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ORIGINAL ARTICLE

Optimization of gamma-aminobutyric acid production by probiotic bacteria through response surface methodology

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ABSTRACT

Background and Objectives: Gamma-aminobutyric acid (GABA) is a non-protein four-carbon amino acid that has many physiological properties, including reducing blood pressure, accelerating protein synthesis in the brain, and treatment of insomnia and depression. This amino acid is produced by a number of lactic acid bacteria, fungi and yeasts. The objective of the present study was to identify probiotic bacteria with the maximum ability to generate GABA and optimize the bacterial culture conditions having the highest potential for GABA production.

Materials and Methods: The potential of GABA production by *Lactobacillus delbrueckii* ssp. *bulgaricus, Lactobacillus rhamnosus, Lactobacillus casei, Streptococcus thermophilus, Lactobacillus brevis* and *Lactococcus lactis* ssp. lactis in the culture medium of MRS broth was assessed by High Performance Liquid Chromatography (HPLC). In order to increase the rate of GABA produced by the bacteria having the highest potential for GABA production, the conditions of the culture medium including pH (3.5 to 6.5) "temperature (25 to 45°C), time (12 to 96 h) and glutamic acid (GA) concentration (25 to 650 mmol) were optimized by the Box-Behnken's Response Surface Method (RSM).

Results: *Lactobacillus brevis* had the highest potential of GABA production (5960.8 mg/l). The effect of time and GA concentration was significant on the amount of GABA production. The best conditions of culture medium to achieve the highest amount of GABA production by *Lactobacillus brevis* (19960 mg/l) were temperature 34.09°C, pH 4.65, GA concentration 650 mmol and time 96 h.

Conclusion: The results showed that by optimization of the culture medium conditions of probiotic bacteria we can produce more GABA in culture medium.

Keywords: Gamma-aminobutyric acid; *Lactobacillus brevis*; Probiotic bacteria; Response surface method

INTRODUCTION

Gamma-aminobutyric acid (GABA) is a non-protein four-carbon amino acid, which is found extensively among organisms and synthesized by the glutamic acid decarboxylase (GAD) (1). Glutamic Acid decarboxylase has been confirmed for a long time in many prokaryotes and all eukaryotes. GABA has several physiological roles including calming, preventing diabetes, reducing blood pressure, soothing, suppressing stress, accelerating protein synthesis in the brain, as well as treatment of insomnia, depression, and neurological anomalies such as epilepsy, Parkinson's disease, schizophrenia, spasm and Alzheimer's disease (2). Using up to 18 g for 4 days or 120 mg for 12 months of GABA in healthy people has positive effects on the sleep disorders and stress (3). GABA is produced by different microorganisms including fungi, yeasts, algae and lactic acid bacteria (4). Research has shown that probiotic lactic acid

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bacteria are the largest group of microorganisms that produce GABA (4). Probiotic lactic acid bacteria (LAB) are among that Gram-positive bacteria that are used in most of the fermented food stuffs such as a variety of fermented vegetables (5) and are found in both human and animals intestine. These bacteria play an important role in producing many important products including linoleic acid, vitamin B6, vitamin B12, aromatic compounds, bacteriocins, enzymes and GABA (6). GABA is produced by different species of probiotic lactic acid bacteria including *L. brevis* (7), *L. casei* (8), *L. buchneri* (4), *L. plantarum*, *L. bulgaricus, Lactococcus lactis, L. paracasei* (9), *Strepetococcus thermophilus* (10) and *L. buchneri* (11). Many researchers have shown that some of probiotic bacteria have the ability to produce GABA. For example, *L. brevis* (12), *L. bulgaricus* and *Lactococcus lactis* (8), in the cheese medium conditions produce 302 mmol, 26.9 mmol, 63 mg/l and 36 mg/l GABA, respectively. Evaluation of potential to produce GABA by probiotic LAB in the food industries is very important because each of these bacteria is different in terms of producing products resulting from fermentation (13). The main factors having impact on the amount of GABA production by microbial fermentation of LAB include the temperature of culture medium, pH, fermentation time, GA concentration, and existence of additives, and nutrient materials in the bacterial growth environment (4).

