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Full Length Research Paper

Response surface modeling for γ-aminobutyric acid production by *Monascus pilosus* GM100 under solid-state fermentation

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***Monascus*, a traditional Chinese fermentation fungus, is used as a natural dietary supplement. As a metabolite of *Monascus*, γ-aminobutyric acid (GABA) had been proven to be a hypotensive agent. In this study, the ability of *Monascus pilosus* to produce GABA was investigated under solid-state fermentation. Plackett-Burman design (PBD) was applied to seek for the crucial parameters that affect the production of GABA. In addition, central composite design (CCD) was used to optimize each crucial variable. As a result, the maximum GABA production predicted by the CCD was 937.61 mg/kg using 60 g sterilized rice and 0.5% ethanol; optimum values of those crucial coefficients were determined to be monosodium glutamate (MSG) 0.714 (37.14 g·kg^{-1}), $CaCl_2 \cdot 2H_2O$ –0.025 (4.4625 g·kg^{-1}), and time of anaerobic treatment with CO_2 ($CO_{2\ Time}$) –0.1947 (43.327 h). Under the theoretical optimal conditions, the actual GABA production was 884.32 mg/kg, approximately 4.9 times than that before optimization.**

Key words: Central composite design, GABA, hypotensive, Plackett-Burman design, solid-state fermentation.

INTRODUCTION

Monascus purpureus, *Monascus ruber* and *Monascus pilosus* are three traditional fermentation fungi used in food for thousands of years in East Asia (Hawksworth and Pitt, 1983). Recently, scientific researches revealed the prominent decreasing blood pressure effect of *Monascus*, and demonstrated its antihypertensive substance, γ-aminobutyric acid (GABA) (Kono and Himeno, 2000; Rhyu et al., 2002; Tsuji et al., 1992; Tsuyoshi et al., 2005). It is well known that GABA, with two receptors-$GABA_A$ and $GABA_B$, is the main suppressive nerve transmitter of the central nervous system (Lauder, 2005). Moreover, GABA-rich food has multiple physiological functions such as antihypertensive (Aoki et al., 2003b; Nakamura et al., 2000; Hayakawa et al., 2004), liver protective and tranquilizer effects (Okada et al., 2000). Therefore, GABA has been applied to clinical medicine for hypertension therapy (Lacerda et al., 2003), Parkinson's disease therapy (Nandi et al., 2002), hypochondria (Petty et al., 1997), and epilepsy therapy (Löscher et al., 1998), etc.

With a view to the source of GABA, it was reported that it could be produced by various fungi other than *Monascus* (Kono and Himeno, 2000) including *Rhizopus* (Akoi et al., 2003a), *Saccharomyces cerevisiae* (Kishimoto and Sodeyama, 2003) and *Aspergillus* (Kato et al., 2002). The GABA production of *M. purpoureus* was reportedly as high as 5,004 mg/kg (Wang et al., 2003). However, there exists one disadvantage of the high GABA production in red yeast rice fermented by *M. purpureus*, which is the severe citrinin contamination.

Citrinin contamination has become a pressing issue in fungi fermented GABA product due to its severe nephrotoxicity and hepatototoxicity (Kitabatake et al., 1993). Blanc et al. (1995) reported that citrinin was definitely detected in the product fermented by *M. purpureus and M. ruber*. Fortunately, as to fungus *M. pilosus*, few citrinin contamination cases were reported in fermented products; these findings undoubtedly doomed the commercial potential of *M. pilosus*. Moreover, GABA-abundant red yeast rice, a product of solid state

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fermentation (SSF) by *M. pilosus*, has already proved a good market as supplements in Japan (Kato et al., 2002). Since the optimization of the solid-state fermentation conditions with *M. pilosus* for the GABA production has not been extensively studied yet, we aimed at optimizing the condition of solid fermentation by *M. pilosus*, so that we can improve the GABA production.

