A Substrate Fed-Batch Biphasic Catalysis Process for the Production of Natural Crosslinking Agent Genipin with *Fusarium solani* ACCC 36223

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The natural crosslinking agent genipin has been applied widely in biomedicines and foods nowadays. Because of the special hemiacetal ring structure in its molecule, it can only be prepared by hydrolysis of geniposide according to biocatalysis. In this research, strategies including aqueous-organic biphasic catalysis and substrate fed-batch mode were adopted to improve the biocatalysis process of genipin. A 10 L ethyl acetate-aqueous biphasic system with geniposide fed-batch led to a satisfying genipin yield. With *Fusarium solani* ACCC 36223, 15.7 g/l genipin in the ethyl acetate phase was obtained, corresponding to space-time yields of 0.654 g l⁻¹ h⁻¹.

**Keywords:** Biphasic bicatalysis, geniposide, genipin, *Fusarium solani* ACCC 36223

**Materials and Methods**

**Chemicals**

Standards of geniposide and genipin were purchased from the National Institute for the Control of Pharmaceutical and Biological Products.

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**Introduction**

Geniposide (1) is one of the major iridoid glycosides of *Gardenia jasminoides* Ellis (Rubiaceae), which has been reported to contain various biological activities, such as hepatic-protective [13], neuroprotective [12], anti-inflammatory [8, 10], and anti-diabetes [21]. Nowadays, its aglucone genipin (2) has attracted a lot of researchers, because of its various applications in food and biomedical industries. Owing to the specific reaction with primary amines to form blue-colored adducts, genipin has been used to produce safe and edible stable pigments that are widely used as food colorants (gardenia blue) in beverages and desserts. Moreover, it has also been applied as a natural crosslinking agent in tissue engineering and drug delivery [4, 11, 14]. Compared with chemical crosslinking agents (e.g., glutaraldehyde), the cytotoxicity of genipin is approximately 5,000–10,000 times less [19]. Apart from these, it can be used in the forensic science as a fingerprint developer [1, 3], and in the textile industry for dyeing [18, 20].

According to the ether bond in the iridoid structure, genipin cannot be prepared easily by hydrolysis of geniposide in acid. Consequently, hydrolysis catalyzed by enzymes or microorganisms was considered as a good solution to this problem [5, 6, 23]. However, the yield of genipin was always affected by many factors (Fig. 1), because of the unstable hemiacetal ring structure. First, genipin would be hydrolyzed to a double aldehyde structure in water [15]. Second, it could crosslink with amino acids and enzymes to form pigments [17]. Hence, genipin is very expensive because of its difficulty and instability in preparation, which also limits its popularization. Therefore, it is necessary to investigate an efficient and economic method for genipin preparation, which can avoid the degradation and blue color formation.

In this research, *Fusarium solani* ACCC 36223 was screened as an efficient enzyme resource for genipin preparation. Moreover, a substrate fed-batch biphasic biocatalysis system was designed for the preparation of genipin with a high yield.
Geniposide extract was prepared by extraction, isolation, and purification from Fructus Gardeniae at the Nanjing Research Institute for Comprehensive Utilization of Wild Plants. The geniposide content was up to 90%, which was detected by HPLC.

Acetonitrile of chromatographic grade was purchased from Sigma (USA). Other chemicals were of analytical grade and obtained from Nanjing Chemical Reagent Co., Ltd.

Microorganism and Screening Test

Fusarium solani was obtained by courtesy of Prof. S. L. Chen of Nanjing Normal University, China. It has been deposited at Agricultural Culture Collection of China with an accession number of ACCC 36223. Aspergillus ochraceus CICC 40330 was obtained by courtesy of Prof. J. Zhang of China Pharmaceutical University, China. Rhizopus chinensis CICC 40927 and Trichoderma viride CICC 13002 were purchased from the China Center of Industrial Culture Collection.

Screening tests were carried out by vaccinating different strains into 5.0 ml of improved Potato Dextrose Agar (PDA) held in petri dishes and incubating at 30°C for 48 h. The improved PDA medium was prepared as follows: 200.0 g of peeled potatoes were cut into pieces, boiled in water for 20 min, and filtered. Glucose 10.0 g, KH$_2$PO$_4$ 3.0 g, NaNO$_3$ 1.5 g, agar 20 g, geniposide 5.0 g, and monosodium glutamate 2.0 g were added into the filtrate and diluted with distilled water to 1,000 ml before sterilization. If the geniposide was hydrolyzed by microorganisms, the medium would change into a bright blue, because of the combination of genipin and monosodium glutamate.

Analytical Methods

Geniposide and genipin were analyzed by HPLC (Agilent 1200) with the chromatographic column Eclipse XDB-C18 (150 × 4.6 mm, 5.0 µm), temperature of 30°C, and detection wavelength of 238 nm. The mobile phase was acetonitrile and water at a ratio of 15:85 and a flow-rate of 1.0 ml/min. The retention time of geniposide and genipin was 4.1 and 8.3 min, respectively.

