Screening and identification of Monascus strain with high TMP production and statistical optimization of its culture medium composition and liquid state fermentation conditions using response surface methodology (RSM)

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Abstract
Teramethylpyrazine (TMP) is a pyrazine alkaloid with a variety of pharmacological effects and widely used in clinical practice. In this study, Monascus strain M-3 with high TMP and low citrinin productions was screened from red yeast rice collected from different areas of Fujian province in China. Monascus strain M-3 was further identified through molecular biological methods based on the sequencing of different gene regions. Results showed that primer set of β-tubulin-F/β-tubulin-R targeting the β-tubulin gene was more suitable for the identification of Monascus strain M-3. Based on comparative analysis of the results obtained by different primer sets, Monascus strain M-3 was finally identified as Monascus purpureus. Then, the composition of culture medium and liquid state fermentation conditions of Monascus strain M-3 were optimized by single-factor experiment and central composite design of response surface methodology, with the purpose of optimizing the TMP productions. The optimum composition of culture medium and liquid state fermentation conditions are as follows: starch 5.1%, peptone 3.51%, MgSO4 0.1%, NaNO3 0.2%, KH2PO4 0.25%, fermentation temperature of 28.23 °C, fermentation time of 9 d, shaker speed of 180 r/min, inoculum size of 7.21% and loading volume of 100 mL/250 mL triangular flask. Under the optimum composition of culture medium and liquid state fermentation conditions, the TMP production of Monascus strain M-3 can reach 13.49 µg/mL. This study is expected to provide a new approach of TMP production through biosynthesis by Monascus strain, and promote the further development of functional foods or medicines from Monascus fermentation products.

Introduction
Teramethylpyrazine (also called 2,3,5,6-Tetramethylpyrazine (TMP), as shown in Figure 1) is one of the main active ingredients of Rhizoma Chuanxiong, Zingiberaceae Curcuma rhizomes and Euphorbiaceae Jatropha stems [1]. Previous studies have evidenced that TMP has pharmacological activity of anti-platelet aggregation and the physiological functions of softening the blood vessels, increasing coronary blood flow, improving microcirculation and scavenging free radicals [2–5]. It has been used in clinical applications as a medicinal agent with high security for more than 30 years, especially in the treatment of patients with cerebral ischemic diseases, because of its neuroprotective effects and global cerebral ischemia [6,7].

Currently, the method of preparation of TMP included chemical synthesis [8], extraction from Chinese traditional medicinal plants (Rhizoma Chuanxiong, Z. Curcuma rhizomes and E. Jatropha stems) [9] and biosynthesis [10,11]. However, chemical synthesis has great limitations because of its high chemical residues, low security, environmental destruction and other factors. Extraction from medicinal plants has great limitations due to the large raw material wastage, time-consuming of extraction process and the needs of large quantities of organic solvents. Therefore, both of chemical synthesis and extraction from medicinal plants still cannot well meet the needs of scientific research and the pharmaceutical industry. Compared with chemical synthesis and extraction from medicinal plants, biosynthesis has obvious advantages, especially for health foods and drugs production. It has been reported that some micro-organisms (such as Bacillus subtilis [12] and Sulfitobacter ponticus [13]) can produce a certain amount of TMP. The application of Monascus...
species in foods, medicine and industry can be traced back to a thousand years ago. The secondary metabolism of Monascus produces various valuable components, including pigments, monacolin K, \( \gamma \)-aminobutyric acid, ergosterol, etc. [14–16]. In the former work of our laboratory, we had discovered Monascus also produces a certain amount of TMP. Compared with \( B. \ subtilis \) and \( S. \ ponticus \), the production of TMP from Monascus sp. is relatively low. The TMP production is closely dependent on Monascus strain, medium composition (carbon source, nitrogen source and other nutritional factors) and environmental factors (oxygen and temperature, etc.). Therefore, it is necessary to further optimize the fermentation process of Monascus sp. for the higher production of TMP.

