Optimisation of Fermentative Parameters for GABA Enrichment by *Lactococcus lactis*

XiaoXue LU[1,2], Chunyan XIE[1] and ZhenXin GU[1]

College of Food Science and Technology, Nanjing Agricultural University, Nanjing, Jiangsu Province, People's Republic of China; [2] Guangxi Agricultural Department, Guilin, Zhuang Autonomous Region, People's Republic of China

Abstract

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Gamma-aminobutyric acid (GABA) has antihypertensive and anti-stress functions on humans. Submerged fermentation of *Lactoccocus lactis* was regarded as an effective method to obtain GABA. In this study, the effects of fermentative parameters on the production of GABA was investigated. Firstly, one-variable-at-a-time experiments were performed in order to investigate the effects of significant factors influencing the GABA yield and monosodium glutamate (MSG) mole transformation percentage, i.e, the culture temperature, initial pH of the medium, MSG addition, and MSG addition time. Then, the response surface methodology (RSM) was applied to determine the optimum fermentative parameters. The results indicated that MSG addition was the most significant factor influencing GABA yield and MSG mole transformation percentage. The optimum parameters obtained by RSM for GABA enrichment by *Lactoccocus lactis* subsp. *lactis* (*L. lactis*) are listed as follows: temperature 31.8°C, pH 7.1 and MSG addition 15 g/l. Under these conditions, the predicted values were: GABA maximum 7.2 g/l and MSG mole transformation percentage 68.4%.

Keywords: γ-aminobutyric acid; *Lactococcus lactis*; fermentative parameters; optimisation

Gamma-aminobutyric acid (GABA), a four-carbon non-protein amino acid, serves as a major inhibitory neurotransmitter in mammalian nervous systems (Krnjevic & Schwartz 1966; Desai *et al*. 2005). It has several physiological functions, such as hypotensive effect (Hayakawa *et al*. 2005), epilepsy therapy (Ueda *et al*. 2007), tranquilising excitement, and enhancing memory (Kayahara & Sugiura 2001). It also has the effects of controlling asthma (Xu & Xia 1999), regulating hormone secretion (Parkash & Kaur 2007), activating liver and kidney function (Sun 2004). Therefore, the preparation and application of GABA have been obsects of concern in recent years.

As a promising compound with bio-functions, GABA can not only be utilised as a drug with significant pharmacological effects, but can also be used as a component of health food. GABA is difficult to extract from natural organisms because of its low content in biological tissues. Therefore, researchers tried to find effective methods to obtain GABA. And in the last two decades, the synthesis based on chemistry and biology has been reported (Abe *et al*. 1995; Kono & Himeno 2000; Takahashi & Naito 2001; Tong *et al*. 2002; Wang *et al*. 2003; Zhao *et al*. 2004; Wang *et al*. 2006; Komatsuzaki *et al*. 2007). However, the chemical synthesis is not very suitable for of the use of corrosive reactants, thus the biological method has become the focal point in the research field. In the biological method, microbial fermentation is regarded as the effective one because of its

convenience and a high ratio of transformation. Several safe microorganisms including lactic acid bacteria (LAB) have been widely applied in GABA production. *Lactobacillus brevis* (Park & Oh 2007a,b), *Lactobacillus paracasei* (Komatsuzaki *et al*. 2005) and *Lactoccocus lactis* (Xu *et al*. 2002) are extensively studied for the production of GABA-rich foods and pharmaceuticals.

This study was aimed at optimising the fermentative parameters to obtain a high production of GABA by *Lactoccocus lactis* subsp. *lactis* (*L. lactis*). On the basis of natural media (made up of brown rice juice, germinated soybean juice, and enzymolysed skim milk) optimisation, one-variable-at-a-time experiments were carried out to investigate the effects of temperature, initial pH, MSG addition, and MSG addition time on both the GABA yield and MSG mole transformation percentage. Afterward, Response Surface Methodology (RSM) was applied to investigate systematically the effects of the parameters selected. Under the optimum fermentative conditions, maximum GABA yield and MSG mole transformation percentage were reached simultaneously.

