Gamma-Aminobutyric Acid Production from a Novel *Enterococcus avium* JS-N6B4 Strain Isolated from Edible Insects

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Gamma-aminobutyric acid (GABA)-producing strains were isolated from four edible insects and subjected to 16S rRNA sequence analysis. Among the four GABA-producing bacteria, *Enterococcus avium* JS-N6B4 exhibited the highest GABA-production, while cultivation temperature, initial pH, aerobic condition, and mono-sodium glutamate (MSG) feeding were found to be the key factors affecting GABA production rate. The culture condition was optimized in terms of glucose, yeast extract, and MSG concentrations using response surface methodology (RSM). GABA production up to 16.64 g/l was obtained under the conditions of 7 g/l glucose, 45 g/l yeast extract, and 62 g/l MSG through the optimization of medium composition by RSM. Experimental GABA production was 13.68 g/l, which was close to the predicted value (16.64 g/l) calculated from the analysis of variance, and 2.79-fold higher than the production achieved with basic medium. Therefore, GABA-producing strains may help improve the GABA production in edible insects, and provide a new approach to the use of edible insects as effective food biomaterials.

Keywords: Edible insects, gamma-aminobutyric acid, *Enterococcus avium*, mono-sodium glutamate, optimization

Introduction

Gamma-aminobutyric acid (GABA) is a four-carbon, non-protein derived amino acid widely expressed in bacteria, plants, and vertebrates. It is one of the major inhibitory neurotransmitters in the mammalian central nervous system and is extensively found in animals, plants, and microorganisms [1, 2]. GABA is primarily biosynthesized by glutamic acid decarboxylase (GAD; E.C. 4.1.1.15), a pyridoxal-5'-phosphate-dependent enzyme that catalyzes the irreversible α -decarboxylation of L-glutamic acid to

GABA through the incorporation of hydrogen ions [3, 4]. GABA exhibits several physiological functions such as antihypertensive, diuretic, and anti-diabetic effects in humans. In addition, GABA exerts tranquilizing effects, particularly related to depression, sleeplessness, and neurological disorders such as Parkinson's disease, Huntington's chorea, and Alzheimer's disease [3–6]. GABA is one of the many signaling molecules in the brain and is an important modulator of the immune function [7]. As a result, studies have been directed toward the development of functional foods containing GABA, including cereals,

sourdough, breads, cheese, fermented sausages, teas, vegetables, legumes, dairy soy products, alcohol beverages, and traditional Asian fermented foods [8].

Edible insects have received considerable attention as effective protein substitutes; however, there are several obstacles for their industrial use. First, people generally have a revulsion about insects and relate consumption with starvation and the only means of survival in developing countries. These misunderstandings are caused by the aversion of Western society to insect consumption [9]. Therefore, it is necessary to increase awareness about edible insects, owing to their having high nutritional value and positive impact on the environment, which may eventually change the perception about insect consumption. Second, large-scale insect breeding systems are warranted to increase the commercial availability of edible insects. Current production systems are very expensive, and conventional studies have shown that the cost of producing mealworm is 4.8 times higher than that of producing regular chicken feed. Labor and housing costs, especially for large-scale insect feed production, are much higher than those for chicken feed [10]. Therefore, it is necessary to increase the awareness about edible insects as an alternative to animal protein by reducing the cost of production in large-scale production systems. This may demand the development of automated systems for breeding and processing. It is necessary to create competitive market prices by continuously producing massive quantities of excellent quality insects [10]. The aim of this study was to increase the industrial usage of edible insects by producing high value-added products, achieved by providing functionality to insect proteins prior to the development of large-scale production systems. In the development of edible insects as protein substitutes, GABA may serve as an attractive biomaterial and impart higher value to the final product.

Several studies have evaluated microorganisms that produce GABA, particularly Korean fermented foods such as kimchi [11, 12], makgeolli [13], fish products [14], cheese [15–17], and sourdough [18] as well as Chinese fermented foods such as paocai [19]. Studies have also been conducted to maximize GABA production from isolated strains, and using recombinant enzymes. However, no report has described the use of strains isolated from edible insects except for the isolation of microbes from honeybees [3]. Domestic edible insects recognized as food materials in South Korea include *Bombyx mori* larvae infected with *Beauveria bassiana* (Bals.) Vuill., *Bombyx mori* larvae, mealworm (*Tenebrio molitor* larvae), *Oxya japonica* Thunberg, *Protaetia*

brevitarsis, *Allomyrina dichotoma*, and the two-spotted cricket (*Gryllus bimaculatus*). It is essential to impart high value to edible insects to increase their value as protein substitutes with the methods such as GABA fortification.

