Optimization of Submerged Fermentation Medium for Matrine Production by *Aspergillus terreus*, an Endophytic Fungus Harboring Seeds of *Sophora flavescens*, Using Response Surface Methodology

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**Abstract** Different endophytes isolated from the seeds of *Sophora flavescens* were tested for their ability to produce matrine production. Response surface methodology (RSM) was applied to optimize the medium components for the endophytic fungus. Results indicated that endophyte *Aspergillus terreus* had the ability to produce matrine. The single factor tests demonstrated that potato starch was the best carbon source and the combination of peptone and NH$_4$NO$_3$ was the optimal nitrogen source for *A. terreus*. The model of RSM predicted to gain the maximal matrine production at 20.67 μg/L, when the potato starch was 160.68 g/L, peptone was 24.96 g/L and NH$_4$NO$_3$ was 2.11 g/L. When cultured in the optimal medium, the matrine yield was an average of 20.63 ± 0.11 μg/L, which was consistent with the model prediction. This study offered an alternative source for the matrine production by endophytic fungus fermentation and may have far-reaching prospect and value.

**Keywords** Endophytic fungi, Matrine, Response surface methodology, *Sophora flavescens*

Due to environmental condition and over-harvesting, some wild medicinal resources of plants are facing with reduction [1]. So, it is important to search for sustainable alternative sources of well-known high value plants. Endophyte is an endosymbiont, which takes the whole or part of its life to reside in the endocellular or intracellular areas of host plants. And, it can protect its hosts from diseases [2-4]. Endophyte can hasten growth of hosts and increase their ability of stress resistance [5]. What's more, some of endophytes have the ability to produce the secondary metabolites generally assumed to be produced by the host plants. Therefore, a lot of researchers are interested in discovering endophytes and using microbial fermentation to meet the ever-growing demand for several life-saving drugs derived-from plants resources. *Sophora flavescens* (Leguminosae) has been used as an herbal medicine or food ingredients in East Asian countries for thousands of years. With the increase of usage, the resources of *S. flavescens* gradually decreases. Matrine (Fig. 1) is a major bioactive component of *S. flavescens*, which has been used in Chinese traditional medicine for its pharmacological effects of anti-inflammatory, anti-bacterial, anti-hypertensive, anti-diabetic and anti-tumor activities.
1), a tetracycloquinolizidine alkaloid, is one of the main active ingredients of *S. flavescens*, *S. alopecuroides*, and *S. subprostrata* [6-8]. Matrine has been used clinically in the treatment of gastric cancer, the study of Li et al. [9] showed that matrine inhibited SGC-7901 cell proliferation by changing miRNA expression in a dose-dependent manner (0.5–2.5 mg/mL). Shao et al. [10] demonstrated that treatment with 3 mM matrine exerted inhibitory effects on the cell viability of estrogen receptor-positive MCF7 cells, human epidermal growth factor receptor 2-positive BT-474 cells and highly metastatic MDA-MB-231 cells, resulting in 76.4–84.5% reduction in cell numbers. Matrine (4–128 μg/mL) could inhibit human enterovirus 71 infection in human RD cells, treatment with 20 mg/kg also could reduce the mortality of mice upon lethal enterovirus 71 challenge [11]. Additionally it is considered a useful agent in treatment of allergy and central nervous system autoimmunity [12, 13].

Response surface methodology (RSM) is an effective statistical method that can build mathematical models to assess the effects of several factors onto a desired response [14]. RSM combines optimization theory with modern mathematic statistic to reduce the number of experiments needed and evaluate multiple variables and their interaction. RSM can address some drawbacks, such as time consumption and high cost [14]. Box-Behnken design (BBD) of RSM is widely used in the optimization of the medium constituents and other fermentation parameters for the secondary metabolites, such as extracellular lipase, curdlan, cordycepin, c-phycocyanin, and extracellular polysaccharide [14-17].

