Improved Gastrodin Production of Biotransformation Conditions by Cultured Cells Armillaria luteo-virens Sacc and the Anti-inflammatory Activity In Vivo

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Abstract

Gastrodin (GAS), the main bioactive component of G. elata Blume, has important pharmaceutical and functional activities. The aim of this study is to produce GAS from p-2-hydroxybenzyl alcohol (HBA) through biotransformation. The conversion of exogenous HBA into GAS compound was conducted using cell suspension cultures of Armillaria luteo-virens Sacc. The bioconversion conditions were fully optimized with response surface methodology (RSM), turning out that the optimal transformation conditions composed of 3 mg/mL HBA, 6.5 g/30 mL inoculum levels, 1.5% Tween 80, pH 4.5, and transformation temperature at 23°C. Under the optimized conditions, the conversion productivity of GAS reached the highest value (5.65 ± 0.45 mg/L). Verified experiments further validated that the optimized conditions were suitable for predicting the actual process of HBA transformation in the resting-cell system. The bioconversion kinetics model was as well simulated with Michaelis–Menten equation, which showing the suitability. The present study proposed the biotransformation pathway of HBA into GAS by resting cells transformation, indicating that the biotransformation process involved glucosylation reaction. Furthermore, Imprinting Control Region (ICR) mice in vivo demonstrated that the identified gastrodin possessed a significant anti-inflammatory activity. The fundamental data in the present work provides an efficient way to produce GAS through the whole-cells biocatalysis.

Keywords: Gastrodin; Armillaria luteo-virens Sacc; Biotransformation conditions; Optimization; Anti-inflammatory activity

Introduction

Gastrodin, the bioactive component of G. elata Blume, has sedative and anticonvulsant functions, neuroprotective effect, facilitating memory consolidation, retrieval, antioxidant and free radical scavenging activities [1,2]. Recent studies have focused mainly on the extraction, identification, quantification and pharmaceutical activity of functional compounds from Gastrodia elata Blume [3-6]. However, the traditional extraction is time-consuming and high-cost. And also, the chemical synthesis of GAS does cause environmental pollutions. As a result, developing a new method to produce GAS more economically and more environmentally friendly is to be an interesting and prospective routine.

Presently, there are still many researches focusing on obtaining GAS from p-2-hydroxybenzyl alcohol (HBA) or p-hydroxybenzaldehyde (HBD) through biotransformation. Among those researches, fungi are not commonly used to the possible biotransformation of HBA. Zhang et al. reported that GAS can be produced by fungi cells, such as A. luteo-virens Sacc [7]. Fan et al. identified that the highest yield of gastrodin was 36 mg/L for Aspergillus foetidus ZU-G1 and 65 mg/L for Penicillium cyclopium AS 3.4513 during the resting cell biotransformation [8]. In general, biotransformation is a useful tool for modifying structures of biologically active compounds owing to its so many good advantages as high stereo- and region-selectivity, mild reaction condition, simple operation procedure [9] and more approached to examine the metabolism of natural products [10] compared with chemical synthesis. The characteristic of biocatalysts as converting exogenous substrates to region- and stereo- selectively useful substances under mild conditions is one of great interests, since some of the resulted products are not easy to prepare through biotransformation or synthetic chemical methods [11]. The main reactions involved include oxidation, reduction, hydroxylation, esterification, methylation, isomerization and glycosylation [12,13]. Glycosylation using in vitro plant cultures, as a one-step enzymatic reaction is useful for the preparation of glycosides rather than chemical glycosylation, which requires tedious steps including the protection and the deprotection of the hydroxyl groups of sugar moieties [14]. More studies concerning the abilities of glycosylation have been observed at plant cell tissue cultures [15,16]. There have been literatures on the glycosylation of exogenously supplied phenolic compounds through the cultured plant cells [17-19].

However, little attention has been paid to the glycosylation of HBA or HBD by cultured microbial cells. Thus, in considering of previous study [7], we conducted the enhance gastrodin production by resting cells transformation of A. luteo-virens Sacc, and evaluated the anti-inflammatory effect of biotransformed gastrodin. The objectives of this study are to optimize transformation conditions for the maximal GAS production by cultured A. luteo-virens Sacc cells, then to simulate a kinetics model of biotransformation process and lastly to evaluate the anti-inflammatory activity of GAS obtained from biotransformation broth.