In this study, GABA-producing bacteria were isolated from four edible insects, and *Enterococcus avium* JS-N6B4 was found as the best GABA producer. The optimum culture conditions for *E. avium* JS-N6B4 growth were investigated using response surface methodology (RSM).

Materials and Methods

Materials

Mono-sodium glutamate (MSG) and GABA at $\geq 96\%$ purity were purchased from Daejung (Korea) and Alfa-Aesar (Thermo Fisher Scientific, USA). Four GABA-producing bacteria were isolated from edible insects such as *G. bimaculatus*, *T. molitor* larvae, *P. brevitarsis* larvae, and *A. dichotoma* larvae provided by the Bio Control Research Center (Korea). All other chemicals used were of reagent grade.

Isolation and 16S rRNA Gene Sequencing of the GABA-Producing Strains from the Edible Insects

The ground insects were subjected to extraction with sterilized water at 25°C for 1 h and incubated on solid MRS medium (Difco, USA) at 37°C for 24 h. To isolate GABA-producing strains, the selected colonies were cultivated in modified MRS broth containing 1% (w/v) MSG at 37°C for 72 h [11]. Extraction of genomic DNA of the four GABA-producing strains was carried out using Chelex 100 beads (Bio-Rad Laboratories, USA). The 16S rRNA gene was subjected to polymerase chain reaction (PCR) based amplification using the chromosomal DNA and universal bacterial primer pair 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGCTACCTTGTTACGACTT-3'). The purified PCR products were sequenced by SolGent (Korea). Multi-alignment of related strains was performed using Clustal W [20].

Qualitative and Quantitative Analyses of GABA Production

Qualitative analysis of GABA production was carried out with thin-layer chromatography (TLC) [21]. Culture supernatants after centrifugation at 11,463 $\times g$ for 10 min were immediately spotted on TLC silica gel plates (Merck, USA) and developed twice using a solvent mixture comprising *n*-butanol/acetic acid/water (5:3:2, v/v/v) followed by the addition of 0.2% (w/v) ninhydrin reagent in ethanol for visualization [22].

Quantitative analysis of GABA was carried out as per the method described by Rossetti and Lombard with some modifications [23]. In brief, 10 μ l of culture supernatants were dissolved in 20 μ l of ethanol/water/triethylamine (TEA) (2:2:1, v/v/v) and evaporated to dryness under vacuum. To form phenyl isothiocyanate (PITC) derivatives, a PITC derivatization reagent (50 μ l) (ethanol/water/TEA/phenyl-isothiocyanate [7:1:1:1, v/v/v/v]) was added to

each sample and allowed to react for 40 min at 25°C. The excess of reagent was removed under vacuum for 20 min, and the dried samples were dissolved in 600 µl of solvent A (1.4 mM sodium acetate, 0.1% TEA, 6% acetonitrile, pH 5.5). Diluted samples were filtered through a 0.20-µm syringe filter (Advantech Toyo Kaisha, Japan), and the derivatized samples were injected onto a high-performance liquid chromatography column (HPLC, Shimadzu, Japan) using an HPLC system equipped with Agilent ZORBAX 300SB-C₁₈ (5 µm, 4.6 × 150 mm, Agilent Technologies, Palo Alto, CA, USA), pumps A and B (LC-20AD, Shimadzu, Kyoto, Japan), and a UV-VIS detector (SPD-20A, Shimadzu, Japan). The column was eluted for 40 min with a linear gradient of 0–100% solvent B (60% acetonitrile) at a flow rate of 0.5 ml/min at 46°C for 40 min. Sample was injected at a volume of 5 µl and monitored at a wavelength of 254 nm. The same method was applied to GABA standard. The PITC derivative of GABA was eluted at 10.6 min. A calibration curve was produced by applying the same procedure to eight standard solutions of GABA (0.156, 0.313, 0.625, 1.25, 2.5, 5.0, 10.0, and 20.0 g/l).

