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Optimization of γ-aminobutyric acid production by *Lactiplantibacillus plantarum* isolated from Vietnamese fermented pork (*nem chua*) using central composite design

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Abstract

Lactic acid bacteria (LAB) capable of biosynthesizing gamma-aminobutyric acid (GABA) have attracted increasing interest due to their status as safe microorganisms in food preservation and processing. In this study, GABA content was quantified using thin-layer chromatography (TLC), and optimal fermentation conditions, including initial concentrations of monosodium glutamate (MSG), Tween 80, and pH, were modeled using response surface methodology (RSM). The results indicated that *Lactiplantibacillus plantarum* can produce GABA at a concentration of 1.533 mg/mL after 72 h of fermentation at 37ºC in de Man, Rogosa, and Sharpe (MRS) broth supplemented with 1% MSG at pH 5.0. RSM analysis using central composite design (CCD) demonstrated that the maximum GABA production of 1.911 mg/mL was achieved in an optimized MRS medium with an initial pH of 4.97, supplemented with 59.72 g/L of MSG and 2.05 g/L of Tween 80.

Keywords: γ-aminobutyric acid, Central Composite Design, Fermented pork, *Lactiplantibacillus plantarum*, *Nem chua*

1. Introduction

Gamma-aminobutyric acid (GABA) is an amino acid that is not involved in protein synthesis. It has the ability to block or inhibit certain brain signals and reduce nervous system activity, functioning as an inhibitory neurotransmitter [2]. GABA exhibits a calming effect when it binds to the brain protein known as the GABA receptor. Specifically, at the benzodiazepine binding site of $GABA_A$ receptors, central-type benzodiazepine (BZ) receptors interact with certain $GABA_A$ receptor subtypes, enhancing physiological GABA responses. The presence of GABA at the $GABA_A$/BZ receptor may promote clinical effects of benzodiazepines (BZDs), including anxiolytic, antiepileptic, muscle relaxant, and hypnotic effects [3]. Consequently, GABA is utilized as an adjunct to help lower blood pressure, combat obesity, improve sleep, and reduce stress, anxiety, and depression [4]. In recent years, GABA has gained recognition as a food and dietary supplement due to these properties. Typical GABA-containing foods include those fermented by lactic acid bacteria (LAB), such as tempeh, kimchi, and cheese [5].

Several LAB strains capable of producing GABA have been isolated from fermented foods, and efforts have been made to create GABA-rich foods that are safe for human consumption [4]. Various species of the genus Lactobacillus, along with a few species from the genera *Enterococcus*, *Leuconostoc*, *Pediococcus*, *Propionibacterium*, and *Weissella*, have been shown to produce GABA [4]. *Lactiplantibacillus plantarum* (formerly known as *Lactobacillus plantarum*) is a probiotic commonly used as a starter culture, playing a crucial

role in producing various fermentation products in the commercial field [6]. The mechanism of glutamate-GABA metabolism in lactic acid bacteria is present in most GABA-producing lactic acid isolates recovered from acid-based fermented foods, such as Korean kimchi, Chinese paocai, and Vietnamese fermented pork. This mechanism contributes to bacterial resilience in acidic environments by deprotonating H+ ions through the decarboxylation of glutamate to GABA [7]. Lactic fermentation products, including yogurt, cheese, meat, and pickled vegetables, have been produced using LAB, a type of beneficial bacteria that humans have long utilized in many areas of food production and preservation.[8]. The direction of research on lactic acid bacteria employed in the manufacture of goods and functional foods containing healthy compounds of safe biological origin is currently a topic of significant global concern [9, 10].

Nem chua, a traditional Vietnamese dish, is prepared through the fermentation of ground pork, boiled sliced pork rind, and roasted rice powder [11]. It has been reported that the most dominant species of LAB in *Nem chua* include *Lpb. plantarum*, *Lpb. pentosus*, *Lpb. brevis*, *Lpb. paracasei*, *Lpb. farciminis*, *Leuconostoc citreum*, and *Pediococcus pentosaceus* [12].

