

## Biotransformation of Rutin to Quercetin by Microorganisms

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**Abstract --** Quercetin is a kind of flavonoid drugs, which is produced mainly by direct extracting from Flos Sophorae or acid hydrolysing from rutin. In this study, microorganism strains that can transfer rutin to quercetin were isolated from soil, screened by TLC and re-screened by HPLC. As a result, strain D97 with the highest transformation ability was obtained. The hydrolyze enzyme was intracellular enzyme and transformation rate was 75.6% within a system comprising of 50mg wet cells in 4mL of 0.3% rutin solution and transformed for 72hr at 25°C. Strain D97 was identified as *Pseudomonas* sp. by integrating the 16S rDNA phylogeny analysis result and a variety of morphological, physiological and biochemical features.

**Key words:** Rutin; Quercetin; Transformation; Identification

### 1. INTRODUCTION

Flos Sophorae is the dried flowers and buds of Chinese scholartree *Sophora japonica* L., which originates in the north of China, and widely distribute in the south and southwest areas nowadays. Traditional Chinese medicine considers that Flos Sophorae has a number of drug effects, such as, cooling blood, hemostasis, clearing away heat and reducing fire and so on. The most important use of it is treating hematochezia, metrorrhagia and metrostaxis and liverfire. Recently researches indicate that Flos Sophorae contains abundant physiological active components and nutrients, and shows remarkable curative effect as natural medicine and high quality feed in clinic. Moreover the water extract of Flos Sophorae has significant effect on anti-bacteria, anti-inflammatory, antiviral, etc (Sun et al., 2009).

The major effective components of Flos sophorae are flavonoids composed of large quantity of rutin (24.46%, w/w) and little amount of quercetin (1.41%, w/w) (Cheng et al., 2004). Both rutin and quercetin are medicines and have many physiological activities. They have similar activities in eliminating free-radicals, antioxidative activities and protective effect in the hypoxia/hypoglycemia model of bacteria precipitation, antioxidative activities in vitro and anti-lipid peroxidation, but quercetin act preponderant function than rutin (Jin et al. 2007, Jin et al. 2009, Su et al. 2002, and Jiang et al. 2007). At present, only little part of Flos sophorae are used for rutin extraction, most of them are wasted. Rutin is often hydrolyzed by acid to obtain the more active quercetin in modern industry production, and large quantity of acidic waste water is produced. Hence, it is necessary to develop a simple, economical, environment friendly and efficient method for quercetin production.

This study aimed to screen potential bacteria that could transform rutin to quercetin from soil samples collected from a decomposed leaves accumulation area of Chinese scholartree woods. If this procedure comes to reality in industry, it will not only solve the waste of Flos sophorae as resources and improves its additional value, but also can reduce the environmental pollution caused by the quercetin acid hydrolysis technology.

## 2. MATERIALS AND METHODS

### 2.1 Samples

Soil samples were collected from a Chinese scholartree woods in Baoding, China.

### 2.2 Medium and reagent

Nutrient agar medium (pH7.0) was used for bacteria isolation and cultivation. Rutin (AR), standard reagents of rutin and quercetin were purchased from Chengdu Must Bio-Technology Co., Ltd.

### 2.3 Isolation of quercetin-producing bacteria

10g soil sample was suspended in sterile water (pH 7.0) with 20 glass beads and oscillated at 25°C for 25 min. Quercetin-producing bacteria were isolated using serial dilution method by spread suitable dilutions on Nutrient agar medium supplemented with 0.1% (w/w) rutin. After incubate for 1d at 25°C, bacterium-like colonies were inoculated, and those colonies with deep-colored hydrolysis zones after sprayed 1.0% AlCl<sub>3</sub>-ethanol solution were selected for screening.

### 2.4 Screening techniques

#### 2.4.1 Preliminary screening

Thin layer chromatography (TLC) method was used to screen the active strains. The isolate was cultured in 4mL of Nutrient broth (pH7.0) in 15×150mm test tubes. Cultivation was conducted at 25°C in a thermostat shaker by shaking at 280r/min for 1d. The liquid culture was centrifuged at 4500r/min for 5min at room temperature (24°C ± 1°C) to separate the cell and the supernatant. The cell pellet was washed with 2mL of 0.1mol/L phosphate buffer (pH 7.0) and centrifuged again to harvest the biomass. Then the bacteria biomass was suspended with 4mL of phosphate buffer (pH7.0). 4mL of cell suspension and the fermentation supernatant were supplemented with 0.3% rutin and react for 3d by shaking at 280r/min and 25°C, repetitively. At last, TLC analysis was performed to check the activity of transferring rutin to quercetin.

