Research Article

pISSN: 2345-5357
eISSN: 2423-4214

OPEN ACCESS

*APPLIED FOOD BIOTECHNOLOGY, 2022, 9 (1):1-8*
*Journal homepage: www.journals.sbmu.ac.ir/afb*

# Isolation, Screening, Identification and Optimization of Culture Parameters to Produce γ-aminobutyric Acid by *Lactiplantibacillus pentosus* R13, an Isolate from Ruoc (Fermented Shrimp Paste)

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## Abstract

**Background and Objective:** Production of γ-aminobutyric acid has recently much interested because of its benefits for health. The objective of this study was to optimize γ-aminobutyric acid production by a novel identified *Lactiplantibacillus pentosus* isolated from a fermented shrimp paste of ruoc.

**Material and Methods:** A species of lactic acid bacterial was isolated from 'ruoc', a high-salt fermented shrimp paste and identified using matrix-assisted laser desorption/ionization-time of flight mass spectrometry. The γ-aminobutyric acid production was optimized using various culture conditions (initial cell density from $5 \times 10^5$ to $5 \times 10^7$ CFU $ml^{-1}$, monosodium glutamate concentration of 0.5-2% (w $v^{-1}$), initial pH of 4-9, incubation temperature of 30-50 °C and incubation time of 24-120 h) with one-factor-at-a-time approach.

**Results and Conclusion:** Of 20 lactic acid bacteria isolated from 'ruoc', four isolates of R1, R3, R12 and R13 produced significant quantities of γ-aminobutyric acid. Isolate R13 produced the highest γ-aminobutyric acid quantity, identified as *Lactiplantibacillus pentosus* using matrix-assisted laser desorption/ionization-time of flight mass spectrometry. A culture media optimization study was carried out for *Lactiplantibacillus pentosus* R13 to improve its γ-aminobutyric acid yield. Results showed that at optimal conditions of 1.5% monosodium glutamate (w $v^{-1}$), initial pH of 7, initial cell density of $5 \times 10^6$ CFU $ml^{-1}$, cultivation temperature of 45 °C and fermentation time of 96 h, *Lactiplantibacillus pentosus* R13 produced 23.34 mM ±0.11 of γ-aminobutyric acid. In conclusion, γ-aminobutyric acid production by this isolate was verified to be heavily dependent on monosodium glutamate concentration, initial cell density, initial pH, incubation temperature and fermentation time.

**Article Information**

**Article history:**
- Received 13 Sep 2021
- Revised 4 Oct 2021
- Accepted 16 Oct 2021

**Keywords:**
- Fermentation
- γ-aminobutyric acid
- *Lactiplantibacillus pentosus*
- Lactic acid bacteria
- Optimization

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**Conflict of interest:** The authors declare no conflict of interest.

**How to cite this article**

*Bich Thuy DT, Nguyen T-A, Vandamme P. Isolation, Screening, Identification and Optimization of Culture Parameters to Produce γ-aminobutyric Acid by Lactiplantibacillus pentosus R13, an Isolate from Ruoc (Fermented Shrimp Paste).Appl Food Biotechnol. 2022; 9 (1): 1-8. http://dx.doi.org/10.22037/afb.v9i1.36103*

## 1. Introduction

Use of lactic acid bacteria (LAB) as starters for food fermentation has become popular as lactic acid is an effective preservative and a flavor enhancer [1]. In addition, LAB are often used for the production of functional foods because of their probiotic potential and production of vitamins (e.g. thiamine, nicotine, folic acid, pyridoxine and vitamin $B_{12}$), free amino acids, exopolysaccharides and short chain fatty acids (SCFA) [2-4]. Naturally, LAB can produce a non-protein amino acid or γ-aminobutyric acid (GABA) [5,6], which acts as inhibitory neurotransmitter, hypotension