There are a variety of fermentation methods in the batch production of micro-organisms, including solid state fermentation, liquid state fermentation, immobilized cells fermentation and so on. They have different applications in the fermental cultivation of Monascus sp., such as red pigment production through immobilized Monascus fermentation, lovastatin production by solid state fermentation. Compared with solid state fermentation, liquid state fermentation has many advantages [17]: (1) the substrates, products and other matters in the liquid are easy to spread and the distribution is uniform, which is beneficial to the growth of micro-organism; (2) liquid culture has the characteristics of convenient operation and control; (3) the separation and purification of the active ingredient in fermented product is more convenient.

To date, no report is available on the optimum liquid state fermentation conditions of Monascus for the production of TMP. In this study, Monascus strain M-3 with high TMP and low citrinin productions was screened from red yeast rice collected from different areas of Fujian province in China, and further identified by molecular biological method based on the sequencing of different gene regions. Furthermore, the composition of culture medium and liquid state fermentation conditions of TMP production by Monascus strain were optimized by means of single-factor experiment and central composite design (CCD). The results would provide a new approach of TMP production through biosynthesis by Monascus strain, and promote the further development of functional foods or medicines from Monascus fermentation products.

**Materials and methods**

**Sample collection**

Twenty samples of dry commercial traditional red yeast rice were obtained from the small-scale factories located in the northern, southern and western areas of Fujian province in China. Samples were stored at 4 °C immediately after collection prior to testing. Ten grams of powdered wine fermentation starter sample were homogenized in 90 mL of 0.85% w/v sterile physiological saline. Then a series of decimal dilutions were made. One millilitre each from the dilutions of \( 10^{-4}, 10^{-5}, 10^{-6} \) and \( 10^{-7} \) was poured onto Czapek-Dox agar (Difco, Detroit, MI, USA) and potato dextrose agar (Difco) supplemented with ampicillin (100 ng/\( \mu \)L, Merck, Darmstadt, Germany) and incubated at 28 °C for 3 d. All the colonies isolated were identified with traditional methods including a macroscopic level (surface colour, reverse side colour, spores and diameter) and microscopic characteristics such as conidiophores, conidia and fertile hyphae. Monascus strains were screened from the isolates and sub-cultured on new Czapek-Dox agar plates (Difco) and purified by repeated streaking. The purified isolates were obtained and maintained on potato dextrose agar (Difco) slants at 4 °C until use.

**Screening of Monascus strains with high TMP production**

Pure cultures were incubated in 250 mL Erlenmeyer flasks containing 50 mL of fermentation medium (10 g/L glycerinen – autoclaved separately at 121 °C for 15 min, 10 g/L peptone, 1 g/L \( \text{MgSO}_4 \), 2 g/L \( \text{KH}_2\text{PO}_4 \) at pH 7.0) at 30 °C for 5 d with shaking at 150 r/min. The fermentation broth was used for TMP production measurement by high-performance liquid chromatography (HPLC) method [18].

**Identification of Monascus strain with the highest TMP production**

DNA extractions for Monascus strain were carried out using a benzyl chloride method [19]. DNA extract was dissolved in 50 \( \mu \)L TE containing RNase. The yields and fragmentation of the DNA were determined by ethidium bromide-UV detection on 1% (w/v) agarose gel. Nucleic acid extracts from each sample were also analysed spectrophotometrically at 260 and 280 nm by using a DU800 spectrophotometer (Beckman Coulter, Fullerton, CA, USA). The DNA extracts were stored in a freezer at −80 °C until analysis. Different gene regions were amplified using different fungal primers (Supplementary material Table S1) under the conditions according to

![Figure 1. The structure of tetramethylpyrazine (TMP).](image-url)
HPLC analysis of citrinin

Fermentation products (0.1 g of weight) were extracted with 3 mL 95% ethanol in 60 °C for 30 min. The extracted solution was filtrated using 0.45 μm filter. The concentration of secondary metabolites was measured by HPLC method using an Agilent 1260 Series LC system equipped with a photodiode array detector (Waters 2996; Waters Ltd., Milford, MA, USA). Chromatographic separation was conducted on Phenomenex® Luna 5μ C18 (2) (250 × 4.6 mm) column. Solvent A (1 L acetonitrile with 0.05% TFA) and solvent B (distilled water with 0.05% TFA) were used as the mobile phase. Solvent A/ solvent B: 75/25 was eluted for 30 min. The eluent was pumped at a flow rate of 0.5 mL/min. The concentration of citrinin was determined by fluorescence detector (emission: 330 nm; emission: 500 nm). The citrinin was identified by comparison of their retention time values and spectra with known standards [25].

