Screening of Gamma Aminobutyric Acid (GABA) and Anti-oxidant Producing *Bacillus* Isolated from the Honey of Bees and Stingless Bees

Benyapa Prakit[1], Patthira Jansiri[2], Kittiya Khongkool[1], Wankuson Chanasit[3], Monthon Lertworapreecha[3*]

[1]Biotechnology program, Faculty of Science, Thaksin University
[2]Microbiology program, Department of Biology, Faculty of Science, Thaksin University
[3]Microbial technology for Agriculture Food and Environment Research Center, Faculty of Science, Thaksin University

*Corresponding author. E-mail: worapreecha@gmail.com

ABSTRACT

GABA is a non-protein amino acid that acts as a neurotransmitter and helps to reduce stress, promote muscle building, and reduce fat accumulation. Some probiotics such as *Bacillus* spp. have been reported as a potential GABA producer and antioxidant activity. In this study, 22 isolates of *Bacillus* spp. isolated from honey and stingless honey samples were investigated the GABA production. The result showed three isolates, namely BPW-SB21, BPW-SB17, BPW-14 were able to produce a maximum amount of GABA of 985.59 ±0.4 ng/mL, 963.65 ±1.3 ng/mL and 962.51 ±1.1 ng/mL, respectively. In addition, antioxidant activity was examined by DPPH assay and the result demonstrated that BPW-SB2, BPW-SB21, BPW-SB1 were able to produce a maximum amount of anti-oxidation of 75.44 ±0.5%, 72.30 ±1.6%, 70.80 ±0.2%, respectively. The results suggest that the newly isolates of *Bacillus* from honey and stingless honey samples may be a promising bacterial candidate for use as a potential GABA producer with antioxidant activity.

Keywords: *Gamma amino butyric acid, Bacillus, Antioxidant, Honey, Stingless bee honey*

Article history:
Received 12 August 2023; Received in revised from 18 August 2023;
Accepted 28 December 2023; Available online 30 December 2023

INTRODUCTION

Gamma-aminobutyrate (GABA) is an amino acid derivative not found in proteins and is commonly observed in germinating grains. Microorganisms such as *Lactobacillus* and *Bacillus* can produce GABA, a process achieved through the decarboxylation of L-glutamic acid (Ngo & Vo, 2019; Wang et al., 2019). The production of GABA is dependent on the process of decarboxylation. This involves stripping the carboxyl group from L-glutamic acid, turning it into GABA. Recognized for its health benefits, GABA can lower blood pressure, promote relaxation, and alleviate stress (Ngo & Vo, 2019). This has led to its supplementation in animal feed, improving productivity and stress management in animals (Yowtak et al., 2013; Zhang et al., 2022). Further, certain probiotic strains, notably *Bacillus*, can also produce antioxidants, enhancing their value as additives in animal feed (Adebayo-Tayo & Fashogbon, 2020; Gangalla et al., 2021). *Bacillus* have been previously identified in honey and stingless bee samples. Considering the vast distances bees, including stingless varieties, travel in their nectar quest, they might introduce an array of microorganisms, possibly affecting nectar purity (Saleem et al., 2021).

Therefore, our study aims to isolate *Bacillus* from honey and stingless honey, focusing on those strains capable of generating GABA and antioxidants.

METHODOLOGY

Bacillus Isolation from Honey and Stingless Nectar Samples

A total of nine samples were collected from the beekeeping community enterprise group located in Phatthalung Province during the months of October and November 2022. This comprised three honey samples and six stingless nectar samples. Each honey and stingless honey sample was first diluted using a 0.85% saline solution. From these diluted solutions, aliquots of 0.1 mL were carefully spread across Glucose-Yeast-Peptone (GYP) agar plates. The inoculated GYP agar plates were subsequently placed in an incubator set at 37°C. The growth of bacterial colonies was monitored over a duration ranging from 24 to 48 hours. Following incubation, bacterial colonies manifesting on the agar were selected at random and streaked onto new GYP agar plates. This sub-culturing process was iteratively performed to ensure the isolation of single colonies, indicative of a pure culture. The isolated pure bacterial cultures were then transferred to vials and stored in a deep freezer at -80°C for future use.

Identification of *Bacillus*

Bacteria were identified by screening for their preliminary properties, i.e., colony appearance, catalase activity, Gram staining and cell morphology. The bacteria that gave a positive catalase test were then selected. Gram-positive and rod shape were further verified using Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometer (MALDI-TOF MS).