inductor, diuretic agent and tranquilizer [7,8]. Therefore, GABA is one of the most commonly used bioactive components in fermented foods [9]. The biochemical can be produced by a variety of species, including animals, plants and microorganisms. The GABA production by LAB is particularly interesting due to their safety, low production costs and narrow space requirements. Hence, several studies have focused on the enrichment of various food systems with GABA from LAB [10-14]. Biosynthesis of GABA in LAB is catalyzed by glutamic acid decarboxylase (GAD), including GAD enzyme and glutamate/GABA antiporter GadC. The L-glutamate can be transported into the cell via GadC and converted to GABA by decarboxylation under catalysis of GAD with pyridoxal-5'-phosphate as cofactor [8]. Then, GABA is exported extracellularly via GadC. Alternatively, L-glutamate can be produced intracellularly from α-ketoglutarate [8] and GABA can be degraded into succinate by GABA aminotransferase and succinate semialdehyde dehydrogenase [7,8]. Several factors, including pH and temperature, were reported to affect activity of GAD as well as GABA production in LAB species [15-17]. Thus, cultivation parameters need to be optimized for the LAB strains to achieve optimal GABA yields. *Ruoc* is a high-salt fermented paste that is produced by mixing small sea shrimps (*Acetes* sp.) with nearly 25% (w $w^{-1}$) of sea salt. Then, this mixture is compressed and incubated at ambient temperature for nearly 9-12 months. During *ruoc* production, proteins are hydrolyzed by intracellular shrimp enzymes, which occurs with microbial secondary metabolite formation simultaneously. This results in typical *ruoc* aroma and flavor. Up-to-date, no studies have been published on the production of GABA by LAB isolated from *ruoc*. Therefore, the aim of this study was to assess GABA-producing LAB isolates from *ruoc* samples in Vietnam. The isolate with the highest GABA producing ability was identified to the species level and its GABA production conditions were optimized.

## 2. Materials and Methods

### 2. Materials and Methods

### 2.1. Isolation of lactic acid bacteria

Commercial *ruoc* samples were purchased from local markets in Hue, Vietnam. Forty-five *ruoc* samples were homogenized in Ringer solution (Sigma-Aldrich, Milan, Italy), decimally diluted, spread on de Man, Rogosa and Sharpe (MRS) agar (Oxoid, Milan, Italy) and anaerobically incubated at 37 °C for 48 h. Isolates were reported as LAB if they were positive to Gram stain and negative to catalase test. The isolates were stored at -80 °C for further analysis.

### 2.2. Preparation of inoculant

Cells were activated in 10 ml of MRS broth at 37 °C for 24 h. The cell biomass collected after centrifuging at 12000× g for 5 min at 4 °C was resuspended in Ringer's solution. The $OD_{600}$ of the cell suspensions was measured and used to standardize cell density before using as inoculants.

### 2.3. Screening of γ-aminobutyric acid-producing lactic acid bacteria

Isolates were cultured in MRS broth containing 1% of monosodium glutamate (MSG) at 37 °C for 24 h with initial pH 6.2. Cultures were centrifuged at 12000× g for 5 min at 4 °C and GABA contents in the supernatants were measured using high-performance liquid chromatography (HPLC).

### 2.4. Identification of lactic acid bacteria

The LAB isolate R13 was identified through MALDI-TOF mass spectroscopy analysis as described before [18] using MBT Compass Explorer Software v.4.1 (Bruker Daltonik, Germany), BDAL Database (MSP-8468) and *in-house* identification databases. Samples for the analysis were prepared based on a method reported in a previous study [18]. The MS profiles of LAB were reported using MALDI TOF/TOF Analyzer 4800 Plus (Applied Biosystems, Framingham, MA, USA). Furthermore, 200-Hz tripled UV Nd:YAG laser was used to generate ions, which were accelerated in a 20-kV electric field through a grid at 19.2 kV. Ions were separated based on their m/z ratio in a 1.5-m linear field-free drift tube. Mass spectra were recorded through scanning in 2000-20000 Da mass ranges. Calibration was carried out before analyses using Protein Calibration Standard I (Bruker Daltonics, Leipzig, Germany). Identification criteria provided by the manufacturer were used to interpret identification scores. Matching between MALDI-TOF MS profiles from novel isolates and MALDI-TOF MS profiles in the reference database was expressed using BioTyper Software as log (score) and color code (green, yellow and red). BioTyper log (score) values greater than 2.3 and green color indicated a highly probable identification at the species level [19].

