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# Medium optimization for enhanced production of cytosine-substituted mildiomycin analogue (MIL-C) by *Streptoverticillium rimofaciens* ZJU 5119*

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**Abstract:** Cytosine-substituted mildiomycin analogue (MIL-C) was produced effectively by supplementing cytosine into the culture of *Streptoverticillium rimofaciens*. In order to improve the yield of MIL-C, statistically-based experimental designs were applied to optimize the fermentation medium for *S. rimofaciens* ZJU 5119. Fifteen culture conditions were examined for their significances on MIL-C production using Plackett-Burman design. The Plackett-Burman design and one-variable-at-a-time design indicated that glucose and rice meal as the complex carbon sources, and peanut cake meal and $NH_4NO_3$ as the complex nitrogen sources were beneficial for MIL-C production in *S. rimofaciens* ZJU 5119. The results of further central composition design (CCD) showed that the optimal concentration of glucose, rice meal and peanut cake meal were 18.7 g/L, 64.8 g/L and 65.1 g/L, respectively. By using this optimal fermentation medium, the MIL-C concentration was increased up to 1336.5 mg/L, an approximate 3.8-fold improvement over the previous concentration (350.0 mg/L) with un-optimized medium. This work will be very helpful to the large-scale production of MIL-C in the future.

**Key words:** Cytosine-substituted mildiomycin analogue (MIL-C), Plackett-Burman design, Response surface methodology, *Streptoverticillium rimofaciens*, Nucleoside antibiotic

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## INTRODUCTION

Mildiomycin (MIL) is an effective nucleoside antibiotic against powdery mildew diseases in many kinds of plants with low toxicity in mammals and fishes, and has been produced in large-scale in Japan (Harada *et al*., 1978; Ye *et al*., 2006; Iwasa *et al*., 1978; Suzuki *et al*., 1982). The effects of ferrous ions and the biosynthetic pathway of mildiomycin were investigated to improve mildiomycin yield in the previous work (Kishimoto *et al*., 1997). It was proposed that serine, arginine and 5-hydroxymethyl cytosine might be the precursors of mildiomycin biosynthesis in *Streptoverticillium rimofaciens*. It was also found that a variety of new mildiomycin analogues were obtained when the analogue of 5-hydroxymethyl cytosine was added into the medium of a mildiomycin-producing microorganism (Takatsuki *et al*., 1981). The corresponding molecular structures of mildiomycin analogues are shown in Fig.1, which were named as cytosine-substituted mildiomycin analogue (MIL-C), flu-cytosine-substituted mildiomycin analogue (MIL-F) and bro-cytosine-substituted mildiomycin analogue (MIL-Br), respectively (Takatsuki *et al*., 1981).

A mildiomycin producing strain, *S. rimofaciens* ZJU-5119, was isolated in this laboratory and conserved in China General Microbiological Culture Collection (CGMCC, with accession No. CGMCC

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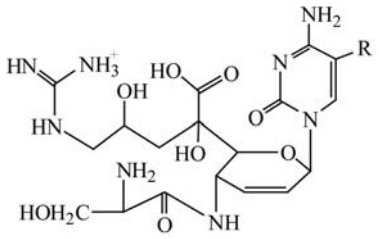

**Fig.1 The structure of mildiomycin cytosine analogues**
MIL: R=$CH_2OH$; MIL-C: R=H; MIL-F: R=F; MIL-Br: R=Br

1503). The mildiomycin (2.6 g/L) was obtained after strain improvement and fermentation conditions optimization (Xie *et al*., 2005). And a series of mildiomycin analogues were biosynthesized by adding different precursors to the medium. Among them, MIL-C performed the highest bioactivity and the lowest toxicity. And after bioactivity evaluation for controlling powdery mildew of plants in greenhouse and field tests, MIL-C showed a higher bioactivity against powdery mildew diseases compared with mildiomycin. However, the yield of MIL-C in *S. rimofaciens* ZJU-5119 was relative low (350.0 mg/L) using the original culture conditions developed by Xie *et al*.(2005), which has limited its wide application in agriculture. Therefore, the optimization of fermentation factors is needed to improve the fermentation efficiency of MIL-C.