GABA Production

Each strain was seed-cultured at 37°C in 10 ml of modified MRS broth containing 1% (w/v) MSG. Cultures were incubated under shaking conditions until the optical density reached 1.0 at 600 nm wavelength. Each strain was sub-cultured into 20 ml modified MRS broth and incubated at 37°C for 96 h. The simplified media containing 10 g/l of a carbon source (glucose, fructose, galactose, xylose, maltose, sucrose, lactose, or mannitol), 10 g/l yeast extract, 10 g/l MSG, and 1 g/l di-potassium phosphate (K₂HPO₄) were prepared to investigate the influence of carbon source on levels of GABA produced by each strain. Each isolated strain was seed-cultured at 37°C for 18 h in 10 ml of modified MRS broth starting from 1% (v/v) inoculum. Strains were then inoculated into 20 ml simplified media supplemented with various carbon sources and incubated at 37°C for 96 h. Supernatants were harvested for the analysis of GABA production.

After the selection of the carbon source that allowed maximum GABA production, the simplified media containing 10 g/l carbon source with the highest GABA production, 10 g/l nitrogen source (yeast extract, beef extract, malt extract, peptone, tryptone, soytone, proteose peptone No. 3, casein, urea, or ammonium chloride), 10 g/l MSG, and 1 g/l K₂HPO₄ were prepared to investigate the effect of nitrogen source on GABA production of each strain. Each strain was seed-cultured at 37°C for 18 h in 10 ml of medium. Strains were then cultured in 20 ml simplified media supplemented with various nitrogen sources at 37°C for 96 h, and the culture supernatants were harvested for the analysis of GABA production.

Culture Conditions for GABA Production

Among the isolated GABA-producing strains, *E. avium* JS-N6B4 isolated from *A. dichotoma* larvae showed maximum GABA production. Further characterization of culture conditions was carried out for this strain. Simplified media (10 g/l carbon source

[glucose, fructose, galactose, xylose, maltose, sucrose, lactose, or mannitol] 10 g/l yeast extract, 10 g/l MSG, and 1 g/l K₂HPO₄) were prepared to investigate the effect of carbon source. *E. avium* JS-N6B4 was seed-cultured at 37°C for 18 h in 10 ml of modified MRS broth containing 1% (w/v) MSG. The strain was then sub-cultured in 20 ml of each basic medium containing various carbon sources, and incubated at 37°C for 96 h. Supernatants were harvested for the analysis of GABA production.

After evaluation of the carbon source that yielded the highest GABA level, the simplified media (10 g/l carbon source with the highest GABA production, 10 g/l various nitrogen sources, 10 g/l MSG, and 1 g/l K₂HPO₄) were prepared to investigate the effect of nitrogen source on GABA production. The strain was seed-cultured at 37°C for 18 h in 10 ml of medium and cultured in 20 ml of each basic medium containing various nitrogen sources at 37°C for 96 h. The supernatants were harvested for the analyses of GABA production.

The effect of incubation temperature on GABA production by *E. avium* JS-N6B4 was investigated. The strain was seed-cultured at 37°C in 10 ml modified MRS broth containing 1% (w/v) MSG. These cultures were used to inoculate 20 ml cultures of basic medium (10 g/l glucose, 10 g/l yeast extract, 10 g/l MSG, and 1 g/l K₂HPO₄) and incubated under shaking conditions at 30°C, 33°C, 37°C, 41°C, and 45°C for 96 h.

The effect of initial pH of the medium on GABA production by *E. avium* JS-N6B4 was evaluated. The strain was seed-cultured at 37°C in multiple 10-ml tubes of modified MRS broth containing 1% (w/v) MSG. Seed cultures were used to inoculate 20 ml cultures containing basic medium (10 g/l glucose, 10 g/l yeast extract, 10 g/l MSG, and 1 g/l K₂HPO₄) adjusted to an initial pH of 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, and 8.0 with 2 M hydrochloric acid or 1 M sodium hydroxide. These cultures were incubated at 37°C under shaking conditions for 96 h.

The effects of aerobic and anaerobic conditions on GABA production were investigated. For aerobic conditions, the strain was seed-cultured in two flasks of 20 ml modified MRS broth and cultured at 37°C for 18 h at 180 rpm. One of the flasks was blocked with a cotton plug, and the other was blocked with foil. Each seed culture was used to inoculate 100 ml of basic medium, and these cultures were then incubated with shaking at 180 rpm, at 37°C for 96 h. For anaerobic conditions, the strain was seed-cultured in 20 ml modified MRS broth, and topped with liquid paraffin. These cultures were covered with foil and cultured at 37°C for 18 h without shaking. The strain was also inoculated into 100 ml basic medium with liquid paraffin, and 100 ml basic medium without liquid paraffin, and incubated at 37°C for 96 h without shaking.