GABA Production Assay

The isolated *Bacillus* was cultured in Luria-Bertani (LB) medium, supplemented with 0.5 g of monosodium glutamate (MSG). The culture was maintained at 30°C with agitation at 100 rpm for a duration of 5 days. Following incubation, the cell-free supernatant was obtained by centrifuging the culture at 5,000 rpm for 10 minutes. Then, the cell-free supernatant was subjected to analysis using the QuickDetect™GABA ELISA kit (ELISA et al., USA). A standard solution was prepared by combining 50 µL of the provided standard GABA and 50 µL of standard diluent. In each well of the ELISA plate, 10 µL of the test sample was added. To the same well, 40 µL of sample diluent was introduced. Subsequently, 100 µL of HRP-conjugate reagent was added to each well. The plate was then incubated at 37°C for 60 minutes. Following the incubation, 50 µL of chromogen solution A and 50 µL of chromogen solution B were added to each well. The contents were gently mixed and incubated at 37°C for 15 minutes in dark conditions. The optical density (OD) was measured at a wavelength of 450 nm.

Antioxidant Activity Assay Using 2,2-Diphenyl-1-picrylhydrazyl (DPPH)

The cell-free supernatant was utilized to assess the scavenging ability through the DPPH assay. Ascorbic acid, acting as the standard antioxidant, was prepared at concentrations of 10, 20, 40, 60, 100, and 200 mg/L, respectively. Into each well of a 96-well plate, 100 µL of the sample was added. To this, 100 µL of a 0.6 mM DPPH solution was added and mixed thoroughly by shaking. The plate was then incubated in the dark for 30 minutes. After incubation, the absorbance of each well was measured using a microplate reader at a wavelength range of 515-517 nm. Presence of antioxidants in the sample would lead to a color change of the solution from purple to yellow. The obtained absorbance values were used to determine the percentage scavenging ability, calculated through a specified equation.

$$\% \text{ scavenging ability} = [(A_{DPPH} - A_{sample}) / A_{DPPH}] \times 100$$

$A_{sample:}$ is the absorbance of the sample set.

$A_{DPPH:}$ is the absorbance of DPPH solution without sample.

RESULTS AND DISCUSSION

Bacillus Isolation

In the present research, *Bacillu*s strains showcasing GABA production and antioxidant activity were isolated from both honey and stingless honey samples sourced from various regions of the country. Zulkhairi Amin et al. (2019) documented the prevalence of *Bacillus* bacteria within honey and stingless bee honey, emphasizing their promising probiotic attributes. Given the diverse floral sources, it was anticipated that honey samples might exhibit a high incidence of GABA-producing *Bacillus* and potential antioxidant contaminants.

From a total of three honey and six stingless honey samples, as many as 115 bacterial isolates were successfully isolated. Notably, among these isolates, 22 were identified as gram-positive, rod-shaped bacteria exhibiting catalase enzyme production. Further validation using the MALDI Biotyper (Bruker Daltonik GmbH, Germany) confirmed these 22 isolates as *Bacillus* spp. Detailed findings associated with these isolates are presented in Table 1.

Table 1. The results of the identification of bacteria by MULDI-TOF

Isolates	Morphology	Gram staining	Catalase test	MULDITOF identification
BPW-SB1	bacilli	Positive	+	*Rummeliibacillus pycnus*
BPW-SB2	bacilli	Positive	+	*Bacillus amyloliquefaciens*
BPW-301	bacilli	Positive	+	*Bacillus amyloliquefaciens*
BPW-3	bacilli	Positive	+	*Bacillus amyloliquefaciens*
BPW-SB4	bacilli	Positive	+	*Bacillus amyloliquefaciens*
BPW-SB5	bacilli	Positive	+	*Bacillus pumilus*
BPW-SB6	bacilli	Positive	+	*Bacillus megaterium*
BPW-7	bacilli	Positive	+	*Bacillus mojavensis*
BPW-SB8	bacilli	Positive	+	*Bacillus licheniformis*
BPW-SB9	bacilli	Positive	+	*Bacillus subtilis*
BPW-g1	bacilli	Positive	+	*Bacillus pumilus*
BPW-702	bacilli	Positive	+	*Bacillus subtilis*
BPW-SB10	bacilli	Positive	+	*Bacillus thuringiensis*
BPW-M	bacilli	Positive	+	*Bacillus subtilis*
BPW-SB12	bacilli	Positive	+	*Bacillus megaterium*
BPW-SB14	bacilli	Positive	+	*Bacillus amyloliquefaciens*
BPW-SB16	bacilli	Positive	+	*Fructobacillus fructosus*
BPW-SB17	bacilli	Positive	+	*Fructobacillus fructosus*
BPW-SB18	bacilli	Positive	+	*Fructobacillus fructosus*
BPW-SB19	bacilli	Positive	+	*Bacillus subtilis*
BPW-SB21	bacilli	Positive	+	*Fructobacillus fructosus*
BPW-SB22	bacilli	Positive	+	*Bacillus amyloliquefaciens*

GABA Synthesis and Antioxidant Activity

The assay revealed that all 22 *Bacillus* isolates synthesized GABA. Among them, BPW-SB21, BPW-SB17, and BPW-SB14 were the most prolific, registering GABA concentrations of 985.59 ±0.4, 963.65 ±1.3, and 962.51 ±1.1 ng/mL, respectively (Figure 1A). The BPW-SB1 exhibited the highest antioxidant activity. The percentage scavenging ability as determined by the DPPH assay was 75.44 ± 0.5, 72.30 ± 1.6, and 70.80 ± 0.2, respectively (Figure 1B). The GABA synthesis in *Bacillus* spp. varies among species due to differences in the presence and activation of the glutamate decarboxylase (gad) gene.