A study of He [18] reported that endophyte *Aspergillus terreus*, isolated from the seeds of *Sapophora flavescens*, was able to produce matrine. But he did not quantify matrine yield because *A. terreus* only produced trace amount of matrine. That offered a new source for the matrine production by endophytic fungus fermentation and may have far-reaching prospect and value. So in present study, we attempt to isolate the endophyte *A. terreus* and optimize the medium components of it using RSM to increase yield of matrine.

**MATERIALS AND METHODS**

**Reagents and instruments.** The reference standard of matrine were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). SSC800 high-speed centrifuge (Hebei DECO Machinery Co. Ltd., Xingtai, China); LRH-250-Z incubator shaker (medical equipment factory in Guangdong, China); peptone, yeast extract (analytical grade, Beijing AoBoXing Bio-Tech Co. Ltd., Beijing, China); agar (analytical grade, Beijing AUKS Technology Co. Ltd., Beijing, China); KH2PO4, MgSO4 · 7H2O, NH4NO3, (NH4)2SO4 (analytical grade, Beijing Chemical Works, Beijing, China); sucrose, maltose, glucose (analytical grade, Aladdin Industrial Corporation, Shanghai, China); methanol (chromatographic grade, Sinopharm Chemical Reagent Co. Ltd., Shanghai, China); triethylamine (chromatographic grade, Shanghai Feather Flower BioTech Co. Ltd., Shanghai, China).

**Endophyte separation.** Healthy *S. flavescens* were collected from Shandong Provinces, China, in October 2014. The endophytes were isolated from the seeds of *S. flavescens* according to He [18] described with some modification. Specific protocol as follows: the seeds were sterilized by sequential immersion in 70% (v/v) ethanol for 60s and 0.1% (v/v) HgCl2 for 180 sec, and three times rinsed with sterile water. The successful sterilization process was confirmed by a plated final wash onto potato dextrose agar (PDA; potato 200 g/L, dextrose 20 g/L, agar 15 g/L) medium and plates were incubated at 30°C for 5 days. If no microbial growth was found, the seeds were used for further analysis. Then cut them into 0.3 cm2 small pieces, and plated on fresh PDA medium with streptomycin (50 U/mL) at 25°C for 10 days. The growing mycelia were transferred and purified on PDA medium by picking mycelia tip, repeatedly above operations to obtain the pure strains. The isolated strains were incubated and the ingredients of fermentation broths were identified by high-performance liquid chromatography (HPLC), respectively. The isolated strains were saved on PDA medium at 4°C respectively and the slants were subcultured every 3 mon. Identification of endophyte which had the ability to produce matrine was carried out by morphological characteristics (scanning optical microscope, Jiangnan Optical Instrument Factory, Nanjing, China) and internal transcribed spacer (ITS) sequences of rDNA. The rDNA ITS region of the endophyte was cloned and sequenced, and the characteristic of ITS sequence was performed by Shanghai GeneCore Bio Technologies Co. Ltd. (Shanghai, China).

**Shake flask culture.** Inoculum was prepared by transferring a few of endophyte *A. terreus* mycelia blocks on PDA medium to an erlenmeyer (250 mL) with 100 mL seed medium (glucose 20 g/L, peptone 5 g/L, yeast extract 5 g/L, MgSO4 · 7H2O 0.5 g/L, KH2PO4, 0.5 g/L). Seeds were cultured at 25°C on a rotary shaker incubator at 150 rpm for 6 days. The fermentation culture was carried out with orbital agitation at 25°C for 15 days in 500-mL erlenmeyer flask with 200 mL of original nutrient solution (potato 200 g/L, glucose 20 g/L). And, the inoculum size was 5% (v/v). Each experiment repeated three times.

