Enzyme and Microbial Technology 57 (2014) 42–47

Contents lists available at ScienceDirect

Enzyme and Microbial Technology

journal homepage: www.elsevier.com/locate/emt

# Efficient glycosylation of puerarin by an organic solvent-tolerant strain of *Lysinibacillus fusiformis*

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ARTICLE INFO

*Article history:*
Received 26 November 2013
Received in revised form 16 January 2014
Accepted 19 January 2014
Available online 28 January 2014

*Keywords:*
*Lysinibacillus fusiformis*
Biotransformation
Puerarin
Puerarin-7-*O*-fructoside
Organic solvent-tolerant

ABSTRACT

A bacterial strain able to glycosylate the plant natural product puerarin was isolated from local soil in Nanjing, China. It was identified as *Lysinibacillus fusiformis*, and deposited in China General Microbiological Culture Collection (CGMCC) under accession number 4913. Incubation of this strain with puerarin led to efficient production (91.6% conversation rate) of puerarin-7-*O*-fructoside, a derivative that possesses improved water solubility and antioxidant activity. A minor product puerarin-7-*O*-isomaltoside was also produced in small amounts, with a conversion rate of less than 1% after 48-h reaction. Both products were characterized based on the spectral data. Among the four tested sugars, sucrose (92.6% conversion rate of puerarin) is the best glycosyl donor for *L. fusiformis* CGMCC 4913, followed by maltose (39.8% conversion rate of puerarin), while glucose and fructose are not appropriate donors for this biotransformation process. *L. fusiformis* CGMCC 4913 can survive in the presence of 10% (v/v) organic solvents such as methanol, ethanol, toluene, cyclohexane, and dimethyl sulfoxide. The biotransformation efficiency of puerarin was increased 2-fold in the presence of 10% ethanol at 12 h compared to the transformation solution without ethanol. The optimum pH and substrate concentration are 8.0 and 4 g/L, respectively. Under the optimal conditions, the final conversion rate of puerarin reached 97.6 ± 2.3% at 48 h in the presence of 10% ethanol. Therefore, *L. fusiformis* CGMCC 4913 represents a new and efficient biocatalyst for the biotransformation of puerarin.



## 1. Introduction

Puerarin (Fig. 1) is an isoflavone from Kudzu (*Pueraria lobata*) that contains a unique C-glucoside. This natural product has shown promising biological activities and has been used to treat coronary heart disease, angina pectoris, cardiac infarction, ocular blood flow problems, cardiovascular disorders and prevent cancer, acting as an antioxidant and scavenging free radicals [1]. However, the low water solubility and poor absorption after oral administration both limit its further clinic use [2]. A lot of studies have been carried out to improve the water solubility and bioactivity of puerarin via structural modification [3–5]. Glycosylation has shown its promise in increasing the water solubility of organic molecules. For example, our previous work on glycosylation of another plant natural product curcumin using *Beauveria bassiana* ATCC 7159 has led to the formation of a new glycoside. The water solubility of this new compound is 39,000-fold higher than the substrate [6]. Another work from us demonstrated that a bacterial strain, *Microbacterium oxydans* CGMCC 1788, has the ability to attach the glucose moiety from sucrose to the 7-OH of the substrate to significantly improve the water solubility. The antioxidant activity of the product is decreased [7], while the vasorelaxing effect remains the same [8]. Yu et al. reported that *M. oxydans* CGMCC 1788 could also attach a fructose moiety to the 7-OH on the A ring of puerarin to form puerarin-7-*O*-fructoside [9], which has 50% increased antioxidant activity [7]. However, the poor water solubility of puerarin limited the biotransformation efficiency of this compound in normal water-based reaction buffers [8]. Thus, discovery of organic solvent-tolerant strains that have the ability to glycosylate puerarin will significantly improve the efficiency of enzymatic preparation of more water-soluble peurarin derivatives. In this work, a strain of *Lysinibacillus fusiformis* (CGMCC 4913) was isolated from a soil sample and characterized. This strain was found to convert puerarin to a major product puerarin-7-*O*-fructoside and

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http://dx.doi.org/10.1016/j.enzmictec.2014.01.009

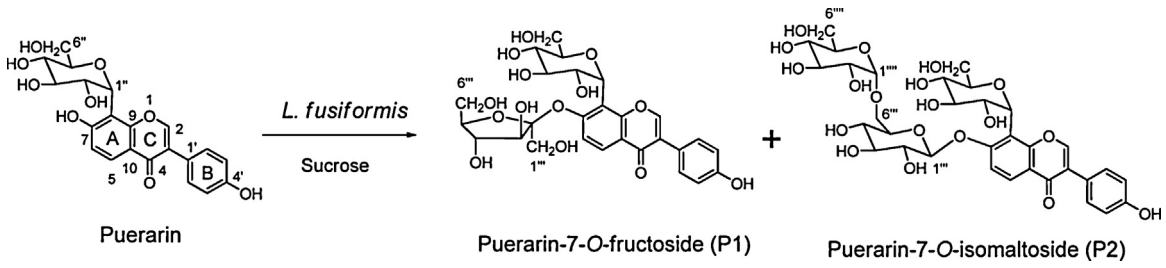


**Fig. 1.** Glycosylation of puerarin by *L. fusiformis* CGMCC 4913.

a minor product puerarin-7-*O*-isomaltoside in the reaction buffer containing organic solvents. In the presence of 10% ethanol, the molar conversion rate can reach 97.6% in 48 h.

