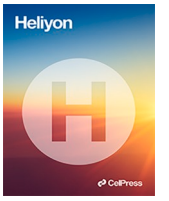
Heliyon 11 (2025) e41884

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon



Research article

Isolation and optimization of *Bacillus thuringiensis* BRB-3 isolated from germinated black upland rice seeds for GABA production

Rungnapha Wannasutta, Sophon Boonlue, Nuntavun Riddech, Wiyada Mongkolthanaruk *

Department of Microbiology, Faculty of Science, Khon Kaen University, 123 Moo 16, Mittraparp Road, Muang Khon Kaen, Khon Kaen, 40002, Thailand

ARTICLE INFO

Keywords:
Black rice seed
Endophytes
GABA
Upland rice

ABSTRACT

Gamma-aminobutyric acid (GABA) is a non-coded amino acid that recently gained increased attention within the functional food industry. This study aimed to isolate endophytic bacteria from sterilized surfaces of germinated black upland rice seeds for GABA production and optimization. A total of 38 isolates were screened for glutamic acid decarboxylase (GAD) activity using a colorimetric method. Among these, thirteen isolates exhibited positive GAD activity. The BRB-3 strain emerged as the most promising, producing the highest GABA levels (4.54 g/L) at an optimal pH of 6.5. However, the BRB-3 strain was sensitive to pH lower than 5.5; so the pH was adjusted to 6.5 through all experiments. Further identification of the BRB-3 strain was performed using 16S rRNA gene sequence, which placed it in *Bacillus cereus* group. Subsequent *rpo*B gene sequence analysis confirmed that the BRB-3 strain was *Bacillus thuringiensis* BRB-3. GABA production was optimized in MRS broth by investigating carbon, nitrogen, monosodium glutamate (MSG), pH and temperature. Interestingly, the concentration of MSG did not impact GABA production of *B. thuringiensis* BRB-3. Under optimal conditions of 3 % soy protein as the nitrogen source, 2 % sucrose, no added MSG at 30 °C, pH 6.5 for 12 days, *B. thuringiensis* BRB-3 achieved a high GABA yield of 25.06 g/L (242.9 mM). Therefore, *B. thuringiensis* BRB-3 demonstrated significant potential for GABA production and could be utilized in various functional food products, including germinated rice seeds.

1. Introduction

Gamma-aminobutyric acid (GABA) is a critical non-protein amino acid found across a wide range of organisms, including plants, animals, fungi, yeast and bacteria. As a major inhibitory neurotransmitter in the central nervous system, GABA plays a crucial role in alleviating several physiological illnesses in humans, such as hypertension, insomnia, diabetes, memory lapse and depression. Its potential as a natural supplement has attracted global interest for application in functional foods, pharmaceuticals and cosmetics [1]. As a result, there is a growing focus on producing GABA through microorganism-based fermentation processes to enhance the nutritional value of various foods. Lactic acid bacteria (LAB) are noted for their high GABA yield and are generally recognized as safe (GRAS) microorganisms, making them ideal for use in food production. LAB strains producing GABA have been isolated from a variety of fermented foods, including Kimchi [2], traditional cheese starter [3], fermented fish from Korea and Vietnam [4], plant-based Thai

* Corresponding author.
E-mail address: wiymon@kku.ac.th (W. Mongkolthanaruk).

https://doi.org/10.1016/j.heliyon.2025.e41884
Received 27 July 2024; Received in revised form 26 December 2024; Accepted 9 January 2025
Available online 11 January 2025

fermented foods [5], Iranian dairy products [6], Thai fermented fish (Plaa-som) [7]. Notable GABA-producing LAB strains include *Levilactobaicllus brevis* F109-MD3, *Lactococcus lactis*, *Lactobacillus plantarum*, *Lactobacillus brevis*, *Lactiplantibacillus plantarum*. Other significant GABA producers include *Bacillus thuringiensis* LH2134, *Lactococcus lactis* LA43, *Bacillus subtilis* and *Enterococcus avium* JS-N6B4, with novel strains also identified in edible insects [8]. Few studies have explored endophytes from rice seeds for GABA production which could be a promising source for GABA enhancing bacteria in rice.

Rice, especially germinated brown rice, is a well-known source of GABA, as it naturally accumulates GABA during germination [9], due to nutritional and biochemical changes. The germination process which is achieved by soaking rice seeds in water at room temperature (25–35 °C) overnight can boost GABA content. Although cooking (typically boiling one-part rice with two parts water for 20 min) may reduce GABA levels by 30–40 %; germinated rice still retained more GABA than non-germinated rice after cooking [10]. Therefore, upland rice, cultivated in dry or well-drained fields, is a choice for adding nutritional value, particularly Maled-Phai cultivar, a non-glutinous, black upland rice, rich in anthocyanins and antioxidant compounds [11]; it has been promoted in north-eastern Thailand for its health benefits. This rice could serve as a valuable source for GABA synthesizing bacteria.

Endophytic bacteria within seeds significantly enhance plant growth by producing phytohormones, siderophores, and enzymes for nutrient absorption and and nitrogen fixation traits [12,13]; that are vertically inherited through plant generations. These bacteria contribute to the germination process, displaying amylase activity and synthesizing indole-related compounds. However, seed endophytes generally exhibit low diversity, varying by host plant. In this study, endophytic bacteria with potential to produce GABA were isolated from germinated black upland rice (Maled-Phai) to identify potential high yield producers. This research highlights these bacteria as a novel source for GABA production and explores optimal conditions for maximizing GABA synthesis which varies based on bacterial species and their growth conditions.

2. Materials and methods

2.1. Isolation of endophytic bacteria from germinated black upland rice seeds

Seeds of Maled-Phai cultivar of black upland rice (*Oryza sativa* subsp. *indica*) were obtained from the Rice project, Faculty of Agriculture, Khon Kaen University, Thailand. Two types of rice seeds, polished and brown rice, were used for isolation of endophytic bacteria. The seeds were surface-sterilized according to the method described by Kaga et al. [14]. First, 2.5 g of rice seeds were rinsed twice with sterilized water, then soaked in 75 % ethanol for 20 s with shaking. The seeds were subsequently transferred to 1 % sodium hypochlorite solution for 1 min and rinsed twice more with sterilized water. The final rinse water was cultured on Luria-Bertani (LB) plates to verify the effectiveness of the surface sterilization. The sterilized rice seeds were then soaked in sterilized water and kept in the dark at room temperature for 24 h to allow germination. The germinated seeds were ground using a sterilized mortar and pestle, diluted with sterilized water, and spread onto LB and MRS® (De Man, Rogosa and Sharpe medium, Himedia) agar plates. The plates were incubated at 37 °C for 2 days, after which bacterial colonies were observed. These bacterial isolates were purified on the same media to obtain single colonies and stock cultures were stored at −20 °C for future use.

2.2. Screening of GABA-producing bacteria using GAD activity test

The activity of Glutamate decarboxylase (GAD) was tested for preliminary screening of GABA-producing bacteria. All bacterial isolates were grown in the medium supplemented with 50 mM MSG [15] at 37 °C for 18 h with shaking at 150 rpm. The bacterial cells were then harvested by centrifugation at 10,000 rpm, 25 °C for 5 min. The cell pellets were washed twice with 0.85 % NaCl solution and resuspended in 500 μl of GAD reagents solution, which contained 1 g/L of L-glutamic acid, 300 μl/L of Triton X-100, 90 g/L of NaCl and 0.05 g/L of bromocresol blue at pH 4.0 [6]. The reaction was incubated at 37 °C for 4 h. After incubation, the color change indicated GAD activity: yellow remained for no activity, greenish for low activity and bluish for high activity.