Cultivation of Fusarium solani ACCC 36223

Fusarium solani ACCC 36223 was cultured in 500 ml flasks containing 150 ml of potato liquid medium in a rotary shaker (200 rpm, 30°C). The potato liquid medium was prepared as follows: 200 g of peeled potatoes were cut into pieces, boiled in water for 20 min, and filtered. Glucose 20 g, KH$_2$PO$_4$ 3 g, and MgSO$_4$·7H$_2$O 1.5 g were added into the filtrate and diluted with distilled water to 1,000 ml before sterilization. After 48 h, the culture was centrifuged (5,000 rpm, 5.0 min) and the supernatant was collected for further use.

Partition Coefficient of Geniposide and Genipin in Four Different Aqueous–Organic Biphasic Systems

With the purpose of screening the best aqueous–organic biphasic system, the partition coefficient of geniposide and genipin in the water phase of different aqueous–organic biphasic systems was analyzed. Geniposide (1.0 mg) and genipin (1.0 mg) were dissolved in 4.0 ml of water in quintuplicate. One of them was the control, and the other four were added to 1.0 ml of petroleum ether, chloroform, ethyl acetate, or n-butanol, respectively, to form four systems (APS, ACS, AES and ABS). After shaking for 5.0 min, the contents of geniposide and genipin in the water phase were detected by HPLC.

Preparation of Genipin in Aqueous Single-Phase System

Geniposide (100 mg) was added into 20 ml of culture supernatant. Then, the substrate-containing culture was fermented in a shaking...
incubator (200 rpm) at 30°C. After 4.0 h, the fermentation broth was analyzed by HPLC, which had been filtered through a 0.45 µm microporous filtering membrane first.

### Preparation of Genipin in Ethyl Acetate–Aqueous Biphasic System
Culture supernatant (120 ml) and ethyl acetate (30 ml) were mixed first. Geniposide (100 mg) was subsequently added into the biphasic system. The substrate-containing biphasic system was cultivated in a shaking incubator (200 rpm) at 30°C. After 4 h, each sample (10 µl) of aqueous phase and ethyl acetate phase were analyzed by HPLC, respectively.

### Optimization of Reaction Conditions in Biphasic System
The effect of reaction pH on the preparation of genipin was determined. The substrate-containing biphasic systems were adjusted to a specified pH (3.5, 4.0, 4.5, 5.0, 5.5, and 6.0) under otherwise similar conditions. Meanwhile, the effect of reaction temperature was examined at different temperatures from 30°C to 70°C by assaying their activities at the respective temperatures under otherwise similar conditions. After 4 h, the content of geniposide in the aqueous phase was analyzed by HPLC, respectively. All experiments were performed in triplicate.

Furthermore, the inhibition of substrate was determined according to the reaction time course of geniposide conversion. Three AES systems with different substrate (geniposide) concentration (w/v: 1%, 3%, and 5%), respectively, were incubated in a shaking water bath (200 rpm) at the optimal pH and temperature. The time courses were formed by the geniposide conversion at 1, 2, 4, 6, 10, 16, and 24 h, respectively.

### 10 L AES Biphasic Catalysis Process with Substrate Fed-Batch
_Fusarium solani_ ACCC 36223 was cultivated in a 25 L fermenter (30°C, pH 7.0, and 200 rpm) with the aeration rate of 1.5 l/min. After 48 h, the fermentation broth was collected and centrifuged (5,000 rpm, 15 min), with a harvest of 13.6 L supernatant. Then, a 10 L AES biphasic system was formed by mixing 8.0 L of fermentation broth supernatant and 2.0 L of ethyl acetate. Geniposide (500 g) was divided into three parts and fed as solid at 0, 4, and 8 h, respectively. The time course of geniposide content and genipin yield in this process was detected by HPLC. The volumetric productivity and space-time yield of this process were also calculated.

### Results

#### Screening Test of Microorganisms
By the screening of four different microbes, we found that the medium of _F. solani_ ACCC 36223 changed into bright blue after 24 h, because of the combination of genipin and glutamic acid (Fig. 2). Therefore, geniposide can be hydrolyzed to genipin efficiently by the enzymes of _F. solani_ ACCC 36223.

#### Partition Coefficient of Geniposide and Genipin in Four Different Aqueous–Organic Biphasic Systems
By comparing the partition coefficient of geniposide and genipin in the aqueous phase of biphasic systems, the AES biphasic system was screened as the best of these four (Fig. 3). In the APS and ACS systems, the partition coefficients of genipin in the aqueous phase were 91.6% and 87.7%. It means genipin could not transfer into these two organic solvents from aqueous phase easily. When the AES and ABS biphasic systems were used, although the partition coefficients of geniposide in water were decreased to 76.5% and 72.8%, those for genipin were decreased much more significantly to 23.7% and 23.2%. Hence, genipin transferred into the organic phase in these two biphasic systems, while geniposide stayed in the water for continuous reaction. Because the emulsification of the ABS biphasic system is serious, the AES biphasic system was screened as the best biphasic system in this research.