**Analytical methods.** The fermentation broth was centrifuged at 10,000 xg for 15 min to separate mycelia and supernatant. The supernatant was concentrated under vacuum to 1/50 of the original volume. The condensed supernatant was applied to measure matrine yield using a waters series HPLC system equipped with a 2996 photodiode array detector. Chromatographic separation was carried out on a ZORBAX Eclipse XDB C8 column (250 x 4.6 mm i.d.; particle 5-μm, Agilent Technologies, Palo Alto, CA, USA). The mobile phase was done according to Li and Wang.
Zhang et al. [19], to be specific, it was composed of 0.01 mol/L KH$_2$PO$_4$ buffer-methanol-triethylamine in the ratio 94 : 6 : 0.01 (v/v/v). Flow rate was 1.0 mL/min, detection wave length was 208 nm and the column temperature was maintain at 40°C. The chromatographic peak of matrine was identified by comparing the retention time with matrine reference standard.

**RSM for optimizing medium constituents.** The variables (potato, peptone and NH$_4$NO$_3$) were selected to study their concentration and their interaction effect on matrine yield using BBD of RSM. The software Design-Expert 8.0.5b trial was used in experimental design, date analysis, and quadratic equation construction. The range of independent variables and their levels were presented in Table 1. The independent variables and their levels were chosen based on the results of our pre-experiments. The response value was matrine production. Triplicates at the center (−1, 0, and 1) of the experimental design were intended to estimate the pure error sum of squares. About the statistical calculation, the independent variables were coded according to the following formula.

\[
x_i = \frac{X_i - X_0}{\Delta X_i}, \quad i = 1, 2, 3
\]

Where $x_i$ was the coded values of $X_i$, while $X_i$ represented the real value of an independent variable, $X_0$ represented the real value of independent variables on the center point and $\Delta X_i$ was the step change value.

The behavior of the system was explained by the following polynomial model equation:

\[
Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j
\]

Where $Y$ was predicted response value, $\beta_0$ was intercept term, $\beta_i$ was linear term, $\beta_{ii}$ was squared term, $\beta_{ij}$ was interaction, $X_i$ and $X_j$ were the coded level of independent variables.

The statistics analysis of the polynomial equation was performed to evaluate analysis of variance (ANOVA). Fisher’s F-test and the coefficient of determination $R^2$ were applied to express the goodness of fit of the polynomial model. The surface and contour plots expressed the fitted polynomial equation, which could show the relationship between experimental level of each independent and the response to deduce the optimal conditions [20, 21].

**RESULTS AND DISCUSSION**

**HPLC chromatogram of matrine.** With the report of Stierle et al. [22] on taxol production by endophyte Taxomyces andreanae, a host of researchers were interested in bioprospecting of endophytes for secondary metabolites and got some breakthrough [23-25]. In present study, five strains were isolate from the seeds of *S. flavescens*. The isolated strains were incubated and the ingredients of fermentation broths were identified by HPLC, respectively. Results indicated that only one of strains could have the ability to produce matrine. Fig. 2A and 2B showed that typical chromatograms of reference standard and fermentation broth sample, respectively. The retention time of matrine was 7.302 min. Comparison the chromatograms of sample with standard indicated that there was matrine production present in fermentation broth of *A. terreus*. Endophyte *A. terreus* had the ability to produce the secondary metabolites generally assumed to be produced by the host *S. flavescens*.

**Identification of endophyte A. terreus.** Identification of endophyte which had the ability to produce matrine

![Fig. 2. Chromatograms of matrine standard (A) and fermentation broth sample (B).](image-url)
was carried out by morphological characteristics and ITS sequences of rDNA. The centre of the strain colony was yellow, while the edge was white. The fruiting bodies of the endophyte had several aerial mycelium with diaphragm. There were lots of branch hyphae with linear conidia chains, and the spores were round. The ITS sequence was BLAST matched with GenBank database on homology, and the similarity with ribosomal rDNA of *A. terreus* was 100%, which indicated that the endophyte had the ability to produce matrine was *A. terreus*. He [18] made the same conclusion according to the analysis of ITS, 16S rDNA sequences and morphological method.

