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Evaluation of factors that influence the L-glutamic and γ -aminobutyric acid production during *Hericium erinaceus* fermentation by lactic acid bacteria

Evaluación de los factores que influyen la producción de los ácidos L-glutámico y γ -aminobutírico durante la fermentación de *Hericium erinaceus* con bacterias ácido-lácticas

Sasimar Woraharn^a, Narissara Lailerd^b, Bhagavathi Sundaram Sivamaruthi^a, Wiwat Wangcharoen^c, Sophon Sirisattha^d, Sartjin Peerajan^e and Chaiyavat Chaiyasut^{a*}

^aDepartment of Pharmaceutical Sciences, Faculty of Pharmacy, Chiang Mai University, Chiang Mai 50200, Thailand; ^bDepartment of Physiology, Faculty of Medicine, Chiang Mai University, Chiang Mai 50200, Thailand; ^cFaculty of Engineering and Agro-Industry, Maejo University, Sansai, Chiang Mai 50290, Thailand; ^dDepartment of Bioscience, Thailand Institute of Scientific and Technological Research, Pathumthani 12120, Thailand; ^eHealth Innovation Institute, Chiang Mai 50200, Thailand

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Fermented plant beverages (FPBs) are non-alcoholic and are produced by lactic acid bacteria (LAB) with different edible plant sources. Owing to the lack of a detailed study on fermented *Hericium erinaceus* with LAB, the current study was employed to exploit and optimize the fermentation condition for the production of L-glutamic acid (GA)- and γ -aminobutyric acid (GABA)-rich fermented *H. erinaceus* beverage by *Lactobacillus brevis* HP2 and *Lactobacillus fermentum* HP3, respectively. Box–Behnken design (BBD) for response surface methodology was employed with cofactor, pH and temperature as independent variables. The results suggested that *L. brevis* HP2 and *L. fermentum* HP3 are good starter cultures for *H. erinaceus* fermentation with enriched GA and GABA production. In this case, GA production was significantly influenced by K_2HPO_4 , pH and temperature whereas GABA production was significantly influenced by pH and temperature. Further thorough and fine optimization is required to fabricate high-quality fermented drinks with low cost.

Keywords: fermented plant beverages; γ -aminobutyric acid; *Hericium erinaceus*; L-glutamic acid; lactic acid bacteria; response surface methodology

Las bebidas de plantas fermentadas (FPB) son sin alcohol y son producidas con bacterias ácido-lácticas (LAB) con diferentes fuentes de plantas comestibles. Debido a la falta de un estudio detallado sobre la fermentación de *Hericium erinaceus* con LAB, se empleó el presente estudio para explotar y optimizar la condición de fermentación para la producción de ácido L-glutámico (GA) y ácido γ -aminobutírico (GABA) en la bebida de *H. erinaceus* ricamente fermentado con *Lactobacillus brevis* HP2 y *Lactobacillus fermentum* HP3, respectivamente. Se empleó El diseño Box-Behnken para la metodología de superficie de respuesta con cofactor, pH y temperatura como variables independientes. Los resultados mostraron que *L. brevis* HP2 y *L. fermentum* HP3 son buenos cultivos de inicio para la fermentación de *H. erinaceus* con producción de ácido L-glutámico y ácido γ -aminobutírico enriquecidos. En este caso, la producción de ácido L-glutámico estuvo influenciada de manera significativa por K_2HPO_4 , el pH, y la temperatura mientras que la producción de ácido γ -aminobutírico estuvo influenciada de manera significativa por el pH y la temperatura. Se requiere una optimización más exhaustiva y precisa para elaborar bebidas fermentadas de alta calidad con un bajo coste.

Palabras clave: bebidas de plantas fermentadas; ácido γ -aminobutírico; *Hericium erinaceus*; ácido L-glutámico; bacteria ácido-láctica; metodología de superficie de respuesta

Introduction

Fermented plant beverages (FPBs) are prominent in East Asian countries such as Thailand, Japan, Korea and China (Kim, Nam, & Friedman, 2013; Lee et al., 2010). FPBs are non-alcoholic and are produced by lactic acid bacteria (LAB) from different edible plants such as medicinal plants, vegetables and fruits (Kantachote & Charemrjiratrakul, 2008a, 2008b; Prachyakij, Charemrjiratrakul, & Kantachote, 2008; Ratanaburee, Kantachote, Charemrjiratrakul, Penjamras, & Chaiyasut, 2011). Fermented plant juices are well known and are considered to be a novel functional food product since they contain live LAB-based probiotics, plenty of nutrients, antioxidants and antimicrobials (Duangjitcharoen, Kantachote, Ongsakul, Poosaran, & Chaiyasut, 2009; Prachyakij et al., 2008; Ratanaburee et al., 2011).

The mushroom *Hericium erinaceus* is one of the famous bases for fermented mushroom beverages (FMBs) and known

for its medicinal values. Fruiting body and mycelia display various pharmacological activities such as anti-cancer activity, immuno-modulating effect, neuro-protective activities, and anti-oxidant and antimicrobial activities (Kim et al., 2013; Lee et al., 2010; Lee, Min, Cho, & Hong, 2009; Wong, Sabaratnam, Abdullah, Kuppusamy, & Naidu, 2009). *H. erinaceus*, also known as monkey's head mushroom, is suitable for use as a natural substrate for glutamic acid (GA) fermentation because it has a high protein content (8.01%) and free amino acid (Choi, Park, Woo, Jeong, & Shin, 2003). The fermented juice with high GA can serve as the best substrate for the production of fermented juice with a high content of Gamma-aminobutyric acid (GABA).