Although GABA production from the *Bacillus* isolates in this study was lower than several reports showcasing values in mg/mL, certain studies, such as those on *B. subtilis* in bioreactor systems, have demonstrated significantly higher outputs, reaching up to 12 mg/mL (Asun et al., 2022). It's pertinent to mention that the mentioned research used an elevated 5% MSG content under optimized conditions, whereas our study incorporated just 0.5% MSG, a tenth of the former. Regarding antioxidant activity, *Bacillus amyloliquefaciens* (BPW-SB2), *Fructobacillus fructosus* (BPW-SB21), and *Rummeliibacillus pycnus* (BPW-SB1) recorded percentages of 75.44 ±0.5, 72.30 ±1.6, and 70.80 ±0.2, respectively. When juxtaposed with literature, this study's *Bacillus* strains manifested greater scavenging abilities than those reported by Kadaikunnan et al. (2015) for *B. amyloliquefaciens*, which approximated 67.12%.

The intriguingly high antioxidant activity in *Bacillus* may be attributed to their growth in nutrient-rich environments like honey and stingless honey. Such medium might supply *Bacillus* with essential nutrients, promoting robust synthesis of antioxidants such as various vitamins. This hypothesis warrants deeper exploration and validation.

Isolating *Bacillus* from honey and stingless honey yielded a rich diversity of *Bacillus* strains, most being high-safety strains. Several strains, like *B. amyloliquefaciens* and *B. subtilis*, are already approved for probiotic use in both livestock and humans (Anadon et al., 2006; FAO., 2017). This underscores the potential to further develop and harness these *Bacillus* isolates for probiotic formulations.

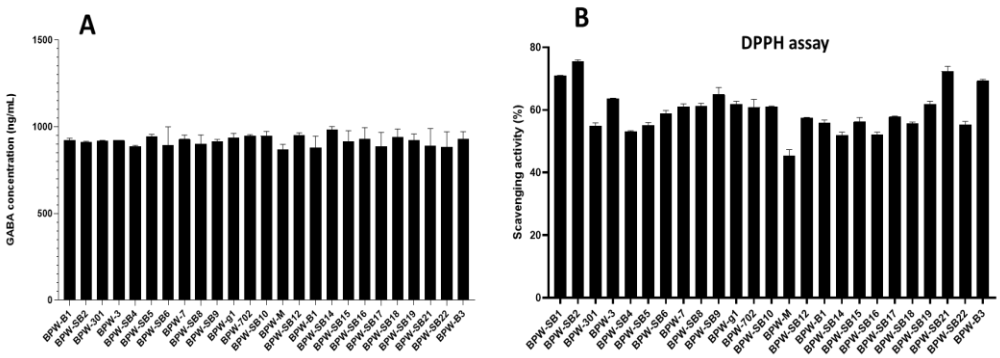

Figure 1 A). Results of GABA production assay in *Bacillus* isolates from honey. and stingless honey by ELISA method. B). Antioxidant activity test results of samples from *Bacillus* supernatants showing antioxidant activity compared to vitamin C as standard antioxidant.

CONCLUSIONS

Through rigorous screening of *Bacillus* strains isolated from honey and stingless honey for gamma-aminobutyric acid (GABA) production and antioxidant activity, distinct strains showcased exemplary performance. *F. fructosus* BPW-SB21, *F. fructosus* BPW-SB17, and *B. amyloliquefaciens* BPW-SB14 emerged as the premier GABA producers, registering concentrations of 985.59 ± 0.4, 963.65 ± 1.3, and 962.51 ± 1.1 ng/mL, respectively. In terms of antioxidant prowess, *B. amyloliquefaciens* BPW-SB2, *F. fructosus* BPW-SB21, and *R. pycnus* BPW-SB1 stood out. Their scavenging abilities, as assessed through the DPPH assay, were 75.44 ± 0.5, 72.30 ± 1.6, and 70.80 ± 0.2, respectively.

Furthermore, this investigation underscores the potential of honey and stingless honey as rich reservoirs of diverse *Bacillus* strains, which bear significant promise for practical applications.