The TLC analysis was carried out by loaded the sample on polyamide film and developed with 75% microemulsion consisted of SDS/n-butyl alcohol/n-heptane/water, 0.27: 0.63: 0.10: 36 (m/m) (Kang et al., 2000). The developed polyamide film was stained by spraying 1% AlCl<sub>3</sub>-ethanol solution and dried under room temperature. TLC polyamide film were scanned and analyzed by UN-scan-it gel scanning software (SIM International Group Co. LTD. U.S.A.).

#### 2.4.2 Secondary screening

After preliminary screening, High Performance Liquid Chromatography (HPLC) was used to re-screen the active strains by comparing the retained time of converted products with quercetin standard. The chromatography conditions were as following: separated by pre-packed Hypersil ODS (C18) column (250×4.6mm, Elite, China) column at 24°C ± 1°C and detected by UV detector at 360nm, the mobile phase composed of acetonitrile: methanol: water: phosphoric acid (100:10:340:0.3, v/v) was used as the eluant at a flow rate of 1.0mL/min, sample volume was 20μL (Jia et al., 2008).

The HPLC sample was prepared as following: the transformed solution was centrifuged to get rid of the cell, and then the supernatant was filtrated by 0.22μm membrane and ready for HPLC.

### 2.5 Transformation rate determination

Diluted standards solution of rutin and quercetin were prepared by using 4.90mg rutin and 5.88mg quercetin and they were used for preparation of different working standards using methanol.

20 $\mu$ L of the standard solution of each concentration (rutin solution: 0.272 $\mu$ g/mL, 0.544 $\mu$ g/mL, 1.089 $\mu$ g/mL, 2.178 $\mu$ g/mL, 4.356 $\mu$ g/mL; quercetin solution: 0.327 $\mu$ g/mL, 0.653 $\mu$ g/mL, 1.307 $\mu$ g/mL, 2.613 $\mu$ g/mL, 5.227 $\mu$ g/mL) were analyzed by HPLC. Standard curves were manufactured with the concentration of standard solutions as abscissa and the average peak area of three times as ordinate.

Transformation of rutin to quercetin was carried out by cell biomass as described in 2.4.1. The HPLC sample was prepared as described in 2.4.2. After injected three times, the average peak area of the sample was obtained from the liquid chromatogram and then the concentration of quercetin was figured out through the standard curve of quercetin and the starting rutin concentration of the transformation system.

### 2.6 Taxonomical Investigations of active strain

The identification of bioactive strain was conducted based on 16S rDNA phylogenetic analysis and morphological observation, as well as determination of physiological and biochemical features.

The genomic DNA of tested strain was extracted with phenol-chloroform method (Marmur, 1961), 16S rDNA was amplified by polymerase chain reaction using universal forward primer 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and reverse primer 1525R (5'-AGAAAGGAGGTGWTCCARCC-3') (Lane, 1991) with described procedure (Lu et al., 2001). Purified PCR products were directly sequenced by Beijing Sunbiotech Corporation. The sequence obtained was initially estimated by the BLAST facility of NCBI ([www.ncbi.nlm.nih.gov/BLAST](http://www.ncbi.nlm.nih.gov/BLAST)) and then aligned with all related sequences obtained from GenBank by BioEdit (Hall, 1999). Evolutionary distance matrices were calculated by using the method of Kimura 2-parameter and a neighbour-joining tree was reconstructed by the Mega 5.1 program (Saitou & Nei, 1987, Tamura et al., 2011).

Cell morphology was examined with a light microscopy (Olympus, BH-2). Presence of spore was examined by staining using Schaeffer-Fulton method, and Gram reaction was determined using the bioMe'rieux Gram Stain kit according to the manufacturer's instructions (Beijing Land Bridge Technology Co., Ltd). All other phenotype determinations were carried out according to Tvrvova' *et al.* (2006).

## 3. RESULTS

### 3.1 Screening result of active strain

25 bacteria strains and 8 fungi strains were isolated on the Nutrient agar plate at 25°C, and four of which exhibited obvious quercetin chromatography dots by TLC screening (Fig.1).

The conversion capabilities of the four strains were re-screened by HPLC with rutin and quercetin standards as control. Retained time of rutin was 5.277 min, and retained time of quercetin was 19.944 min (Fig.3). The results suggested that rutin and quercetin could be separated effectively under the experiment condition. Among the four positive strains of preliminary screened, strain D97 had an evident elution peak corresponding to quercetin and showed the highest conversion ability (Fig.2).

Thus strain D97 was selected for further analysis quantitatively.

After the cultivation of strain D97 in the Nutrient broth for 24h, both the fermentation supernatant and suspension of cell precipitation were prepared and checked conversion activity. The quercetin was only found in cell precipitation transformation system, which suggested that the active enzyme was intracellular enzyme.

