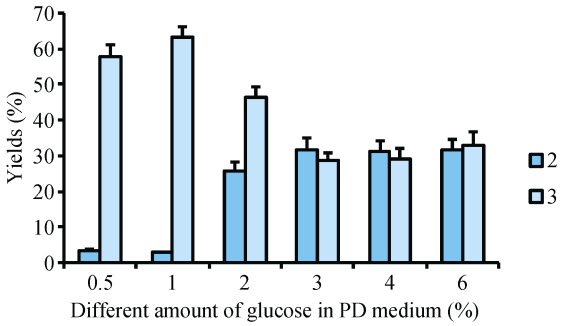


Fig. 6 The yields of 2 and 3 at 12 h in PD medium with different contents of glucose

concentration of glucose, the yield of **2** gradually increased and that of **3** decreased on the contrary. When the culture medium contained 3% of glucose, the yield of **2** reached up to 31% and the yield of **3** reduced to 28%. The results showed little changes in the ratio of **2** and **3** since the concentration of glucose was more than 3%.

Influences of metal ion on the microbial biotransformation

The fundamental essence of biotransformation is chemical reactions catalyzed by enzymes. Metal ions can influence the active sites and play an important role in enzymatic reaction. The coordination of flavonol to the active site metal ion is the first step in the enzymatic flavonol degradation mechanism [25]. Thus, we selected five metal ions in the biotransformation study. As shown in Fig. 7, with the additional metal ions, the yields of **2** reduced to 1/5−1/3 of the control and the yields of **3** nearly doubled. These changes suggested that Ca^{2+}, Fe^{2+}, Mn^{2+}, Mg^{2+}, and Zn^{2+} could significantly promote dioxygenation whereas the glycosylation was inhibited remarkably.

Biotransformation of analogs of quercetin

To explore the promiscuity of this culture, we firstly chose rutin (**6**) as substrate, which has been widely used as a substrate to study the quercetin-2,3-dioxygenase and glycosidase of different biotransformation cultures [26-28]. It was very strange that rutin could not be metabolized by this culture at all, which was quite different from the previous reports [24, 29]. Then a series of flavonols with hydroxyl groups at different

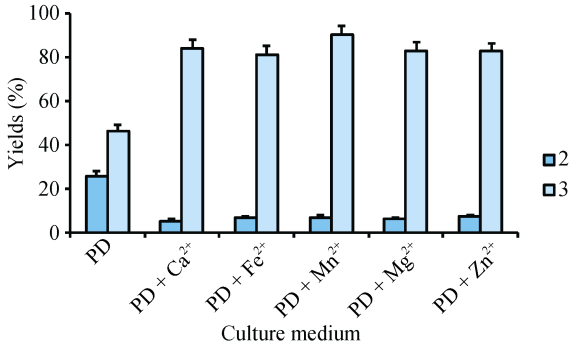
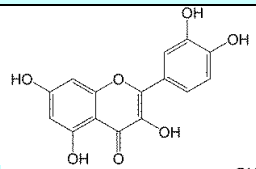
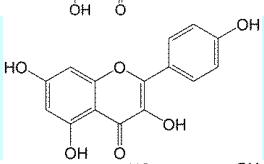
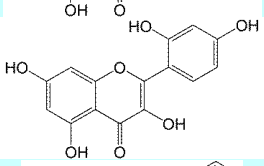
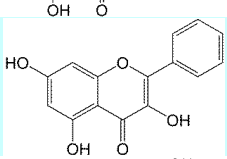
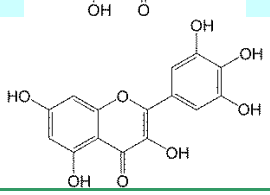


Fig. 7 Effects of the additional metal ions on the formation of product 2 and 3

carbon positions of ring-B were subjected to microbial transformation by *G. deliquescens* NRRL 1086. The microorganism exhibited regio-selectivity glycosylation towards flavonol compounds and generated 3-*O*-glycoside. After 24 h of fermentation, the yields of their corresponding glycosylation products are shown in Table 2. Compound **13** showed the highest yields among the five flavonols, with the value being 44.0%. Products of 2,3-dioxygenation were also found in the biotransformation of **7**, **8**, and **10**, and their yields in biotransformation are shown in Table 2. This result suggested that most

Table 2 Biotransformation of flavonols by *G. deliquescens* NRRL 1086

	Structure	Yields of glycosylation products (%)	Yields of dioxygenation products (%)
1		21.6	42.0
7		34.7	47.3
8		10.3	58.0
9		44.0	–
10		15.8	52.3

1: Quercetin; **7**: Kaempferol; **8**: Morin; **9**: Galangin; **10**: Myricetin

of substrates in initial 24 h went towards dioxygenation just the same as Compound **1**. It was very interesting that Compound **9** could only be converted to its corresponding glycoside, and no oxidized metabolites were detected, which implied that the hydroxyl groups on the B ring might play a crucial role in the dioxygenation reaction of flavonols.

Discussion

Microbial transformation of quercetion have long been investigated and reviewed, and a wide array of microbial glycosylation, oxidation, sulfation, methylation, hydroxylation and aromatic ring degradation have been reported [30]. In our research, two co-existing metabolic routes, regio-selectivity glycosylation and quercetin 2,3-dioxygenation, were discovered for the first time.