GA is widely used in various industries such as food, pharmaceutical, biochemical, medical, cosmetics and analytical industries (Vijayalakshmi & Sarvamangala, 2011). GA is also a

*Corresponding author. Email: chaiyavat@gmail.com

precursor of GABA, a four-carbon non-protein amino acid, widely distributed in nature, and serves as the major inhibitory neurotransmitter in mammalian nervous system (Lu, Xie, & Gu, 2009). GA has been used to treat hepatic coma (McDermott, Adams, & Riddell, 1954), mental retardation (Zimmerman, Burgemeister, & Putnam, 1946), cancer (act as an adjuvant to anticancer drugs) and antivincristine-induced neurotoxicity prevention (Kulkarni, Kulkarni, & Hamsa, 2005). GA has been produced through hydrolysis of L-glutamine by L-glutaminase (Jeya Prakash, Poorani, & Anantharaman, 2010). L-glutaminase acts as proteolytic endopeptidase and is the cellular enzyme deaminating the substrate L-glutamine, which hydrolyses the peptide bonds of protein molecules (Nathiya, Sooraj, Angayarkanni, & Palaniswamy, 2011).

GABA is extensively distributed in the environment (Ueno, 2000), which has notable physiological functions, for instance, as an anti-stress molecule (Vaiva et al., 2004), suppresses the propagation of cancer cells (Park & Oh, 2007), acts as an anti-hypertensive (Yoshimura et al., 2010) and it prevents diabetes (Soltani et al., 2011). GABA production in microbes has been reported through glutamic acid decarboxylase (GAD) (Li & Cao, 2010). GABA production is the resultant of an acid-resistance mechanism of bacteria, via decarboxylation of L-glutamate by GAD (Cotter & Hill, 2003).

We screened and identified the potential LAB, *Lactobacillus brevis* HP2 and *Lactobacillus fermentum* HP3 isolated from Thai fermented foods, which can produce extracellular glutaminase and GAD enzyme, respectively (Woraharn et al., 2014). There have been no comprehensive reports regarding the development of fermented *H. erinaceus* juice (FHJ) with high amounts of GABA using glutaminase and GAD-producing LAB.

Thus, the current study was conceived and carried out to explore the use of identified enzyme producing LAB strains for the production of FHJ with high content of GABA and to evaluate the impact of cofactor, pH and temperature on GA and GABA production. Use of LAB provides added advantage – it may also act as a probiotic. We adapted the response surface methodology (RSM), a collection of statistical techniques for researching multiple process variables with fewer experimental trials. The Box–Behnken design (BBD) is one such popular RSM used to identify the optimum conditions for production with optimum values of multiple variables. Thus, in this study, we applied RSM with BBD (Box & Behnken, 1960) to estimate the influence of treatment variables on GA and GABA production using *H. erinaceus* as the substrate for FMB.

Materials and methods

Materials

H. erinaceus, in a dry form, was purchased from an organic farm at Nakhonratchasima province, northeast of Thailand. Cane sugar and potable water were received from the local market. *L. brevis* HP2 and *L. fermentum* HP3 strain were used, which were isolated from Thai fermented foods and identified by 16S rRNA sequencing and deposited in the culture collection centre of faculty of Pharmacy, Chiang Mai University, Thailand (Woraharn et al., 2014). These strains were selected for fermentation since they produce significantly higher amounts of glutaminase and GAD (Woraharn et al., 2014). The selected LAB was cultured in MRS broth for seed culture preparation.

Methods

Description of the experimental design

Designing of an experiment for enzyme production has been influenced by several factors such as cofactors, temperature, etc. Based on the previous literature survey for GA production, we have selected three factors for experimental design – initial concentration of cofactor, initial pH and incubation temperature. K_2HPO_4 was used as a cofactor in the concentration range of 0–100 mM. pH (6–8) and temperature (30–40°C) were also selected as independent variables based on the previous literature (Katikala, Bobbarala, Tadimalla, & Guntuku, 2009; Vermeulen, Gänzle, & Vogel, 2007; Weingand-Ziadé, Gerber-Décombaz, & Affolter, 2003). Second-stage fermentation for GABA production also involved the selection of three factors including pyridoxal-5-phosphate (PLP), cofactor, concentration (0–0.05 mM) (Bai et al., 2009; Komatsuzaki, Nakamura, Kimura, & Shima, 2008), pH (4.5–6.5) (Cho, Chang, & Chang, 2007; Komatsuzaki et al., 2008) and temperature (30–40°C) (Cho et al., 2007; Lu et al., 2009; Ratanaburee et al., 2011).

The optimal combination of initial cofactor, initial pH and temperature for the production of GA and GABA in *H. erinaceus* fermentation by selected strains was examined using different concentrations of initial cofactor (X_1) (w/v), initial pH (X_2) and temperature (X_3) (°C). A BBD was used to describe the character of the response surface in the normal experimental region. The levels of the independent variables (X_1 , X_2 and X_3) were studied at three levels and coded as –1, 0 and +1 for low, middle and high levels, respectively (Table 1). In order to statistically optimize the optimal condition and assess the main effects, interaction effects and quadratic effects of the three factors on the production of GA and GABA, a design with three factors and three levels including five replicates at the centre point was used (Tables 2 and 5). For assessment of the three factors, Equation (1) was used.

