Optimized cultural conditions of functional yogurt for γ -aminobutyric acid augmentation using response surface methodology

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ABSTRACT

Yogurt, a functional dairy food product, is an effective medium for delivering beneficial functional ingredients. One ingredient, γ -aminobutyric acid (GABA), has growing appeal in the development of functional foods for its potential in reducing the risk of diabetes, hypertension, and stress as a bioactive agent. However, the concentration of GABA in existing food products is remarkably low. We developed a functional yogurt rich in GABA using *Streptococcus thermophilus* fmb5. The GABA yield of yogurt was enhanced by optimization of culture conditions using single factor and response surface methods. The results showed that culture temperature, monosodium glutamate concentration, and culture time are the 3 main factors that affect GABA yield. The optimal culture conditions were determined as follows: 38.8°C for culture temperature, 20 g/L of monosodium glutamate, and 120 h of culture time. Under the above optimal conditions, the actual yield of GABA production was maximized at 9.66 g/L, which was 1.2 times or higher than that of from any single factor treatment. The GABA concentration, viable bacteria number, and water-holding capacity of GABA-rich yogurt were stable throughout the whole storage time. The results show that producing yogurt with *Streptococcus thermophilus* fmb5 and the optimized culture conditions will achieve high GABA concentrations that maximize health benefits to consumers.

Key words: γ -aminobutyric acid, functional yogurt, optimizing conditions, response surface methodology

INTRODUCTION

Yogurt, with its ability to support live cultures, is a common and preferred dairy product for introduc-

ing probiotics and beneficial functional ingredients into the digestive system (Lecomte et al., 2016). Yogurt's beneficial ingredients are derived from either the initial strains during the production of yogurt (*Streptococcus thermophilus* and *Lactobacillus delbrueckii*), or from additional strains that offer a wide array of probiotic function (Linares et al., 2016). With high probiotic function and bioactive ingredients, yogurt can serve as a highly effective functional food. *Streptococcus thermophilus* is a safe lactic acid bacteria (LAB) that is one of the most widely applied LAB in the dairy industry (van Baarlen et al., 2013). The feasibility of *S. thermophilus* is due to its rapid conversion of lactose into lactic acid (Iyer et al., 2010). This results in a swift reduction in pH, causing a coagulation of milk proteins. Therefore, *S. thermophilus* can be easily used to prepare yogurt and exhibits beneficial health effects based on previous studies (Uriot et al., 2017).

γ -Aminobutyric acid (GABA) is a major inhibitory neurotransmitter in the central nervous system of mammals. γ -Aminobutyric acid widely exists in plants, animals, and microorganisms. γ -Aminobutyric acid and GABA-rich foods can alleviate hypertension and depression in humans (Abdou et al., 2006; Ko et al., 2013; Ng'ong'ola-Manani et al., 2014; Wu and Shah, 2017). Previous studies also showed that GABA can improve the quality of sleep and could serve as a treatment for epilepsy (Cossart et al., 2005; Yamatsu et al., 2016). In addition, GABA can be used as an antidiabetic agent to control blood glucose levels (Ligon et al., 2007; Soltani et al., 2011; Tian et al., 2011; Chen et al., 2016). These health benefits have attracted the interest of the medical, chemical, and food industries to develop more functional supplements of GABA. Many foods rich in GABA, such as rice germ (Karladee and Suriyong, 2012), functional beverages (Kang et al., 2011), tea (Liao et al., 2017), and dairy products (Hayakawa et al., 2004; Wu and Shah, 2017), have successfully been developed, but the GABA yield in existing products is still quite low. Therefore, it is of great value to develop a new functional food with high levels of GABA.