**Optimization of carbon source and nitrogen source.** Different strains have different living habits. Carbon source and nitrogen source, two major ingredients of medium, play significant roles in synthesis of secondary metabolites and the growth of cells [26]. Their types and concentration have effect on yield of secondary metabolites [27, 28]. So, the optimum carbon source and nitrogen source for matrine yield secreted by endophyte *A. terreus* were screened using single factor tests. In this study, glucose (Aladdin Industrial Corporation), maltose (Aladdin Industrial Corporation), sucrose (Aladdin Industrial Corporation), soluble starch (Tianjin Dingshengxin Chemical Industry Co. Ltd., Tianjin, China), potato starch and corn meal (Shandong Zhenhua Biotechnology Co., Ltd., Binzhou, China) (50 g/L, respectively) were applied to elect the optimum carbon source for endophyte *A. terreus*. The yield of matrine was assayed and the results were exhibited in Fig. 3, which indicated that all kinds of carbon sources could increase matrine production. But their influences on matrine yield were different. Potato starch and glucose were better suited for *A. terreus* to produce matrine than others. From the perspective of economic, potato starch was used as optimal carbon source in following experiment.

In order to search for the optimal nitrogen source for *A. terreus*, organic nitrogen source (peptone, yeast extract, soybean meal [Beijing AoBoXING Bio-Tech Co., Ltd.]), inorganic source (NH$_4$NO$_3$, (NH$_4$)$_2$SO$_4$ [Beijing Chemical Works]) or the combination were performed and the results were shown in Table 2. The combination of peptone and NH$_4$NO$_3$ was preferable for matrine production. A considerable body of evidence indicated that the combination of organic source and inorganic source was more suitable for strains to produce secondary metabolites [29-31]. So, the combination of NH$_4$NO$_3$ and peptone were used in following experiments.

A host of studies increased the yield of secondary metabolites by optimizing the formula of media. Zong *et al.* [29] selected peptone, (NH$_4$)$_2$SO$_4$, and glucose as the most influential parameters to optimize the medium constituents for ε-poly-L-lysine. Under the optimal culture condition, the value of ε-poly-L-lysine was 1.75 times than that of original medium. Tanyol *et al.* [32] reported that peptone, ammonium sulphate and sunflower oil cake (carbon source) were the most influential parameters in lipase production, the maximum lipase activity of 10.8 U/L was obtained when sunflower oil cake was 11.10% (w/v), peptone was 1.18% (w/v), ammonium sulphate was 0.83% (w/v). A report by Wang *et al.* [20] indicated that potato, glucose, and wheat bran were the most influential parameters in the production of extracellular polysaccharide from *Agaricus blazei* Murrill. A 1.79-fold increase in production was obtained under the optimal medium.

**Optimizing medium components by RSM.** RSM is an effective optimization technology and it has been widely used in many fields [33-35], especially in biological engineering. In this study, the BBD of RSM was used to estimate the influence of the individual factors including potato starch content (X$_1$), peptone treatment (X$_2$), NH$_4$NO$_3$ addition (X$_3$) and their interaction effects on matrine production. In the 17 experiments of BBD, the experimental and predicted values of matrine under different treatment conditions were presented in Table 3.

The predicted response $Y_{matrine}$ for matrine production in terms of coded factors was expressed as follow:

$$Y_{matrine} = 20.24 + 1.11 X_1 + 1.26 X_2 + 0.10 X_3$$
$$+ 0.043 X_1 X_2 + 0.030 X_1 X_3 + 0.13 X_2 X_3$$
$$- 2.65 X_1^2 - 1.29 X_2^2 - 0.81 X_3^2$$

Where $Y_{matrine}$ was the response variable of matrine, and $X_1$, $X_2$, and $X_3$ were the concentration of potato starch, peptone and NH$_4$NO$_3$, respectively.