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 \quad (1)$$

where Y is the predicted response; β_0 is the model constant; β_i (β_{1-3}) are the linear coefficients; β_{ii} (β_{11-33}) are the quadratic coefficients; β_{ij} (i.e. β_{12}) are the cross-product coefficients; and X_1 , X_2 and X_3 are independent variables.

Table 1. Codes of independent variables and their actual values used in RSM optimization for L-glutamic acid and GABA production.

Tabla 1. Códigos de variables independientes y sus valores reales utilizados en la optimización RSM para la producción de ácido L-glutámico y ácido γ -aminobutírico.

Independent variables	Symbol	Code level		
		–1	0	+1
<i>L-glutamic acid production</i>				
K_2HPO_4 (mM)	X_1	0	50	100
pH	X_2	6	7	8
Temperature (°C)	X_3	30	35	40
<i>GABA production</i>				
PLP (μ M)	X_1	0	25	50
pH	X_2	4.5	5.5	6.5
Temperature (°C)	X_3	30	35	40

Table 2. Box–Behnken design for optimization of three factors for GA production during *H. erinaceus* juice fermentation. Results are the outcome of three trials (mean \pm SD).

Tabla 2. Diseño Box-Behnken para la optimización de tres factores para la producción de ácido L-glutámico durante la fermentación de zumo de *H. erinaceus*. Los datos son los resultados de tres pruebas (promedio \pm SD).

Trial No.	Factors			L-Glutamic acid (mg/ml)	
	X ₁ K ₂ HPO ₄ (mM)	X ₂ pH	X ₃ Temperature (°C)	Actual	Predicted
1	-1 (0)	-1 (6)	0 (35)	0.10 \pm 0.02 ^a	0.13
2	1 (100)	-1 (6)	0 (35)	0.10 \pm 0.01 ^a	0.22
3	-1 (0)	1 (8)	0 (35)	0.10 \pm 0.02 ^a	0.06
4	1 (100)	1 (8)	0 (35)	1.28 \pm 0.10 ^d	1.32
5	-1 (0)	0 (7)	-1 (30)	0.10 \pm 0.01 ^a	0.14
6	1 (100)	0 (7)	-1 (30)	0.23 \pm 0.03 ^{ab}	0.18
7	-1 (0)	0 (7)	1 (40)	0.15 \pm 0.05 ^a	0.05
8	1 (100)	0 (7)	1 (40)	1.55 \pm 0.20 ^e	1.36
9	0 (50)	-1 (6)	-1 (30)	0.12 \pm 0.02 ^a	-0.03
10	0 (50)	1 (8)	-1 (30)	0.18 \pm 0.03 ^{ab}	0.11
11	0 (50)	-1 (6)	1 (40)	0.14 \pm 0.04 ^a	0.13
12	0 (50)	1 (8)	1 (40)	0.97 \pm 0.20 ^c	1.04
13	0 (50)	0 (7)	0 (35)	0.32 \pm 0.10 ^b	0.31
14	0 (50)	0 (7)	0 (35)	0.32 \pm 0.02 ^b	0.31
15	0 (50)	0 (7)	0 (35)	0.30 \pm 0.05 ^b	0.31
16	0 (50)	0 (7)	0 (35)	0.31 \pm 0.01 ^b	0.31
17	0 (50)	0 (7)	0 (35)	0.31 \pm 0.06 ^b	0.31

Notes: The small alphabets indicate that the values are significantly different in L-glutamic acid content among the trial ($p < 0.05$, $n = 3$). The alphabet "a" represents the low L-glutamic acid group and significantly differs from b, c, d and e. The maximum yield of L-glutamic acid is represented in the e group.

Nota: Las letras pequeñas indican que los valores son significativamente diferentes en el contenido de ácido L-glutámico entre las pruebas ($p < 0.05$, $n = 3$). La letra "a" representa el grupo inferior de ácido L-glutámico y difiere significativamente de b, c, d y e. La mayor cantidad de ácido L-glutámico se representa en el grupo e.

Statistical analyses of RSM

The behaviour of the response surface was evaluated for the response function (Y) using the regression polynomial equation. After 17 runs of BBD arrangement, we obtained the coefficients of regression equation (β_0 , β_1 , β_2 , β_3 , β_{11} and so on) by SPSS 16.0 for Windows[®] (2007 SPSS, Inc.). The significances of all independent variable in terms of the polynomial were detected statistically by calculating the *F*-value at a probability level (*p*) of 0.05. Only the independent variable terms, found statistically significant ($p < 0.05$), were included in the reduced models. The model is resubmitted for statistical analysis to neglect all terms that are statistically non-significant ($p > 0.05$). Regression coefficients are used to create the regression equation and generate a response surface map and the acceptance of the model was determined using model *p*-value ($p < 0.05$), lack-of-fit *p*-value test ($p > 0.05$) and coefficient of determination (R^2). The nearness of the coefficient of determination (R^2) to 1 or 100% ensures the satisfactory adjustment of the quadratic model to the experimental data. The surface plot based on two independent variables was generated by keeping the third independent variable at a constant level (middle value) with response value (GA or GABA). The behaviour of the response surface was created using a regression equation by Statistica 6.0 (the demo version from <http://statistica.soft-ware.informer.com/6.0/>).

