https://doi.org/10.48048/tis.2024.8514

Reusable Immobilized *Lactobacillus futsaii* CS3 for Enhanced GABA Synthesis using Low-Cost Substrates in Fermenter-Scale Batch and Fed-Batch Fermentations

Kanokwan Thongruck and Suppasil Maneerat*

Center of Excellence in Innovative Biotechnology for Sustainable Utilization of Bioresources, Faculty of Agro-Industry, Prince of Songkla University, Songkhla 90110, Thailand

(*Corresponding author's e-mail: suppasil.m@psu.ac.th)

Received: 1 June 2024, Revised: 9 July 2024, Accepted: 16 July 2024, Published: 20 October 2024

Abstract

On the industrial scale, the cost of commercial culture media for the production of gamma-aminobutyric acid (GABA) is a very important factor. This study employed a low-cost substrates and by-product from agri-food industry for GABA synthesis by *Lact*. *futsaii* CS3 at the fermenter-scale. *Lact*. *futsaii* CS3 cells were immobilized in 3 % (w/v) sodium alginate and employed for GABA synthesis in the optimal modified MRS medium (3.48 % (w/v) cane sugar, 3.84 % (w/v) tuna condensate waste and 10.77 % (w/v) monosodium glutamate (MSG)) with the initial pH medium of 5, fermentation temperature at 37 °C and agitation speed at 30 rpm. During the 60th h of batch fermentation without pH control, immobilized *Lact. futsaii* CS3 efficiently transformed MSG into the highest GABA of 19.05 g/L, achieving the volumetric productivity of 0.32 g/L/h with a bioconversion rate of 29.01 %. To further enhance GABA production, MSG (7.5 % w/v) was fed into the fermenter during the 48th h of fed-batch fermentation, aiming to amplify GABA synthesis. The maximum GABA (26.28 g/L) was synthesized at 84 h of fed-batch fermentation, and the volumetric productivity and a bioconversion rate of 0.31 g/L/h and 23.59 %, respectively, were obtained. Fed-batch fermentation significantly outperformed batch fermentation, resulting in a 37.95 % increase in GABA production. Moreover, immobilized *Lact. futsaii* CS3 cells could be reused in 2 batch cycles. In the 1st reusability, the maximum GABA synthesis reached 25.94 g/L, and the volumetric productivity and a bioconversion rate of 0.31 g/L/h and 23.29 %, respectively, were achieved at 84 h of fed-batch fermentation. While in the 2nd reusability, GABA synthesis decreased to 23.29 g/L, with the volumetric productivity and a bioconversion rate of 0.28 g/L/h and 20.91 %, respectively, at 84 h of fed-batch fermentation.

Keywords: Biosynthesis compound formation, Fermentation technology, Gamma-aminobutyric acid, Lactic acid bacteria, Microbial encapsulation, Renewable waste substrate, Tuna condensate waste

Introduction

GABA is a potent bioactive compound, a 4-carbon free amino acid, soluble in water, and widely distributed in microorganisms, plants and animals [1]. Predominantly found in the mammalian brain [2], GABA acts as a crucial inhibitory neurotransmitter in the mammalian central nervous system [1-4]. Beyond its neurological role, GABA exhibits potential health benefits, including the promotion of brain cell metabolism by increasing oxygen supply, activating cerebral blood flow and inhibiting vasopressin secretion [2]. Additionally, GABA has shown promise in health treatments, such as improving

sleeplessness and depression [1,4,5], enhancing immunity, modulating blood pressure [1], regulating cardiovascular function [2], promoting growth hormone secretion, preventing diabetes [4,5] and serving as a treatment for stroke [2]. Furthermore, GABA plays a role in regulating neurological disorders like Parkinson's disease, Huntington's chorea and Alzheimer's disease [2,6].

Microorganisms, especially lactic acid bacteria (LAB), are significant sources of GABA, synthesizing it from glutamate through pyridoxal-5'-phosphate (PLP)-dependent glutamate decarboxylase (GAD) [4-6]. Various LAB, including species of *Lactobacillus* [1,6-9] and others genera like *Streptococcus*, *Enterococcus*, *Leuconostoc*, *Pediococcus*, *Propionibacterium* and *Weissella*, have been identified as GABA producers [1,10]. Strategies for efficient GABA production by LAB have been developed, including various fermentation techniques (batch, fed-batch and mixed fermentations), as well as resting and immobilized cells technologies [11]. Immobilized cell technology, which involves embedding and coating techniques, is particularly promising for its potential benefits, such as increased microbial cells viability [12], simplified purification processes and enhanced long-term operational stability [11,13,14]. Materials commonly used for entrapment immobilization include calcium alginate, agar, agarose, kappa-carrageenan, collagen, chitosan, cellulose, maltodextrin and whey protein isolate [15,16]. Alginate consists of calcium, magnesium and sodium salts of alginic acid [17], a naturally derived polysaccharide extracted from brown algae cell walls, and is composed of β-D-mannuronic and α-L-guluronic acids [18]. Alginate has been extensively used for immobilization in forms such as xanthan-alginate beads, alginate-polyacrylamide gels, calcium alginate beads and sodium alginate beads promoting enzymatic activity, microbial cells viability and reusability in both laboratory and industrial processes [17,19].

Despite the potential of microbial GABA production using glutamate as a substrate, challenges including the high costs of the commercial culture media and the proficiency of GABA production. The De Man, Rogosa and Sharpe (MRS) medium, commonly used for lactobacilli cultivation [20], is a simple medium for GABA production by LAB. However, to improve the overall economy of the production process, alternative low-cost substrates and by-products from the agri-food industry are necessary. Tuna condensate waste or tuna precooking water is a by-product from the precooking process of tuna fish at 100 °C for about 1 h in the tuna canning industry. It is suggested as a potential nitrogen source due to its nutrient-rich compositions, abundant N-containing compounds and omega-3 fatty acids [21,22].

Previous studies have shown that *Lact. futsaii* CS3 (accession no. AB839950), isolated from *Kong-Som*, a Thai traditional fermented shrimp, has the ability to produce GABA. *Kong-Som* is rich in glutamic acid, which serves as the substrate for GABA biosynthesis by LAB [23]. In our previous work, *Lact. futsaii* CS3 demonstrated high GABA production using cane sugar as a carbon source and tuna condensate waste as a nitrogen source in an optimized modified MRS medium under optimal GABA-producing conditions [22]. However, challenges related to microbial cells viability, such as pH, acids, osmolarity [24] and other factors within the tuna condensate waste [22], necessitated the immobilization of *Lact. futsaii* CS3 cells. This study aims to present new strategies involving low-cost substrates and by-product for GABA production, utilizing cane sugar and tuna condensate waste in an optimized modified MRS medium, with MSG as a substrate. The process involves the fermentation of immobilized whole cells of *Lact. futsaii* CS3 at the fermenter-scale, encompassing batch and fed-batch fermentation processes.

