Full Paper

Genome analysis and optimization of γ-aminobutyric acid (GABA) production by lactic acid bacteria from plant materials

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Running title GABA production and genome analysis

The DDBJ accession number for the 16S rRNA gene sequence of strain SL9-6 is LC496807. The GenBank/EMBL/DDBJ accession number of the draft genome of strain SL9-6 is VSFI00000000.
Summary

Gamma-aminobutyric acid (GABA) plays a key role as an inhibitory neurotransmitter in the mammalian sympathetic nervous system and has other health benefits. Molecular characterization, genome analysis, and optimization were investigated to improve GABA production of a selected strain of lactic acid bacteria. Eleven isolates from plant materials were screened for GABA productivity and were identified based on phenotypic and genotypic characteristics. The most potent strain was chosen for genome analysis and GABA production optimization using the response surface methodology (RSM). Each of the two strains was closely related to Lactobacillus plantarum, Lactobacillus brevis, Weissella cibaria, Leuconostoc pseudomesenteroides while each strain was similar to Lactobacillus pentosus, Enterococcus, and Leuconostoc mesenteroides. They produced GABA ranging from 0.036±0.000 to 17.315±0.171 g/L at 72 h-cultivation. Among them, the most potent strain, SL9-6, showed the highest GABA production (17.315 g/L) when cultivated with 10% (v/v) inoculum for 48 h. The draft genome sequence of strain SL9-6 exhibited 96.90% average nucleotide identity value and 74.50% digital DNA-DNA hybridization to Lactobacillus brevis NCTC 13768T. This strain contained a glutamate decarboxylase gene system (gadA, gadB and gadC). Optimal culture conditions were determined as 40.00 g/L glucose, 49.90 g/L monosodium glutamate, pH 5.94, and 31.10°C by RSM, giving maximum GABA production of 32.48 g/L. Results from RSM also indicated that monosodium glutamate concentration, pH, and temperature were significant variables. GABA production significantly improved here could promise further application of strain SL9-6.

Key words: Glutamate decarboxylase gene; Gamma-aminobutyric acid; Lactic acid bacteria; Lactobacillus brevis; Response surface methodology; Silage

Introduction

Gamma-aminobutyric acid (GABA), commonly found in microorganisms and products of plants and animals (Ueno, 2000), plays an important role in the cardiovascular function, immunomodulation, alleviation of psychotic disorders and other health benefits (Wong et al., 2003). GABA is produced by some strains of lactic acid bacteria (LAB) in the genus Lactobacillus (Lb.), which are generally found in fermented foods such as Lb. brevis (Li et al., 2010b), Lb. senmaizukei (Hiraga et al., 2008), Lb. plantarum (Tajabadi et al., 2015), Lb. rhamnosus (Lin, 2013), Lb. paracasei (Komatsuzaki et al., 2008), Lb. namurensis (Ratanaburee et al., 2013), and Lb. buchneri (Zhao et al., 2015). In addition, strains of other LAB genera also produce GABA including Lactococcus (Lc.) lactis from Chinese cabbage kimchi (Lu et al., 2009), Pediococcus (P.) pentosaceus isolated...
GABA is synthesized by glutamate decarboxylase (GAD: EC 4.1.1.15) with pyridoxal 5’ phosphate (the active form of vitamin B6) as a coenzyme that catalyzes the irreversible α-decarboxylation to convert L-glutamate to GABA. GAD is encoded by gadA or gadB genes in bacterial cells. Glutamate is transported into a cell through an antiporter, and then the decarboxylation occurs. Finally, the GABA product is secreted from the cell by the glutamate/GABA antiporter, which is encoded by gadC (Gao et al., 2019). Bacterial GABA fermentation is affected by various factors including substrates, cultivation pH and temperature. Carbon source and monosodium glutamate (MSG) are substrates for cell growth and GABA conversion, and thus they are the major factors of GABA production. Besides, pH had a significant effect on GAD activity and biomass production (Li et al., 2010b). In addition, temperature significantly affects GABA production in Lb. brevis (Tajabadi et al., 2015).

Changing only one-factor-at-a-time (OFAT) is the simplest statistical method to study one key factor; however, this lacks diagnosability on interaction between factors. Response surface methodology (RSM) using central composite design (CCD) is often used for the development of mathematical models to estimate the relationship between response and factors. Advantages of RSM include a reduced number of experiments to evaluate multiple factors and their interactions, thus saving experimental resources and time (Li et al., 2008). RSM is a proven and effective tool for determining the optimal condition using the appropriate polynomial equations. This research focused on the selection, identification, genome analysis, and culture condition optimization of a GABA-producing LAB strain.