The effect of MSG on GABA production was evaluated. The strain was inoculated in 20 ml of modified MRS broth and cultured at 37°C for 18 h. A 100-ml flask of basic medium containing an initial MSG concentration of 10 g/l was inoculated and incubated for 96 h. During cultivation, 10 g/l of MSG was added every 24 h to achieve a final MSG concentration of 40 g/l. Another 100-ml flask initially containing 40 g/l MSG was

inoculated and cultured at 37°C for 96 h without any further addition of MSG. A third 100-ml flask of basic medium initially containing 40 g/l MSG was inoculated and cultured at 37°C for 96 h. An additional 20 g/l of MSG was added every 24 h to achieve a final MSG concentration of 100 g/l. A fourth flask of basic medium containing an initial MSG concentration of 100 g/l was inoculated and cultured at 37°C for 96 h without further additions of MSG.

Optimization for GABA Production with RSM

Based on the results obtained from the preliminary screening for GABA production, a central composite design (CCD) experiment was performed with concentrations in the vicinity of optimum range to determine the precise optimum concentrations of glucose, yeast extract, and MSG. The CCD matrix was generated using Design-Expert software (Stat-Ease Inc., USA). Each factor in the design was studied at five different levels (-1.682, -1, 0, +1, and +1.682). The design matrix corresponding to the CCD is shown in Table 1.

Analysis of variance (ANOVA) was used with the response function, and the relationships and interactions between variables

Table 1. The central composite design matrix of independent variables.

Runs	Glucose (A, g/l)	Yeast extract (B, g/l)	MSG (C, %w/v)	GABA production (g/l)	
				Experimental	Predicted
1	2	15	2	3.53	2.51
2	7	15	2	10.47	9.72
3	2	45	2	8.97	7.25
4	7	45	2	11.44	11.93
5	2	15	7	1.64	0.31
6	7	15	7	10.08	10.95
7	2	45	7	8.45	8.36
8	7	45	7	16.29	16.48
9	0.30	30	4.5	2.52	4.60
10	8.70	30	4.5	18.39	17.50
11	4.5	4.77	4.5	4.18	5.10
12	4.5	55.23	4.5	13.47	13.74
13	4.5	30	0.30	2.70	4.08
14	4.5	30	8.70	6.24	6.05
15	4.5	30	4.5	8.83	9.75
16	4.5	30	4.5	9.79	9.75
17	4.5	30	4.5	9.95	9.75
18	4.5	30	4.5	10.93	9.75
19	4.5	30	4.5	9.47	9.75
20	4.5	30	4.5	9.73	9.75

Variable values are correlated with experimental and predicted GABA concentrations produced by *E. avium* JS-N6B4.

were determined using the following second-order polynomial equation:

$$y = \beta_0 + \sum \beta_i x_i + \sum \beta_{ii} x_i^2 + \sum \beta_{ij} x_i x_j$$

where y is the predicted response, β_0 is the offset term, β_i is the linear effect, β_{ij} is the quadratic effect when $i = j$, and the interaction effect when $i < j$, β_{ii} is the squared term, and x_i is the i th independent variable. The data were statistically analyzed with Design-Expert 7.1 (Stat-Ease Inc.), and response surface and contour plots were constructed to evaluate interaction effects of the parameters as well as the optimum value of each variable. A set of optimized experimental conditions was tested to evaluate GABA production and confirm the validity of RSM results. Fermentation was carried out in a 100-ml flask containing the optimized medium at 37°C for 96 h. Samples were aseptically obtained and analyzed for cell growth (optical density_{600nm}), pH, and GABA production (g/l).

Statistical Analysis

All experiments were conducted in duplicate. The data were analyzed for statistical significance with ANOVA followed by Duncan's multiple-range test using SPSS version 18.0 (USA). The level of significance was set at $p < 0.05$.

