Rayavarapu et al. Beni-Suef University Journal of Basic and Applied Sciences (2021) 10:14
https://doi.org/10.1186/s43088-021-00100-3

Beni-Suef University Journal of Basic and Applied Sciences

RESEARCH Open Access

Optimization and comparison of γ-aminobutyric acid (GABA) production by LAB in soymilk using RSM and ANN models

Bhargavi Rayavarapu, Padmavathi Tallapragada^ and Usha MS*

Abstract

Background: γ-Aminobutyric acid (GABA) is a non-proteinaceous amino acid. In the mammalian nervous system, GABA functions as an inhibitory neurotransmitter. The present study focused on screening and optimization of γ-aminobutyric acid (GABA) yield by lactic acid bacteria by using soymilk as basal media. *Lactobacillus fermentum* (*Lb. fermentum*) was isolated from sourdough. The qualitative confirmation of GABA production by *Lb. fermentum* was observed by detecting colored spots on thin layer chromatography plate (TLC) and comparing it with standard GABA spot. The GABA from bacteria is confirmed by its molecular mass using mass spectrophotometry analysis (MS analysis). Single variable experiments were conducted for various physical and nutritional parameters, and determined the GABA content produced from *Lb. fermentum*, viable bacterial count, and pH of the fermented soymilk medium. Experimental data were authenticated by using response surface method (RSM) and artificial neural network (ANN) model.

Results: The results demonstrated that through single variable experiments, the yield of GABA and the viable bacterial cells increased in soymilk containing one percent of glucose, monosodium glutamate (MSG), and inoculum volume incubated at 37 °C, 48 h at pH 5. According to RSM results, the interaction of the highest concentration of MSG (1.5%) and mid glucose concentration (1.156%) yielded maximum GABA (5.54 g/L). The experimental data were in good agreement with two optimization models. The RSM models showed less error percentage than that of the ANN model.

Conclusion: This study indicates that soymilk is the best basal substrate for GABA production and growth of *Lb. fermentum* compared to synthetic media. *Lb. fermentum* can be explored further by food and pharmaceutical industries for the development of functional foods and therapeutic purposes.

Keywords: *Lb. fermentum*, Response surface methodology, γ-Aminobutyric acid, ANN, Soymilk

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1 Background

Gamma-aminobutyric acid (GABA) is a well-known apo-protein, bioactive nitrogenous compound (amino acid), which was first observed in potato tuber [1] and later in mammalian brains [2]. Since then, the GABA is noticed in many life forms like fungi, bacteria, animals, and plants [3, 4]. GABA is ubiquitous and plays many essential functions. In *Neurospora crassa* and *Bacillus megaterium*, GABA plays a vital role in the germination of spores [5]. In the central nervous system of mammals, it is a neurotransmitter inhibitor. It is involved in many physiological functions such as hypotensive, relaxant, antidiabetic, lowers the blood cholesterol levels, enhances immunity under stress conditions, and prevents cancer cell proliferation [6]. It enhances the concentration of plasma, synthesis of protein in the brain, and hormones for growth. Due to its biological functions, many food and pharmaceutical industries use GABA in the preparation of functional foods and medicines. Due to its many functions, the production of GABA increased commercially. The biosynthetic method is more promising than chemical synthesis because of its high potential in reacting as a catalyst, simplicity in the reaction process, and environmentally congenialness [5]. GABA is produced by an irreversible chemical reaction by decarboxylation of glutamic acid using glutamate decarboxylase (GAD) [7].

In Japan, Pharma Foods International Limited used lactic acid bacteria (*Lactobacillus hilgardii* K-3) to convert monosodium glutamate resulting in the production of GABA [8]. Many strains of *Lactococcus* and *Lactobacillus* have been isolated from different fermented foods and screened for GABA yield, such as *Lb. delbruskii* subsp., *Lb. lactis*, *Lb. plantarum*, *Lb. brevis*, *Lb. helviticus*, *Lb. bulgaricus*, and *Lb. paracasei* [9]. Sourdough consists of flour and wheat also it can be fermented spontaneously with the stable association of LAB and yeast. For example, hydrolysis of maltose by *Lb. sanfranciscencis* favors the growth of *Saccharomyces exiguous* or *Candida humilis* in San Francisco sourdough [10]. Predominant LAB observed in wheat sourdough are *Lb. plantarum*, *Lb. sanfranciscensis*, *Lb. acidophilus*, *Pediococcus pentosaceus*, *Lb. alimentarius*, *Lb. amylovorus*, *Lb. pontis*, *Lb. brevis*, *Lb. paralimentarius*, and *Lb. fermentum* [11]. Recently, Villegas et al. [12] isolated *Lb. brevis* CRL 1942 from quinoa sourdough. It produced 270 mM of GABA.

Soybeans are rich in proteins and oils. They contain 32-46% of crude protein, 15-24% of fat, 4.5–6.4% of crude ash, 5.12% of crude fiber, minerals (Ca, p, Mg, K, Na), and vitamins (folic acid, niacin, biotin). Soybeans are the substantial source for amino acids, either essential as isoleucine, leucine, and lysine, or non-essential types like aspartic and glutamic acids [13].

The present work aims to screen and optimization of growth parameters for enhancing the GABA yield from lactic acid bacteria in potential basal substrate, which was isolated from sourdough. The experimental data were authenticated statistically by RSM and ANN models.

2 Methods

2.1 Preparation of sourdough

Sourdough samples were prepared, according to Saez et al. [14] with some minor modifications that chickpeas were collected from local market and made to flours. In this study directly five different wheat flour samples were collected from the local market in Bangalore City, Karnataka State, India. Doughs were prepared by mixing 50 g of flour and 50 mL of sterile warm water and incubated at 37 °C for 24 h. After incubation, the same step was repeated five times by inoculating 10% of ripen sourdough. Five back sloping was performed daily into fresh water-flour mixture (95 ml of water and 95 g of flour) and incubated at 37 °C. Sample from the 5th cycle of back slopping sourdough was taken for isolation of bacterial strains.

2.2 Isolation and identification of bacterial strains from sourdough

Ten grams of sourdough sample was dissolved in 90 mL of sterile water. One milliliter of sample was serially diluted for 10^{-9} dilution and spreaded on MRS media and incubated for 48 h at 37 °C. The most predominant colonies were isolated and subcultured for pure colonies. Further, the colonies were identified morphologically by observing colony characteristics on MRS plate and biochemically by performing various tests like indole, methyl red vogus proskauer (MR-VP), catalase, and carbohydrate fermentation. Further, the bacterial strain was identified by 16S rRNA sequencing method. Approximately 1.5 KB (kilobase) of bacterial DNA was isolated by using a DNA isolation kit (GeNei ™) procured from Bangalore GeNei Company. Isolated bacterial genome was subjected to polymerase chain reaction (PCR) using forward (5′ AGAGTTTGATCM TGGCTCAG 3′) and reverse (5′ AGAGTTTGATCM TGGCTCAG 3′) primers. PCR reaction mixture contains 1 μL of bacterial genomic DNA, 400 ng each primers, 10X PCR assay buffer (10 μL), 3 U Taq polymerase enzyme (1 μL), 50 mM $MgCl_2$, and final volume made up to 100 μL by adding nucleic acid-free water. The used for PCR protocol included the following details: cycling was initial denaturation at 94 °C for 3 min, 30 cycles at 94 °C for 3 s: annealing (60 °C for 3 s), extension (72 °C for 1 min), final extension at 72 °C for 10 min, and holding temperature at 4 °C for infinity. After PCR cycling, the amplified samples were resuspended in

distilled water and subjected to electrophoresis in an ABI 3730xl capillary sequencer for DNA analysis. BLAST (basic local alignment search tool) was performed to the amplified sequence using the NCBI database gene bank. Based on the BLAST result, phylogenetic tree was constructed (data was not shown).

