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Optimization of process parameters for GABA and L-DOPA production on fermented coconut drink

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Abstract

Gamma-aminobutyric acid (GABA) and levodopa (L-DOPA) are compounds that can potentially treat hypertension and Parkinson's diseases. The purpose of this study was to optimize the fermentation parameters for GABA and L-DOPA production by *Lactobacillus acidophilus* co-cultured with *Lactobacillus brevis* (LALB) in mature coconut water (MCW). In this study, the response surface methodology (RSM) with central composite design (CCD) was applied to optimize the concentration of sucrose, culture temperature and incubation time. The optimum conditions for the yield of GABA and L-DOPA were found to be: 1 g sucrose at 27°C for 28 hrs of incubation time under static conditions with the predicted accuracy of 95.4% (352 mg GABA/L and 94.7% (89 mg L-DOPA/L). This work was conducted to add value to the by-product of the coconut milk industry through the production of fermented coconut drinks. The MCW fermented with LALB offers a potential functional drink for hypertension and Parkinson's disease treatment and prevention measures.

1. Introduction

Coconut (*Cocos nucifera* L.) is a 'versatile tree' due to its multifunctional attributes that have generated a wide range of uses from food to cosmetics, and household to industrial products. One of the interesting parts about the coconut tree is coconut water (CW), which is sterile when remains in its envelope and is widely consumed around the world. It is refreshing, high in nutritional values, naturally fat-free and contains many potential therapeutic properties. Therefore, it becomes the latest trendy drink in the global beverage market. Currently, mature coconut water (MCW) offers a new raw material to create high value products in the market. This project was carried out to exploit MCW as a new substrate to the beverages and nutraceutical industry in Malaysia, as it offers new business opportunities. The CW is rich in bioactive compounds, including amino acids, minerals, sugars, sugar alcohols, lipids, phenolic acids, organic acids, and enzymes and can be applied in the food, health and cosmetics industries (Arditti, 2008). Biotechnological process, particularly microbial fermentation was used to enhance the concentration of bioactive compounds (Oliveira *et al*., 2012). Microbes can break down or hydrolyze complex raw materials into smaller units using enzymes that they secrete. Thus, it enhances the nutrition values and improves digestibility.

Previous findings showed CW has several functional activities such as antioxidant, anti-hypertensive, anti-carcinogenic, anti-thrombotic, anti-aging properties and possesses an inhibitory effect on acetylcholinesterase, which can be used to treat Alzheimer's disease (Campbell-Falck *et al*., 2000). A by-product from industrial food production should be considered to be one of the best raw materials for producing inexpensive, yet high value products. Perhaps, MCW has the best potential for this because it is considered a by-product waste from coconut milk production. It is enriched with nutrients which is suitable for microbial cultivation. The use of MCW as a substrate for the fermentation process would be worth investigating as it is a cheap, readily available by-product from the coconut industry. The use of starter cultures is recommended to produce fermented products with higher benefits than those from spontaneously fermented products. Lactic acid bacteria (LAB) were used in the production of industrial chemicals, biological products and food preservatives. Therefore, LAB was chosen to investigate the potential GABA and L-DOPA production in MCW. Previous findings have proven that fermented foods have more nutrients, and gain popularity and product acceptance because of their functional benefits. The incorporation of LAB in food and beverages is a global trend and the

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functional properties of these products have been scientifically demonstrated.

GABA, a non-protein amino acid, possess well-known physiological functions such as neurotransmission, induction of hypotension and diuretic and tranquillizer effects (Siragusa *et al*., 2007). GABA also showed the potential of lowering blood pressure in spontaneously hypertensive rats (SHR) and hypertensive humans (Kajimoto *et al*., 2004). There are many well-characterized physiological functions associated with GABA including relaxation and immunity enhancement (Abdou *et al*., 2006). Generally, the human body can produce its own supply of GABA. However, GABA production is sometimes inhibited by a lack of estrogen, zinc or vitamins, or by an excess of food additives (Aoshima *et al*., 1997). The GABA content in the daily human diet is relatively low. Therefore, GABA enriched food is needed. GABA is formed by the irreversible a-decarboxylation reaction of L-glutamic acid or its salts, catalyzed by the glutamic acid decarboxylase (GAD) enzyme (Bertoldi *et al*., 1999). Meanwhile, L-DOPA (L-3-,4-dihydroxyphenylalanine) is the precursor of the neurotransmitter dopamine and its synthesis involves the use of tyrosinase to convert tyrosine to L-DOPA, which is a useful drug in the treatment of Parkinson's disease (Azlina *et al.,* 2015).

