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EVALUATING THE GAMMA-AMINOBUTYRIC ACID PRODUCING CAPACITY OF LACTIC ACID BACTERIA AND THEIR APPLICATION IN A COCONUT MATRIX

Phuong Hong Le[1*], Kha Mong Thi Phan[1], Phuong Thanh Thi Nguyen[1], Thu Anh Thi Nguyen[2], Hien Minh Nguyen[3]

[1]*Department of Chemical Engineering, Faculty of Chemical Engineering and Food Technology, Nong Lam University*

[2]*Ho Chi Minh City University of Industry and Trade*

[3]*Department of Food Microbiology and Product Development, Faculty of Chemical Engineering and Food Technology, Nong Lam University*

*Email: *phuonghle@hcmuaf.edu.vn*

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ABSTRACT

Gamma-aminobutyric acid (GABA) is a non-protein amino acid that provides numerous health benefits. GABA is produced from Glutamic acid (Glu) by Glutamate decarboxylase, an enzyme excreted by certain microorganisms. This study focused on the bioconversion of Glu in mature-coconut-water and young-coconut-kernel to develop GABA enriched food. First, seven strains of lactic acid bacteria (LAB) were screened for their GABA-producing capacity on de Man-Rogosa-Sharpe medium supplemented with Glu. Both thin layer chromatography and spectrophotometric results confirmed the ability of LAB to convert Glu to GABA. *Lactobacillus plantarum-H* was selected for application on the coconut substrate. The growth of colonies and the decrease in pH demonstrated the survival of *L. plantarum-H* on this food matrix. The fermentation of the mixture of mature coconut water:young coconut kernel (2:1, v/w) resulted in a GABA yield of 926.18 ± 25.71 mg/L. These findings highlight the potential to enhance the value of the coconut substrates and to develop GABA-enriched fermented foods, such as coconut yogurt, using GABA-producing LAB.

Keywords: Coconut substrate, fermentation, gamma-aminobutyric acid (GABA), lactic acid bacteria.

1. INTRODUCTION

Gamma-aminobutyric acid (GABA), a non-protein amino acid, works as an inhibitory neurotransmitter in mammalians. Several health benefits of GABA have been reported such as regulating blood pressure, improving neurological disorders, alleviating Alzheimer's and Parkison's diseases, and providing anti-diabetic effects [1–4]. Consequently, GABA supplementation in foods and the production of GABA-enriched products have become a trend in the food industry.

GABA is synthesized from Glutamate (Glu) by glutamate decarboxylase (GAD). This enzyme is produced by microorganisms or activated during seeds germination. Certain microorganisms, including mold and bacteria, have been reported for their GABA-producing

capacity, in which lactic acid bacteria (LAB) are the most produced strains [5,6]. Food materials rich in Glu content are suitable materials to produce GABA enriched foods by fermentation such as soy-yogurt, black raspberries fruit juice, tempeh and meat seasoning [7–10]. The yield of GABA produced strongly depends on the GABA-producing capacity of strains and fermentation conditions such as number of cultures inoculated, initial pH, fermentation temperature and time as well as the supplementation of precursor [5,11].

In Vietnam, the coconut (*Cocos nucifera*) is a key tree species that adapts well climate change and mangrove environments. This has led to the strategic development of coconut products to balance the government's responsibilities with generating profits for farmers. Young coconut water is consumed fresh, but the young coconut kernel (YCK) is not. In contrast, the mature coconut kernel is primarily used to produce coconut milk, while mature coconut water (MCW) has limited applications. In some countries, several coconut-based products have been developed, such as a refreshing beverage from mature coconut water blended with lemon juice [12], reduced-fat set coconut milk yogurt [13], and a fermented beverage from mature coconut water [8].

MCW has been evaluated for its high content of amino acids, carbohydrates, vitamins and minerals, which serve as a nutrient medium for the growth of microorganisms. Notably, Glu is the most abundant amino acid, with a concentration of 280.52 mg/100mL [8]. The high content of Glu makes it a potential precursor for synthesizing GABA, which offers significant health benefits. The possible conversion of Glu to GABA in MCW has been reported in previous studies [8,14,15].