2.3 Screening and confirmation of GABA by mass spectrometry analysis

The bacterial strains were screened for GABA production by inoculating LAB in MRS broth supplemented with 1% of monosodium glutamate (MSG). After incubation, the supernatant was collected from centrifuging the culture broth at 8000 g for 10 min. Supernatant (5 μL) was spotted on thin layer chromatography (TLC) 60F254 aluminum sheets (Merck, Darmstadt, Germany) using n-butanol, acetic acid, and water (5:3:2) as mobile phase. The presence of GABA on TLC sheet was confirmed by observing a red color spot and it was compared with standard GABA after spraying 0.8% of ninhydrin reagent and it was incubated at 60 °C [12]. Mass analysis was carried out by using Shimadzu Class VP version 5.0 with acetonitrile/water (65:35 v/v pH -3.5) as a mobile phase, and the flow rate was set to 1 ml/min and detected at 235 nm by triple quadrupole mass spectrometer detector (TQD) with electrospray ionization (ESI) interface. Detection performed in positive multiple reaction monitoring (MRM) mode [15].

2.4 Colorimetric estimation of GABA

GABA was estimated by colorimetric method, as described by Dikshit and Tallapragada [16]. Culture (24 h) was inoculated into MRS broth supplemented by 1% MSG and incubated at 37 °C for 48 h. After incubation, the culture broth was centrifuged (8000 rpm) and the supernatant was collected. Supernatant (2 μL) was spotted on TLC plate and allowed to run using n-butanol, acetic acid, and water (5: 3: 2). The purified GABA spots were scrapped and placed on a glass test tube containing 3 mL of borate buffer (pH 7) and 0.5 mL of ninhydrin reagent (0.8%; dissolved in acetone), then incubated in water bath at 70 °C for 20 min and the absorbance was read at 570 nm under UV-Visible spectrophotometer. Standard GABA with different concentrations were prepared (10 μg/mL-10 mg/mL) and estimated by the above protocol. Blank was prepared as above without addition of the sample [17].

2.5 Preparation of soybean milk as a basal substrate

Fresh soybeans (*Glycine max*) were obtained from local place at Bangalore, India. Nutritional values of soybeans like fat and protein content were determined by using the Association of Official Analytical Chemists (AOAC) accepted methods [18]. Thoroughly washed soybeans were immersed in water overnight and cooked at 100 °C for 50 min. The fully cooked beans were allowed to cool down and grinded by using a blender. Double-layered cheesecloth was used to extract the milk. The extracted soymilk was autoclaved and taken as a basal substrate for growth of bacteria [19].

2.6 Standardization of cultural parameters

2.6.1 Effect of sources of nitrogen and carbon on culture media

Various carbon sources like glucose, sucrose, lactose, and maltose of same concentration were added to soymilk media and autoclaved at 121 °C for 15 min. After, autoclaving 24 h, old bacterial culture was inoculated into autoclaved soymilk and incubated at 37 °C for 48 h. Similarly, different nitrogen compounds like peptone, yeast extract, beef extract, and monosodium glutamate of same concentration (1%) were supplemented to media (soybean milk). After incubation, GABA was estimated from the supernatant.

Varied concentrations of highest GABA yielded carbon (0.5, 1, 1.5, and 2%) and nitrogen (1, 2, 3, and 4%) were tested as above. Final pH, viable count of bacterial cells, and GABA concentrations were estimated in the supernatant. The pH was estimated by pH meter. Viable cells of *Lb. fermentum* were determined by colony-forming unit (cfu/mL) and GABA concentration was estimated using the protocol mentioned above.

2.6.2 Effect of pH and temperature

Various ranges of pH and temperatures were optimized for GABA production in soymilk. pH ranges of 4, 5, 6, and 7 were maintained in different soymilk flasks. Bacterial culture (24 h) was inoculated into media and incubated for 48 h. Similarly, bacterial cells inoculated soymilk media was maintained at different temperatures like 25, 30, 35, 37, and 40 °C. GABA concentration, pH, and viable cell count in media were estimated in the supernatant.

2.6.3 Inoculum volume and incubation period

Different concentrations of inoculum volumes 1–4% were optimized for GABA production. Soymilk medium was inoculated with different concentrations of inoculum and incubated at 37 °C for 48 h. After incubation, GABA concentration, pH, and viable count of bacteria were determined. Similarly, soymilk media inoculated with bacteria cells were incubated for different time periods (24, 48, 72, and 96 h). At every time period, pH, viable cells, and GABA yield were determined in the fermented culture broth.

2.6.4 Time course for GABA production

Time course of GABA production was determined in soymilk broth by maintaining all optimized parameters:

1% glucose, MSG, inoculum volume, and pH 5 incubated for 48 h at 37 °C. During fermentation process, pH, viability, and GABA yield were determined at regular time intervals from the culture broth. At the same time, another soymilk flask was maintained with unoptimized conditions (no carbon and nitrogen source, 0.5% of inoculum volume at pH 7, and 30 °C temperature) was considered as control [20].

2.6.5 Response surface methodology

Central composite design (CCD) at three-level of RSM performed with the three independent variables (MSG, glucose, and incubation period) and one dependent variable (GABA) (Table 1). A set of 20 experiments were conducted by taking independent variables at five different levels (−2, −1, 0, + 1, + 2) (Table 2). The relative effect of two independent variables on the response was studied on the 3D contour plots. MATLAB software was used to analyze the regression studies, and data of the experiments to give their respective contour plots and graphs [16].

2.6.6 Artificial neural network

ANN is a computational program that is attracted by science researchers. ANNs explain the interaction and the relationship between the vast data points. Initially, this program acquires the information by identifying the patterns and relationships in the data by learning or training or by experience. ANNs are connected by several hundred units of artificial neurons or processing elements (PE). These artificial neurons are formed as single or multiple layers. ANN covers several architectures includes CNN (convolutional neural network) and RNN (recurrent neural networks). ANN model is a feed-forward direction, and the data flows through neuronal layers, in which initially the input data points get into the primary neuronal layer, and further, the data was introduced to hidden layers by weights (coefficients). Each neuron in hidden layers is interconnected and resulted in output layer GABA yield. Finally, they add all the weighted inputs in the hidden layers, including bias by using Eq. (1):

$$\text{sum} = \sum_{i=1}^{n} x_i w_i + \theta \tag{1}$$

Where w_i (i = 1, n) represents connection weights, n is the bias, and x_i represents the input parameter.

The output coefficient finally passed through an activation function. The activation function shifts the space in the nonlinearity of input data. The logistic output function used in this work is shown in Eq. (2):

$$f(\text{sum}) = \frac{1}{1 + \exp(-\text{sum})} \tag{2}$$

From the 20 experimental data (100%) points, ANN used fourteen (70%) data points for training and learning the patterns between data. From the remaining data points, six (30%) were split evenly for validation and testing [21]. The error function root mean square error (RMSE) in this model is calculated by the Eq. (3):

$$\text{RMSE} = \sqrt{\frac{\sqrt{\sum_{i=1}^{N}\sum_{n=1}^{M}\left(y_n^i - \hat{y}_n^i\right)2}}{\text{NM}}} \tag{3}$$

Here, N represents the number of training patterns used; M denotes output nodes number; input pattern (vector) index by I, while the desired (target) and predicted outputs of the nth output node are respectively denoted by y^i and by.

