J Ind Microbiol Biotechnol (2012) 39:299–305
DOI 10.1007/s10295-011-1031-7

BIOCATALYSIS

# Biotransformation of puerarin into puerarin-6″-*O*-phosphate by *Bacillus cereus*

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Received: 3 June 2011 / Accepted: 16 August 2011 / Published online: 1 September 2011


**Abstract** The biotransformation of puerarin catalyzed by *Bacillus cereus* NT02 was studied. A primary screening was carried out using 307 strains of bacteria isolated from soil which were able to grow in the presence of puerarin. Strain NT02, identified as *B. cereus*, was able to convert puerarin into puerarin-6″-*O*-phosphate. Under the optimum conditions, resting cells of *B. cereus* NT02 converted 27% of added 0.4 g/l puerarin into puerarin-6″-*O*-phosphate that was characterized by MS, $^{13}$C NMR, $^{31}$P NMR. The activity of puerarin-6″-*O*-phosphate was 25 times higher than that of puerarin in the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging system. The water solubility of puerarin-6″-*O*-phosphate was 85.4 times higher than that of puerarin.

**Keywords** *Bacillus cereus* · Biotransformation · Puerarin · Puerarin-6″-*O*-phosphate · Antioxidant

## Introduction

Puerarin, a naturally occurring isoflavonoid C-glycoside, is isolated from *Pueraria lobota*, one of the most popular Chinese herbal medicines, which is traditionally used to treat coronary heart disease, cardiac infarction, problems in ocular blood flow [14], sudden deafness, and alcoholism [1]. It has been suggested that puerarin plays a role in the management of various cardiovascular disorders, enhancing vascular relaxation independent of the endothelium [22]. In addition, puerarin may prevent cancer, by acting as an antioxidant and free radical scavenger [2], lower serum cholesterol levels, and have antiallergic activities. However, puerarin's efficacy is limited by its low aqueous solubility and poor biological activities [16]. In order to increase the pharmacological and biological activities of puerarin, methods of improving its physical and chemical characteristics have been studied in recent years [7, 8, 11, 15, 21]. Li et al. [15] demonstrated that glycosylation of puerarin in vitro by maltogenic amylase resulted in the formation of two puerarin glycosides, α-D-glucosyl-(1→6)-puerarin and α-D-maltosyl-(1→6)-puerarin, which have a 14- and 168-fold higher solubility than puerarin, respectively. Jiang et al. [11] used *Microbacterium oxydans* CGMCC 1788 to biotransform puerarin into puerarin-7-*O*-glucoside and puerarin-7-*O*-isomaltoside which have an 18- and 100-fold higher solubility than puerarin, respectively. Previous studies revealed that the fungus *Trichoderma harzianum* NJ01 strain could transform puerarin into 3′-hydroxypuerarin which has a 20-fold increased antioxidant activity and slightly improved water solubility [21]. Moreover, phosphorylation of flavonoids affords derivatives with wide-ranging bioactivities and increased aqueous solubility [4, 23]. In this study, puerarin was phosphorylated at the 6″-position to yield puerarin-6″-*O*-phosphate by using *Bacillus cereus* CGMCC 4326. Puerarin-6″-*O*-phosphate exhibited superior water solubility and higher antioxidant activity compared with puerarin.

## Materials and methods

### Materials

Puerarin (98%) used in this study was purchased from Shandong Lv Ye Pharmacological Co. (Shandong, China).

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DL-α-Tocopherol and 1,1-diphenyl-2-picrylhydrazyl (DPPH) were purchased from Sigma Chemical Co. (Louis, USA). Tris(hydroxymethyl)aminomethane (Tris), dimethyl sulfoxide (DMSO), ethanol, and acetic acid (HPLC grade) were purchased from Wako Pure Chemical Industries (Osaka, Japan). Methanol (HPLC grade) was obtained from Kokusan Chemical Co. Ltd. (Tokyo, Japan).