Materials and methods

Isolation and screening of GABA-producing LAB

Sixty-five LAB were isolated from five fruit samples collected from Chiang Mai, Chiang Rai, Surin and Maha Sarakham Provinces and seven flower samples collected from Prachinburi Province. Another seven flower samples were collected from Suphan Buri Province and one silage sample was sourced from Nan Province, Thailand. Each sample (0.25 g) was enriched in 5 mL of de Man Rogosa and Sharpe (MRS) broth (Difco, France) and incubated at 30°C for 24 h. The culture solution was streaked on MRS-CaCO₃(0.3%) agar plates and cultivated at 30°C for 24 h. Colonies surrounded by a clear zone were selected for purification on the same medium and preserved in 10% (w/v) skimmed milk solution at -80°C or lyophilized for long-term preservation.
An inoculum of 65 isolates was prepared by culturing in 5 mL of MRS broth at 30°C for 24 h. Each inoculum (10%, v/v) was transferred to the production medium of glucose-yeast extract-peptone broth (Lyu et al., 2018) supplemented with 3% (w/v) MSG and incubated at 30°C for 72 h. The culture solution was centrifuged at 8,000 × g for 5 min, and the supernatant (2 µL) was spotted onto thin layer chromatography (TLC) plates (Aluminum TLC Plates Silica gel 60 F254, Merck, Germany). The plates were developed using the mobile phase including acetic acid: n-butanol: distilled water (5:3:2), and detected by spraying with 0.4% (w/v) ninhydrin dissolved in ethanol, with heating at 100°C until spots were appeared (Villegas et al., 2016).

**Quantitative analysis of GABA by High-Performance Liquid Chromatography (HPLC)**

Derivatization of GABA was performed by mixing 100 µL of supernatant or standard solution with 20 µL of 5 mg/mL O-phthalaldehyde (Kutlan and Molnar-Perl, 2003), 75 µL of 0.1 M borate buffer (pH 9.3) and 5 µL of mercaptopropionic acid, followed by vortexing and standing at room temperature (25°C) for 1 min (Silva et al., 2009). GABA derivative was analyzed by HPLC (Varian Prostar, Granite Quarry, NC, USA) equipped with a 3 µm particle size C18 analytical column (150 mm × 4.6 mm, ID; Hibar-Futigsanle RT) at 25°C and eluted with a mobile phase of 85% solution A [0.05M sodium acetate, tetrahydrofuran, and methanol (50:1:49, v/v/v)] and 15% solution B (methanol) at a flow rate of 1 mL/min. The fluorescence detector model 363 was set at an excitation wavelength of 337 nm and an emission wavelength of 454 nm.

**Identification of isolates**

**Phenotypic characterization**

Morphological and cultural characteristics including Gram reaction, cell morphology and colonial appearance of the selected strains (Table 1) were determined after culturing cells grown on MRS agar at 30°C for 24 h. Physiological and biochemical characterization on growth at different parameters of pH (3, 6, and 9), temperature (15, 30, and 45°C), NaCl concentrations (2, 4, 6, and 8%, w/v), catalase activity, nitrate reduction, gas production, arginine hydrolysis, and acid production from carbohydrates were performed as previously described (Tanasupawat et al., 1998).

**Genotypic characterization**

The 16S rRNA gene sequencing of the selected strains was carried out using the primers 20F (5’-GAGTTTGATCCTGGCTCAG-3’) and 1530R (5’-GTACCTTGTACGACTT-3’) as previously reported (Suriyachadkun et al., 2009). The 16S rRNA gene amplicons were sequenced on a DNA sequencer (Macrogen,
Korea) using the universal primers as described earlier (Lane, 1991). Sequence similarity values between the selected strains and the related reference strains were evaluated on the EzBiocloud server (Yoon et al., 2017). A phylogenetic tree based on the neighbor-joining method (Saitou and Nei, 1987) was constructed using MEGA 7.0 (Kumar et al., 2016). Bootstrap analysis was performed to determine the confidence values of individual branches in the phylogenetic tree with 1,000 replications (Felsenstein, 1985). The identified sequences were deposited in the DDBJ (DNA Data Bank of Japan, Mishima, Japan).

Crude DNA of the selected strains was extracted from the cell pellet using the alkaline-polyethylene glycol-based method (Chomczynski and Rymaszewski, 2006). DNA fingerprinting was performed based on the (GTG)_5-PCR method. PCR amplification, gel electrophoresis, and analysis methods were conducted as previously described (Tolieng et al., 2018). *Lb. plantarum* subsp. *plantarum* NRIC 1067\textsuperscript{T}, *Lb. pentosus* NRIC 1069\textsuperscript{T} and *Lb. brevis* NRIC 1684\textsuperscript{T} were used as reference strains.

**Genome analysis insight to strain SL9-6**

Genomic DNA of strain SL9-6 was extracted and purified by a phenol-chloroform method following the procedure of Tamaoka (1994). Genome sequencing of strain SL9-6 was implemented with an Illumina MiSeq platform (Illumina, Inc., San Diego, US-CA) using 2 × 250 bp paired-end reads. The reads were assembled to contigs using SPAdes 3.12 (Bankevich et al., 2012). Open reading frames were annotated using the RAST server (Aziz et al., 2008) in accordance with the NCBI Prokaryotic Genome Annotation Pipeline and compared in the SEED Viewer (Aziz et al., 2012). Average nucleotide identity by BLAST (ANIb) values between strain SL9-6 and the related type strains were calculated on the JSpeciesWS web-tool (Richter et al., 2016). The draft genome of strain SL9-6 is deposited to GenBank (accession no. VSFI00000000). Nucleotide data of the close type strains were obtained from the GenBank database.