The production of GABA by LAB is primarily catalyzed by the enzyme glutamate decarboxylase (GAD), which converts L-glutamic acid, a compound present in monosodium glutamate (MSG), into GABA [4]. The expression of the GAD gene, and consequently GABA synthesis, is influenced by various factors determined by the natural environment of each LAB isolate [13]. Among these, pH value is a key environmental factor affecting GABA production, bacterial growth, and GAD activity. Some studies have shown that acidic conditions (optimal pH 3.5-5.5) enhance GAD activity, while near-neutral pH (around 7.0) leads to a significant decrease in its activity. Additionally, while Tween 80 can interact with metal ions to stimulate GAD activity [15], MSG serves as the primary precursor for GABA production [4]. However, excessive MSG levels may suppress cell growth and diminish yields [16].

In light of these issues, this study employed response surface methodology (RSM) based on central composite design (CCD) to assess GABA production by *Lpb. plantarum* CP1.2 isolated from *nem chua* (Vietnamese fermented pork) in culture media. The research aims to evaluate and optimize the culture conditions of *Lpb. plantarum* CP1.2 to achieve maximum GABA production.

2. Materials and methods

2.1 GABA-producing Lactiplantibacillus plantarum

In a previous study, *Lpb. plantarum* CP1.2, an isolate from Vietnamese fermented pork rolls, was identified as having the highest capacity for producing GABA and was preserved at −20 °C in the Laboratory of Industrial Microbiology, Institute of Food and Biotechnology, Can Tho University (Can Tho City, Viet Nam). The isolate was reactivated by cultivating 20% (v/v) bacterial biomass in Man Rogosa and Sharpe (MRS) broth and incubating at 37 °C for 24-48 h to reach a cell concentration of 10^9 cells/mL [17].

2.2 Effects of culture temperature and fermentation time on GABA production

The effect of culture temperature (30 ºC and 37 ºC) and incubation time (24, 48, and 72 h) on GABA yield was determined using fixed fermentation parameters: an initial MSG concentration of 1% (w/v), an initial pH of 5.0, and bacterial isolates at 1% (v/v) with a density of 10^9 cells/mL. Selected *Lpb. plantarum* strains were first proliferated in liquid MRS medium for 48 h at 37 °C. Then, 1 mL of the pre-culture at a cell density of 10^9 cells/mL was inoculated into 99 mL of MRS medium supplemented with 1% MSG in a 250-mL flask. The flask was gently shaken to ensure uniform distribution of the bacteria in the culture medium, followed by static fermentation at 30 °C and 37 °C. GABA concentration in the medium was assessed by thin-layer chromatography (TLC) at 24, 48, and 72 h at pH 5.0 [18].

2.3 Optimization of fermentation conditions influencing GABA production

This experiment evaluated three independent variables of initial pH, MSG, and Tween 80 concentration to determine optimal conditions for GABA production. Three The variables with the coded and actual values are shown in Table 1. The experiment was designed according to the central composite design (CCD), comprising 20 treatments, three replicates. Initially, 7.9 mL of liquid MRS medium was prepared, followed by the addition of the designated volume of Tween 80. These media were sterilized and supplemented with sterilized MSG corresponding to each treatment in an aseptic environment. After 48 h of fermentation, 100 µL of a bacterial suspension (10^9 cells/mL) was inoculated. The samples were then cultured statically under controlled temperature and time conditions. TLC was used to determine the GABA concentration in the culture medium [18].

Table 1 Levels and codes of the CCD experimental design.

Variable factor	Code level				
	-α	- 1	0	+1	-α
A- Initial pH	3.3	4.0	5.0	6.0	6.6
B- MSG (g/L)	16.3	30	50	70	83.6
C- Tween 80 (g/L)	0.3	1.0	2.0	3.0	3.6

Note: MSG - monosodium glutamate; α is star or axial point for orthogonal CCD in the case of three independent variables.