### Screening, isolation, and identification of bacterium

Bacterial strains were isolated from soil samples collected in Haohe River, Nantong, Jiangsu, China, by using Luria–Bertani (LB) agar medium plates (pH 7.0). Single colonies of bacteria were inoculated in 10 ml of LB medium (yeast extract, 5 g/l; peptone, 10 g/l; and NaCl, 10 g/l, pH 7.5) in 50-ml flask and cultivated at 35°C and 220 rpm until the late-exponential phase cultures appeared ($OD_{600} = 3.0$). After cells were harvested and washed with 50 mM $Na_2HPO_4/KH_2PO_4$ buffer (pH 7.5), they were resuspended in 10 ml of the biotransformation reaction mixture [50 mM $Na_2HPO_4/KH_2PO_4$ buffer (pH 7.5), with 0.2 g/l of puerarin] and incubated at 35°C under shaking conditions for 4 days. Culture control without added puerarin and substrate control without bacterial cells in the biotransformation reaction mixture were incubated under the same conditions. The supernatant samples from 1 ml of reaction mixtures at 24, 48, 72, and 96 h were subsequently subjected to HPLC. New peaks, along with the peak for puerarin, were selected for further LC–MS analysis. The strain NT02 with biotransformation activity was characterized and identified using standard biochemical tests [3] and then confirmed by amplification and sequencing of the 16S rRNA gene [17]. The obtained sequence was compared with sequences in GenBank by using an online Blastn alignment tool.

### Cultivation of strain NT02, biotransformation and purification of transformed product

The single colony of NT02 growing on LB agar was inoculated into 50 ml of LB medium in 250-ml flasks and incubated at 35°C and 160 rpm for 12 h ($OD_{600} = 3.0$). Cells were then harvested for biotransformation by centrifugation (6,000 × *g*, 5 min at room temperature). Cells (26 mg) from about 10 ml of cultivation broth were suspended in 50-ml biotransformation reaction mixture containing 50 mM $Na_2HPO_4/KH_2PO_4$ buffer (pH 7.5) and 0.4 g/l of puerarin in 50-ml flasks, and incubated at 35°C and 180 rpm for 48 h for the first biotransformation cycle. Then, the cells were treated for the second and third biotransformation cycles according to the above method. The biotransformation mixtures were sampled and analyzed by HPLC at designated times. Each result is the mean ± SD from three independent experiments with three replicates. After bacterial cells in the biotransformation mixture were removed by centrifugation (10,000 × *g*, 5 min, room temperature), the reaction mixture was heated at 100°C for 10 min and centrifuged again. The transformation broth was extracted twice with an equal volume of *n*-butanol. The concentrated extracts were purified by using a Shimadzu SPD-6AV preparative HPLC system equipped with a Shim-pack prep ODS (10 μm, 250 × 30 mm) column. The column was eluted with methanol/water (28:72, v/v) at a flow rate of 15 ml/min. Elution was monitored at 254 nm with a Shimadzu SPD-10A UV detector and then the eluted fractions were concentrated using a vacuum rotary evaporator until colorless needles were produced.

### HPLC analysis

The concentrations of the compounds during the biotransformation were quantitatively determined with an Agilent 1100 HPLC system equipped with a Zorbax ODS column (5 μm, 250 × 4.6 mm). Elution was carried out at a flow rate of 1.0 ml/min with methanol/water (28:72, v/v) and monitored at 254 nm with an Agilent G1314A UV detector. The concentrations of puerarin and its derivative were calculated from their standard curves.

### Spectral analytical methods

LC/ESI–MS and GC/EI-MS were carried out on an Agilent 1100 LC-MSD and a Waters GC–TOF mass spectrometer, respectively. $^{13}C$ NMR and $^{31}P$ NMR spectra of the product were recorded in DMSO-$d_6$ with a Bruker AV-400 spectrometer (Switzerland) at 400 MHz. Chemical shifts are stated in ppm relative to internal TMS.

### Solubility determination of puerarin and its derivative

The solubility of puerarin and its derivative was determined as described by Li et al. [15].

### Measurement of DPPH radical-scavenging activity

The DPPH radical-scavenging activity was measured according to the method of Yamaguchi et al. [20].

### Statistical analysis

Data are presented as the mean ± SD. The data were evaluated by one-way analysis of variance (ANOVA) using the SPSS program, and the differences between the means were assessed using Duncan's multiple-range test. Statistical significance was considered at $P < 0.05$.