Standard curves were manufactured by Excel2003 in the further quantitative study. The regression equation of rutin was  $y=12002x+946.85$  ( $R^2=0.9977$ ), and the regression equation of quercetin was  $y=26141x+1812.8$  ( $R^2=0.9974$ ) (Fig.3). The results showed that rutin and quercetin had good linearities within the tested concentration range. The transformation rate was calculated based on the standard curve and the starting concentration of rutin, as well as the final concentration of quercetin. A high transformation rate of 75.6% was reached when transformed by the 50mg wet cell for 72hr at 25°C in the system of 4mL containing 0.3% rutin.

### 3.2 Identification result of strain D97

Nearly complete 16S rDNA sequence of 1491bp of D97 strain was obtained. When comparing the 16S rDNA sequence with the GenBank database, the greatest similarity of 99.2% was found between strain D97 and *Pseudomonas moraviensis* (AY970952). In the neighbour-joining phylogenetic tree, strain D97 and *Pseudomonas moraviensis* CCM 7280<sup>T</sup>(AY970952) clustered together with a high bootstrap value of 95% (Fig.4).

Strain D97 is Gram negative and rod bacterium in size of 0.7~0.9×2.4~3.0 μm. The colony is round and wet with irregular edge and in white or translucent color. No spore is formed. Motile by polar flagella. Growth occurs at 10–37°C, with optimum growth at 28–35°C. Oxidase is positive, urease and DNase are negative. Tween 80, gelatin and tyrosine are hydrolysed, but not aesculin or starch. Lecithinase and indole are not produced. Fermentation of D-glucose and assimilation of adipate are positive. Malate is assimilated, but not citrate. Can use glycerol, L-arabinose, D-glucose, D-fructose, D-lyxose as sole carbon sources, but not D-trehalose, D-maltose, D-lactose, D-melibiose and D-sucrose. Tween 80, gelatin and starch are hydrolyzed. Can not reduce nitrate to nitrite. All these phenotype characteristics serve to classify strain D97 as a member of the genus *Pseudomonas* sp..

## 4. DISCUSSION

TLC method was widely used in separation and determination of flavonoids including rutin and quercetin (Zhou *et al.*, 2006). In this research, we obtained four strains that had the potential activity of transforming rutin to quercetin. But when they were checked by HPLC, only strain D97 exhibited a strong activity. The result indicated that TLC method only fit for preliminary screening of active strains. Although the transformation rate of strain D97 from rutin to quercetin is not as high as the industrial production needed, this wild type bacterium will illustrate a good prospect of application after breeding and optimization of transformation conditions.

The active strain D97 of this research was identified to be *Pseudomonas* sp., i.e. a bacterium, which is different from other strains that reported mainly fungus and actinomycetes. From another point of view, we found a new microbial resource for biotransformation of rutin to quercetin, and will offer a new technology for quercetin production. However, the enzymology characteristics, conversion conditions and breeding of the higher active strain ect, further studies are still needed to be carried out for this

strain.

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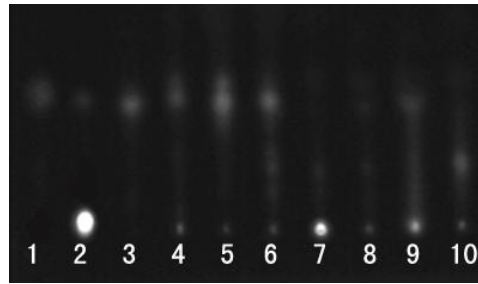


Fig 1. TLC determination result of some bacterial samples

1 rutin, 2 quercetin, 3 negative control, 4-10 samples (7. D97)