2.7 Statistical analysis

Every experiment was conducted in triplicates and the mean values and standard error values were calculated. IBM MATLAB software was used for RSM study design. ANN model was performed using the JAVA program.

3 Results

3.1 Bacterial strains isolation from sourdough

For 72 h of incubation, twenty predominant colonies were isolated on MRS plates, and isolates were identified morphologically and biochemically. Morphologically, the colonies were white, mucoid, smooth, and elevated. From the biochemical test, all isolates exhibited indole, methyl red-vogues proskauer, catalase-negative, gram-positive, and non-sporing bacteria. Further, these isolates were screened for GABA production in MRS medium.

3.2 Screening and estimation of GABA production by *Lb. fermentum*

Qualitatively, the GABA production by bacterial isolates was determined by observing red-colored spots on thin-layer chromatography (TLC) plate after treating with

Table 1 Experimental range and levels of independent variables

	Actual factor level at coded factor levels at					
Variables with designates	**Code**	**−2**	**− 1**	**0**	**+1**	**+ 2**
Glucose (%)	X1	0.5	0.75	1	1.25	1.5
MSG (%)	X2	0.5	0.75	1	1.25	1.5
Incubation period (h)	X3	24	36	48	60	72

Table 2 Central composite design of factors in coded for GABA yield in fermented soya milk by *Lb. fermentum*

				Experimental values	RSM predicted values	ANN predicted values
S. no.	Glucose (X1)	MSG (X2)	IP (X3)	GABA (g/L)	GABA (g/L)	GABA (g/L)
1	−1	−1	− 1	2.51	2.1971	2.4940
2	1	−1	− 1	2.58	2.9284	2.6396
3	−1	1	−1	2.96	2.7409	2.6368
4	1	1	−1	4.15	3.9771	2.7822
5	−1	−1	1	2.47	2.1984	3.7984
6	1	−1	1	2.18	1.9546	3.9439
7	−1	1	1	4.77	3.9771	4.0867
8	1	1	1	4.37	4.2384	3.1448
9	−2	0	0	1.37	1.946	3.4359
10	2	0	0	3.07	2.9385	3.1476
11	0	−2	0	2.54	2.5485	3.4331
12	0	2	0	4.94	5.376	1.9735
13	0	0	−2	1.68	1.636	4.5947
14	0	0	2	1.41	1.8985	3.2904
15	0	0	0	4.53	3.9341	3.2904
16	0	0	0	3.28	3.9341	3.2904
17	0	0	0	4.47	3.9341	3.2904
18	0	0	0	3.98	3.9341	3.2904
19	0	0	0	3.79	3.9341	3.2904
20	0	0	0	3.11	3.9341	3.2904

0.8% of ninhydrin reagent (Fig. 1). Among all these isolates, three isolates (S1, S2, and S3) showed GABA production, and R_f value (0.54) of the sample was matched with standard GABA. In MRS media S1, S2, and S3 produced maximum GABA 6.38, 4.56, 4.24 g/L. These strains were screened on different substrates like agro wastes (rice bran, wheat bran, coconut oil cake, sesame oil cake) and cereal milks (chickpea, soymilk, and mung bean milk). Among all the substrates, bacterial isolates produced maximum GABA in soymilk (data was not shown). S1 produced maximum GABA (2.88 g/L) compared with S2 (2.26 g/L) and S3 (2.44 g/L) in soymilk as a best basal substrate. From the 16S rRNA sequencing method, the isolate S1 was identified as *Lb. fermentum*. As per the literature survey, the present study is the first report on gamma-aminobutyric acid produced by *Lb. fermentum* from sourdough.

3.3 GABA Confirmation using mass spectrometry analysis

Based on the mass spectral analysis, GABA is confirmed by determining the mass of the compound. The elemental composition of the GABA is $C_4H_9NO_2$, which has a molecular weight of 103. Liquid chromatography-MS, which is positive ionization, employed for the analysis, and from the results, it was clear that mass/atomic number (*m/z*) for GABA as 104.95 (103 + H^+) produced by *Lb. fermentum* (Fig. 2).

3.4 Standardization of cultural parameters in soymilk by *Lb. fermentum* for GABA yield

The effect of cultural growth parameters on GABA production in soymilk from *Lb. fermentum* was shown in Table 3, and viable count of *Lb. fermentum* and pH was also determined. Highest yield of gamma-aminobutyric acid was noticed for glucose, MSG, and inoculum size at 1% of concentration, temperature at 37 °C, and pH 4 for 48 h of the incubation period. The carbon (glucose), and nitrogen (MSG) source strongly affects the gamma-aminobutyric acid production and *Lb. fermentum* cell population in soymilk. Maximum GABA production was observed in soymilk supplemented with glucose compared with other carbon sources (sucrose, maltose, and lactose). Different concentrations of glucose from 0.5–2% were screened for GABA yield. The yield of GABA was increased from 3.97 to 4.74 g/L, with an increase in the concentration of glucose from 0.5 to 1%. Further, increase in glucose concentration from 1 to 2%, resulted in significant decrease in GABA yield from 4.74 to 0.79 g/L. Concentration of glucose strongly affects the viable

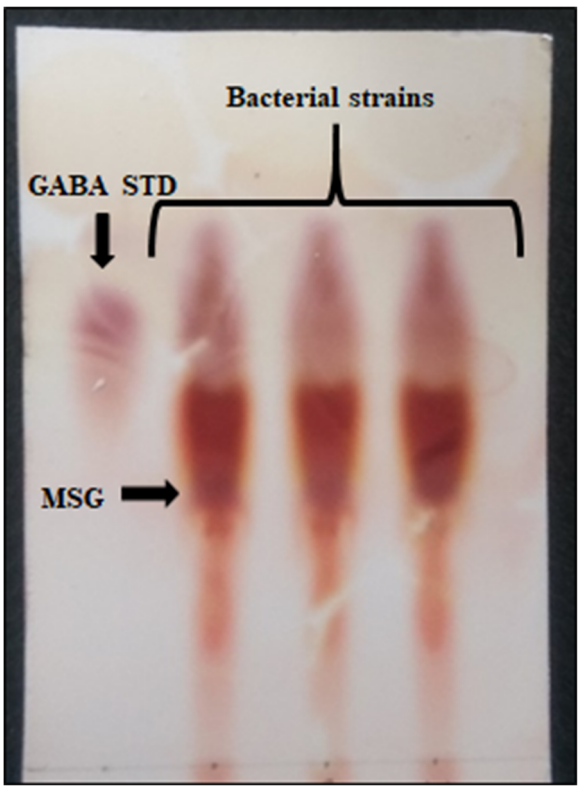

Fig. 1 Qualitative detection of GABA (red color spots) produced by bacterial strains on TLC plate

count of *Lb. fermentum*. As increase in glucose concentration from 0.5-1%, *Lb. fermentum* cell count was increased from 1.2 × 10^9-6.26 × 10^9 CFU/mL in soymilk. Later, reduction of viable count was observed due to high glucose concentration. In all the tested concentrations, pH was constant in soymilk after fermentation.