### 2.5. Assessment of optimal conditions for γ-aminobutyric acid production

The optimal cultivation parameters for GABA production by *Lactiplantibacillus* (*L.*) *pentosus* R13 were assessed using one-factor-at-a-time method. Moreover, GABA accumulated in the culture media was assessed using HPLC. Cell growth was assessed by measuring $OD_{600}$ of the culture media, which was normalized to log CFU $ml^{-1}$ using cell density standard curve. Cultivation parameters investigated in this study included an initial cell density from $5 \times 10^5$ to $5 \times 10^7$ CFU $ml^{-1}$, a monosodium glutamate concentration of 0.5-2% (w $v^{-1}$), initial pH of 4-9, incubation temperature of 30–50 °C and incubation time of 24-120 h.

### 2.6. High-performance liquid chromatography quantification of γ-aminobutyric acid

Sample preparation and HPLC analysis of GABA were carried out as described previously [17]. Briefly, GABA in

culture media was purified via centrifugation and elimination of proteins with sulfosalicylic acid, followed by dabsylation using 4-dimethylaminoazobenzen-4-sulfonyl chloride. Dabsyl-GABA was quantified using HPLC featured with C18 column (250 × 4.6 mm, 5-µm particle size), 465 nm of detection wavelength, isocratic eluent mode and mobile phase of 25 mM ammonium acetate buffer containing 0.1% of acetic acid:acetonitrile at a ratio of 26:74 (v $v^{-1}$). All analyses were carried out in triplicate.

### 2.7. Statistical analysis

Data were reported as mean ±SD (standard deviation) of triplicate analyses. One-way ANOVA followed by Tukey's HSD test were used to assess differences between the means. Differences were reported significant if $p \leq 0.05$. All statistical analyses of data were carried out using SPSS Software v.16 (SPSS, Chicago, USA).

## 3. Results and Discussion

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### 3.1. The γ-aminobutyric acid-producing ability of lactic acid bacteria

Ability of GABA production by LAB strains depends on activity of GAD (EC 4.1.1.15) system as well as L-glutamate dehydrogenase (GDH) [7,8]. Variations in the activity of these enzymes results in differences in GABA production by the strains. Siragusa et al. [20] reported that GABA yields were different between *Lactobacillus paracasei* PF6, *L. plantarum* C48 and *L. delbrueckii* subsp. *bulgaricus* PR1 isolated from cheese at similar conditions. Therefore, it is crucial to screen for the most potential LAB candidates for GABA enrichment in particular food products. In the current study, a total of 20 LAB isolates (data not shown) from *ruoc* were inoculated in MRS broth containing 1% MSG (w $v^{-1}$) at initial pH 6.2, which were then incubated at 37 °C for 24 h to assess their GABA-producing ability. Of these isolates, R1, R3, R12 and R13 isolates showed significant GABA production (Figure 1). Isolate R13 produced the highest quantity (14.69 mM ±0.16) of GABA. Therefore, this isolate was further identified and its culture conditions were optimized to maximize GABA production.

### 3.2. Identification of lactic acid bacteria R13 isolate

Isolate R13 was identified with a high identification log score (2.57) *L. pentosus* using MBT Compass Explorer Software v.4.1. (Bruker Daltonik, Germany) and therefore this identification was considered highly valid [19]. The identification result has verified several previous reports on the usefulness of MALDI-TOF MS for the species level identification of LAB isolates from various sources [19,21,22].