MATERIALS AND METHODS

Material. *L. lactis* strain B was isolated from Chinese traditional cabbage kimchi. Brown rice was supplied by Mayang Oil Co. Ltd. in Mayang County, Hunan Province, China. Yoghurt, germinated soybean, and kimchi (made from Chinese cabbage and radish) were bought from the local supermarket (Weigang, Nanjing city, Jiangsu Province, China). Alpha-amylase (4000 U/g), glucoamylase (100 000 U/g) and papain (2000 U/mg) were purchased from Beijing Double Spin Microbial Medium Plant, Shanghai Chemical Company and Nanjing Scigene Technology Co. Ltd. in China, respectively. Skim milk powder was obtained from Bright Dairy Co. Ltd., China. All the other chemicals used were commercial products.

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Fermentation substrate producing. Brown rice was milled into fine powder passing through a 60-mesh sieve. After adding five volumes of double-distilled water, the rice powder was gelatinised at 95°C for 40 minutes. After cooling to 60°C, the rice paste was mixed with 0.5% (w/w) α-amylase, and then incubated at 60°C for 60 minutes. The liquefied rice slurry was homogenised twice, in a colloid mill. The resulting mixture was adjusted to pH 4.5 with citric acid solution, 0.3% (w/w), then glucoamylase was added and the mixture was incubated at 60°C for 2 hours. The mixture was subsequently adjusted to pH 7.0 with NaOH solution, then papain 0.1% (w/w) was added and the mixture was incubated at 60°C for 3 hours. Finally, the above enzymolysed liquid was centrifuged at 4000 rpm for 10 minutes. The supernatant was withdrawn and adjusted to 4 Brix.

Germinated soybeans were boiled with the same weight of distilled water for 10 min to inactivate lipoxygenase and wet-milled by a triturator for 30 second. The crushed materials were treated with 0.1% (w/w) papain at 60°C for 3 hours. Germinated soybean juice was obtained by centrifugation at 4000 rpm for 10 minutes. The supernatant was adjusted to 4 Brix.

Skim milk (12%, w/v) was mixed with 0.1% papain and incubated at 60°C for 3 hours. Then the enzymolysed milk was centrifuged at 4000 rpm for 10 minutes. The supernatant was adjusted to 4 Brix as a fermentation substrate.

Fermentation conditions. The medium for *L. lactis* seed culture was MRS broth. A 250 ml flask containing 100 mL MRS broth was inoculated and incubated at 30°C for 20 hours. The seed culture contained approximately 10^8 CFU/ml. Brown rice juice, germinated soybean juice, and enzymatically degraded skim milk were mixed at a ratio of 33: 58: 9 (v: v: v), respectively (Lu *et al*. 2008) and used as the fermentation substrate for GABA synthesis by *L. lactis*. MSG was sterilised and added to the substrate before inoculation. The initial pH of the medium was adjusted with 0.1 mol/l NaOH solution. After the inoculation with 1% (v/v) of the seed liquid, 100 ml fermentation medium in 250 ml conical flasks was incubated for 6 days by static culture. All the media were sterilised in an autoclave at 121°C for 20 min before use.

Experimental design. One-variable-at-a-time experiments were performed in order to analyse the effects of the four parameters (temperature, initial pH, MSG addition, and MSG adding time) on GABA yield and MSG mole transformation percentage by *L. lactis*. Then, RSM was applied to analyse further the effects of several main independent factors on the GABA enrichment.

Table 1. Independent variables and their coded and actual values used in RSM optimisation

Independent variables	Symbol	Code levels		
		–1	0	1
Temperature (°C)	X_1	30	34	38
Initial pH	X_2	6.5	7.5	8.5
MSG addition (g/l)	X_3	1.25	20.00	38.75

Response surface methodology (RSM), originally described by Box and Wilson (Box & Wilson 1951), is a collection of mathematical and statistical techniques which are useful for designing experiments, building models, and analysing the effects of several independent factors. The main advantage of RSM is the reduced number of experiments needed to evaluate multiple factors and their interactions. Also, the study of the individual and interactive effects of these factors will be helpful in the effort to find the target value. Therefore, RSM provides an effective tool for investigating the factors affecting the desired response if there are many factors and their interactions in the experiment. RSM can be employed to optimise the experimental process by determining a suitable polynomial equation for describing the response surface.