First-stage fermentation for GA production

L. brevis HP2 was propagated in culture medium (50% of MRS medium (Himedia, Cat No. M369) and 50% of 10% (w/v) cane sugar) for 18 h at 37°C. The cells were pelleted out by centrifuge at 6000 rpm for 10 min at 4°C and were washed twice with 0.85% (w/v) NaCl. The cell pellet was resuspended in sterile distilled water to approximately 10⁹ CFU/mL and was used as inoculum (starter culture). Dried *H. erinaceus*, cane sugar and potable water were mixed at a ratio of 10:5:85 (w:w:v), respectively. The cane sugar and K₂HPO₄ were dissolved in the potable water and were boiled. Then the mixture was cooled down to 80–90°C and dried mushroom was added to the mixture for pasteurization (75°C for 10 min), then the pH was adjusted according to Table 2 using 5 M citric acid and 5 M NaOH. Bacterial load of the mixture was also assessed before fermentation to ensure the sterility (data not shown). The mixture of the fermentation after inoculating 10% of the initial culture had a total volume of 16 L in a 20 L plastic bucket. All fermentation containers were incubated at 30 \pm 2°C for 30 days to monitor changes in the amount of GA. Fermentation without starter served as the control. Finally, the optimum condition for GA production was used to prepare the *H. erinaceus* medium for the next step of fermentation.

Second-stage fermentation for GABA production

Starter culture of *L. fermentum* HP3 was prepared as described in first-stage fermentation. The FHJ with high GA was used as substrate medium for GABA synthesis. First-stage FHJ was added with PLP and pasteurized (75°C for 10 min), then pH was adjusted according to Table 5 using 5 M citric acid and 5 M NaOH. Bacterial load of the mixture was also assessed before fermentation to ensure the sterility (data not shown). After adding 10% of inoculum to the substrate medium, fermentation containers were incubated at 30 \pm 2°C for 30 days. Fermentation without starter served as control.

Analysis of L-GA and GABA by HPLC

Centrifugation was performed for 1 mL of culture at 6000 rpm for 10 min and the supernatant was collected for L-GA and GABA analysis. The standard solutions of GA, GABA and sample were determined by pre-column derivatization of phenylthiocarbonyl-GABA (PTC-GABA) from phenyl isothiocyanate (PITC) according to the method detailed by Cho et al. (2007). Briefly, PITC solution was added to samples to form PTC-GABA and PTC-GA. The standard curves for GABA and L-GA were determined by following the same procedure to four concentrations of standards. Standards and samples were analysed for GA and GABA by HPLC (LC-10ADVP, Shimadzu, Japan) with C18 reversed-phase column (5 μ m dia), 4.6 \times 250 mm. Mobile phase A consisted of 70 mM sodium acetate buffer solution (pH 5.8) and mobile phase B was acetonitrile. The gradient system was optimized for a good separation. UV detector at 254 nm was used.

Statistical analysis

All experiments were performed in triplicate and all data are expressed as means \pm SD. Dissimilarities between the mean values of multiple groups were evaluated by one-way analysis

of variance (ANOVA). The differences were considered significantly different when $p < 0.05$.

Results and discussion

GA production

In the current study, RSM was engaged for the optimization of GA production using *H. erinaceus* as substrate and *L. brevis* HP2 as the starter culture. RSM helps determine the main and interaction effects of growth and GA production. Codes for independent variables for L-GA production were allotted and tabulated (Table 1). The diversity of each response variable was evaluated as a function of linear, quadratic and interaction terms of cofactor, K_2HPO_4 (X_1), pH (X_2) and temperature (X_3). Phosphate is the reported cofactor of glutaminase (Yuasa, Okamura, & Kataoka, 2003). Use of sodium phosphate in beverage may not be appropriate for people with hypertension. Thus, we used potassium phosphate as the cofactor in this study. Seventeen combinations of parameters were selected based on BBD of experiment and all the combinations were tested for GA production. The actual productions of GA and predicted values are tabulated in Table 2. Regression coefficients for L-GA concentration in first-stage fermentation were estimated using SPSS and are represented in Table 3. Results suggested that the quadratic model was fitted for the GA production because of its best statistic data such as maximum R^2 (97.9%) and maximum adjusted R^2 (97.2%), and the lowest predicted residual sum of square (3.104) and lack of fit (0.187), implying that the model

Table 3. Estimated regression coefficients of L-glutamic acid concentration during first-stage fermentation.

Tabla 3. Coeficientes de regresión estimados de la concentración de ácido L-glutámico durante la 1ª etapa de fermentación.

Term	Parameters estimate	p-Value
Constant	0.312	0.000
K_2HPO_4	0.339	0.000
pH	0.259	0.000
Temperature	0.272	0.000
$K_2HPO_4 \times K_2HPO_4$	0.119	0.039
pH \times pH	-0.036	0.469
Temperature \times Temperature	0.076	0.148
$K_2HPO_4 \times$ pH	0.295	0.000
$K_2HPO_4 \times$ Temperature	0.318	0.000
pH \times Temperature	0.192	0.005

Table 4. Analysis of variance for the quadratic model of GA and GABA production in fermented *H. erinaceus* juice.

Tabla 4. Análisis de la varianza para el modelo cuadrático de la producción de ácido L-glutámico y ácido γ -aminobutírico en zumo de *H. erinaceus* fermentado.

Source of variation	DF	Sum of squares		Mean square		F-value		p-Value	
		GA	GABA	GA	GABA	GA	GABA	GA	GABA
Model	9	3.038	0.923	0.338	0.103	36.188	29.206	0.000	0.000
Residue	7	0.065	0.025	0.009	0.004				
Lack of Fit	3	0.065	0.014	0.022	0.005	2.621	1.735	0.187	0.298
Pure Error	4	0.033	0.011	0.008	0.003				
Total	16	3.104	0.948						

Notes: GA, $S = 3.104$, $R^2 = 97.9\%$, R^2 (Adjusted) = 97.2%.