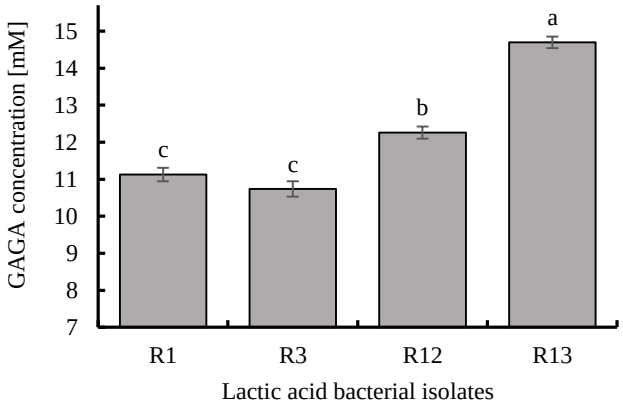


**Figure 1.** The γ-aminobutyric acid production by lactic acid bacteria from *ruoc*. All isolates were grown for 24 h at 37 °C in de Man, Rogosa and Sharpe broth containing 1% (w $v^{-1}$) of monosodium glutamate at initial pH 6.2. The γ-aminobutyric acid content was measured by high performance liquid chromatography. Data are means ±SD of triplicate experiments. Means that do not share a common letter differ significantly at $p \leq 0.05$

### 3.3. Optimization of cultivation parameters for γ-aminobutyric acid production by *Lactiplantibacillus pentosus* R13

#### 3.3.1. Initial cell density

Cell suspensions of *L. pentosus* R13 with cell count of $5\times10^5$ to $5 \times 10^7$ CFU $ml^{-1}$ were inoculated into MRS broth supplemented with 1% (w $v^{-1}$) MSG at initial pH 6.2 and incubated for 24 h at 37 °C. The highest GABA yield (15.31 mM ±0.17) was achieved at an initial cell density of $5 \times 10^6$ CFU $ml^{-1}$ (Figure 2).

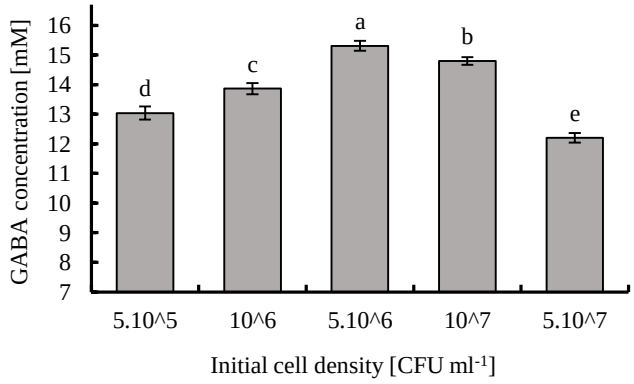


**Figure 2.** The γ-aminobutyric acid production by *Lactiplantibacillus pentosus* R13 at different initial cell densities. Cell suspensions with different initial densities were grown for 24 h at 37 °C in de Man, Rogosa and Sharpe broth containing 1% (w $v^{-1}$) of monosodium glutamate at initial pH 6.2. The γ-aminobutyric acid content was measured by high-performance liquid chromatography. Data are means ±SD of triplicate experiments. Means that do not share a common letter differ significantly at $p \leq 0.05$

Lower initial cell densities ($5 \times 10^5$ and $10^6$ CFU $ml^{-1}$) were possibly suboptimal to reach a maximum GABA production, while higher initial cell densities ($10^7$ and $5 \times 10^7$ CFU $ml^{-1}$) might be inhibited by a lack of nutrients, which could force cells to use GABA as a carbon and energy source. This result was similar to that from a previous study [17], which showed that *Pediococcus pentosaceus* MN12 produced higher quantities of GABA at an initial density of $5 \times 10^6$ CFU $ml^{-1}$, compared to higher initial cell densities of $10^7$ or $5 \times 10^7$ CFU $ml^{-1}$. Similarly, *Levilactobacillus namurensis* NH2 produced a higher GABA yield at a lower initial cell density (6 log CFU $g^{-1}$), compared to higher cell densities (7 and 8 log CFU $g^{-1}$) [23].

### 3.3.2. Monosodium glutamate concentration

Since glutamate is a source of carbohydrate for GABA synthesis by LAB [8], supplementation of glutamate into culture media is essential for achieving high GABA yields. In this study, effects of MSG supplementation on GABA production by *L. pentosus* isolate R13 were investigated using various MSG concentrations of 0-2% (w $v^{-1}$) with 0.5% increments while culture temperature, initial pH and fermentation time were 37 °C, 6.2 and 24 h, respectively. Concentration of GABA in the culture media increased with the increase of MSG concentration from 0 to 1.5% (w $v^{-1}$), reaching a maximum of 14.02 mM ±0.09 (Figure 3). However, GABA concentration decreased when the MSG concentration exceeded 1.5% (w $v^{-1}$). High MSG concentrations in the culture media might impose an extreme osmotic stress on the cells, interrupting GABA synthesis [8].