2.3. Determination of GABA-producing bacterial isolates

A total of 13 bacterial isolates exhibiting high GAD activity were selected for further testing of GABA production. The following isolates, LBRB-1, LBR-1, LWRB-1, LBRN-2 and LBRN-4, were cultured in LB medium supplemented with 2 % glucose and 50 mM MSG. The isolates WRG-1, WRB-2, BRG-2, BRG-4, BRB-1, BRB-2, BRB-3 and BRB-4 were cultivated in MRS® broth supplemented with 50 mM MSG. All cultures were incubated at 37 °C, 150 rpm for 24 h, after which GABA production was measured using a colorimetric method. Moreover, growth and pH changes in the potential strains were determined over 6 days during GABA production.

2.4. Optimization of GABA production in the highest GABA-producing strain

In this experiment, ΔMRS medium was prepared without ammonium ferric citrate to avoid interference with GABA detection by the colorimetric method. The endophytic bacterium BRB-3, identified as the highest GABA-producing strain in MRS® broth supplemented with 50 mM MSG, was studied to optimize GABA production. The optimal temperature for GABA production was tested at 30 °C and 37 °C, while optimal pH was evaluated at pH 5.5, 6.5 and 7.5 for 14 days. Additionally, the influence of nutritional effects including carbon sources, nitrogen sources, and MSG concentration on GABA production was assessed. The bacterium was cultured in ΔMRS medium with 2 % of different sugars as carbon sources and in ΔMRS medium containing 5 g/L of yeast extract along with different nitrogen sources at 10 g/L. The impact of varying MSG concentration on GABA production was also determined in ΔMRS

medium. Throughout the experiments, the culture pH was maintained at 6.5 and bacterial growth was measured by optical density at 600 nm.

2.5. GABA determination by colorimetric method

GABA concentration was measured using a modified Bertherod-colorimetric method as previously described by Zhang et al. [16]. To minimize interference from sugars in the medium, the supernatant of bacterial cell culture was collected by centrifugation and diluted 10-fold prior to the addition of Bertherod reagents. The reaction mixture was prepared by combining 0.2 mL of 0.2 M borate buffer (pH 9.0) with 1 mL of 6 % phenol solution, followed by the addition of 0.5 mL of the diluted supernatant. This mixture was left to stand for 10 min. Next, 0.5 mL of 7 % sodium hypochlorite was added, and the mixture was heated in a water bath at 100 °C for 10 min. After that, the reaction was halted by cooling it in an ice water bath for 10 min and mixed thoroughly using a vortex mixer. Finally, 1.9 mL of 60 % ethanol was added, and the solution was mixed well and left at room temperature for 10 min to allow the color to stabilize. The absorbance of the reaction was measured at 645 nm using a spectrophotometer (GENESYS 10S UV–Vis; Thermo Fisher Scientific). GABA concentration was calculated based on the GABA standard curve (Sigma-Aldrich) with a linear range from 0.5 to 7.5 mM ($R^2 = 0.999$; $y = 0.1223x$).

2.6. TLC analysis

The end-product of bacterial fermentation (GABA) was analyzed using Thin-layer chromatography (TLC) as described by Jo et al. [8]. GABA standard and MSG were prepared as reference substances at a concentration of 5 μg/μL. A 2 μL samples was spotted on a TLC silica plate (Merck, USA), while 1 μL of the GABA-MSG standard mixture was spotted alongside. The plate was then allowed to dry at room temperature. The mobile phase used consisted of a mixture of n-butanol, acetic acid and water in a ratio of 5:3:2 by volume. After the TLC plate was developed in the mobile phase, it was dried at 50 °C. To visualize the spots, the plate was sprayed with 1 % ninhydrin in ethanol and then heated at 100 °C for 30 min.

2.7. Bacterial identification using 16S rRNA gene and rpoB gene analysis

The bacterial isolates were cultivated in MRS® or LB broth at 37 °C for 18 h. The cell pellets were collected by centrifugation at 10,000 rpm for 10 min. The genomic DNA was extracted using PureLink®Genomic DNA kit (Thermo Fisher Scientific) and used as a DNA template for 16S rRNA gene amplification using universal primers 27f (5′-AGATTTGATCCTGGCTCAG-3′) and 1492r (5′-GGCTACCTTGTTACGACTT-3′). PCR reaction consisted of 1x reaction buffer, 0.2 mM each of deoxynucleoside triphosphates, 2 mM $MgCl_2$, 0.5 μM each primer, 1 U of Phusion™ Plus DNA polymerase (Thermo Fisher Scientific) and 1 ng of template DNA. PCR condition was pre-denaturation at 94 °C for 3 min, 34 cycles of denaturation at 92 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 3 min and post-extension at 72 °C for 10 min. PCR products were purified by GeneJET PCR Purification kit (Thermo Fisher Scientific) and were sequenced by ATGC Co., Ltd (Thailand). Moreover, the *rpo*B gene was amplified to distinguish species from *Bacillus cereus* group by *Bacillus* genus-specific primers, *rpo*B forward: 5′-AGGTCAACTAGTTCAGTATGGAC-3′and *rpo*B reverse: 5′-AAGAACCATAACCGGCAAC TT-3′ [5]. The PCR reaction was the same as described above; the PCR condition was also the same except for annealing at 50 °C for 30 s. PCR products were purified and sequenced by ATGC Co., Ltd (Thailand). The DNA sequence was analyzed using BLAST tool and submitted to GenBank of NCBI database.

2.8. Statistical analysis

All experiments were performed in triplicate with standard deviation. Data were subjected to analysis of variance (ANOVA) using STATISTIX 10 software. Multiple comparisons of ANOVA with the least significant difference (LSD) at $P < 0.001$ were performed for optimization of bacterial fermentation.

3. Results

3.1. Isolation of GABA-producing endophytic bacteria

Endophytic bacteria were isolated from germinated black upland rice seeds (Maled Phai cultivar), yielding 38 isolates for study. All isolates were screened for GAD activity and 28 isolates demonstrated positive results. Among bacteria from brown rice seeds, 80 % showed positive GAD activity, while 67 % of those from polished rice seeds were GAD positive. Thirteen isolates exhibited strong GAD activity, indicated by greenish-blue to blue color, including LWRB-1, LBR-1, LBRB-1, LBRN-2, LBRN-4, WRG-1, WRB-1, BRG-2, BRG-4, BRB-1, BRB-2, BRB-3 and BRB-4 (Table 1). The GABA yield of these 13 isolates was measured, ranging from 0.063 to 0.949 g/L after 24 h of cultivation at 37 °C in the medium supplemented with 50 mM MSG and 2 % glucose. The GAD-producing bacteria were identified using 16S rRNA gene sequencing, revealing that 7 isolates belonged to *Bacillus* genus, including BRG-2 (*B. tropicus*), LBRN-2 (*B. safensis*), BRB-4 (*B. amyloliquefaciens*), and 4 isolates from *Bacillus cereus* group (BRG-4, BRB-1, BRB-2 and BRB-3). Additionally, 2 isolates were identified as *Priestia* genus: WRG-1 (*P. megaterium*) and WRB-1 (*P. aryabhattai*). Four isolates belonged to *Pantoea* sp. (LBRB-1, LBR-1, LWRB-1 and LBRN-4). Lastly, BRN-2, a negative GAD strain, was identified as *Staphylococcus pasteuri*. These findings confirmed that rice seeds are a novel source of GABA-producing bacteria. Moreover, BRB-3 strain, belonging to *Bacillus cereus* group,

was further identified as *Bacillus thuringiensis* BRB-3 through *rpoB* gene sequencing (Supplementary Fig. S1).

