Screening and Identification of a Quercetin-Producing Bacterium *Paenibacillus glucanolyticu* D3

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Abstract

Quercetin is a kind of flavonoid, which has outstanding drug action. To develop the production technology of quercetin by microorganism, strains that can transform rutin to quercetin were isolated from soil, screened by TLC and re-screened by HPLC. As a result, strain D3 with the highest transformation ability was obtained. The hydrolyze enzyme was intracellular enzyme and transformation rate was 41.6% within a system comprising of 50mg wet cells in 4mL of 0.1% rutin solution and transformed for 72hr at 25°C. Strain D3 was identified as *Paenibacillus glucanolyticus* by integrating the 16S rDNA phylogeny analysis result and a variety of morphological, physiological and biochemical features.

Keywords: 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) (Fig.1). 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 quercitin act preponderant function than rutin (Jin *et al*., 2007; Jin *et al*., 2009; Su *et al*., 2002; Jiang *et al*., 2007). At present, only little part of *Flos sophoraes* are used for rutin extraction, most of them are wasted.

It had been reported that rutin could be degraded by microorganisms, such as *Aspergihs flavus* (Krishnamurty *et al*., 1970), *Penicillum rugulosum* (Narikawa *et al*., 2000) and *Thermoactinomyces vulgaris* (Yang *et al*., 2009), and so on. These microorganisms produced β-glucosidases which converted rutin (quercetin-3-O-β-D-glucose-α-L-rhamnose) to quercetin. But till now quercetin is obtained mainly by acid hydrolyzed in modern industry production, and large quantity of acidic waste water is produced. The reason is due to the low activity of the rutin degrade enzymes and the limited solubility of the rutin, which lead to a very low productivity. 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 can produce novel type of rutinosidase and transform rutin to quercetin with high efficiency from soil samples collected in accumulation area of Chinese scholartree. 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 decomposed leaves accumulation area of Chinese scholartree in Hebei University campus.

2.2 Medium and reagent

Beef extract-peptone 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 beades and oscillated at 25°C for 25 min. Quercetin-producing bacteria were isolated using serial dilution method by spread suitable dilutions on Beef extract-peptone 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% $AlCl_3$-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 Beef extract-peptone 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 0.1% 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 transforming 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%$AlCl_3$-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 by concentrating 4mL of converted liquid to 200μL at 40°C, supplemented with 300μL of methanol, centrifuged at 4500r/min for 5min to wipe off the precipitation, and made up to 500μL.

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.

20μL of the standard solution of each concentration (rutin solution: 0.272μg/mL, 0.544μg/mL, 1.089μg/mL, 2.178μg/mL, 4.356μg/mL; quercetin solution: 0.327μg/mL, 0.653μg/mL, 1.307μg/mL, 2.613μg/mL, 5.227μg/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.

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 substrate concentration.

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'-AGAAAGGAGGTGWT CCARCC-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) 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 test methods were based on those of Gordon *et al*. (Gordon, 1973) and have been described previously (Priest, 1988).

3. Results

3.1 Screening result of active strain

From the soil samples, 80 bacteria strains were isolated first on the beef extract peptone medium plate at 25°C. Three strains, B12, C20 and D3, with potential activity of transforming rutin to quercetin were obtained after TLC determination (Figure 2).

The conversion capabilities of the three 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 (Figure 3). The results suggested that rutin and quercetin could be separated effectively under the experiment condition. Among the three positive strains of preliminary screened, strain D3 had an evident elution peak corresponding to quercetin and showed the highest conversion ability (Figure 3). Thus strain D3 was selected for further analysis quantitatively.

After the cultivation of strain D3 in the rutin-Beef extract-peptone 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 transfer 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) (Figure 4 and Figure 5). The results showed that rutin and quercetin had good linearities within the tested concentration range. After injected three times, the average peak area of quercetin was 10767498 in D3 sample. According to the quercetin standard curve, quercetin concentration of D3 sample was 0.412mg/mL, and transformation rate was 41.6% when transferred by the 50mg wet cell for 72hr at 25°C in the system of 4mL containing 0.1% rutin.