Materials and Methods

Chemicals and reagents

Standard GAS (98% purity) was purchased from Chinese Pharmaceutical Bio-product identification Institute (Beijing, China). HBA (99%) was purchased from Sigma-Aldrich Chemical Co., (St.
Sacc QH (CGMCC 1884) was inoculated into a 250 mL flask containing 30 mL of culture medium were inoculated with cells before subculture. After additional 5 days of subculture, the cultures were incubated with the shaking speed of 100 r/min at 23°C for 3 days. After that, the culture broth was centrifuged at 10,000 g for 30 min, and the cultured cells were washed with sterilized water for three times, collected and stored at 4°C for further use [7].

**Microorganism and preparation of cultured suspension cells**

*Armillaria luteo-virens* Sacc QH (CGMCC 1884) was inoculated and incubated at 23°C for 3 days. Erlenmeyer flasks (250 mL) containing 30 mL of culture medium were inoculated with cells before 5 days of subculture. The cultures were incubated with the shaking speed of 100 r/min at 23°C for 3 days. After that, the culture broth was centrifuged at 10,000 g at 4°C for 30 min, and the cultured cells were washed with sterilized water for three times, collected and stored at 4°C for the following use.

**Biotransformation and structure identification of GAS**

The suspended cells were cultivated in a 250 mL flask containing 30 mL liquid transformation broth (3% glucose and sterilized water, initial pH 5-6). The prepared substrate solution dissolved in absolute ethanol (1 mL) was added to one flask with resting cell culture, and one additional flask without substrate as the control. After additional 5 days of incubation at 23°C in darkness, the cell cultures were filtered under 4000 r/min at 23°C for 3 days. After centrifugation at 10,000 g at 4°C, the suspension cells were then re-suspended into 5 L fermenter containing 3 L biotransformation medium. Time-course experiment and mathematical model simulation

The accumulation of fungus mycelia was firstly carried out in batch through submerged cultivation with 2 L stirred tank fermenter. After mycelia growth reached a stationary phase (72 h), cells were harvested by centrifugation at 10,000 g for 20 min (4°C). The cells were washed with sterile deionised water and re-centrifuged. The obtained cells from the submerged culture were re-suspended into 5 L fermenter containing 3 L biotransformation medium. Time-course experiment and mathematical model simulation

The accumulated mycelia of *A. luteo-virens* was inoculated into 2 L stirred tank fermenter. After mycelia growth reached a stationary phase (72 h), cells were centrifuged at 10,000 g for 20 min (4°C). The cells were washed with sterile deionised water and re-centrifuged. The obtained cells from the submerged culture were then re-suspended into 5 L fermenter containing 3 L biotransformation medium. Time-course experiment and mathematical model simulation

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**Optimization experiment design and response surface analysis**

To develop suitable transformation conditions for GAS production, a series of statistically designed studies involving kinds of impact factors were conducted. The optimization process firstly entailed to identify the factors influencing GAS production by varying one factor at a time while keeping the others constant. Then response surface methodology (RSM) was applied to optimize the main factors that selected from the first step experiment. Central composite design (CCD) is a powerful tool for rapid optimization of influencing factors. CCD was used to investigate the factors influencing the production of GAS [21]. In the CCD, a leading to 16 sets of experiments, performed in duplicate, was used to optimize most significant factors affecting GAS production. The variables were coded according to the following equation:

\[
x_i = \frac{(X_i - X_0)}{\Delta X_i}
\]

Where \(X_i\) is the coded value of an independent variable, \(X_0\) is the real value of an independent variable, \(X_i\) is the real value of an independent variable at the center point, and \(\Delta X_i\) is the step change value.

In this study, the range and the level of variables both coded values and natural values were given in Table 1. GAS (\(Y_1\) and \(Y_2\)) were considered as the dependent variable or response (\(Y\)). In order to fit the empirical second-order model, a CCD with five coded levels was developed. The model proposed for the response (\(Y\)) was shown in Equation 2:

\[
y = b_0 + \sum b_i X_i + \sum b_{ij} X_i^2 + \sum b_{ij} X_i X_j
\]

Where \(y\) is the response variable, \(b_0, b_i, b_{ij}, b_{ij}\) are the regression coefficient variables, for intercept, linear, quadratic and interaction terms, respectively, and \(X_i\) is independent variables.