3.2. Characterization of bacterial culture during GABA production

The potential isolates (LBRN-2, BRG-2, BRB-3 and BRB-4) with high GABA production for 24 h (Table 1) were further evaluated for GABA production at 37 °C for 6 days in LB/ΔMRS medium supplemented with 50 mM MSG and 2 % glucose, starting at an initial pH of 6.5. After 24 h, the pH dropped due to sugar consumption (Fig. 1). LBRN-2 and BRB-4 showed a subsequent pH increase above 7, resulting in GABA yields of approximately 2.50 g/L (Fig. 1A and B). In contrast, BRB-3 and BRG-2 exhibited lower GABA production due to their sensitivity to pH levels below 5.5 (Fig. 1C and D), with reduced growth observed after 48 h, as confirmed by bacterial counts (Supplementary Fig. S2). To address this issue, the culture pH was adjusted back to 6.5 every 24 h. As the result, BRG-2's GABA yield increased to 4.05 g/L at 144 h (Fig. 1E), with the pH dropping early in the fermentation and rising after 72 h. Similarly, BRB-3 produced 4.54 g/L of GABA at 120 h when the pH was regularly adjusted (Fig. 1F). These finding demonstrate the critical role of pH control in supporting bacterial growth and optimizing GABA production during fermentation. Based on these results, BRB-3 was selected as the most promising high GABA-producing strain for further investigation.

3.3. pH optimization and time of fermentation

B. thuringiensis BRB-3 demonstrated high GABA production, strongly influenced by pH conditions. The culture pH was investigated to optimize GABA yield; it was adjusted to specific points (pH 5.5, 6.5 and 7.5) every 24 h using sterile NaOH and HCl under aseptic conditions with pH monitored using a pH meter. At pH 5.5, the pH remained stable, but GABA production was minimal due to limited bacterial growth (Fig. 2A). In contrast, fermentation at pH 6.5 and 7.5 resulted in a gradual increase in GABA content (Fig. 2B and C). The pH initially dropped within the first 24 h, followed by an increase above pH 7.0. The optimal pH for GABA production was

Table 1
Quantitative GAD activity and GABA production of endophytic bacteria isolated from geminated upland rice seeds.

No.	Strain Code	Species (Accession number)	Source	GAD activity	Color result	GABA production (g/L)
1	BRG-1	ND	Brown rice	++++	Greenish blue	–
2	BRG-2	*Bacillus tropicus* (PQ044493)	Brown rice	+++++	Blue	0.801 ± 0.017
3	BRG-3	ND	Brown rice	-	Yellow	–
4	BRG-4	*Bacillus cereus* (PQ044494)	Brown rice	++++	Greenish blue	0.358 ± 0.006
5	BRB-1	*Bacillus cereus* (PQ044489)	Brown rice	++++	Greenish blue	0.270 ± 0.013
6	BRB-2	*Bacillus cereus* (PQ044490)	Brown rice	+++++	Blue	0.536 ± 0.011
7	BRB-3	*Bacillus thuringiensis* (PQ044491)	Brown rice	++++	Greenish blue	0.700 ± 0.006
8	BRB-4	*Bacillus amyloliquefaciens* (PQ044492)	Brown rice	+++++	Blue	0.236 ± 0.019
9	WRB-1	*Priestia aryabhattai* (PQ044501)	Polished rice	+++	Green	0.084 ± 0.006
10	WRB-2	ND	Polished rice	+++	Green	–
11	WRB-3	ND	Polished rice	+++	Green	–
12	WRB-4	ND	Polished rice	-	Yellow	–
13	WRG-1	*Priestia megaterium* (PQ044502)	Polished rice	++++	Greenish blue	0.063 ± 0.002
14	WRG-2	ND	Polished rice	+++	Green	–
15	WRG-3	ND	Polished rice	+++	Green	–
16	WRG-4	ND	Polished rice	+++	Green	–
17	WRG-5	ND	Polished rice	-	Yellow	–
18	LBRB-1	*Pantoea* sp. (PQ044497)	Brown rice	++++	Greenish blue	0.544 ± 0.019
19	LBRB-2	ND	Brown rice	-	Yellow	–
20	LWRB-1	*Pantoea stewartii* (PQ044500)	Polished rice	++++	Greenish blue	0.514 ± 0.01
21	LWRB-2	ND	Polished rice	-	Yellow	–
22	LBRN-1	ND	Brown rice	+	Yellow	–
23	LBRN-2	*Bacillus safensis* (PQ044498)	Brown rice	++++	Greenish blue	0.949 ± 0.004
24	WR-1	ND	Polished rice	++	Lemon green	–
25	WRN-1	ND	Polished rice	-	Yellow	–
26	WRN-2	ND	Polished rice	-	Yellow	–
27	BRN-1	ND	Brown rice	+++	Green	–
28	BRN-2	*Staphylococcus pasteuri* (PQ044495)	Brown rice	-	Yellow	–
29	LWRN-1	ND	Polished rice	++++	Greenish blue	–
30	LWRN-2	ND	Polished rice	++++	Greenish blue	–
31	LWR-1	ND	Polished rice	-	Yellow	–
32	LWR-2	ND	Polished rice	++	Lemon green	–
33	LBR-1	*Pantoea stewartii* (PQ044496)	Brown rice	++++	Greenish blue	0.460 ± 0.006
34	LBR-2	ND	Brown rice	-	Yellow	–
35	LBRN-3	ND	Brown rice	+++	Green	–
36	LBRN-4	*Pantoea stewartii* (PQ044499)	Brown rice	++++	Greenish blue	0.565 ± 0.008
37	LBRN-5	ND	Brown rice	+++	Green	–
38	LBRN-6	ND	Brown rice	+++	Green	–

ND means not detected as no activity. The meaning of strain code indicates as follow: L: LB medium, no L: MRS medium, BR: brown rice seed, WR: Polished rice seed (white rice), G: grind rice, B: broth (liquid of grind rice), N: non surface sterilization.

determined at the condition pH of 6.5, yielding 6.12 (g/L) of GABA on day 8, compared to 4.55 g/L at pH 7.5. The results underscore the importance of maintaining the pH at 6.5 during fermentation. Interestingly, GABA production continued to increase after 8 days of fermentation. Thus, GABA production of *B. thuringiensis* BRB-3 was conducted using MRS® and ΔMRS (without ammonium ferric citrate) media at the optimal pH (6.5) and two incubation temperature (30 °C and 37 °C). The bacterial growth and GABA production were slightly higher in MRS® medium (Fig. 3A). The best condition for GABA production was at 30 °C for 14 days, where GABA yield was nearly twice as high as at 37 °C (9.0 ± 0.5 g/L GABA at 120 h). The results indicated that ΔMRS medium was as effective as MRS® for GABA optimization in further experiments (Fig. 3B).

3.4. Nutrient optimization for GABA production

Nutritional factors were examined to enhance GABA production in *B. thuringiensis* BRB-3. The bacterium was able to utilize all sugars for growth and GABA production with no significant differences observed between dextrose, glucose and fructose (Fig. 4A). These sugars did not promote GABA production including maltose. Interestingly, GABA production from xylose was higher than from dextrose, likely due to the origin of *B. thuringiensis* BRB-3 from upland rice seeds which contain xylose in the embryo [8]. GABA production progressively increased when sucrose, mannitol and lactose were used as carbon sources.