Materials and methods

Microbial preparation

Lact. futsaii CS3 (accession no. AB839950) was previously isolated from *Kong-Som* (Thai traditional fermented shrimp) based on its capacity to produce high concentrations of GABA from MSG [23]. This strain was cultured in MRS broth (2 % D-glucose, 0.4 % yeast extract, 1 % peptone, 0.8 % beef extract, 0.5 % CH_3COONa, 0.2 % K_2HPO_4, 0.1 % Tween 80, 0.2 % $C_6H_{17}N_3O_7$, 0.02 % $MgSO_4 \cdot 7H_2O$, 0.005 % $MnSO_4 \cdot 4H_2O$ and 2 % NaCl) at 37 °C for 24 h without agitation.

Immobilization of *Lact. futsaii* CS3

Cell preparation

The 1 % (v/v) seed culture of *Lact. futsaii* CS3 was cultivated in 300 mL MRS broth in a 500-mL Erlenmeyer flask and cultivated without agitation at 37 °C for 24 h to achieve a cell density of 10^{10} cfu/mL. *Lact. futsaii* CS3's cells were harvested by centrifugation at 8,000 rpm for 20 min at 4 °C. Then, cell pellets were washed with 0.85 % (w/v) sterile NaCl (pH 5.5).

Immobilization of *Lact. futsaii* CS3

Cell pellets were suspended in varying concentrations of sodium alginate (1, 2, 3 and 4 % w/v) (Wako Pure Chemical Industries, Osaka, Japan), then stirred on a magnetic stirrer plate (600 rpm, 30 min). After that, cell suspension was dropped through a 27G syringe needle into 0.1 M $CaCl_2$ (the distance between the syringe needle and $CaCl_2$ solution was 5 cm) by using a peristaltic pump with a flow rate of 0.5 mL/min. The resulting gel beads were allowed to stand at room temperature for 1 h to ensure complete gelification. Immobilized *Lact. futsaii* CS3 beads were harvested by filtration (Whatman No. 4, filter paper, Fisher Scientific) and rinsed with 0.85 % (w/v) sterile NaCl (pH 5.5) and stored in 0.1 % (w/v) peptone water (pH 5.5) at 4 °C for further analysis. The immobilized beads made from various concentrations of sodium alginate, which showed the strongest structure under fermenter conditions, were chosen for further GABA production.

Enumeration of various immobilized *Lact. futsaii* CS3

One g of the immobilized beads was mixed in phosphate buffer saline (pH 7.0). This step aimed to homogenize the microencapsules, breaking the polymer formed and releasing the immobilized culture into this buffer. The released cells were diluted to appropriate concentrations and enumerated using MRS medium at 37 °C for 24 h. Enumeration was performed using the spread-plate method, and the immobilized *Lact. futsaii* CS3 cells were enumerated as cfu/g.

GABA production by immobilized whole cells of *Lact. futsaii* CS3 in the fermenter by batch and fed-batch fermentation processes

GABA production with immobilized whole cells of *Lact. futsaii* CS3 in both batch and fed-batch fermentation processes were performed by using the optimized modified MRS medium contained 3.47 % (w/v) cane sugar (commercial grade; Mitr Phol, Bangkok, Thailand), 3.84 % (w/v) tuna condensate waste (Chotiwat Manufacturing Co., Ltd., Songkhla, Thailand), 10.77 % (w/v) MSG (commercial grade; Ajinomoto, Bangkok, Thailand), 0.5 % (w/v) CH_3COONa, 0.2 % (w/v) K_2HPO_4, 0.1 % (w/v) Tween 80, 0.2 % (w/v) $C_6H_{17}N_3O_7$, 0.02 % (w/v) $MgSO_4 \cdot 7H_2O$, 0.005 % (w/v) $MnSO_4 \cdot 4H_2O$ and 2 % (w/v) NaCl [22]. The batch fermentation, 10 % (w/v) of encapsulated cells contained approximately 10^{11} cfu/g in gel beads was inoculated into a 2-L of the optimized modified MRS medium (pH 5) in a 3-L fermenter (BEM-MDL Series, B.E. Marubushi Co., Ltd., Chiyoda, Japan). The optimized modified MRS medium

(pH 5) was used for GABA production at 37 °C with agitation speed 30 rpm for 72 h of batch fermentation [22]. The fed-batch fermentation was also carried out in a 3-L fermenter under following conditions: 2-L of the optimized modified MRS medium (pH 5), 10 % (w/v) of encapsulated cells (~10^{11} cfu/g), temperature at 37 °C, agitation speed of 30 rpm and fermentation time of 96 h. When the MSG concentration in the fermented broth was reduced to less than 10 % (w/v), sterile MSG (7.5 % w/v) was added to the fermenter at 48 h during the fed-batch fermentation process. This was done to enhance GABA production, without inhibit GAD enzyme activity and microbial cell growth. GABA content and residual MSG concentrations in the fermented broths at each time of batch and fed-batch fermentation processes were determined by HPLC analysis. Moreover, bacterial cells in gel beads were enumerated as cfu/g.

Fed-batch fermentation reusability and cell survivability of the immobilized whole cells of *Lact. futsaii* CS3 on GABA production

Fed-batch fermentation reusability was performed by decanting the spent optimized modified MRS medium for every 84 h and replaced the fresh optimized modified MRS medium with initial pH values of 5 after washing the immobilized whole cells of *Lact. futsaii* CS3 with 0.85 % (w/v) sterile NaCl. Only completed immobilized beads were used for this reusability experiment. The fermentation process was repeated in a 3-L fermenter under following conditions: 2-L of the optimized modified MRS medium (pH 5), temperature at 37 °C, agitation speed of 30 rpm, sterile MSG (7.5 % w/v) was fed into the fermenter at 48 h of fed-batch fermentation and fermentation time of 84 h until the immobilized beads was disintegrated. GABA content and residual MSG concentrations were determined by HPLC analysis and microbial cells survivability in gel beads after each cycle was enumerated as cfu/g.

Analysis of GABA

High-performance liquid chromatography (HPLC) analysis of GABA and MSG

The concentrations of GABA and residual MSG were analyzed using HPLC by derivatizing the culture broth supernatant with ortho-phthalaldehyde (OPA) (Sigma-Aldrich, Steinheim, Germany) solution. HPLC determination was operated with an Agilent Technologies 1200 series binary pump, autosampler and a fluorescence detector (FLD) equipped with an automatic liquid sampler and injector program. Hypersil ODS C18 column (particle size 5 μm, length 250 mm, internal diameter 4.6 mm) was used as the HPLC column, which was thermostatted at 40 °C. The HPLC mobile phase was comprised potassium phosphate buffer (1M, pH 7) as mobile phase A, while acetonitrile as mobile phase B. Absorbance was detected at 330 nm (λ excitation) and 440 nm (λ emission) [22].