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Chemical synthesis, plant enrichment, and microbial fermentation methods are the 3 main methods of producing GABA. The microbial fermentation method of producing GABA has many advantages such as low production cost, mild reaction conditions, and efficient production of GABA compared with the chemical synthesis and plant enrichment methods. Moreover, the microbial fermentation method of producing GABA has been widely studied in recent years, and numerous studies have shown that LAB is quite efficient at producing a high yield of GABA (Li et al., 2008; Yang et al., 2008; Sun et al., 2009; Lee et al., 2010; Kim et al., 2014). Lactic acid bacteria, a type of probiotic, is being used to produce GABA as demand increases. Previous studies showed that the traditional mutagenesis and LAB-strain screening methods were not quite efficient for GABA production. Villegas et al. (2016) implied that culture conditions such as time, temperature, and monosodium glutamate (MSG) concentration greatly influence GABA production, as their results indicated that GABA production by *Lactobacillus brevis* in quinoa sourdoughs was enhanced through optimization of all 3 factors where maximum GABA concentration was achieved with 270 mM MSG at 30°C for 48 h incubation time. Seo et al. (2013) found that *Lactobacillus brevis* 877G as a GABA producer co-fermented with skim milk and 29.57 mM MSG, resulting in a significant increase of GABA production (22.51 mM). Nejati et al. (2013) showed that *Lactobacillus plantarum* PU11 produced 77.4 mg/kg of GABA after 120 h of milk fermentation. In addition, Shan et al. (2015) optimized the culture conditions of yogurt and increased the yield of GABA up to 314.56 mg/100 g by using response surface methodology (RSM). In this study, we carried out both a single factor experiment and RSM to enhance GABA production using *Streptococcus thermophilus* fmb5 as a starter culture, and it is of great value to develop a new functional yogurt with high levels of GABA.

MATERIALS AND METHODS

Bacterial Strains and Culture Method

The strain *Streptococcus salivarius* ssp. *thermophilus* fmb5 with high-yielding GABA production was screened by nitrosoguanidine mutagenesis from the *Streptococcus salivarius* ssp. *thermophilus* STX2 (Chen et al., 2016). *Lactobacillus delbrueckii bulgaricus* was stored in the Enzyme Engineering Laboratory of Nanjing Agricultural University. *Streptococcus thermophilus* fmb5 was activated twice at 37°C with a skim milk (Fonterra Ltd., Auckland City, New Zealand) pre-culture. Then 2% (vol/vol) of activated strain was inoculated into de Man, Rogosa, and Sharpe broth (Thermo Fisher Scien-

tific, Waltham, MA) for seed liquid preparation. Skim milk (10%) and sodium glutamate (1.2%) were mixed and sterilized in an autoclave for 5 min at 115°C, then cooled to 42°C. The sterile mixture was then inoculated with the 2% seed liquid and fermented at 37°C for 72 h. After fermentation, the fermented milk was cooled and stored at 4°C.

Optimized Fermentation Condition of Functional Yogurt

The mixture of skim milk (10% and sodium glutamate (1.2%) is considered as the basic component of the culture medium. The single factor of medium ingredients includes skim milk (8, 10, 12, and 14%), pyridoxal phosphate (0, 200, 400, 600, 800, and 1,000 $\mu\text{mol/L}$), and sodium glutamate (6, 8, 10, and 12 g/L) individually. The fermentation conditions include 3 factors, namely, initial pH of medium (6.5, 7.0, and 7.5), fermentation temperature (35, 40, and 45°C), and proportions of bacterium (*S. salivarius* ssp. *thermophilus* fmb5: *L. delbrueckii bulgaricus*) for different fermentation times, respectively.

Response Surface Methodology

Response surface methodology is a systematic analytical method for investigating the effects of an independent variable (fermentation temperature, MSG, and time) and is an optimization tool widely used in production processing. In this work, RSM was used to optimize the fermentation medium and condition for GABA-rich yogurt. Based on the results of the single factor experiment above, the effect of each factor on GABA yield was assessed, the 3 most effective factors (X_1 : fermentation temperature; X_2 : MSG, and X_3 : fermentation time) on GABA yield was then selected, and 3 different coded levels of the 3 factors (−1, 0, and +1) were determined with the Box-Behnken design as shown in Table 1. The optimal fermentation conditions were calculated by testing the empirical second-order polynomial regression model. The ANOVA, regression coefficients, model fitting to the quadratic model of the response factor were determined with Design Expert software (version 8.0.5, Stat-Ease Inc., Minneapolis, MN). The significance of all terms was analyzed statistically at $P < 0.05$.

GABA Analysis and Chromatographic Conditions

The milk solid was separated from yogurt through centrifugation at 12,000 rpm for 10 min. The supernatant parts were collected and derived by dansyl chloride for 1 h (Huang, 2006).

