Production of gamma-aminobutyric acid by lactic acid bacteria of marine origin from Vietnam

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Abstract: Gamma-aminobutyric acid (GABA) is a non-proteinogenic amino acid, which functions as a neurotransmitter in the central nervous system. GABA has been shown to have several positive effects on human health, including reducing anxiety and promoting relaxation. In this study, various fermented fish products were screened for their ability to produce GABA. A total of 35 acid lactic producing strains were isolated from 8 samples of marine organism, 10 strains were found to have GABA-producing abilities. These strains were identified using 16S rDNA sequencing. The GABA concentrations produced by the 10 isolates ranged from 725 to 5590 mg/L. *Lactobacillus brevis* GB111 is the best isolated GABA producing strain, with a conversion rate up to 90.3%, which suggests that these strains may have potential applications in the food and pharmaceutical industries as a source of GABA.

1 Introduction

Gamma-aminobutyric acid (GABA) is a naturally occurring amino acid found in a variety of organisms, including bacteria, plants, and vertebrates. Its formation is primarily facilitated by the catalysis of glutamic acid decarboxylase, which transforms L-glutamic acid or its salts into GABA [1]. GABA constitutes a major inhibitory neurotransmitter in the sympathetic nervous system and has antidepressant [2], antihypertensive [3], positive antioxidant, and anti-diabetic effects in humans [4]. Additionally, GABA has been found to delay or inhibit the invasion and metastasis of various types of cancer cells, including those found in the mammary gland, colon, and liver [4]. Due to its multiple physiological functions and positive effects on human health, GABA is considered a bioactive natural compound that is found in many foods. However, the amount of GABA in the typical daily human diet is relatively low, which has led to an increasing demand for GABA-enriched food products [5].

Lactic acid bacteria (LAB) have become a popular subject of interest for the food industry, particularly because they are generally regarded as safe (GRAS) for human consumption. The production of GABA using (LAB) has enormous potential in the field of food science and health promotion. Several LAB strains have been identified as GABA-producers, including *Lactobacillus fermentum*, which has been shown to have a high GABA-

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producing ability [7]. *Lactobacillus brevis* is another well-studied GABA-producing LAB strain, with numerous studies reporting its ability to produce GABA [2,8,9,10]. Other LAB strains that have been identified as GABA-producers include *Lactococcus lactis* [11], *Lactobacillus paracasei* [12], and *Lactobacillus plantarum* [13]. The discovery of these GABA-producing LAB strains has provided a valuable tool for the production of natural and safe GABA, with numerous potential health benefits. Marine LAB have gained significant attention in recent years as a valuable source of novel bioactive compounds. They possess unique traits and biological activities that differentiate them from their terrestrial counterparts. One key factor that contributes to their distinctiveness is their ability to thrive under the challenging physicochemical conditions of the marine environment. This endurance gives them a competitive edge over terrestrial LAB and may account for the distinctive features observed in marine LAB by-products [14]. Therefore, further studies are needed to fully explore the potential of these bacterial strains and their possible applications.

The aim of this study was to screen various types of GABA-producing bacteria from marine fish in Vietnam. The findings of this study would contribute to our understanding of the unique characteristics of marine LAB and their potential for biotechnological applications.

2 Materials and methods

2.1 Materials

- Samples: 08 marine fish samples were collected from Vietnam
- Media: De Mann, Rogosa Sharpe (MRS) medium agar and MRS broth with 1% mono sodium glutamate (MSG).

2.2 Methods

Isolation of GABA-Producing LAB from Fish Intestines: The fish were kept on ice (~0 °C) and were then dissected aseptically. The fish intestine contents were homogenized with 0.1% peptone water [10]. The serial dilutions were subsequently prepared in sterile PBS and inoculated onto MRS medium followed by incubation at 37°C for 48 h.

Screening of GABA-Producing LAB Using Thin-Layer Chromatography (TLC): The isolated strains were incubated in MRS broth with 1% MSG at 37 °C without shaking for 48 h. The GABA-containing cell culture was centrifuged and spotted 2 µL bacterial culture supernatant 2 µL GABA solution (1 mg/ml), and 2 µL MRS medium (1%) onto TLC plates. The TLC running solvent contained n-butanol, acetic acid, and distilled water (5:3:2) with 1,2% ninhydrin. After TLC analysis, the TLC plates were dried at 105 °C.