Determination of the volumetric productivity

The volumetric productivity (g/L/h) was calculated by the following formula:

$$\text{Volumetric productivity} = \text{GABA content (g/L)}/\text{GABA production times (h)} \quad (1)$$

Determination of a bioconversion rate

The bioconversion rate (%) was calculated by the following formula:

$$\text{Bioconversion rate (\%)} = \text{Final GABA concentration (mM)} \times 100/\text{Initial MSG concentration (mM)} \quad (2)$$

Statistical analysis

All experiments were conducted in triplicate, and the results were reported as mean ± standard deviation (mean ± SD). The Statistical Package for Social Sciences (IBM SPSS Statistic) program was used to analyze significant differences ($p < 0.05$, indicating statistical significance). Microsoft Excel 2010 software was employed to create column and line graphs.

Results and discussion

GABA production by immobilized whole cells of *Lact. futsaii* CS3 in the fermenter by batch and fed-batch fermentation processes

Lact. futsaii CS3, isolated from *Kong-Som*, a traditional Thai fermented shrimp rich in glutamic acid, exhibited robust GABA-producing capabilities [23]. LAB, including *Lact. futsaii* CS3, catalyze the irreversible α-decarboxylation of L-glutamate through a GAD system, contributing to pH homeostasis and releasing GABA into the culture medium [11]. In previous studies, *Lact. futsaii* CS3 demonstrated high efficiency in converting MSG in MRS broth to GABA, achieving over 99 % conversion within 72 h [23,25]. Factors such as temperature, pH, cultivation time and culture media significantly influenced GABA production by *Lact*. *futsaii* CS3. Optimal conditions, identified in previous work, involved the use of low-cost substrates (cane sugar, tuna condensate waste and MSG) in an optimized modified MRS medium without pH control [22]. Under these conditions, GABA concentrations of 20.63 and 17.24 g/L were obtained in flask-scale and fermenter-scale on batch fermentation at 48 h, respectively. Fed-batch fermentation further increased GABA concentrations to 23.01 g/L at 72 h [22].

The present work utilized these optimal conditions to achieve high GABA concentrations from immobilized *Lact. futsaii* CS3 cells (approximately 1.34×10^{11} cfu/g) in batch and fed-batch fermentations. In this study, 3 % (w/v) sodium alginate was selected for immobilizing whole cells of *Lact. futsaii* CS3, resulting in spherical beads approximately 0.2 cm in diameter. These beads demonstrated the highest strength when applied in an agitated fermenter. Sodium alginate concentration of 1 and 2 % (w/v) also produced spherical beads, but these beads had thinner walls and could not withstand the agitation speed in the fermenter, leading to bead breakage and acidification of fermented broth. On the other hand, 4 % (w/v) sodium alginate resulted in a highly viscous, making it difficult thoroughly without forming bubbles. This produced spherical beads, but hindered MSG diffusion into the gel beads. Immobilized cells in sodium alginate beads exhibited GABA production increasing with fermentation time. In batch fermentation without pH control, GABA production increased steadily, reaching 19.05 g/L at 60 h, with the volumetric productivity of 0.32 g/L/h and a bioconversion rate of 29.01 %. Notably, GABA levels plateaued after 60 h, corresponding to a bacterial cell count of 9.3×10^{8} cfu/g and remaining MSG concentration of 12.74 g/L (**Table 1** and **Figure 1(a)**).

A pH decreased from 5 to 3.98 during batch fermentation correlated with increased GABA production. This aligns with findings in *Lact. buchnerii* and *Lact. plamtarum* FNCC 260, where optimal GABA production occurred at a pH below 4.0 [26]. Microbial GAD activity, crucial for GABA formation, thrives in the pH range of 3.5 - 5.0 [1]. The pH drop observed here was attributed to lactic and acetic acids formation during microbial cultivation [27].

In fed-batch fermentation (without pH control), where substrate feeding compensates for initial limitations, GABA production was enhanced. MSG was supplemented into a fermenter when MSG concentration in the fermented broth was reduced to less than 10 % (w/v). The addition of MSG (7.5 % w/v) at 48 h led to a maximum GABA synthesis of 26.28 g/L at 84 h, with the volumetric productivity of 0.31 g/L/h and a bioconversion rate of 23.59 %. The residual MSG in the fermented broth remained at 9.34 g/L, and bacterial cells within the sodium alginate beads were enumerated as 1.10×10^{8} cfu/g.

Compared to the batch fermentation process, fed-batch fermentation demonstrated a 37.95 % increase in GABA production (**Table 2** and **Figure 1(b)**). **Figure 2** displays the GABA contents in the fermented broths of batch and fed-batch fermentations as measured by the HPLC analysis.

Batch fermentation, though common, has limitations, such as substrate inhibition and lower substrate concentrations. Fed-batch fermentation, introduced in this study, compensated for these limitations by feeding substrates during the process. The approach effectively increased GABA productivity and demonstrated its advantages in sustaining microbial cells viability [28]. GABA production via microbial cells faces challenges, including GABA transaminase (GABA-T)-mediated conversion of GABA to succinic semialdehyde (SSA) [1,8,14,26,27]. Fed-batch fermentation mitigates these challenges by improving microbial cell viability [8]. Adjusting the pH to optimal alkaline conditions was crucial for blocking GABA-T activity, ensuring sustained GABA production [2,14].

Supplementation of MSG in GABA production is crucial, as LAB typically do not synthesize enough L-glutamate [1,29]. MSG, acting as a substrate and pH buffer, prevents medium pH decrease [10,30]. The optimal MSG concentration varies among microorganisms. Excessive MSG can inhibit GABA production, as observed in this study and in previous research [2,10,22,31].

Interestingly, GABA production by immobilized *Lact. futsaii* CS3 cells remained constant after 60 and 84 h in batch and fed-batch fermentations, respectively. This stability suggested that GABA was not utilized as a nutrient during prolonged cultivation, possibly due to the absence of GABA-T in *Lact. futsaii* CS3, which degrades GABA. The study observed the necessity of adjusting the pH to an optimal alkaline range to block GABA-T activity and ensure sustained GABA production.

In conclusion, the results underscore the potential of immobilized *Lact. futsaii* CS3 for efficient GABA production in fed-batch fermentations, offering insights into the factors influencing GABA synthesis and the advantages of fed-batch strategies. The study contributes valuable information for optimizing GABA production processes and advancing the industrial application of immobilized microbial cells in biocatalytic conversions.

Table 1 GABA production from immobilized whole cells of *Lactobacillus futsaii* CS3 in the optimized modified MRS medium (pH 5.0) without pH control during batch fermentation at the fermenter-scale.