The GABA from all yogurt samples was identified and quantified using an Agilent 1290 Infinity HPLC system coupled to a diode array detector from Agilent Technologies (Palo Alto, CA) by comparison of retention time with the authentic commercial GABA standard. Briefly, GABA was separated using a reversed phase C18 Luna column (150 × 4.6 mm; particle size 5 μm) from Phenomenex (Torrance, CA) at ambient temperature (between 22 and 27°C). The separation was achieved using a binary gradient with 2 solvents. Solvent A was methanol and solvent B was the mixture of tetrahydrofuran, methanol, and sodium acetate (volume ratio is 5:75:420). The linear gradient consisted of 80% (B) to 50% (B) for 25 min, followed by 0% (B) for 9 min, then gradually increased from 0% (B) to 80% (B) for 5 min and held at 80% (B) for 7 min. The flow rate was set to 1 mL/min. The detection wavelength was 254 nm, and 20 μL was the injection volume.

Sensory Evaluation of Yogurt

The sensory analysis was carried out by a trained panel of 20 members that are familiar with basic sensory evaluation techniques. The sensory evaluation considered 4 main areas of focus: color, aroma, odor and texture. Aroma was scored on a 1–5 scale, where 1 = no aroma and 5 = extremely strong aroma. Color, taste, and texture quality was evaluated with a 9-point hedonic scale, where 9 = like extremely, 5 = neither like nor dislike, and 1 = dislike extremely (Meilgaard et al., 1991).

Variation of Yogurt Indicators During Storage

The produced yogurt with GABA was stored in 4°C for 20 d to test the storage stability.

Method of Counting Viable Bacteria. The yogurt was diluted by 0.85% sterilized physiological saline, and 3 appropriate gradients were made on the de Man, Rogosa, and Sharpe plate count agar. The culture plates were placed in a constant temperature incubator at 37°C for 48 h. Generally, 30 to 300 cfu Petri dishes were chosen to count.

Measurement of Water-Holding Capacity. After transferring 10 mL of sample in a centrifuge tube,

the weight of the sample (W_0) was measured. The sample was placed in a centrifuge at 3,000 rpm for 20 min, and then was standing for 10 min. The supernatant was removed, and then the weight of residue (W) was measured. The calculation formula of WHC is as follows:

$$\text{WHC} = (W/W_0) \times 100. \quad [1]$$

RESULTS

Effect of Skim Milk, Sodium Glutamate, Pyridoxal Phosphate, and Initial pH on GABA Yield

The effect of skim milk content on GABA yield is shown in Figure 1A. During the fermentation of 0 to 72 h, the concentration of GABA increases rapidly. As the content of skim milk increases, the production rate of GABA increases as well. During the fermentation stage of 72 to 120 h, the concentration of GABA increases at a slow rate. The 8% of skim milk at 120 h fermentation obtained the highest GABA yield (7.74 g/L) among all the skim concentrations. In general, there are no significant differences of GABA yields among all different concentrations of skim milk during fermentation.

The effect of MSG concentration on GABA yield is presented in Figure 1B. During the first 24 h of fermentation, no significant differences in GABA yield were observed among all different concentrations of MSG. At 48 h of fermentation, 10 and 12 g/L of MSG obtained higher GABA production than 6 and 8 g/L of MSG. Furthermore, at 84 h of fermentation, 12 g/L of MSG had the highest concentration of GABA (8.04 g/L) among all the treatments. These results indicated that GABA yield increased when MSG concentration was also increased.

Pyridoxal phosphate (PLP) is the coenzyme of transaminase and decarboxylase in AA metabolism, which can promote the decarboxylation of glutamic acid and enhance the generation of GABA. The effect of PLP addition to culture medium on GABA yield is shown in Figure 1C. The 800 μmol/L of PLP at 120 h of fermentation obtained the greatest GABA yield (7.74 g/L) among all the PLP concentrations. Our results indicated that PLP has a certain promoting effect

Table 1. Codes and levels of factors chosen for Box-Behnken experiments

Variable	Symbol		Coded level		
	Actual	Coded	−1	0	1
Culture temperature (°C)	X_1	x_1	35	40	45
Sodium glutamate (g/L)	X_2	x_2	8	14	20
Culture time (h)	X_3	x_3	72	96	120

on GABA yield, but the concentration of GABA with different concentrations of PLP had no significant difference.