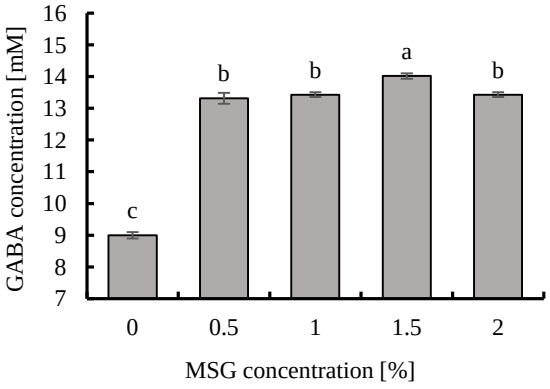


**Figure 3.** The γ-aminobutyric acid production by *Lactiplantibacillus pentosus* R13 at different monosodium glutamate concentrations. Cell suspensions with initial density of $5 \times 10^6$ CFU $ml^{-1}$ were grown for 24 h at 37 °C in de Man, Rogosa and Sharpe broth containing different monosodium glutamate concentrations at initial pH 6.2. The γ-aminobutyric acid content was measured by high-performance liquid chromatography. Data are means ±SD of triplicate experiments. Means that do not share a common letter differ significantly at $p \leq 0.05$

A similar dose dependency was reported by Tajabadi et al. [24] using *Lactiplantibacillus plantarum* Taj-Apis362, a honeybee isolate and by Komatsuzaki et al. [25] using *Lacticaseibacillus paracasei* NFRI 7415, a funa-sushi (Japanese traditional fermented fish food) isolate.

### 3.3.3. Initial pH

Technically, pH of the culture media is a key parameter for the production of GABA by LAB as it affects cell growth and GAD activity [8]. In this study, effects of initial pH on GABA synthesis by *L. pentosus* R13 were investigated by adjusting media pH from 4 to 8 prior to inoculation, while MSG supplement, initial cell density, culture temperature and growth time were maintained at 1.5% (w $v^{-1}$), $5 \times 10^6$ CFU $ml^{-1}$, 37 °C and 24 h, respectively. Results showed that GABA production by *L. pentosus* R13 increased with the increase of initial pH and reached a maximum yield of 16.72 mM ±0.16 at initial pH 7.0 (Figure 4).

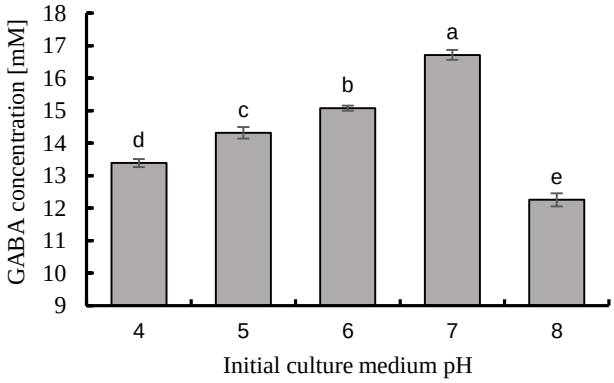


**Figure 4.** The γ-aminobutyric acid production by *Lactiplantibacillus pentosus* R13 at different initial pH values. Cell suspensions with initial density of $5 \times 10^6$ CFU $ml^{-1}$ were grown for 24 h at 37 °C in de Man, Rogosa and Sharpe broth containing 1.5% (w $v^{-1}$) monosodium glutamate at various medium pH values. The γ-aminobutyric acid content was measured by high-performance liquid chromatography. Data are means ±SD of triplicate experiments. Means that do not share a common letter differ significantly at $p \leq 0.05$