## 2. Materials and methods

### 2.1. Chemicals

Puerarin (99% purity) was purchased from Qingdao Jinfeng Pharmaceutical Co., Ltd. (Qingdao, China). Methanol and acetic acid (HPLC grade) were purchased from Tedia (Fairfield, OH, USA). Analytical grade glucose, fructose, maltose, sucrose, ethanol and other reagents were purchased from Shanghai Fine Chemical Co., Ltd. (Shanghai, China). Ultrapure water for HPLC was produced in the laboratory.

### 2.2. Screening and isolation of the bacterium

The organic solvent-tolerant strains were isolated from the soil sample from a local gas station in Nanjing, China by pour plate method after culturing in Luria–Bertani (LB) media (yeast extract, 5 g/L; peptone, 10 g/L; and NaCl, 10 g/L, pH 7.2) containing 5% (v/v) ethanol. Single colonies were picked to 30 mL of LB with 5% (v/v) ethanol in 100-mL flasks and grown at 30 °C and 220 rpm until the $OD_{600}$ reached 3.0. The culture was then amplified to 30 mL of LB with 10% (v/v) ethanol in 100-mL flasks. Cells were grown until the $OD_{600}$ reached 3.0. 10 mL of the cells were collected by centrifugation and then washed briefly with 0.07 M $Na_2HPO_4/KH_2PO_4$ buffer (pH 8.0). After that, the cells were resuspended in 10 mL of biotransformation reaction buffer [0.07 M $Na_2HPO_4/KH_2PO_4$ buffer, pH 8.0, 4 g/L puerarin, 60 g/L sucrose]. Biotransformation was conducted at 30 °C, 220 rpm for 48 h. The culture control (without puerarin) and substrate control (without cells) were conducted under the same conditions. The reaction mixture was sampled (1 mL) at 12, 24, 36, and 48 h and analyzed by HPLC.

### 2.3. Identification of the bacterium

The only selected bacterial isolate with biotransformation activity was characterized and identified using standard biochemical tests mentioned in Bergey's Manual of Determinative Bacteriology [10], Biolog GEN III GN/GP Identification Microstation system test [11], and the structure of cell and spores were observed with a transmission electron microscope (H7650, Japan). Then, the result was confirmed by amplification and sequencing of the 16S rRNA gene [12]. The sequence was compared to the database of NCBI GenBank. The strain was deposited in China General Microbiological Culture Collection (CGMCC) under accession number 4913.

### 2.4. Cultivation

A single colony of *L. fusiformis* CGMCC 4913 on LB agar was inoculated into 30 mL of LB medium in 100-mL flasks and incubated at 30 °C and 220 rpm for 12 h ($OD_{600}$ = 3.0, $3.5 \times 10^8$ cells/mL). Cells were collected for biotransformation by centrifugation (6,000 × *g*, 5 min at room temperature). For organic solvent-tolerance experiment, *L. fusiformis* CGMCC 4913 was grown in LB medium and re-suspended in the reaction buffer with 10% (v/v) different organic solvents. LB medium and reaction buffer without organic solvents served as the control. The biotransformation samples were analyzed by HPLC. Each result is shown as mean ± SD from three independent experiments with three replicates.

### 2.5. Biotransformation

Cells from 10 mL of culture were re-suspended in 10 mL of biotransformation reaction buffer containing 0.07 M $Na_2HPO_4/KH_2PO_4$ buffer (pH 8.0), 4 g/L of puerarin, 60 g/L of sucrose, and $3.5 \times 10^8$ cells/mL in a 100-mL flask and incubated at 30 °C and 220 rpm for 48 h. After reaction, bacterial cells in the biotransformation mixture were removed by centrifugation (10,000 × *g*, 5 min, room temperature). Then, the reaction mixture was heated at 100 °C for 10 min and centrifuged again. The supernatant was collected and diluted 20 times for HPLC analysis. In the experiment of testing the specificity of the sugar donors, other saccharides, including glucose, fructose and maltose, were individually used as the donor at a concentration of 60 g/L, with sucrose severing as the control group at the same time. For time course analysis, cells from 25 mL of culture were suspended in 25 mL of biotransformation reaction buffer in a 100-mL flask. The reaction mixture was sampled (1 mL) at 12, 24, 36 and 48 h, and the supernatant samples were analyzed by HPLC, after cell removal using the same method described above. To determine the tolerance of the strain to organic solvents, *L. fusiformis* CGMCC 4913 cells were suspended in reaction buffers containing 10% (v/v) methanol, ethanol, toluene, xylene, cyclohexane, chloroform or dimethyl sulfoxide (DMSO), respectively. The maximum tolerable concentration of ethanol was determined by adding 5–20% (v/v) ethanol in the reaction buffer. The optimum pH for biotransformation was determined at 30 °C. The optimum concentration of puerarin was determined by dosing different amount of puerarin (2–10 g/L) in the reaction mixture at pH 8.0 and 30 °C. Each result is presented as mean ± SD from three independent experiments with three replicates.