2.4 Analytical methods

2.4.1 Determination of GABA by thin-layer chromatography

The production of GABA by bacterial isolates was first identified using the TLC method [18]. The bacterial culture medium was centrifuged at 10,000 rpm at 4 ºC for 15 min to remove cells. Then, two microliters of bacterial supernatants were injected into silica gel 60 F_{254} TLC panels (Merck KGaA, Darmstadt, Germany). The TLC panels were dried at 90 ºC for 30 min and left at room temperature for 3 minutes. The TLC-activating solvent was prepared by mixing n-butanol, acetic acid, and water in a 5:3:2 (v/v) ratio and supplemented with 1.2% (w/v) ninhydrin (Xilong, China). The TLC panels were placed in a container and 10 mL of TLC-activated solvent was poured for analysis until the solvent line reached 0.5 cm from the top of the TLC panels. The panels were then dried at 70 ºC for 80 min to check for spots corresponding to analyzed GABA standard (Merck KGaA, Darmstadt, Germany).

2.4.2 Quantification of GABA by spectrophotometry method

Quantification of GABA concentration followed the protocol established by Li et al. (2009) [19]. Standard GABA solutions with concentrations ranging from 0.05 to 1.5 mg/mL were prepared using commercial GABA. Then, 0.5 mL of standard GABA solutions were mixed with 0.5 mL of 1.2% (w/v) ninhydrin solution. The mixtures were heated at 70 ºC in a water bath for 5 minutes. Subsequently, 0.25 mL of the mixture was injected into tubes containing 4.75 mL of 0.6% (w/v) $CuSO_4.5H_2O$ prepared in 75% (v/v) ethanol. The reaction mixtures were incubated in a rotational shaking incubator at 200 rpm for 15 minutes. The absorbance at a wavelength of 512 nm was measured to establish the standard correlation between absorbance and GABA concentration. The standard correlation was determined as $y = 1.5607x - 0.3061$ ($R^2 = 0.9625$), where x is GABA concentration (mg/mL) and y represents the absorbance at 512 nm. The GABA contents of the samples were determined using the same protocol based on the established correlation.

2.5 Statistical analysis

All assays were performed in triplicate to enable analysis of variance (ANOVA), and the results presented are the mean of three replicates. Duncan's multiple range test was also used, when appropriate, to elaborate on statistical differences between data. Differences were considered statistically significant at $p<0.05$. Data were analyzed using ANOVA using the Statgraphics Centurion XV software (Statpoint Technologies Inc., USA). The CCD model and RSM were constructed using Design-Expert 11 software (Stat-Ease, Inc., USA).

3. Results and discussion

3.1 Effects of temperature and fermentation time on GABA production by Lactiplantibacillus plantarum

The results of TLC visually indicated the presence of GABA spots in each treatment. These spots were extracted, and an optical density (OD) measurement was performed at a wavelength of 512 nm. The OD values achieved were then applied to the established linear equation and adjusted to the total volume used for standard curve construction to determine the final GABA concentration in each treatment, as provided in Table 2.

Figure 1 reveals the mean GABA yields of *Lpb. plantarum* in MRS media supplemented with a fixed MSG concentration of 1% and an initial pH of 5.0, demonstrating the effect of temperatures (30 °C and 37 °C) and times (24, 48, 72 h) on GABA production.

Table 2 GABA production calculated from TLC results.