Lu et al. optimized the bacterial fermentation environment for maximum production of GABA in terms of the ratio of brown rice, soy sauce and skim milk. They found that optimal conditions for producing the maximum amount of GABA were observed in a mixture of brown rice, soy sauce, and skim milk with the ratio of 33, 58 and 9 mmol, respectively. In another research (14), Tung et al. examined the optimal conditions for GABA production by *L. plantarum*. Independent variables in this research included concentration of skim milk, concentration of monosodium glutamate, and temperature of culture medium. The results showed that the highest amount of GABA was produced at 37°C and in the presence of skim milk having 8 to 12% solid fatless materials and 6 to 1% monosodium glutamate (15). Zarei et al. optimized the conditions for GABA production by the probiotic bacteria extracted from traditional Iranian dairy products (doogh, yogurt and cheese). They showed that *Lactobacillus* extracted from dough at 37°C, incubation time of 60 h, pH 5, and glutamic acid concentration of 50 mmol had the highest ability to produce GABA (16). Up to now, many studies have been done on the production of GABA by probiotic LAB (10, 14, 17, 20), but there is a lack of studies that investigate the potential of GABA production by probiotic bacteria and optimize its culture medium conditions. The objective of this research was to examine the potential of GABA production by probiotic bacteria in the MRS broth and optimization of the culture medium conditions for the growth of probiotic bacteria with the highest potential for GABA production by Response Surface Method (RSM).

MATERIALS AND METHODS

Materials. *L. delbrueckii* ssp. *bulgaricus* (IBRC-M10730) and *Lactococcus lactis* ssp. *lactis* (IBRC-M10629) were purchased from the National Center for Genetic and Biological Reserves of Iran, and *L. casei* (TD3), *L. rhamnosus* (LAR-7), *L. brevis* (TD10) and *S. thermophilus* (SAX-107) were supplied from Takgene Zist Laboratories, Tehran, Iran. Yeast and meat extracts, glucose, potassium phosphate, sodium acetate, ammonium citrate, magnesium sulfate, manganese sulfate and poly sorbate (Tween 80), sodium citrate salt, triethyl amine, acetonitrile, sodium dihydrogen phosphate, acetonitrile, and methanol were supplied from the Merck Company, Germany. Furthermore, phenethyl isothiocyanate, and Ortho Phthalic Aldehyde (OPA), GABA standard and MRS broth were purchased from Sigma-Aldrich (USA).

Selection and inoculation of probiotics bacteria in the culture media. It has been reported that *L. delbrueckii* ssp. *bulgaricus* (9), *Lactococcus. lactis* ssp. *lactis* (5), *L. casei* (20), *L. rhamnosus* (21), *L. brevis* (19) and *St. thermophilus* (22) have the highest amount of GABA production. An amount of single colony from each of the six microorganisms of *L. delbrueckii* ssp. *bulgaricus* and *Lactococcus. lactis* ssp. *Lactis, L. casei, L. rhamnosus, L. brevis,* and *St. thermophilus* was added to the MRS broth containing 50 mmol GA (23) and kept for 24-48 h incubated at 37°C. To determine the number of inseminated bacteria in every ml of the suspension, the standard of 0.5 McFarland was used. One of the advantages of this method is that the number of cells existing in a liquid is counted very rapidly. In addition, working is easy with it, and the approximate number of bacteria can be determined in

the shortest time possible. To provide 0.5 McFarland, 0.05 ml of 1.175% barium chloride was blended with 1% sulfuric acid and the created opacity was equal to 5.1×10^8 cfu/ml of bacteria cells (24).

Measurement of GABA using reverse phase HPLC. The amount of GABA produced by the probiotic bacteria was determined by an Agilent 1200 series HPLC system (Agilent Tech, Waldron, Germany). The derivation was conducted according to the method of Bartolomeo et al. (25). After centrifugation (12000 g, 10 min and 25°C) of the culture medium, 20 µl of the separated sample was poured into a 2-ml vial and 20 µl of borate buffer was added and completely blended. Then 10 µl of OPA was added. After 1 min, 5 µl of 5% acetic acid was added. After derivation, 20 µl of each specimen was injected into a C18 reverse-phase column with 5 µm diameter, 250 mm length and 4.6 mm internal diameter (Thermo Fisher Scientific Co., Waltham, MA, USA) by detector in λ= 338 nm. The mobile phase of A was 40 mmol sodium dihydrogen phosphate (pH=7.8), while the mobile phase of B was acetonitrile/methanol/water (10:45:45, v/v/v).