The response surface methodology (RSM) using a central composite design (CCD) with the statistical experimental approach was used to optimize the process parameter variables for GABA and L-DOPA production in MCW. Optimization through factorial design and response surface analysis is a common practice in biotechnology (Giovanni, 1983). Various researchers have applied this technique, especially for the optimization of culture conditions (Kalil *et al*., 2000), lipase (Rathi *et al*., 2002) and xylanase (Park *et al*., 2002). Hence, the purpose of this study was to investigate the potential of MCW as a medium growth for GABA and L-DOPA production by optimizing process parameter conditions to produce fermented coconut drink enriched with GABA and L-DOPA.

2. Materials and methods

2.1 Standards and chemicals

GABA and L-DOPA were purchased from Sigma Aldrich (St. Louis, MO, USA). *Lactobacilli* MRS broth was purchased from Difco (Detroit, Mich., USA). Chromatographic grade solvent/chemical: acetonitrile and formic acid were purchased from Merck (Darmstadt, Germany) and ultrapure Mili-Q water (Waters, Milford, MA, USA) was used throughout the study.

2.2 Coconut water preparation

Fresh mature coconut water (MCW) was obtained from mature coconut fruits harvested from the Malaysian Agricultural and Research Development Institute (MARDI) coconut plantation in Perak, Malaysia. The coconuts were thoroughly disinfected with alcohol to reduce the risk of contamination. Then, the coconut water was extracted and pasteurized at 105°C for 10 mins before being subjected to fermentation.

2.3 Strains and culture preparation

Two strains of lactic acid bacteria (LAB), consisting of *Lactobacillus acidophilus* (B0258) and *Lactobacillus brevis* (designed as LALB) were obtained from the Centre of Functional Food Cultures (CFFC), MARDI. The starter cultures were kept with 20% glycerol at -80ºC in a 1 mL Eppendorf tube as stock culture. Both LAB strains were revised on MRS plate for 24 hrs at 37ºC before being used as an inoculum for the production of fermented mature coconut water (MCW). The number and viability of the lactobacilli were determined by anaerobic cultivation on MRS plate.

2.4 Model design of experiments

Response surface methodology (RSM) is a reliable and useful statistics approach, usually used for investigating the optimal growth conditions. Experimental design and statistical analysis were analyzed using Minitab software. Central composite design (CCD) with the quadratic model of three independent variables was employed to study the variable's combination effect. The crucial factors considered in this study included sucrose concentration (1st variable, range 2-5 g), culture temperature (2nd variable, range 30-45ºC) and incubation time (3rd variable, range 12-36 hrs) with five variable levels. Inoculum size was fixed in this study. A design matrix of 20 experiments with central points was generated in triplicate. The central point of the design experiment was 3.5 g sucrose, 37.5ºC temperature and 24 hrs of incubation time. A second-order polynomial function was fitted to correlate the relationship between the independent variables and the responses (GABA and L-DOPA). The quality of the fit to the model equations was expressed by the coefficient of determination, R^2.

2.5 Fermentation condition

All runs were conducted in a 150 mL shake flask containing 100 mL of MCW with 3% (v/v) inoculum. All samples were incubated under static conditions by varying sucrose concentration, culture temperature and incubation time as designed in the 3-factorials-5-levels response surface methodology. The GABA and L-DOPA

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content were monitored every 4, 12, 24, 36 and 44 hrs of cultivation. Non-fermented MCW was used as the negative control. The colony-forming unit (CFU/mL) and pH of the medium were determined at the respective times. The samples were centrifuged at 10,000 rpm for 10 mins to collect the supernatant. The supernatant was further filtered through a 0.22 µm membrane filter before being subjected to chromatographic analysis using Ultra Performance Liquid Chromatography (UPLC). The filtrates were kept at -80°C for further analysis. All experiments were performed in triplicate.