Factor		OD_{512nm} value			GABA content (mg/mL)		
Temperature (°C)	Time (h)	1st	2nd	3rd	1st	2nd	3rd
30	24	0.088	0.099	0.125	1.264	1.297	1.380
	48	0.107	0.062	0.124	1.322	1.181	1.380
	72	0.181	0.146	0.133	1.564	1.447	1.405
37	24	0.138	0.159	0.216	1.422	1.489	1.672
	48	0.133	0.137	0.112	1.405	1.422	1.339
	72	0.218	0.140	0.159	1.680	1.430	1.489

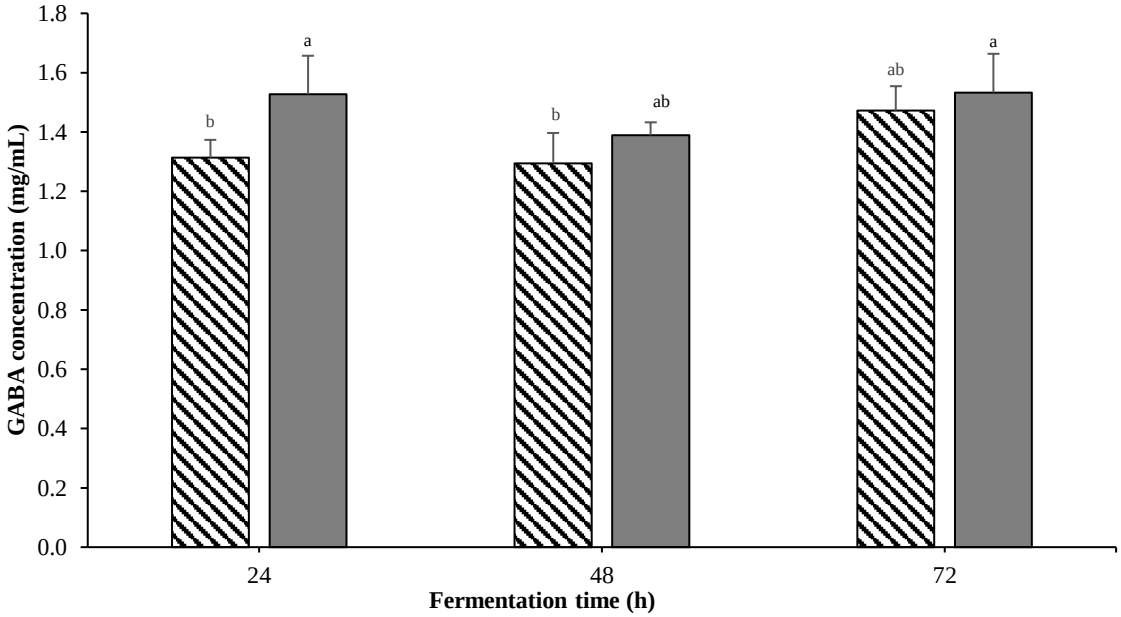

Figure 1 GABA production by *Lactiplantibacillus plantarum* after a 72-h fermentation. The isolate was cultivated in MRS medium supplemented with 1% (w/v) monosodium glutamate (MSG) at pH 5.0 and incubated at 30 ºC (cross bars) and 37 ºC (gray bars) Triplicate experiments; means ± error bars. Columns sharing a superscript letter are not significantly different ($p > 0.05$).

Overall, GABA yield by *Lpb. plantarum* after 48 h was the lowest compared to the figures from 24 h or 72 h. GABA production decreased slightly at 48 h relative to 24 h at both 30 °C and 37 °C, although the decrease was not statistically significant ($p>0.05$). As expected, GABA concentration increased to higher levels of 1.472 mg/mL (30 °C) and 1.533 mg/mL (37 °C) after 72 h. The extended fermentation likely supported physiological adaptation and the synthesis of components that favor GABA production. Consistent with these findings, a study on GABA production in functional grape-must beverages reported a high GABA content of 4.83 mM produced by *L. plantarum* at an optimal fermentation time of 72 h [20].