Optimization for culture medium for higher TMP productions

The effects of different carbon source, nitrogen sources and their proportions on the TMP production were studied. The fermentation conditions were as follows: inoculum size 10%, shaker speed 150 r/min, fermentation temperature 30 °C; the content of TMP in the fermented products was determined after fermentation of 5 days.

Optimization of liquid state fermentation conditions for higher TMP productions

Flask experiments were performed in 250 mL Erlenmeyer flasks containing 50 mL of modified optimum medium (51 g/L starch – autoclaved separately at 121 °C for 15 min, 35.1 g/L peptone, 1 g/L Mg2SO4, 2 g/L KH2PO4). First, the best fermentation time was selected by examining the TMP producing ability at different fermentation times by HPLC. The experiment conditions were: 10.0% inoculum size of in modified optimum medium at pH 7.0, 30 °C, shaking at 150 r/min. The selected fermentation time was applied in the study of other conditions.

The effects of oxygen supply on TMP production were tested shaking at 90, 120, 150, 180 and 210 r/min, respectively. Different incubation temperatures (26, 28, 30, 32 or 34 °C) or inoculum size (of 5%, 7%, 9%, 11% or 13%) were examined individually in the modified optimum medium (pH 7.0). All experiments were performed in triplicate, and the mean and standard deviations were determined.

The culture medium and fermentation conditions of Monascus strains with high TMP production were optimized by the test design. First, the factors that affect the yield of TMP were selected by single-factor experiment. Then, the selected factors were further optimized using central composite experimental design of response surface methodology (RSM).

Statistical analyses

The central composite experimental design of RSM was used to study the variables independently for their interactions and quadratic effects. The experimental data were analysed by Design Expert© 9.0 software. The two degree polynomial of the relationship between the response variable and the independent variable was obtained. Model can be described as

\[
y = b_0 + \sum b_i x_i + \sum b_{ij} x_i^2 + \sum b_{ij} x_i x_j
\]

where \(y\) is the predicted response values; \(x_i\) for the independent variables of the code value; \(b_0\) is intercept; \(b_i\) is the linear coefficient; \(b_{ij} (i \neq j)\) is interaction coefficient; \(b_{ij}\) is square coefficient. The multiple repeat of centre point provided a sufficient degree of freedom for test error estimation. The experimental design references were carried out, and the experimental data were analysed by Design Expert© 9.0 software.

Results and discussion

Characteristics and screening of Monascus strain with high TMP production

Twenty strains were isolated from red yeast rice collected from different areas of Fujian province in China. Their colonial morphology is shown in Figure 2. The TMP productions of different Monascus strains were measured by HPLC method. Results showed that the TMP productions of different Monascus strains were significantly different (as shown in Figure 3) (\(p < 0.01\)). The TMP yields of vast majority of strains were relatively low (<5.0 μg/mL). It is noteworthy that the TMP yields of Monascus strain M-3, M-9 and M-10 were higher than 8.0 μg/mL. Parallel tests also indicated that the TMP productions of Monascus strain M-3, M-9 and M-10 were relatively stable (data not shown). What is of importance,
Monascus-fermented products may be contaminated by citrinin, initially named monascidin A, which could damage the kidneys and liver. Previous studies by others have pointed out that most of Monascus sp. excretes certain amount of citrinin [26]. In this study, the amount of citrinin produced by the twenty strains isolated from red yeast rice were also determined through HPLC-FD method. Results showed that a lower amount of citrinin produced by Monacus strain M-3 compared to Monascus strains M-9 and M-10. Therefore, Monacus strain M-3 was selected for further study.