Isolation was done with a flow rate of 1 ml/min using a gradient program for 35 min. Then the washing operation continued in B (33%) to reach the time of 7 h. Then standard curves in the concentrations of 50, 100, 200, 300, 400 mg/l were drawn in view of retention time and the area under the curves comparing them with relevant standards. GABA concentration was stated in mg/l.

Optimization of GABA production by RSM. Considering that the conditions of culture medium (temperature, time, GA concentration and pH) have a significant effect on increase of GABA production, the conditions of bacterial culture medium having the highest potential for GABA production were optimized. To this end, the temperature ranges of 25-45°C, pH of 3.5-6.5, time of 12-96 h and GA concentration of 25-650 mmol were selected according to Table 1. To design the treatments, Box-Behnken's Response Surface Method (RSM) was used. Thus, 27 treatments were designed, and the concentration of the GABA produced by *L. brevis* bacteria grown in the MRS broth culture medium was optimized.

Statistical analysis. To analyze the results related to the potential of GABA production by probiotic bacteria, the Duncan's one-way variance analysis (ANOVA) was applied, and to optimize the conditions of GABA production, the RSM in the Minitab software (ver. 16) was used.

RESULTS

Examining the potential of GABA production by probiotic bacteria. The amount of GABA produced by *L. delbrueckii* ssp. *bulgaricus, Lactococcus. Lactis* ssp. *Lactis, L. casei, L. rhamnosus, L. brevis*, and *St. thermophilus* in the MRS broth is shown in Table 2, and its chromatogram is presented in Fig. 1.

According to the results, *L. brevis* bacteria showed the highest amount of GABA production in the culture medium of MRS broth (5960.8 mg/l) that had a significant difference with the other treatments ($P \leq 0.05$). The lowest amount of GABA production was observed for *L. delbrueckii* ssp. *bulgaricus* (36.07 mg/l).

Optimization of the conditions of culture medium of *L. brevis* on GABA. Results given in Table 2 show that *L. brevis* had the highest potential

Table 1. Independent variables levels in order to optimize the culture condition for the production of GABA

Independents variables	Model	+1	0	-1
Temperature	A	45	35	25
pH	B	6.5	5	3.5
Time	C	96	54	12
Glutamic Acid	D	650	337.5	25

Where A is culture temperature, B is pH, C is glutamic acid concentration and D is incubation

Table 2. GABA production in MRS broth (mg/l)

Microorganisms	GABA (mg/l)
Lactobacillus delbrueckii ssp. *bulgaricus*	36.07 ± 3.1^{c}
Lactococcus. lactis ssp. *lactis*	741.14 ± 45.4^{b}
Lactobacillus casei	53.82 ± 5.8^{c}
Lactobacillus rhamnosus	51.08 ± 3.00^{c}
Lactobacillus brevis	5960.8 ± 10.8^{a}
Streptococcus thermophilos	116.4 ± 12.5^{c}

Different small letters in each column show significant differences ($P \leq 0.05$)