At higher initial pH, GABA accumulation in culture media decreased significantly; as previously reported for *P. pentosaceus* MN12 [17]. During the fermentation, lactic acid formation causes decreases in pH and induces GABA synthesis by GAD as this enzyme contributes to acid tolerance ability of the LAB by utilizing hydrogen ions [26]. In contrast, LAB such as *L. brevis* CGMCC 1306, *L. plantarum* C48, *Lb. paracasei*, *Streptococcus salivarius* subsp. *thermophilus* Y2 and *Lactococcus lactis* PU1 produce maximum quantities of GABA at pH 4-5 [14,20,25,27,]. Possibly an initial pH of 7.0 was favorable for the acclimatization of *L. pentosus* R13 and *P. pentosaceus* MN12 [17] cells in the culture media, which then accelerated cell growth and faster transferred the cells to the stationary phase, compared to other initial pH values. It is noteworthy that the media pH decreased to ~4.8 after 24 h of fermentation, which was the optimal pH for GAD activity [8].

### 3.3.4. Culture temperature

The GABA concentration achieved after 24 h of fermentation increased with increasing the fermentation temperature (Figure 5). The optimal temperature was 45 °C; at which, a GABA yield of 20.53 mM ±0.10 was achieved. Further increasing the incubation temperature to 50 °C decreased GABA yield to 13.70 mM ±0.07. This might be due to the high temperature stress on the metabolism of *L. pentosus* R13, particularly on the activity of GAD system. An optimal temperature of 45 °C was higher than that reported for other LAB, including 30-40 °C [25,27].

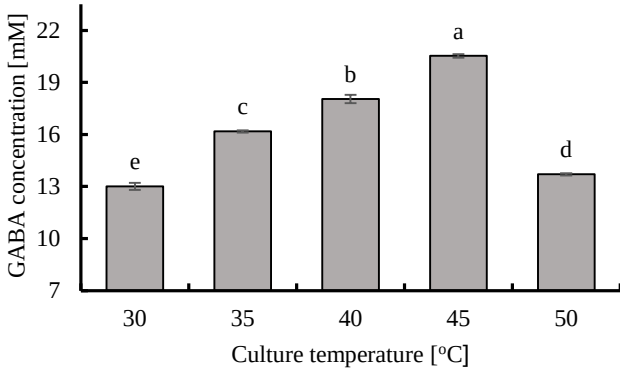


**Figure 5.** The γ-aminobutyric acid production by *Lactiplantibacillus pentosus* at different temperatures. Cell suspensions with initial density of $5 \times 10^6$ CFU $ml^{-1}$ were grown for 24 h in de Man, Rogosa and Sharpe broth containing 1.5% (w $v^{-1}$) monosodium glutamate at initial pH 7 and at different temperatures. The γ-aminobutyric acid content was measured by high-performance liquid chromatography. Data are means±SD of triplicate experiments. Means that do not share a common letter differ significantly at $p \leq 0.05$

### 3.3.5. Changes of pH, cell growth and γ-aminobutyric acid production by *Lactiplantibacillus pentosus* R13 during fermentation

To investigate kinetics of GABA production by *L. pentosus* R13, pH, cell density and GABA content in the media were assessed every 24 h during fermentation at optimal conditions. Within the first 24 h of incubation, pH of the media decreased from 7.0 to 4.8 (Figure 6a), with a sharp increase in the cell numbers from 6.70 to 9.12 log CFU $ml^{-1}$ (Figure 6b) and a rapid accumulation of GABA to 21.02 mM ±0.19 (Figure 6c). Changes in pH, cell yield and GABA content were similar to those reported by Lin [28] for *L. rhamnosus* YS9. From 24 to 96 h of fermentation, the cell number of *L. pentosus* R13 steadily increased with a gradual GABA accumulation to a maximum of 23.34 mM ±0.11.