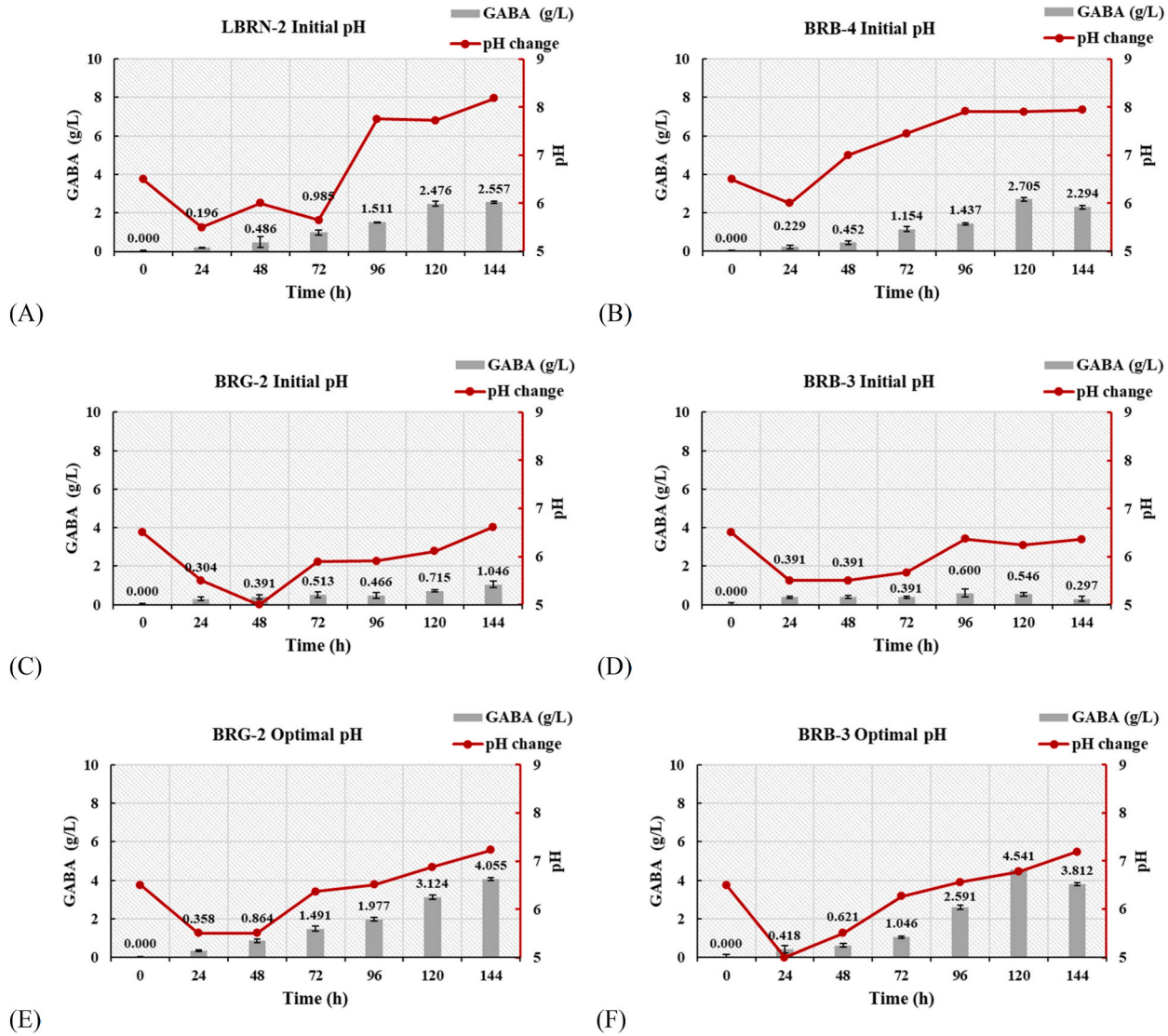


Fig. 1. GABA production and pH changes during cultivation of four isolates in LB/ΔMRS medium supplemented with 50 mM MSG and 2 % glucose, incubated at 37 °C for 6 days. (A) LBRN-2, (B) BRB-4, (C) BRG-2, (D) BRB-3, with an initial fermentation pH of 6.5 without pH adjustment. (E) Optimal pH for BRG-2, and (F) Optimal pH for BRB-3, where optimal pH refers to adjusting the culture to pH 6.5 every 24 h. Numbers indicate GABA content.

Nitrogen sources were very important for producing the amino acid intermediates required for GABA synthesis. Yeast extract, commonly used as a major nitrogen source in many studies [17,18]; it was retained, while peptone and beef extract were substituted with various nitrogen sources in ΔMRS medium. Peptone was identified as the most effective nitrogen source for GABA production, yielding higher levels of GABA compared to tryptone and the control (Fig. 4B). However, beef extract and soy protein did not enhance GABA production, resulting in lower yields than the control. Since glutamic acid is a key precursor in GABA synthesis; therefore, MSG concentration in ΔMRS broth was varied to optimize GABA production. No significant differences were observed between 25 and 50 mM of MSG, and GABA levels remained consistent in MSG ranging from 75 to 150 mM (Fig. 4C). Surprisingly, *B. thuringiensis* BRB-3 was capable of producing GABA without MSG as a precursor. Also, the high MSG concentration (200 mM) actually decreased GABA yield, suggesting that excessive nutrients may negatively impact production. The effect of sucrose concentration was also evaluated; lower sucrose concentration resulted in higher GABA yields. There was no significant difference in GABA content for all sucrose concentrations (Fig. 4D). These findings suggest that optimizing nutrient levels in the medium is essential for GABA production efficiency.

3.5. Relationship of carbon, nitrogen and MSG for GABA synthesis in bacterial fermentation

Soy protein was chosen to use in the ΔMRS medium with sucrose as the carbon source to optimize the ratio of carbon and nitrogen (C:N), both with and without MSG (0 mM and 50 mM). The experiment was designed in 16 conditions as shown in Fig. 5. The GABA-producing bacterium, *B. thuringiensis* BRB-3 produced the highest GABA content under conditions with high nitrogen and either low or high sucrose without the addition of MSG (condition 4 and 8); this indicated that nitrogen was the major source for amino acid conversion to GABA. When nitrogen was high and sucrose was low, the presence of MSG appeared to enhance GABA production (condition 11 and 12). In contrast, under low nitrogen conditions, the bacterium required MSG for GABA production, regardless of sucrose levels (condition 9, 10, 13 and 14). The sucrose was primarily used for bacterial growth, as both low and high sucrose conditions resulted in the lowest GABA production when nitrogen and MSG were absent (condition 1 and 5). These results revealed that the optimal medium of *B. thuringiensis* BRB-3 to achieve high GABA production was either the C:N ratio of 1:1.5 without 50 mM MSG (condition 8) or the C:N ratio of 1:6 with 50 mM MSG.