This study, therefore, aimed to evaluate the fermentation of a combination of MCW and YCK by LAB to produce a coconut product enriched with GABA. Several LAB strains were screened to identify the strain having the highest GABA-producing capacity. Subsequently, this strain was applied to a coconut substrate to assess its ability to produce GABA within this matrix.

2. MATERIALS AND METHODS

2.1. Chemicals

DeMan-Rogosa-Sharpe was supplied by Himedia, India. GABA and monosodium glutamate (MSG) were supplied by Sigma-Aldrich, USA. All other chemicals used for the research were of analytical grade.

2.2. Microbiology preparation

LAB used in this study (Table 1) was supplied by Chr. Hansen and Danmark DHG Pharma, Vietnam. They were kept in the refrigerator at -18^{o}C for use. To prepare the stock culture, lyophilized culture was reactivated by inoculating in the MRS broth (Himedia, India) and incubating at their optimum growth temperature for 24 h. After that, this stock culture was used to prepare the working culture, which was incubated in the same conditions.

Initially, the LAB working cultures were assessed for their ability to produce GABA in MRS broth supplemented with MSG at concentrations ranging from 1% to 5%. Fermentation was carried out for 36 hours at the cultures' optimal growth temperature. The GABA content in the resulting fermented broths was determined using thin-layer chromatography and spectrophotometric techniques. The strain with the highest GABA production was selected for application on the coconut substrate.

Table 1. Lists of lactic acid bacteria studied

Number	Strain	Abbreviation	Optimum growth temperature
1	*Lactobacillus plantarum* - H	C1	30
2	*Probiotic Califlora* F- B19	C2	37
3	*Lactobacillus acidophilus*	C3	37
4	*Lactobacillus plantarum* LB – 1	C4	30
5	*Streptococcus thermophilus* STY12	C5	37
6	*Lactobacillus delbrueckii* YL- L812	C6	37
7	*Probiotic* ABY-10	C7	37

The culture used to ferment coconut substrate, which was called the starter culture, was prepared following the previous protocol [12]. Briefly, 1 mL of working culture was added into 10 mL of pasteurized coconut milk and incubated at the optimum growth temperature for 24 hours. This culture was prepared 1 day before performing the fermentation on coconut substrate [13].

2.3. Preparation of yogurt-like product from coconut milk

MCW and YCK were bought at the local market (Thu Duc, Ho Chi Minh City, Vietnam). The outside brown skin of the coconut kernel was gently removed and then washed again by sterilized water. Coconut substrate was prepared by grinding MCW with YCK (2:1, v/w) using a grinder (HR2108, Philip, Netherlands) for 2 min. After that, the mixture was pasteurized at 90^{o}C for 15 min, then cooled to room temperature to use as the substrate for the fermentation.

To evaluate the capacity of LAB fermentation on coconuts, the experiment was carried out on three media including MRS, coconut water and a mixture of MCW:YCK (2:1, v/w). The ratio of culture and substrate was set at 1%, 3% and 5% (v/w). The fermentation was performed for 6 h at the growth temperature of selected strain and then the final products were stored in the fridge at 4^{o}C for further analysis, including viable cells count, pH and GABA content.

2.4. pH and viable cell count

The pH was measured using a pH meter (Hanna, HI2002-02, Hungary). To determinate the viable cells count, samples were diluted in a 10-fold series, spread on MRS agar plates and incubated at growth temperature for 48 h. The number of LAB was counted, and results were expressed as log colony-forming units per mL (log CFU/mL).

2.5. Thin layer chromatography

Thin-layer chromatography (TLC) was used to screen the GABA-producing capacity of LAB [16]. Initially, cultures were inoculated into MRS broth containing MSG and incubated for 38h at the optimum temperature. Following incubation, the cultures were centrifuged at 4^{o}C for 10 min to obtain the supernatant. Subsequently, 10 µL of culture supernatant was spotted onto a TLC plate (Silica gel 60 F254, Merk, Germany). After that, n-butanol:acetic

acid:water (4:1:1, v/v/v) was used as the solvent system to separate the spots. The plate was left for a moment to allow the solvent to evaporate before being treated with 0.2% ninhydrin and heated at 65°C to visualize the spots.