Temperature was a key determinant of total GABA yield. With a fixed initial inoculum of 10^9 CFU/mL, GABA production in MRS broth at 37 °C exceeded that at 30 °C, suggesting better growth and GABA biosynthesis at 37 °C. The highest concentration at 37 °C after 72 h was 1.533 mg/mL, not significantly different from 1.472 mg/mL at 30 °C after 72 h. At 37 °C, the 24- and 48-h values were 1.528 and 1.389 mg/mL, respectively. These results indicate that 37 °C and 72 h were the most suitable conditions for this isolate. The activity of glutamate decarboxylase (GAD), which catalyzes α-decarboxylation of L-glutamic acid to GABA, is promoted at

temperatures at or above 30 °C [4, 21]. For example, L. brevis exhibited an optimal GABA production temperature of 37 °C [22], and *L. plantarum* DSM19463 produced the highest GABA at 30-35 °C. Temperature also influences the thermodynamic equilibrium of enzyme activity, with bacteria requiring a specific range to maximize GABA production [23]. Accordingly, 37 °C and 72 h were selected for subsequent experiments.

3.2 Optimal conditions for GABA production by Lactiplantibacillus plantarum using central composite design

3.2.1 Response surface methodology analysis

RSM was used to model the effects of initial pH, MSG concentration, and Tween 80 concentration on GABA synthesis by *Lpb. plantarum*. Fixed conditions at the design center were initial pH 5.0, MSG 50 g/L, and Tween 80 at 2 g/L.

Table 3 GABA production in treatments constructed by the Central Composite Design matrix.

Run	Experimental factor			GABA content (mg/mL)	
	A- pH	B- MSG (g/L)	C- Tween 80 (g/L)	Predicted	Observed
1	5.0	16.3	2.0	1.416	1.383 ± 0.141
2	6.0	30.0	1.0	1.635	1.677 ± 0.202
3	6.0	70.0	1.0	1.623	1.720 ± 0.077
4	6.0	30.0	3.0	1.556	1.668 ± 0.193
5	5.0	50.0	2.0	1.852	1.794 ± 0.099
6	5.0	83.6	2.0	1.706	1.629 ± 0.272
7	5.0	50.0	2.0	1.852	1.943 ± 0.189
8	4.0	30.0	1.0	1.402	1.386 ± 0.081
9	5.0	50.0	2.0	1.852	1.839 ± 0.045
10	5.0	50.0	2.0	1.852	1.833 ± 0.074
11	5.0	50.0	2.0	1.852	1.913 ± 0.051
12	5.0	50.0	2.0	1.852	1.807 ± 0.023
13	6.0	70.0	3.0	1.636	1.730 ± 0.071
14	4.0	70.0	1.0	1.667	1.633 ± 0.042
15	4.0	30.0	3.0	1.408	1.389 ± 0.039
16	6.6	50.0	2.0	1.696	1.528 ± 0.052
17	5.0	50.0	0.3	1.584	1.568 ± 0.041
18	3.3	50.0	2.0	1.608	1.666 ± 0.307
19	5.0	50.0	3.6	1.600	1.506 ± 0.048
20	4.0	70.0	3.0	1.765	1.801 ± 0.042

Note: MSG - monosodium glutamate.

Observed GABA concentrations ranged from 1.383 ± 0.141 to 1.943 ± 0.189 mg/mL; predicted values ranged from 1.402 to 1.852 mg/mL. Variation among triplicates was small. The highest observed GABA content (1.943 ± 0.189 mg/mL) compares favorably with the findings of Cai et al. for *L. plantarum* FRT7 (1.2 mg/mL at pH 7, 3% MSG, 48 h in MRS) and exceeds yields reported by Tajabadi et al. for *L. plantarum* Taj-Apis362 isolated from honey (7.15 mM with 497.97 mM L-glutamate at 36 °C, initial pH 5.31, 60 h) [24, 25]. Collectively, prior studies support the conclusion that culture-condition optimization enhances GABA production by LAB, in line with the present results.