3.2 Identification result of strain D3

Nearly complete 16S rDNA sequence of 1501bp of D3 strain was obtained. When comparing the 16S rDNA sequence with the GenBank database, the greatest similarity of 99.47% was found between strain D3 and *Paenibacillus glucanolyticus* DSM5162^T (AB073189). In the neighbour-joining phylogenetic tree, strain D3 and *Paenibacillus glucanolyticus* DSM5162^T clustered together with a high bootstrap value of 100% (Fig.6).

Colonies of the strain D3 were flat, smooth, and opaque and were motile during growth on nutrient agar plates. Cells were 3.0~3.5μm×0.8~0.9μm, Grampositive, and produced oval terminal spores that markedly distend the sporangium. In physiological and biochemical experiments, strain D3 did not produce gas from glucose, and produced acid from cellobiose, D-fructose, D-galactose, D-glucose, lactose, maltose, D-mannose, sucrose, trehalose, D-xylose. In Voges-Proskauer broth the pH was 5.3. The strain hydrolyzed arbutin, DNA, pullulan, ribonucleic acid and starch, and did not hydrolyze allantoin, chitin, elastin, pectin, tyrosine, or urea. Strain D3 could grow in the presence of 5% NaCl but not in the presence of 10% NaCl and was negative for indole production and the Voges-Proskauer reaction. All these phenotype characteristics serve to classify strain D3 as a member of the species *Paenibacillus glucanolyticus*. There for this active strain was identified as *Paenibacillus glucanolyticus* D3 on species level.

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 three strains that had the potential activity of transforming rutin to quercetin. But when they were checked by HPLC, only strain D3 exhibited a strong activity. The result indicated that TLC method only fit for preliminary screening of active strains. Although the transformation rate of strain D3 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 D3 of this research was identified to be *Paenibacillus glucanolyticus*, i.e. a bacillus, which is different from other strains that reported mainly fungi and actinobacteria. 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.

Moreover, the rutin degrade enzyme of *P. glucanolyticus* D3 is produced constitutively, contrasting to the reported enzymes of *Aspergillus, Penicillum, Thermoactinomyces* and *Streptococcus*, which are induced rutin degrade enzymes of different types (Krishnamurty *et al*., 1970; Narikawa *et al*., 2000; Yang *et al*., 2009; Mamma *et al*., 2004). This characteristic enables *P. glucanolyticus* D3 with lower cost in enzyme production. However, the enzymology characteristics, conversion conditions and breeding of the higher active strain ect, further studies are still needed to be carried out.

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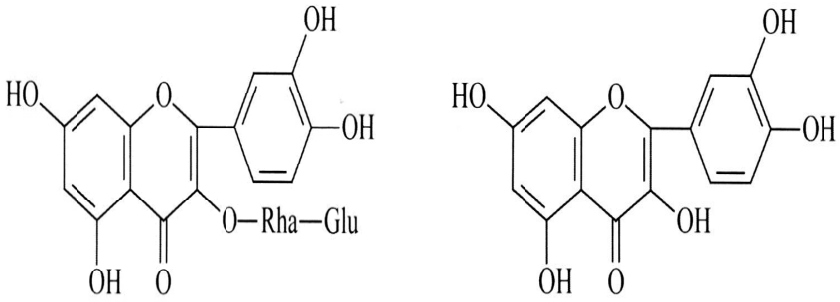

Figure 1. Chemical structures of rutin (left) and quercetin (right)

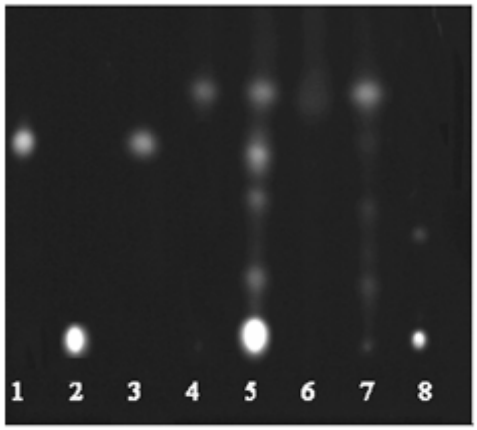

Figure 2. TLC determination result of some bacterial samples

1 rutin, 2 quercetin, 3 negative control, 4-8 samples (5. D3)