Plackett-Burman design (PBD) is a well-established, widely used statistical design technique for key factors screening, out of a large number of solid-state fermentation (SSF) parameters, without numerous experiments. Response surface methodology (RSM) is very useful to test multiple process variables because fewer experimental trials are needed compared to the study of one variable at a time. In addition, interactions between variables could be identified and quantified by such a technique. Another design, central composite design (CCD), was conducted in the optimum vicinity to locate the true optimum values of the multiple variables. Therefore, RSM and CCD are increasingly used for optimization of many fermentation conditions (Casas López et al., 2004; Chang et al., 2006; Sadik et al., 2007). RSM, as an efficient experimental strategy, was used to seek the optimal conditions for multivariable system and factors affecting the objects were evaluated with the PBD and the CCD. In all, this work was meant to optimize the SSF parameters of *M. pilosus* for the GABA production to be used for antihypertensive and diuretic studies.

MATERIALS AND METHODS

Microorganism and growth conditions

M. pilosus GM100 (a citrinin non-producer) was originally isolated from Chinese traditional food, red fermented rice, and deposited in the Institute of Brewing, Zhejiang University of Technology, Hangzhou, China. Stock culture was kept on slants of potato dextrose agar (PDA) (*Difco*, Detroit, USA) at 4℃. Spores were harvested using sterilized solution (0.9% NaCl, 0.05% Tween 80) and the fungi were allowed to grow on PDA slants for 5 days at 30℃ (Hajjaj et al., 2001).

Solid-state fermentation

A suspension of 2×10^6 spores was inoculated in a 500 ml Erlenmeyer flask containing 100 ml seed culture (rice powder, 30 g; glucose, 30 g; $NaNO_3$, 2 g; KH_2PO_4, 1.5 g; $MgSO_4{\cdot}7H_2O$, 1 g in 1000 ml distilled water; adjusted pH to 6.0). The seed culture was modified from the liquid medium previously described by Su et al. (2003). Cultures were incubated at 30℃ for 48 h at 200 rpm. The SSF procedure was carried out according to the method of Xu et al. (2005) with slight modification. Sixty gram of sterilized rice in the 500 ml Erlenmeyer flask was inoculated with 10 ml seed culture. The inoculated substrate was maintained at 32℃ for the first 3 days and then continued to be cultivated at 26℃ for 11 days. After cultivation, the substrate was incubated with CO_2 under 0.1 Mpa pressure in the airproof tank, and dried at 60℃ for 24 h.

GABA estimation

One gram of dried red yeast rice powder was extracted with 5 ml water at 60℃ for 2 h with vigorously shaking. After 12,000 × g centrifuging for 20 min at 4℃, 400 µl aliquot of supernatant (or standard solution of GABA) was vacuum-dried. The residue was dissolved in 50 µl ethanol-water-triethylamine (2:2:1) solution, and the mixture was then evaporated to dryness under vacuum until dry and redissolved again in 40 µl ethanol-water-triethylamine-phenylisothiocyanate solution (6:1:1:1). The final mixture was allowed to react for 20 min at room temperature to form phenylisothiocyanate-GABA (PTC-GABA).

Procedure of HPLC analysis described by Wang et al. (2004) was slightly modified. Briefly, the dry residue containing PTC-GABA was dissolved by adding 400 µl mobile phase that consisted of 80% solution A (aqueous solution of 8.205 g sodium acetate, 0.5 ml triethylamine, 0.7 ml acetic acid, and 5.0 ml acetonitrile in 1000 ml distilled water, pH 5.8) and 20% solution B (acetonitrile-water, 60:40, pH 5.8). Chromatographic separation was conducted on a Shim-pack VP-ODS C_{18} column (4.6 × 150 mm *i.d.*, 5 µm). The eluent was pumped at a flow rate at 0.6 ml/min. Temperature of column oven was 46℃ and UV detection wavelength was set at 254 nm.

Table 1. Effects of carbon sources on GABA production by *M. pilosus* GM100 using SSF.

Carbon source	Concentration (w/w)	GABA mg/kg)
Starch	0.5 %	203.46 ± 8.37
Maltose	0.5 %	299.45 ± 10.53
Glucose	0.5 %	223.87 ± 6.38
Ethanol	0.5 %	385.32 ± 12.42
Sucrose	0.5 %	284.59 ± 9.66
Lactose	0.5 %	321.45 ± 11.67
Blank	–	180.32 ± 3.23.

The basal medium consists of 60 g sterilized rice with 1.0 % (w/w) peptone in a 500 ml Erlenmeyer flask.