3.6. Quantitative analysis of GABA by TLC

GABA production from *B. thuringiensis* BRB-3 was visualized on TLC (Fig. 6). The MSG spots appeared in all samples arranged in order of increasing sucrose concentration; also the GABA spots were present in all samples at the same position as the GABA standard,

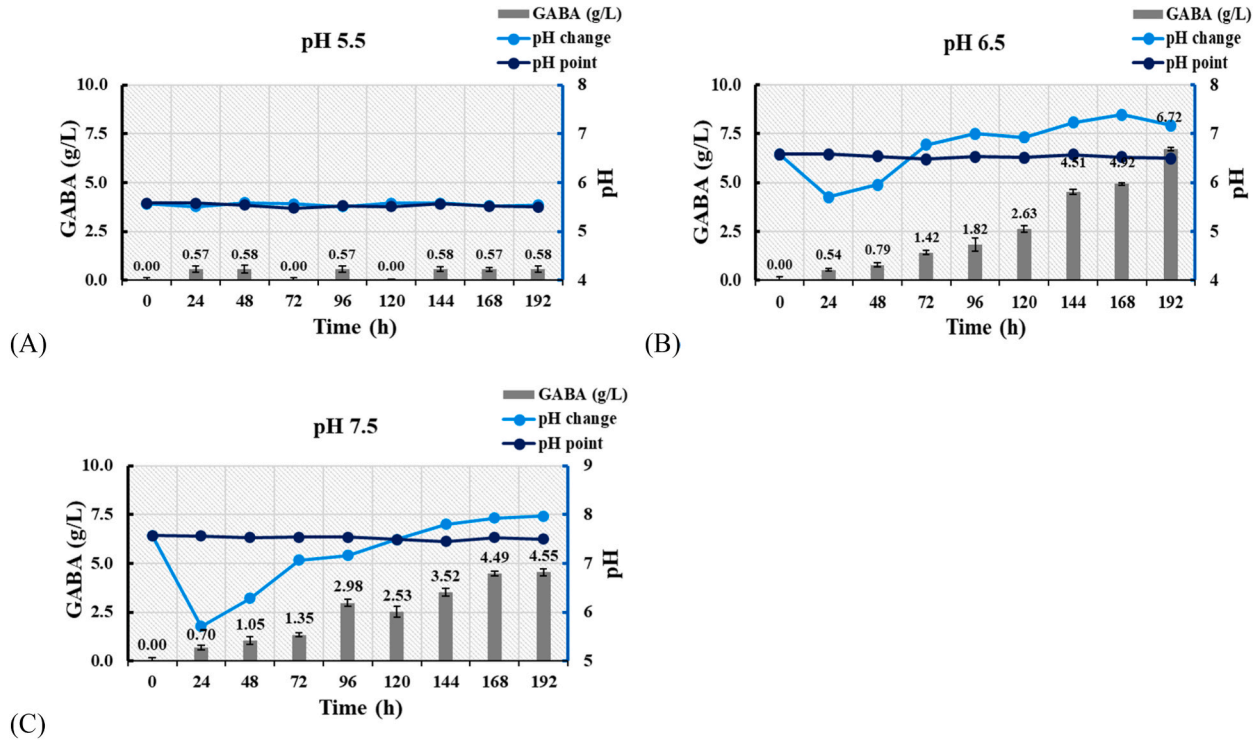


Fig. 2. Optimal pH during cultivation of *B. thuringiensis* BRB-3 in ΔMRS broth for GABA production. The culture was determined at three different pH levels: (A) pH 5.5 (B) pH 6.5 and (C) pH 7.5, incubated at 37 °C for 6 days. GABA content and pH changes were measured.

except in the negative control. This result confirmed the GABA produced during fermentation of *B. thuringiensis* BRB-3. Moreover, a faint MSG band was observed in the low sucrose medium (lane 1), suggesting that the bacterium utilized MSG for GABA synthesis.

4. Discussion

Bacterial groups identified in rice seedings were predominantly from Phyla Bacteroidetes, Firmicutes and Proteobacteria, with Proteobacteria being the most abundant group [19]. The diversity of endophytic bacteria showed variation across the stages of seed maturation: early maturation stages exhibited higher bacterial diversity, while later stages revealed a reduction in diversity [20], where only the genus *Bacillus* was detected. These bacteria are known for their desiccation-resistant spores [21] and amylolytic activity, which enables them to degrade starch. In this study, the endophytic bacteria isolated from upland rice seeds (Maled Phai cultivar) were identified as gram-positive bacteria from Phylum Firmicutes (*Bacillus* and *Priestia*) and gram-negative bacteria belonging to Gammaproteobacteria (*Pantoea*), exhibiting low diversity. This low diversity aligns with findings on rice seed endophytes. Moreover, the isolated bacteria also showed potential for GABA production, indicating that they may play a role in GABA accumulation in rice seeds. Previous studies have reported starch hydrolysis activity in seed endophytes [19], which may facilitate GABA production by utilizing nutrients available during seed germination. Certain *Bacillus* species, such as *B. subtilis*, *B. thuringiensis*, *B. velezensis* DMB06 and *B. licheniformis* 0DA23-1, have been reported to produce GABA from substrates like brewer's spent grain, using hemicellulose as a primary carbohydrate source [22]. Our findings confirmed that *B. thuringiensis* BRB-3 is a GABA producer, and its GABA synthesis during rice germination will be explored further.

In the present work, various sugars were tested as carbon sources for in vitro GABA production. The results revealed that pH changes during GABA production significantly affected both bacterial growth and GABA yield. *B. thuringiensis* BRB-3 metabolized all tested sugars via glycolysis, converting pyruvate into organic acids, such as lactic acid, acetic acid or formic acid. Sugars like dextrose, glucose, and maltose caused a rapid pH drop within 24 h, whereas sucrose, lactose, mannitol, xylose, and fructose led to a pH increase (Supplement Fig. S3). After 4 days, all cultures became alkaline due to nitrogen consumption by the bacteria. The acidic condition inhibited bacterial growth; thus, the medium pH was adjusted to 6.5. Under these controlled conditions, sucrose emerged as the most effective carbon source for GABA production, possibly due to *B. thuringiensis* BRB-3's ability to produce sucrolytic enzymes. *Bacillus* species typically utilize sucrose through the phosphoenolpyruvate (PEP)-dependent phosphotransferase system (PTS), which requires high energy input. This system transports sucrose into the cell and converts it into sucrose-6-phosphate, which is then broken down into fructose and glucose-6-phosphate before entering glycolysis [23]. Sucrose utilization also depends on media components; for instance, *B. licheniformis* TKU004 showed optimal sucrolytic enzyme production at pH 6.7 with pomelo (*Citrus maxima*) albedo powder as an agro-byproducts [24]. This study suggests that *B. thuringiensis* BRB-3 uses a similar mechanism to utilize sucrose for GABA production.

Amino acids, especially glutamate and arginine, are key precursors for GABA synthesis [22]. Yeast extract, which provides various biologically active ingredients, was an effective nitrogen source [2]. Among tested nitrogen sources, peptone was the most effective, while tryptone, beef extract, and soy protein were less beneficial for GABA production. The peptone and tryptone contain amino acids including glutamate and arginine [25] related to GABA synthesis. The optimum nitrogen source depended on the abilities of different bacterial species; for example, *Lactiplantibacillus plantarum* FBT215 preferred tryptone for GABA production [26]. Soy protein containing various amino acids yielded lower GABA levels due to its limited glutamate content. Interestingly, the additional MSG did not enhance GABA production, possibly because the fermentation medium already contained sufficient nutrients. *Bacillus* species employ two main pathways for GABA synthesis: (1) conversion of glutamate to GABA by glutamate decarboxylase, and (2) conversion of arginine to GABA via putrescine through gamma-glutamyl-gamma-aminobutyrate hydrolase [27]. Thus, if the medium provides adequate glutamate and arginine, extra MSG may not be required, as verified by experiments testing different ratios of carbon, nitrogen, and MSG.