The effect of pH of culture medium on GABA yield is presented in Figure 1D. The pH of culture medium has no significant influence on the formation of GABA. The pH of culture medium is naturally 6.5; therefore, there is no need to adjust the initial pH of culture medium.

Effect of Fermentation Temperature and Proportions of Bacterium on GABA Yield

The effect of fermentation temperature on GABA yield is shown in Table 2. The curd time of yogurt shortens as the fermentation temperature rises. At 30°C, yogurt took the longest time to produce curds in comparison with the other temperatures, and the texture of yogurt is rarefied at this temperature. Although the curd time of yogurt is shortened at 43°C compared with 37°C, the texture of yogurt is significantly better at 37°C. In addition, the concentration of GABA is lower at 30°C than at 37 and 43°C. This could be explained as the growth of the bacteria being slower,

Table 2. Effect of fermentation temperature on GABA yield

Temperature (°C)	Curd time (h)	GABA ¹ yield (g/L)
30	14	0.241 ± 0.024
37	10	2.079 ± 0.127
43	5	2.124 ± 0.161

¹GABA = γ-aminobutyric acid. Mean value ± SD (n = 3).

so glutamic acid decarboxylase (GAD) activity, which transfers MSG to GABA by decarboxylation, was less at 30°C than at 37 and 43°C. However, no difference in the GABA yield at 37 and 43°C was observed, indicating that higher temperatures might promote GABA production from MSG.

The effect of proportions of bacterium on GABA yield is shown in Table 3. The results indicated that the strain *Lactobacillus delbrueckii bulgaricus* was not able to increase GABA productivity. The concentration of GABA (2.75 g/L) was higher in fermented yogurt when using *Streptococcus thermophilus* fmb5 alone compared with using both strains.

Figure 1. Effect of different single factors on γ-aminobutyric acid (GABA) yield. (A) Effect of different concentrations of skim milk on the yield of GABA. (B) Effect of different concentrations of monosodium glutamate (MSG) on the yield of GABA. (C) Effect of different concentrations of pyridoxal phosphate on the yield of GABA. (D) Effect of different initial pH on the yield of GABA. Error bars represent SD.

Table 3. Effect of different proportions of *Streptococcus thermophilus* and *Lactobacillus delbrueckii bulgaricus* on the yield of γ-aminobutyric acid (GABA)

<i>S. thermophilus</i> : <i>L. delbrueckii</i>	GABA yield ¹ (g/L)	
	24 h	32 h
Proportion 1:0	1.644 ± 0.124	2.746 ± 0.171
Proportion 1:1	1.443 ± 0.092	2.252 ± 0.105
Proportion 1:2	1.180 ± 0.058	1.893 ± 0.107
Proportion 2:1	1.585 ± 0.093	2.764 ± 0.125
Proportion 3:2	1.576 ± 0.140	2.496 ± 0.156

¹Mean value ± SD (n = 3).

Response Surface Methodology to Enhance GABA Yield

Box-Behnken design was used to optimize the fermentation conditions (Table 4). Using the designed experimental data, the polynomial model for GABA yield (Y) was regressed by considering only the significant terms, as represented below:

$$Y = 8.49 - 1.71x_1 + 0.68x_2 + 0.65x_3 + 0.33x_1x_2 - 0.30x_1x_3 + 0.71x_2x_3 - 3.45x_1^2 - 0.54x_2^2 - 0.68x_3^2, [2]$$

where Y is the predicted GABA yield, x_1 is the culture temperature, x_2 is the concentration of MSG, and x_3 is the culture time.

The results of variance analysis are shown in Table 5. The *P*-values of the model indicated that the obtained experimental data fitted well with the model ($P < 0.0001$). The values of R^2 and adjusted R^2 were 0.9849 and 0.9654, respectively. Only 3.57% of the variation

cannot be predicted by the model. These values indicated that the accuracy and general ability of the polynomial model were exceptional.