**Determination of GAS by Reverse-phase HPLC (RP-HPLC)**

The crude samples with two-step purification were analyzed by RP-HPLC according to method by Zhang et al. [7]. The RP-HPLC system used throughout the current study consisted of two Waters 510 pumps (Waters, Milford, MA, USA), a sample injector (Rheodyne, Cotati, CA, USA) equipped with a 20 µL loop, and a Waters 996 photodiode array detector. Evaluation and quantification were made on a Millennium Chromatography Data System (Waters). The column was a Reversed-phase Symmetry C18 (150 mm × 3.9 mm, i.d., 5 µm, Waters). The mobile phase was filtered, degassed by sonication and pumped through the system at a flow rate of 1.0 mL/min under 35°C. The normal operating pressure was 90–120 MPa and the analytical time was 20 min. The mobile phase consisted of eluent A (methanol) and eluent B (0.02% phosphoric acid, pH 2.6) in the aso-gradient mode as follows: 95% A and 5% B, using a flow rate of 0.5 mL/min and 220 nm was used as the preferred wavelength. Routine sample calculations were made by comparison of the peak area with the standard curve. GAS was analyzed and identified with the combination of HPLC-MS and NMR methods [7].

**Mycelia measurement and the residual reduced sugar analysis**

Fungal mycelia were collected with centrifugation at 10,000 g and weighed at 4°C for measurement. Residual reducing sugar was measured with 3,5-dinitrosalicylic acid colorimetric method (DNS) [20].

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course experiments were performed by a procedure similar to normal transformation experiments using the optimized conditions and non-optimized conditions. At regular time interval (12 h), one of the flasks was determined to evaluate the bioconversion yields of GAS by HPLC as described above. The advantage of constructing a model from its overall reaction is that all neglected variables and rate constants can be recovered if evidences negating the validity underlying one or more of the assumptions emerge [22].

**Anti-inflammatory activity evaluation in vivo of the biotransformed product**

180 ICR (Imprinting Control Region) mice (18–22 weeks) were purchased from Animal Center, Zhejiang Chinese Medical University, China. The test animals were maintained in a standard environmental condition and fed with rodent diet and water. All the procedures were in strict accordance with the China legislation on the use and care of laboratory animals and with the guidelines established by the Institute for Experimental Animals, and also were approved by the Committee for Animal Experiments.

**Xylene-induced ear edema**

Mice were divided into five groups, with each group consisting of eight animals. Thirty minutes after i.p. injection of the extract, namely, diclofenac, 0.05 mL of xylene was applied to the anterior and posterior surfaces of the right ear. The left ear was considered as the control. One hour after xylene application, mice were killed and both ears were removed. Circular sections were taken, using a cork borer with a diameter of 8 mm, and weighed. The increase in weight caused by the irritant was measured by subtracting the weight of the untreated left ear section from that of the treated right ear sections. The formula for computing percent inhibition was: average writhes in the control group (normal saline) minus writhes in the drug group divided by writhes in the control group times 100%. The control group received normal saline (10 mL/kg), diclofenac (10 mg/kg) were used as reference drug.

**Cotton pellet-induced granuloma**

The experimental mice were divided into five groups, with each group consisting of eight animals. After shaving the fur, the mice were anesthetized with ether and 20 mg of sterile cotton pellets were surgically inserted into the groin region. Each of the three groups including the GAS samples, indomethacin (10 mg/kg, p.o.) and the control (only saline) was administered for 7 consecutive days from the day of the cotton pellet implantation. The animals were anesthetized on the 8th day and the cotton pellets were removed surgically so that the mice were made free from extraneous tissues. The pellets were incubated at 37°C for 24 h and dried at 60°C for constant weight. Increment in the dry weight of the pellets was taken as measure of granuloma formation [23].

**Statistical analysis**

All the experiments were carried out in triplicate. Treatment effects were analyzed using analysis of variance to determine the differences between the treatment means of GAS concentration. Differences were considered to be significant at P<0.05 level throughout this study.

**Results and Discussion**

**Optimization of biotransformation conditions for GAS production**

The leading factors that affect GAS production to a great extent were chosen to optimize the maximum production of GAS through the resting-cells suspension transformation. Based on the preliminary results and reported data, [7] the initial pH, precursor concentration, inoculums level and Tween 80 had significant effects on HBA conversion. In this work, we conducted CCD to evaluate the effects of four factors on GAS formation. The levels of four factors designed are shown in Table 1. The experimental design and its results were demonstrated in Table 2. Response surface design is appropriate when the optimal region for running the process has been identified. The optimization of GAS production was carried out using Box-Wilson CCD with four star points and five replicates at center point for each of the four factors, with other factors fixed at suitable levels. X1, X2, X3 and X4 were further optimized in order to obtain the optimal conditions.