	Fermentation time				
	24 h	**36 h**	**48 h**	**60 h**	**72 h**
Residual MSG (g/L)	22.05 ± 0.13^{a}	21.91 ± 0.07^{a}	19.27 ± 0.02^{b}	12.74 ± 0.07^{c}	12.50 ± 0.27^{c}
GABA (g/L)	2.41 ± 0.01^{d}	9.70 ± 0.06^{c}	13.09 ± 0.09^{b}	19.05 ± 0.10^{a}	19.00 ± 0.05^{a}
Volumetric productivity (g/L/h)	0.10 ± 0.03^{c}	0.27 ± 0.05^{b}	0.27 ± 0.06^{b}	0.32 ± 0.08^{a}	0.26 ± 0.02^{b}
Bioconversion rate (%)	3.67 ± 0.02^{d}	14.77 ± 0.04^{c}	19.93 ± 0.05^{b}	29.01 ± 0.07^{a}	28.93 ± 0.03^{a}
Gel beads (cfu/g)	1.04×10^{10a}	9.70×10^{9b}	1.30×10^{9c}	9.30×10^{8d}	5.05×10^{8e}

Note: [a]Values represent the mean ± SD of triplicate determinations (n = 3). Different superscripts in the same column of each factor indicate significant differences ($p < 0.05$).

Table 2 GABA production from immobilized whole cells of *Lactobacillus futsaii* CS3 in an optimized modified MRS medium (pH 5) during fed-batch fermentation process without pH control.

	Fermentation time						
	24 h	**36 h**	**48 h**	**60 h**	**72 h**	**84 h**	**96 h**
Residual MSG (g/L)	22.26 ± 0.08^{a}	21.95 ± 0.02^{a}	19.15 ± 0.11^{b}	11.82 ± 0.19^{c}	9.43 ± 0.03^{d}	9.34 ± 0.12^{d}	9.32 ± 0.07^{d}
GABA (g/L)	2.26 ± 0.06^{f}	9.82 ± 0.03^{e}	14.39 ± 0.28^{d}	19.16 ± 0.08^{c}	21.78 ± 0.05^{b}	26.28 ± 0.13^{a}	26.19 ± 0.13^{a}
Volumetric productivity (g/L/h)	0.09 ± 0.05^{c}	0.27 ± 0.07^{b}	0.30 ± 0.04^{a}	0.32 ± 0.09^{a}	0.30 ± 0.06^{a}	0.31 ± 0.09^{a}	0.27 ± 0.07^{b}
Bioconversion rate (%)	3.44 ± 0.07^{f}	14.95 ± 0.08^{e}	21.91 ± 0.11^{b}	17.20 ± 0.04^{d}	19.55 ± 0.06^{c}	23.59 ± 0.14^{a}	23.51 ± 0.08^{a}
Gel beads (cfu/g)	1.02×10^{10a}	9.75×10^{9b}	1.56×10^{9c}	9.20×10^{8d}	4.90×10^{8e}	1.10×10^{8f}	9.60×10^{7g}

Note: [a]Values represent the mean ± SD of triplicate determinations (n = 3). Different superscripts in the same column of each factor indicate significant differences ($p < 0.05$).

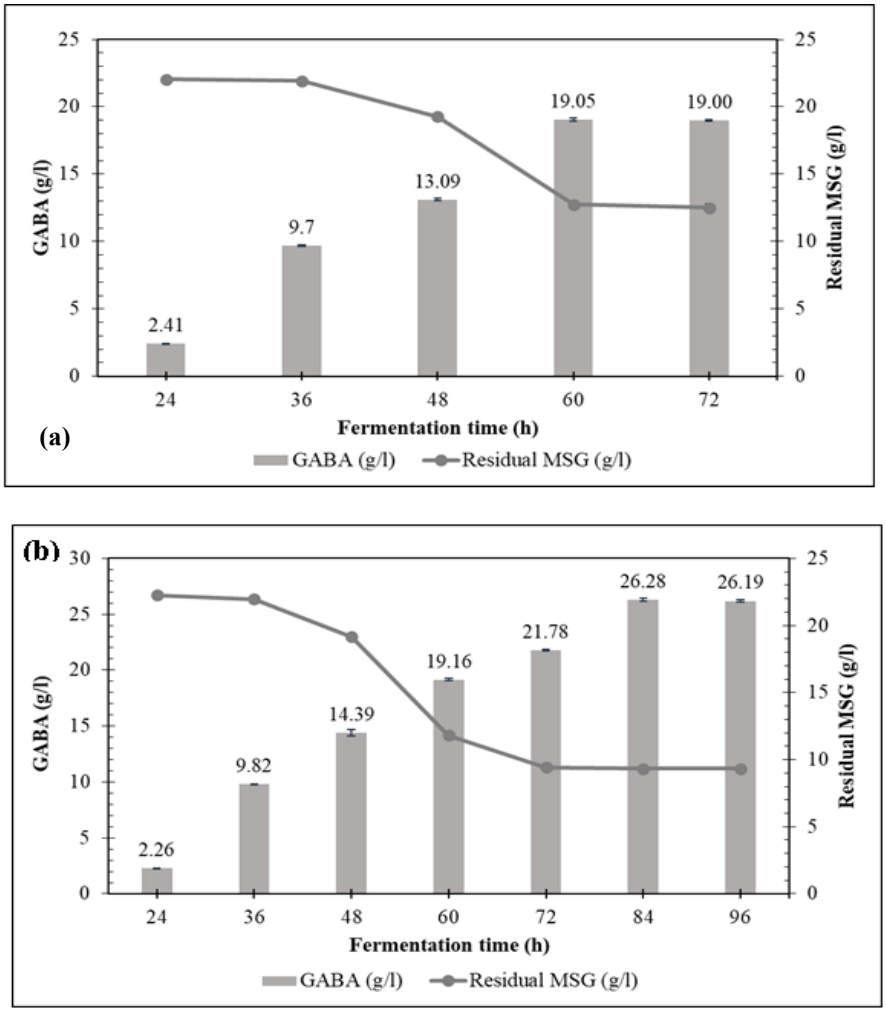

Figure 1 GABA production from immobilized whole cells of *Lactobacillus futsaii* CS3 in the optimized modified MRS medium (pH of 5) without pH control during batch fermentation (a) and fed-batch fermentation (b) at the fermenter-scale. Data presented are the mean of independent triplicates, and the error bars on data points represent standard errors.