Results and Discussion

Phylogenetic Identification of GABA-Producing Strains from Edible Insects

We investigated the GABA production ability of 87 isolates from four edible insects. Most strains showed a very low level of GABA production. Among 87 isolates, four bacterial strains were subjected to 16S rRNA sequencing. The isolated strains had the lowest sequence similarity value based on the 16S rRNA gene sequence, suggesting that these may be classified as *E. avium* or *Proteus* sp. As shown in Fig. 1, the strain isolated from *G. bimaculatus* was identified as *Proteus* sp. L-G7, while the strains isolated from *T. molitor* larvae, *P. brevitarsis* larvae, and *A. dichotoma* larvae were identified and designated as *E. avium* No-22-1, *Enterococcus avium* HJ-N6, and *Enterococcus avium* JS-N6B4, respectively. Most GABA-producing microbes have been isolated from traditional fermented foods such as kimchi [11, 12], makgeolli [13], fish products [14], cheese [15–17], sourdough [18], and paocai [19]. These sources generally provide an acidic environment and contain high levels of glutamate, a GABA precursor. The environmental conditions that the potential sources provide are, therefore, important in designing screen methods to isolate GABA-producing microbial strains [24]. Although several microbes are capable of producing GABA, the most notable sources are lactic

Fig. 1. Neighbor-joining phylogenetic trees based on the 16S rRNA sequence analysis of GABA-producing strains isolated from edible insects and other related taxa.

(A) *Proteus* sp. L-G7 isolated from two-spotted cricket, (B) *E. avium* HJ-N6 isolated from *P. brevitarsis* larvae, (C) *E. avium* No-22-1 isolated from mealworm, and (D) *E. avium* JS-N6B4 isolated from *A. dichotoma* larvae.

acid bacteria (LAB) such as *Lactobacillus* or *Leuconostoc*. The environmental conditions with high concentrations of glutamate and acidic pH may facilitate the production of GABA [25].

GABA production by microbes in edible insects may be facilitated in the environment naturally formed by glutamate contained in the feed ingredients. Although many GABA-producing strains have been identified from various fermented foods, four strains with GABA-producing

ability were successfully isolated from edible insects. To the best of our knowledge, this is the first report on GABA-producing bacteria isolated from edible insects. The identification of microbial sources of GABA may expand the applications and flexibility of edible insects [24].

GABA Production by GABA-Producing Strains

The GABA production from each isolated strain was compared using MRS broth containing 1% (w/v) MSG

Fig. 2. Comparison of GABA production between four isolates from edible insects.

(A) Qualitative analysis of GABA from four isolates using thin-layer chromatography (TLC). Lane 1, 10 g/l MSG standard; lane 2, 1.25 g/l GABA standard; lane 3, MRS medium containing 1% (w/v) MSG (control); lane 4, GABA production with *Proteus* sp. L-G7; lane 5, GABA production with *E. avium* HJ-N6; lane 6, GABA production with *E. avium* No-22-1; lane 7, GABA production with *E. avium* JS-N6B4. (B) Growth and GABA production of GABA-producing strains isolated from edible insects. (□), Cell growth (optical density, 600 nm); (■), GABA production (g/l).

(Fig. 2). Among the isolated strains, *E. avium* JS-N6B4 derived from *A. dichotoma* larvae showed maximum GABA production (3.24 g/l), followed by the strain isolated from *G. bimaculatus* (0.47 g/l). This difference may arise owing to the differences in expression levels and kinetic characteristics of GAD expressed in each GABA-producing strain. These differences affect the rate of bio-conversion of L-glutamate to GABA.

Due to the complex components in MRS broth, the effect of carbon sources on GABA production was investigated with a simplified basic medium (10 g/l yeast extract, 10 g/l MSG, and 1 g/l K_2HPO_4) supplemented with 1% (w/v) of different carbon sources. The carbon and nitrogen sources that yielded the highest GABA production were investigated. As shown in Fig. 3, the switch from modified MRS broth to basic medium resulted in a 2.08-fold increase in GABA production (from 0.47 to 0.98 g/l) with *P. sp.* L-G7, 1.86-fold increase (from 0.33 to 0.60 g/l) with *E. avium* No-22-1, 10.11-fold increase (from 0.23 to 2.32 g/l) with *E. avium* HJ-N6, and 1.98-fold increase (from 3.24 to 6.40 g/l) with *E. avium* JS-N6B4. Overall, each strain showed an increase in GABA production in response to the change in the medium composition from MRS broth to basic medium. Medium components such as carbon and nitrogen sources exert a strong influence on GABA production [26].

Therefore, GABA production could be improved by the optimization of carbon and nitrogen sources for each strain. Here, carbon and nitrogen sources for optimum GABA production by the four species described herein

Fig. 3. Improvement in GABA production through the optimization of carbon and nitrogen sources for the growth of edible insect-derived strains in culture media.