GABA, $S = 0.948$, $R^2 = 97.4\%$, R^2 (Adjusted) = 94.1%.

equation was adequate for predicting the production of GA (Table 4). Increased concentration of K_2HPO_4 resulted in an increased production of GA, whereas pH and temperature showed a linear effect in GA production. Linear interaction reveals that K_2HPO_4 concentration affects the GA production compared to pH and temperature ($0.339 > 0.259$ and 0.272) (Table 3 and Equation (2)). Besides, co-interaction of variables, $K_2HPO_4 \times$ temperature and $K_2HPO_4 \times$ pH, resulted in synergistic interaction with GA production and the influence of pH \times temperature interaction was found to be less (Table 3 and Equation (2)). The concentration of cofactor (K_2HPO_4), pH and temperature of the reaction as 100 mM, 7 and 40°C, respectively, has been identified as optimum conditions for the maximum production of GA in both predicted and actual categories. Based on the regression coefficient values predicted, GA production was calculated by Equation (2). Based on the surface contour plot results, the optimum condition for L-GA enrichment during *H. erinaceus* fermentation by *L. brevis* HP2 included the following parameters: 100 mM K_2HPO_4 , pH 8.0 and temperature 40°C (2.20 mg/mL of L-GA from Equation (2)) (Figure 1). The GA production in control fermentation (fermentation without *L. brevis* HP2) yielded about 0.10 mg/mL in all 17 trials, which suggested that pH, temperature and cofactor are not influenced. However, in the presence of starter culture, *L. brevis* HP2, GA production was promoted with the influence of the tested factors.

$$\begin{aligned} \text{L-glutamic acid (mg/mL)} = & 0.312 + 0.339X_1 + 0.259X_2 \\ & + 0.272X_3 + 0.119X_1^2 \\ & + 0.295X_1X_2 + 0.318X_1X_3 \\ & + 0.192X_2X_3 \end{aligned} \quad (2)$$

The impact of concentration of the cofactor on GA production in FHJ was correlated with other factors also. For example, an increase in production of GA was observed in trial no. 8, whereas very less amount of GA production was recorded in trial no. 2, with the same concentration of cofactor. GA production was not significantly affected by the pH of the medium, except trial no. 8. This data indicates that pH is one of the rate-limiting factors for GA production with selected LAB strain, but its impact was correlated with cofactor and temperature too (Figure 1). Thus these data indicated that all parameters are necessary and they should be in precise levels to produce high amounts of GA in *H. erinaceus* fermentation with *L. brevis* HP2.

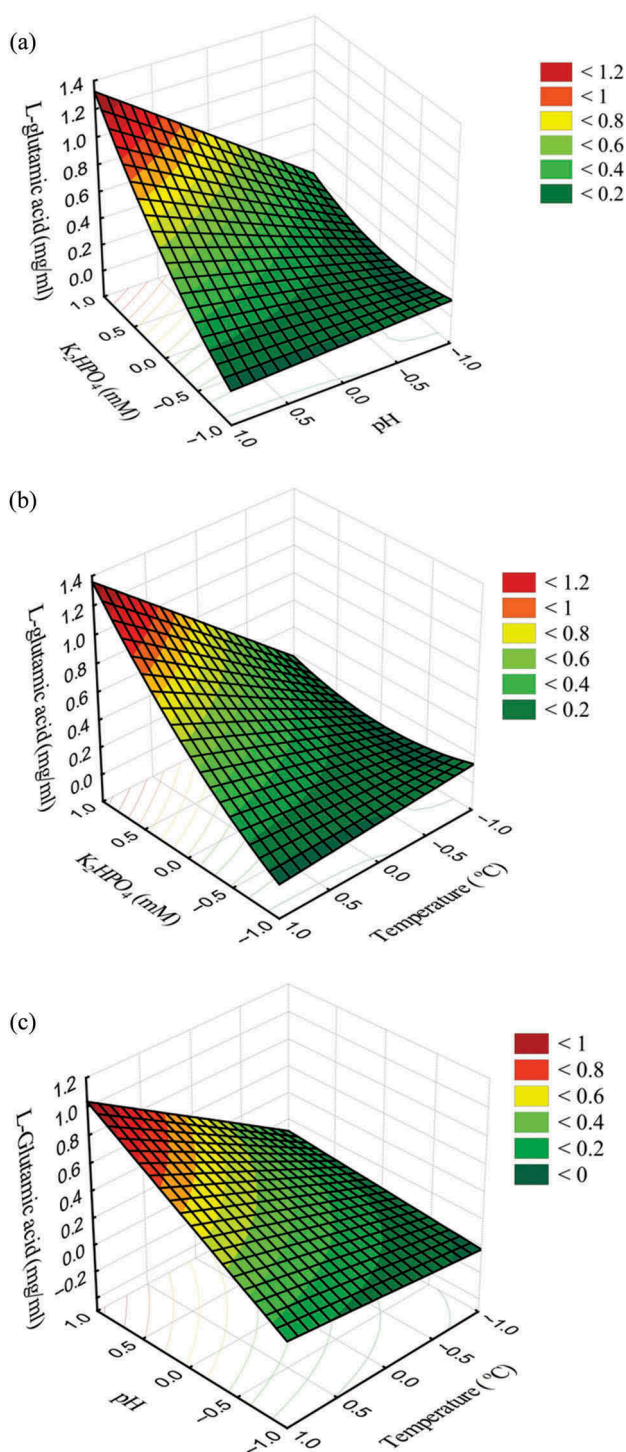


Figure 1. Response surface for the effect of cofactor (K_2HPO_4), pH and temperature on L-glutamic acid (GA) concentration (mg/mL) after the first fermentation of *H. erinaceus* by glutaminase-producing *L. brevis* HP2: (a) effect of concentration of K_2HPO_4 (mM) and pH on GA production, (b) effect of concentration of K_2HPO_4 (mM) and temperature on GA production and (c) effect of pH and temperature on GA production.