In this study, three factors including the culture temperature, initial pH, and MSG addition were selected as independent variables. The GABA yield and MSG mole transformation percentage in the culture broth were the dependent variables. A three-factor and three-level Box-Behnken design (BBD) of RSM was chosen to evaluate the combined effect of three independent variables. The temperature, initial pH, and MSG addition were coded as X_1, X_2, and X_3, respectively (Table 1). The three levels (–1, 0, +1) were set up according to the results of single factor experiments. The values for the temperature were set at 34–38°C, initial

Table 2. Three-level and three-factor Box-Behnken design arrangements and responses

Trial No.	Factors			GABA yield (g/l)		MSG mole transformation (%)	
	X_1	X_2	X_3	experimental	predicted	experimental	predicted
1	–1	–1	0	8.09	7.83	57.94	56.25
2	1	–1	0	6.50	6.24	45.72	45.52
3	–1	1	0	7.34	7.12	52.17	48.8
4	1	1	0	4.81	5.53	32.81	38.07
5	–1	0	–1	2.10	2.21	96.66	99.87
6	1	1	–1	2.06	1.70	94.32	89.15
7	–1	0	1	7.85	8.20	29.90	31.75
8	1	0	1	5.65	5.53	20.91	21.02
9	0	–1	–1	2.12	2.52	98.45	98.09
10	0	1	–1	1.96	1.81	88.33	90.65
11	0	–1	1	7.32	7.43	27.72	29.97
12	0	1	1	7.07	6.72	26.73	22.52
13	0	0	0	7.83	7.82	55.90	55.85
14	0	0	0	7.87	7.82	56.24	55.85
15	0	0	0	7.80	7.82	55.71	55.85
16	0	0	0	7.80	7.82	55.71	55.85
17	0	0	0	7.80	7.82	55.71	55.85

pH of medium to 6.5–8.5, and MSG addition to 1.25–38.75 g/l. To avoid bias, 17 treatments were performed in a random order in which 12 axial points (treatment 1–12) and 5 center points (treatment 13–17) were considered (Table 2). The experimental design and statistical analysis were performed using Stat-Ease software (Design-Expert 6.0.10 Trial, Delaware, USA, Echip, 1993).

The optimisation data are fitted to a second order polynomial regression model which contains the coefficients of linear, quadratic, and two factors interaction effects. The model equation of the response (Y) of the three process variables (X_1, X_2, X_3) is:

$$Y = \beta_0 + \sum_{i=1}^{k} B_i X_i + \sum_{i=1}^{k} B_{ii} X^2 + \sum_{i>k}^{k} B_{ij} X_i X_j \quad (1)$$

where:

β_0 – constant
B_i – linear coefficient
B_{ii} – quadratic coefficient
B_{ij} – cross product coefficient
X_i, X_j – levels of the independent variables
k – number of the factors tested (k = 3)

The analysis of variance (ANOVA) table is generated and the regression coefficients of the individual linear, quadratic, and interaction terms are determined. The significances of all terms in polynomial are judged statistically by computing the F-value at a probability level (P) of 0.01 or 0.05. The model is then submitted to statistical analysis to neglect all terms that are statistically insignificant (P > 0.05). Regression coefficients are used to generate a contour map for the regression model.

Measurement of GABA content. GABA contents in the culture broth were measured using an amino acids analyser (Hitachi L-8900, Japan, 4.6 mm × 60 mm, at 57°C of the column temperature and 136°C of the reactor temperature). The cells were removed from the culture media by centrifugation at 10 000 rpm and 4°C for 10 minutes. Then 0.5 ml of the supernatant was diluted with 0.5 ml of 10% trichloroacetic acid and centrifuged at 10 000 rpm for 10 min, and then further diluted with 200-fold volume of distilled water. 10 ml samples were injected into the amino acids analyser.

Calculation method of MSG mole transformation percentage. MSG mole transformation percentage (MMTP) was calculated according to the formula as follows:

$$\text{MMTP (\%)} = \frac{M/103.12 - 5.13/1000}{W/169.13 + 8.22/1000} \times 100 \quad (2)$$

where:

M – GABA yield in the culture broth after fermentation (g/l)
W – MSG addition in the culture medium (g/l)
103.12 – represented the molecular weight of GABA (g/l)
169.13 – molecular weight of MSG (g/mol)

The molar concentrations of GABA and MSG in the culture broth after autoclaving before inoculation were 5.13 mmol/l and 8.22 mmol/l, respectively.