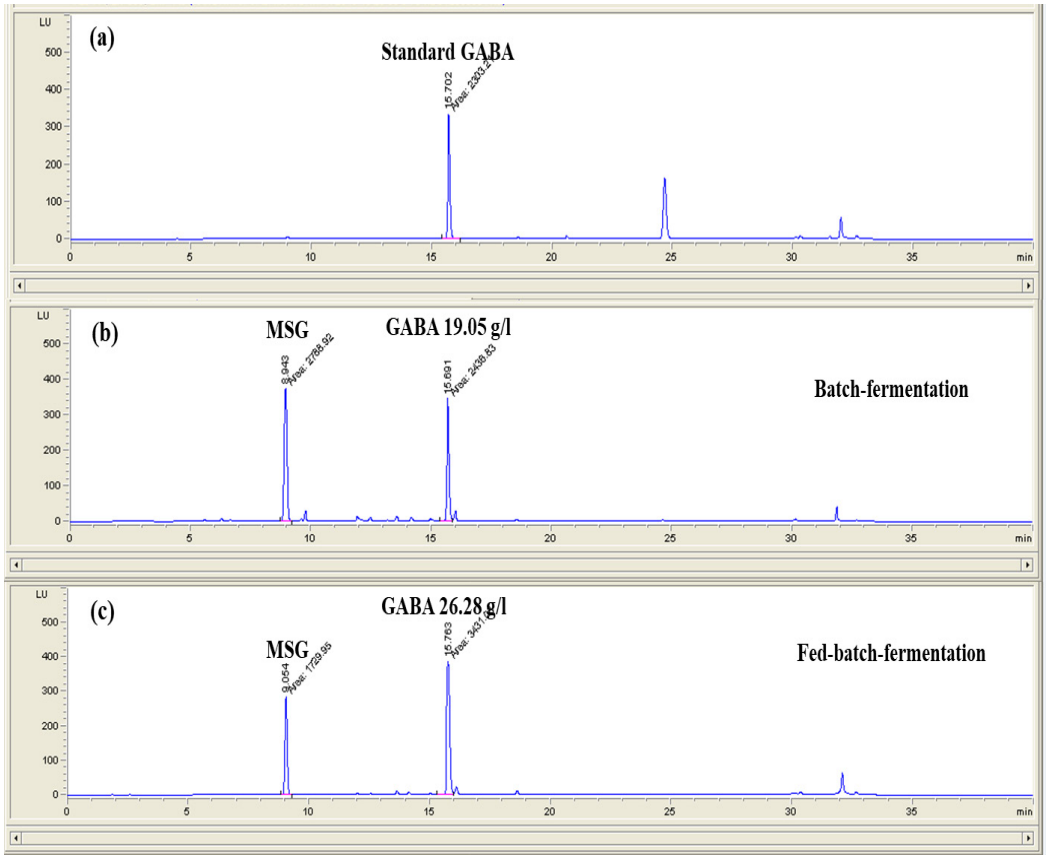

Figure 2 HPLC chromatograms of standard GABA (a) and GABA produced by immobilized *Lactobacillus futsaii* CS3 in the optimized modified MRS medium (pH 5.0) without pH control at 60 h of batch fermentation (b) and at 84 h of fed-batch fermentation (c) at the fermenter-scale.

Fed-batch fermentation reusability and cell survivability of the immobilized whole cells of *Lact. futsaii* CS3 on GABA production

The investigation into the reusability and survivability of *Lact*. *futsaii* CS3's immobilized cells during GABA production under fed-batch fermentation revealed a gradual decrease in bacterial cell counts within sodium alginate beads over cultivation time. HPLC analysis confirmed GABA production by the reused immobilized *Lact*. *futsaii* CS3 cells, with a retention time of GABA at ~15.7 min (**Figure 3**).

In the 1st reusability cycle, maximum GABA synthesis reached 25.94 g/L, with a volumetric productivity of 0.31 g/L/h and a bioconversion rate of 23.29 % at 84 h. Bacterial cells within the sodium alginate beads were enumerated as 2.03×10^6 cfu/g, and residual MSG in the fermented broth remained at 9.34 g/L. However, during the 2nd reusability cycle with the same immobilized cells (2.03×10^6 cfu/g) after the 1st cycle, GABA synthesis decreased to 23.29 g/L, with a volumetric productivity of 0.28 g/L/h and a bioconversion rate of 20.91 %. Residual MSG in the fermented broth remained at 12.05 g/L at 84 h of fed-batch fermentation (**Table 3** and **Figure 4**). The decline in the 2nd cycle may be attributed to decreased cell viability within sodium alginate beads (4.80×10^3 cfu/g) (**Table 3**). Similar studies have reported a decrease in GABA production by immobilized *Enterococcus faecium* CFR 3003 after multiple batch cycles, attributed to a decline in bacterial cell count within gel beads [13]. In the 2nd reusability

cycle, the immobilized beads of *Lact*. *futsaii* CS3 started to disintegrate at 60 h, completely disintegrating by 84 h of fed-batch fermentation. This suggests that the immobilized whole cells of *Lact*. *futsaii* CS3 in 3 % (w/v) sodium alginate beads can only be effectively reuse for 2 batch cycles in fed-batch fermentation for GABA production. Entrapment technique limitations include gel bead disruption from pressure generated by dividing microbial cells and the accumulation of inhibitory products due to diffusion restrictions within and outside the gel layer [16]. Additionally, alginate beads are sensitive to the acidic environment [18]. Nevertheless, this defect can be compensated by mixing alginate with other polymer compounds, coating the beads by another compound, or applying structural modification of alginate by using different additives [32].

In both batch and fed-batch fermentations, immobilized whole cells of *Lact*. *futsaii* CS3 produced more GABA than *Lact*. *futsaii* CS3's seed culture. However, cultivation time for GABA synthesis with immobilized whole cells was longer due to mass transfer limitations between bacterial cells in sodium alginate beads and the substrate, MSG. The highest GABA concentrations, 19.05 and 26.28 g/L, were achieved from *Lact*. *futsaii* CS3's immobilized cells at 60 h of batch fermentation and 84 h of fed-batch fermentation, respectively. In comparison, the maximum GABA concentrations from the seed culture of *Lact*. *futsaii* CS3 were 17.24 g/L at 48 h of batch fermentation and 23.01 g/L at 72 h of fed-batch fermentation [22].

The use of immobilized cells provides advantages in enhancing process stability during cultivation in challenging environments [33,34]. Immobilized cells offer potential for simpler and more cost-effective commercial implementation. However, the reusability of immobilized cells is a crucial factor influencing their industrial application. The entrapping material nature impacts microbial enzyme activity; for instance, gellan gum demonstrated increased GAD activity compared to agar and κ-carrageenan [11,15]. Alginate, a cost-effective and non-toxic gel matrix, is commonly used for immobilization due to its ease of handling and the ability to release cells by sequestering calcium ions. Alginate is derived from brown algae cell walls and is recognized as a food additive [19]. Sodium alginate has been widely employed in various forms for immobilization, demonstrating improved enzyme activity, cell viability and reusability [17].