2.6. GABA assay by spectrophotometric method

GABA was spectrophotometrically determined following the method described previously [17]. Firstly, the samples (inoculated LAB in MRS broth or coconut substrate) were extracted with EtOH 50% for 30 min at room temperature on the shaker (Dlab SK-O330, Korea). The extract was then centrifuged for 15 min at room temperature, in which the supernatant was collected and boiled in a water bath at 80 °C. This was followed by the addition of 0.5 mL distilled water, then 2 mL of 0.6% phenol and 0.4 mL of borate buffer (pH 9) were added, shaken well and cooled in ice for 5 min. Next, 0.4 mL of 8% NaOCl was added to the solution, mixed thoroughly and cooled in ice again. Finally, the solution was boiled in a water bath at 100°C for 10 min and then cooled to room temperature using tap water. The absorbance of the solution was spectrophotometrically detected at a wavelength of 630 nm (Spectro UV11, Germany) and the GABA content was calculated using the external standard curve [17].

2.7. Statistical analysis

Data was presented as mean ± standard deviation. One-way and two-way analysis of variance was carried out using SPSS 20 (IBM, USA) with the significant difference at $p < 0.05$. Tukey multiple range test was used to analyze the difference between treatments.

3. RESULTS AND DISCUSSION

3.1. Screening LAB for their GABA-producing capacity

3.1.1. Evaluating GABA production by thin layer chromatography

LAB collected from the laboratory was initially screened for its GABA-producing capacity using a qualitative method. MSG at concentration of 1%, 3% and 5% was added to MRS broth, which served as the cultivation medium for LAB. After 38 h of growth, GABA production was visualized using ninhydrin solution on TLC. Compared to line 1, the other lines appeared a round spot in the same row as the Glu and GABA standards, indicating the presence of GABA in the fermented product (Figure 1). This result demonstrated the role of Glu as a precursor for GABA production. The production of GABA by LAB in substrates rich in Glu was reported previously [8,18]. However, the appearance of a Glu spot suggested an excess amount of Glu in the MRS broth, preventing complete conversion into GABA by LAB.

Figure 1. Thin layer chromatography shows the GABA production of LAB

3.1.2. Evaluating GABA production of LAB by spectrophotometer

The initial viable cell counts of these LAB in MRS broth ranged from 6.90 ± 0.47 log CFU/mL to 8.31 ± 0.18 log CFU/mL (Table 2), with the variation influenced by the amount of MSG supplemented ($P < 0.05$). However, the average viable cell counts across these strains showed no significant difference ($P > 0.05$), indicating similar their growth patterns in the broth. These results demonstrate that LAB can survive in a medium supplemented with MSG. This finding aligns with Kook et al. (2010), which showed that LAB could grow in a medium with up to 15% MSG supplementation. However, when the MSG supplementation exceeded 7%, a decrease in cell growth was observed [19].

The growth of LAB was accompanied by a decrease in the pH of the medium, dropping from 6 to a range of 4.16 – 4.61, depending on the strains (Table 2). This finding aligns with previously obtained data, which indicate that bacterial survival is associated with lactic acid production, leading to a reduction in pH. Although fluctuations in pH were observed, no significant differences were detected ($P > 0.05$). Our results are consistent with prior research, which reported that the pH of MRS broth supporting the growth of GABA-producing LAB ranged from 4.2 to 5.2 [20]. Thus, the viable cell counts and the decrease in pH confirm the survival of LAB in MRS broth supplemented with MSG.

However, increasing the concentration of MSG supplemented in MRS broth did not lead to consistent changes in pH, contrasting findings from a previous study [21], which reported an increase in pH with higher MSG levels. MRS broth is as an optimal nutrient medium for LAB. In this study, extensive modifications to the medium's composition may have negatively affected LAB growth, limiting their ability to further reduce the medium's medium.