Different strategies can be used for the optimization of cultivation conditions (Mao *et al*., 2007; Farid *et al*., 2000). Conventional "one-variable-at-a-time" approach was used more often than other approaches, but it is time-consuming and likely leads to confusion in understanding the process parameters (Kumar and Satyanarayana, 2007). Some statistical techniques, such as Plackett-Burman design and response surface methodology (RSM), were proved to be useful for developing, improving and optimizing processes, and were extensively used in the industries and in bioprocesses including the formulation of culture medium for bacteria and fungi (Didier *et al*., 2007).

The medium was optimized systematically in the present work for improving the production of MIL-C. The effects of various sources of carbon and nitrogen were evaluated on the biosynthesis of MIL-C. Then, a Plackett-Burman design was adopted to determine the most important factors that affect MIL-C production. And a central composite design (CCD) was used to optimize the levels of these controllable factors in order to formulate an optimal medium to increase the concentration of MIL-C by *S. rimofaciens* ZJU 5119.

## MATERIALS AND METHODS

### Strains and stock culture

*S. rimofaciens* ZJU 5119 used in this study was conserved in China General Microbiological Culture Collection (CGMCC, with accession No. CGMCC 1503).

The culture was maintained on slant medium (20 g soluble starch, 1.0 g $KNO_3$, 0.5 g NaCl, 0.5 g $K_2HPO_4{\cdot}3H_2O$, 0.5 g $MgSO_4{\cdot}7H_2O$, 5.0 g peptone, 20 g agar, per 1 L) at 28 °C and subcultured every 2 weeks.

### Cultivation of *S. rimofaciens* ZJU 5119

The seed culture was prepared by inoculating a loopful of stock culture into 250-ml shake-flask containing 30 ml of seed medium (20 g glucose, 30 g soybean cake meal, 5.0 g yeast extraction, 1.0 g $(NH_4)_2SO_4$, 0.5 g $MgSO_4{\cdot}7H_2O$, 3.0 g $CaCO_3$, per 1 L) and incubating the flask in a rotary shaker for 40 h at 28 °C, 200 r/min. Then 3 ml of seed culture was transferred into a 250-ml shake-flask containing 30 ml fermentation medium (the same as the slant medium, but eliminating 20 g agar and adding 40 g soybean cake meal, 0.75 g cytosine, per 1 L). This culture was incubated for another 8 d under the same condition. The supernatant was used for yield determination of MIL-C after centrifuge (4000×g for 10 min). All of these experiments were carried out in triplicate.

### Analyses of MIL and MIL-C

The concentrations of MIL and MIL-C in the broth were determined by HPLC (Liang *et al*., 2006; Chen *et al*., 2006). An Agilent 1100 series HPLC System equipped with a Hypersil BDS $C_{18}$ column (250 mm×4.6 mm) was employed to analyze the MIL-C concentration (Liang *et al*., 2006). The column temperature was kept at 25 °C. The mobile phase consisted of methanol/trichloroacetic acid (1.0%, w/v)/$H_2O$ (80/100/820, v/v/v), and the flow-rate was 1.0 ml/min. The wavelength of UV detector was set at

279 nm and the volume of each injection was 10 μl. The standards of MIL (99.5%, w/w) and MIL-C (96%, w/w) were obtained by preparative HPLC in our laboratory.

**Medium optimization with statistically-based experiment designs**

1. Plackett-Burman design

A Plackett-Burman design was used to determine the most important factors influencing MIL-C production and remove the dispensable ones to conclude a smaller and more manageable set of factors (Prakash and Srivastava, 2005). The different factors were prepared in two levels, −1 for low level and +1 for high level based on Plackett-Burman design (Table 1).

**Table 1 Factors and levels in Plackett-Burman design**

| Factors | Abbr. | Low level (−1) | High level (+1) |
|---|---|---|---|
| Glucose (g/L) | A | 20 | 30 |
| Rice meal (g/L) | B | 60 | 80 |
| Peanut cake meal (g/L) | C | 40 | 60 |
| $NH_4NO_3$ (g/L) | D | 1 | 1.25 |
| $K_2HPO_4{\cdot}3H_2O$ (g/L) | E | 0.4 | 0.5 |
| NaCl (g/L) | F | 0 | 0.5 |
| $MgSO_4{\cdot}7H_2O$ (g/L) | G | 0.3 | 0.4 |
| $FeSO_4{\cdot}7H_2O$ (g/L) | H | 0.01 | 0.02 |
| Original pH value | J | 6 | 7 |
| Precursor (g/L) | K | 0.75 | 1 |
| Time for adding the precursor (h) | L | 0 | 24 |
| *N*,*N*-dimethylacetamie (ml/L) | M | 1 | 1.25 |
| Dosage of seeds (ml) | N | 0.5 | 1 |
| Seed age (h) | O | 30 | 40 |
| Beading | P | None | Add |