It is noteworthy that utilizing cane sugar as a carbon source and tuna condensate waste as a nitrogen source enabled the immobilized cells of *Lact*. *futsaii* CS3 to achieve remarkably high GABA levels in both batch and fed-batch fermentations (19.05 to 26.28 g/L). This outperforms reported concentrations for other immobilized strains, such as *Lact*. *brevis* $GadA_{\blacktriangle C14}$ immobilized cells produced GABA concentrations of 9.13, 7.95 and 7.41 g/L with gellan gum, κ-carrageenan and agar, respectively [11]. Another study with immobilized *Lact*. *brevis* CGMCC 1306 cells in calcium-alginate beads produced GABA at a concentration of approximately 80 mM [14].

In conclusion, this study sheds light on the 2-batch cycles reusability of *Lact*. *futsaii* CS3's immobilized cells in GABA production and emphasizes the impact of the entrapping material on cell viability. The study's innovative use of cane sugar and tuna condensate as substrates contributes to the advancement of GABA production processes, highlighting the potential for industrial applications of immobilized microbial cells.

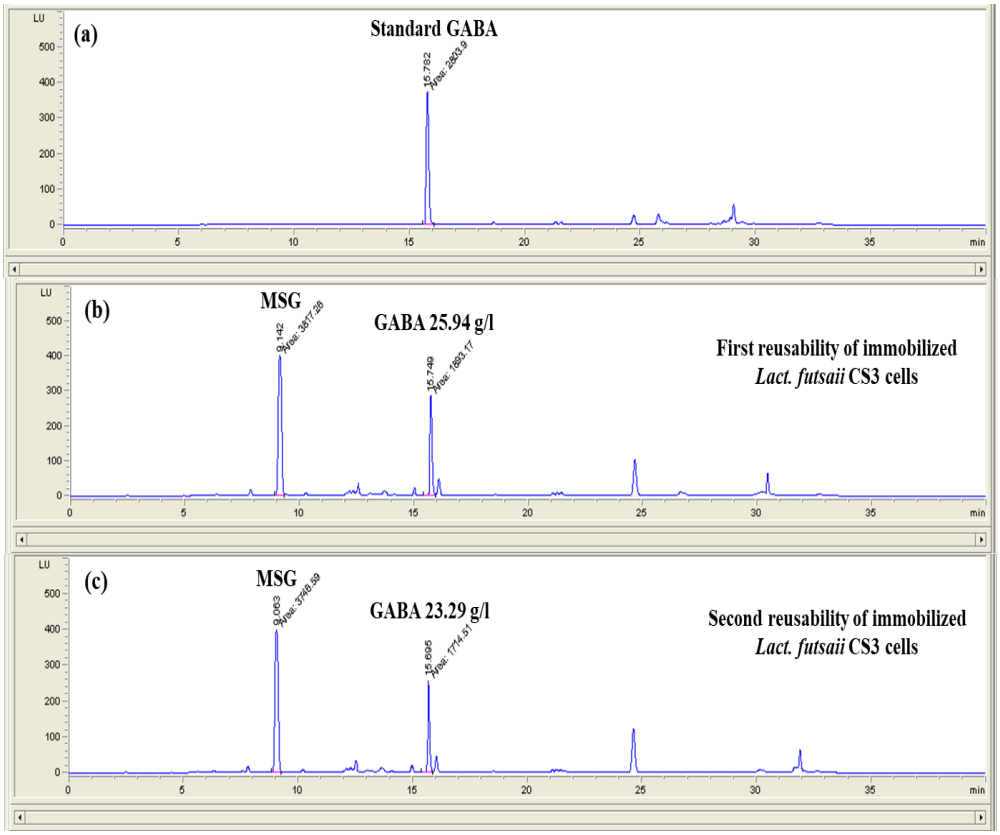

Figure 3 HPLC chromatograms of standard GABA (a) and GABA produced by reused immobilized *Lactobacillus futsaii* CS3 in the optimized modified MRS medium (pH 5.0) without pH control at 84 h of fed-batch fermentation at the fermenter-scale (b) and (c).

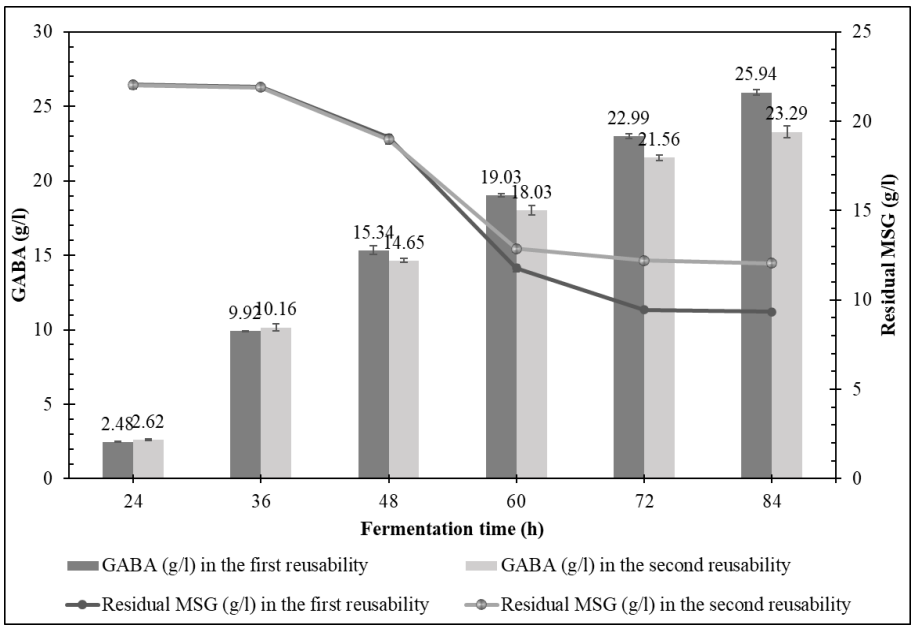

Figure 4 Fed-batch fermentation reusability of immobilized *Lactobacillus futsaii* CS3 in the optimized modified MRS medium (pH 5.0) without pH control at the fermenter-scale. Data presented are the mean of independent triplicates, with error bars representing standard errors.

Table 3 Fed-batch fermentation reusability and cell survivability of the immobilized *Lactobacillus futsaii* CS3 on GABA production in an optimized modified MRS medium (pH 5.0) without pH control at the fermenter-scale.