### 2.6. Analysis and identification of the biotransformation products

The biotransformation samples were analyzed on an Agilent 1100 HPLC system with an Agilent HC-C18 column (5 μm, 250 mm × 4.6 mm), eluted with 30% aqueous methanol (v/v) 0.1% (v/v) acetic acid at 1 mL/min. The products were detected at 254 nm.

To isolate the products for structure elucidation, 20 mL of the supernatant of the reaction mixture was subjected to a HP-20 column, washed by pure water to remove the sugars. It was then successively eluted by 5%, 10%, 20%, 50% and 100% methanol. The 10% and 20% methanol fractions, which contain target compounds, were concentrated and further separated on an Agilent 1220 HPLC equipped with a XDB-C18 column (5 μm, 250 mm × 4.6 mm). The column was eluted with 20% aqueous methanol (v/v) with 0.1% (v/v) trifluoroacetic acid at 1 mL/min to yield the products P1 and P2 in pure form (32.0 mg and 3.9 mg). These purified products were used to establish the standard curve to calculate the yields and ESI-MS spectra of the purified compounds were acquired on an Agilent 6130 quadrupole LC–MS in the positive and negative modes. NMR spectra were recorded in DMSO-$d_6$ on a JEOL NMR instrument (300 MHz for $^1$H NMR and 75 MHz for $^{13}$C NMR). Optical rotations were measured at 23 °C on a Rudolph Autopol IV automatic polarimeter with a 10-cm cell.

P1: $[\alpha]_D = -15.4^\circ$ (*c* 0.28, $H_2O$). $^1$H NMR (300 MHz, DMSO-$d_6$) (*J* in Hz): $\delta$ 9.51(1H, s, 4′-OH), 8.30 (1H, s, H-2), 7.94 (1H, d, 8.9, H-5), 7.40 (2H, d, 8.6, H-2′, H-6′), 6.99 (1H, d, 8.9, H-6), 6.80 (2H, d, 8.3, H-3′, H-5′), 4.81 (1H, d, 9.6, H-1″), 3.92 (1H, t, 6.9, H-2″), 3.95 (1H, m, H-4‴), 3.86 (2H, m, H-6‴), 3.79 (1H, m, H-5‴), 3.51 (1H, m, H-3‴), 3.46 (2H, m, H-1‴), 3.44–3.32 (5H, overlap, H-3″-H-6″). $^{13}$C NMR (75 MHz, DMSO-$d_6$): $\delta$ 174.9 (C-4), 160.9 (C-7), 157.1 (C-9 and C-4′), 152.6 (C-2), 130.0 (C-2′ and C-6′) 126.2 (C-5), 123.1 (C-3), 122.5 (C-1′), 116.3 (C-10), 114.9 (C-3′ and C-5′), 112.5 (C-8), 104.2 (C-2‴), 82.1 (C-3‴), 79.7 (C-5″), 78.5 (C-3″), 76.7 (C-4‴), 75.1 (C-5‴), 73.5 (C-1″), 70.8 (C-2″), 70.4 (C-4″), 62.4 (C-6‴), 61.5 (C-1‴), 61.1 (C-6″).

P2: $[\alpha]_D = -84.4^\circ$ (*c* 0.13, $H_2O$). $^1$H NMR (300 MHz, DMSO-$d_6$) (*J* in Hz): $\delta$ 9.53 (1H, s, 4′-OH), 8.35 (1H, s, H-2), 7.94 (1H, d, 8.9, H-5), 7.42 (2H, d, 8.6, H-2′ and H-6′), 6.99 (1H, d, 8.9, H-6), 6.78 (2H, d, 8.4, H-3′ and H-5′), 4.78 (1H, d, 7.3, H-1″), 4.62 (2H, brs, H-1‴ and H-1⁗), 3.10–4.00 (30H, overlap, H-2″-H-6″, H-2‴-H-6‴, H-2⁗-H-6⁗).

### 2.7. Determination of the water-solubility of the major biotransformation product

The purified product P1 was tested for its water solubility as previously described [4]. Briefly, the compound was mixed with 200 μL of distilled water in an Eppendorf tube at 25 °C. An ultrasonic cleaner (Soniprep 150, Sanyo) was used to maximize the solubility. After 1 h of sonication and centrifugation at 9,000 × *g* for 10 min to remove insoluble material, the solution was diluted, filtered through a 0.45-μm membrane, and used for HPLC analysis to determine the compound concentrations in the solution.

## 3. Results and discussion

### 3.1. Isolation and identification of a puerarin-biotransforming strain

Biocatalysis and whole-cell biotransformation have numerous advantages such as mild reaction conditions, high selectivity