2. Central composite design (CCD)

The maximum yield of MIL-C was investigated using a CCD with three variables (Maddox and Richert, 1977; Shi *et al*., 2006). Each factor in the design was studied at five different levels ($-\alpha$, −1, 0, +1, $+\alpha$), which is shown in Table 2. Based on the Plackett-Burman design, the processing variables (factors) including the concentrations of glucose ($X_1$), rice meal ($X_2$) and peanut cake meal ($X_3$) were chosen for the CCD.

**Table 2 Factors and levels in CCD**

| Variables | Range and levels | | | | |
|---|---|---|---|---|---|
| | −1.682 | −1 | 0 | 1 | 1.682 |
| Glucose (g/L) | 15.73 | 17.40 | 20.00 | 22.60 | 24.27 |
| Rice meal (g/L) | 57.40 | 52.50 | 60.00 | 67.50 | 76.60 |
| Peanut cake meal (g/L) | 46.55 | 52.00 | 60.00 | 68.00 | 73.45 |

As shown in Table 3, a set of 20 experiments was carried out. All variables were taken at a central coded value considered as zero, which was determined by the "one-variable-at-a-time" approach. The minimum and maximum ranges of variables were investigated and the full experimental plan with respect to their values in actual and coded forms was also listed in Table 3. Upon completion of experiments, the yield of MIL-C was taken as the response ($Y$). A second order polynomial equation was then fitted to the data by a multiple regression procedure. The equation resulted in an empirical model that relates the measured response to the independent variables of the experiment. When several factors are involved, the model is expressed as follows:

$$Y = \beta_0 + \beta_i \sum x_i + \beta_{ij} \sum x_i x_j + \beta_{ii} \sum x_{ii}^2 , \quad (1)$$

**Table 3 The experimental design and results of CCD**

| | $x_1$ | $x_2$ | $x_3$ | $Y$ (mg/L) |
|---|---|---|---|---|
| 1 | −1 | −1 | −1 | 1324.0 |
| 2 | −1 | −1 | +1 | 680.6 |
| 3 | −1 | +1 | −1 | 1048.0 |
| 4 | −1 | +1 | +1 | 736.2 |
| 5 | +1 | −1 | −1 | 998.6 |
| 6 | +1 | −1 | +1 | 1026.0 |
| 7 | +1 | +1 | −1 | 633.5 |
| 8 | +1 | +1 | +1 | 426.1 |
| 9 | −1.682 | 0 | 0 | 530.6 |
| 10 | +1.682 | 0 | 0 | 1127.9 |
| 11 | 0 | −1.682 | 0 | 873.8 |
| 12 | 0 | +1.682 | 0 | 1090.1 |
| 13 | 0 | 0 | −1.682 | 548.6 |
| 14 | 0 | 0 | +1.682 | 609.8 |
| 15 | 0 | 0 | 0 | 1167.8 |
| 16 | 0 | 0 | 0 | 1139.4 |
| 17 | 0 | 0 | 0 | 1109.8 |
| 18 | 0 | 0 | 0 | 1103.5 |
| 19 | 0 | 0 | 0 | 1153.4 |
| 20 | 0 | 0 | 0 | 1138.7 |

$x_1$, $x_2$, and $x_3$ are coded independent variables for concentrations of glucose ($X_1$), rice meal ($X_2$) and peanut cake meal ($X_3$)

where $Y$ is the measured response; $\beta_0$, $\beta_i$, $\beta_{ij}$, and $\beta_{ii}$ are the intercept term, linear coefficient, interactive coefficient, and quadratic coefficient, respectively; and $x_i$ is the coded independent variable ($i$=1, 2, 3). Low and high factor settings are coded −1 and +1, and the midpoint is coded 0. The factor setting of trials that ran along axes drawn from the middle of the cube through the center of each face of the tube is coded +1.682 or −1.682. An Expert-Design software (Design Expert Version 6.0.5, Stat-Ease Inc., Minneapolis, USA) was used to analyze the results.