To reduce costs and improve compatibility with industrial applications, soy protein was chosen in this study as a nitrogen source, similar to previous findings where *B. subtilis* BBEL02 utilized soybean hydrolysate from food industry waste with 5 % MSG, yielding 12.5 g/L GABA [28]. Similarly, *Bacillus cereus* KBC produced 3.39 g/L GABA in MRS with 0.5 % MSG [29]. For *B. thuringiensis* BRB-3,

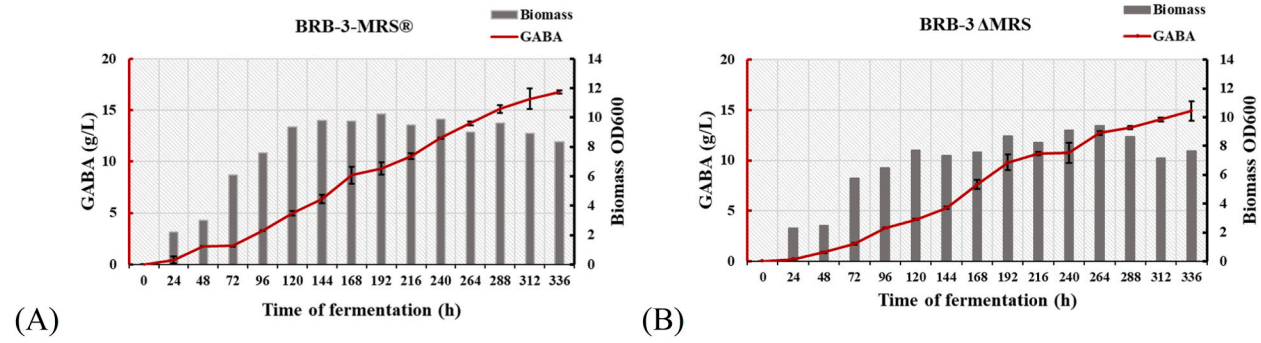


Fig. 3. (A) Fermentation time for GABA production by *B. thuringiensis* BRB-3 in MRS® and (B) ΔMRS (without ammonium ferric citrate) with pH adjusted to 6.5, at 30 °C over 14 days.

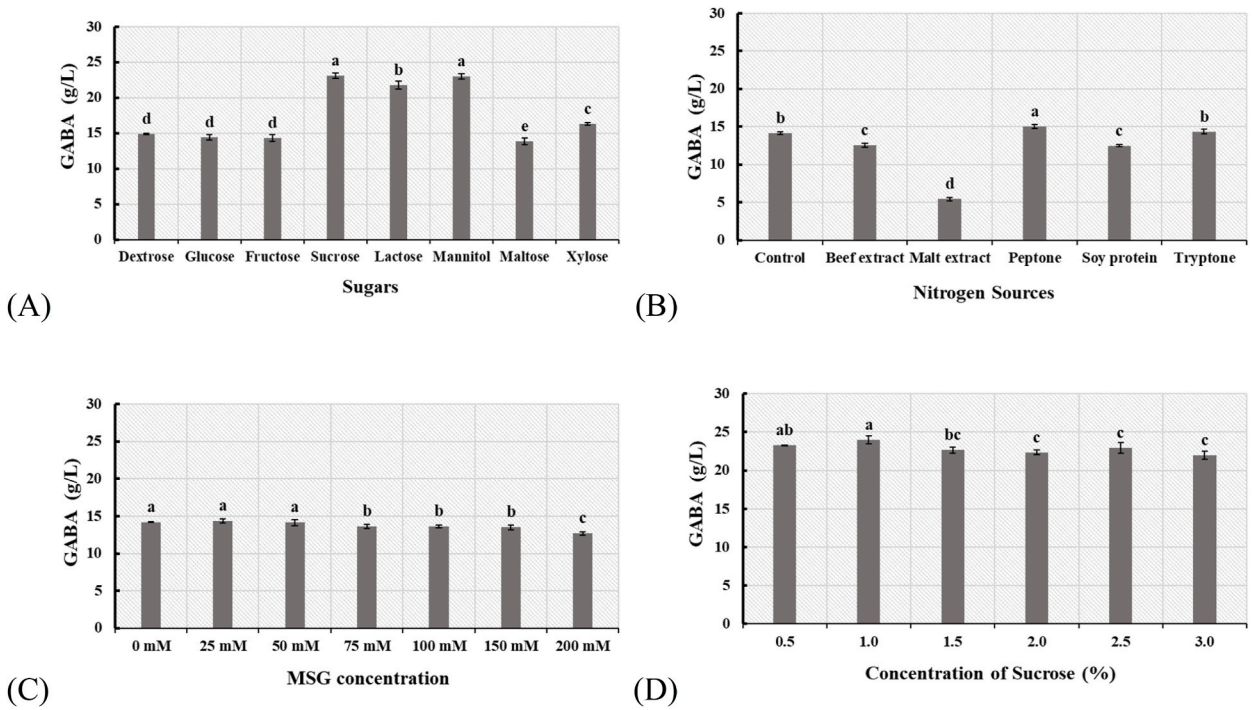
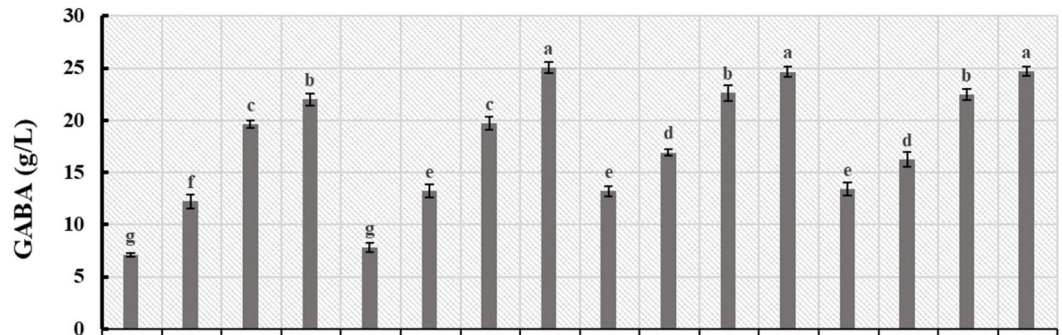


Fig. 4. Effect of nutrients on GABA yield by *B. thuringiensis* BRB-3 in ΔMRS broth under optimal conditions (30 °C, pH 6.5, 150 rpm) for 14 days. (A) different carbon sources (2 %) in ΔMRS broth with 50 mM MSG, (B) different nitrogen sources (1 %) in ΔMRS broth with 2 % dextrose and 50 mM MSG, (C) various MSG concentrations in ΔMRS broth with 2 % dextrose, (D) various sucrose concentrations in ΔMRS broth with 50 mM MSG. Different letters on each bar indicate significant differences at $p < 0.001$.

medium composition was optimized by varying carbon and nitrogen levels with and without MSG. The high nitrogen content facilitated GABA production even with low sugar levels, particularly under conditions of 0.5 % sucrose and 3 % soy protein (condition 4 and 8). At higher sucrose levels, the soy protein was required at a ratio of 1.5:1 relative to sucrose, as amino acids from soy protein directly supported GABA synthesis. Additionally, MSG enhanced GABA production when both sucrose and soy protein were present at low levels (condition 9), resulting in 2-fold increase compared to the condition without MSG (condition 1). Therefore, *B. thuringiensis* BRB-3 demonstrates potential for cost-effective GABA production using sucrose and soy protein, which can be sourced from industrial by-products, e.g. molasses, whey, brewer's spent grain, without the need for added MSG.