**Identification of Monacus strain M-3 through sequencing of different gene regions**

The extracted genomic DNA of Monacus strain M-3 is shown in Figure 4(a). The electrophoretic patterns of PCR amplification products with four primer sets are shown in Figure 4(b). In all cases, sequence similarities approached 99% with publicly available sequences at the GenBank (as shown in Table 1). Monacus strain M-3 was identified as Monascus ruber, Monascus purpureus or Monascus kaoliang through the sequencing of PCR products by primers ITS1/ITS4, and identified as M. purpureus, M. kaoliang, Monascus aurantiacus or Monascus rutilus through the sequencing of PCR products by primers ITS4/ITS5. A variety of fungal species can be differentiated by analysis of sequences of the 26S rRNA gene region, the 5’ end of the large subunit rDNA encompassing the D1 and D2 expansion domains [27]. In this study, Monacus strain M-3 was identified as M. ruber, M. purpureus or Monascus eremophilus using the sequencing of PCR products by primers NL1/NL4. Therefore, it is difficult to accurately determine the taxonomic status of Monacus strain M-3 only using one of these primers (ITS1/ITS4, ITS4/ITS5 and NL1/NL4). Targets for the genus/species level classification of Monascus strains have included the 18S rRNA gene, β-tubulin gene, polyketide biosynthesis gene and the internal transcribed spacer (ITS) regions [28]. Comparative sequence analyses based on sequence divergence of specific genes have been conducted in our previous research work, which indicated that the resolution of 18S rRNA gene sequences and ITS-5.8S rRNA gene sequences were insufficient for the identification of Monascus strains at the species level.
Identification of *Monacus* strain M-3 by primer set \( \beta \)-tubulin-F/\( \beta \)-tubulin-R showed that the sequence of \( \beta \)-tubulin gene of *Monacus* strain M-3 was most similar to *M. purpureus* (with 100% sequence similarity) (as shown in Table 1). Based on the above identification through four primer sets and their comparative analysis, *Monacus* strain M-3 can be finally identified as *M. purpureus*.

**Optimization for culture medium for higher TMP productions**

**Single-factor tests for carbon and nitrogen source**
Carbon is the main component of microbial cells and their metabolites. The carbon source provides the necessary energy for the fermentation process, and is one of the main components of the medium [29]. Effects of
several different carbon sources on the yield of TMP are shown in Figure 5(a). When starch was used as carbon source, Monascus strain M-3 had the highest TMP yield. Therefore, starch was used as carbon source in the following experiment. In order to determine the optimum amount of starch, the effect of different starch amount on the yield of TMP was determined. As shown in Figure 6(a), the TMP yields reached the highest point when adding 3% starch. Too much or too little starch addition was not conducive to TMP synthesis.

### Central composite experimental design

The results obtained from the run of 13 experimental sets, suggested by CCD models of RSM were analysed by Design Expert® 9.0. Analysis of variance (ANOVA), regression coefficients and polynomial regression equation were obtained. Table 2 shows the relationship between TMP production and independent variables, its ANOVA results are tabulated in Table 3. The model has a \( R^2 \) value of 60.14, which is large enough and implies that the model is significant. The ‘Coefficient of Determination’ \( R^2 \) value of the study was 0.9773. The high \( R^2 \) value suggests that strong correlation exists between the observed values and the values predicted by the CCD model [31]. The ‘adjusted R-squared’ value was 0.9610. The quadratic polynomial equation obtained by multiple regression analysis depicting the effect of parameters \( (X1 \) and \( X2) \) and their interactions in the response (TMP production) is described in the following equation:

\[
Y = 13.71 - 1.09 \times X1 - 1.13 \times X2 - 2.61 \times X1 \times X2 - 3.04 \times X2 + 0.56 \times X1 \times X2.
\]

Figure 8(a) shows the 2D contour plots and 3D surface plots of the RSM study, which disclose the mutual interaction in between parameters and response. The validation of RSM result was done by the numerical optimization of CCD model. The predicted optimum values for the parameters are shown in Table 2. An experiment was performed using parameters suggested by numerical optimization, then TMP production was measured manually in triplicates with the help of Archimedes principle. The maximum value of TMP production observed was 13.48 ± 0.94 \( \mu \)g/mL, which is very close to the value 13.06 ± 1.31 \( \mu \)g/mL predicted by RSM. This maximum TMP production was found against the 5.1% starch, 3.51% peptone. This experiment validates that the RSM method is applicable for the parameter optimization to medium composition.