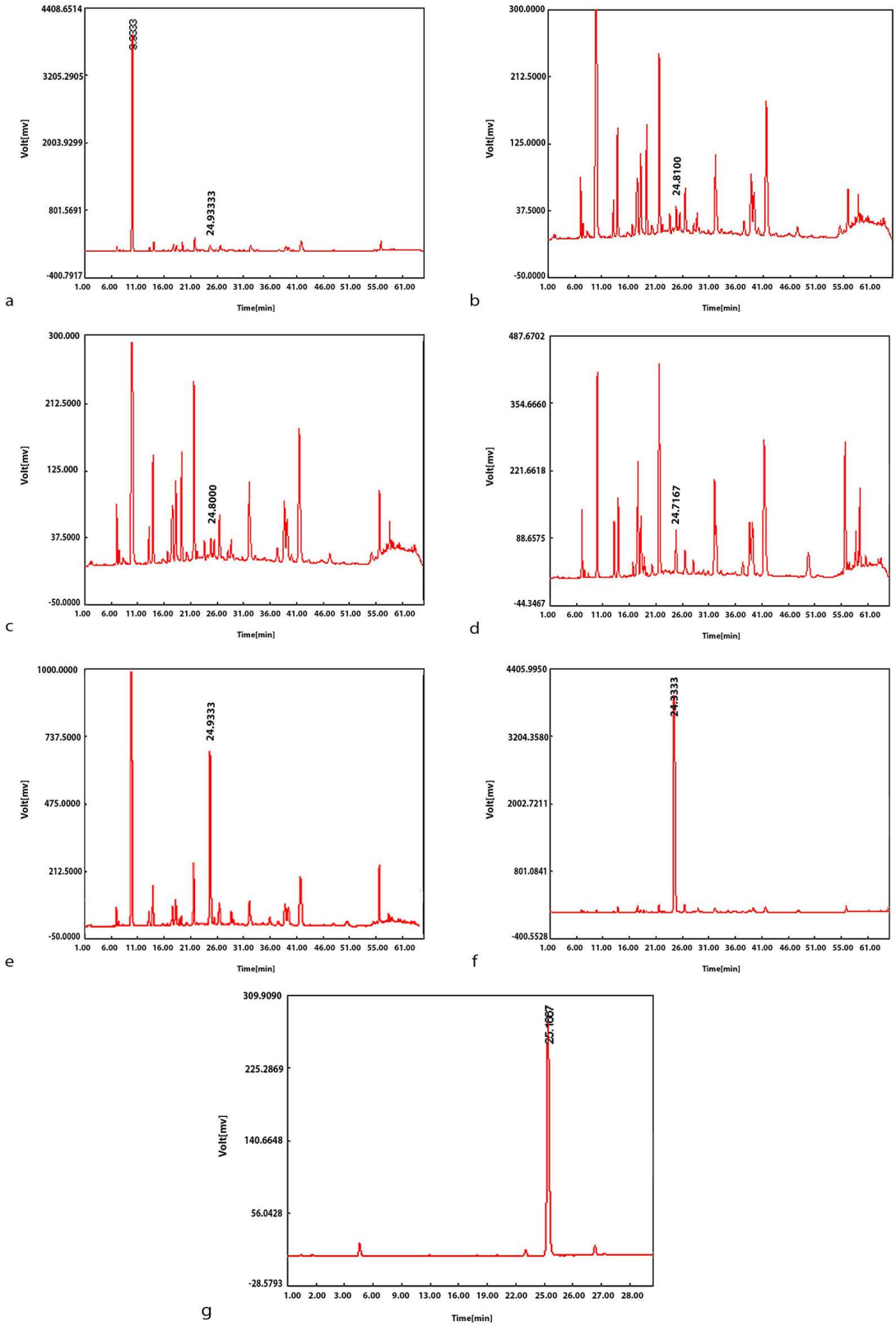

Fig. 1. Chromatogram of GABA production by lactic acid bacteria in MRS broth, a) *Lactobacillus. delbrukii bulgaricus*, b) *Lactobacillus. rhamnosus* c) *Lactobacillus.casei* d*) Steteptococoos. thermophilos* e) *Lactococcus lactis. lactis* f) *Lactobacillus. Brevis* g) standard sample

of GABA production (5960.82 mg/l) in the MRS broth. Therefore, in order to increase the amount of GABA production, the conditions of the culture of the mentioned bacteria were optimized and its regression model was designed as follows. In this model the effect of four independent variables namely temperature (A), pH (B), time (C) and GA concentration (D) on GABA production by *L. brevis* (y) is shown.

y=12489.6+5519.2A+272.2B-335.5C+1113.9D-130.8A2-1988.2B2-2103.1C2 +217.2 D2-179.4 AB-487.7 AC+614.3 AD-305.3 BC+57.5 BD-175.8CD

Analysis of variance and GABA production by *L. brevis*. The analysis of variance (ANOVA) results are reported in Table 3. The linear effect of GA (D), time (C), pH and temperature on the amount of GABA produced by *L. brevis* in the MRS broth was significant ($p \leq 0.05$). However, the effect of other factors was not significant. Closeness of R^2=97.35% to 100% suggests a desirable correlation of the experimental model and the data resulting from the tests, so the precision of the mentioned results was confirmed.