	0.0 M; 0.5 S; 0.5 N	0.0 M; 0.5 S; 1.0 N	0.0 M; 0.5 S; 2.0 N	0.0 M; 0.5 S; 3.0 N	0.0 M; 2.0 S; 0.5 N	0.0 M; 2.0 S; 1.0 N	0.0 M; 2.0 S; 2.0 N	0.0 M; 2.0 S; 3.0 N	50 M; 0.5 S; 0.5 N	50 M; 0.5 S; 1.0 N	50 M; 0.5 S; 2.0 N	50 M; 0.5 S; 3.0 N	50 M; 2.0 S; 0.5 N	50 M; 2.0 S; 1.0 N	50 M; 2.0 S; 2.0 N	50 M; 2.0 S; 3.0 N
GABA (g/L)	7.08	12.23	19.63	22.00	7.82	13.25	19.73	25.06	13.22	16.93	22.64	24.65	13.40	16.25	22.47	24.68
Condition	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16

Fig. 5. Effect of carbon, nitrogen and MSG ratios on GABA production by *B. thuringiensis* BRB-3 in ΔMRS broth, cultivated at 30 °C, 150 rpm with pH adjusted to 6.5. M: MSG concentration (mM), S: sucrose concentration (%), N: soy protein concentration (%). GABA content was measured at 12 days. Different letters on each bar indicate significant differences at $p < 0.001$.

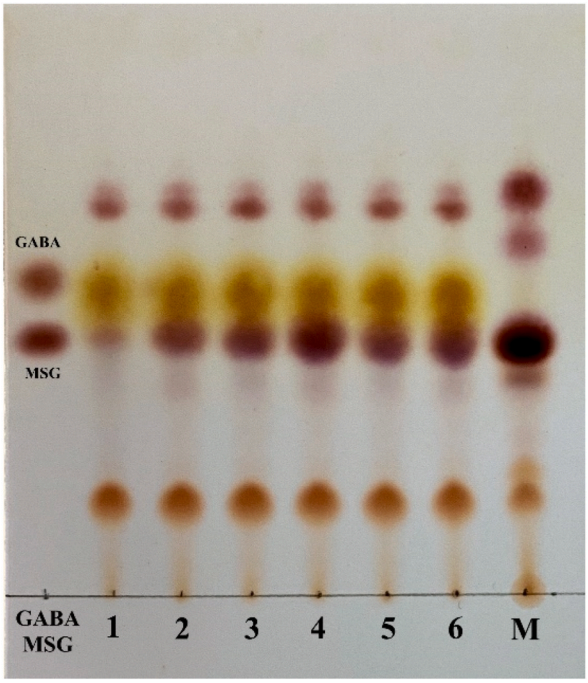


Fig. 6. TLC analysis of GABA production from *B. thuringiensis* BRB-3 cultured in ΔMRS with 2 % soy protein, 50 mM MSG and varying sucrose concentrations on day 10. GABA/MSG: standard mixture of GABA (7.5 μg/μl) and MSG (2.5 μg/μl). Lanes: L1: 0.5 % sucrose, L2: 1 % sucrose, L3: 1.5 % sucrose, L4: 2 % sucrose, L5: 2.5 % sucrose, L6: 3 % sucrose, M: ΔMRS broth as a negative control.

5. Conclusion

A novel GABA producing strain, the endophytic *Bacillus thuringiensis* BRB-3 was successfully isolated from germinated black upland rice seeds using MRS® medium. Screening for GAD activity highlighted its strong potential. Under optimized conditions (ΔMRS broth containing 3 % soy protein, 2 % sucrose at 30 °C for 12 days), *B. thuringiensis* BRB-3 achieved a maximum GABA yield of 25.06 g/L (242.97 mM). Controlling pH to 6.5 was important for bacterial growth and GABA production. Additionally, the concentration of MSG influenced GABA production particularly in low nitrogen and carbon sources. This is the first to report the high GABA yield from *B. thuringiensis* BRB-3, an endophytic bacteria from rice seeds. Future applications, *B. thuringiensis* BRB-3 could use in enhancing rice seed germination or as an ingredient in function foods enriched with GABA.

CRediT authorship contribution statement

Rungnapha Wannasutta: Writing – original draft, Methodology, Investigation, Data curation. **Sophon Boonlue:** Visualization. **Nuntavun Riddech:** Visualization. **Wiyada Mongkolthanaruk:** Writing – review & editing, Funding acquisition, Conceptualization.

Data availability

Supplement data can be found online at.

Funding

This research was supported by the Fundamental Fund of Khon Kaen university from the National Science, Research and Innovation Fund (NSRF).

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2025.e41884.

References

[1] T. Zotta, I. Faraone, M. Giavalisco, E. Parente, L. Lela, L.V. Storti, A. Ricciardi, The production of γ-aminobutyric acid from free and immobilized cells of *Levilactobacillus brevis* cultivated in anaerobic and aerobic conditions, Microorganisms 10 (11) (2022) 2184, https://doi.org/10.3390/microorganisms10112184.

[2] W. Liu, L. Liu, K. Ko, I. Kim, Screening of gamma-aminobutyric acid producing lactic bacterial and the characteristic of glutamate decarboxylase from *Levilactobacillus brevis* F109-MD3 isolated from Kimchi, J. Appl. Microbiol. 132 (3) (2022) 1967–1977, https://doi.org/10.1111/jam.15306.

[3] N. Lacroix, D. St-Gelais, C.P. Champagne, J.C. Vuillemard, Gamma-aminobutyric acid producing ability of *Lactococcal* strains isolated from old style cheese starter, Dairy Sci. Technol. 93 (2013) 315–327, https://doi.org/10.1007/s13594-013-0127-4.

[4] T.T. Vo, H.J. Park, Characteristics of potential gamma-aminobutyric acid-producing bacteria isolated from Korean and Vietnamese fermented fish products, J. Microbiol. Biotechnol. 29 (2) (2019) 209–221, https://doi.org/10.4014/jmb.1811.09072.

[5] A. Pakdeeto, S. PhuengJayaem, T. Arayakarn, C. Phitchayaphon, A. Tungkajiwangkoon, S. Tanaupawat, Identification of gamma-aminobutyric acid (GABA)-producing lactic acid bacteria from plant-based Thai fermented foods and genome analysis of *Lactobacillus brevis*7-4, Sci. Asia 48 (2022) 254–262, https://doi.org/10.2306/scienceasia1513-1874.2022.037.

[6] M.R.E. Dovom, M.B.H. Najafi, P.R. Vosogh, N. Norouzi, S.J.E. Nezhad, B. Mayo, Screening of Lactic acid bacteria strains isolated from Iranian traditional dairy products for GABA production and optimization by response surface methodology, Sci. Rep. 13 (2023) 440, https://doi.org/10.1038/s41598-023-27658-5.

[7] V. Tanamool, P. Hongsachart, W. Soemphol, Screening and characterization of gamma-aminobutyric acid (GABA) producing lactic acid isolated from Thai fermented fish (Plaa-som) in Nong Khai and its application in Thai fermented vegetables (Som-pak), Food Sci. Technol. Int. 40 (2) (2020) 483–490, https://doi.org/10.1590/fst.05419.

[8] M.H. Jo, S.J. Hong, H.N. Lee, H.H. Ju, B.R. Park, J.H. Lee, J.E. Kim, Y.J. Wee, Y.M. Kim, Gamma-aminobutyric acid production from a novel *Enterococcus avium* JS-N6B4 stain isolated from edible insects, J. Microbiol. Biotechnol. 29 (6) (2019) 933–943, https://doi.org/10.4014/jmb.1905.05001.