**Statistical analysis of data**

The data of the production of MIL-C were subjected to analysis of variance (ANOVA) using Expert-Design software to estimate $t$-value, $P$-value and confidence levels. Optimal values of MIL-C were estimated using the solver function of Expert-Design software.

## RESULTS AND DISCUSSION

**Effects of different nitrogen sources on MIL-C production**

In order to examine the effects of different nitrogen sources, three-level concentrations (20, 30 and 40 g/L) of different organic nitrogen sources were set, and the concentration of soluble starch was fixed at 40 g/L as the sole carbon source in all tested media. As shown in Fig.2 and Fig.3, among all the tested nitrogen sources, peanut cake meal was super for the production of MIL-C, achieving as high as 650 mg/L MIL-C by adding 60 g/L peanut cake into the fermentation medium. In order to explore the syntactic effect of inorganic nitrogen sources, different concentrations of amine sulphate nitre and amine nitrate were supplemented into the peanut cake meal-containing medium, respectively. As shown in Fig.4, the yield of MIL-C was improved by approximately 5% with the supplement of 1 g/L amine nitrate, but the production was not further enhanced by supplementing higher concentrations of this inorganic nitrogen source.

Of all nitrogen sources, inorganic nitrogen sources (such as amine sulphate, nitre and amine nitrate) are regarded as quick metabolized nitrogen sources, which are beneficial for fast microorganism growth relieving the need of long-time accumulation of product. Simultaneously, some organic nitrogen sources, including peanut cake meal, soybean cake meal and peptone, are sustainable nitrogen sources, which are beneficial for steady product accumulation (Choi *et al*., 2000). So, a mixed nitrogen source consisted of both peanut cake meal and amine nitrate was adopted in the medium with different ratios (Fig.4). The fermentation was divided into two stages in such formulated culture medium. At the first stage, namely biomass accumulation, amine nitrate can be metabolized quickly to improve the growth of microorganism. Consecutively at the stage of metabolism, peanut cake meal can be decompounded by organisms and keep the levels of usable nitrogen sources in the medium steady.

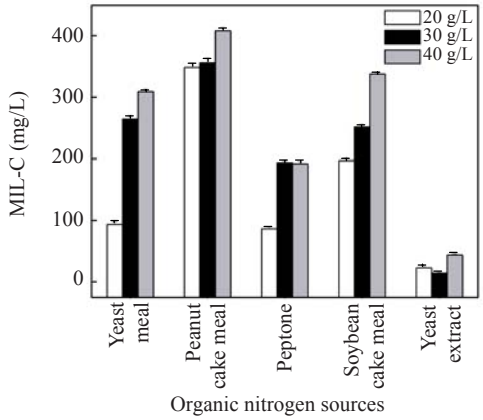


**Fig.2 Comparison of MIL-C concentration by cultivating *S. rimofaciens* ZJU 5119 in different organic nitrogen sources**

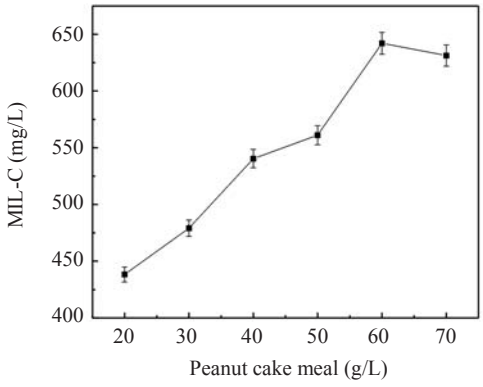


**Fig.3 The effect of peanut cake meal concentration on MIL-C production by *S. rimofaciens* ZJU 5119**
The concentration of soluble starch was fixed at 40 g/L as the sole carbon source in all the tested media