## Results and discussion

### Isolation, screening, and identification

Approximately 307 strains of bacteria that could grow on LB medium containing 0.4 mg/ml of puerarin were isolated from local soils, among which only strain NT02 could convert puerarin into a new product detected by HPLC. The retention times of puerarin and its product (P) on the column were 11.866 and 4.607 min (Fig. 1), respectively.

Strain NT02 was characterized by standard biochemical tests. The results showed that NT02 was sporulating, Gram positive, and catalase positive. It could grow on lactose, glucose, rhamnose, sucrose, maltose, and dextrin in the presence of 5.5% NaCl. The 16S rRNA gene analysis of strain NT02 (GenBank accession no. HM345945) showed 100% similarity with that of *B. cereus*. Consequently, strain NT02 was identified as *B. cereus* (deposited in the bacterial collection of the China General Microbiological Culture Collection Center, CGMCC, accession no. 4326).

### Isolation and purification of transformed product

In a 5-l mini-jar fermentor system, 27% of puerarin in the transformation broth was converted to the product by *B. cereus* CGMCC 4326. By extraction, concentration, and HPLC preparative purification, 0.12 g of the biotransformation product with a purity of 96.5% was obtained from 1.4 g puerarin. ESI–MS analysis of the biotransformation product exhibited an $[M + H]^+$ peak at *m*/*z* 496.1 (Fig. 2), whereas the peak of puerarin was at *m*/*z* 416. The fragment ion at *m*/*z* 80 suggested the presence of a phosphate or sulfate ester.

### NMR spectral data of transformed product

The $^{13}C$ NMR (400 MHz, DMSO-$d_6$, $\delta$ ppm) data of puerarin and its biotransformation product are listed in