2.6 Determination of GABA and L-DOPA content

The GABA and L-DOPA were analyzed using UPLC according to a method described by Azlina *et al.* (2015). Briefly, 10 µL of the sample was derivatized with 70 µL of AccQ-Tag™ Ultra borate buffer first before mixing vigorously and vortexed. Then, 20 µL of AccQ-Tag™ Fluor agent was added and continued mixing for 1 min, followed by heating at 55°C for 10 min. The 1 µL of the mixture was injected into the UPLC system. An AccQ-Tag™ Ultra column (2.1 mm x 100 mm, 1.7 µm) with a flow rate of 0.7 mL/min. The column temperature was set to 55°C with the UV spectra wavelength of 260 nm. The elution solvents consisted of (A) AccQ-Tag™ Ultra Eluent A and (B) acetonitrile:formic acid (98:2). The gradient elution strategy was performed as follows: 99.9% A was maintained from 0.00 to 0.54 min; followed by a linear gradient of A from 99.9 to 90.9% at 0.54 to 5.74 min; linear gradient of A from 90.9 to 78.8% at 5.74 to 7.74 min; linear gradient of A from 78.8 to 40.4% from 7.74 to 8.50 min; the constant ratio of A at 40.4% for 0.30 min; linear gradient of A from 40.4 to 99.9% at 8.80 to 8.90 min and finally, a constant ratio of A at 99.9% for 2.10 min. The analyses were performed in triplicates.

2.7 Statistical analysis

Minitab software was used to analyze the relationship of the variables to the response using the regression model with the significance level, $\alpha = 0.05$. The *P*-value is a tool for evaluating the significance. Thus, the quadratic models were selected by considering the *P*-value ($P < 0.05$), lack of fit and the test statistics (low value of standard deviation press but high value of *R* and adjusted R^2) combination. The high value of *F* but the low value of *P* indicated a good model. The soundness of the regression models can be checked by the determination coefficient, R^2, and the adjusted R^2. Data were presented as mean ± standard deviation for each sample with three replications.

3. Results and discussion

3.1 Growth of LALB n the fermentation medium of mature coconut water

To determine the changes in growth and pH value after 44 hrs incubation, 3% LALB was inoculated in coconut water at 1% (w/v) sucrose with the culture temperature at 37°C. Within 4 hrs of incubation time, the cell number has reached log CFU/mL of 8.04 and pH value of 5.7. At the 24-hour incubation, the cell number had increased to log CFU/mL 9.54 and reached log CFU 9.86 after the 44-hour incubation period. On the other hand, the pH value was found significantly decreased from 5.7 to 3.6 after the coconut water was fermented for 44 hrs (Figure 1).

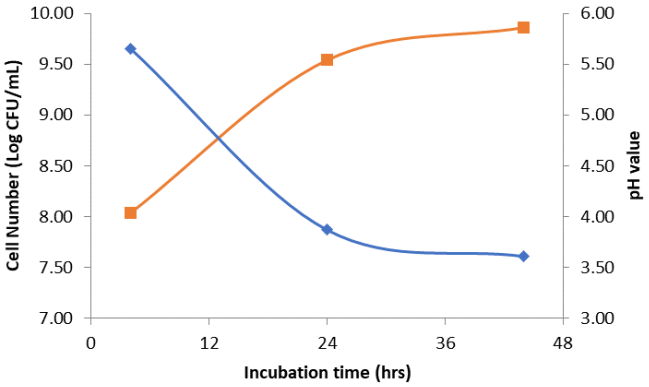

Figure 1. Effect of LALB cultivation time for the changes in pH values and microbe growth performance (log CFU/mL) on fermented MCW

3.2 Effects of different experimental conditions on the production of GABA and L-DOPA in MCW

The RSM was used to identify optimal fermentation conditions for GABA and L-DOPA productions in MCW by LALB. In a previous full factorial design (FFD) study, we examined the effects of main and interactive factors on GABA and L-DOPA production in sterile MCW. The effects of pyridoxal phosphate (PLP) and glucose were chosen and tested for both productions. The results suggested that glucose was a major parameter that influenced GABA and L-DOPA production. Therefore, glucose was chosen but it was replaced with sucrose due to its low cost. Temperature and incubation time simultaneously affected GABA and L-DOPA productions in MCW. Therefore, it was chosen as the 2nd and 3rd variables for the RSM optimization model. The inoculum size of 3% (v/v) was a fixed parameter for the whole experiment.