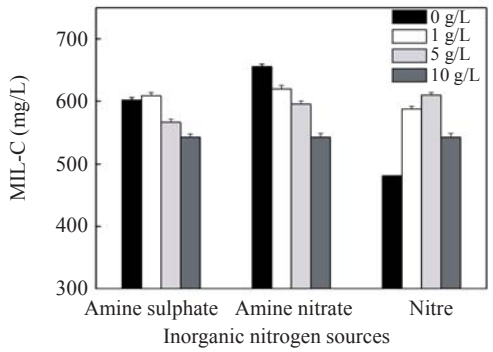


**Fig.4 Comparison of MIL-C concentration by cultivating *S. rimofaciens* ZJU 5119 in different mixed nitrogen sources**

The concentration of peanut cake meal was fixed at 60 g/L

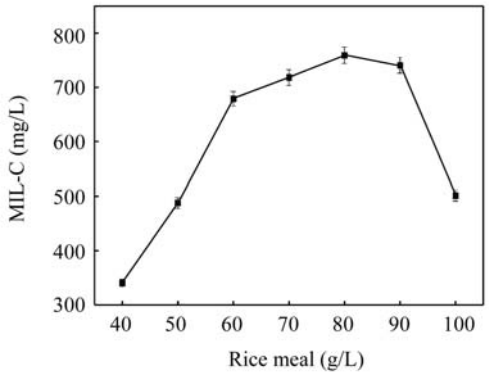


**Fig.6 The effect of rice meal concentration on MIL-C production by *S. rimofaciens* ZJU 5119**

The concentrations of nitrogen sources were fixed: 60 g/L peanut cake meal and 1 g/L amine nitrate

## Effects of different carbon sources on MIL-C production

The effects of different carbon sources on MIL-C production were examined with a fixed level of nitrogen sources (60 g/L peanut cake meal and 1 g/L amine nitrate) in all the tested media. As shown in Fig.5, among six carbon sources, cornstarch and rice meal were super for the production of MIL-C, and a range of rice meal (60~90 g/L) in the medium could lead to high production of MIL-C (Fig.6). Further tests showed that the concentration of MIL-C was further improved by 18% with the supplement of 20 g/L glucose into the rice meal-included medium, and a high concentration of MIL-C (1189 mg/L) in the broth was achieved (Fig.7).

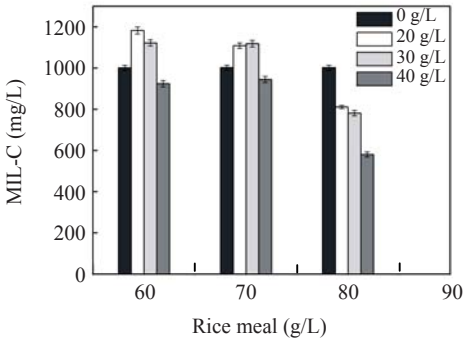


**Fig.7 Comparison of MIL-C concentration by cultivating *S. rimofaciens* ZJU 5119 in different mixed carbon sources**

The concentrations of nitrogen sources were fixed: 60 g/L peanut cake meal and 1 g/L amine nitrate; The different column colors show the concentrations of glucose

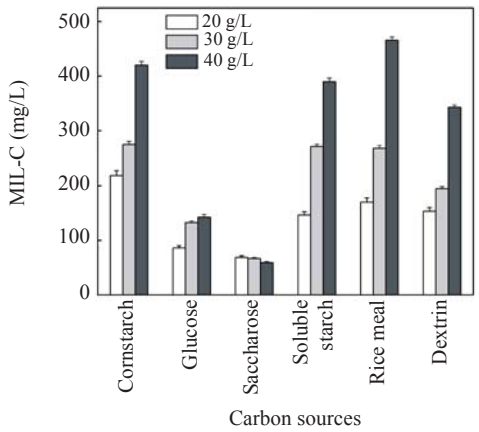


**Fig.5 Comparison of MIL-C concentration by cultivating *S. rimofaciens* ZJU 5119 in different carbon sources**

The concentration of nitrogen source was fixed: 60 g/L peanut cake meal

Similarly, all carbon sources can be divided into two groups, quickly-metabolized carbon sources and sustainable carbon sources. Of which, glucose and saccharose are regarded as quickly-metabolized carbon source, whereas rice meal, cornstarch, dextrin and soluble starch are sustainable carbon sources. The same to nitrogen sources, the quickly-metabolized carbon sources and sustainable carbon sources are beneficial to the growth of the microbe and increase the aimed production.

## Screening of important factors by Plackett-Burman design

Plackett-Burman design offers an effective screening procedure and computes the significance of a large number of factors in one experiment, which is time saving and maintains convincing information on

each component (Sharma and Satyanarayana, 2006). The experimental data of MIL-C production in the screening Plackett-Burman experiments were listed in Table 4, and the results illustrated a wide variation of concentrations from 220.3 to 1064.8 mg/L, which reflected the importance of medium optimization to attain higher yields.