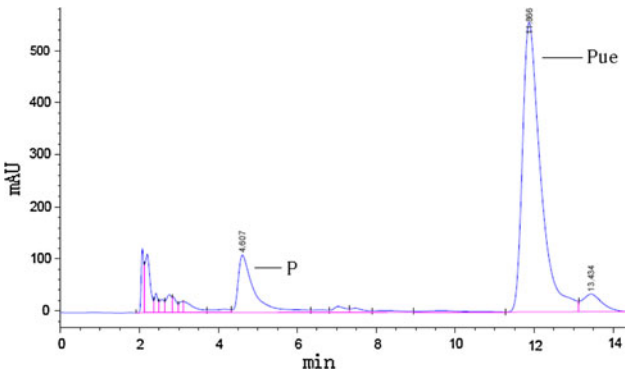



**Fig. 1** HPLC chromatogram of biotransformation of puerarin with *B. cereus*

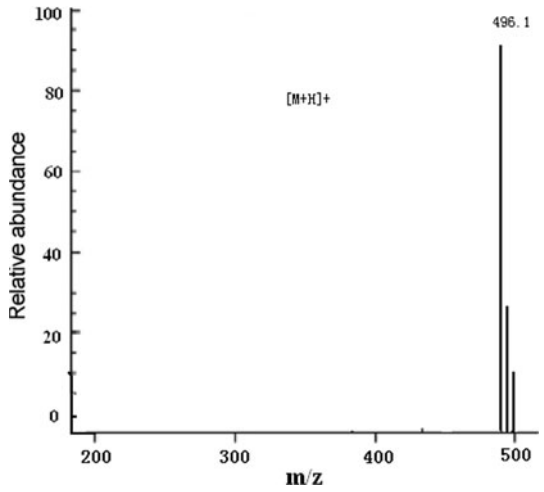



**Fig. 2** Mass spectrum of the biotransformation product of puerarin

**Table 1** $^{13}C$ NMR data of puerarin and its transformed product ($\delta$ in ppm)

| Ring | Number of atoms | Puerarin $\delta$ | Puerarin-6″-*O*-phosphate $\delta 1$ | Difference $\delta 1 - \delta$ |
|---|---|---|---|---|
| Daidzein | 2 | 152.7 | 153.0 | 0.3 |
| | 3 | 123.1 | 123.4 | 0.3 |
| | 4 | 175.0 | 175.4 | 0.4 |
| | 5 | 126.3 | 126.6 | 0.3 |
| | 6 | 115.2 | 115.9 | 0.7 |
| | 7 | 161.2 | 161.9 | 0.7 |
| | 8 | 112.7 | 112.9 | 0.2 |
| | 9 | 156.6 | 156.7 | 0.1 |
| | 10 | 116.8 | 117.1 | 0.3 |
| | 1′ | 123.0 | 123.1 | 0.1 |
| | 2′ | 130.1 | 130.4 | 0.3 |
| | 3′ | 115.0 | 115.4 | 0.4 |
| | 4′ | 157.2 | 157.6 | 0.4 |
| | 5′ | 115.0 | 115.4 | 0.4 |
| | 6′ | 130.1 | 130.4 | 0.3 |
| *β*-D-Glucosyl-(1–8)- | 1″ | 73.5 | 74.1 | 0.6 |
| | 2″ | 70.8 | 71.1 | 0.3 |
| | 3″ | 78.8 | 78.8 | 0 |
| | 4″ | 70.5 | 70.4 | −0.1 |
| | 5″ | 81.9 | 81.1 | −0.8 |
| | 6″ | 61.5 | 64.9 | 3.4 |

Table 1. Acylation of a hydroxy moiety as a sulfate or phosphate usually leads to a downfield shift of the adjacent carbon atom [13]. As shown in Table 1, large downfield shifts (bottom row in Table 1) were observed for C-6″ in the glucose moiety of puerarin, from 61.5 to 64.9 ppm, implying that the transferred sulfate or phosphate unit was

attached to C-6″ in the glucose moiety of puerarin. The $^{31}P$ NMR spectrum of the biotransformation product had signals at 2.27 ppm. The product was therefore identified as puerarin-6″-*O*-phosphate ester. This is the first reported example of puerarin having a monophosphate at the 6″-position. A previous study with *B. subtilis* NCI-21011 demonstrated that in similar isoflavonoids the phosphate was located at the 7-position [13].

## Optimal conditions for strain cultivation and puerarin transformation

The time-course experiment was used to investigate the biotransformation of puerarin by *B. cereus* CGMCC 4326. The kinetic data in Fig. 3 show that production of puerarin-6″-*O*-phosphate (P) increased linearly until 18 h with a stoichiometric decrease of puerarin. Puerarin-6″-*O*-phosphate accumulated in the reaction mixture at the highest rate during 18–20 h and thereafter degraded. The highest productivity was 27.0%. As shown in Scheme 1,

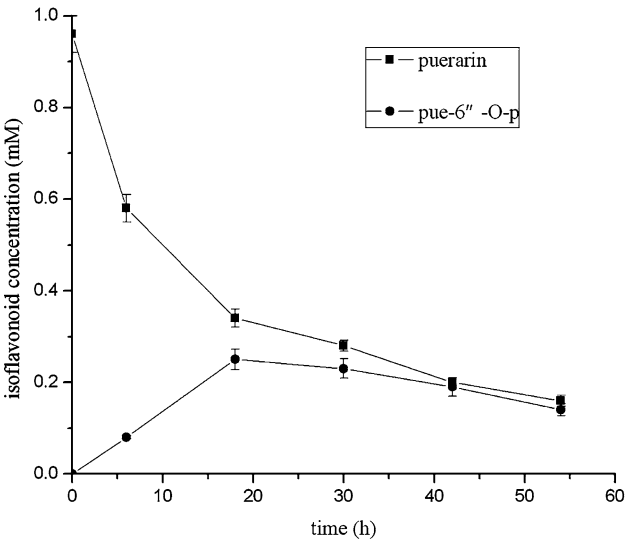
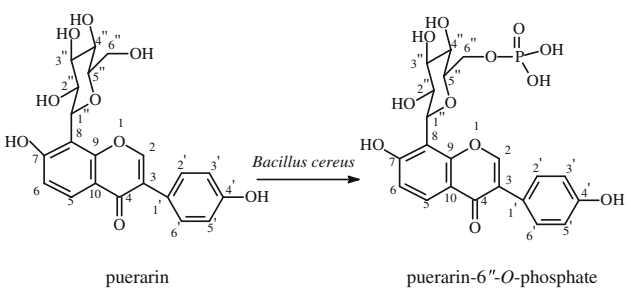



**Fig. 3** Time course of the biotransformation of puerarin with resting cells of *B. cereus* CGMCC 4326. Values are means ± SD of three separate experiments