3.2.2 Optimal conditions for GABA production by Lactiplantibacillus plantarum using CCD

The CCD design and the experimental data are presented in Table 3. ANOVA and quadratic polynomial regression yielded the following model (Equation 1) describing the effects of the three factors on GABA production.

$$[\text{GABA, mg/mL}] = 1.85 + 0.0259A + 0.0862B + 0.005C - 0.0693AB - 0.0213AC + 0.023BC - 0.0706A^2 - 0.1028B^2 - 0.0918C^2 \quad (1)$$

Where: A is the initial pH value, B is MSG concentration, and C is Tween 80 concentration.

The ANOVA results (Table 4) indicate that the model is statistically significant (F = 4.92, p = 0.0102), demonstrating a robust relationship between the factors and GABA production. The coefficient of determination was high (R^2 = 0.8157), indicating that 81.57% of the total variation in GABA yield is explained by the model. Lack of fit was not significant (p = 0.0583), supporting model adequacy.

Table 4 ANOVA for quadratic model for central composite design.

Source	Sum of squares	df	Mean square	F-value	p-value	Note
Model	0.4475	9	0.0497	4.92	0.0102	significant
A- pH	0.0092	1	0.0092	0.9069	0.3634	
B- MSG	0.1016	1	0.1016	10.04	0.0100	
C- Tween 80	0.0003	1	0.0003	0.0332	0.8590	
AB	0.0384	1	0.0384	3.79	0.0801	
AC	0.0036	1	0.0036	0.3572	0.5633	
BC	0.0042	1	0.0042	0.4185	0.5323	
A^2	0.0718	1	0.0718	7.10	0.0237	
B^2	0.1522	1	0.1522	15.04	0.0031	
C^2	0.1214	1	0.1214	12.01	0.0061	
Residual	0.1011	10	0.0101			
Lack of fit	0.0833	5	0.0167	4.66	0.0583	not significant
Pure error	0.0179	5	0.0036			
Cor. total	0.5487	19				

As shown in Figure 2, most data points lie within the 95% confidence bands, and the optimal region is near the design center. The optimum level of each variable and their interactions for maximum GABA production are illustrated by plotting the 3D response surface in Figure 3, with Tween 80 fixed at 2 g/L.

A combination of ANOVA (Table 4) and 3D plots (Figure 3) revealed a significant effect of MSG concentration on GABA production. Although initial pH and Tween 80 were not individually significant (p > 0.05), both contributed to model performance. As MSG increased to ~50 g/L, GABA reached a maximum; at ~70 g/L, yield decreased slightly. Similar MSG-dependent trends have been reported, e.g., LAB from indigenous fermented buffalo milk showed increased GABA up to 500 mM MSG and a decrease beyond 550 mM [26]. In general, MSG supplementation effects are strain-dependent and must be empirically determined [27]. Although pH and Tween 80 were not significant main effects, Figures 2 and 3 indicate higher GABA at pH 5.0 and ~2 g/L Tween 80, consistent with studies showing that pH 5.0-6.0 supports GABA production by L. plantarum [28]. Acidic pH maintains GAD activity, whereas near-neutral pH leads to loss of activity [4].

Although the effects of pH and Tween 80 contents were not significant, Figures 2 and 3 indicate that higher GABA contents were achieved at a pH of 5.0 and Tween 80 concentration of approximately 2 g/L. Higher GABA production at a pH value of 5.0 was similar to the results of a study on GABA production using *L. plantarum*, which indicated that a pH of 5.0 to 6.0 is suitable for GABA production [28]. Acidic pH levels are necessary to maintain the GABA production activity of the glutamate decarboxylase enzyme responsible for converting glutamate to GABA, while activity is lost at neutral pH [4]. Based on these values, pH levels and Tween 80 contents were designed in the model verification test discussed in the next section of this study.