On analyses of regression coefficients, *F*-value and *P*-value of 15 ingredients (Table 4), the factors with positive effect for MIL-C production are rice meal, peanut cake meal, $MgSO_4$, pH value of the medium and dosage of seeds. The most important factor was determined by the *P*-value and *F*-value evaluation of each individual effect. Of all the 15 variables, concentrations of peanut cake meal, rice meal, glucose and the number of beads were regarded as the most significant factors, with contributions of 36.1%, 29.1%, 4.2% and 2.1% to MIL-C production, respectively. It appeared that the number of beads in the medium brought negative effects, which was similar to the previous conclusion in the similar fermentation process (Peter *et al*., 2006). It indicated that the growth of *S. rimofaciens* was sensitive to the shear stress. Compared with other factors, the concentrations of glucose ($X_1$), rice meal ($X_2$), and peanut cake meal ($X_3$) were considered significant for MIL-C yield. However, the interactions between these variables were not examined. Thus a linear regression equation was obtained by applying the following fractional factorial equation:

$$Y=951.8-1.605X_1+6.664X_2+9.072X_3. \quad (2)$$

Based on the results of Plackett-Burman design, the concentrations of peanut cake meal, rice meal and glucose were selected for the further optimization subjects. The corresponding original medium for MIL-C production applied to RSM was as following (g/L): glucose 20, rice meal 60, peanut cake meal 60, $KNO_3$ 0.50, $K_2HPO_4{\cdot}3H_2O$ 0.45, NaCl 0.25, $MgSO_4{\cdot}7H_2O$ 0.35, $FeSO_4{\cdot}7H_2O$ 0.015, cytosine 0.88, *N*,*N*-dimethylacetamine 1.13, no bead addition and preadjusted the pH value to 6.0. The precusor (cytosine) was added after 12 h cultivation of *S. rimofaciens*.

**Table 4 Experimental design and results of Plackett-Burman design**

| | A | B | C | D | E | F | G | H | J | K | L | M | N | P | O | MIC-C (mg/L) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 40 | 20 | 40 | 0 | 0.5 | 0.5 | 0.4 | 0.02 | 6 | 0.75 | 24 | 1.25 | 0.5 | Add | 60 | 634.3 |
| 2 | 40 | 30 | 40 | 1 | 0.4 | 0 | 0.4 | 0.02 | 6 | 1 | 24 | 1 | 0.5 | None | 40 | 866.1 |
| 3 | 0 | 30 | 40 | 1 | 0.5 | 0 | 0.3 | 0.02 | 7 | 0.75 | 24 | 1.25 | 0.5 | None | 40 | 954.7 |
| 4 | 40 | 30 | 0 | 0 | 0.4 | 0 | 0.4 | 0.01 | 7 | 0.75 | 24 | 1.25 | 1 | None | 60 | 1064.8 |
| 5 | 0 | 20 | 40 | 0 | 0.5 | 0 | 0.4 | 0.02 | 7 | 1 | 0 | 1 | 1 | None | 60 | 820.3 |
| 6 | 40 | 20 | 0 | 1 | 0.5 | 0 | 0.4 | 0.02 | 6 | 0.75 | 0 | 1 | 1 | Add | 40 | 561.3 |
| 7 | 0 | 20 | 0 | 0 | 0.4 | 0 | 0.3 | 0.01 | 6 | 0.75 | 0 | 1 | 0.5 | None | 40 | 651.2 |
| 8 | 40 | 20 | 0 | 0 | 0.4 | 0.5 | 0.3 | 0.02 | 6 | 1 | 24 | 1.25 | 1 | None | 40 | 470.6 |
| 9 | 40 | 30 | 40 | 0 | 0.4 | 0.5 | 0.4 | 0.01 | 7 | 1 | 0 | 1 | 0.5 | Add | 40 | 892.8 |
| 10 | 0 | 20 | 0 | 1 | 0.4 | 0.5 | 0.3 | 0.02 | 7 | 1 | 24 | 1 | 0.5 | Add | 60 | 463.5 |
| 11 | 0 | 30 | 40 | 0 | 0.5 | 0.5 | 0.3 | 0.01 | 6 | 0.75 | 24 | 1 | 1 | Add | 40 | 686.4 |
| 12 | 40 | 30 | 0 | 1 | 0.5 | 0 | 0.3 | 0.01 | 6 | 1 | 0 | 1.25 | 0.5 | Add | 60 | 314.5 |
| 13 | 40 | 20 | 40 | 1 | 0.4 | 0 | 0.3 | 0.01 | 7 | 0.75 | 24 | 1 | 1 | Add | 60 | 684.3 |
| 14 | 40 | 20 | 40 | 1 | 0.5 | 0.5 | 0.3 | 0.01 | 7 | 1 | 0 | 1.25 | 1 | None | 40 | 991.6 |
| 15 | 0 | 30 | 0 | 1 | 0.5 | 0.5 | 0.4 | 0.01 | 6 | 1 | 24 | 1 | 1 | None | 60 | 640.5 |
| 16 | 0 | 30 | 40 | 0 | 0.4 | 0 | 0.3 | 0.02 | 6 | 1 | 0 | 1.25 | 1 | Add | 60 | 753.2 |
| 17 | 40 | 30 | 0 | 0 | 0.5 | 0.5 | 0.3 | 0.02 | 7 | 0.75 | 0 | 1 | 0.5 | None | 60 | 688.1 |
| 18 | 0 | 30 | 0 | 1 | 0.4 | 0.5 | 0.4 | 0.02 | 7 | 0.75 | 0 | 1.25 | 1 | Add | 40 | 440.0 |
| 19 | 0 | 20 | 0 | 0 | 0.5 | 0 | 0.4 | 0.01 | 7 | 1 | 24 | 1.25 | 0.5 | Add | 40 | 220.3 |
| 20 | 0 | 20 | 40 | 1 | 0.4 | 0.5 | 0.4 | 0.01 | 6 | 0.75 | 0 | 1.25 | 0.5 | None | 60 | 896.5 |
| *r* | −64.18 | 90.72 | 266.50 | −6.90 | −67.10 | −8.64 | 37.88 | −39.08 | 74.58 | −82.82 | −32.40 | −21.40 | 53.10 | −239.40 | −23.00 | |
| *F* | 1.65 | 3.01 | 26.20 | 0.02 | 1.64 | 0.02 | 0.54 | 0.58 | 2.09 | 2.58 | 0.37 | 0.18 | 1.07 | 21.30 | 0.18 | |
| *P* | 0.12 | 0.30 | 0.18 | 0.01 | 0.91 | 0.29 | 0.89 | 0.52 | 0.50 | 0.24 | 0.21 | 0.59 | 0.71 | 0.06 | 0.02 | |