The yield of gamma-aminobutyric acid (4.46 g/L), and viable count of *Lb. fermentum* (6.8 × 10^9 CFU/mL) was high at MSG level 1%. Further, increases of MSG concentration from 1 to 4%, reduction in GABA yield (3.74 g/L) and cell count of *Lb. fermentum* in soymilk were observed. The pH 4.4 was remained constant in all soymilk containing tested MSG concentrations. From the above result, concentration of MSG strongly affects the GABA yield and *Lb. fermentum* growth in soymilk.

In the soymilk fermentation medium, GABA yield (4.2 g/L) and *Lb. fermentum* bacterial cells (6.1 × 10^9 CFU/mL) were maximum at 37 °C temperature compared with 25, 30, 35, and 40 °C. As temperature increased from 37 to 40 °C, GABA production and *Lb. fermentum* cell count was decreased. The pH (4.4) was remained constant in all tested temperatures. The maximal yield of GABA (2.58 g/L) and bacterial population 4.3 × 10^9 CFU/mL were counted at initial test pH 5 compared with pH 4, 6, and 7. There is no significant GABA yield observed at pH 5 and 6. As pH increased from 4 to 7, the GABA yield (1.46 g/L) was decreased.

Incubation period strongly affected the GABA production from *Lb. fermentum* in soymilk. Maximum GABA production (4.27 g/L) was detected for 48 h of incubation period compared with 24, 72, and 96 h. As the incubation period (96 h) is increased, reduction in GABA yield 0.86 g/L was noticed. The highest bacterial population (6.9 × 10^9 CFU/mL) was recorded at 48 h of incubation period. Significant high GABA production (3.79 g/L) was determined at 1% (v/v) of inoculum size. As inoculum size increased from 1 to 4%, there is no significant change in GABA production in soymilk. By change in the inoculum size, no significant change in GABA yield from *Lb. fermentum*.

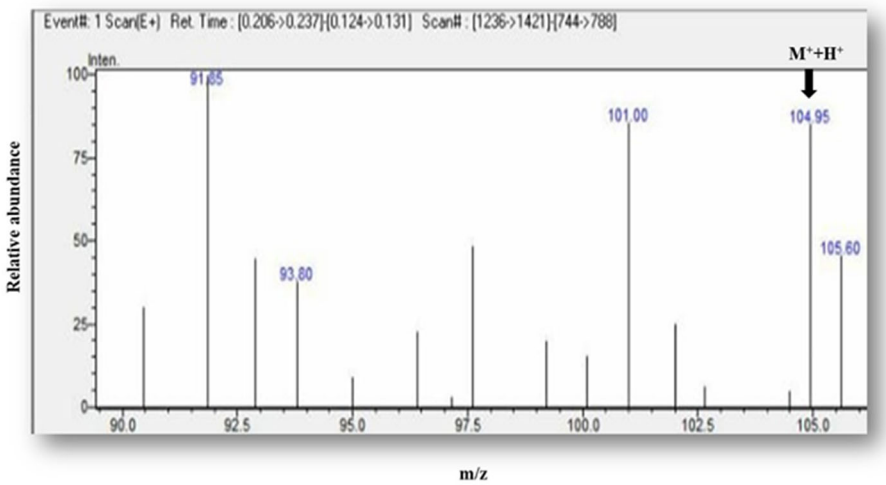

Fig. 2 Chromatogram of mass spectral analysis (m/z) for conformation of GABA produced by *Lb. fermentum*

Table 3 Effect of physical and nutritional parameters on GABA production by *Lb. fermentum* in soymilk: carbon source, MSG concentration, temperature, pH, incubation period, inoculum size

Carbon source					
	Control	Glucose	Sucrose	Maltose	Lactose
GABA (g/L)	0.46 ± 0.03	4.74 ± 0.03	2.4 ± 0.02	1.37 ± 0.02	3.96 ± 0.03
Concentration of glucose (%)					
	Control	0.5	1	1.5	2
GABA (g/L)	0.42 ± 0.02	3.97 ± 0.02	4.74 ± 0.03	2.43 ± 0.03	0.79 ± 0.04
Viable count (log cfu/ml)	1.2 ± 0.03	4.73 ± 0.05	6.26 ± 0.1	5.33 ± 0	3.8 ± 0
pH	4.9 ± 0.3	4.5 ± 0.6	4.5 ± 0.2	4.3 ± 0.5	4.4 ± 0.5
Concentration of MSG (monosodium glutamate (%))					
	Control	1	2	3	4
GABA (g/L)	0.45 ± 0.02	4.46 ± 0.05	4.21 ± 0.01	4.01 ± 0.03	3.74 ± 0.03
Viable count (log cfu/ml)	1.1 ± 0.01	6.8 ± 0.4	6.2 ± 0.5	5.3 ± 0.4	4.8 ± 0.4
pH	4.7 ± 0.4	4.4 ± 0.5	4.4 ± 0	4.4 ± 0	4.4 ± 0
Temperature (°C)					
	Control (37)	25	30	35	40
GABA (g/L)	4.2 ± 0.04	2.83 ± 0.2	3.31 ± 0.2	4.01 ± 0.03	3.86 ± 0.1
Viable count (log cfu/ml)	6.1 ± 0.3	4.0 ± 0.5	4.5 ± 0.2	5.5 ± 0.3	4.2 ± 0.1
pH	4.4 ± 0	4.4 ± 0.5	4.4 ± 0	4.4 ± 0	4.4 ± 0
pH					
	Control	4	5	6	7
GABA (g/L)	0.38 ± 0.02	2.35 ± 0.02	2.58 ± 0.04	2.59 ± 0.03	1.46 ± 0.1
Viable count (log cfu/ml)	0.8 ± 0.02	2.1 ± 0	4.3 ± 0.2	1.4 ± 0.1	1.1 ± 0.1
pH	5.2 ± 0	3.1 ± 0	3.4 ± 0	3.4 ± 0	4.1 ± 0
Incubation period (no. of days)					
	Control	1	2	3	4
GABA (g/L)	0.48 ± 0.02	4.02 ± 0.01	4.27 ± 0.02	1.18 ± 0.02	0.86 ± 0.02
Viable count (log cfu/ml)	1.0 ± 0.02	6.7 ± 0.05	6.9 ± 0.02	4.8 ± 0.04	3.74 ± 0.06
pH	4.9 ± 0	4.4 ± 0	4.4 ± 0.1	4.4 ± 0.05	4.2 ± 0
Inoculum size (%)					
	Control	1	2	3	4
GABA (g/L)	0.43 ± 0.04	3.79 ± 0.01	3.71 ± 0.05	2.62 ± 0.05	2.12 ± 0.04
Viable count (log cfu/ml)	1.2 ± 0.02	5.8 ± 0.3	6.4 ± 0.4	TNC	TNC
pH	5 ± 0	4.4 ± 0	4.4 ± 0	4.4 ± 0	4.4 ± 0

TNC too numerous to count

3.5 Time course of GABA production by isolate *Lb. fermentum*

Figure 3 depicted the GABA production, bacterial population, and pH in the fermented soymilk basal substrate under all optimized parameters, and a separate flask of soymilk under unoptimized conditions was used as control. *Lb. fermentum* was multiplied till 24 h, and reached to stationary phase from 24 to 48 h containing 6.1×10^9 CFU/mL. It was reported that in probiotic foodstuff to exert therapeutic benefits in vivo, the bacterial population should be 10^6 CFU/mL [12]. GABA content was slowly observed at 24 h, and maximum concentration (4.6 g/L) was seen at 48 h of incubation period. It shows that GABA yield was independent at log phase of *Lb. fermentum*, and produced at the stationary phase. Under optimized conditions in soymilk, 1.59-fold increase in GABA content from *Lb. fermentum* was noticed compared with unoptimized conditions of soymilk. The pH of the medium was dropped from 5 to 4.2 after 48 h of the incubation period.