### Table 1. Identification of Monascus strain M-3 through sequencing of different gene regions.

| Primer       | Closest relative species       | Accession number | Total scores | % Identity |
|--------------|--------------------------------|------------------|--------------|------------|
| ITS 1/ITS 4  | Monascus ruber                 | HQ857600         | 985          | 99%        |
|              | Monascus purpureus             | AY750726         | 976          | 99%        |
|              | Monascus koaliang              | HQ659499         | 974          | 99%        |
| ITS 4/ITS 5  | Monascus purpureus             | JN942661         | 977          | 99%        |
|              | Monascus koaliang              | AB477250         | 977          | 99%        |
|              | Monascus auranticus            | DQ978995         | 977          | 99%        |
| NL1/NL4      | Monascus purpureus             | DQ978997         | 977          | 99%        |
|              | Monascus purpureus             | JN940514         | 1018         | 99%        |
| β-tubulin-F/ | Monascus purpureus             | JQ221438         | 1759         | 100%       |
| β-tubulin-R  | Monascus purpureus             | JX221439         | 1757         | 100%       |
|              | Monascus eremophilus           | JX221432         | 1709         | 100%       |
|              | Monascus purpureus             | AY498598         | 1550         | 100%       |
|              | Monascus purpureus             | AY498599         | 1550         | 100%       |
Figure 5. Effects of proportion of carbon (a) and nitrogen (b) source on TMP production.

Figure 6. Effects of proportion of carbon (a) or nitrogen (b) on TMP production.
Optimization of liquid state fermentation conditions for higher TMP productions

Single-factor tests for liquid state fermentation conditions

After determining the optimum amount of carbon source and nitrogen source in the culture medium, the effects of fermentation time, fermentation temperature, shaker speed and inoculation amount on the yield of TMP were further investigated. As shown in Figure 7(a), the TMP production of Monacus strain M-3 reached maximum value (12.31 μg/mL) after 9 d of fermentation. As shown in Figure 7(b), the TMP production increased with the increase of temperature, and reached a maximum of 11.93 μg/mL at 28 °C. However, when the temperature continued to increase, the TMP production appeared to decline, indicating that the optimum fermentation temperature was 28 °C. Besides, the TMP production increased with the increase of shaking speed, reaching a maximum of 10.38 μg/mL at 180 r/min (Figure 7(c)), and then remained at comparable levels (210 r/min). This result indicated that oxygen supply is positively associated with TMP production. The amount of TMP production increased with an increase in inoculated volume from 5% (7.85 g/L) to 7% (11.36 g/L) (Figure 7(d)). However, when the inoculum continued to increase, the TMP production appeared to decline, indicating that the optimum fermentation inoculum size of was 5%.

Central composite experimental design

According to the single-factor experiment, temperature and inoculation amount (the two main factors affecting the yield of TMP) were chosen to do the response surface test design of two factors and five levels. The results by central composite experimental design (CCD models of RSM) were analysed by Design Expert® 9. ANOVA, regression coefficients and polynomial regression equation were obtained. Table 4 shows the relationship

Table 2. Central composite design (CCD) of culture medium and the results for TMP production.

| Runs | X1 | X2 | Experimental | Predicted |
|------|----|----|--------------|-----------|
| 1    | 0  | 0  | 12.68        | 13.06     |
| 2    | -1 | -1 | 10.65        | 11.29     |
| 3    | 1  | -1 | 8.13         | 7.80      |
| 4    | 0  | 0  | 12.73        | 13.06     |
| 5    | 0  | 0  | 13.48        | 13.06     |
| 6    | 1.414 | 0 | 7.84         | 8.42      |
| 7    | 0  | -1.414 | 9.09 | 8.92      |
| 8    | 0  | 0  | 13.48        | 13.06     |
| 9    | 0  | 1.414 | 4.64 | 4.60      |
| 10   | 0  | 0  | 12.94        | 13.06     |
| 11   | -1 | 1  | 5.43         | 5.97      |
| 12   | -1.414 | 0 | 10.93        | 10.14     |
| 13   | 1  | 1  | 7.44         | 7.02      |

Table 3. ANOVA results of the central composite design (CCD) of culture medium for TMP extraction.