**Optimization of GABA production**

**Effect of cultivation time and inoculum size on cell growth and GABA production**

An inoculum of the highest GABA-producing strain SL9-6 was maintained at a cell number of 8 log CFU/mL. Different inoculum sizes (1, 5, and 10%, v/v) were transferred to the production medium and incubated at 30°C. Samples were withdrawn every 24 h until 120 h. Bacterial cell growth was measured using a spectrophotometer (CLARIOstar Plus, BMG Labtech) at OD600. GABA content was tested using the HPLC method.
Response surface methodology (RSM) with Central composite design (CCD)

RSM based on CCD was applied to investigate the effects of four factors (glucose concentration: \( X_1 \), MSG concentration: \( X_2 \), initial pH: \( X_3 \), and temperature: \( X_4 \)) with five levels of each independent variable (−2, −1, 0, +1, +2) on GABA production by strain SL9-6. To control the error, 24 runs were showed in an axial point with six replications at the center to estimate the pure error (Zhang et al., 2012). All variables were taken to the coded values, and each run of experimental is shown in Table 2. The optimization value of the response was calculated by the following quadratic equation:

\[
Y = \beta_0 + \sum_{i=1}^{4} \beta_iX_i + \sum_{i=1}^{2} \sum_{j=i+1}^{4} \beta_{ij}X_iX_j + \sum_{i=1}^{4} \beta_i^2X_i^2
\]  

(Eq. 1)

where, \( Y \) is the predicted response; \( \beta_0 \), constant; \( \beta_i \), linear coefficients; \( \beta_{ij} \), interaction coefficients; and \( \beta_i^2 \), squared coefficients. The symbols \( X_i \) and \( X_j \) represent the levels of independent variables.

Statistical analysis

All experiments to determine GABA production from the selected LAB strains were performed in triplicate, with statistical analysis implemented using SPSS 15 (IBM, USA). Data were presented as the mean with standard deviation. One-way analysis of variance (ANOVA) was used to compare each factor, followed by Tukey’s and Duncan’s multiple range tests which were pairwise comparisons tested at a \( p \)-value of 0.05. RSM data were processed with CCD for Eq. 1 using the trial version of Design-Expert program version 12, including ANOVA to obtain the interactive effects between the process factors and the responses. Quality of the quadratic model was investigated by the coefficient of determination \( R^2 \) and the lack of fit, and its statistical significance was verified by the \( F \)-test.

Results and discussion

Isolation and screening for GABA-producing LAB

Sixty-five isolates of lactic acid bacteria were isolated from 20 samples of fruits, flowers, and silage in Thailand. All isolates were qualitatively screened for GABA production using the TLC method. Eleven isolated strains, Br1-9, Di1-6, LCH1-4, LCH1-6, LCH2-3, LCH2-4, LPC1-1, LSP4-4, LSP6-3, SL9-1, and SL9-6 showed positive spots at \( R_f = 0.57 \), corresponding to the spot of GABA standard (10 mg/mL) (Fig. S1).
Quantification of GABA using HPLC

GABA production of the 11 strains was quantitatively determined using HPLC. The GABA standard curves showed a linear relationship ($R^2 = 0.9811$) between peak areas and GABA concentrations ranging from 2 to 64 mg/L of GABA (Fig. S2). The 11 strains were divided into five groups based on the capability of GABA production (Table S1-2). Strain SL9-6 exhibited the highest GABA production of 17.3 g/L followed by isolate SL9-1 with GABA production of 17.13 g/L. Other isolates including Br1-9, Di1-6, LCH1-4, LCH1-6, LCH2-3, LCH2-4, LPC1-1, LSP4-4 and LSP6-3 showed GABA production ranging from 36 to 315 mg/L.

Strain SL9-6 could produce a quietly high level of GABA compared with *Lb. brevis* strain IFO-12005 (Yokoyama et al., 2002), HYE1 (Lim et al., 2017), CRL 1942 (Villegas et al., 2016) and GABA100 (Kim et al., 2009) that produced 1.05, 2.21, 26.30 and 26.9, respectively. Different strains of *Lb. brevis* NPS-QW-145, NPS-QW-171, NPS-QW-177, NPS-QW-193, NPS-QW-216, NPS-QW-242, NPS-QW-255, and NPS-QW-267 from kimchi had produced GABA in the range 19.07-25.83 g/L (Wu and Shah, 2017). *Lb. rhamnosus* (Song and Yu, 2018), YS9 (Lin, 2013), *L. fermentum* (Rayavarapu et al., 2019), *Lb. plantarum* Taj-Apis 362 (Tajabadi et al., 2015), and *Lc. lactis* B (Lu et al., 2009) produced 1.13, 19.28, 5.34, 0.74, and 7.2 g/L of GABA, respectively. Thus, strain SL9-6 displayed high potential, and therefore it was selected to determine optimal conditions for GABA production.

Identification of isolates

All selected strains formed milky white, circular, convex and opaque colonies. The 11 strains produced acid from arabinose, fructose, glucose, maltose, ribose, and xylose and grew at 30-37°C, pH 3 and 2% (w/v) NaCl but showed negative results for catalase and nitrate reduction. Their differential characteristics are presented in Table 1.