Based on the 3-factorials-5-levels response surface design, the effects of sucrose concentration (1, 2, 3.5, 5 and 6 g), culture temperature (25, 30, 37.5, 45 and 50°C) and incubation time (4, 12, 24, 36 and 44 hrs) on GABA and L-DOPA production were investigated. After 44 hrs,

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the effect of each factor and their interaction were analyzed using the analysis of variance (ANOVA). The statistical significance of the model was checked using different criteria. Both GABA and L-DOPA models were good with a high *F*-value but low *P*-value of < 0.05 indicating the models are significant. The regression equation with insignificant lack-of-fit values at 0.4552 and 0.6301 indicated that each model was adequate for predicting the GABA and L-DOPA content, respectively. Lack of fit refers to the fact that the simple linear regression model may not adequately fit the data (Table 1a and 1b). Table 2 summarizes the experimental and predicted data for 3 factorial-5-levels response surface analysis on GABA and L-DOPA. The R^2 values for both response variables were higher than 0.90, indicating that a good regression model was achieved. The value of the adjusted R^2 at 90.69 and 99.73% for GABA and L-DOPA production in MCW, respectively further supports the predicted model.

The quadratic equation of regression for the GABA and L-DOPA responses was represented by a three-dimensional surface as shown in Figure 2. The response surface of LALB optimal fermentation for GABA and L-DOPA production was evaluated and used to analyze the interacting effects of three variables; sucrose concentration, temperature and incubation time. The individually optimum conditions for the yield of GABA were found to be: 1 g sucrose at 37.5°C with 24 hrs of incubation time, while the optimal conditions for L-DOPA were 5 g sucrose at 30°C with 36 hrs of incubation time under static condition (Table 2). Both GABA and L-DOPA production was found to increase with the decreasing temperature in the studied range, suggesting that fermentation temperature was the main determining factor for both productions by LALB. On the contrary, the sucrose concentration showed no significant effect on GABA and L-DOPA production. By utilizing the RSM optimization model, the production of GABA and L-DOPA in LALB-fermented MCW significantly increased by 7.6 and 89.4%, respectively when compared with the negative control (non-fermented MCW).

Table 1a. Analysis of ANOVA for GABA production in LALB fermented MCW

Source	Sum of Squares	DF	Mean Square	F value	Prob > F	
Model	635.47	9	70.61	21.57	< 0.0001	significant
A	33.56	1	33.56	10.25	0.0095	
B	77.12	1	77.12	23.57	0.0007	
C	2.60	1	2.60	0.79	0.394	
A^2	44.40	1	44.40	13.57	0.0042	
B^2	11.58	1	11.58	3.54	0.0894	
C^2	354.86	1	354.86	108.43	< 0.0001	
AB	1.13	1	1.13	0.34	0.5707	
AC	21.13	1	21.13	6.45	0.0293	
BC	66.13	1	66.13	20.20	0.0012	
Residual	32.73	10	3.27			
Lack of Fit	17.23	5	3.45	1.11	0.4552	not significant
Pure Error	15.50	5	3.10			

Table 1b. Analysis of ANOVA for L-DOPA production in LALB fermented MCW

Source	Sum of Squares	DF	Mean Square	F value	Prob > F	
Model	957.10	9	1063.01	767.41	< 0.0001	significant
A	1.60	1	1.60	1.16	0.3070	
B	4356.12	1	4356.12	3144.78	< 0.0001	
C	1351.61	1	1351.61	975.76	< 0.0001	
A^2	1.98	1	1.98	1.43	0.2600	
B^2	1782.11	1	1782.11	1286.54	< 0.0001	
C^2	2328.52	1	2328.52	1681.01	< 0.0001	
AB	0.13	1	0.13	0.090	0.7700	
AC	6.13	1	6.13	4.42	0.0618	
BC	15.13	1	15.13	10.92	0.0080	
Residual	13.85	10	1.39			
Lack of Fit	5.85	5	1.17	0.73	0.6301	not significant
Pure Error	8.00	5	1.60			

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Table 2. Central composite design matrix on GABA and L-DOPA production in LALB fermented MCW