Table 2. Effects of nitrogen sources on GABA production by *M. pilosus* GM100 using SSF.

Nitrogen sources	Concentration (w/w)	GABA (mg/kg)
$(NH_4)_2SO_4$	1.0 %	403.32 ± 10.31
MSG	1.0 %	502.39 ± 14.46
$NaNO_3$	1.0 %	390.16 ± 12.45
Yeast extract	1.0 %	451.25 ± 9.85
Soybean powder	1.0 %	406.49 ± 12.64
Malt peptone	1.0 %	426.47 ± 9.88
Blank	□	376.28 ± 12.36

The basal medium consists of 60 g sterilized rice with 0.5 % (w/w) ethanol in a 500 ml Erlenmeyer flask.

Optimization of carbon sources and nitrogen sources

Six carbon sources and 6 nitrogen sources were individually added into 500 ml Erlenmeyer flask containing 60 g sterilized rice. The optimal carbon source and nitrogen source were evaluated by the effects on the GABA production (Table 1 and 2).

Plackett-Burman design

In this part, the optimum carbon and nitrogen sources were further

Table 3. Variables and their levels for the CCD during GABA production by *M. pilosus* GM100.

X_1-MSG (g/kg)		X_3-$CaCl_2$ (g/kg)		X_6-CO_2 Time (h)	
Coded value	**Actual value**	**Coded value**	**Actual value**	**Coded value**	**Actual value**
-1.68	13.2	-1.68	1.98	-1.68	7.68
-1	20	-1	3.0	-1	24
0	30	0	4.5	0	48
+1	40	+1	6.0	+1	72
+1.68	46.8	+1.68	7.02	+1.68	88.32

Table 4. Experimental design and results of the CCD during GABA production by *M. pilosus* GM100.

Number [a, b]	X_1	X_3	X_6	GABA (mg/kg)	
				Predicted	**actual**
1	20	3.0	24	822.06	786.37
2	40	3.0	24	815.54	764.18
3	20	6.0	24	812.54	770.32
4	40	6.0	24	773.62	732.19
5	20	3.0	72	845.92	823.25
6	40	3.0	72	825.54	773.45
7	20	6.0	72	875.32	824.29
8	40	6.0	72	779.23	719.96
9	13.2	4.5	48	718.65	769.43
10	46.8	4.5	48	764.43	724.67
11	30	1.98	48	835.04	790.89
12	30	7.02	48	822.35	787.62
13	30	4.5	7.68	775.06	693.28
14	30	4.5	88.32	883.75	827.34
CP^c	30	4.5	48	933.32	836.17
CP^c	30	4.5	48	938.94	826.29
CP^c	30	4.5	48	943.04	832.78
CP^c	30	4.5	48	948.92	817.65
CP^c	30	4.5	48	916.63	821.48
CP^c	30	4.5	48	926.88	827.67

[a]Experiments were run in a random order; [b]Experiments were repeated in triplicate for accuracy; CP^c: central point.

optimized together with other variables: X_1-MSG (monosodium glutamate), X_2-ethanol, X_3-$CaCl_2 \cdot 2H_2O$, X_4-$MgSO_4 \cdot 7H_2O$, X_5-KH_2PO_4, X_6-$CO_{2\ Time}$ (time of anaerobic treatment with CO_2) and X_7-temperature.

Central composite design

The CCD was adopted to optimize the crucial variables selected by the PBD. A three-factor-five-level CCD with six star points (α=1.68) and six replicates at the centre points was carried out according to Table 3. The experimental design is shown in Table 4. The experiments were performed in triplicate. A second order polynomial, equation (1), which included all interaction terms, was used to calculate the predicted response:

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

Where, γ represents response variable; β_0 is the interception coefficient; β_i is the coefficient of the linear effect; β_{ii} is the coefficient of quadratic effect and β_{ij} is the coefficient of interaction effect; χ_i and χ_j denote the coded levels of variable X_i and X_j investigated in the experiment. The variable X_i is coded as χ_i according to the equation (2):

$$x_i = \frac{X_i - X_0}{\Delta X} \quad (2)$$

Where, χ_i is coded value of the variable X_i, X_0 is the real value of X_i at the center point (zero) level, and the ΔX_i is the step change value.