**Scheme 1** Conversion of puerarin to puerarin-6″-*O*-phosphate by *B. cereus* CGMCC 4326

phosphorylation of puerarin by *B. cereus* CGMCC 4326 occurred preferentially at the 6″-position. The impacts of temperature, pH, and air on bioconversion rates were investigated. As shown in Fig. 4, the optimal biotransformation temperature was 35°C (Fig. 4a) and the optimal pH was 7.5 (Fig. 4b). In the 100-ml Erlenmeyer flask, transformation activities increased with an increase of the shaking rate, but decreased with an increase of reaction mixture volume over 25 ml. This indicates the $O_2$ requirement of the phosphorylation process. Transformation activities were the highest at 180 rpm in 25 ml broth in the 100-ml shaking flask (Fig. 5a, b). The biotransformation with resting cells of *B. cereus* CGMCC 4326 was also assayed in the presence of increasing puerarin concentrations, showing the highest conversion yields with 1.5 g/l of substrate, whereas a marked activity decrease was observed at higher substrate concentrations (Fig. 6). This result suggests that puerarin may be toxic toward the biotransformation strain, because the strain was not able to abundantly grow at concentrations of puerarin up to 2.0 g/l when puerarin was the only carbon source in the solid medium.

## Reutilization of resting cells and their residual activity in each biotransformation cycle

Table 2 shows the abilities of the resting cells to convert puerarin into puerarin-6″-*O*-phosphate in each biotransformation cycle. The concentration of puerarin-6″-*O*-phosphate in the first biotransformation cycle was 0.25 mM, whereas the concentrations of puerarin-6″-*O*-phosphate in the second and third biotransformation cycles were 0.092 and 0.032 mM, respectively, about 0.36 and 0.12 times that of the concentration of puerarin-6″-*O*-phosphate in the first biotransformation cycle. This suggests that the activity of the reutilized resting cells greatly decreased with each biotransformation cycle.

## Solubility and antioxidative activity of puerarin-6″-*O*-phosphate

Compared with puerarin, which has a limited water solubility of 11.8 ± 0.20 mM, puerarin-6″-*O*-phosphate has a significantly higher ($P < 0.01$) water solubility of 1,008.3 ± 19.3 mM, i.e., about 85.4 times.

In the radical-scavenging system of DPPH, the activity of puerarin-6″-*O*-phosphate was more than 25 times higher than that of puerarin and also 3.7 times higher than that of a standard positive control, the common antioxidant α-tocopherol (Fig. 7). This result provides some insight into the relationship between puerarin's structure and its activity.

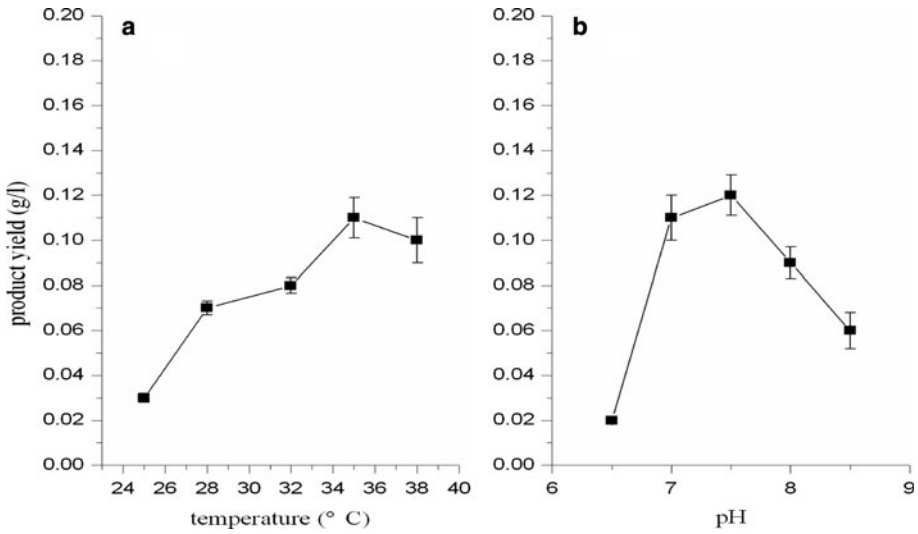



**Fig. 4** Effect of reaction temperature (**a**) and pH (**b**) on the product yield. Values are means ± SD of three separate experiments