| Source | Sum of squares | DF | Mean square | F-value | Prob  |
|--------|----------------|----|-------------|---------|-------|
| Model  | 110.84         | 5  | 22.17       | 60.10   | <0.0001|
| X1     | 2.98           | 1  | 2.98        | 8.24    | 0.0240|
| X2     | 18.61          | 1  | 18.61       | 51.51   | 0.0002|
| X1X2   | 24.68          | 1  | 24.68       | 68.30   | <0.0001|
| X1²    | 68.75          | 1  | 68.75       | 190.23  | <0.0001|
| X2²    | 5.13           | 1  | 5.13        | 14.20   | 0.0070|
| Residual | 2.53          | 7  | 0.36        |         |       |
| Lack of fit | 1.97       | 3  | 0.66        | 4.70    | 0.0844|
| Pure error | 0.56       | 4  | 0.14        |         |       |
| Cor total | 113.37      | 12 |             |         |       |

Figure 7. Effects of different fermentation conditions on TMP production.
between TMP yields and independent variables, its ANOVA results were tabulated in Table 5. The model has an ‘F-value’ of 54.35, which is large enough and implies that the model is significant. The ‘Coefficient of Determination’ ($R^2$) value of the study was 0.9749. The high $R^2$ value suggests that strong correlation exists between the observed values and the values predicted by the CCD model the ‘adjusted $R$-squared’ value of 0.9570. The quadratic polynomial equation obtained by multiple regression analysis depicting the effect of parameters ($X_1$ and $X_2$) and their interactions in the response (TMP production), and is described in the following equation:

$$Y = 13.71 - 1.09 \times 1 - 1.13 \times 2 - 2.61 \times 12 - 3.04 \times 22 + 0.56 \times 1 \times X_2. \quad (2)$$

Figure 8(b) shows the 2D contour plots and 3D surface plots of the RSM study, which disclose the mutual interaction in between parameters and response. The predicted optimum values for the parameters are shown in Table 4. The optimized value of TMP productions from the second-order quadratic model defined by Equation (2) was $13.71 \pm 1.01 \mu g/mL$ under the optimum composition of culture medium and liquid state fermentation conditions: starch 5.1%, peptone 3.51%, Mg$_2$SO$_4$ 0.1%, NaNO$_3$ 0.2%, KH$_2$PO$_4$ 0.25%, fermentation temperature 28.23 °C, fermentation time of 9 d, shaker speed of 180 r/min, inoculum size of 7.21% and loading volume of 100 mL /250 mL triangular flask. The experiment was carried out under the above-predicted optimum conditions in order to check the validity of the model, and a yield of $14.32 \pm 1.01 \mu g/mL$ was obtained. This demonstrated a good match between the experimental and predicted values, thus substantiating the proposed model. The results validate that the RSM method is applicable for the parameter optimization to culture medium composition and liquid state fermentation conditions.

**Conclusions**

In summary, Monascus strain M-3 with the highest TMP production ability was screened from red yeast rice collected from different areas of Fujian province in China. The target Monascus strain M-3 was identified as
M. purpureus by molecular biological method. The liquid state fermentation conditions of Monascus strain M-3 were further determined by the single-factor test and CCD of RSM; starch 4.4%, peptone 3.51%, MgSO₄ 0.1%, NaNO₃ 0.2%, KH₂PO₄ 0.25%, culture temperature of 28.23 °C, fermentation time of 9 d, shaker speed of 180 r/min, inoculum size of 7.21% and loading volume of 100 mL/250 mL triangular flask. Under the optimum composition of culture medium and liquid state fermentation conditions, the TMP production could be as high as 13.49 µg/mL.

The study is expected to provide a new approach of TMP production through biosynthesis by Monascus strain. The results would promote the further development of functional foods or medicines from Monascus fermentation products, and provide reference for China’s traditional food industry. The final yield of TMP by Monascus strain M-3 is still lower compared to Bacillus sp. strains reported by others. Optimization to higher TMP production is required, which could be achieved by selecting and breeding the excellent strains through physical or chemical mutagenesis. In addition, further studies also need to be focused on the specific metabolic pathways of TMP production by Monascus.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This work was financially supported by National Natural Science Foundation of China (grant number 31601466); Project of China Postdoctoral Science Foundation (grant number 2016T90591); [grant number 2015MS70549]; Fund for outstanding young scientific talents of Fujian Agriculture and Forestry University [grant number XJQ201707]; Natural Science Foundation of Fujian Province [grant number 2016J01095].

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