3.6 RSM

From the above results of single variable tests, three variables, i.e., glucose, MSG, and incubation period (independent) were

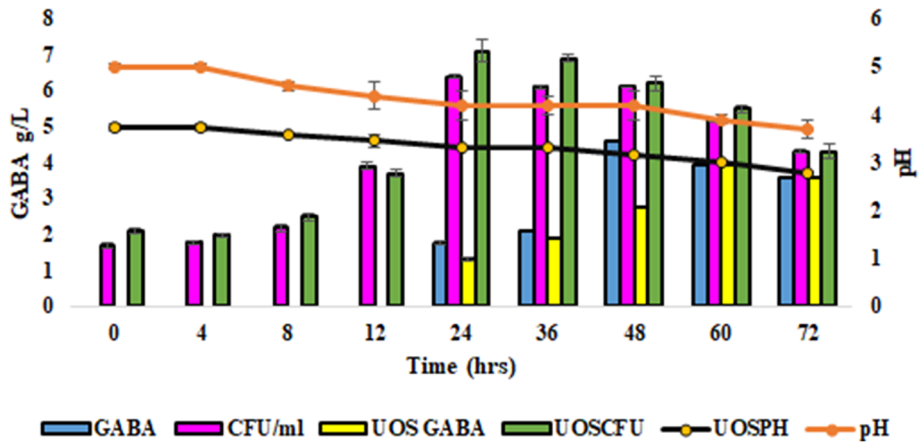

Fig. 3 GABA production, bacterial growth, and pH values during time course of soymilk fermentation with *Lb. fermentum* under optimized conditions. Blue, pink, and red bars indicate GABA, bacterial growth, and pH values from soymilk with optimized conditions. Yellow, green, and black bars indicate GABA, bacterial growth, and pH values from soymilk with unoptimized conditions

considered for CCD (central composite design). Glucose, MSG, and incubation period were coded as X1, X2, and X3. Equation (4) explains the relationship and interactions between the three independent variables on the output yield of GABA produced by *Lb. fermentum*.

$$\begin{aligned} GABA\ yield\ \left(\frac{g}{L}\right) = & -11610.2273 + 14807.0455 \\ & \times X1 - 4357.9545 \times X2 \\ & + 344.9384 \times X3 \\ & + 2020.0000 \times X1 \\ & \times X2 - 81.2500 \times X1 \times X3 \\ & + 102.9167 \times X2 \\ & \times X3 - 5967.2727 \times X_1^2 \\ & + 112.2727 \times X_2^2 + 127.5 \\ & \times X_3^2 \end{aligned} \quad (4)$$

The data for GABA yield by *Lb. fermentum* was obtained from experimentation, and the CCD model was shown in Table 3. The GABA yield from *Lb. fermentum* was studied by 3D surface counterplots for two variables and maintained one factor at its middle level constant (0). Figure 4 illustrates the glucose concentration increases from 0.5 to 1.5%; the yield of GABA was raised initially and then reduced. Near mid glucose concentration (1-1.2%), the GABA yield was maximum, which meant that moderate concentration of glucose is amicable for higher GABA yield. As the MSG concentration increased from 0.5 to 1.5%, the GABA yield increased steadily but more rapidly decreased with a higher concentration of glucose. The maximum yield observed at 1.5% of MSG. From the interaction of MSG and glucose, it was observed that the highest concentration of MSG (1.5%) and mid glucose concentration (1.156%) yields maximum GABA concentration (5.543 g/L).

Figure 5 3D plot showed the typical bell shape. It depicts that individually increasing the glucose concentration and incubation hours; initially, the GABA yield was increased and declined later. It indicates that the moderate level of glucose and incubation period (IP) results in more GABA production. The interaction of the incubation period (at 47.17 h) and glucose concentration of (1.086%) leads to intense GABA yield (3.974 g/L).

From this plot (Fig. 6), noticed that there is no significant change in GABA yield with a higher concentration of MSG, and less incubation period, whereas there is an abrupt rise in the yield of GABA at a higher incubation period. As concerned with the effect of the incubation period with MSG concentration, the GABA yield increased as the incubation period increased until the modest incubation period, and then a sudden fall of GABA yield was observed. The interaction of both incubation periods (at 50.48 h) and MSG concentration (of 1.5%) resulted in a high GABA yield (5.494 g/L).

Table 4 presents the regression coefficients of data obtained from the CCD model. The p value and t are higher in magnitude, indicating the corresponding significant coefficient. It depicts that the quadratic effect as concerns; the incubation period was the most significant variable. Furthermore, the concentration of glucose, the combination of MSG (X2), and the incubation period (X3) were highly significant compared with other interactions. From the above results, it was depicted that the incubation period (X3) influences the GABA yield produced by *Lb. fermentum*.

Considering the ANOVA, the p value explains the high significance of the model for GABA yield. The

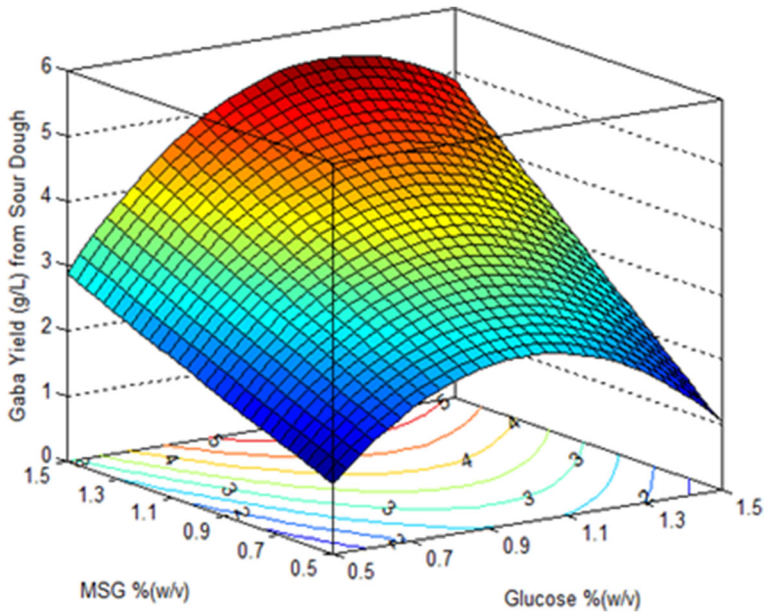

Fig. 4 3D plot for interactive effect of glucose and MSG concentration on the GABA Yield (g/L) by *Lb. fermentum* keeping incubation period at its constant central level (0)

value 0.8487, determination coefficient (R^2) was enumerating the experimental values and was best fitted to the CCD model. R^2_{adj} is 0.71, and it is closer to R^2 value, which confirms that the model was significant. From the R^2 value, the model understood 84.87% of experimental data. Only 15.13% of experimental data were not explained by model. From the above ANOVA results, the experimental model was validated and explained the actual connection between the independent factors and GABA outcome (Table 5).