Table 3. Analysis of variance for GABA production by *L. brevis* (mg/l)

Source	F value	F value
Regression	31.48	0.000*
Linear	97.63	0.000*
Temperature (A)	0.91	0.360
pH (B)	1.55	0.237
Time (C)	0.91	0.002*
Glutamic Acid (D)	372.87	0.000*
Square	11.77	0.000*
Temperature × Temperature (A^2)	21.50	0.001*
pH × pH (B^2)	24.06	0.000*
Time × Time (C^2)	0.26	0.622
Glutamic Acid × Glutamic Acid (D^2)	0.90	0.766
Interaction	0.53	0.778
Temperature × pH (A×B)	0.38	0.549
Temperature × Time (A×C)	0.01	0.909
Temperature × Glutamic Acid (A×D)	0.13	0.723
pH × Time (B×C)	0.13	0.729
pH × Glutamic Acid (B×D)	0.97	0.344
Time × Glutamic Acid (C×D)	1.54	0.238
Lack-of-Fit	390.42	0.003

* Significant difference ($p \leq 0.05$)

As shown in Table 4, there is no significant difference between the produced GABA in the culture medium and the predicted GABA by *L. brevis*. The results confirmed that by increase of time from 54 to 96 h and GA concentration from 25 to 650 mmol, the amount of produced GABA increased from 5124.540 to 18345.120 mg/l. Although the linear effect of pH on GABA production was not significant but the highest GABA production was observed in pH 5 and the lowest amount of GABA production was in pH 6.5. Furthermore, the linear effehighest and lowest amounts of GABA production were observed at 35°C.

Optimization of culture conditions for GABA production. The optimization of MRS broth conditions in order to achieve the highest amount of GABA production by *L. brevis* is shown the highest amount of GABA production in the optimum conditions of 34.09°C, time of 96 h, pH of 4.65 and GA concentration of 650 mmol was obtained as 19960 mg/l (with 91.59% desirability). The predicted results were confirmed by the experiments with twice replication, and no significant difference was observed between the predicted and experiment data.

DISCUSSION

The potential of GABA production by probiotic bacteria. The *L. brevis* had the highest amount of GABA production. It can be said that *L. brevis* has the ability to produce GAD that is an intra-cell enzyme responsible for irreversible catalytic decarboxylation of L-glutamate for GABA production (19). Although the reaction of decarboxylation for GAD of the lactic acid bacteria is similar, but the initial structure in the N-terminal and C-terminal regions is significantly different. Difference in the initial structure of this enzyme can influence on its ability to produce GABA (26). Moreover, in most of lactic acid bacteria, the active form of glutamate decarboxylase is in dimer form but the active form of this enzyme in *L. brevis* is in tetramer form that can lead to more GABA production comparing to other lactic acid bacteria (27). Siragusa et al. reported that the best GABA producing species include *L. paracasei* PF6, *L. delbrueckii*. subspecies of *bulgaricus* PR1, *Lactococcus lactis* PU1 and *L. brevis* PM17 isolated from cheese (8).

Table 4. Comparison between the amounts of produced GABA in culture medium and predicted GABA by *Lactobacilus brevis*

Treatment	Temperature (°C)	pH	Time (h)	Glutamic Acid (mmol)	GABA (mg/l)	GABA Fit (mg/l)	Residual
1	35	3.5	54	650	16754.870	16618.186	136.684
2	45	5	12	337.5	9870.230	9275.079	595.151
3	35	5	54	337.5	12435.750	12489.580	-53.830
4	35	3.5	54	25	5124.540	4604.228	520.312
5	35	6.5	12	337.5	9055.980	9310.157	-254.177
6	35	6.5	54	650.	14546.560	14931.628	-385.068
7	35	5	54	337. 5	12545.450	12489.580	55.870
8	25	5	12	337.5	10453.330	9934.531	518.799
9	35	5	96	650	18345.120	19823.380	1521.740
10	25	5	54	25	5875.130	4944.230	930.900
11	35	6.5	96	337.5	11457.230	11186.227	271.003
12	25	5	96	337.5	11587.340	12047.246	-459.906
13	35	5	96	25	6875.780	7556.357	680.577
14	25	5	54	650	15453.670	16341.459	-887.789
15	45	3.5	54	337.5	9234.540	8786.922	447.618
16	35	3.5	96	337.5	11980.220	12248.925	-268.705
17	45	5	96	337.5	11234.230	11617.784	-383.554
18	35	5	12	650	17435.320	16367.105	1068.215
19	35	5	54	337.5	12487.540	12489.580	-2.040
20	35	6.5	54	25	4867.230	4868.669	-1.439
21	45	5	54	650	13984.450	15438.232	-1453.782
22	35	5	12	25	5423.110	6557.212	-1134.102
23	25	3.5	54	337.5	8678.760	8720.784	-42.024
24	35	3.5	12	337.5	8875.690	9669.575	-793.885
25	25	6.5	54	337.5	8560.340	8620.320	-59.980
26	45	5	54	25	5123.450	4758.544	364.906
27	45	6.5	54	337.5	7894. 930	7465. 269	429.661

The effect of culture medium temperature on the amount of GABA production by *L. brevis.* The results showed that the best temperature for GABA production by *L. brevis* is in the range of 30-35°C that varies depending on the concerned strain, while for the other probiotics, it may increase up to 37°C. The reason can be looked for in the optimum temperature for activity of glutamate decarboxylase that is in the range of 30-50°C (28).