Quantification of GABA-producing bacteria GABA concentrations in cultured broths were determined by pre-staining paper chromatography [14]. A 2 µl of supernatants were spotted onto cellulose plates and developed at 300 C with n-butanol-acetic acid-water (5:3:2) containing 1.2% ninhydrin. After development, GABA spots were scratched out from the paper and were extracted with 5 ml of 75% alcohol (v/v):0.6% cupric sulfate (w/v) (38:2) at 400 C. The absorbance was read using spectrophotometer at 512 nm. The purity of GABA was also determined by HPLC as reference procedures.

DNA extraction: Genomic DNA was isolated using Zymo Research Kit (USA).

Sequence analysis: A 1 µl of supernatantwas used as a template in PCR. The 16S rRNA genes were amplified using a pair of universal primers corresponds to positions 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTT ACGACTT-3'). The PCR cycling conditions were: initial denaturation at 95°C for 5 min, 35 cycles of

denaturation at 95°C for 30 sec, annealing at 55°C for 30 sec, and extension at 72°C for 30 sec, and a final extension at 72°C for 5 min. Sequence comparisons were performed using the Basic Local Alignment Search Tool (BLAST) available at the National Center for Biotechnology Information (NCBI; National Institutes of Health, USA) and phylogenetic tree was performed using MEGA X.

Identification of GAD gene sequence: using a pair of primers: GAD forward primer (5' - ATGGCAATGTTATACGGTAAACAC- 3') and GAD reverse primer (5' – TCAGTGTGTGAATAGGTATTTCTTAGGT-3').

3 Results and discussions

3.1 Isolation and screening of GABA-producing strains

The isolation and identification of bacterial strains are crucial for studying their characteristics and their potential applications in various fields. We isolated 35 bacterial strains from the intestines of five fish on MRS agar plates. The colonies were presumptively identified as putative LAB (Table 1).

In order to isolate LAB producing GABA, *35* isolates selected on MRS agar plates were firstly screened to isolate GABA-producing strains with 1% MSG, resulting that 10 isolates were identified as showing spots at the position corresponding to GABA on the TLC plates (Fig 1). This result indicates that these 10 strains have the potential to produce GABA.

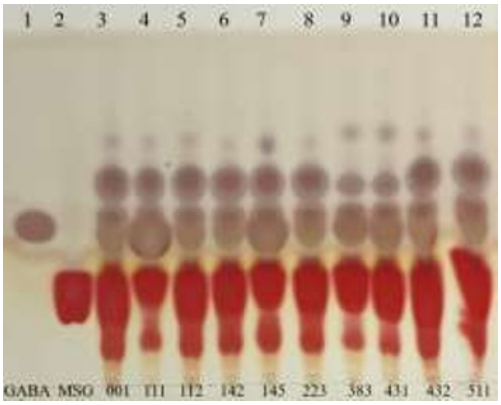

Fig. 1. Screening of GABA producing bateria using TLC plate. Lane 1: GABA standard at concentration of 1 mg/ml; lane 2: MSG at concentration of 1%; lane 3-12: presented bacterial isolated cultured in MRS broth with 1% MSG

Table 1. Isolation of GABA-producing strains from marine fish from Vietnam

№	Source isolated	№ of isolates	№ of GABA production
1	Silver pomfret *(Pampus argenteus)*	3	1
2	Black pomfret *(Parastromateus niger)*	4	1
3	Smudgespot spinefoot *(Siganus canaliculatus)*	6	2
4	Milk fish (*Chanos chanos)*	5	2
5	Shortfin saury *(Saurida umeyoshii)*	4	1
6	Indian mackerel (*Rastrelliger kanagurta)*	5	1
7	Round scad ***(****Decapterus punctatus****)***	5	2
8	Giant tiger prawn *(Penaeus monodon)*	3	0
Total		**35**	**10**