The regression analysis was performed to fit the response with the experimental data. The statistical significance of the second-order equation was checked by an F-test (Table 3). Among various treatments, the highest bioconversion yield (5.73 mg/L) was from trial 21 and the smallest conversion (only 0.45 mg/L) was from trial 18. Coefficients of a full model were evaluated by regression analysis. The insignificant coefficients were eliminated stepwise on the basis of the F value. As indicated in Table 3 (Y), the model was significant (P<0.05). The fit value, termed R2 (determinant coefficient), of the polynomial model was calculated to be 0.92, indicating that 92% of the variability in the response could be explained by the second-order polynomial prediction model. Thus, the final polynomial equation is suitable to explain the actual biotransformation process of HBA (Equation 3).

Based on the statistical and mathematical analysis, the yield of GAS reached the maximum point at a combination of uncoded levels, namely, HBA concentration of 3 mg/mL, inoculums level of 6.5 g/30 mL, 1.5% Tween 80, initial pH 4.5, temperature at 23°C. This model

| Factors | Levels |
|---------|--------|
| X1      | Initial pH |
| X2      | Tween 80 (%) |
| X3      | HBA concentration (mg/mL) |
| X4      | HBA levels (g/30 mL) |
| runs    | table 2: CCD experiments |
| Y runs  | 1       |
| 1       | 0.61    |
| 2       | 0.25    |
| 3       | 1.26    |
| 4       | 0.26    |
| 5       | 0.71    |
| 6       | 0.44    |
| 7       | 0.35    |
| 8       | 0.30    |
| 9       | 0.35    |
| 10      | 0.75    |
| 11      | 0.12    |
| 12      | 1.00    |
| 13      | 0.42    |
| 14      | 0.39    |
| 15      | 0.67    |
| 16      | 0.71    |
| 17      | 0.95    |
| 18      | 0.15    |
| 19      | 0.77    |
| 20      | 0.60    |
| 21      | 1.05    |

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predicted the maximal response value of 5.65 ± 0.45 mg/L at 120 h, which also can be confirmed by the response surface plot presented in Figure 1.

\[ Y = 1.75 + 0.37X_1 - 0.14X_2 - 0.23X_3 - 0.71X_4 + 0.80X_1^2 + 0.41X_2^2 - 0.31X_3^2 + 0.86X_4^2 - 1.40X_1X_2 + 0.05X_1X_3 - 1.11X_1X_4 + 1.51X_2X_3 + 1.13X_2X_4 - 1.04X_3X_4 \]

(3)

**Model verification and biotransformation kinetics simulation**

To validate the predicted results, experiments using the improved formula were performed and the observed values are shown in Figure 2a and 2b. On the basis of the solutions given by this design, two runs of experiments were established at the fixed conditions. The experiment values were found to be in reasonable agreement with the predicted ones, which confirmed the validity and adequacy of the predicted model. Therefore, it could be concluded that the optimization of mycelia-catalyzed synthesis of GAS was successfully developed through the current design.

![Figure 1: Response surface plots of initial pH, Tween 80, HBA concentration and inoculums level against gastrodin (GAS) productivity.](image)

| Source | Mean squares | F-Value | Prob>F |
|--------|--------------|---------|--------|
| Model  | 5.04         | 4.68    | 0.0337 |
| $X_1$  | 0.79         | 0.73    | 0.0425 |
| $X_2$  | 0.11         | 0.11    | 0.0755 |
| $X_3$  | 0.70         | 0.65    | 0.0452 |
| $X_4$  | 2.84         | 2.84    | 0.0156 |
| $X_1^2$| 9.49         | 8.81    | 0.0250 |
| $X_2^2$| 2.56         | 2.38    | 0.1739 |
| $X_3^2$| 1.43         | 1.33    | 0.2925 |
| $X_4^2$| 10.96        | 10.18   | 0.0188 |
| $X_1X_2$| 6.50        | 6.04    | 0.0493 |
| $X_1X_3$| 0.02        | 0.02    | 0.8865 |
| $X_1X_4$| 4.10        | 3.81    | 0.0989 |
| $X_2X_3$| 18.12       | 16.83   | 0.0063 |
| $X_2X_4$| 4.21        | 3.91    | 0.0952 |
| $X_3X_4$| 8.67        | 8.05    | 0.0297 |

*R^2=0.92.