ACKNOWLEDGEMENTS

This work was supported by National Higher Education, Science, Research and Innovation Policy Council, Thaksin University (Research project grant) Fiscal Year 2022. Special thanks to the Microbial Technology for Agriculture, Food, and Environment Research Center, Faculty of Science, Thaksin University, for the generosity and laboratory research tools.

REFERENCES

Adebayo-Tayo, B., & Fashogbon, R. (2020). In vitro antioxidant, antibacterial, *in vivo* immunomodulatory, antitumor and hematological potential of exopolysaccharide produced by wild type and mutant *Lactobacillus delbureckii* subsp. bulgaricus. Heliyon, 6(2), https://doi.org/10.1016/j.heliyon.2020.e03268

Anadon, A., Martinez-Larranaga, M. R., & Aranzazu Martinez, M. (2006). Probiotics for animal nutrition in the European Union. Regulation and safety assessment. Regul Toxicol Pharmacol, 45(1), 91-95. doi:10.1016/j.yrtph.2006.02.004

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

Bora, S., Sarma, K., Das, S., & Barooah, M. (2016). Structural and Functional analysis of Glutamate Decarboxylase System in *Bacillus aryabhattai*. Research journal of biotechnology, 11, 1-11.

FAO. (2017). Probiotics in animal nutrition: production, impact and regulation: FAO.

Gangalla, R., Sampath, G., Beduru, S., Sarika, K., Govindarajan, R. K., Ameen, F., . . . Thampu, R. K. (2021). Optimization and characterization of exopolysaccharide produced by *Bacillus aerophilus* rk1 and its in vitro antioxidant activities. Journal of King Saud University-Science, 33(5), 101470. https://doi.org/10.1016/j.jksus.2021.101470

Kadaikunnan, S., Rejiniemon, T. S., Khaled, J. M., Alharbi, N. S., & Mothana, R. (2015). In-vitro antibacterial, antifungal, antioxidant and functional properties of *Bacillus amyloliquefaciens*. Annals of Clinical Microbiology and Antimicrobials, 14(1), 9. doi:10.1186/s12941-015-0069-1

Liu, Q., Cheng, H., Ma, X., Xu, N., Liu, J., & Ma, Y. (2016). Expression, characterization and mutagenesis of a novel glutamate decarboxylase from *Bacillus megaterium*. Biotechnology Letters, 38. doi:10.1007/s10529-016-2070-y

Ngo, D.-H., & Vo, T. S. (2019). An updated review on pharmaceutical properties of gamma-aminobutyric acid. Molecules, 24(15), 2678. https://doi.org/10.3390/molecules24152678

Saleem, M., Malik, S., Mehwish, H. M., Ali, M. W., Hussain, N., Khurshid, M., . . . Chen, Y. (2021). Isolation and functional characterization of exopolysaccharide produced by *Lactobacillus plantarum* S123 isolated from traditional Chinese cheese. Arch Microbiol, 203, 3061-3070.

Sun, L., Bai, Y., Zhang, X., Zhou, C., Zhang, J., Su, X., . . . Tu, T. (2021). Characterization of three glutamate decarboxylases from *Bacillus* spp. for efficient γ-aminobutyric acid production. Microbial Cell Factories, 20(1), 153. doi:10.1186/s12934-021-01646-8

Wan-Mohtar, W. A. A. Q. I., Sohedein, M. N. A., Ibrahim, M. F., Ab Kadir, S., Suan, O. P., Weng Loen, A. W., . . . Ilham, Z. (2020). 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(6), 652. Retrieved from https://www.mdpi.com/2227-9717/8/6/652

Wang, H., Huang, J., Sun, L., Xu, F., Zhang, W., & Zhan, J. (2019). An efficient process for co-production of γ-aminobutyric acid and probiotic *Bacillus subtilis* cells. Food Sci Biotechnol, 28(1), 155-163. doi:10.1007/s10068-018-0461-7

Yowtak, J., Wang, J., Kim, H. Y., Lu, Y., Chung, K., & Chung, J. M. (2013). Effect of antioxidant treatment on spinal GABA neurons in a neuropathic pain model in the mouse. PAIN®, 154(11), 2469-2476.

Zhang, S., Zhao, J., Hu, J., He, H., Wei, Y., Ji, L., & Ma, X. (2022). Gama-aminobutyric acid (GABA) alleviates hepatic inflammation via GABA receptors/TLR4/NF-κB pathways in growing-finishing pigs generated by super-multiparous sows. Animal Nutrition, 9, 280-290.

Zulkhairi Amin, F. A., Sabri, S., Ismail, M., Chan, K. W., Ismail, N., Mohd Esa, N., . . . Zawawi, N. (2019). Probiotic Properties of *Bacillus* Strains Isolated from Stingless Bee (*Heterotrigona itama*) Honey Collected across Malaysia. Int J Environ Res Public Health, 17(1). doi:10.3390/ijerph17010278