The 11 strains were divided into five groups based on their phenotypic characteristics and 16S rRNA gene sequences (Fig. 1). Group I consisted of three isolates, Br1-9, Di1-6, and LPC1-1. They grew in 2-6% (w/v) NaCl [only LPC1-1 grew in 8% (w/v)], pH 3-6, and at 15-45°C. Based on the analysis of the 16S rRNA gene sequence, isolates Br1-9 and Di1-6 had 100% sequence similarity to *Lb. plantarum* subsp. *plantarum* ATCC 14917T (Bringel et al., 2005), while isolate LPC1-1 had 99.57% sequence similarity to *Lb. pentosus* DSM 20314T (Zanoni et al., 1987) (Fig. 1).
Group II contained two isolates, SL9-1 and SL9-6, which grew in 2-6% (w/v) NaCl, pH 3-6 and at 15-45°C, and grew weakly in 8% (w/v) NaCl and pH 9. The isolates SL9-1 and SL9-6 were closely related to *Lb. brevis* due to the 16S rRNA gene sequences with 99.75 and 99.57% similarity to ATCC 14869², respectively (Fig. 1).

Group III comprised only one isolate, LSP6-3. It grew in 2-8% (w/v) NaCl, pH 3-6 and at 15-45°C. Based on the 16S rRNA gene sequence, LSP6-3 closely related to *E. lactis* due to 99.79% sequence similarity to BT159² (Morandi et al., 2012) (Fig. 1).

Group IV encompassed two isolates, LCH1-6 and LCH2-4. They grew in 2-8% (w/v) NaCl, at pH 6 and 15-30°C, and weakly grew at pH 3 and 9 and 45°C. These two isolates were closely related to *W. cibaria* with 100% similarity of the 16S rRNA gene sequences to KACC 11862² (Bjorkroth et al., 2002) (Fig. 1).

Group V included three isolates, LCH1-4, LCH2-3, and LSP4-4. They grew in 2-6% (w/v) NaCl, at pH 3-6 (LCH2-3 and LSP4-4 tolerated pH 9), and 15-30°C (LSP4-4 did not grow at 15°C). Based on the 16S rRNA gene sequence, LCH1-4 and LCH2-3 had 99.93 and 99.57% sequence similarity, respectively, to *Leu. pseudomesenteroides* NRIC 1777² (Farrow et al., 1989). LSP4-4 had 99.85% sequence similarity to *Leu. mesenteroides* subsp. *jonggajibkimchii* DRC 1506² (Jeon et al., 2017) (Fig. 1).

The (GTG)₅-PCR analysis was used to detect the intraspecific variability of the strains. The number of detectable amplicons varied from 10 to 20 with different DNA band sizes ranging from 300-10,000 bp. UPGMA dendrogram for DNA fingerprinting using the (GTG)₅ primer clustered the 11 strains and the three reference strains into seven groups (Fig. 2). From the fingerprints and dendrogram, Br1-9 and Di1-6 were identified as *Lb. plantarum* subsp. *plantarum*, and LPC1-1 was identified as *Lb. pentosus*. SL9-1 and SL9-6 were also confirmed as *Lb. brevis*, while the remaining isolates LCH1-6, LCH2-4, LSP4-4 and LSP6-3 exhibited unique profiles.

**Genome analysis insight to strain SL9-6**

The size of the draft genome of SL9-6 was 2.52 Mb with 2,502 coding sequences. Strain SL9-6 exhibited an ANIb value of 96.90% and digital DNA-DNA hybridization value of 74.50% with *Lb. brevis* NCTC 13768T. Over 95% of ANIb threshold clearly indicated that the strain was identified as the same species (Richter et al., 2016). The genomic features of strain SL9-6 are shown in Table S3.
The genome information revealed that strain SL9-6 had two GAD genes, *gadA* (469 aa) and *gadB* (480 aa), and a glutamate/GABA antiporter gene, *gadC* (502 aa) (Fig. 3A). The genes, *gadA*, *gadB* and *gadC* had amino acid sequence similarity with other LAB strains in the range 67.66-99.57, 52.47-100, and 43.33-100%, respectively (Fig. 3A). Furthermore, glutamyl-tRNA synthetase (*gts*, 504 aa) and transcriptional regulator (*gadR*, 197 aa) also presented in the SL9-6 genome (Fig. 3B). A comparison of the GAD-related genes between strain SL9-6 and other LAB species indicated that the *gadB* gene was located next to the *gadC* gene. The whole operon, including *gts* and *gadR* was similar to *Lb. brevis* strains (Li et al., 2013). Both *gadB* and *gadC* were found in *Lb. lactis* but the *gadC* gene was absent in *Lb. plantarum*. Differences in genome information containing GAD-related genes and GABA production between SL9-6 and the other LAB strains are summarized in Table S4.

**Effects of cultivation time and inoculum size on cell growth and GABA production**

The time course of cell growth, GABA production, and MSG utilization are shown in Fig. 4. Inoculum size and cultivation time had a significant effect on GABA production (Table S5). Among the five periods without 0 h of cultivation time, 48 and 72 h provided the highest GABA yield, however, yields of GABA at 96 and 120 h significantly decreased (Fig. 4A, Table S6). For inoculum size, 10% (v/v) gave significantly high average GABA level of 15.34 g/L followed by the inoculums of 5% (v/v) and 1% (v/v) with GABA yields of 14.15 and 13.48 g/L, respectively (Table S7). Cell growth gradually increased after 24 h, and prolonged time from 24 to 96 h resulted in the exponential phase with maximum cell growth found at 96 h. GABA production was maximized in the middle of the log phase (48 h) according to the result of the relationship between cell growth, cultivation time, and GABA production (Fig. 4A). MSG was rapidly consumed after 24 h, slightly decreased from 48 to 72 h, with no significant change from 72 h to 120 h (Fig. 4B), while different inoculum sizes had no important effect on MSG utilization (Tables S11-S13). These results suggested that 48 h of cultivation and 10% inoculum size were optimal, and these parameters were used for further production of GABA by *Lb. brevis* SL9-6. Results also concurred with the maximum GABA production (1005.81 mM = 103.72 g/L) by *Lb. brevis* NCL912 cultivated for 48 h at 10% inoculum (Li et al., 2010b).