Run	Independent variables (coded-level)			GABA (mg/L)		L-DOPA (mg/L)	
	Sucrose conc. (g)	Temperature (°C)	Incubation time (hrs)	Actual	Predicted	Actual	Predicted
1	5.00	30.00	36.00	356±2.12	357	94±0.71	94
2	2.00	30.00	36.00	355±3.54	356	92±0.71	92
3	3.50	37.50	24.00	360±2.83	361	89±0.035	90
4	3.50	37.50	24.00	362±0.71	361	88±3.20	90
5	6.00	37.50	24.00	364±1.41	364	90±0.71	90
6	3.50	37.50	44.00	349±1.41	346	71±1.41	71
7	1.00	37.50	24.00	369±1.41	368	91±0.71	92
8	3.50	37.50	24.00	360±1.50	361	90±0.08	90
9	5.00	30.00	12.00	361±1.41	359	75±0.71	75
10	3.50	37.50	24.00	363±2.12	361	91±0.71	90
11	2.00	45.00	36.00	357±2.12	358	60±0.63	60
12	3.50	37.50	24.00	364±2.83	361	91±1.41	90
13	3.50	37.50	24.00	360±1.71	361	91±1.76	90
14	3.50	25.00	24.00	363±2.12	364	88±0.49	89
15	3.50	50.00	24.00	355±2.12	356	28±0.71	28
16	3.50	37.50	4.00	346±0.01	345	36±0.08	37
17	5.00	45.00	12.00	350±0.12	348	37±0.57	36
18	2.00	30.00	12.00	367±1.41	364	78±0.71	77
19	2.00	45.00	12.00	357±1.41	355	39±0.71	39
20	5.00	45.00	36.00	356±1.41	358	60±0.71	60