Figura 1. Superficie de respuesta para el efecto del cofactor (K_2HPO_4), el pH y la temperatura en la concentración de ácido L-glutámico (GA) (mg/mL) después de la primera fermentación de *H. erinaceus* mediante *L. brevis* HP2 con producción de glutaminasa (a) efecto de la concentración de K_2HPO_4 (mM) y el pH en la producción de ácido L-glutámico (b) efecto de la concentración de K_2HPO_4 (mM) y la temperatura en la producción de ácido L-glutámico (c) efecto del pH y la temperatura en la producción de ácido L-glutámico.

Zareian, Ebrahimpour, Mohamed, and Saari (2013) have reported the production of GA and GABA by *L. plantarum* with optimization by BBD of experiment. The authors selected four different parameters to study and optimize the production of GA and GABA. They succeeded with the improvement of 3–10-fold higher productivity upon optimization. Another study revealed the use of BBD for the production of glucuronic acid from grape juice and the authors improved the production up to 178 g/L, which is higher than normal productivity (Yavari, Assadi, Moghadam, & Larijani, 2011).

Gamma amino butyric acid production

The second fermentation process was carried out with *L. fermentum* HP3 as starter culture and the resultant of the first fermentation as substrate. This fermentation process also employed the use of BBD of experiment for the production of GABA. Codes for independent variables for GABA production were allotted (Table 1). Each response variable was evaluated as a function of linear, quadratic and interaction terms of cofactor, PLP (X_1), pH (X_2) and temperature (X_3). Seventeen combinations of parameters were generated based on BBD of experiment and all the combinations were tested for GABA production. The actual production of GABA and the predicted values are tabulated in Table 5. Regression coefficients for GABA production were estimated using SPSS and are represented in Table 6. All independent

Table 5. Box–Behnken design for optimization of three factors for GABA production during *H. erinaceus* juice fermentation. Results are the outcome of three trials (mean \pm SD).

Tabla 5. Diseño Box–Behnken para la optimización de tres factores para la producción de ácido γ -aminobutírico durante la fermentación de zumo de *H. erinaceus*. Los datos son los resultados de tres pruebas (promedio \pm SD).

Trial No.	Factors			GABA (mg/mL)	
	X_1 PLP (μ M)	X_2 pH	X_3 Temperature ($^{\circ}$ C)	Actual	Predicted
1	-1 (0)	-1 (4.5)	0 (35)	1.67 \pm 0.2 ^{abcd}	1.68
2	1 (50)	-1 (4.5)	0 (35)	1.79 \pm 0.1 ^{abcd}	1.84
3	-1 (0)	1 (6.5)	0 (35)	2.11 \pm 0.4 ^d	2.07
4	1 (50)	1 (6.5)	0 (35)	1.92 \pm 0.3 ^{abcd}	1.91
5	-1 (0)	0 (5.5)	-1 (30)	1.66 \pm 0.2 ^{abcd}	1.75
6	1 (50)	0 (5.5)	-1 (30)	1.78 \pm 0.2 ^{abcd}	1.75
7	-1 (0)	0 (5.5)	1 (40)	2.04 \pm 0.4 ^{cd}	1.98
8	1 (50)	0 (5.5)	1 (40)	1.97 \pm 0.3 ^{bcd}	1.98
9	0 (25)	-1 (4.5)	-1 (30)	1.70 \pm 0.2 ^{abcd}	1.65
10	0 (25)	1 (6.5)	-1 (30)	1.72 \pm 0.2 ^{abcd}	1.72
11	0 (25)	-1 (4.5)	1 (40)	1.72 \pm 0.1 ^{abcd}	1.72
12	0 (25)	1 (6.5)	1 (40)	2.05 \pm 0.3 ^d	2.11
13	0 (25)	0 (5.5)	0 (35)	1.38 \pm 0.3 ^a	1.42
14	0 (25)	0 (5.5)	0 (35)	1.43 \pm 0.4 ^{ab}	1.42
15	0 (25)	0 (5.5)	0 (35)	1.47 \pm 0.4 ^{abc}	1.42
16	0 (25)	0 (5.5)	0 (35)	1.35 \pm 0.3 ^a	1.42
17	0 (25)	0 (5.5)	0 (35)	1.46 \pm 0.4 ^{ab}	1.42

Notes: The small alphabets indicate that the values are significantly different in GABA content among the trial ($p < 0.05$, $n = 3$). The alphabet “a” represents the low GABA group and it significantly differs from b, c, d and e. The maximum yield of GABA is represented in the “d” group.