	Fermentation time					
	24 h	**36 h**	**48 h**	**60 h**	**72 h**	**84 h**
Reusability I						
Residual MSG (g/L)	22.06 ± 0.14^{a}	21.94 ± 0.02^{a}	19.10 ± 0.12^{b}	11.78 ± 0.18^{c}	9.46 ± 0.06^{d}	9.34 ± 0.08^{d}
GABA (g/L)	2.48 ± 0.02^{f}	9.92 ± 0.03^{e}	15.34 ± 0.28^{d}	19.03 ± 0.13^{c}	22.99 ± 0.15^{b}	25.94 ± 0.16^{a}
Volumetric productivity (g/L/h)	0.10 ± 0.05^{c}	0.28 ± 0.03^{b}	0.32 ± 0.13^{a}	0.32 ± 0.09^{a}	0.32 ± 0.07^{a}	0.31 ± 0.11^{a}
Bioconversion rate (%)	3.78 ± 0.03^{e}	15.11 ± 0.02^{d}	23.36 ± 0.09^{a}	17.08 ± 0.11^{c}	20.64 ± 0.07^{b}	23.29 ± 0.11^{a}
Gel beads (cfu/g)	1.06×10^{8a}	9.60×10^{7b}	2.55×10^{7c}	9.70×10^{6d}	5.05×10^{6e}	2.03×10^{6f}
Reusability II						
Residual MSG (g/L)	22.03 ± 0.20^{a}	21.89 ± 0.05^{a}	18.98 ± 0.27^{b}	12.87 ± 0.20^{c}	12.21 ± 0.02^{c}	12.05 ± 0.08^{c}
GABA (g/L)	2.62 ± 0.05^{f}	10.16 ± 0.25^{e}	14.65 ± 0.14^{d}	18.03 ± 0.33^{c}	21.56 ± 0.19^{b}	23.29 ± 0.41^{a}
Volumetric productivity (g/L/h)	0.11 ± 0.07^{c}	0.28 ± 0.15^{b}	0.31 ± 0.09^{a}	0.30 ± 0.17^{a}	0.30 ± 0.08^{a}	0.28 ± 0.16^{b}
Bioconversion rate (%)	3.99 ± 0.09^{d}	15.47 ± 0.13^{c}	22.31 ± 0.09^{a}	16.19 ± 0.16^{c}	19.85 ± 0.11^{b}	20.91 ± 0.23^{b}
Gel beads (cfu/g)	2.03×10^{6a}	1.14×10^{6b}	1.05×10^{5c}	5.10×10^{4d}	1.31×10^{4e}	4.80×10^{3f}

Note: [a]Values represent the mean ± SD of triplicate determinations (n = 3). Different superscripts in the same column of each factor indicate significant differences ($p < 0.05$).

Conclusions

This study unveils a novel avenue for enhancing GABA production by utilizing immobilized bacterial cells, particularly leveraging agri-food industry by-products. The fermenter-scale optimization, encompassing 3.48 % (w/v) cane sugar, 3.84 % (w/v) tuna condensate waste and 10.77 % (w/v) MSG at an initial pH of 5, with a temperature of 37 °C, agitation speed of 30 rpm and without pH control, showcased the exceptional GABA production capabilities of immobilized *Lact. futsaii* CS3 cells in 3 % (w/v) sodium alginate. This resulted in the highest GABA yields of 19.05 g/L at 60 h in batch fermentation and 26.28 g/L at 84 h in fed-batch fermentation. Furthermore, the study demonstrated the practicality of reusing immobilized *Lact. futsaii* CS3 cells in 2 successive batch cycles during fed-batch fermentation, with peak GABA concentrations of 25.94 and 23.29 g/L in the 1st and 2nd batch cycles, respectively. This signifies a sustainable approach that holds promise for cost reduction in GABA production by tapping into low-cost substrates derived from renewable resources.

The significant GABA output achieved from both *Lact. futsaii* CS3's seed culture and immobilized *Lact. futsaii* CS3 cells underscored the potential for large-scale GABA formation. These findings lay the groundwork for future endeavors, including the purification of GABA from both sources. The purified GABA holds promise for applications in the development for functional food products, aligning with the growing interest in bioactive compounds for health-oriented food formulations.

Acknowledgements

The authors gratefully acknowledge the financial support from Thesis Research Grant, and the PSU-Ph.D. Scholarship, Graduate School, Prince of Songkla University, Thailand.

References

[1] Y Cui, K Miao, S Niyaphorn and X Qu. Production of gamma-aminobutyric acid from lactic acid bacteria: A systematic review. *Int. J. Mol. Sci.* 2020; **21**, 995.

[2] SB Sarasa, R Mahendran, G Muthusamy, B Thankappan, DRF Selta and J Angayarkanni. A brief review on the non-protein amino acid, gamma-amino butyric acid (GABA): Its production and role in microbes. *Curr. Microbiol.* 2020; **77**, 534-44.

[3] J Kim, YW Yoon, MS Kim, MH Lee, GA Kim, K Bae and SS Yoon. Gamma-aminobutyric acid fermentation in MRS-based medium by the fructophilic *Lactiplantibacillus plantarum* Y7. *Food Sci. Biotechnol.* 2022; **31**, 333-41.

[4] V Tanamool, P Hongsachart and W Soemphol. Screening and characterization of gamma-aminobutyric acid (GABA) producing lactic acid bacteria isolated from Thai fermented fish (*Plaa-som*) in Nong Khai and its application in Thai fermented vegetables (*Som-pak*). *Food Sci. Tech.* 2020; **40**, 483-90.

[5] H Altaib, T Kozakai, Y Badr, H Nakao, MAM El-Nouby, E Yanase, I Nomura and T Suzuki. Cell factory for γ-aminobutyric acid (GABA) production using *Bifdobacterium adolescentis*. *Microb. Cell Fact.* 2022; **21**, 33.

[6] A Santos-Espinosa, LM Beltrán-Barrientos, R Reyes-Díaz, MÁ Mazorra-Manzano, A Hernández-Mendoza, GA González-Aguilar, SG Sáyago-Ayerdi, B Vallejo-Cordoba and AF González-Córdova. Gamma-aminobutyric acid (GABA) production in milk fermented by specific wild lactic acid bacteria strains isolated from artisanal Mexican cheeses. *Ann. Microbiol.* 2020; **70**, 12.

[7] Q Wang, X Liu, J Fu, S Wang, Y Chen, K Chang and H Li. Substrate sustained release-based high efficacy biosynthesis of GABA by *Lactobacillus brevis* NCL912. *Microb. Cell Fact.* 2018; **17**, 80.

[8] IBA Yogeswara, S Maneerat and D Haltrich. Glutamate decarboxylase from lactic acid bacteria - A key enzyme in GABA synthesis. *Microorganisms* 2020; **8**, 1923.

[9] Y Zhang, M Zhu, W Lu, C Zhang, D Chen, NP Shah and C Xiao. Optimizing *Levilactobacillus brevis* NPS-QW 145 fermentation for gamma-aminobutyric acid (GABA) production in soybean sprout yogurt-like product. *Foods* 2023; **12**, 977.