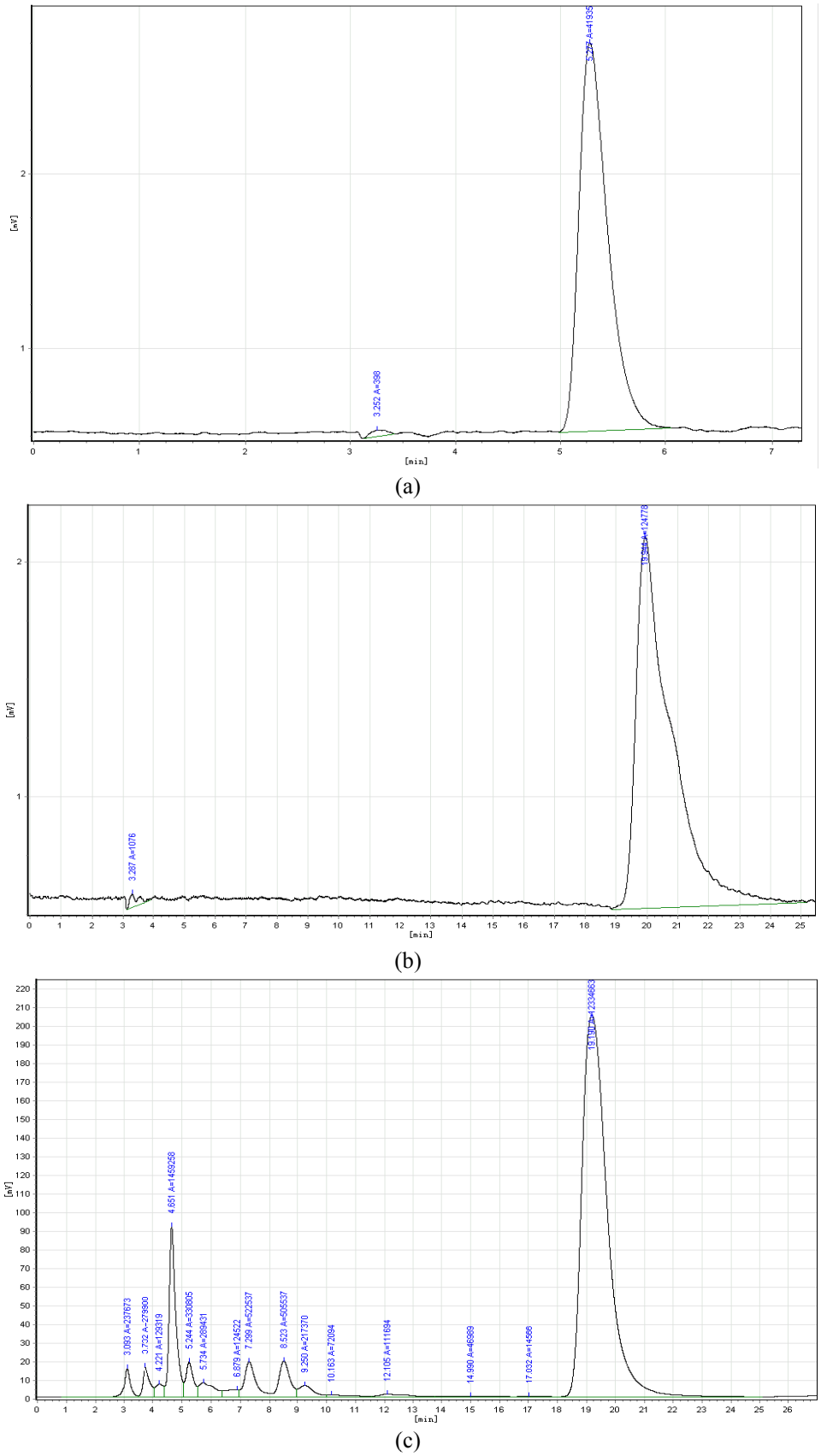

Figure 3. HPLC chromatograms of rutin standards and tested sample

(a) rutin standard, (b) quercetin standard, (c)tested sample D3

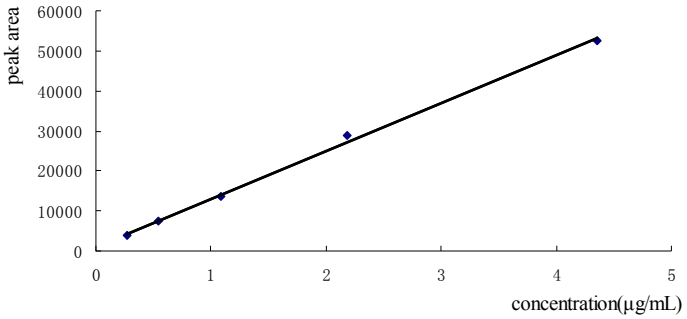

Figure 4. Standard curve of rutin by HPLC

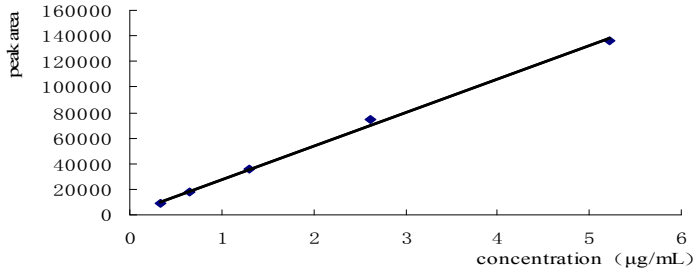