*Lb. brevis* AN1-5, AN2-2, AN3-5, AN4-5, ANP7-6, and SB109, *Lb. buchneri* AN1-1 and SB21, and *Ws. hellenica* SB101 and SB105 had been completely utilized glutamic acid within 96 h and produced maximum GABA production from 5 to 8 g/L (Barla et al., 2016), while *Lb. brevis* HYE1 had given maximum GABA of 16.94 mM after 36 h incubation (Lim et al., 2017). *Lb. buchneri* WPZ001 with 10% inoculum size had produced
GABA of 75.5 g/L after 60 h incubation cell growth, and the GABA concentration had not increased regardless extension of incubation time to 72-96 h (Zhao et al., 2015).

**Optimization of key factors for GABA production using RSM with CCD**

Triplicate actual GABA production for each run, along with the predicted values are summarized in Table 2. Maximum GABA production of 29.59 g/L was obtained from the run number 28 consisting of 3.00% glucose and 5.00% MSG with an initial pH of 6.00 at 30.00°C. The predicted value was calculated as 30.29 g/L based on the following quadratic equation (Eq. 2).

\[
Y = 20.56 + 0.52X_1 + 4.19X_2 + 1.81X_3 - 1.24X_4 + 1.48X_1^2 + 0.34X_2^2 - 4.17X_3^2 - 3.69X_4^2 + 0.05X_1X_2 - 0.71X_1X_3 - 0.56X_1X_4 - 1.36X_2X_3 + 1.94X_2X_4 + 3.70X_3X_4
\]  

(Eq. 2)

The positive coefficients of glucose (0.52), MSG (4.19) concentrations, and pH (1.81) indicated that a high level of these factors contributed to GABA production. On the contrary, the negative coefficient of temperature (-1.24) indicated that low temperature encouraged GABA accumulation. Statistical analysis suggested that the optimization condition for GABA production as 4.00% glucose, 4.99% MSG, and pH 5.94 at 31.10°C, produced the maximum predicted value of GABA concentration of 32.67 g/L. To verify the quadratic equation, GABA production by *Lb. brevis* SL9-6 under the optimal conditions was analyzed in triplicate. As a result, the actual value of 32.48±1.57 g/L obtained was almost similar (99.42%) to the predicted value and was improved from 87% for the first screening.

The significance of the regression coefficient was proved using ANOVA (Table 3). The *F*-value of 50.89 with Model Prob > *F* less than 0.0001 implied that the quadratic model was highly significant. In this case, *X_2*, *X_3* and *X_4* were significant factors for GABA production, with *X_2* and *X_3* as the most significant factors with less than 0.0001 as Model Prob > *F*. This implied that the interaction between each factor *X_2X_3*, *X_2X_4*, and *X_3X_4* had significance. The *R*² and adjusted *R*² values were 0.979 and 0.9601, respectively, which indicated that the model sufficiently represented an accurate relationship between the selected factors. The “Lack of Fit *F*-value” of 2.51 and *p*-value of 0.1607 implied that the Lack of Fit was not significantly related to the pure error. There was a 16.07% chance that such a large Lack of Fit *F*-value could occur due to noise. Non-significant lack of fit also indicated that the model fitted. Therefore, this quadratic model suitably predicted GABA production of *Lb. brevis* SL9-6. Optimal levels of each factor and the effects of their interactions on GABA production are illustrated by three-dimensional contour plots (Fig. 5).
The effects of glucose and MSG concentration (Fig. 5A), glucose concentration and pH (Fig. 5B), and glucose concentration and temperature (Fig. 5C) on GABA production were determined when the other factors were fixed at the center point. Results illustrated that increased glucose concentration did not increase GABA production when MSG concentration was at a low level. GABA production gradually increased with an increase of MSG concentration at moderate pH value (4-6) and temperature (20-30°C). This phenomenon was supported by the result from ANOVA, that glucose was not significant, while MSG concentration had strong significant for GABA production (Table 3). Interaction between MSG concentration and pH (Fig. 5D) on GABA production was examined when the other factors were constant at a center point. GABA production was low at low or high pH correlated with MSG concentration. Although GABA production increased in the range of initial pH from 4 to 6, it dramatically decreased when the initial pH was higher than 6. GABA production significantly increased by enhancing MSG concentration from 1 to 5% at pH 6 (Fig. 5D). The effects of MSG concentration and temperature on GABA production (Fig. 5E) explained that a high level of MSG concentration at temperature of 25 to 35°C was suitable for GABA production. MSG is generally regarded as a required substrate for GABA generation, and a high level of MSG concentration is needed to enhance GABA production (Li et al., 2010b), it have been reported that a high level of MSG (6 to 15%) suppressed GABA production (Li et al., 2010b; Tajabadi et al., 2015). The relationship between pH and temperature on GABA production clearly demonstrated the optimal pH and temperature at 5-7 and 25-35°C, respectively (Fig. 5F). These results were in accordance with those of Lb. brevis NCL912 that had not produced GABA at pH 3.0 in spite of the growth at 45°C (Li et al., 2010b). Many strains of LAB producing GABA have been optimized within the range of pH 4 to 6 including pH 5.3 for Lb. plantarum Taj-Apis362 (Tajabadi et al., 2015) and pH 4.74 for Lb. brevis HYE1 (Lim et al., 2017). From the other reports, the optimum pH values for maintaining the activity of GADs were pH 4.0 to 5.0 (Huang et al., 2007; Komatsuzaki et al., 2008). Lc. lactis produced the highest GABA of 7.2 g/L at pH ranging from 7.5 to 8.0, while productivity decreased when pH was higher than 8.0 (Lu et al., 2009). Optimal conditions to produce GABA were different between LAB species and strains. The pH, neutralization and alkalization by proton import associated with GABA export greatly affected cell growth and GAD-related enzyme activity, namely GABA production (Li et al., 2010b). Not only pH, MSG concentration and temperature would also have impacted cell growth and GAD-related enzyme activity depending on LAB species and strains.