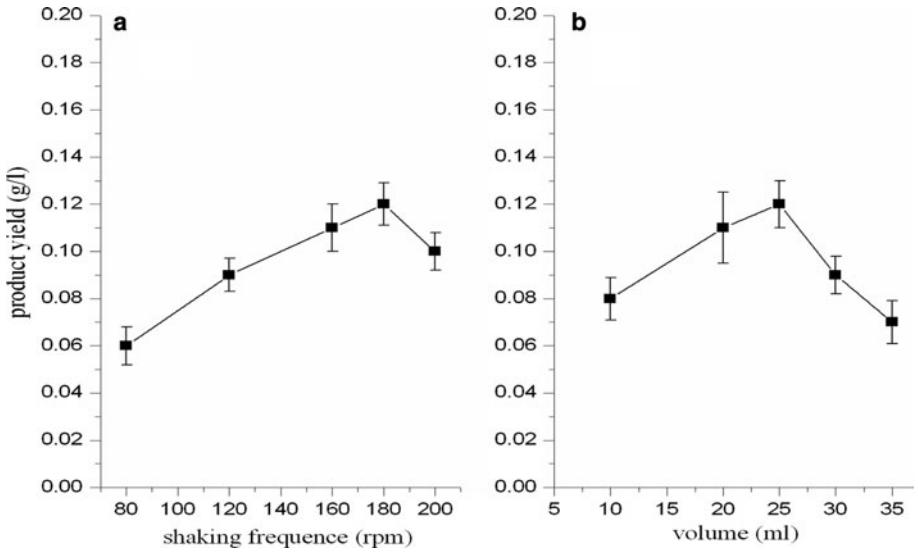



**Fig. 5** Effect of reaction shaking frequency (**a**) and volume of transformation broth (**b**) on the product yield. Values are means ± SD of three separate experiments

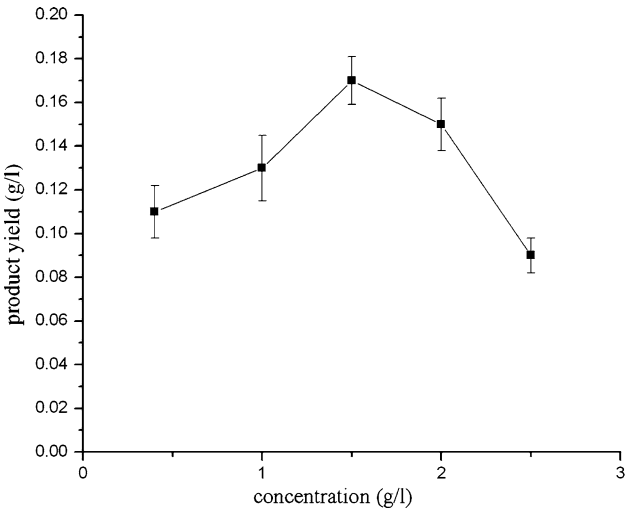



**Fig. 6** Effect of reaction concentration on the product yield. Values are means ± SD of three separate experiments

**Table 2** Reutilization of resting cells of *B. cereus* for the conversion of puerarin into puerarin-6″-*O*-phosphate

| Biotransformation cycles | Puerarin-6″-*O*-phosphate concentration (mM)[a] | Relative concentration |
|---|---|---|
| 1 | 0.25 ± 0.022 | 1 |
| 2 | 0.09 ± 0.005 | 0.36 |
| 3 | 0.03 ± 0.002 | 0.12 |

[a] Mean ± SD ($n = 3$)

Microbial modification of (iso)flavonoids such as puerarin and daidzein has been reported. The main goal of these studies was the identification of microbial species able to convert these substrates to valuable products, such as 3-hydroxypuerarin or puerarin-7-*O*-glucoside or daidzein 7-*O*-phosphate [11, 13, 21]. In the pharmaceutical industry, phosphate esters are often used as prodrugs to increase the water solubility and bioavailability of the active agent [12].