**Table 3: ANOVA result of GAS production ($Y_1$) with CCD optimization**.

![Table 3](image)
A comparison between the optimized transformation conditions and the non-optimized one was carried out (Figure 2). Generally, the optimized conditions were much more beneficial than that of the non-optimized for GAS production. The highest content of GAS was observed at 144 h biotransformation time (>550 μg/100 mL), but at that time GAS from the non-optimized condition was found to be lower than 300 μg/100 mL. The conversion rate of HBA under the optimal condition was as well higher than that of the non-optimized. However, the glucose consuming rate showed insignificant difference between the positive sample and the non-optimized one. The bioconversion yield of HBA followed the Michaelis–Menten equation, suggesting that the transformation rate varied with substrate concentration was similar to the behavior of many enzymes reaction. The bioconversions of hydrophobic compounds often meet with two serious obstacles: limited substrate accessibility to the biocatalyst as a result of the low aqueous solubility of most organics; inhibition or toxicity of both substrate and product exerted upon the microorganism [24]. The reaction rate for GAS was increasing while transformation time lasting, implying that the formation of GAS in the re-suspended cells system followed the Michaelis–Menten equation. The simulated plots between the kinetic model and the actual biotransformation data were presented in Figure 3. The constructed model well explained the transformation process of HBA whether under the optimized or non-optimized condition (R²>0.98, P<0.05). This study will provide the preliminary evidences for understanding the glucosylation of HBA through resting-cells bioconversion. However, to obtain the highest GAS production, the regulation of metabolism activity should be elucidated as well.

Proposed biotransformation pathway of GAS

The novel one-step biotransformation from HBA will be economic for industrial scale production and can be applied to the C-11 hydroxylation of any other steroidal drugs or related compounds. Glucosylation reactions are of special interest as they facilitate the conversion of water-insoluble compounds to water-soluble compounds, i.e., detoxification reaction and bioactivity improvement [8]. Till now, limited is available with respect to the glucosylation process of HBA by aerobic microorganisms. Only a few transformation pathways by several cultured plant cells have been proposed [25]. According to the relevant reports [26,27], uridine diphosphate glucose (UDPG) was formed during biotransformation and thus the glucosylation process should be included. On the basis of this study, the presence of UDPG formed during biotransformation and thus the glucosylation process should be included. On the basis of this study, the presence of UDPG measured throughout the biotransformation process confirmed this supposition (data not shown). Combined the data with other findings [28,29], the biotransformation pathway of GAS formation using the developed transformation system was proposed (Figure 4). The process of glycosylation has been studied extensively in prokaryotes but many questions still remain unanswered. Glycosyltransferase is the enzyme which mediates glycosylation and has its preference for the target glycosylation sites as well as for the type of glycosylation. Through lots of reported studies, we can speculate that glycosyltransferase and UDPG-producing enzymes may play important roles in GAS biosynthesis, while not further discussed in this investigation. Future study should focus on the enzymes identification and molecular elucidation of the related enzymes that involved into GAS formation.

Anti-inflammatory activity of GAS from the transformed broth

As previously reported, GAS derived from Chinese medicinal orchid *Gastrodia elata* plant possesses many beneficially clinical and pharmacological functions, such as sedative and anticonvulsant functions, neuroprotective effect, etc. [30] To evaluate the bioactive or pharmaceutical activity of GAS produced through biotransformation procedure, the anti-inflammatory effects were determined by both xylene-induced ear oedema and cotton pellet granuloma test in mice. As indicated in Figure 5, GAS extracted from bioconversion broth at 8 mg/kg dosage displayed the significant inhibiting effect on xylene-induced ear swelling in mice in comparison with other dosages (P<0.05),
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Effects of GAS and the reference drug (diclofenac) on xylene-induced granuloma in ICR mice (HBA). The administration of GAS in a dose of 8 mg/kg orally resulted in significant anti-inflammatory activity against cotton pellet granuloma in ICR mice (P<0.05). The data were comparable with the standard anti-inflammatory drug indomethacin, indicating that GAS isolated from the biotransformation procedure had strong potential to be exploited for anti-inflammatory purposes.

Conclusions

In summary, a novel one-step biotransformation of HBA into GAS using resting cells of A. luteo-virens Sacc has been successfully demonstrated. The transformation conditions were optimized for maximal GAS production by response surface methodology. Statistical methodology in experimental design of biotechnological processes is confirmed to overcome the limitations of conventional practice of varying one factor at a time [31], as a consequence, proving to be a powerful tool for the optimization of GAS production by A. luteo-virens Sacc. The highest yield of GAS was 550 ± 25.2 μg/100 mL under the optimized conditions. The present results described herein suggested that the glucosylation of HBA was a promising research aspect, particularly in the resting-cell conversion. The biotransformation kinetic model was simulated and constructed in the present context. The results of anti-inflammatory activity evaluation provided the evidence for the biotransformed product uses. All before mentioned results demonstrate a good potential for GAS production since the chemical synthesis of GAS from HBA or HBD is difficult and environmentally harmful, and as also the traditional extraction from plant sources is expensive and low-efficiency. The bioactive activity of GAS against inflammation process is evaluated to be highly interesting for potential application.

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