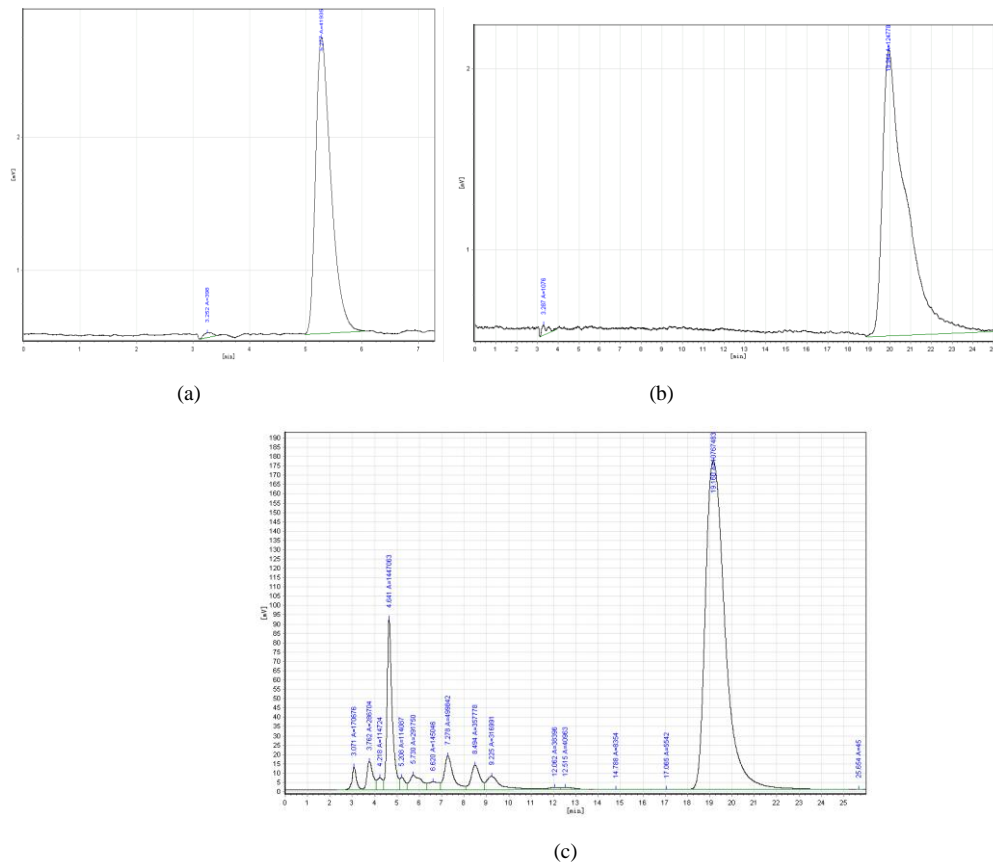


Fig 2. HPLC chromatograms of rutin standards and tested sample

(a) rutin standard, (b) quercetin standard, (c) tested sample D97

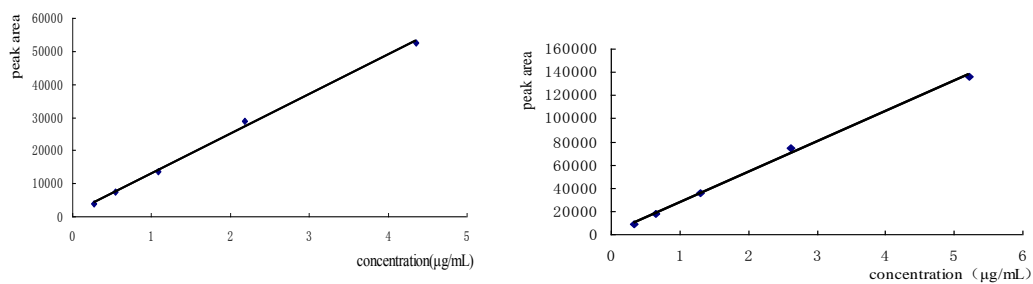


Fig.3 Standard curves of rutin (left) and quercetin (right) by HPLC

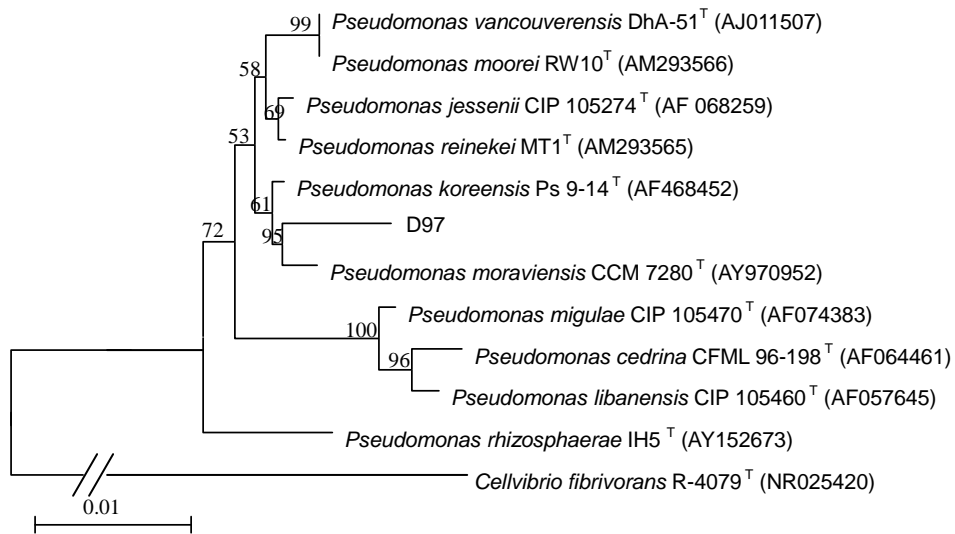


Fig 4. Neighbour-joining phylogenetic tree of strain D97 and related *Pseudomonas* representatives based on almost complete 16S rDNA sequences using *Cellvibrio fibrivorans*(NR025420) as outgroup