Fig. 4. Effect of carbon sources (A), nitrogen sources (B), incubation temperature (C), initial pH (D), aerobic culture and anaerobic culture (E), and mono-sodium glutamate concentrations (F) on the growth and GABA production of *E. avium* JS-N6B4. (□), Cell growth (optical density, 600 nm); (■), GABA production (g/L).

were determined. *E. avium* JS-N6B4 was selected as the best GABA-producing bacterium strain for further experiments.

Effect of Culture Conditions on GABA Production

The effects of various carbon sources on GABA production

by *E. avium* JS-N6B4 were investigated in basic medium. As shown in Fig. 4A, carbon sources influenced both the growth and GABA production of *E. avium* JS-N6B4. Among the tested carbon sources, glucose served as an economical and cheap source and proved as the optimal carbon source for this strain. The effects of nitrogen sources on GABA production were evaluated using 10 g/l glucose as the carbon source. Among the tested nitrogen sources (Fig. 4B), yeast extract resulted in the highest GABA level (5.14 g/l), followed by beef extract (2.38 g/l). The change in the optimum nitrogen and carbon sources resulted in minor differences in the amount of GABA produced by *E. avium* JS-N6B4, as evident from the production level of (5.14 g/l with glucose-yeast extract) and (5.14 g/l with sucrose-beef extract). Although there would be various factors in GABA production, we could not accurately interpret the effects of each ingredient from the mixed nitrogen sources. Finally, GABA production by *E. avium* JS-N6B4 was carried out in the presence of glucose and yeast extract as carbon or nitrogen source, respectively.

The effect of culture temperature on GABA production by *E. avium* JS-N6B4 was investigated in basic medium. As shown in Fig. 4C, GABA production was maximum at 37°C (5.63 g/l), followed by 33°C (5.60 g/l). However, a similar level of production was achieved at temperatures of 33°C–37°C. GABA production significantly decreased at 41°C or higher owing to cell growth inhibition. Culture temperature is a major factor affecting GABA production depending on the expression or catalytic activity of GAD. Therefore, high cell density and appropriate culture temperature are essential for the efficient conversion of glutamate to GABA [27]. From the above results, we concluded that the growth and GABA production of *E. avium* JS-N6B4 were stable at 33°C–37°C. We selected 37°C as the optimum temperature. We tested the effect of initial pH on the GABA production ability of *E. avium* JS-N6B4 (Fig. 4D), and found better GABA production at pH values from 6.5 (5.69 g/l) to 7.0 (5.67 g/l). Therefore, the initial pH was not adjusted for basic medium in subsequent experiments. The effects of

Table 2. Effect of various initial MSG concentrations and feeding regimens on GABA production by *E. avium* JS-N6B4 using basic medium.

MSG (g/l)				Optical density (600 nm)	GABA production (g/l)
Initial	Feeding				
	24 h	48 h	72 h		
40				1.14 ± 0.05	12.47 ± 0.29
10	10	10	10	1.16 ± 0.03	10.15 ± 0.44
100				0.66 ± 0.01	4.02 ± 0.63
40	20	20	20	0.84 ± 0.01	11.02 ± 0.95

aerobic and anaerobic conditions on GABA production by *E. avium* JS-N6B4 were investigated in basic medium. No statistically significant difference in cell growth and GABA production was observed between aerobic and anaerobic cultures. GABA production was slightly better in shaking cultures (4.85 g/l) than in non-shaking cultures (4.80 g/l) blocked with foil (Fig. 4E). Furthermore, GABA production in aerobic cultures (4.69 g/l) was slightly higher than that in anaerobic cultures (4.57 g/l).

The effects of MSG concentration and feeding regimens on GABA production of *E. avium* JS-N6B4 were investigated in basic medium. At 40 g/l MSG concentration, batch supply resulted in more effective GABA production (12.47 g/l), than fed-batch supply (10.15 g/l). At 100 g/l MSG concentration, the cell growth was significantly inhibited with both feeding types, and GABA production was affected. These results suggest that *E. avium* JS-N6B4 restricts high initial MSG concentration, which may directly induce toxic effects such as apoptosis [28]. As shown in Table 2 and Fig. 4F, 40–50 g/l of MSG was chosen as the suitable concentration range for GABA production to avoid growth inhibition of *E. avium* JS-N6B4. GABA production has been previously carried out using a two-stage fermentation strategy, wherein maximum biomass production was achieved in the first stage and biotransformation of MSG into GABA was achieved in the second stage [29].