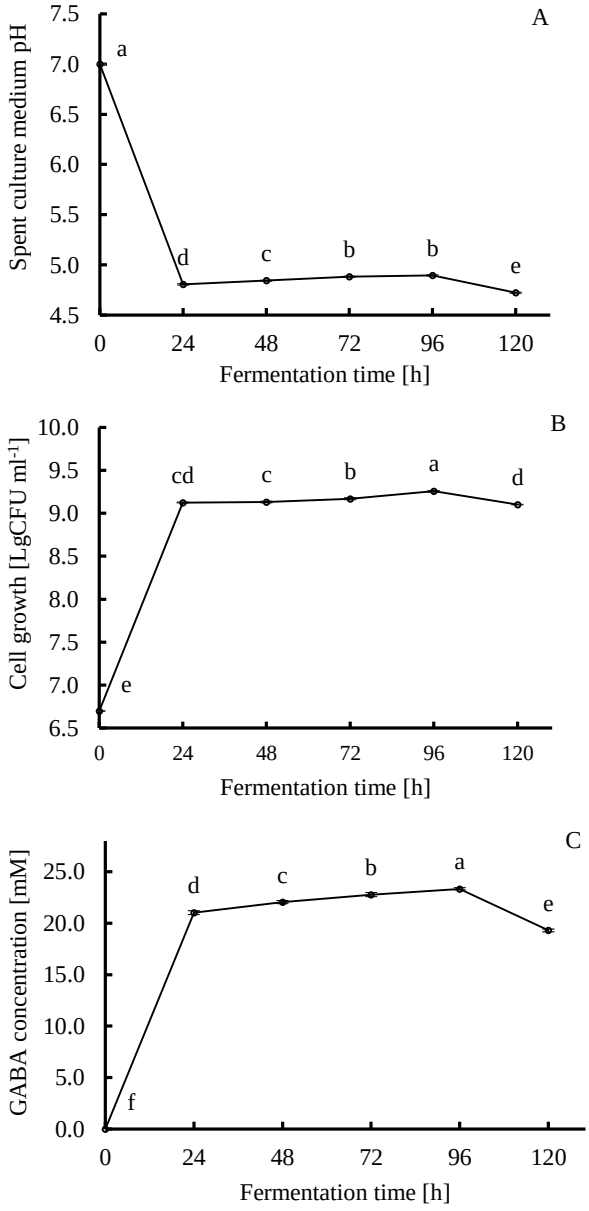


**Figure 6.** Changes of culture media pH (A), cell growth (B) and γ-aminobutyric acid production (C) during fermentation. Cell suspensions with initial density of $5 \times 10^6$ CFU $ml^{-1}$ were grown for 120 h at 45 °C in de Man, Rogosa and Sharpe broth containing 1.5% (w $v^{-1}$) monosodium glutamate at initial pH 7. The final pH, cell growth and γ-aminobutyric acid concentration were monitored at 24 h intervals. Data are means ±SD of triplicate experiments. Means that do not share a common letter differ significantly at $p \leq 0.05$

After 96 h, nutrient depletion likely triggered decreases in the cell growth and GABA production. Further decreases of media pH after 96 h were correlated to decreases in GABA concentration. Naturally, decarboxylation of glutamate to produce GABA utilizes hydrogen ions and the exchange of further alkaline GABA for glutamate increases the pH of environment [29]. Therefore, decreases in GABA production after 96 h might explain further decreases of media pH.

## 4. Conclusion

In the present study, four LAB isolates from *ruoc*, a high-salt fermented sea-shrimp paste, included capacities to produce GABA. Isolate R13 produced higher quantities of GABA and was identified as *L. pentosus* using MALDI-TOF MS analysis. Initial cell density of $5 \times 10^6$ CFU $ml^{-1}$ in broth media containing 1.5% (w $v^{-1}$) MSG, initial pH of 7.0, cultivation temperature of 45 °C and incubation time of 96 h were verified as the optimal production parameters, yielding 23.34 mM ±0.11 of GABA in culture media. Further studies are needed to use these results for the production of functional foods containing GABA as a primary bioactive component.

## 5. Acknowledgements

We are grateful to Margo Cnockaert at the Laboratory of Microbiology, Ghent University, Belgium, for her help with the identification of LAB isolates.

## 6. Conflict of Interest

The authors report no conflicts of interest.