A, B, C, D, E, F, G, H, J, K, L, M, N, P and O are the abbreviations for the factors as shown in Table 1

**Optimization of important medium components by RSM**

A CCD was used for screening out the combinations of the process variables that would lead the high concentration. Based on a regression analysis of the data from CCD experiments, the effects of three independent variables (glucose, rice meal and peanut cake meal) on MIL-C production were predicted by a second-order polynomial function:

$$Y = 1135.79 - 21.88X_1 - 60.16X_2 - 44.06X_3 + 77.66X_1^2 - 41.35X_2^2 - 11.54X_3^2 + 12.10X_1X_2 + 96.90X_1X_3 + 93.08X_3X_2, \quad (3)$$

where $X_1$, $X_2$, $X_3$ are the concentrations of glucose, rice meal and peanut cake meal, respectively. The corrective measures for estimating the regression equation are the multiple correlation coefficients $R$ and the determination coefficient $R^2$. The closer the $R$ value is to 1, the better the correlation between the observed and the predicted values will be. Results were considered as significant ($P$=0.0228, $P$<0.05) from Eq.(3). The value of the determination coefficient ($R^2$=0.935) indicated a high degree of correlation between the observed and the predicted values. The effects of glucose, rice meal and peanut cake meal on MIL-C yield were shown in Fig.8. Maximum MIL-C concentration was obtained when the initial concentrations of glucose, rice meal and peanut cake meal were 18.7 g/L, 64.8 g/L, and 65.12 g/L, respectively. The maximum MIL-C yield of 1276.15 mg/L was predicted by using CCD. The corresponding optimal medium composition for efficient MIL-C production was finalized as following (g/L): glucose 18.7, rice meal 64.8, peanut cake meal 65.12, $KNO_3$ 0.50, $K_2HPO_4{\cdot}3H_2O$ 0.45, NaCl 0.25, $MgSO_4{\cdot}7H_2O$ 0.35, $FeSO_4{\cdot}7H_2O$ 0.015, pH 6, cytosine 0.88, *N*,*N*-dimethylacetamine 1.13.