#### Difference of Geniposide Hydrolysis in Aqueous Single Phase and Ethyl Acetate–Aqueous Biphasic System Catalysis
While geniposide was hydrolyzed in the aqueous single

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**Fig. 2.** Screening test of _Fusarium solani_ ACCC 36223 (A: top; B: bottom).

**Fig. 3.** Distribution of geniposide and genipin in the aqueous phase of different biphasic systems.
phase and AES biphasic system for the same time, the concentration of geniposide in each phase was analyzed by HPLC. Geniposide was transformed efficiently in both reaction systems, but there were some obvious differences (Fig. 4). First, a significant by-product was detected in the aqueous single phase reaction system. It was considered to be pigments produced by the cross-linking reaction of genipin and amino acid [6]. Second, there was only a little genipin detected in the aqueous phase; otherwise, a large number was detected in ethyl acetate. All of these evidence indicated that genipin transferred from the aqueous phase to ethyl acetate immediately in the AES biphasic system, which protected it efficiently from side reactions in the aqueous phase.

Effects of Reaction Conditions in AES Biphasic System

Optimization of the reaction conditions was assayed at different reaction temperatures (30°C, 40°C, 50°C, 60°C, and 70°C) and pH values (3.5, 4.0, 4.5, 5.0, 5.5, and 6.0). As shown in Fig. 3, the optimum conditions were determined to be 50°C and pH 5.0, and the influence of temperature was more significant (Fig. 5).

Using the optimum temperature and pH condition, time courses of this reaction in different geniposide concentrations were formed by the geniposide conversion, respectively. The conversion of geniposide at 24 h decreased from 85.6% to 47.0% while its concentration increased from 1% to 5% (Fig. 6). Therefore, inhibition of substrate concentration in the preparation of genipin is indubitable.

Yield of 10 L AES Biphasic Catalysis Process with Substrate Fed-Batch

Substrate conversion and product yield are very important factors for the reaction conditions optimization in laboratory-scale preparation. In industrial production, volumetric

Fig. 4. Hydrolysis of geniposide catalyzed by β-glucosidase for 2 h in water or biphasic catalysis. a: blank; b: catalysis in single aqueous phase; c: aqueous phase of AES biphasic catalysis; d: ethyl acetate of AES biphasic catalysis.

Fig. 5. Effects of reaction pH (A) and temperature (B) on the geniposide hydrolysis activity.

Fig. 6. Time course profile for the hydrolysis of geniposide in the aqueous phase of biphasic catalysis.
productivity and space–time yield will be more attractive. With the purpose of increasing these two factors, a 10 L AES biphasic catalysis process with substrate fed-batch was designed. Geniposide (500 g, 5% (w/v)) was divided into three parts and fed at a certain time. Downward-facing arrows indicate addition of substrate at each time.

Discussion

A lot of research on the transformation of geniposide to gardenia blue has been done [2], but only several researches investigated on the preparation of genipin. Xu et al. [23] investigated the microbial transformation of geniposide to genipin with a very high conversion rate of genipin (95%) at 120 h by *Penicillium nigricans*. However, the isolation and purification of genipin after fermentation by the combined application of macroporous resin and silica gel columnation are complex. Khanal et al. [9] chose human intestinal microflora as the enzyme resource in the biotransformation of geniposide. Comparing with microbial use, more researchers applied enzymes in the preparation of genipin for its high efficiency, such as β-glucosidase and amygdalase. Gong et al. [7] purified a β-glucosidase from *A. niger* and applied it in the biotransformation of geniposide. Niu et al. [16] optimized the process of genipin preparation by beta-glucosidase with a conversion rate of 85.8% at 20 h using a high ratio of β-glucosidase/geniposide (1/1). Zheng et al. [25] applied amygdalase in the process and optimized by quadratic general rotation design. The conversion rate was 36.4% at 1.5 h with amygdalase/geniposide (1/10). By decreasing the enzyme ratio and reaction time, there was less blue sediment of by-product [25]. As we know, biphasic catalysis was only used in two of them. Winotapun et al. [22] reported a one-pot reactor to extract and hydrolyze geniposide at the same time, catalyzed by the cellulase from *A. niger* [22]. Yang et al. [24] reported the usage of immobilized β-glucosidase in phase-transfer catalysis, with the purpose of protecting the enzyme from the organic solvent [24]. In our research, whole-cell biphasic catalysis was used in the preparation of genipin for the first time. According to this, the product genipin was protected from the side reaction by transferring into ethyl acetate. It can also be easily purified by recrystallization without chromatography. In fact, the optimum reaction conditions (50°C and pH 5.0) of *Fusarium solani* ACCC 36223 is very similar with β-glucosidase [5]. Thus, it may be a new and good resource of β-glucosidase. Besides this, a biphasic catalysis process with substrate fed-batch was also investigated for genipin preparation. According to this, the volumetric productivity and space-time yield of genipin in 10 L scale are 15.7 g/l and 0.654 g l⁻¹ h⁻¹, respectively. Hence, genipin can be prepared efficiently by AES biphasic catalysis of *Fusarium solani* ACCC 36223 with substrate fed-batch.

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References