Statistics. The GABA yield was determined by actual response value. The data reported represented its mean. Statistical significance was evaluated using Student's t-test and $P < 0.05$ was considered as significant. Second-order polynomial regressed equations were established on the basis of the experimental data. Optimum parameters were defined by the Experimental Design-Expert 6.0.10 Software.

RESULTS AND DISCUSSION

Single factor experiments

Effect of temperature on GABA yield. Figure 1 shows the effect of the culture temperature on GABA yield. A significant increase of GABA yield was observed over the culture temperature range (18–34°C). Maximum GABA yield of 4.39 g/l was reached at 34°C but the yield declined when the temperature surpassed 34°C. Obviously, 34°C was the optimum temperature to produce GABA using *L. lactis*. The result was approximately the same as the yield obtained in the research by Qing and Huiyuan (Li *et al*. 2004).

Effect of initial pH on GABA yield. The effect of the initial pH of the medium on GABA yield is shown in Figure 2. GABA yield increased in the pH range from 5.5 to 7.0 and remained constantly at the maximum at pH 7.0–8.0, but it declined when pH increased above 8.0. This suggested that the initial pH of 7.0 to 8.0 was optimal for the GABA synthesis by *L. lactis*.

Effect of MSG adding time on GABA yield. Significant differences in GABA yield were observed between various times of MSG addition as seen in Figure 3. The culture broth contained the highest GABA content when MSG was added at the beginning of fermentation (0 h). However, GABA yield declined quickly when MSG was added at a

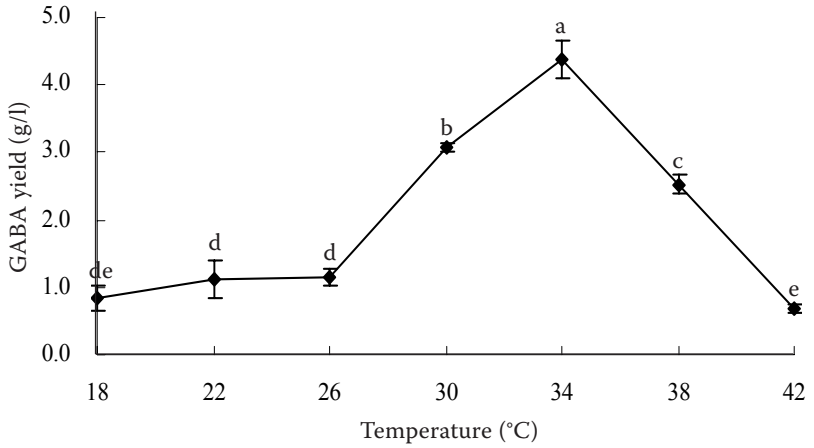

Figure 1. GABA yields at various temperatures by *L. lactis*. The vertical bars represent the standard deviation (S.D., n = 3). Values marked by the same letter are not significantly different (P > 0.05). Culture conditions were: initial pH 7.0, MSG addition 10 g/l, MSG adding time 0 hours

later time. Consequently, MSG was added at 0 h in the following exploration.

Effect of MSG addition on GABA yield and MSG mole transformation percentage. MSG can dissociate into Na^+ and L-glutamic acid (L-Glu) which is the substrate for GABA synthesis. GABA yield and MSG mole transformation percentage at different MSG additions are presented in Figures 4 and 5, respectively. A remarkable increase of GABA yield was observed when MSG addition increased from 0 g/l to 20 g/l (Figure 4). GABA yield was up to 6.83 g/l at 20 g/l MSG addition. Further additions resulted in a decrease until 80 g/l. Increased MSG addition at lower levels (0–20 g/l) can increase GABA yield. But too great MSG addition (20–8.0 g/l) may increase the osmotic pressure of the cells and disturb the bacteria metabolism, resulting in a decrease of GABA yield. This suggests that the MSG addition of 20 g/l was the optimum dose for GABA yield. A significant increase of MSG mole transformation percentage was observed with the MSG addition range of 0 g/l to 5 g/l, and it reached maximum with additions around 5 g/l to 10 g/l MSG (Figure 5). And then the MSG transformation was declined when the additions were above 10 g/l. This suggested that the MSG additions of 5 g/l to 10 g/l were the optimum ones to obtain maximum MSG mole transformation percentage.