These experiments indicated that the initial concentrations of carbon and nitrogen sources in the culture medium were important for achieving high MIL-C concentration in *S. rimofaciens* ZJU 5119. According to the proposed pathway of MIL biosynthesis, MIL was synthesized from 5-hydroxymethycytosine, serine and arginine (Kishimoto *et al*., 1997). Both serine and arginine can be obtained from the hydrolysis of proteins, or produced from the metabolism of carbon sources, whereas cytosine in the medium was not enough for MIL-C synthesis and had to be supplemented as the precursor for high production of MIL-C.

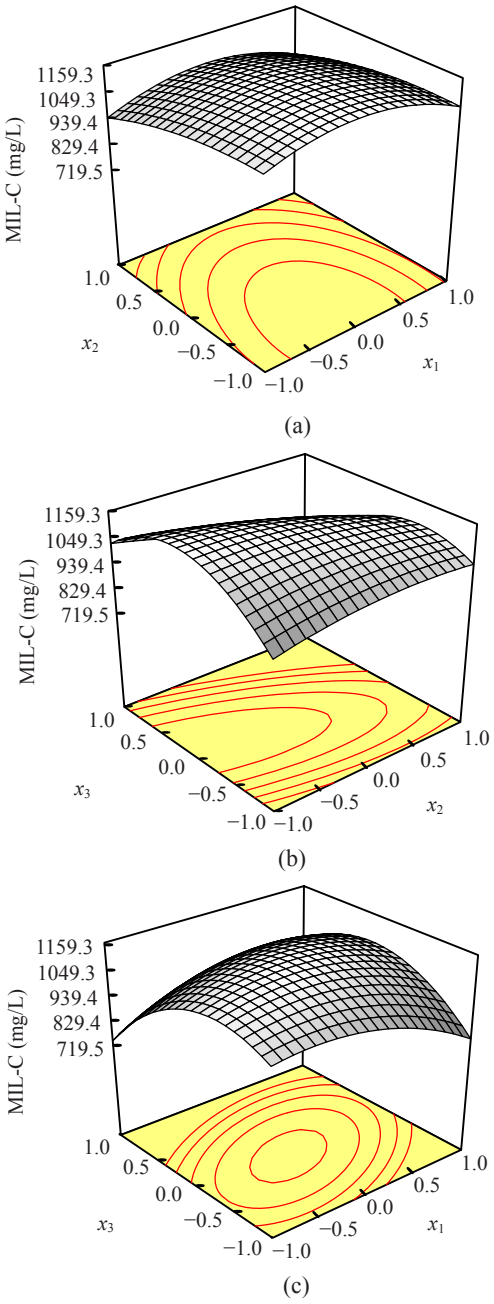


**Fig.8 Response surface plots for the combinatory effects. (a) Glucose ($x_1$) and rice meal ($x_2$). Fixed level: peanut cake meal ($x_3$)=0 (60 g/L); (b) Peanut cake meal ($x_3$) and rice meal ($x_2$). Fixed level: glucose ($x_1$)=0 (20 g/L); (c) Glucose ($x_1$) and peanut cake meal ($x_3$). Fixed level: rice meal ($x_2$)=0 (60 g/L)**

**Verification of the predicted concentration in the optimal medium**

The formulated optimal medium from RSM experiments was verified experimentally and compared with the predicted data from the model. The average concentration of MIL-C in the broth was 1336.5 mg/L from triple-duplicated experiments, which suggests the accuracy of the model is over 95%.

## CONCLUSION

The fermentation medium was optimized systematically with regard to the production of MIL-C with *S. rimofaciens* ZJU 5119. The experiments of Plackett-Burman design and one-variable-at-a-time design indicated that glucose and rice meal as the complex carbon sources, and peanut cake meal and $NH_4NO_3$ as the complex nitrogen were beneficial for MIL-C production. Furthermore, the CCD formulated an optimal fermentation medium, and a high concentration of MIL-C (1336.5 mg/L) was achieved after 8-d cultivation at 28 °C. Compared with the production of MIL-C in un-optimized medium, the systematically optimized medium produced a high yield of MIL-C (1336.5 mg/L), an approximate 3.8-fold improvement, which has never been reported before in the literature. This work will support a further process development to produce this new mildiomycin analogue in large scales.

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