Figure 5. Standard curve of quercetin by HPLC

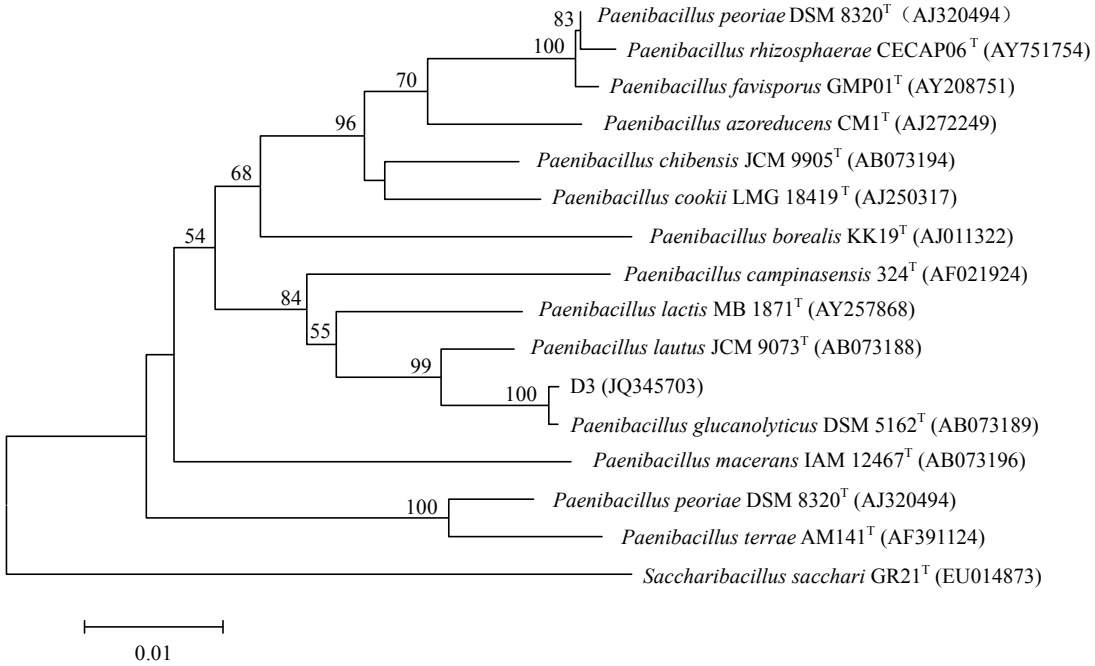

Figure 6. Neighbour-joining phylogenetic tree of strain D3 and related *Paenibacillus* representatives based on almost complete 16S rDNA sequences using *Saccharibacillus sacchari* (EU014873) as outgroup