Table 2. Morphological and biochemical characteristics of LABs

Strains	Group I	Group II	Group III	Group IV
Number of isolates	2	5	1	2
Colody	Light yellow colored, small, circular, smooth, and slightly raised structure on agar plates	Cream colored, small, circular, convex structure on agar plates	White colored, small, circular, smooth, and slightly raised structure on agar plates	Creamy white colored, small, circular, convex structure on agar plates
Shape	Short and rod-shaped with rounded ends, occurring singly or in pairs, length of 0.5 to 1.0 µm, width of 0.5 to 0.8 µm	rod-shaped and occur singly, in pairs or in chains, length of 1.0 to 1.5 µm, width of 0.5 to 1.0 µm	rod-shaped and occur singly or in pairs, length of 0.5 to 2.5 µm, width of 0.5 to 0.8 µm	rod-shaped and occur singly or in pairs length of 0.5 to 1.5 µm, width of 0.5 to 0.8 µm
Gram stain	G^+	G^+	G^+	G^+
Catalase	-	-	-	-
Oxidase	-	-	-	-
Motility	-	-	-	-
Fermentation type	hetero	homo	hetero	homo
Spore forming	-	-	-	-
Growth at temperature 15-45 °C	+	+	+	+
Growth at pH 3 -9	+	+	+	+
Growth in NaCl at concentration of 1-10%	+	+	+	+
GABA production yield (mg/L)	2185 - 5590	725 - 1620	835	755 - 910
GABA conversion yield (%)	35.3 - 90.3	11.7 - 26.2	13.5	12.2 - 14.7

Gram: G^+, Gram-positive, G^-,Gram-negative; Catalase: +, producing hydrogen peroxide; -, no hydrogen peroxide produced; Fermentation type: Homo, homofermentative; Hetero, heterofermentative; Motility and Spore forming: -, non ability, +, ability; Growth: +, normal growth; -, no growth

3.2 Morphological and biochemical characteristics of LABs isolated

In this study, the 10 GABA-producing bacteria were grouped into four groups based on several characteristics such as their colony morphology, cell shape, size, motility, and their ability to ferment different carbohydrates. Additionally, their enzymatic activities, pH, and temperature tolerance may have been evaluated.

The results showed that all 10 isolated strains exhibited morphological and biochemical characteristics typical of *Lactobacillus*, such as Gram positive, rod-shaped cells, non-motility, non-spore-forming, and the ability to grow at a low pH.... Apart from morphology, the study also identified the homo- and hetero-fermentative properties of these bacterial strains. The ability of the bacteria to grow in media containing varying concentrations of NaCl, ranging from 0% to 10%, was also observed in this study Furthermore, our study revealed that the majority of the isolates were able to grow at both low (15°C) and high (45°C) temperatures, indicating their potential adaptability to a wide range of environmental conditions.

All 10 isolates of GABA-producing bacteria were capable of producing GABA at varying concentrations, with the range spanning from 725 mg/l to 5590 mg/l. Interestingly, three isolates, GB111, GB145 and GB384, exhibited high GABA production capabilities, with GABA content exceeding 1000 mg/L. The isolate GB111, which was obtained from the Smudgespot spinefoot, was identified as the highest producer among all the isolates with a conversion rate up to 90.3%

3.3 Molecular characterization of LABs using 16S rRNA sequence analysis

The 16S rRNA gene sequence analysis is an essential tool to identify bacterial species. In this study, all 10 isolated strains were analyzed using the BLAST search program at the NCBI website. The 16S rRNA gene sequencing of all 10 isolated were identified from 99 to 100%. Two strains from group I (GB111 and GB145) were highly similar to *Lactobacillus brevis*. Group II identified as the *Lactobacillus plantarum* with five strain (GB001, GB112, GB223, GB384, GB431). Two LABs from group IV (GB142 and GB432) belonged to *Lactobacillus paracasei*, and the only strain from group III (GB511) was *Lactobacillus buchneri*. The use of 16S rRNA sequence analysis is critical in ensuring the reliability, quality assurance, and safety of bacteria, enabling accurate differentiation between pathogenic and non-pathogenic bacteria. The dendrogram displays the phylogenetic relationships between the LABs and closely related bacteria (Fig 2).