Wu et al. in a study on the efficiency of GABA production by *L. brevis* RK03 observed that in the culture temperature of 25-45°C, the highest amount of GABA production was at 30°C (29). Li et al. reported that growth of *L. brevis* NCL912 was enhanced with the increase of temperature (up to 35°C) and then reduced by the increase of temperature (30).

The effect of culture medium pH on the amount of GABA production by *L. brevis.* Several species and subspecies of LAB having the ability to produce GABA. Almost all the strains isolated from the traditional fermented foods such as cheese, tea, sour paste, Kimchi and Paocai had acidic pH (31). The reason is that the optimum level of pH to keep glutamate decarboxylase active is 4-5 (30). Wu et al. in a study on the efficiency of GABA production by *L. brevis* AK03 observed that the highest amount of GABA production was in pH 4.5, which was 983 and 25359 mmol, respectively in the two culture media of MRS and GA containing MRS (29). Zarei et al. observed that the highest amount of GABA production by *L. plantarum* was in pH 5 (16). A research by Komatsuzaki et al. showed that the desirable pH for GABA production with *L. plantarum* is 5-5.5 (26). In addition, the amount of effective pH for maximum production of GABA completely depends to species (24).

The effect of *L. brevis* cultivation time on the amount of GABA production. The results of current research showed that by increasing the cultivation time, the amount of GABA production increased significantly. Evaluation of the effect of *L. brevis* RK03 growth time in GABA production showed that the cells grown in an optimal culture medium containing 650 mmol monosodium glutamate had the maximum GABA production (mg/l 62523) in 88 h and the minimum GABA production (mg/l 57591) was in 96 h (29). Villegas et al. reported that *L. brevis* CRL1942 by adding 270 mmol monosodium glutamate and fermentation for 48 h produced 26.3 g/L GABA with the conversion rate of 90% (32).

The effect of the GA concentration of culture medium on the amount of GABA production by *L. brevis*. The GA concentration is the most important factor in GABA production. Literature review indicates that all sources of GABA isolation contained a high amount of glutamate. Therefore, traditional fermented food stuffs enriched with glutamate are important sources for isolation and selection of LAB-producing GABA (33). Villegas et al. found that addition of 270 mmol monosodium glutamate to the MRS culture medium resulted in production of maximum amount of GABA by *L. brevis* CRL194 (32).

Optimization of conditions for GABA production. The results showed that by optimization of the conditions of culture medium of *L. brevis*, the amount of produced GABA increased from 5960.82 mg/l to 19960 mg/l. Binh et al. observed that GABA production by *L. brevis*, K203 increased by optimization of the fermentation conditions at the initial pH of 5.25 and temperature of 37°C (1). Zarei et al. reported that by optimizing the culture media conditions, the amount of GABA produced by *L. plantarum* in the MRS broth increased from 115.24 mg/l to 170.492 mg/l (16).

CONCLUSION

In this research, the potential of GABA production by probiotic bacteria (*L. delbrueckii* ssp. *bulgaricus, Lactococcus lactis* ssp. *Lactis, L. casei, L. rhamnosus, L. brevis,* and *Streptococcus thermophilus*) in the culture medium of MRS broth was evaluated and the results showed that *L. brevis* had the highest amount of GABA production (5960.82 mg/l), and *L. delbrueckii* (the subspecies of *bulgaricus*) had the lowest amount of GABA production (36.07 mg/l). In order to increase the amount of GABA production, the conditions of *L. brevis* culture medium (temperature, time, pH and GA concentration) were optimized. The results showed that by optimizing the conditions of *L. brevis* culture medium (temperature=34.09°C, pH=4.65, time 96 h and GA concentration of 650 mmol), we could increase GABA production amount from 5960.82 to 19960 mg/l.

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