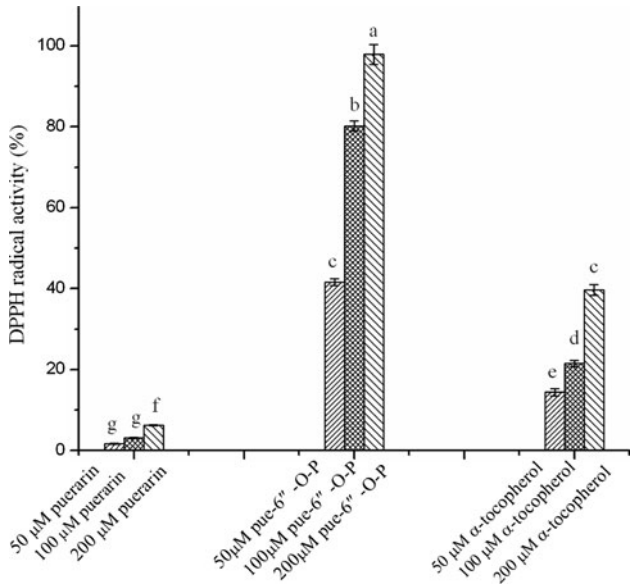



**Fig. 7** Antioxidative activity of puerarin, puerarin-6″-*O*-phosphate, and α-tocopherol in the radical-scavenging system using DPPH. Values are means ± SD of three separate experiments. *Different letters* indicate that compound concentrations are significantly different ($P < 0.05$, Duncan test)

Chen et al. [6] synthesized derivatives of puerarin to increase its pharmacological and biological activities. Zhang et al. [23] reported that chrysin-7-yl phosphate (CPE) and the tetraethyl bis-phosphoric ester of chrysin (CP) could effectively inhibit cell proliferation and induce apoptosis in Hela cells. Calias et al. [4] discovered that the solubility of inositol 2-phosphate-quercetin was 15,000 times higher than that of quercetin. Genistein halogenations were observed with *Actinoplanes* sp. HBDN08. The antioxidant activities of 3′,8-dichlorogenistein and 8-chlorogenistein were about twice that of genistein [18]. Sulfation reactions of flavonoids have been notably observed. With respect to flavanones, fermentation of naringenin with the fungus *Cunninghamella elegans* yielded naringenin 7-*O*-sulfate [9]. *Streptomyces fulvissimus* converted 5-hydroxyflavone into the more polar 4′,5-dihydroxyflavone-4′-sulfate [10]. Flavonoids sulfates exhibit many biological activities. In plants, sulfation of flavonoids was presumed to represent a way of inactivating harmful waste products and it may play a role in the transfer of sulfur from an inorganic to organic state [19]. Prunin (naringenin 7-glucoside), a flavanone glucoside resulting from the hydrolysis of naringin, showed no inhibitory effect against the microorganisms, but prunin 6″-*O*-lauroyl ester inhibited *Escherichia coli, Salmonella enterica*, *Bacillus* sp., and *Listeria monocytogenes* strains [5]. Microorganisms are known to hydroxylate flavonoids [21]; however, phosphorylation of flavonoids in microbial systems has rarely been observed. By combining the morphological and physiological description with chemotaxonomy and the 16S rDNA sequence, strain NT02 was identified as a member of *B. cereus*. Results obtained from this study unequivocally show that *B. cereus* is capable of phosphorylating puerarin. It is of great importance that puerarin-6″-*O*-phosphate is more soluble than puerarin, and phosphorylation contributes to an enhancement of puerarin antioxidant activity, although the yield of this bioconversion was relatively low (ca. 27%). Consequently, the development of the microbial phosphorylation of puerarin is of particular importance in medicinal chemistry. Further studies on the pharmacological effects of puerarin-6″-*O*-phosphate will accelerate the development of the method of converting puerarin to puerarin-6″-*O*-phosphate. Microbial biotransformation occurring mildly and often more conveniently is an important tool to convert natural resources into commercially valuable products.

**Acknowledgments** We are thankful for the support from the project funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD). We are also grateful to Dr. Wei-Min Zhao from Shanghai Institute of Materia Medica, Chinese Academy of Sciences, and Dr. Jia-Hong Zhou from Nanjing Normal University for their contribution to MS and NMR studies.

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