[9] N. Komotsuzaki, K. Tsukahara, H. Toyoshima, T. Suzuki, N. Shimizu, T. Kimura, Effect of soaking and gaseous treatment on GABA content in germinated brown rice, J. Food Eng. 78 (2) (2007) 556–560, https://doi.org/10.1016/j.jfoodeng.2005.10.036.

[10] T. Toyoizumi, T. Kosugi, Y. Toyama, T. Nakajima, Effects of high-temperature cooking on the gamma-aminobutyric acid content and antioxidant capacity of germinated brown rice (*Oryza sativa* L.), CyTA J. Food 19 (1) (2021) 360–369, https://doi.org/10.1080/19476337.2021.1905721.

[11] B. Sripanidkulchai, J. Junlatat, S. Tuntiyasawasdikul, N. Fangkrathok, J. Sanitchon, S. Chankaew, Phytochemical and bioactivity investigation of Thai pigmented-upland rice: dam-Mong and Ma-Led-Fy varieties, Agric. Nat. Resour. 56 (5) (2022) 889–898, https://doi.org/10.34044/j.anres.2022.56.5.03.

[12] K.N. Nghi, A. Tagliani, L. Mariotti, D.A. Weits, P. Perata, C. Pucciariello, Auxin is required for the long coleoptile trait in japonica rice under submergence, New Phytol. 229 (2021) 85–93, https://doi.org/10.1111/nph.16781.

[13] S. Compant, A. Samad, H. Faist, A. Sessitsch, A review on the plant microbiome: ecology, functions, and emerging trends in microbial application, J. Adv. Res. 19 (2019) 29–37, https://doi.org/10.1016/j.jare.2019.03.004.

[14] K. Kaga, H. Mano, F. Tanaka, A. Watanabe, S. Kaneko, H. Morisaki, Rice seeds as sources of endophytic bacteria. Microbes, Environ. Times 24 (2) (2009) 154–162, https://doi.org/10.1264/jsme2.ME09113.

[15] S. Sharafi, L. Nateghi, Optimization of gamma-aminobutyric acid production by probiotic bacteria through response surface methodology, Iran. J. Microbiol. 12 (6) (2020) 584–591, https://doi.org/10.18502/ijm.v12i6.5033.

[16] Q. Zhang, J. Xiang, L. Zhang, X. Zhu, J. Evers, W. Werf, L. Duan, Optimizing soaking and germination conditions to improve gamma-aminobutyric acid content in japonica and indica germination brown rice, J. Funct.Foods 10 (2014) 283–291, https://doi.org/10.1016/j.jff.2014.06.009.

[17] H. Harnentis, N. Nurmiati, Y. Marlida, F. Adzitey, N. Huda, γ-Aminobutyric acid production by selected lactic acid bacteria isolate of an Indonesian indigenous fermented buffalo milk (dadih) origin, Vet. World 12 (2019) 1352–1357, https://doi.org/10.14202/vetworld.2019.1352-1357.

[18] M.C. Kook, M.J. Seo, C.I. Cheigh, Y.R. Pyun, S.C. Cho, H. Park, Enhanced production of γ-aminobutyric acid using rice bran extracts by *Lactobacillus sakei* B2-16, J. Microbiol. Biotechnol. 20 (4) (2010) 763–766, https://doi.org/10.4014/jmb.0911.11016.

[19] G.D. Ahumada, E.M. Gómez-Álvarez, M. Dell'Acqua, I. Bertani, V. Venturi, P. Perata, C. Pucciariello, Bacterial endophytes contribute to rice seedling establishment under submergence, Front. Plant Sci. 13 (2022) 908349, https://doi.org/10.3389/fpls.2022.908349.

[20] S. Okunishi, K. Sako, H. Mano, A. Imamura, H. Morisaki, Bacterial flora of endophytes in the maturing seed of cultivated rice (*Oryza sativa*), Microb. Environ. 20 (3) (2005) 168–177, https://doi.org/10.1264/jsme2.20.168.

[21] S. Truyens, N. Weyens, A. Cuypers, J. Vangronsveld, Bacterial seed endophytes: genera, vertical transmission and interaction with plants, Environ. Microbiol. Rep. 7 (1) (2015) 40–50, https://doi.org/10.1111/1758-2229.12181.

[22] T. Kim, S. Heo, H.I. Na, G. Lee, J.H. Lee, J.Y. Kim, D.W. Jeong, Increased production of γ-aminobutyric acid from brewer's spent grain through *Bacillus* fermentation, J. Microbiol. Biotechnol. 33 (4) (2023) 527–532, https://doi.org/10.4014/jmb.2210.10051.

[23] J. Feng, Y. Gu, Y. Quan, W. Gao, Y. Dang, M. Cao, X. Lu, Y. Wang, C. Song, S. Wang, Construction of energy-conserving sucrose utilization pathways for improving poly-γ-glutamic acid production in *Bacillus amyloliquefaciens*, Microb. Cell Fact. 16 (2017) 98, https://doi.org/10.1186/s12934-017-0712-y.

[24] C.T. Doan, T.N. Tran, T.T. Nguyen, T.P.H. Tran, V.B. Nguyen, T.D. Tran, A.D. Nguyen, S.L. Wang, Production of sucrolytic enzyme by *Bacillus licheniformis* by the bioconversion of pomelo albedo as a carbon source, Polymers 13 (2021) 1959, https://doi.org/10.3390/polym13121959.

[25] F. Davami, F. Eghbalpour, L. Nematollahi, F. Barkhordari, F. Mahboudi, Effects of peptone supplementation in different culture media on growth, metabolic pathway and productivity of CHO DG44 cells; a new insight into amino acid profiles, Iran. Biomed. J. 19 (4) (2015) 194–205, https://doi.org/10.7508/ibj.2015.04.002.

[26] J. Kim, M.H. Lee, M.S. Kim, G.H. Kim, S.S. Yoon, Probiotic properties and optimization of gamma-aminobutyric acid production by *Lactoplantibacillus plantarum* FBT215, J. Microbiol. Biotechnol. 32 (6) (2022) 783–791, https://doi.org/10.4014/jmb.2204.04029.

[27] M. Jang, D.W. Jeong, G. Heo, H. Kong, C.T. Kim, J.H. Lee, Genetic background behind the amino acid profiles of fermented soybeans produced by four *Bacillus* spp, J. Microbiol. Biotechnol. 31 (3) (2021) 447–455, https://doi.org/10.4014/jmb.2012.12051.

[28] A.C. Asun, S.T. Lin, H.S. Ng, J.C. Lan, Production of gamma-aminobutyric acid (GABA) by *Bacillus subtilis* BBEL02 fermentation using nitrogen-rich industrial wastes as crude feedstocks, Biochem. Eng. J. 187 (2022) 108654, https://doi.org/10.1016/j.bej.2022.108654.

[29] W.A.A.Q.I. Wan-Mohtar, M.N.A. Sohedein, M.F. Ibrahim, S. Ab Kadir, O.P. Suan, A.W. Weng Loen, S. Sassi, Z. Ilham, Isolation, identification, and optimization of γ-aminobutyric acid (GABA)-producing *Bacillus cereus* strain KBC from a commercial soy sauce *moromi* in submerged-liquid fermentation, Processes 8 (2020) 652, https://doi.org/10.3390/pr8060652.