Analysis of Response Surface Methodology (RSM)

Based on the single factor experiments, the temperature, initial pH, and MSG addition were considered as variables in the box-behnken response surface design. GABA yield and MSG mole transformation percentage were the responses. To optimise the fermentative parameters of GABA enrichment by *L. lactis*, the culture temperature of 34°C, initial pH of 7.5, and MSG addition of 20 g/l were chosen as the central conditions of the RSM.

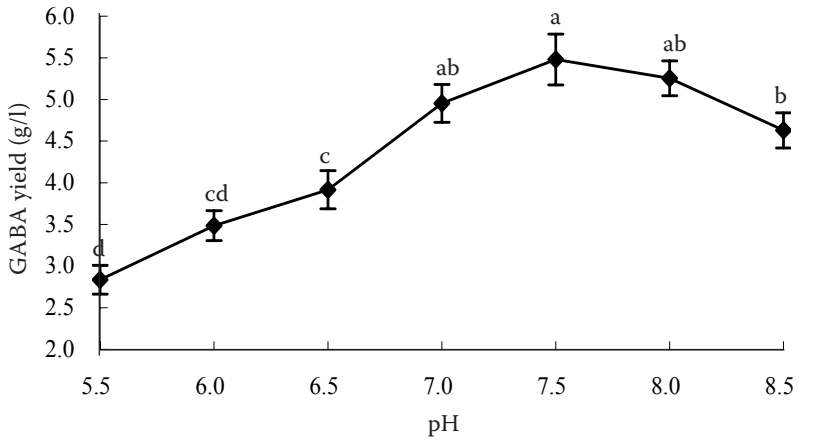

Figure 2. GABA yields by *L. lactis* at various initial pHs of medium. The vertical bars represent the standard deviation (S.D., n = 3). Values marked by the same letter are not significantly different (P > 0.05). Culture conditions were: temperature 34°C, MSG addition 10 g/l, MSG adding time 0 hours

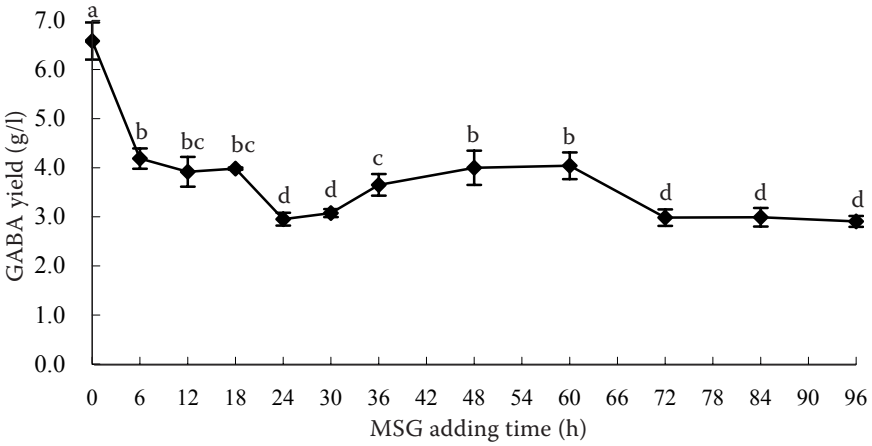

Figure 3. GABA yields at various MSG adding time by *L. lactis*. The vertical bars represent the standard deviation (S.D., $n = 3$). Values marked by the same letter are not significantly different ($P < 0.05$). Culture conditions were: temperature 34°C, MSG addition 10 g/l, initial pH 7.5

Analysis of the effects of temperature, medium initial pH, and MSG addition on GABA yield

Stepwise regression analysis was performed on the experimental data. The coefficients of the model were evaluated for significance with the student *t*-test. The final predictive equation obtained is given in Eq. 3. The cross-product coefficients of X_1X_2 and X_2X_3 were eliminated in the refined equation as their effects were not significant ($P > 0.05$).