One-degree term x_1 , x_2 , and x_3 were extremely significant ($P < 0.01$), suggesting that culture temperature, concentration of MSG, and culture time have a significant linear effect on GABA yield. Interaction item x_2x_3 was significant ($P < 0.05$), but interaction items x_1x_2 and x_1x_3 were not significant. Results indicated that the interaction effect between culture temperature and culture time is significant.

The shape of the contour map can reflect the interaction effect among various factors. An oval shape indicates the interaction between factors is stronger, and a circular shape represents weak interactions between factors. Response surface and contour plots for the effect of cross-interaction are presented in Figure 2. The result indicated that interaction effect of culture temperature and culture time is significant.

Based on the regression equation, the optimal culture conditions are the culture temperature at 38.8°C, the concentration of MSG at 20 g/L, and the culture time at 120 h. Under these culture conditions, the predicted GABA yield is 9.50 g/L. To verify the reliability of this model, the experiments were repeated 3 times under optimal conditions, producing an average yield of 9.66 g/L for GABA. This indicated that experimental and predicted values of GABA yield corresponded with each other. Results suggested that the model is reliable, and the GABA yield of the optimal model was 1.2 times higher than the highest GABA yield (8.04 g/L) obtained from 12 g/L of MSG at 96 h of fermentation among all the single factor treatments.

Table 4. Experiment design and results of response surface methodology

No.	Temperature (X_1)	MSG ¹ (X_2)	Culture time (X_3)	GABA ² yield (g/L)	
				Actual value	Predicted value
1	−1	−1	0	6.32	5.87
2	1	−1	0	2.05	1.79
3	−1	1	0	6.30	6.56
4	1	1	0	3.35	3.80
5	−1	0	−1	5.04	5.12
6	1	0	−1	2.40	2.30
7	−1	0	1	6.91	7.01
8	1	0	1	3.08	3.00
9	0	−1	−1	6.28	6.65
10	0	1	−1	6.94	6.59
11	0	−1	1	6.18	6.53
12	0	1	1	9.66	9.30
13	0	0	0	8.66	8.49
14	0	0	0	8.26	8.49
15	0	0	0	8.84	8.49
16	0	0	0	8.42	8.49
17	0	0	0	8.24	8.49

¹MSG = monosodium glutamate.

²GABA = γ-aminobutyric acid.

Table 5. Analysis of variance for response surface quadratic model

Source ¹	Sum of squares	df	Mean square	F-value	P-value ($P > F$)
Model	88.83	9	9.87	50.56	<0.0001
x ₁	23.40	1	23.40	119.86	<0.0001
x ₂	3.67	1	3.67	18.78	0.0034
x ₃	3.34	1	3.34	17.12	0.0044
x ₁ x ₂	0.43	1	0.43	2.22	0.1794
x ₁ x ₃	0.35	1	0.35	1.80	0.2211
x ₂ x ₃	1.99	1	1.99	10.20	0.0152
x ₁ ²	50.00	1	50.00	256.16	<0.0001
x ₂ ²	1.21	1	1.21	6.22	0.0414
x ₃ ²	1.96	1	1.96	10.06	0.0157
Residual	1.37	7	0.20		
Lack of fit	1.10	3	0.37	5.44	0.0677
Pure error	0.27	4	0.067		
Cor total	90.19	16			
			R = 0.9924	R ² = 0.9849	R ² _{adj} = 0.9654

¹x₁ = the culture temperature, x₂ = the concentration of MSG, and x₃ = the culture time. Cor total = total of model and residual values such as sum of squares by ANOVA for response surface quadratic model. R²_{adj} = percentage of variation explained by only the independent variables that actually affect the dependent variable. Adjusted R² also judges goodness of model in multiple variables. The formula of R² and R²_{adj} is as follows: R²_{adj} = 1 - [(1 - R²)(n - 1)/(n - k - 1)].

Sensory Evaluation of Yogurt

The overall evaluation of yogurt was conducted by a trained panel. The aroma as well as the color, taste, and texture of GABA-rich yogurt were examined. The GABA-rich yogurt had a milky and full-bodied aroma (3.0). The yogurt's color (7.0) and texture (7.5) were exceptional and smooth with no whey separation. The long fermentation time may cause the yogurt to acquire a sour taste (5.0), but it was still acceptable.