All experimental designs and statistical data were analyzed by using software Design-Expert® 7.0.0 (Stat-Ease Inc., Minneapolis, MN, USA).

RESULTS

When ethanol was added into the basal medium of 60 g

Table 5. ANOVA for CCD during GABA production by *M. pilosus* GM100.

Analysis of variance	Value
Regression	
Sum of squares	82958.07
Df	9
Mean squares	9217.56
F ratio	11.0
P	0.0004
Residual	
Sum of squares	8380.94
Df	10
Mean squares	838.09
Correlation coefficient (R^2)	0.9082
Coefficient of variation (CV %)	3.41

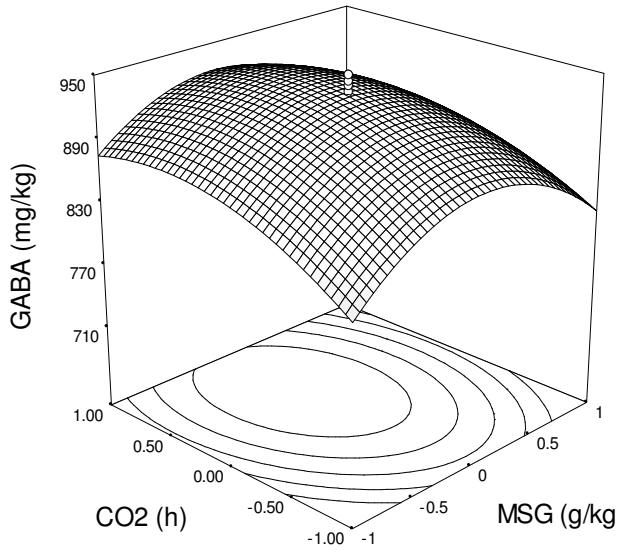

Figure 1. Effects of MSG (X_1) and $CO_{2\ Time}$ (X_6) on GABA production by *M. pilosus* GM100 (Y_1) with other variables set at centre level.

sterilized rice with 1.0% (w/w) peptone, the GABA production was accordingly increased to 385.32 mg/kg, approximately 2.14 times than that obtained from the blank (Table 1). Similarly, the GABA production was increased to 502.39 mg/kg when nitrogen source was monosodium glutamate (MSG) (Table 2). As a result, ethanol and MSG were found to be the best complementary carbon source and nitrogen source for the GABA production.

The crucial parameters screened by PBD: X_1-MSG, X_3-$CaCl_2$ and X_6-$CO_{2\ Time}$ were used for the central composite design (CCD) (Table 3), while the others, X_2-ethanol, X_4-$MgSO_4 \cdot 7H_2O$, X_5-KH_2PO_4, X_7-temperature, were non-significant in the PBD. A second order response surface experiment was formulated using CCD. CCD and its experimental results were presented in Table 4. A mathematical model showing the significant importance was described as follows:

$$\text{GABA production} = +933.75 - 6.22 \times X_1 - 6.52 \times X_3 + 20.87 \times X_6 - 13.52 \times X_1 \times X_3 - 8.88 \times X_1 \times X_6 + 4.32 \times X_3 \times X_6 - 62.56 \times X_1^2 - 31.75 \times X_3^2 - 31.50 \times X_6^2 \quad (3)$$

Where, X_1, X_3, and X_6 indicate MSG, $CaCl_2$, and $CO_{2\ Time}$, respectively.

In this case, the value of R (0.9529) indicates a high agreement between the experimental and predicted values. The value of determination R^2 (0.9082) suggested that the response model be accounted for 90.82% variations of the total. The value of adjusted determination coefficient (R^2_{Adj}=0.8257) was also high enough, which indicated the significance of this model.

The corresponding analysis of variance (ANOVA) is given in Table 5. The *F*-value is a measure of the variation of the mean data. Generally, if the model was a good prediction of the experimental result and the estimated factor effects were real, then the calculated *F* value should be several times greater than the tabulated *F* value. In this case, the ANOVA of the regression model demonstrated that the model is highly significant due to the evident from the calculated *F* value (11.00) and a very low probability value ($P > F$=0.0004).