![Fig. 3](image-url) Effect of different carbon source (50 g/L, respectively) on matrine yield.

| Nitrogen source       | Concentration (g/L) | Matrine (μg/L) |
|-----------------------|---------------------|---------------|
| Peptone               | 10                  | 6.214         |
| Yeast extract         | 10                  | 3.356         |
| Soybean meal          | 10                  | 4.783         |
| NH$_4$NO$_3$          | 2                   | 1.523         |
| (NH$_4$)$_2$SO$_4$    | 2                   | 1.232         |
| Peptone + NH$_4$NO$_3$| 10 + 2              | 9.621         |
| Yeast extract + NH$_4$NO$_3$ | 10 + 2 | 5.368         |
| Soybean meal + NH$_4$NO$_3$ | 10 + 2 | 7.234         |
Table 4 showed the ANOVA for response surface quadratic model and the statistical significance of the regression model, which indicated that the model was highly significant for the matrine production, as was evident from the F-value (model = 19.43) with a very low probability value (p > F) [36]. The p-value less than 0.05 declared that model term was significant. The values of $R^2$ (0.9615) and adjusted $R^2$ (0.9120) were closer to 1 and they also implied the high efficacy of Eq. (3). Adequate precision ratio, used to measure the ratio of signal to noise, was generally desirable to be more than 4 [37]. Adequate precision ratio of 13.364 indicated an adequate signal and could be used to navigate the design space. The coefficient of variation value was 3.39% and indicated a great precision and accuracy of this study. Above all, the regression model for matrine production was a good prediction of the experimental results and the factor effects were real.

Fig. 4A, 4B, and 4C represented the three dimension surface (3D-surface) plot and two dimensional response projection (2D-projection) as a function of potato starch addition, peptone addition and NH$_4$NO$_3$ treatment on matrine yield. The 3D-surface and 2D-projection intuitively showed the response over a region of independent variables and the relationship between experimental levels of each factor. Fig. 4A showed the effect of potato starch addition and peptone treatment on matrine yield. The 3D-surface showed evidence that marine yield increased upon increasing potato starch addition and peptone treatment. A maximal matrine production was obtained when the potato starch was 160.68 g/L and peptone was 24.96 g/L. Afterwards, the matrine production decreased with potato starch and peptone increase. Fig. 4B showed that increasing the content of NH$_4$NO$_3$ to more than 2.11 g/L caused shrink of matrine yield. A quadratic effect of peptone and NH$_4$NO$_3$ in the response was observed in Fig. 4C. The curves of Fig. 4C bear some similarity to that in Fig. 4A and 4B, respectively. Thus, the best content of X$_1$, X$_2$, and X$_3$ were found to be 160.68 g/L, 24.96 g/L, and 2.11 g/L, respectively.

The predicted matrine yield obtained from the model using the optimal medium was 20.67 μg/L. The average result of verification tests was 20.63 ± 0.11 μg/L, which was 1.67 times higher than that of initial media (potato 200 g/L, glucose 20 g/L).

With $S$. flavescens over harvesting, the wild resources of $S$. flavescens could not meet the ever-growing demand. $A$. terreus, an endophytic fungus harboring seeds of $S$. flavescens, had the ability to produce matrine, which may be the alternative source of $S$. flavescens and have far-reaching prospect and value.

The yield of matrine produced by $A$. terreus was very little, further studies were required to optimize the other culture conditions (PH, temperature, precursors, mineral ion, oxygen...
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supply, and light condition) or obtain mutations by a high-energy (~MeV) proton beam to increase the yield of matrine [38]. The biosynthetic pathway of matrine secreted by *A. terreus* also will be clarified in the days ahead.

In this study, endophyte *A. terreus* which had the ability to produce matrine was isolated from the seeds of *S. flavescens*. Which offered an alternative source for matrine production by endophytic fungus fermentation. The single factor tests demonstrated that potato starch was the best carbon source and the combination of peptone and NH$_4$NO$_3$ was the optimal nitrogen source for *A. terreus*. The model of RSM predicted to gain the maximal matrine production at 20.67 μg/L, when the potato starch was 160.68 g/L, peptone was 24.96 g/L and NH$_4$NO$_3$ was 2.11 g/L. The average result of three verified experiments was 20.63 ± 0.11 μg/L, which was consistent with the model prediction.

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