The microorganism exhibited strict regio-selective glycosylation ability towards flavonols at C-3 hydroxyl. As known to all, the number and location of sugar moieties on flavonoid have essential impacts on the bioactivities. Several studies have reported that flavonol 3-*O*-glycosides offer unique advantages of biological benefits, such as bioactivity enhancement including antibacterial [31-33], anti-HIV [34-35], anti-HSV [36], and anti-obesity activities [37] as well as aldose reductase inhibition [38]. The main strategies which have been used to obtain flavonoid glycosides include chemical synthesis, microbial biotransformation, and enzymatic glycosylation [39]. Chemical synthesis of specific flavonoid glycosides usually needs tedious protection /deprotection procedures [40-42]. Previous studies have reported some microbes can glycosylate quercetin at its C-3 hydroxyl, with varying efficiency. For instance, *Bacillus cereus* catalyzes 3-*O*-glycosylation to form isoquercitrin, with the yield of 20% (fermentation time was unknown) [43]; *Cunninghamella elegans* ATCC 9245 transforms quercetin into quercetin 3-*O*-*β*-D-glucopyranoside with a yield of 55.7%, but needs 72 h of fermentation [44]. Enzymatic glycosylation can generate flavonoid glycosides with regio-selectivity and high-efficiency; however the fundamental works of abtaining specific enzymes are profound and complex. Thus, *G. deliquescens* NRRL 1086 can be developed as a versatile microbial tool to obtain flavonols 3-*O*-glycosides in short time. Based on the yields of these flavonol glycosides (Table 2), it was suggested that the hydroxyl groups of ring-B hindered the generation of glycosylated products. This might be related to the steric hindrance in the acceptor binding pocket of enzyme and affected the binding affinity. Generally, enzymatic glycosylation of flavonoids has a tendency to generate its corresponding 7-*O*-glycoside or 3-*O*-glycoside preferentially *in vitro* [39]. The high regio-selectivity displayed here suggested that there were flavonol 3-*O*-glucosyltransferases in *G. deliquescens* NRRL 1086. The corresponding research of enzyme cloning is ongoing in our lab.

Another metabolic route in the biotransformation is related to 2,3-dioxygenation. It is commonly considered that this step is catalyzed by quercetinase (quercetin 2,3-dioxygenase,

E.C. 1.13.11.24). This enzyme catalyzes the oxidative cleavage of quercetin, forming a depside and carbon monoxide. It has been reported that quercetinase could be developed as a food preservative to reduce off-flavour effectively [45]. Studies with enzyme-catalyzed reaction mechanism suggested a catalytic pathway in which the substrate was bound to the metal center, and Glu subsequently served as the active site base abstracting the proton from the reactive C3-OH group of the substrate, and activated the complex for electrophilic attack by O_2 [46-47]. Some moulds, such as *Aspergillus* [29, 48], *Penicillium* [49], *Pullularia* [50], and *Fusarium* [51] species, could pocess the oxidative cleavage and degradation when they grew on rutin as a carbon source. The metabolic pathway presented in *G. deliquescens* NRRL 1086 showed some differences. First, this fungal couldn't efficiently hydrolyze rutin, indicating the absence of relative glycosidase. Second, the biotransformation of quercetin showed bidirectional characteristics, which catalyzed glycosylation and glycoside hydrolysis in one microbial reactor at the same time. From the results, we could deduce that the fungal has glycosidase which can hydrolyze isoquercitrin and/or the glycosyltransferase could also catalyze reversible reaction [52]. And third, the gap between the yields of compounds **4** and **5** at later stage suggested that further degradation might be involved in the biotransformation. There were some other oxidases to degrade the aromatic rings.

Considering the great effects of glucose on microbial energy metabolism and on the generation of activated sugar donor, we investigated the factor of glucose concentration. Increasing amount of glucose in medium could promote glycosylation reaction in *G. deliquescens* NRRL 1086 by affording more activated sugar donor, which was the other substrate of glycosyltransferase. Therefore, oxidative cleavage of quercetin was inhibited to some degree and the yields of **2** was reached up to 31%. We could harvest higher amount of **2** by regulating the concertration of glucose in culture medium. On the other hand, for quercetinase which contains 3-His-1-Glu metal binding site, metal cofactors play a functional role in this enzymes and significantly influence the catalytic reaction. Five kinds of metal ions, Ca^{2+}, Fe^{2+}, Mn^{2+}, Mg^{2+} and Zn^{2+}, leaded the yields of **2** reduced to 1/5-1/3 of the control, suggesting that these metal ions had an inhibitory effect on glycosylation or had notable enhancements on oxidative cleavage. Previous studies had reported the copper- [53-54], manganese- [55], and magnesium- [56] containing dioxygenases which were involved in the pathway of aromatic and heteroatomatic compounds degradation. Our results validated that the metal ions played important roles in catalytic activity site of dioxygenase and could stimulate the improvement of enzyme activities.

In conclusion, our study revealed that *G. deliquescens* NRRL 1086 was able to highly regio-selectively glycosylate the C-3 phenolic hydroxyl of flavonols and maitained abundant enzymes which were involved in flavonol oxidative cleavage and degradation process. Much work about *G. deliquescens* NRRL 1086 such as the transcriptome analysis, cloning, and expressing relative enzymes are still in progress which will provide a better understanding of the microorganism.

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