The three dimensional (3D) response surface plots were employed to determine the variables and the optimum levels, which significantly affected the GABA production. The response surface plots were shown in Figures 1-3 which illustrated the relationship between the response and the experimental data. Figures 1 and 2 suggested the GABA production was predominantly affected by the action of $CO_{2\ Time}$. The GABA production decreased simultaneously with decreasing of $CO_{2\ Time}$, which agrees with the result obtained form the PBD. When $CO_{2\ Time}$ was held zero level, and only the other two parameters varied, MSG and $CaCl_2 \cdot 2H_2O$, the GABA production was in different ways affected, data are shown in Figure 3.

The predicted optimum levels of the tested variables, namely X_1-MSG, X_3-$CaCl_2 \cdot 2H_2O$ and X_6-$CO_{2\ Time}$ were determined by applying regression analysis of equation (3) with Design Expert® 7.0.0.

The optimum values of those crucial coefficients were determined to be: X_1-MSG= 0.714 (37.14 $g \cdot kg^{-1}$), X_3-$CaCl_2 \cdot 2H_2O$ = –0.025 (4.4625 $g \cdot kg^{-1}$), and X_6-$CO_{2\ Time}$= –0.1947 (43.327 h). These values predicted that the GABA production under this model can reach the maximal value of 937.613 mg/kg (9.38%). Simultaneously, to verify the experiment we carried out, we analyzed the actual GABA production, which showed 884.32 mg/kg (8.84%), less than 5.7% deviation from the theoretic maximal value.

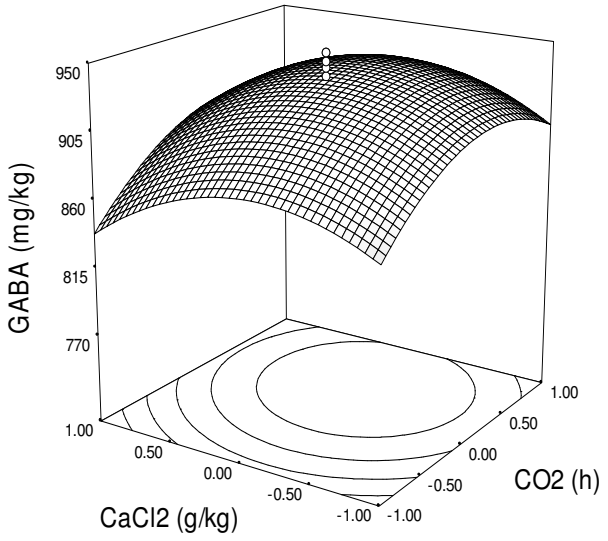

Figure 2. Effects of $CaCl_2 \cdot 2H_2O$ (X_3) and $CO_{2\ Time}$ (X_6) on GABA production by *M. pilosus* GM100 (Y_1) with other variables set at centre level.

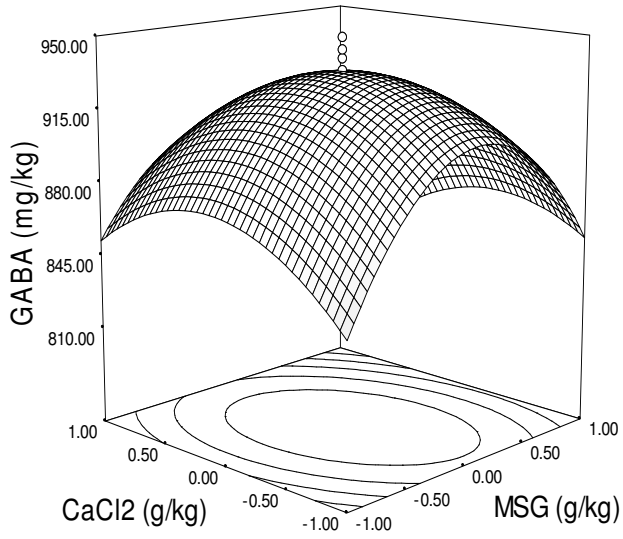

Figure 3. Effects of MSG (X_1) and $CaCl_2 \cdot 2H_2O$ (X_3) on GABA production by *M. pilosus* GM100 (Y_1) with other variables set at centre level.