Scant research has been conducted using RSM to diagnose optimal conditions for GABA production. Lb. brevis strain HYE1 (Lim et al., 2017) and NCL912 (Li et al., 2010b) had produced maximum GABA production of 2.21 and 35.66 g/L, respectively. It was also reported that Lb. plantarum Taj-Apis362 (Tajabadi et
al., 2015), *Lb. fermentum,* and *Lb. rhamnosus* GG (Song and Yu, 2018) produced GABA of 0.74, 5.34, and 1.13 g/L, respectively at optimized conditions using RSM. However, several studies have addressed optimization of the conditions for GABA production by LAB using OFAT (Kim et al., 2009; Lin, 2013; Villegas et al., 2016) (Table 4).

The RSM test results for GABA production by *Lb. brevis* SL9-6 compared favorably with the other researches. In this study, the potential strain and the significant model for GABA production were found successfully.

**Conclusions**

In this research, the selection, identification, genome analysis, and optimization of culture conditions to improve GABA production of *Lb. brevis* SL9-6 were carried out. Out of 65 isolates, strain SL9-6 was determined as having the most prominent for GABA production. Such phenotypic and genotypic characterization, strain SL9-6 grew at pH 3-6, 2-6% (w/v) NaCl, and at 15-30°C, and was identified as *Lb. brevis* by the 16S rRNA gene sequence. *Lb. brevis* SL9-6 was isolated from silage with high ability for GABA production. *Ws. cibaria* and *Le. pseudomesenteroides* were also identified as GABA-producing strains. The whole-genome of *Lb. brevis* SL9-6 consisted of the genes *gadA, gadB* and *gadC* for GABA production, indicating that it had the capability to produce GABA. To improve GABA production, the effects of cultivation time and inoculum size on cell growth and GABA production were investigated by the OFAT method, while RSM was applied to examine optimal GABA production conditions. Results indicated that MSG concentration, pH, and temperature were significant variables, while glucose concentration was not significant for GABA production from *Lb. brevis* SL9-6. The optimal condition was culture medium containing 4.00% glucose and 4.99% MSG at initial pH 5.94, and 48 h cultivation at 31.10°C with 10% inoculum. At the optimal condition, GABA was produced at 32.48 g/L from SL9-6. GABA production increased 87% from the initial experiment. The high potential of GABA producer *Lb. brevis* SL9-6 obtained in this study will be useful for further applications.

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**Funding** This research was supported by a Grant for International Research Integration: Research Pyramid, Ratchadaphiseksomphot Endowment Fund (GCURP_58_01_33_01), and by Ratchadapiseksomphot Endowment Fund, Chulalongkorn University through a Postdoctoral Fellowship to Dr. Sukanya Phuengjayaem.

**Compliance with ethical standards**

**Conflicts of interest.** The authors declare no conflicts of interest.

**References**


### Table 1 Phenotypic characteristics and GABA production of the isolates

<table>
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<tr>
<th>Characteristics</th>
<th>Group I</th>
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<th>Group III</th>
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<td>0.043±0.000</td>
<td>0.036±0.000</td>
<td>17.131±1.69</td>
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**Source**

- Fruit
- Flower
- Silage

**Province**

- Maha Sarakham
- Prachinburi
- Nan
- Suphanburi
- Chiang Mai
- Chiang Rai

1. *Myristica fragrans* Houtt. (Nutmeg);
2. *Ixora coccinea* L. (Ixora);
3. Corn silage;
4. *Hibiscus rosa-sinensis* L. (Chinese rose);
5. *Prunus domestica* L. (Prunus);
6. *Physalis peruviana* L. (Cape Gooseberry); and
7. *Celosia argentea* L. var. cristata (L.) Kuntze (Cockscomb).