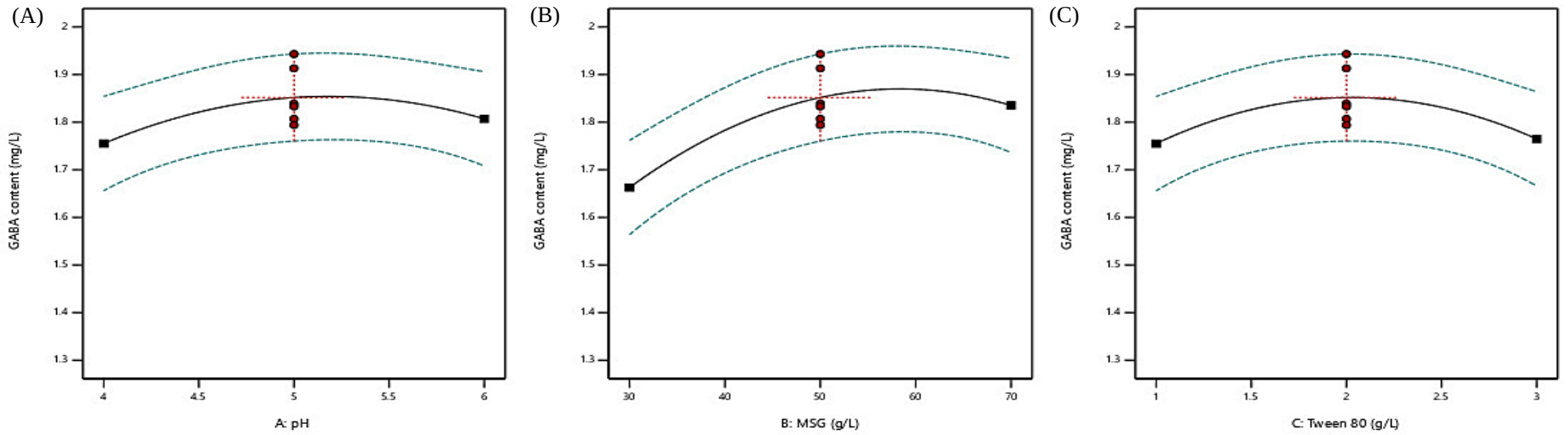

Figure 2 Effects of pH (A), monosodium glutamate (MSG) concentration (B), and Tween 80 concentration (C) on GABA synthesis by *Lactiplantibacillus plantarum.*