DISCUSSION

RSM has been extensively explored for optimizing the medium compositions and operating conditions in many bioprocesses (Ambati and Ayyanna, 2001; Chang et al., 2002). In the current study, RSM has been demonstrated to be an efficient approach to optimize the cultivation conditions that affected the secondary metabolites of *Monascus* spp.

Medium ingredients and cultivation conditions affected the growth of filamentous fungi and the production of the secondary metabolites (Bode et al., 2002; Wang et al., 2003). In this study, properly adding a relative low concentration ethanol (0-0.5%) would increase the GABA production of *M. pilosus* by SSF, which agreed with the findings from Wang et al. (2003). Although Su et al. (2003) showed that $(NH_4)_2SO_4$ played a negative role in the GABA production, the results we obtained illustrated the opposite effect. This is probably a consequence of the increasing decarboxylase (GAD) activity induced by adding sulfate ions. As is well known, GABA is primarily produced from the α-decarboxylation of L-glutamic acid (Glu) that is catalyzed by the enzyme glutamate decarboxylase (GAD) (Reed et al., 2002). Moreover, the glutamate decarboxyse (GAD) activity would be increased by sulfate ions in a dose-dependent manner (Ueno et al., 1997).

We also found that anaerobic treatment can contribute to the GABA production, which supported the results of Aoki et al. (2003a). The results might be due to two reasons: one is the metabolic path from GABA to the succinic acid, which was blocked under the stress (low atmospheric oxygen) (Shelp et al., 1999). Another is the cytosolic acidification that may occur under the anaerobic incubation (Janzen et al., 2001), in which the intracellular pH shifted to the optimum pH of glutamate decarboxylase activity (Kono and Himeno, 2002). Under this condition, the glutamate decarboxylase (GAD) activity is increased because it would be at the maximal point when pH is 5.8 (Bown and Shelp, 1997). Consequently, the GABA production is also improved by the decarboxylation of glutamic acid with GAD. Moreover, an acid protease and an acid carboxypeptidase were secreted with the growth of *Monascus* during red yeast rice fermentation (Narajara, 1994). Subsequently, with the cytosolic acidification occurring, the GAD activity improved. This might be due to the low pH-dependent and consumed H^+ characteristic of GAD (Mazzucotelli et al., 2006). In this study, we also found that a certain amount of MSG contributed to the GABA production, which is consisted with the results form Su et al. (2003). Cholewa et al. (1997) and Scott-Taggart et al. (1999) indicated that GAD activity could be regulated through glutamate in the substrate (Cholewa et al., 1997; Scott-Taggart et al., 1999). We also found that GAD activity can be activated by adding a small amount of $CaCl_2$. As a Ca^{2+}-dependent calmodulin(CaM)-binding protein (Kinnersley and Turano, 2000), GAD activity is rapidly stimulated by increasing the cytosolic Ca^{2+} (Bouché and Fromm, 2004).

RSM can be expressed in terms of a mathematic function using experimental design, algorithm deduction and system analysis. It demonstrated the effect of individual factor on the results of multi-factor experiment and sought the optimum condition for each variable. Since anaerobic treatment would contribute remarkably to the GABA accumulation (Aoki et al., 2003a; Kang et al., 2006; Kono and Himeno, 2002; Shelp et al., 1999), we exposed the substrate to carbon dioxide in this study. The experi-

mental results proved this viewpoint. The predicted GABA production by the fungus *M. pilosus* GM100, 937.613 mg/kg, was 7 and 4 folds higher than that pro- duced by *M. pilosus* 4520 (Kono and Himeno, 2000) and *Aspergillus oryzae* (Kato et al., 2002). Although the GABA production in this study was lower than that reported by Wang et al. (2003), GABA-rich red yeast rice fermented by *M. pilosus* GM100 could be used directly as a dietary supplements and nutraceutical without citrinin contamination.

Recently, it was reported that GABA accumulation was affected by many stress conditions such as cold accli- mation and freezing, darkness and hypoxia (Mazzucotelli et al., 2006). The effects of other stress (salt stress, anaerobic function of inert gas, heat and cold hurt, etc.) on GABA production by *M. pilosus* should be considered in future research.

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