3.7 ANN (artificial neural network (deep learning))

The accuracy of the RSM model was verified in correlation with the deep learning model hypothetically. ANN predicted values were shown in Table 2. Figure 7 represents the feed-forward propagation of the ANN model. It shows the initial stage of the ANN model in which the model is understanding patterns of data, where all orange and blue data points were scattered. In this model, the predicted outcome values have resulted from the addition of all input weights. Later, these weights were

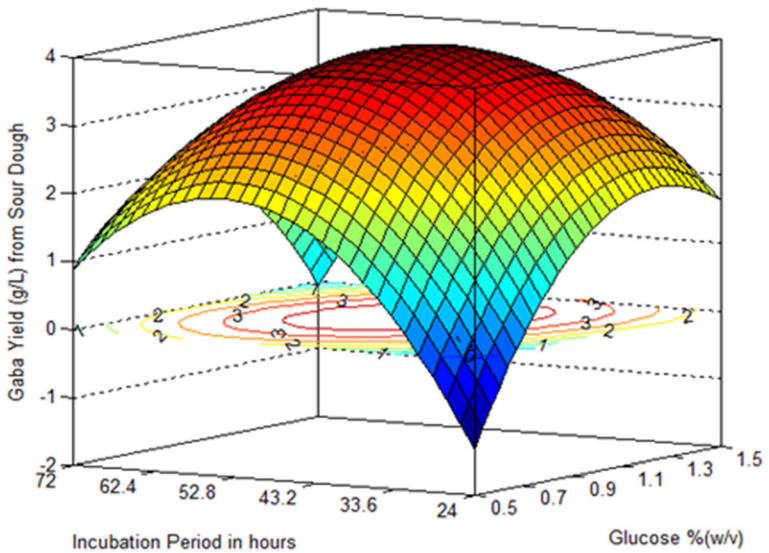

Fig. 5 3D plot for interactive effect of glucose concentration and incubation period on the GABA yield (g/L) by *Lb. fermentum* keeping MSG concentration at its constant central level (0)

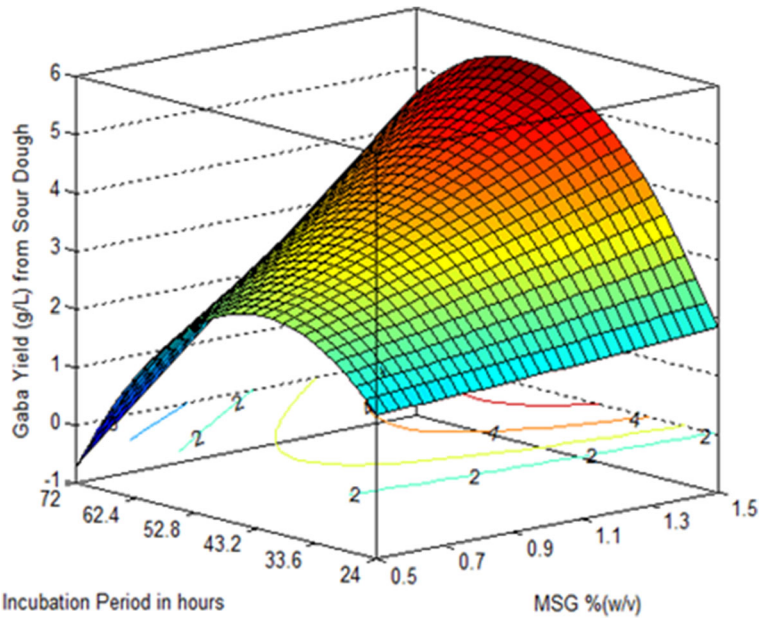

Fig. 6 3D plot for interactive effect of MSG concentration and incubation period on the GABA yield (g/L) by *Lb. fermentum* keeping glucose concentration at its constant central level (0)

used as an analytical tool for the analysis of a new set of input data points. The error in prediction values is decreased through several training cycles until it reaches the accuracy level. In this study, the error loss was constant at 150 epoch (Fig. 8). The comparison analysis for GABA data from experimental and values predicted obtained from both models was seen in Fig. 9. The root mean square error (RMSE) values for data tested for RSM and ANN models are 0.22 and 0.63, respectively. The fitness of data in the ANN model is shown in Fig. 10. RSM model shows less error percentage compared with the ANN model. The accuracy of the RSM model validated by observing the closeness RSM with ANN predicted values.

4 Discussion

In all fermented foods, *Lb. fermentum* is prominent in its number. In Italian wheat sourdough obligatory heterofermentative *Lb. brevis*, *Lb. fructivorans*, *Lb. fermentum*, and *Lb. sanfranciscensis* are dominant microflora [10]. Diana et al. [22] isolated the highest GABA producing *Lb. brevis* from the sourdough medium.

The analysis of GABA through high-performance liquid chromatography (HPLC) is more extravagant and higher in price. A cost-effective method, which involves the use of ninhydrin reagent for estimating the GABA using a colorimeter was employed. It is precise and very simple method. The existence of GABA as zwitterions with a carboxyl group that is a de-protonated and an

Table 4 Regression co-efficient results from the data of CCD (central composite design) experiments for GABA (γ-aminobutyric acid) production

Component	Model coefficient	Standard error	*t* value	*P* value
Constant	−11610.2273	7.0788	−1.6401	0.13201
Glucose (g)	14807.0455	6.1864	2.3935	0.03773
MSG (g)	−4357.9545	6.1864	−0.7044	0.49723
IP (days)	344.9384	0.1289	2.6764	0.02324
Glucose × MSG	2020.0000	3.4034	0.5935	0.56602
Glucose × IP	−81.2500	0.0709	−1.1459	0.27852
MSG × IP	102.9167	0.0709	1.4515	0.17729
Glucose × glucose	−5967.2727	1.9198	−3.1083	0.01109
MSG × MSG	112.7273	1.9198	0.0587	0.95433
IP × IP	127.5000	0.0008	−4.5147	0.00112

MSG monosodium glutamate, *IP* incubation period

Table 5 ANOVA (analysis of variance) for response quadratic model for GABA production

Sum of squares	Degrees of freedom	*f* value	*P* value	Mean square error	R^2	Adj. R^2
3.6198	9	6.2321	0.00424	0.3620	0.8487	0.7125

amino group that is protonated. The ninhydrin reagent reacts with the GABA amino group and leads to the formation of a purple color compound called Ruhemann's purple. Hosseinimehr et al. [17], Bali, and Guar [23] used this method for the analysis of baclofen and pregabalin in brain samples. Baclofen and pregabalin are the analogs for GABA. Li et al. [24] reported the yield of GABA (103.5 g/L) by *Lb. brevis* NCL912, which was isolated from paocai a fermented food in China containing various vegetables.