Nota: Las letras pequeñas indican que los valores son significativamente diferentes en el contenido de ácido γ -aminobutírico entre las pruebas ($p < 0.05$, $n = 3$). La letra “a” representa el grupo inferior de ácido γ -aminobutírico y difiere significativamente de b, c, d y e. La mayor cantidad de ácido γ -aminobutírico se representa en el grupo “d”.

Table 6. Estimated regression coefficients of GABA concentration for second-stage fermentation.

Tabla 6. Coeficientes de regresión estimados de la concentración de ácido γ -aminobutírico para la 2ª etapa de fermentación.

Term	Parameters estimate	p-Value
Constant	1.418	0.000
PLP	-0.002	0.908
pH	0.115	0.001
Temperature	0.115	0.001
PLP \times PLP	0.260	0.000
pH \times pH	0.195	0.000
Temperature \times Temperature	0.185	0.000
PLP \times pH	-0.078	0.035
PLP \times Temperature	-0.048	0.153
pH \times Temperature	0.077	0.035

variables influenced the production of GABA as shown in the quadratic effect (X_1^2 , X_2^2 and X_3^2) (Table 6 and Equation (3)). Increase in pH and temperature positively regulates the GABA production. The PLP \times pH and PLP \times temperature had a destructive influence on GABA production, whereas pH \times temperature showed a synergistic interaction (Table 6 and Equation (3)). The quadratic model is well opted for GABA production in this study, since it produces the greatest statistic data of maximum R^2 (97.4%) and maximum adjusted R^2 (94.1%), and the lowest predicted residual sum of square (0.948) and lack of fit (0.298), implying that the model equation was adequate for predicting the production of GABA (Table 4).

Optimum predicted production of GABA was noted with the combination of cofactor (PLP), pH and temperature ($^{\circ}\text{C}$) at 25 μM , 6.5 and 40, respectively. However, the results of wet lab studies indicated that pH 6.5 and temperature 35 $^{\circ}\text{C}$ without cofactor can produce a greater amount of GABA (2.11 \pm 0.4 mg/mL) by *L. fermentum* HP3. This condition was noted as the second best condition for the predicted production (2.07 mg/mL). Based on the predicted regression coefficient values, GABA production was calculated in Equation (3). Based on the surface contour plot results, the optimum condition for GABA production during *H. erinaceus* fermentation by *L. fermentum* HP3 is 0 μM of PLP, pH 6.5 and temperature 40 $^{\circ}\text{C}$ (2.44 mg/mL of GABA from Equation (3)) (Figure 2). The GABA production in control fermentation (fermentation without *L. fermentum* HP3) yielded about 0.26 mg/mL in all 17 trials, which suggested that pH, temperature and cofactor are not influenced. However, in the presence of starter culture *L. fermentum* HP3, GABA production was promoted with the influence of the tested factors.

$$\begin{aligned} \text{GABA (mg/mL)} = & 1.418 + 0.115X_2 + 0.115X_3 \\ & + 0.260X_1^2 + 0.195X_2^2 + 0.185X_3^2 \\ & - 0.078X_1X_2 + 0.077X_2X_3 \end{aligned} \quad (3)$$

The cofactor in GABA production is not necessarily important for the optimum production of GABA. The third maximum production condition also revealed the unimportance of PLP (Table 5). Regarding pH, increased GABA production was observed in the trials in the pH range of 5.5–6.5. The optimum production (2.11 \pm 0.4 mg/mL) of GABA was observed at pH 6.5 in the case of both prediction and real. Reducing the pH up to 5.5 did not have a huge