Table 3. Analysis of variance for the response surface methodology parameters fitted to a second-order polynomial equation.

Source	Sum of square	Degree of freedom	Mean square	F-value	P-value >F
Regression model	355.098	9	39.455	22.172	<0.0001
Residual	17.795	10	1.780		
Lack of fit	15.437	5	3.087	6.544	0.0299
Pure error	2.359	5	0.472		
Corrected total	372.893	19			

$R^2 = 0.952$; CV = 15.068%; Adjusted- $R^2 = 0.909$; Predicted- $R^2 = 0.669$.

Optimization of GABA Production Based on RSM

GABA production of *E. avium* JS-N6B4 was optimized based on medium composition to increase the economic availability. To determine the optimum concentrations of the combination of key variables such as glucose, yeast extract, and MSG, a second-order polynomial equation was fitted to the responses (Table 1). Results of the ANOVA of the second-order response surface model fitting are shown

Fig. 5. Three-dimensional response surface plots for GABA production showing the interactive effects of glucose, yeast extract, and MSG concentration.

in Table 3. To test the quality of the model, the regression equation and coefficient of determination (R^2) were evaluated. The model presented a high coefficient of determination ($R^2 = 0.95$), explaining 95% variability in the response. The adjusted coefficient of determination was also high, indicative of the strong significance of the model. The coefficients of the regression equation were calculated using Design-Expert statistical package software, and the following regression equation was obtained.

$$y(\text{GABA}) = 9.748273 + 3.834204A + 2.566301B + 0.586154C - 0.63138AB + 0.8585AC + 0.8275BC + 0.459972A^2 - 0.11614B^2 - 1.65463C^2$$

where A , B , and C are glucose (g/l), yeast extract (g/l) and MSG (%-w/v) concentrations, respectively.

The three-dimensional response surface plots are graphical representations of the regression equation. The main objective of response surface analysis was to efficiently determine the optimum values of variables in combination to maximize the response. The response surface and contour plots obtained in this study are presented in Fig. 5. The optimized values of each parameter to maximize GABA production were obtained by regression. The optimum concentrations of each variable according to this analysis were as follows: glucose (A) = 7 g/l, yeast extract (B) = 45 g/l, MSG (C) = 6.21% (w/v). The predicted GABA production level under these optimized conditions was 16.64 g/l. The predicted optimum levels were tested in a

Fig. 6. GABA production, pH, and growth curve of *E. avium* JS-N6B4 under optimized and basic medium.

●, GABA production on optimized medium; ○, GABA production on basic medium; ▲, cell growth on optimized medium; △, cell growth on basic medium; ■, pH of the optimized medium; □, pH of the basic medium.

flask-scale experiment incorporating all other optimized conditions to assess the adequacy of this quadratic model in predicting GABA production. As shown in Fig. 6, maximum GABA concentration was 13.68 g/l, which was closely related to the predicted value (16.64 g/l). This result indicates that the regression model developed in this study shows good agreement between actual and predicted responses. After medium optimization with RSM, GABA production of *E. avium* JS-N6B4 increased by 2.79-fold (13.68 g/l) compared with the 4.90 g/l level obtained with basic medium. Cells entered stationary phase after 24 h and GABA production failed to increase owing to the absence of cell growth. Therefore, to promote GABA production, feeding glucose after 24 h is essential to induce cell growth. Furthermore, the pH of the medium increased over time, making it unfavorable for the microbial production of GABA. Proper acidification of the culture medium is needed for the conversion of glutamate to GABA [29]. It is necessary to continuously adjust the pH to promote GABA production.

In conclusion, a GABA producing-bacterium, *E. avium* JS-N6B4G, was successfully isolated from an edible insect, *A. dichotoma*, and its culture medium was optimized for GABA production using RSM. The optimized cultivation method resulted in a 2.79-fold increase in GABA levels as compared with the basic medium. The GABA-producing strain may expand the industrial value of edible insects as protein alternatives or functional pharmaceutical biomaterials. Although *Enterococcus avium* was isolated from fermented sea food [12, 29] or human intestine [30], *E. avium* is most commonly isolated from the gastro intestinal and urinary tracts [31]. For application in various areas, current research is focused on the genomic approach and bacterial safety.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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