+ Positive reaction; w, weakly positive reaction; - , negative reaction; ^standard deviation of GABA production from triplicate samples.
Table 2 Central composite design with actual and predicted values of GABA production

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<th>Run number</th>
<th>A: Glucose (%)</th>
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<th>D: Temperature (°C)</th>
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Table 3 Analysis of variance (ANOVA) for GABA production

| Source   | Sum of squares | DF  | Mean square | F value | Prob > F |  |
|----------|----------------|-----|-------------|---------|----------||
| Model    | 1814.23        | 14  | 129.59      | 50.89   | < 0.0001 | significant |
| X_1      | 6.43           | 1   | 6.43        | 2.52    | 0.1329   | |
| X_2      | 422.33         | 1   | 422.33      | 165.83  | < 0.0001 | |
| X_3      | 78.93          | 1   | 78.93       | 30.99   | < 0.0001 | |
| X_4      | 36.61          | 1   | 36.61       | 14.38   | 0.0018   | |
| X_1^2    | 60.02          | 1   | 60.02       | 23.57   | 0.0002   | |
| X_2^2    | 3.08           | 1   | 3.08        | 1.21    | 0.2887   | |
| X_3^2    | 475.97         | 1   | 475.97      | 186.90  | < 0.0001 | |
| X_4^2    | 373.70         | 1   | 373.70      | 146.74  | < 0.0001 | |
| X_1X_2   | 0.04           | 1   | 0.04        | 0.02    | 0.8978   | |
| X_1X_3   | 8.08           | 1   | 8.08        | 3.17    | 0.0951   | |
| X_1X_4   | 5.01           | 1   | 5.01        | 1.97    | 0.1812   | |
| X_2X_3   | 29.61          | 1   | 29.61       | 11.63   | 0.0039   | |
| X_2X_4   | 59.92          | 1   | 59.92       | 23.53   | 0.0002   | |
| X_3X_4   | 219.22         | 1   | 219.22      | 86.08   | < 0.0001 | |
| Residual | 38.20          | 15  | 2.55        |         |          | |
| Lack of Fit | 31.86      | 10  | 3.19        | 2.51    | 0.1607   | not significant |
| Pure Error | 6.34         | 5   | 1.27        |         |          | |
Table 4 Comparison of the efficiency and optimization condition for GABA production of LAB strains

<table>
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<tr>
<th>Strain</th>
<th>Source</th>
<th>Factors</th>
<th>Statistical Method</th>
<th>Cultivation time (h)</th>
<th>Optimum condition</th>
<th>GABA production</th>
<th>Reference</th>
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<td><em>Lb. brevis</em> SL9-6</td>
<td>Silage</td>
<td>Glucose 1,2,3,4,5%, MSG 1,2,3,4,5%, pH 4, 5, 6, 7, 8, Temperature 25-25°C</td>
<td>CCD</td>
<td>0-120 h (48 h)</td>
<td>Glucose 4% MSG 4.99% pH 5.94 Temperature 31.1°C</td>
<td>32.48 g/L</td>
<td>This study</td>
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<td><em>Lb. brevis</em> HYE1</td>
<td>Kimchi</td>
<td>Maltose 0.2, 2, 4%, Tryptone 1, 3, 5%, MSG 0.1, 1, 4%, pH 4, 7, 9</td>
<td>BBBD</td>
<td>0-60 h (48 h)</td>
<td>Maltose 2.14% Tryptone 4.01% MSG 2.38% pH 4.74</td>
<td>21.44 mM (2.21 g/L)</td>
<td>(Lim et al., 2017)</td>
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<td><em>Lb. brevis</em> NCL912</td>
<td>Paocai</td>
<td>Glucose 2.5, 5, 7.5%, Soya peptone 1, 2.5, 5%, Tween-80 1, 2, 3 mL/L, MnSO4 4H2O 0.005, 0.010, 0.015 g/L</td>
<td>BBBD</td>
<td>48 h</td>
<td>Glucose 5.525% Soya peptone 3.025% Tween-80 1.38 mL/L, MnSO4 4H2O 0.0061 g/L</td>
<td>345.83 mM (35.66 g/L)</td>
<td>(Li et al., 2010a)</td>
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<tr>
<td><em>Lb. brevis</em> CRL 1942</td>
<td>Real Homillos quinoa</td>
<td>MSG 0-400 mM Temperature 22-37°C</td>
<td>OFAT</td>
<td>0-144 h (48 h)</td>
<td>MSG 270 mM Temperature 30°C</td>
<td>255 mM (26.30 g/L)</td>
<td>(Villegas et al., 2016)</td>
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<td><em>Lb. brevis</em> GABA100</td>
<td>Palm wine</td>
<td>MSG 2% Temperature 25, 30, 37°C Glucose 0.5, 0.75, 1, 1.25, 1.5%, MSG 0.5, 0.75, 1, 1.25, 1.5% Incubation time 24, 36, 48, 60, 72 h</td>
<td>OFAT</td>
<td>0-15 Day (12 days)</td>
<td>pH 5.5 Temperature 30°C</td>
<td>26.9 g/L</td>
<td>(Kim et al., 2009)</td>
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<td><em>Lb. fermentum</em> YS9</td>
<td>Chinese traditional</td>
<td>MSG 10-200 mM Temperature 30-43°C PLP 0-200 µM pH 3-5</td>
<td>OFAT</td>
<td>0-96 h (84 h)</td>
<td>MSG 200mM PLP 200 µM pH 4.5</td>
<td>187 mM (19.28 g/L)</td>
<td>(Lin, 2013)</td>
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<td><em>Lb. plantarum</em> Taj-Apis362</td>
<td>Honey stomach</td>
<td>Glutamate 0-600 mM pH 4-7 Temperature 30-45°C Cultivation time 24-72 h</td>
<td>CCD</td>
<td>24-72 h (60 h)</td>
<td>Glutamate 497.97 mM pH 5.31 Temperature 36°C</td>
<td>7.15 mM (0.74 g/L)</td>
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<td><em>Lc. lactis</em> B</td>
<td>Chinese traditional</td>
<td>MSG 1.25, 2, 3.875% pH 6.5, 7.5, 8.5 Temperature 30-38°C</td>
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<td>0-6 Days</td>
<td>MSG 1.5% pH 7.1 Temperature 31.8°C</td>
<td>7.2 g/L</td>
<td>(Lu et al., 2009)</td>
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<td><em>Lb. namurensis</em> NH2 and <em>P. pentosaceus</em> HNS</td>
<td>Fermented meats</td>
<td>Starter 6, 7, 8 log CFU/g NaCl 2, 2.25, 2.5%, MSG 0, 0.25, 0.5%, MSG 1, 2, 3% Pyridoxine 0.1, 0.2, 0.3%</td>
<td>CCD</td>
<td>0-96 h</td>
<td>Starter 6 log CFU/g NaCl 2.12% MSG 50%</td>
<td>3805 mg/kg</td>
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<td><em>Lb. rhamnosus</em> GG</td>
<td>BCRC</td>
<td>Galactose 1, 1.5, 2%, MSG 1, 2, 3% Pyridoxine 0.1, 0.2, 0.3%</td>
<td>BBBD</td>
<td>0-60 h (36 h)</td>
<td>Galactose 1.44% MSG 2.27% Pyridoxine 0.2%</td>
<td>1.13 g/L</td>
<td>(Song and Yu, 2018)</td>
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*a* Optimal cultivation time is shown in parenthesis; NP: Not provided; BBD: Box-Behnken design; BCRC: Bioresource and Collection Center of the Food Industry Research and Development Institute (Hsin Chu, Taiwan); CCD: Central Composite Design; OFAT: One Factor At a Time.
Figure legends