Table 2. Viable cell counts (log CFU/mL), pH and GABA content produced by LAB in MRS broth

Strains / Properties	MSG add (%)	C1	C2	C3	C4	C5	C6	C7
Viable cell count	0	7.66 ± 0.86^{A}	7.42 ± 0.58^{A}	7.67 ± 0.11^{A}	7.51 ± 0.36^{A}	7.41 ± 0.37^{A}	7.31 ± 0.28^{A}	8.31 ± 0.18^{A}
	1	7.80 ± 0.05^{AB}	7.53 ± 0.61^{AB}	7.20 ± 0.68^{AB}	7.32 ± 0.30^{AB}	7.35 ± 0.46^{AB}	7.20 ± 0.34^{AB}	7.85 ± 0.71^{AB}
	3	6.90 ± 0.47^{B}	6.92 ± 0.04^{B}	7.02 ± 0.13^{B}	6.84 ± 0.03^{B}	6.86 ± 0.38^{B}	6.74 ± 0.23^{B}	7.07 ± 0.32^{B}
	5	7.42 ± 0.13^{A}	7.77 ± 0.16^{A}	7.81 ± 0.91^{A}	7.38 ± 0.60^{A}	7.30 ± 0.22^{A}	7.67 ± 0.73^{A}	8.01 ± 0.46^{A}
pH	0	4.38 ± 0.48	4.32 ± 0.30	4.34 ± 0.15	4.61 ± 0.34	4.38 ± 0.04	4.29 ± 0.16	4.38 ± 0.16
	1	4.33 ± 0.35	4.16 ± 0.03	4.41 ± 0.29	4.50 ± 0.29	4.40 ± 0.02	4.29 ± 0.12	4.61 ± 0.24
	3	4.43 ± 0.35	4.26 ± 0.13	4.36 ± 0.24	4.42 ± 0.33	4.36 ± 0.04	4.26 ± 0.07	4.51 ± 0.23
	5	4.48 ± 0.4	4.23 ± 0.03	4.33 ± 0.24	4.30 ± 0.25	4.29 ± 0.08	4.25 ± 0.06	4.56 ± 0.16

Values are presented as mean ±SD
Different superscript capital letters (A and B) in the same column indicate the significant differences in viable cell counts among MSG supplementation ($P < 0.05$).

Since TLC results revealed the GABA-producing capacity of LAB, the spectrophotometric method was used to quantify the amount of GABA produced and data are presented in Figure 2. The rate of GABA production over time varied depending on LAB strains and fermentation conditions [6,11,22]. Previous studies reported a dramatic increase in GABA content after 10 h of fermentation [23]. In the preliminary test, only a slight increase in GABA was observed after 14 h of fermentation (data not shown), leading to the decision to extend the fermentation period to 38 h (Figure 2). Data analysis revealed significant

differences in the amount of GABA produced among strains, influenced by the concentration of MSG supplementation ($P < 0.05$).

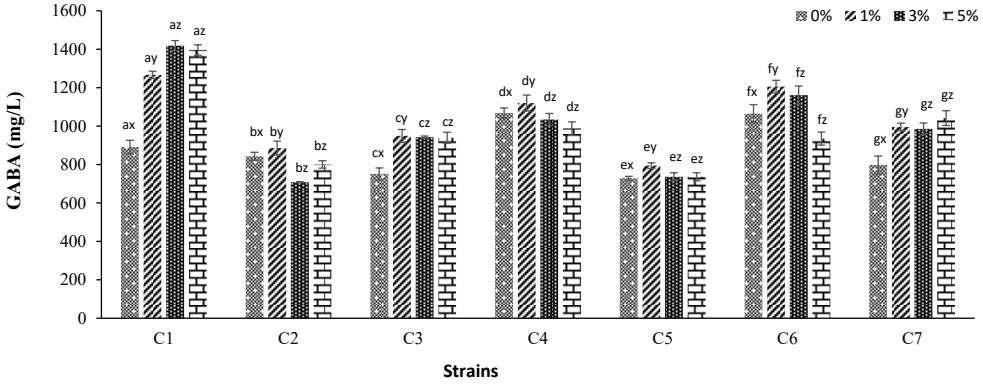

Figure 2. GABA content produced by bacterial strains at different MSG supplementation levels (0, 1, 3 and 5%)

Different superscript letters (a, b, c, d, e, f and g) indicate the significant differences in GABA content among strains (P < 0.05).