$$\text{GABA} = 2.80X_1 + 6.63X_2 + 6.87X_3 - 0.04X_1^2 - 0.47X_2^2 - 0.78X_3^2 - 0.07X_1X_3 - 68.05 \quad (3)$$

According to the analysis of variance (ANOVA) for RSM (Table 3), the determination coefficient (R^2) of the model was 0.99, which indicated that the model had adequately represented the real relationships between the parameters chosen. For any of the terms in the models, a large *F*-value and a small *P*-value indicate a more significant effect on the respective response variables (Li & Fu 2005). Accordingly, all the linear (X_1, X_2, and X_3) coefficients were significant ($P < 0.05$). The variable with the largest effect on GABA yield were the linear term (X_3) and quadric term of MSG addition (X_3^2) ($P < 0.01$), followed by the linear term of temperature (X_1) ($P < 0.01$). The results of ANOVA indicated that the three independent variables all displayed quadratic effects on the response. MSG addition had the most important effect on GABA yield.

In Figure 6, the three-dimensional contour plot and response surface graph are displayed according to Eq. 3. The graph determined the contribution of the temperature and MSG addition on GABA yield at a fixed initial pH of 7.5 and further illuminated their interactions in this model, which made the results of statistical and mathematical analysis evident. Figure 6 indicates that the temperature exerted a slight effect on GABA yield and MSG addition exerted a great one. GABA yield increased as the temperature (30–31°C) increased at a fixed MSG addition and reached the peak at nearly 31°C. Then, it decreased when the temperature

Table 3. Analysis of variance (ANOVA) for the regression Eq. (3)

Source	SS	DF	MS	*F* value	Prob > *F*
Model	91.63	7	13.09	91.08	< 0.0001
X_1	5.05	1	5.05	35.14	0.0002
X_2	1.01	1	1.01	7.02	0.0265
X_3	48.28	1	48.28	335.91	< 0.0001
X_1^2	1.90	1	1.90	13.19	0.0055
X_2^2	0.91	1	0.91	6.35	0.0328
X_3^2	31.50	1	31.5	219.21	< 0.0001
X_1X_3	1.17	1	1.17	8.14	0.0190
Residual	1.29	9	0.14		
Corelation total	92.92	16			

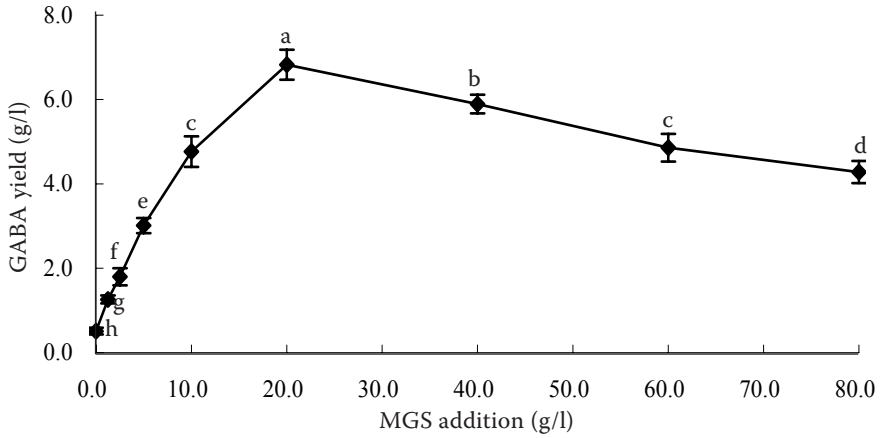

Figure 4. GABA yields at various MSG additions by *L. lactis*. The vertical bars represent the standard deviation (S.D., $n = 3$). Values marked by the same letter are not significantly different ($P > 0.05$). Culture conditions were: temperature 34°C, initial pH 7.5, MSG adding time 0 hours

increased continuously. Similarly, the increase in MSG addition (0–30 g/l) at a fixed temperature led to an increase in GABA yield which reached maximum at 30 g/l MSG addition. When the MSG additions were above 3 %, GABA yield decreased. At lower temperatures (30–34°C), higher GABA yields could be reached at lower MSG additions while at higher culture temperatures (34–38°C) higher MSG amounts had to be added into the medium to obtain the same GABA yield. The optimum reaction temperatures for GABA yield were between 30–32°C and the optimum MSG additions were between 25–35 g/l.