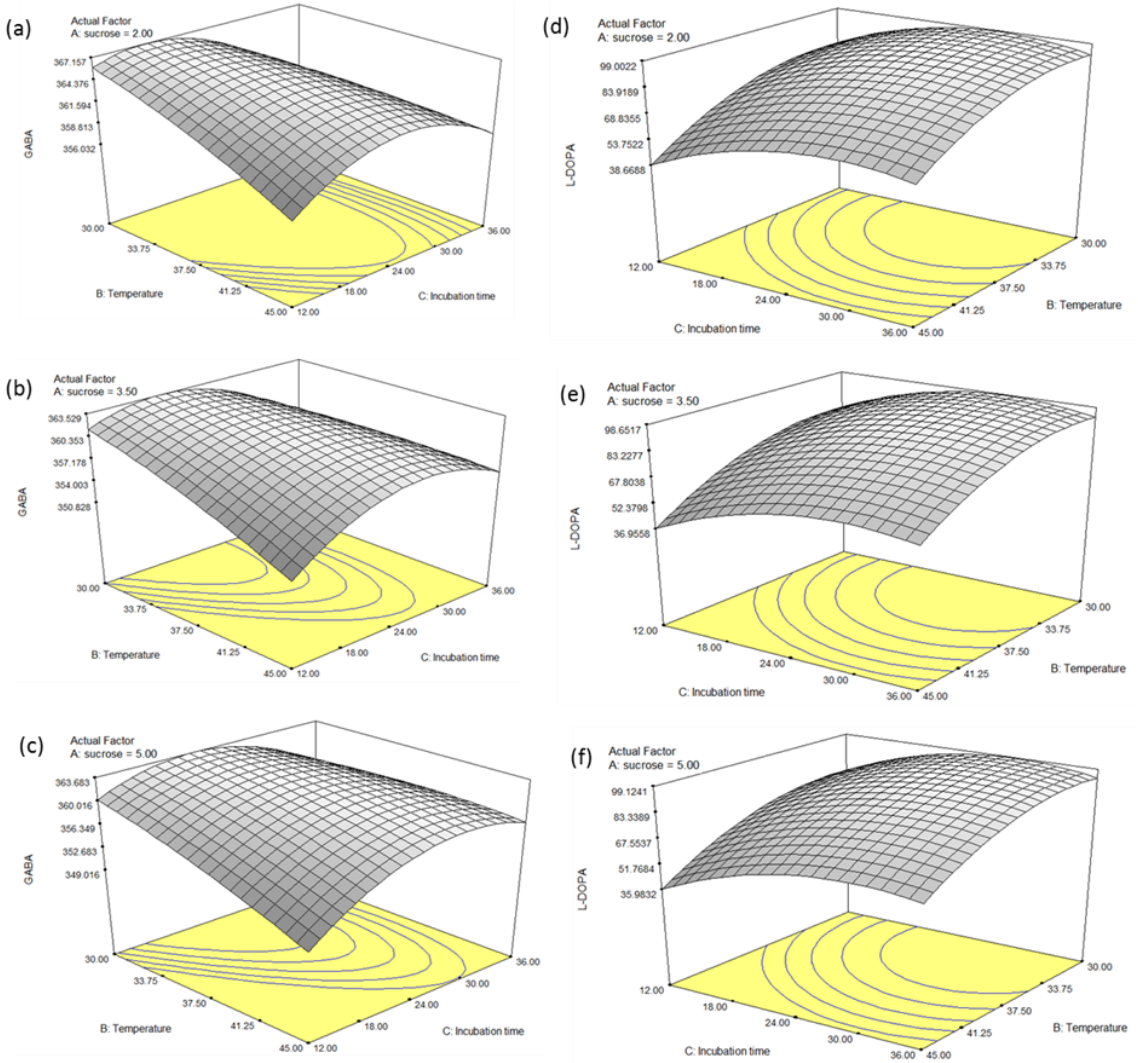

Figure 2. Three-dimensional response surface illustrating the value of GABA (a, b, c) and L-DOPA (d, e, f) in LALB fermented mature coconut water. Each graph displays the interactive effects of two variables while the third variable was fixed.

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3.3 Optimization and model verification for both GABA and L-DOPA production by LALB

According to the RSM results for GABA and L-DOPA production (Table 2 and Figure 2), the optimal condition for the highest GABA and L-DOPA production was investigated in the present study. By using RSM optimization modelling, the optimal fermentation conditions generated from RSM analysis indicated that the optimal production condition of GABA and L-DOPA production by LALB were found to be 1 g sucrose with an incubation temperature of 27°C for 28 hrs under static conditions. These incubation conditions were predicted to obtain the optimal amount of GABA (369 mg/L) and L-DOPA (94 mg/L). To verify the RSM optimization model. The experiment was conducted in triplicate and success to achieve the predicted accuracy predicted accuracy of 95.4% and 94.7% respectively for GABA and L-DOPA based on the recommended optimal fermentation process parameter.

Many studies indicated that several lactic acid bacteria have the potential to produce GABA, including *L. helveticus* ND01 (Sun *et al*., 2009) (Table 3). Kook *et al*. (2010)'s finding showed that *L. sakei* B2-16 significantly increased GABA production up to 17,014 mg/L when 3% of MSG concentration was added to substrate fermentation. Another study demonstrated the potential of *L. plantarum* C48 to produce GABA at a concentration of 125.9 mg/L (Rizzello *et al*., 2008). However, in our study, co-cultured *L. acidophilus* and *L. brevis* yielded 352 mg GABA/L. Therefore, GABA-producing ability is varied widely among the strains of lactic acid bacteria and it is significantly affected by culture conditions and medium composition. Thus, it is important to optimize these conditions to enhance GABA production by LALB. In this study, the optimal conditions for GABA fermentation are varied among different lactic acid bacteria strains, and the main factors affecting GABA production for LALB were characterized by sucrose concentration, culture temperature and incubation time.

4. Conclusion

In summary, our findings demonstrated that the fermentation of MCW with *L. acidophilus* co-cultured with *L. brevis* increased the concentration of GABA and L-DOPA simultaneously. The use of specific lactic acid bacteria strains in the fermentation process can enhance the levels of functional bioactive compounds to provide a beneficial effect on health, particularly to promote the development of value-added products using mature coconut water. Through RSM optimization and verification modelling, the optimal conditions for producing optimal GABA and L-DOPA in MCW by LALB were 1 g sucrose with an incubation temperature of 27°C for 28 hrs under static conditions with the predicted accuracy of 95.4% (352 mg GABA/L) and 94.7% (89 mg L-DOPA/L), respectively. The fermented MCW drink produced using LALB fermentation process offers a potential functional drink market to the by-product of the coconut milk industry for the treatment of hypertension and Parkinson's diseases with the presence of GABA and L-DOPA.

Conflict of interest

The authors declare no conflicts of interest

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Table 3. Studies on GABA production using lactic acid bacteria

Substrate	Starter culture	GABA (mg/L)	References
Milk	*L. helveticus* ND01	113.4	Sun *et al*. (2009)
Rice bran extracts	*L. sakei* B2-16	17014.0	Kook *et al*. (2010)
Sourdough	*L. plantarum* C48	125.9	Rizzello *et al*. (2008)
Matured coconut water	*L. acidophilus* co-cultured with *L. brevis*	369.0	Current study

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