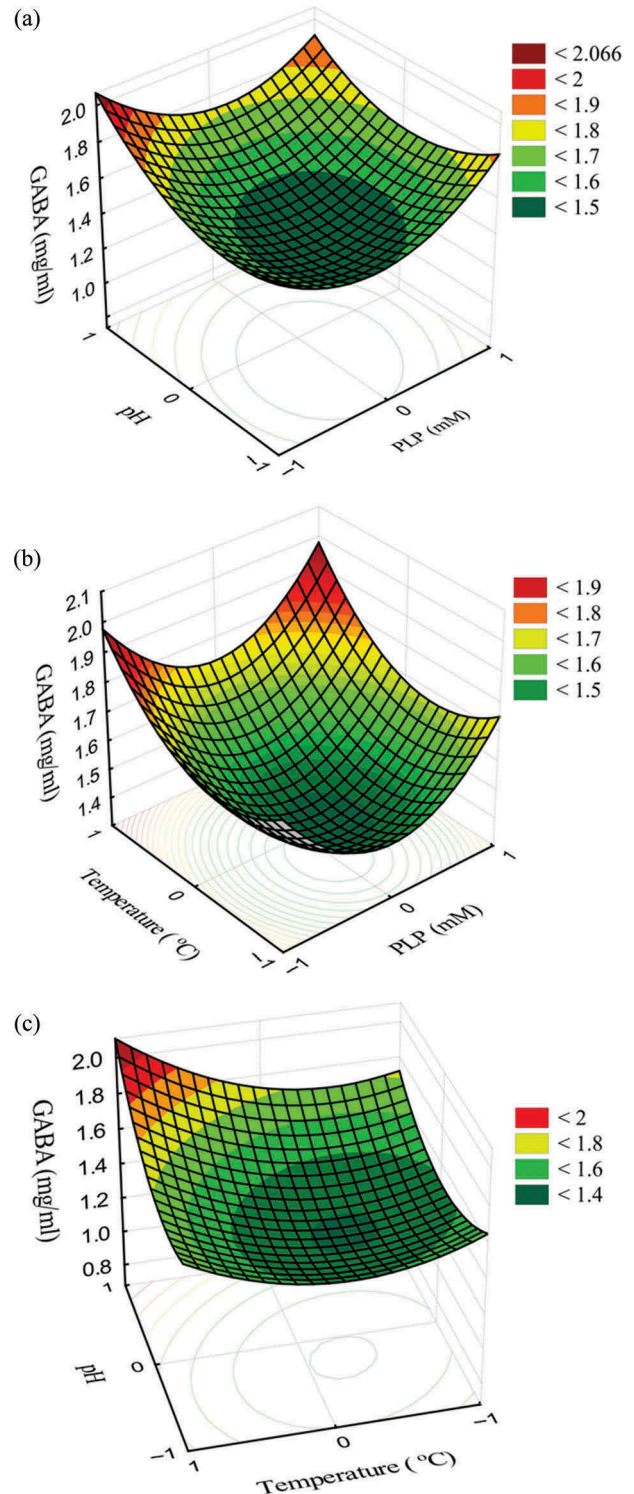


Figure 2. Response surface for the effect of cofactor (PLP), pH and temperature on γ -aminobutyric acid (GABA) concentration (mg/mL) after second-stage fermentation of *H. erinaceus* by *L. fermentum* HP3: (a) effect of concentration of PLP (μM) and pH on GABA production, (b) effect of concentration of PLP (μM) and temperature on GABA production and (c) effect of pH and temperature on GABA production.

Figura 2. Superficie de respuesta del efecto del cofactor (PLP), el pH y la temperatura en la concentración de ácido γ -aminobutírico (GABA) (mg/mL) después del segundo estado de fermentación de *H. erinaceus* con *L. fermentum* HP3 (a) efecto de la concentración de PLP (μM) y el pH en la producción de ácido γ -aminobutírico (b) efecto de la concentración de PLP (μM) y la temperatura en la producción de ácido γ -aminobutírico (c) efecto del pH y la temperatura en la producción de ácido γ -aminobutírico.

impact on GABA production (2.04 ± 0.4 mg/mL) (Table 5 and Figure 2). Improved GABA production was obtained in trials in the temperature range of 35–40°C. These results indicated that all the parameters are necessary for the optimum production of GABA by *L. fermentum* HP3 using fermented *H. erinaceus* with *L. brevis* HP2 as substrate.

Lu et al. (2009) studied the culture temperature, initial pH of the medium, monosodium glutamate (MSG) addition and MSG addition time affecting GABA production by *Lactococcus lactis* subsp. *lactis* (*L. lactis*) and they found that temperature 31.8°C, pH 7.1 and MSG 15 g/L were optimum. Influence of MSG on *Lactobacillus plantarum* DW12-mediated GABA production from red seaweed was reported (Ratanaburee et al., 2011). *L. plantarum* isolated from kimchi – a fermented Korean dish made of vegetables – produced 170.42 µg/mL of GABA in the presence of 3% MSG (Park, Lee, & Lim, 2014).

Another study by Cho et al. (2007) also explains the optimum GABA production by another kimchi isolate of LAB – *Lactobacillus buchneri* – and they reported that 5% MSG, 1% NaCl and 1% glucose, with pH of 5.0 and temperature at 30°C, can yield about 94% GABA conversion rate after 36 h. These results were fairly far from the results of the current investigation. Although starter cultures are isolated from food sources and they are LAB, the ability of enzyme production and the optimum fermentation condition are varied between LAB strains and also depend on the substrate for the fermentation process.

GA production was increased up to threefold under the condition of pH 4.5, temperature 37°C, 12% (w/v) glucose and 0.7% (w/v) ammonium nitrate; however, GABA production enhancement was reported up to 10-fold under the condition of pH 4.5, temperature 37°C, 6% (w/v) glucose and 0.7% (w/v) ammonium nitrate by *Lactobacillus plantarum* MNZ (Zareian et al., 2013). MSG (1%), sucrose (6%) and an initial pH 6 are found as optimal conditions for the production of fermented red seaweed beverage with GABA (Ratanaburee et al., 2011). Guo, Hagiwara, Masuda, and Watabe (2011) reported that *Pichia anomala* MR-1 strain produced high GABA (350 mg/100 mL of media) with the supplementation of 5% glucose and 1% MSG, but improper temperature profile and reaction mixture reduce the concentration's desired product (120 mg/100 mL of media).

Conclusions

In conclusion, production of GA and GABA by LAB strains depends on the starter culture and substrate for the fermentation. The factors manipulating GA and GABA were studied by RSM. The results suggested that cofactor (K_2HPO_4) is the significant cause, which greatly influences the GA production rather than pH and temperature. About 100 mM of K_2HPO_4 , with pH 7 and temperature at 40°C were found as the best conditions for the maximum production of GA. Increase in pH (4.5–6.5) and temperature (30–40°C) positively regulates the GABA production, whereas cofactor concentration (PLP) did not influence the GABA production during *H. erinaceus* fermentation by *L. fermentum* HP3. The results suggested that pH 6.5, temperature at 35°C and without factor (PLP) were found as the standard conditions for the maximum production of GABA.

The current study demonstrated and revealed the best conditions for the production of GA and GABA during *H.*

erinaceus fermentation using *L. brevis* HP2 and *L. fermentum* HP3 as safe starter cultures. Further detailed and subtle optimization will provide the information for the production of plant-based, probiotic, high-quality fermented drinks with low cost.

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