The GAD gene is responsible for the production of gamma-aminobutyric acid (GABA) in lactic acid bacteria (LAB). To confirm the GABA-producing ability of two different LAB strains, genomic DNA was extracted from their cultures. Then, PCR was performed using the optimized GAD primer set to amplify the GAD gene fragment from the genomic DNA. The amplified PCR products were analyzed by electrophoresis on an agarose gel with a concentration of 1%, which allowed the visualization of the DNA fragments based on their size. By comparing the band intensity and size of the PCR products, the GABA-producing ability of the two strains could be determined based on the presence or absence of the expected PCR product size. The gel electrophoresis image showed that the primer could amplify a gene fragment of approximately 1400 bp, which is consistent with the size of the published specific gene group, indicating that the primer was effective in amplifying the GAD gene fragment [15].

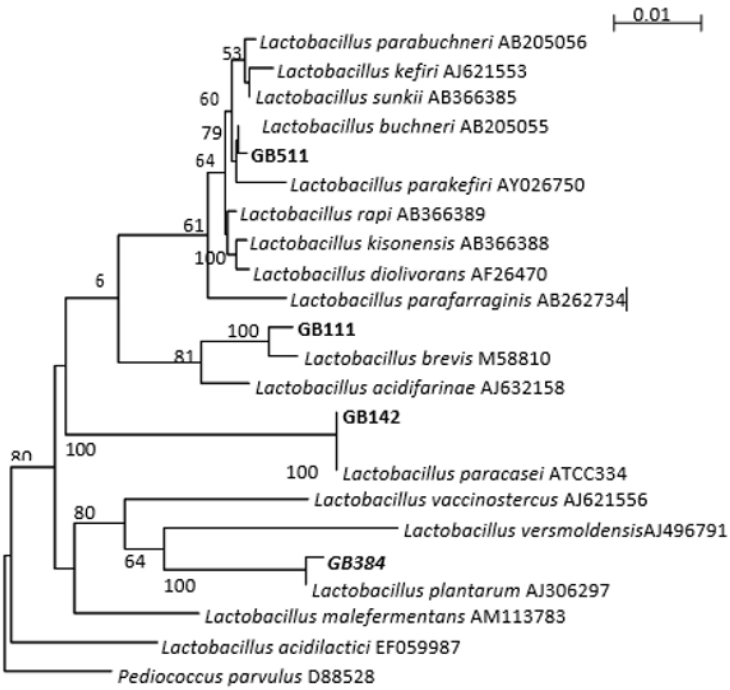

Figure 2. Neighbor-joining tree based on 16S rRNA gene sequences of representative strains

Furthermore, when the PCR reaction was carried out using the GAD primer set and DNA samples from lactic acid bacteria that do not produce GABA (GB110), no DNA bands were observed on the gel electrophoresis image, indicating the presence of the GAD gene in the isolated. The identification of the GAD gene sequence in LABs is also important as it provides insights into the genetic basis of GABA production. By conducting further studies to optimize GABA production by this strain under conditions reinforced with glutamate, we hope to improve the yield of GABA and develop a better understanding of the factors influencing GABA production. This could ultimately lead to the development of more efficient and cost-effective methods for producing GABA using microbial fermentation.

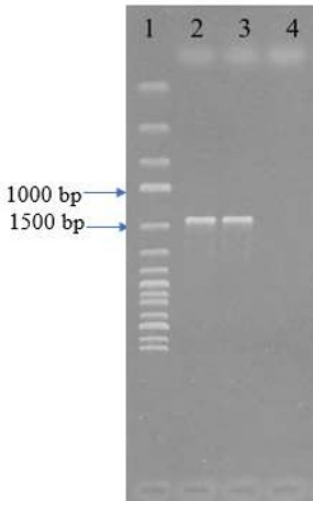

Fig. 3. PCR amplification of the GAD gene from isolated with the primer pair GAD forward/ reverse. Lane 1: Marker; Lane 2: GB111; Lane 3: GB384; Lane 4: GB110

4 Conclusions

Ten LAB strains from marine fish were isolated and identified according to their physiologycal and biochemical characteristic. Overall, our taxonomical and fermentation potential analysis of the isolated GABA producers provided valuable insights into the diversity and functional capabilities of these bacterial strains, which could have potential applications in various industrial and biotechnological processes. Further studies are needed to explore the full potential of these strains and their possible applications.

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