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Research Article
pISSN: 2345-5357
eISSN: 2423-4214
OPEN ACCESS
APPLIED FOOD BIOTECHNOLOGY, 2022, 9 (1): 1-8
Journal homepage: www.journals.sbmu.ac.ir/afb

# جداسازی، غربالگری، شناسایی و بهینه سازی پارامترهای کشت به منظور گاما γ-آمینوبوتیریک اسید توسط *لاکتی‌پلانتی باسیلوس پنتوسوس* $R_{13}$ ، جداشده از *Ruoc* (خمیرتخمیر شده میگو)

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**تاریخچه مقاله**
دریافت ۱۳ سپتامبر ۲۰۲۱
داوری ۴ اکتبر ۲۰۲۱
پذیرش۱۶ اکتبر ۲۰۲۱

**واژگان کلیدی**
- تخمیر، γ- آمینوبوتیریک اسید
- *لاکتی پلانتی باسیلوس پنتوسوس*
- باکتری‌های لاکتیک اسید، بهینه-سازی

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## چکیده

**سابقه و هدف:** اخیرا تولید γ- آمینوبوتیریک اسید، به لحاظ فواید سلامت بخش متعدد بسیار مورد توجه قرار گرفته است. هدف این مطالعه بهینه سازی تولید γ- آمینوبوتیریک اسید توسط *لاکتی‌پلانتی باسیلوس پنتوسوس* جداشده از خمیر تخمیر شده میگوی ruoc بود.

**مواد و روش ها:** سویه‌های لاکتیک اسید باکتری‌ها از ruoc، خمیر تخمیر شده و بسیار شور میگو، جدا و توسط یونش/دفع لیزر به کمک ماتریس طیف سنج جرمی زمان پرواز [1] به همراه لیزر شناسایی شد. تولید γ- آمینوبوتیریک اسید با استفاده از شرایط گوناگون کشت (چگالی اولیه سلولی از $10^{5}$ تا $10^{7}$ CFU $ml^{-1}$ ×۵، غلظت مونوسدیم گلوتامات (w $v^{-1}$)٪ ۲-۰/۵، pH اولیه ۹-۴، درجه حرارت گرمخانه‌گذاری ۵۰-۳۰ °C و مدت زمان گرمخانه‌گذاری ۲۴ الی ۱۲۰ ساعت) با روش اثر یک عامل- در زمان بهینه شد.

**یافته‌ها و نتیجه‌گیری:** از ۲۰ باکتری لاکتیک اسید جدا شده از ruoc، چهار سویه جداسازی‌شده R1، R3، R12و R13 مقادیر قابل توجهی γ- آمینوبوتیریک اسید تولید کردند. R13 بیشترین میزان تولید γ- آمینوبوتیریک اسید را تولید و با استفاده از یونش/دفع لیزر به کمک ماتریس طیف سنج جرمی زمان پرواز به عنوان *لاکتی‌پلانتی باسیلوس پنتوسوس* شناسایی شد. به منظور بهبود راندمان تولیدγ - آمینوبوتیریک اسید توسط *لاکتی‌پلانتی باسیلوس پنتوسوس* R13 ، محیط کشت بهینه سازی شد. نتایج نشان داد تحت شرایط بهینه (غلظت مونوسدیم گلوتامات (w $v^{-1}$) ٪ ۱/۵، pH اولیه۷، چگالی اولیه سلولی از $10^{6}$ CFU $ml^{-1}$ ×۵ ، درجه حرارت کشت ۴۵ °C و مدت زمان تخمیر ۹۶ ساعت)، *لاکتی‌پلانتی باسیلوس پنتوسوس* R13، ۰/۱۱±۲۳/۳۴ mM γ- آمینوبوتیریک اسید تولید کرد. در نتیجه، تولید γ- آمینوبوتیریک اسید توسط این سویه جداسازی شده، وابستگی شدیدی به غلظت مونوسدیم گلوتامات، چگالی اولیه سلولی، pH اولیه، مدت زمان و درجه حرارت گرمخانه‌گذاری دارد.

**تعارض منافع:** نویسندگان اعلام می‌کنند که هیچ نوع تعارض منافعی مرتبط با انتشار این مقاله ندارند.

[1] matrix-assisted laser desorption/ionization-time of flight mass spectrometry or MALDI/TOF MS