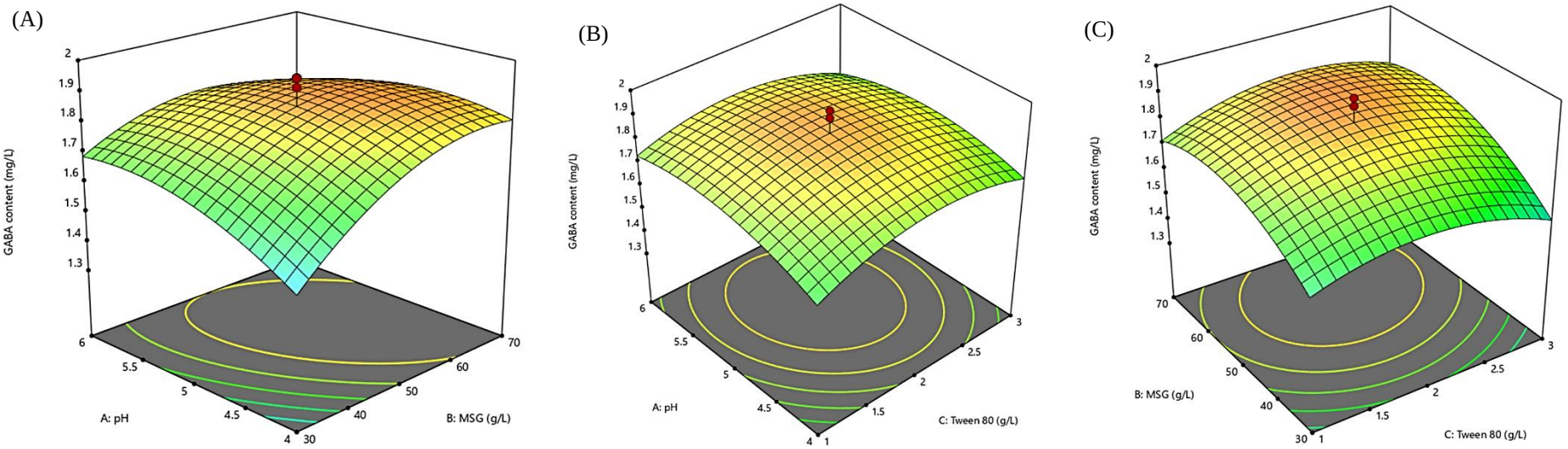

Figure 3 3D response surface plots showing the effect of pH, MSG concentration, and Tween 80 concentration on GABA synthesis by *Lpb. plantarum*. Graphs indicate the interactions of (A) pH with monosodium glutamate (MSG) contents, (B) pH with Tween 80 contents, and (C) MSG and Tween 80 contents on GABA production.

3.2.3 Optimization model verification

Five solutions predicted to yield the highest GABA content were selected for verification (Table 5). Initial pH ranged from 4.61 to 5.22, MSG from 53.58 to 62.60 g/L, and Tween 80 from 1.60 to 2.22 g/L (predicted by Design-Expert 11 software using the quadratic model). Observed GABA contents among the five runs did not differ significantly, with values from 1.849 to 1.911 mg/mL, closely matching predictions. The highest GABA concentration, 1.911 ± 0.027 mg/mL, was obtained at initial pH 4.97, MSG 59.72 g/L, and Tween 80 2.05 g/L, near the design center, and was close to the predicted 1.870 mg/mL.

Table 5 Results of GABA production by fermentation using the suggested optimal conditions.

Run	Experimental factor			GABA content (mg/mL)	
	A- pH	B- MSG (g/L)	C- Tween 80 (g/L)	Predicted	Actual
1	4.97	59.72	2.05	1.870	1.911 ± 0.027
2	4.61	60.02	2.03	1.862	1.855 ± 0.037
3	4.74	62.60	1.82	1.861	1.888 ± 0.020
4	5.22	53.58	2.22	1.860	1.849 ± 0.029
5	4.81	62.41	1.60	1.850	1.860 ± 0.019

Note: MSG - monosodium glutamate.

4. Conclusions

This study demonstrated the production of GABA using *Lpb. plantarum* in MRS medium supplemented with MSG and Tween 80. The suitable temperature and fermentation time for GABA production were determined to be 37 ºC and 72 h, respectively. The RSM analysis according to the CCD model showed that MSG content in the culture medium significantly affected GABA yield. The highest GABA concentration of 1.911 mg/mL was obtained after fermentation by *Lpb. plantarum* in an optimal MRS medium with an initial pH of 4.97, supplemented with 59.72 g/L MSG and 2.05 g/L Tween 80.

5. Acknowledgements

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6. Conflicts of interest

The authors declare no conflict of interest.

7. Author contributions

Thanh, NN.: Conceptualization, Methodology, Investigation, Formal analysis, Writing – original draft; Duyen, TH.: Investigation, Formal analysis, Writing – original draft; Binh, NNH.: Investigation, Formal analysis; Chau, LM.: Investigation, Formal analysis, Data curation; Viet, LQ.: Resources, Formal analysis, Data curation; Long, BHD.: Formal analysis, Data curation, Visualization; Pilap, W.: Data curation, Visualization; Thanonkeo, S.: Visualization, Supervision; Thanonkeo, P.: Supervision, Writing – review & editing; Phong, HX.: Supervision, Funding acquisition, Project administration, Writing – review & editing.

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