Analysis of the effects of temperature, initial pH, and MSG addition on MSG mole transformation percentage

A polynomial model describing the correlation between the MSG mole transformation percentage and three variables of conditions (culture temperature, initial pH, and MSG addition) is obtained as follows:

$$MMTP = 16.84X_1 + 62.53X_2 - 28.26X_3 - 0.27X_1^2 - 4.42X_2^2 + 2.52X_3^2 - 381.67 \quad (4)$$

By applying ANOVA (Table 4) for the mode (Eq. 4), the established model was found to be very significant ($P < 0.01$) and could be used to predict MSG mole transformation percentage in Table 2. MSG mole transformation percentages predicted by the above regression Eq. 4 were close to the observed ones ($R^2 = 0.989$) on the basis of the *F*-test. The ANOVA results (Table 4) indicated that the three independent variables all displayed quadratic effects on the response. The variable with the greatest effect on MSG mole transformation percentage was the linear term of MSG addition (X_3) ($P < 0.01$), followed by the quadric term of MSG addition (X_3^2) ($P < 0.01$) and then by the linear term of the culture temperature (X_2) ($P < 0.01$) (Table 4). The linear term of the initial pH

Table 4. Analysis of variance (ANOVA) for the regression Eq. (4)

Source	SS	DF	MS	*F* value	Prob > *F*
Model	10089.08	6	1681.51	132.49	< 0.0001
X_1	230.16	1	230.16	20.32	0.0011
X_2	110.93	1	110.93	10.76	0.0101
X_3	9282.03	1	9282.03	728.71	< 0.0001
X_1^2	77.02	1	77.02	6.45	0.0250
X_2^2	82.15	1	82.15	7.48	0.0215
X_3^2	331.31	1	331.31	23.06	0.0003
Residual	110.96	10	11.10		
Corelation total	10200.04	16			

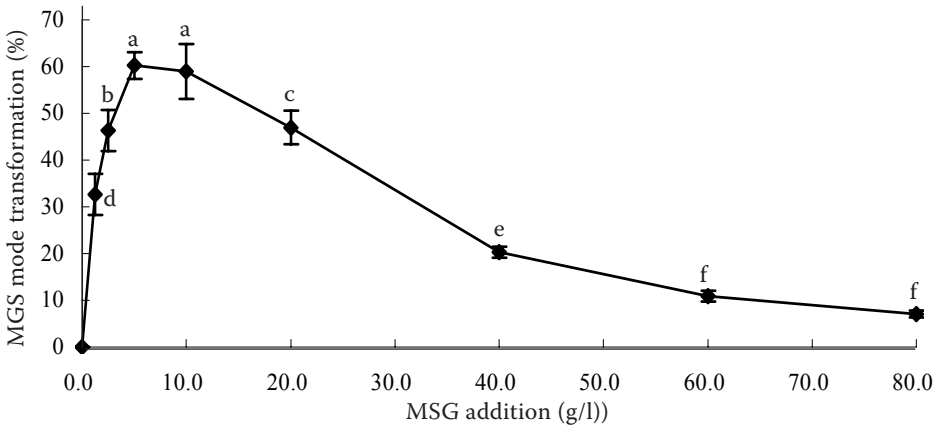

Figure 5. MSG mole transformation percentage at various MSG additions by *L. lactis*. The vertical bars represent the standard deviation (S.D., n = 3). Values marked by the same letter are not significantly different ($P > 0.05$). Culture conditions were temperature 34°C, initial pH 7.5, MSG adding time 0 hour

(X_2) ($P < 0.05$), the quadric term of temperature (X_1^2) ($P < 0.05$), and the quadric term of the initial pH (X_2^2) ($P < 0.05$) also had significant effects on MSG mole transformation percentage. The cross-product coefficients of X_1X_2, X_1X_3, and X_2X_3 were eliminated in the refined equation as their effects were not significant ($P > 0.05$).