Different superscript letters (x, y and z) indicate the significant differences in GABA content among Glu supplementation (P < 0.05).

C1, C2, C3, C4, C5, C6 and C7 are *L. plantarum* – H, *Probiotic Califlora* F- B19, *L. acidophilus, L. plantarum* LB – 1, *S. thermophilus* STY12, *L. delbrueckii* YL- L812, *Probiotic* ABY-10, respectively.

Due to interference from components in the w-amino acid group (β-alanine, taurine, δ-aminovaleric acid, ε-aminocaproic acid, α and β-diaminopropionic acid, α and γ-diaminobutyric acid, α-hydroxy-γ-aminobutyric acid and 2-aminoethylphosphonic acid), a certain amount of GABA was detected in MRS broth [24]. When 1% MSG was added to the broth and fermentation was conducted, Glu was decarboxylated to produce GABA, resulting in a significant increase in GABA content. Among these strains, *L. plantarum* - H. produced the highest amount of GABA, reaching a value of 1267.43 ±18.27 mg/L whereas *Califlora* F-B19 exhibited the lowest conversion capacity. With MSG supplementation increased to 3%, *L. plantarum* - H. was the only strain capable of continuing the conversion of Glu to GABA, achieving 1419.47 ± 26.46 mg/L. At 5% MSG supplementation, GABA production by *Califlora* F- B19 and *Probiotic* ABY-10 increased slightly, while other strains (*L. plantarum* – H, *L. acidophilus*, *L. plantarum* LB-1 and LY-L812) either experienced a decline in GABA synthesis or remained constant (*S. thermophilus* STY12). Therefore, based on these results, *L. plantarum* – H was chosen to study the GABA producing by LAB in a coconut substrate.

3.2. GABA production in the coconut substrates

With the aim of valorizing coconut kernel and coconut water, two media - MCW and a mixture of MCW:YCK (2:1, v/w) - were fermented using *L. plantarum-H.* As a control, MRS medium supplemented with MSG was also used. Table 3 presents the viable cell counts across the three samples after 6 h of fermentation. These counts showed significant differences influenced by the concentration of *L. plantarum*-H applied ($P < 0.05$). Colony counts ranged from 7.21 to 8.84 log CFU/mL, depending on the substrate, with the highest observed in the MRS medium and the lowest in MCW. This variation reflects MRS as an optimal medium for the growth of LAB, while MCW may lack essential nutrients necessary for their proliferation.

Another study similarly noted that variations in substrate composition result in differences in probiotic growth [25].

The initial pH of MRS, MCW and the mixture of MCW:YCK were 6.0, 6.5 and 6.67, respectively. After 6 hours of fermentation, the pH of three media dropped significantly, influenced by the concentration of LAB used ($P < 0.05$). The decrease in pH value of these substrates indicated that the nutrients in coconut were suitable for the growth of *L. plantarum*-*H*, aligning with prior results. At low pH, coconut yogurt-like products were formed, characterized by a white, soft and homogenous texture. These samples were then stored in the fridge at 4 ^{o}C and their pH values remained almost constant. The use of 3% and 5% LAB resulted in similar pH values, which was significantly different from the substrate with 1% LAB. In this case, the higher the concentration of *L. plantarum*-H inoculated into the substrate, the greater the decrease in pH.

Table 3. pH and viable cell count of substrate fermented by different concentration of *L. plantarum*-H.