Fig. 1 Neighbour-joining tree based on the 16S rRNA gene sequences showing relationships among the isolates and related species. Numbers on branches indicate percentage bootstrap values of 1,000 replicates. Only values of more than 50% are indicated. Bar, 0.02 substitutions per nucleotide position.

Fig. 2 (GTG)₅-PCR banding patterns of GABA-producing strains and related type strains. The dendrogram was generated after cluster analysis of the digitized fingerprints and derived from UPGMA linkage of Pearson correlation coefficients.

Fig. 3 Comparison of GAD genes in strain SL9-6 and related strains.

A. Maximum-likelihood tree based on amino acid sequences of GAD genes. Numbers on branches indicate percentage of bootstrap values of 1,000 replicates. Length and GenBank accession numbers of GAD genes from each strain are shown in brackets. B. Comparison of arrangements of GABA-producing genes in \textit{Lb. brevis} SL9-6 and related strains.

Fig. 4 GABA production of strain SL9-6 cultivated at 30°C for 120 h.

A. Effect of cultivation time and inoculum size on cell growth and GABA production. B. Effect of cultivation time and inoculum size on MSG utilization.

Fig. 5 Response surface plots using different variables on GABA production for strain SL9-6.

A. The combined effects of glucose and MSG concentration. B. The combined effects of glucose and pH. C. The combined effects of glucose and temperature. D. The combined effects of MSG concentration and initial pH. E. The combined effects of MSG and temperature. F. The combined effects of initial pH and temperature.
Fig. 1

---

**Group I**
- Br1-9 (LC496794)
- DH-6 (LC496795)
- *Lactobacillus plantarum* subsp. *plantarum* DSM 20171 (EF488099)
- *Lactobacillus plantarum* subsp. *argentoratensis* DR022 (AJ640078)
- LPC1-1 (LC496806)
- *Lactobacillus pentosus* DSM 20314 (AB289240)
- SL1-6 (LC496807)
- SL-9-1 (LC496806)
- *Lactobacillus brevis* ATCC 14869 (EF488097)
- *Lactobacillus hammersii* TMW 1.1226 (A3632219)
- *Lactobacillus yonginensis* THK-V8 (JN128640)

**Group II**
- LSP6-3 (LC496805)
- *Enterococcus lacris* BT159 (GU983697)
- *Enterococcus faecium* DSM 20477 (MF544640)
- *Enterococcus durans* NBRC 100479 (AB681177)

**Group III**
- *Weissella orientae* SG2 (AB690345)
- LCH2-4 (LC496799)
- LCH1-6 (LC496797)
- *Weissella cibaria* LMG 17699 (AJ295989)
- *Weissella confusa* JCM 1093 (AB023241)

**Group IV**
- LSP4-4 (LC496802)
- *Leuconostoc mesenteroides* subsp. *jouggaei* USM 15960 (NR157602)
- LCH1-4 (LC496796)
- LCH2-3 (LC496798)

**Group V**
- LCH1-4 (LC496796)
- *Leuconostoc pseudomesenteroides* NRIC 1777 (X95979)
Fig. 2

Sample name

Br1-9
*Lb. plantarum subsp. plantarum* NRIC 1067ᵀ
D1-6
LCH1-4
LCH2-3
LPC1-1
*Lb. pentosus* NRIC 1069ᵀ
LCH1-6
LCH2-4
SL9-1
*Lb. brevis* NRIC 1084ᵀ
SL9-6
LSP4-4
LSP6-3

(GTG)$_2$-PCR
Fig. 3
Fig. 4
Fig. 5