The above results indicated that maximum GABA by *Lb. fermentum* produced in soymilk substrate compared with other substrates. This might be due to soybean meal that contains the highest glutamic acid content (74.5 g N) than other amino acids [13]. In our findings, low and high glucose levels severely affecting the gamma-aminobutyric acid production from *Lb. fermentum* in soymilk. *Lb. fermentum* yields maximum GABA in soymilk supplemented with 1% of glucose concentration. Our results were in agreement with Cho et al. [25]. He reported that *Lb. buchneri* produced high GABA (230 mM) in MRS broth supplemented with 1% of glucose. Li et al. [24] reported the maximum yield of gamma-aminobutyric acid from *Lactobacillus brevis* NCL912 at 2.5% of glucose than maltose. In recent studies, the *Cornybacterium glutamicum* produced maximum GABA in the EFB solution by utilizing both glucose and xylose [26]. The liberation of GABA by decarboxylation reaction involved in Krebs cycle utilizing glutamate as a substrate. GABA is an end product in the GABA shunt pathway where malic acid from the Krebs cycle is converted into succinic acid. Succinic acid converted into succinic semi-aldehyde through succinate semi-aldehyde dehydrogenase (Gad D). Finally, by GABA aminotransferase (Gad T) drives the conversion of succinic semi-aldehyde into GABA. Proteins of Gad D, and Gad T help succinate directed to GABA, and Gad C (Glu/GABA antiporter) helps in releasing the GABA out. These enzymes form domain-ligand interaction and localized through a synthetic protein scaffold complex [27]. Yang et al. [28] reported that a high concentration of glucose (> 15 g/L) inhibits cell growth (*Enterococcus avium*), thus resulting in a decrease in GABA production. The high concentration of glucose leads to shrinking in the cells due to its high osmotic pressure, which may inhibit the cell growth, and GABA production [29].

Glutamate is converted into GABA, and the GAD enzyme catalyzes the reaction. *Lb. fermentum* produced maximum GABA at 1% of MSG in soymilk. The addition of MSG to the cultural medium directly enters bacterial cells by their antiporter system present in the cell membrane, and increases GABA production [30]. The results were in agreement with Lin [31] that *Lb. rhamnosus* YS9 in culture media yielded maximum GABA at 200 mM of MSG, and with further increase in MSG concentration, GABA yield was decreased. Similar results were reported by Song et al. [32]. He observed *Lb. rhamnosus* YS9 produced maximum GABA yield (0.64 mg/ml) at 2% of MSG in adzuki bean milk, and further increase in MSG concentration leads to a decline in cell growth. Tung et al. [33] reported that *Lb. plantarum* NTU 102 yielded maximum GABA production (33 mg/L) at 0.5% of MSG at 37 °C temperature for 24 h.

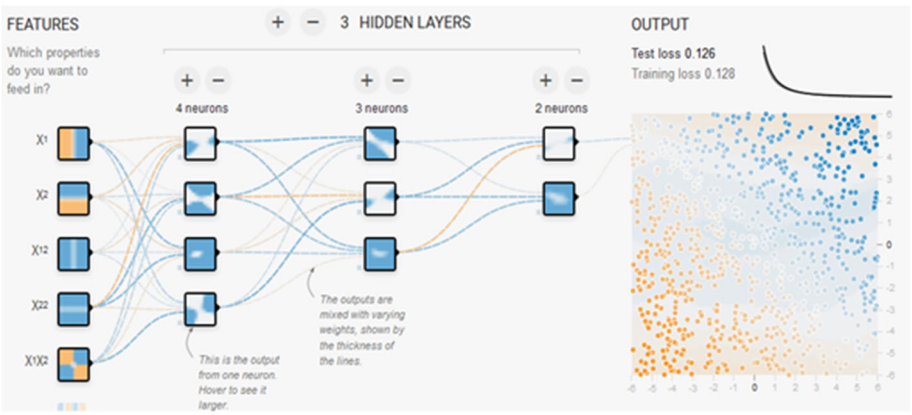

Fig. 7 Initial stage of learning the data in ANN model

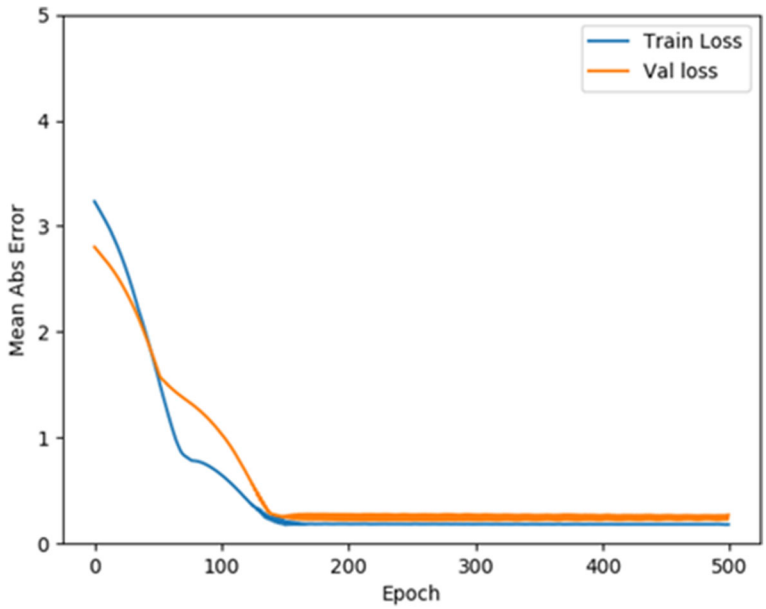

Fig. 8 Changes of mean square error in relate to epoch numbers when best ANN with 150 epoch is trained

The hydrated form of MSG leads to generate Na + and L-glutamic acid. This glutamate acts as a substrate for the synthesis of GABA. The addition of MSG to the fermentation medium aimed to stimulate the production of GABA by activating GAD enzyme via GABA shunt [32]. Li et al. [24] reported that increased concentration of glutamate (0.25-1 M) leads to a decrease in the growth of *Lb. brevis* NCL912 and biomass. It was apparent that high glutamate concentration inhibits bacterial cell growth and leads to a reduction of the GABA yield. At 1% of inoculum concentration yields maximum concentration. With the increase in inoculum volume, there is no significant yield in the GABA. Ko et al. [19] reported similar findings that *Lactobacillus brevis* FPA 3709 had a bacterial population 1.2 × 10^8 CFU/ml after incubation of 24 h in fermented black soymilk.

From the above results, *Lb. fermentum* yielded maximum GABA at pH 5 and 37 °C for 48 h. Lu et al. [34] reported *Lactococcus lactis* yielded the highest GABA at pH 5-7 in fermentation medium containing brown rice juice, soybean juice, and enzymatically degraded skim milk in the ratio of 33:58:9 (v/v). These results are good agreement with a previous study by Villegas et al. [12]. The decrease in pH was due to the production of