Substrates	Concentration of *L. plantarum*-H used (%)			Concentration of *L. plantarum*-H used (%)		
	1	3	5	1	3	5
	pH			Viable cell count (log CFU/mL)		
MRS	5.54 ± 0.06^{AX}	5.23 ± 0.05^{AY}	4.42 ± 0.08^{AY}	8.18 ± 0.12^{ax}	8.84 ± 0.15^{ay}	8.50 ± 0.43^{ax}
MCW:YCK	4.23 ± 0.02^{BX}	4.34 ± 0.02^{BY}	4.29 ± 0.06^{BY}	8.12 ± 0.20^{bx}	8.42 ± 0.01^{by}	7.86 ± 0.12^{bx}
MCW	5.35 ± 0.10^{CX}	4.32 ± 0.02^{CY}	5.31 ± 0.05^{CY}	7.21 ± 0.40^{cx}	7.63 ± 0.01^{cy}	7.54 ± 0.01^{cx}

Different superscript capital letters (A, B and C) indicate the significant differences in pH among substrate ($P < 0.05$).

Different superscript capital letters (X and Y) indicate the significant differences in pH among ratio of bacteria used ($P < 0.05$).

Different superscript letters (a, b and c) indicate the significant differences in viable cell count among substrate ($P < 0.05$).

Different superscript letters (x and y) indicate the significant differences in viable cell count among ratio of bacteria used ($P < 0.05$).

The GABA content obtained in the media varied significantly and was influenced by the concentration of LAB inoculated ($P < 0.05$). The highest amount of GABA was obtained in the MCW:YCK substrate, whereas MCW yielded the lowest GABA content (Figure 3). After 6 h of fermentation with 1% LAB, the GABA content in MRS reached 575.2 ± 41.65 mg/L, and continued to increase with a higher LAB concentration, reaching 833.82 ± 50.50 mg/L. In contrast, the amount of GABA synthesized in MCW was very low, approximately 31.08 ± 12.23 mg mg/L, which was 18.5 times lower than the amount of GABA produced in MRS. When the LAB concentration increased from 1% to 3%, the amount of GABA produced in MCW rose from 31.08 ± 12.23 mg/L to 79.51 ± 17.35 mg/L. The conversion of Glu in coconut water to GABA by *L. plantarum* was also reported in another study [14]. MCW fortified with 0.5% GSM produced 100.1 – 128 mg/L of GABA after 48 h of fermentation by *L. plantarum* DW12 [15].

When MCW was combined with YCK, the GABA content produced in this substrate increased dramatically. The nutrients in YCK supported the growth of *L. plantarum*-H. Indeed, YCK is rich in amino acids, with Glu being the most abundant one [8]. The GABA content in

the MCW:YCK mixture reached 789.90 ± 51.08 mg/L and 926.18 ± 25.71 mg/L at LAB concentrations of 1% and 3%, respectively, due to the conversion of Glu to GABA. However, a further increase in the concentration of *L. plantarum*-H to 5% resulted in a lower amount of GABA produced across three media, likely due to insufficient nutrients for the growth of *L. plantarum-H*. Thus, the low production of GABA was attributed to a lack of nutrients or Glu precursor in the substrates.

The attempt to ferment coconut substrate by LAB to develop coconut yogurt-like products has been reported previously [25,26]. Several characteristics of final products were assessed such as pH, color, viscosity, probiotic viability, syneresis and sensory evaluation. According to the literature, Glu is the most abundant amino acid found in coconut. However, fewer studies have investigated the bioconversion of Glu to GABA. In this study, utilizing the GABA-producing capacity of LAB, 926 mg/L of GABA was obtained from the coconut substrate, presenting an opportunity to develop GABA-enriched products from coconut water and coconut kernel.