Optimisation of parameters for GABA enrichment

To find optimum fermentative parameters for GABA enrichment by *L. lactis*, maximum GABA yield and maximum MSG mole transformation percentage should be obtained at the same time as a lower MSG mole transformation percentage would result in the waste of material and rise of the production cost. According to the results of RSM test, when GABA yield and MSG mole transformation percentage reached their maximums simultaneously, the values of the fermentative parameters were: temperature 31.8°C, pH 7.1, and MSG addition 15 g/l. Under these conditions, the predicted GABA yield and MSG mole transformation percentage were 7.2 g/l and 68.4%, respectively.

CONCLUSIONS

MSG addition strongly affected GABA yield and MSG mole transformation percentage. Optimum

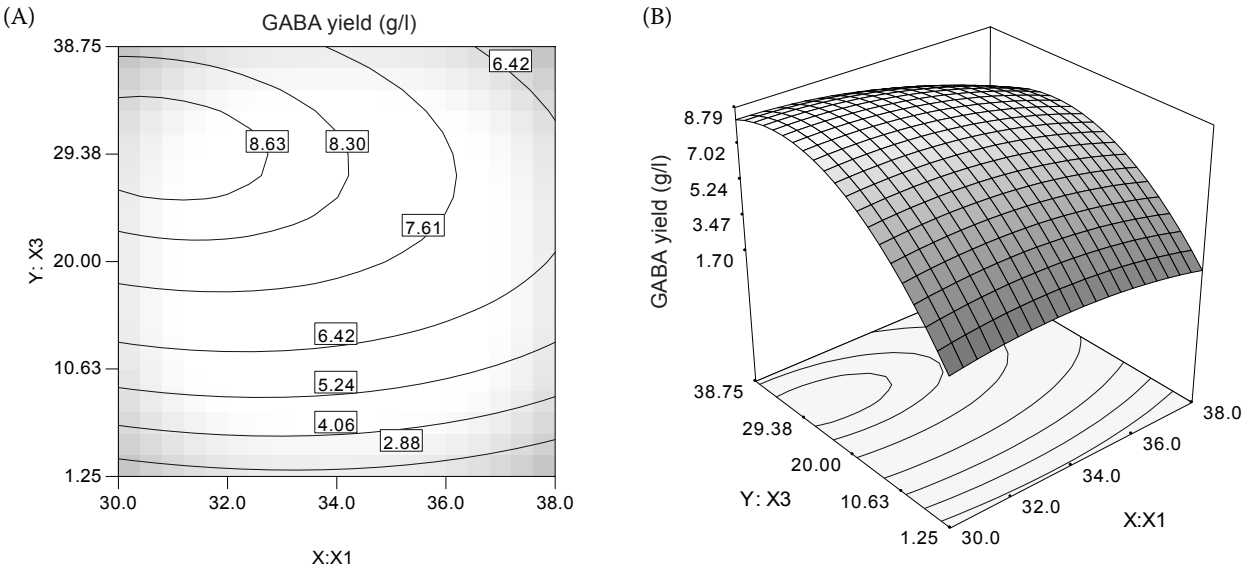

Figure 6. Contour plots (A) and response surface (B) for the effects on GABA yield of culture temperature (°C) (X_1) and MSG addition (g/l) (X_3) at initial pH 7.5

conditions obtained by RSM for GABA enrichment by *L. lactis* included the following parameters: temperature 31.8°C, pH 7.1, and MSG addition 15 g/l. Under these conditions, the model predicted a maximum response of 7.2 g/l GABA yield and 68.4% mole transformation percentage of MSG. On the other hand, two polynomial regression equations were found to be very significant ($P < 0.0001$) and could be used to predict GABA yield and MSG mole transformation percentage. All this illuminated that the method and results were feasible and efficacious. The experiment results are of commercial value for producing GABA functional foods and pharmaceuticals through *L. lactis* fermentation.

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Corresponding author:

Dr. Zhenxin Gu, Nanjing Agricultural University, College of Food Science & Technology, 210095 Nanjing, Jiangsu Province, People's Republic of China
tel./fax: + 86 25 843 962 93, e-mail: xchunyan@gmail.com