GABA Yield, Viable Bacteria Number, and Water-Holding Capacity of Yogurt During Storage

Variation of GABA yield, viable bacteria number, and water-holding capacity of yogurt during storage are shown in Figure 3. The GABA yield is stabilized during storage, which indicates that GABA is stable in yogurt. The viable bacteria number of yogurt declined slowly during 20 d of storage. The number of *Streptococcus thermophilus* fmb5 was 8.4×10^7 cfu/mL at d 20, which was perfect for the regulation of viable bacteria number of yogurt ($>10^6$ cfu/mL). The water-holding capacity of yogurt was quite stable during the whole storage, maintained at about 50%.

DISCUSSION

As mentioned above, functional dairy products are significant to the human diet and show health-beneficial effects (Shiby and Mishra, 2013; Yerlikaya, 2014). This work studied GABA-rich yogurt, which diversified dairy products ranges by tailoring specific functional-

ities. In this work, the strain *Streptococcus salivarius* ssp. *thermophilus* fmb5 with high-yielding GABA production was improved by nitrosoguanidine mutagenesis from the strain *Streptococcus salivarius* ssp. *thermophilus* STX2. The use of mutagenic treatment is a safe way to increase the production of any targeted product, and the strain of *Streptococcus thermophilus* fmb5 is generally recognized as safe.

To facilitate the production of GABA, the culture medium must contain the necessary precursors of GABA for the uses of LAB. Glutamate decarboxylase (GAD) is an enzyme that catalyzes the decarboxylation of glutamate to GABA. Komatsuzaki et al. (2005) reported that addition of PLP could increase the GAD activity of *Lactobacillus paracasei* NFR17415 in traditional fermented foods, whereas Shan et al. (2015) also indicated that PLP could increase the GAD activity in yogurt. In this work, PLP has a certain promoting effect on GABA yield, which is consistent with previous works.

Culture of LAB requires a rich nutrient source that skim milk and yeast extract can entirely provide. The mixture of skim milk, yeast extract, and MSG provide a suitable environment for *Streptococcus thermophilus* fmb5. The artificial addition of MSG can significantly increase the GABA yield of the yogurt in our study.

Many studies indicated that GABA-rich foods could reduce blood pressure in spontaneously hypertensive rats (Aoki et al., 2003; Hayakawa et al., 2004), and Inoue et al. (2003) showed that consumption of only 10 mg of GABA daily for 12 wk was effective for people with mild hypertension. In our study, the yield of GABA reached 9.66 g/L, which means that the intake of moderate GABA-rich yogurt could potentially

decrease blood pressure. Thus, consuming a certain amount of yogurt can satisfy the optimal GABA needs of consumers.

CONCLUSIONS

In this study, culture temperature, MSG concentration, and culture time were the 3 main factors that

affected GABA yield based on the single factor methodology and RSM. The optimal culture conditions include culture temperature at 38.8°C, MSG concentration at 20 g/L, with a culture time of 120 h. With these optimal culture conditions, the predicted GABA yield was 9.50 g/L. As a verification, the actual production of GABA showed a consistent yield of GABA production (9.66 g/L) compared with the predicted value. Our

Figure 2. Response surface and contour plots for the effect of cross-interaction between (A) culture temperature and monosodium glutamate (MSG) concentration, (B) culture temperature and culture time, and (C) culture time and MSG concentration on the yield of γ -aminobutyric acid (GABA).

Figure 3. (A) Variation of γ -aminobutyric acid (GABA) yield and viable bacteria number of yogurt during storage. (B) Variation of water-holding capacity of yogurt during storage. Error bars represent SD.

results suggested that the model is reliable, and the GABA yield of using RSM was 1.2 times higher than that of any single factor treatment. In addition, the GABA concentration, the viable bacteria number, and the water-holding capacity of GABA-rich yogurt were stable during the 20-d storage period at 4°C, indicating that the quality of this GABA-rich yogurt was consistent during storage time. Therefore, yogurt produced by *Streptococcus thermophilus* fmb5 with its optimal conditions can maximize GABA yield and stabilize during long storage periods, thus making this functional dairy product effective for consumers in achieving the health benefits of GABA.

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