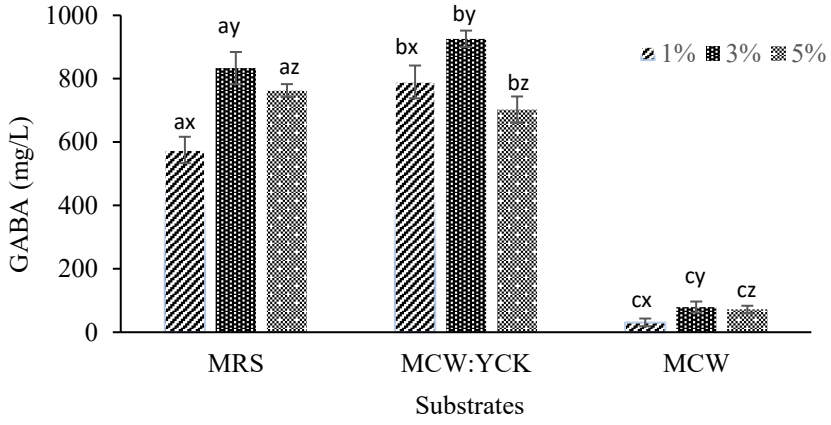

Figure 3. GABA production in MRS and coconut substrates.

Different superscript letters (a, b and c) indicate the significant differences among substrates (P < 0.05). Different superscript letters (x, y and z) indicate the significant differences among ratio of bacteria used. (P < 0.05).

4. CONCLUSION

This study successfully demonstrated that most LAB possess the capacity to produce GABA. The strains studied were able to survive in a medium supplemented with Glu and converted it into GABA, which offers numerous functional properties. When applied to a coconut substrate, *L. plantarum*-H grew well and transformed the natural Glu source found in coconut into 926 mg/L of GABA. The findings of this study highlight the potential for producing GABA-enriched fermented foods from coconut, such as yogurt-like products made from coconut milk.

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TÓM TẮT

ĐÁNH GIÁ KHẢ NĂNG SINH TỔNG HỢP AXIT GAMMA-AMINOBUTYRIC CỦA VI KHUẨN LACTIC VÀ ỨNG DỤNG TRÊN CƠ CHẤT DỪA

Lê Hồng Phượng[1*], Phan Thị Mộng Kha[1], Nguyễn Thị Thanh Phương[1], Nguyễn Thị Anh Thư[2], Nguyễn Minh Hiền[3]

[1]Bộ môn Công nghệ Hóa học, Khoa Công nghệ Hóa học và Thực phẩm, Trường Đại học Nông Lâm TP. Hồ Chí Minh

[2]Phòng Khoa học Công nghệ, Trường Đại học Công Thương Thành phố Hồ Chí Minh

[3]Bộ môn Vi sinh và Phát triển sản phẩm, Khoa Công nghệ Hóa học và Thực phẩm, Trường Đại học Nông Lâm TP. Hồ Chí Minh

*Email: *phuonghle@hcmuaf.edu.vn*

Axit gamma-aminobutyric (GABA) là một axit amin không tham gia trong cấu trúc của protein có nhiều lợi ích cho sức khỏe. GABA được sinh tổng hợp từ axit glutamic (Glu) nhờ vào xúc tác của glutamate decarboxylase, là một loại enzyme được tiết ra bởi một số vi sinh vật. Nghiên cứu này tập trung vào quá trình chuyển đổi sinh học của Glu trong nước dừa già và cơm dừa non để phát triển thực phẩm giàu GABA. Đầu tiên, bảy chủng vi khuẩn lactic (LAB) được sàng lọc về khả năng sản xuất GABA trên môi trường de Man-Rogosa-Sharpe có bổ sung Glu. Cả kết quả sắc ký lớp mỏng và quang phổ cho thấy các LAB này có khả năng chuyển đổi Glu thành GABA. *Lactobacillus plantarum*-H đã được chọn để ứng dụng trên cơ chất dừa. Kết quả tổng số khuẩn lạc và pH giảm cho thấy khả năng phát triển của *L. plantarum*-H trên nền cơ chất này. Quá trình lên men hỗn hợp nước dừa già:cơm dừa non (2:1, v/w) tạo ra sản phẩm có hàm lượng GABA là 926,18 ± 25,71 mg/L. Kết quả của nghiên cứu này cho thấy tiềm năng có thể phát triển thực phẩm lên men giàu GABA từ cơ chất dừa, chẳng hạn như sữa chua dừa, bằng cách sử dụng LAB để tổng hợp GABA.

Từ khóa: Axit gamma-aminobutyric (GABA), cơ chất dừa, lên men, vi khuẩn lactic.