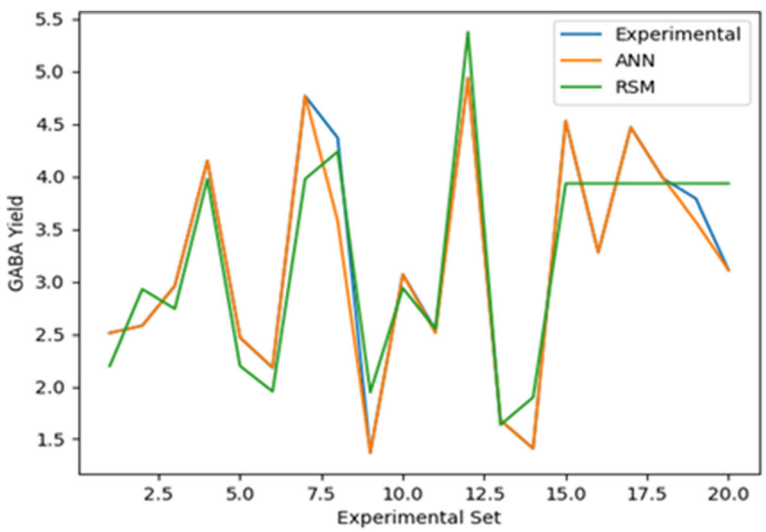

Fig. 9 Comparative plots of experimental data with RSM and ANN models

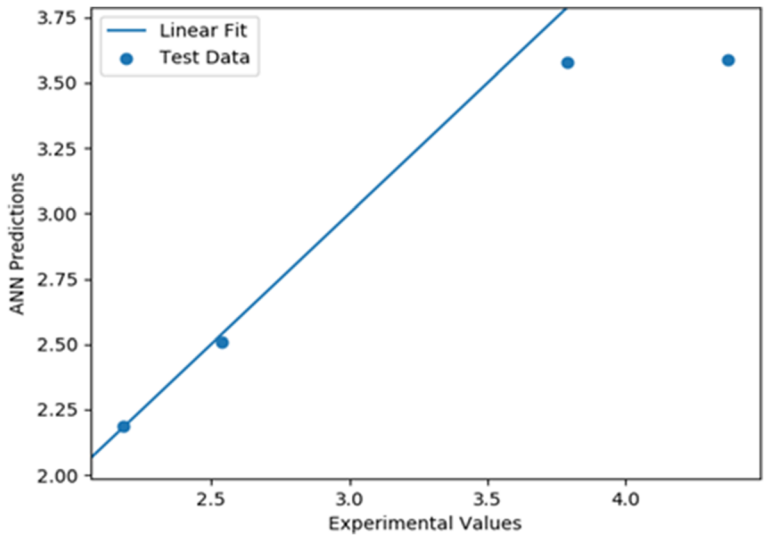

Fig. 10 Linear fit for the results of test data from ANN model

organic acids by lactic acid bacteria during fermentation. GABA confers tolerance to acidic pH in *Lactococcus lactis*, *E.coli*, *Listeria monocryogenes*, *Clostridium perfringes*, and *Mycobacteria* [5]. The production of GABA is closely related to the pH of the cultural medium. Biosynthesis mechanism of GABA is responsible for maintaining the pH within the cell, because glutamate consumes one proton by decarboxylation and increases the pH in the cell [30]. As per previous studies, GABA in lactic acid bacteria is catalyzed by enzyme glutamate decarboxylase (GAD). GAD enzyme is effective at pH 4 and 5 [35]. Hence, in our study the optimum pH for GABA production was selected at 5.

In our results, the maximum yield of GABA was observed at 37 °C temperature. Li et al. [20] reported similar results, *Lb. plantarum* produced maximum GABA (350 mg/L) at 37 °C temperature in fermented chickpea milk. Di Cagno et al. [36], Lu et al. [34], and Komatsuzaki et al. [37] reported that *Lb. plantarum* DMS 19463, *Lb. lactis*, and *Lb. paracasei* NFRI 7415 produced highest GABA (497 mg/L, 0.27 mg/mL, and 31. 14 mg/mL) at 30, 34, and 37 °C. Thus, different lactic acid bacteria have different temperatures for GABA production. Higher temperatures than 37 °C causes inactivation of enzymes and cell aging in bacteria. This leads to a reduction in the bacterial cell growth and GABA production. Cho et al. [28] reported similar results that *Lb. buchneri* produced the highest GABA at 48 h of incubation period, and maximum bacterial population observed at 36 h. From the fish intestine, *Lactobacillus brevis* FPA 3709 yielded highest GABA (5.42 mg/mL) in cooked black soybean milk for 48 h incubation period supplemented with 1% MSG, 1% brown sugar, and 0.1% of peptone [19]. GABA yield from *Lb. fermentum* was 1.59-fold increase in soymilk under optimized conditions compared with soymilk under unoptimized conditions.

The data from the above single variable parameters were validated under RSM and ANN models. There is no significant error difference observed in both the models. Our results were in good agreement with *Lb. plantarum* Taj-Apsis362, which was isolated from honeycombs of honeybees. After single variable experiments, *Lb. plantarum* Taj-Apsis362 produced 1.67-fold high GABA in MRS media [38]. According to Song et al. [32], RSM results suggested that the medium culture at optimal conditions helps increase in GABA yield in milk of adzuki bean constituted with 0.2% pyridine, 2.27% MSG, and 1.44% galactose. RSM and ANN models of solid-state fermentation of GABA by *Monascus sanguineus* were studied by Dikshit and Tallapragada [16]. Shan et al. [9] reported that *Lb. plantarum* NDC75017 showed 1.92-fold enhanced GABA yield (314 mg/100 g) in yogurt under RSM optimized conditions (80 mM MSG, and 18 μM Pyridixylpyrophosphate at 36 °C) than that of unoptimized fermentation conditions. Maximum GABA production (7.2 g/L) was observed from *Lactococcus lactis* under RSM optimized conditions at 31.8 °C temperature, 7.1 pH, and 15 g/L MSG [18]. Meena et al. [39] studied RSM and ANN models on the growth characteristics of a mixed culture of *B. bifidium* and *Lb. acidophilus* to produce high cell biomass for commercial use.

5 Conclusion

From the previous literature survey, and with author knowledge, there is no report on GABA production by *Lactobacillus fermentum* isolated from sourdough. From this study, it was identified that *Lb. fermentum* is a strain

that is efficient for the production of GABA. Our study indicates the use of soybean milk is the best basal substrate for GABA production as compared with synthetic (chemical) media. *Lb. fermentum* produced 1.67-fold higher GABA yield that was observed under optimized conditions compared with unoptimized conditions in soymilk. Results of RSM and ANN models were similar to experimental data. For large scale production of GABA, coupling of statistical RSM model to computational ANN model may result in better yield. These favorable results showed that *Lb. fermentum* strain acts as a good starter culture for industrial purpose. Still, further study is required for using *Lb. fermentum* in pharmaceutical and food industries for development of efficient mass production of GABA.

Abbreviations

GABA: Gamma aminobutyric acid; RSM: Response surface methodology; ANN: Artificial neural network; ANOVA: Analysis of variance; MSG: Monosodium glutamate; CCD: Central composite design; CFU: Colony-forming unit; RMSE: Root mean square error; IP: Incubation period; TLC: Thin layer chromatography; LAB: Lactic acid bacteria; HPLC: High-pressure liquid chromatography; RNA: Ribonucleic acid; GAD: Glutamate decarboxylase; EFB: Empty fruit bunch; KB: Kilobase; BLAST: Basic local alignment search tool; NCBI: National center for biotechnology information; MRS: De Man Rogosa and Sharpe

Acknowledgements

Thanks to Dr. Veranna Gowda and Ms. Ankhita from Bangalore Biotechnology Innovation Center (BBC), Bangalore, Karnataka, India, for providing MS analysis facility.
Thanks to Diwakar Devupalli from Curl Analytics Bangalore, Karnataka, India, for suggestions for analysis of ANN data.
Thanks to Jain University for providing lab facility.

Authors' contributions

BR contributed toward lab work, interpretation of data, and has drafted the work. TP contributed to the research guidance and design of work. MS contributed to the research guidance, data analysis, and manuscript suggestions. All authors read and approved the final manuscript.

Funding

This research did not receive any specific grant from any funding agencies in the public, commercial, or not-for-profit sectors.

Availability of data and materials

All data provided in the manuscript is available upon request.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Non applicable.

Competing interests

Authors declare that they no competing interests.

Received: 13 December 2019 Accepted: 13 January 2021
Published online: 26 February 2021

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