[10] SJ Park, DH Kim, HJ Kang, M Shin, SY Yang, J Yang and YH Jung. Enhanced production of γ-aminobutyric acid (GABA) using *Lactobacillus plantarum* EJ2014 with simple medium composition. *LWT Food Sci. Tech.* 2021; **137**, 110443.

[11] CJ Lyu, L Liu, J Huang, WR Zhao, S Hu, LH Mei and SJ Yao. Biosynthesis of ɣ-aminobutyrate by engineered *Lactobacillus brevis* cells immobilized in gellan gum gel beads. *J. Biosci. Bioeng.* 2019; **128**, 123-8.

[12] S Thangrongthong, N Puttarat, B Ladda, T Itthisoponkul, W Pinket, K Kasemwong and M Taweechotipatr. Microencapsulation of probiotic *Lactobacillus brevis* ST-69 producing GABA using alginate supplemented with nanocrystalline starch. *Food Sci. Biotechnol.* 2020; **29**, 1475-82.

[13] G Divyashri and SG Prapulla. Mass transfer characterization of gamma-aminobutyric acid production by *Enterococcus faecium* CFR 3003: Encapsulation improves its survival under simulated gastro-intestinal conditions. *Bioprocess Biosyst. Eng.* 2015; **38**, 569-74.

[14] J Huang, LH Mei, H Wu and DQ Lin. Biosynthesis of γ-aminobutyric acid (GABA) using immobilized whole cells of *Lactobacillus brevis*. *World J. Microbiol. Biotechnol.* 2007; **23**, 865-71.

[15] P Pandey and HN Mishra. Co-microencapsulation of γ-aminobutyric acid (GABA) and probiotic bacteria in thermostable and biocompatible exopolysaccharides matrix. *LWT Food Sci. Tech.* 2021; **136**, 110293.

[16] Y Zhang, W Cong and SY Shi. Repeated fed-batch lactic acid production in a packed bed-stirred fermentor system using a pH feedback feeding method. *Bioprocess Biosyst. Eng.* 2011; **34**, 67-73.

[17] S Datta, LR Christena and YRS Rajaram. Enzyme immobilization: An overview on techniques and support materials. *3 Biotech* 2013; **3**, 1-9.

[18] J Burgain, C Gaiani, M Linder and J Scher. Encapsulation of probiotic living cells: From laboratory scale to industrial applications. *J. Food Eng.* 2011; **104**, 467-83.

[19] V Chandramouli, K Kailasapathy, P Peiris and M Jones. An improved method of microencapsulation and its evaluation to protect *Lactobacillus* spp. in simulated gastric conditions. *J. Microbiol. Methods* 2004; **56**, 27-35.

[20] T Nguyen-Sy, GY Yew, KW Chew, TDP Nguyen, TNT Tran, TDH Le, CT Vo, HKP Tran, M Mubashir and PL Show. Potential cultivation of *Lactobacillus pentosus* from human breastmilk with rapid monitoring through the spectrophotometer method. *Processes* 2020; **8**, 902.

[21] P Prasertsan, M Jaturapornpipat and C Siripatana. Utilization and treatment of tuna condensate by photosynthetic bacteria. *Pure Appl. Chem.* 1997; **69**, 2439-45.

[22] K Thongruck and S Maneerat. Enhanced production of gamma-aminobutyric acid (GABA) from *Lactobacillus futsaii* CS3 using agri-food industries by-products under batch and fed-batch fermentation. *Indian J. Microbiol.* 2023; **63**, 467-82.

[23] C Sanchart, O Rattanaporn, D Haltrich, P Phukpattaranont and S Maneerat. *Lactobacillus futsaii* CS3, a new GABA-producing strain isolated from Thai fermented shrimp (*Kung-Som*). *Indian J. Microbiol.* 2016; **57**, 211-7.

[24] A Picot and C Lacroix. Encapsulation of bifidobacteria in whey protein-based microcapsules and survival in simulated gastrointestinal conditions and in yoghurt. *Int. Dairy J.* 2004; **14**, 505-15.

[25] C Sanchart, O Rattanaporn, D Haltrich, P Phukpattaranont and S Maneerat. Technological and safety properties of newly isolated GABA-producing *Lactobacillus futsaii* strains. *J. Appl. Microbiol.* 2016; **121**, 734-45.

[26] IBA Yogeswara, S Kittibunchakul, ES Rahayu, KJ Domig, D Haltrich and TH Nguyen. Microbial production and enzymatic biosynthesis of γ-aminobutyric acid (GABA) using *Lactobacillus plantarum* FNCC 260 isolated from Indonesian fermented foods. *Processes* 2021; **9**, 22.

[27] SH Han, KB Hong and HJ Suh. Biotransformation of monosodium glutamate to gamma-aminobutyric acid by isolated strain *Lactobacillus brevis* L-32 for potentiation of pentobarbital-induced sleep in mice. *Food Biotechnol.* 2017; **31**, 80-93.

[28] H Li, T Qiu, G Huang and Y Cao. Production of gamma-aminobutyric acid by *Lactobacillus brevis* NCL912 using fed-batch fermentation. *Microb. Cell Fact.* 2010; **9**, 85.

[29] DTB Thuy, AT Nguyen, KS Khoo, KW Chew, M Cnockaert, P Vandamme, YC Ho, ND Huy, HH Cocoletzi and PL Show. Optimization of culture conditions for gamma-aminobutyric acid production by newly identified *Pediococcus pentosaceus* MN12 isolated from 'mam nem', a fermented fish sauce. *Bioengineered* 2021; **12**, 54-62.

[30] DH Kim, C Dasagrandhi, SK Park, SH Eom, MK Huh, JS Mok and YM Kim. Optimization of gamma-aminobutyric acid production using sea tangle extract by lactic acid bacterial fermentation. *LWT Food Sci. Tech.* 2018; **90**, 636-42.

[31] TTT Binh, WT Ju, WJ Jung and RD Park. Optimization of γ-amino butyric acid production in a newly isolated *Lactobacillus brevis*. *Biotechnol. Lett.* 2013; **36**, 93-8.

[32] W Krasaekoopt, B Bhandari and H Deeth. Evaluation of encapsulation techniques of probiotics for yoghurt. *Int. Dairy J.* 2003; **13**, 3-13.

[33] XH Chen, XT Wang, WY Lou, Y Li, H Wu, MH Zong, TJ Smith and XD Chen. Immobilization of *Acetobacter* sp. CCTCC M209061 for efficient asymmetric reduction of ketones and biocatalyst recycling. *Microb. Cell Fact.* 2012; **11**, 119.

[34] AG Peredo, CI Beristain, LA Pascual, E Azuara and M Jimenez. The effect of probiotics on the viability of encapsulated probiotic bacteria. *LWT Food Sci. Tech.* 2016; **73**, 191-6.