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Statistical optimization of γ-aminobutyric acid production by response surface methodology and artificial neural network models using *Lactobacillus fermentum* isolated from palm wine

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ABSTRACT

The aim of this study was to screen and optimize gamma amino butyric acid (GABA) production by *Lactobacillus fermentum* (*Lb. fermentum*) in soybean milk and De Man- Rogosa Sharp (MRS) media. The GABA yield was confirmed by thin layer chromatography and quantitatively estimated by ninhydrine method. Response surface methodology (RSM), a statistical tool was applied for optimization of GABA yield. The accuracy of RSM predicted results were demonstrated by a non-statistical (hypothetical) model using Artificial Neural Network (ANN). On TLC plate GABA produced by *Lb. fermentum* showed same R_f value (0.54) compared with standard GABA. *Lb. fermentum* produced 6.94 g/L and 3.07 g/L of GABA in MRS media and soymilk respectively. After optimization there was 1.7 fold increase in the GABA yield in optimized conditions when compared to unoptimized soybean milk. In single variable optimization Monosodium glutamate (MSG), glucose and incubation period (IP) were found to favor for GABA production. Through RSM model the maximum predicted GABA yield (5.34 g/L) was observed with a concentration of 1.5% of MSG and 1.19% of glucose with 48 h of incubation. This is the first report on production of GABA in soybean milk by *Lb. fermentum* isolated from palm wine. Production of GABA by *Lb. fermentum* in soymilk as a basal substrate, is economical in comparison with synthetic media (MRS). This convincing results from this study could be a touchstone for exploring *Lb. fermentum* strain to obtain GABA enriched functional food for human consumption.

1. Introduction

Palm wine is a fermented sap obtained from *Borassus flabellifer* (Palmyra palms or Toddy palms) belonging to family *Arecaceae* which is widely distributed in India, Bangladesh, Cambodia, China South-Central, Sri Lanka, Thailand and Vietnam. These are very common in South India especially Andhra Pradesh, Telangana and Tamil Nadu. The sugary syrup also called as toddy, can be collected from young inflorescence either male or female. Fermented toddy is called arrack formed due to microbial fermentation. Okolie et al. (2013) reported 62.5% of LAB (Lactic acid bacteria) which was identified by 16s rRNA sequencing method. The predominant species are *Lactobacillus plantarum, Leuconostoc mesenteriodes sp. dextranicum, Leuconostoc lactis, Lactobacillus casei strain zhang, Lactobacillus sp* and *Pediococcus parvulus.* Lactic acid produced by lactic acid bacteria is the main cause for acidic condition in palm wine (Santiago-Urbnia and Ruiz-Teran, 2014). Many microorganisms such as fungi and bacteria are capable of producing GABA. But GABA production using lactic acid bacteria (LAB) is mainly focused due to their food grade nature and generally regarded as safe (GRAS).

γ-amino butyric acid (GABA) is a 4- carbon, non-protein amino acid widely distributed in prokaryotic and eukaryotic organisms. It acts as inhibitory neurotransmitter in brain and spinal cord in mammals and also has physiological functions such as antioxidant, antidiabetic, anti-carcinogenic, anti-inflammatory, hypolipidemic, diuretic and also used in treatment for stroke and as pain relief (Liao et al., 2013). GABA is formed by decarboxylation reaction of L-glutamic acid catalyzed by glutamate decarboxylase (GAD) enzyme (EC 4.1.1.15). Genes encoding for GAD enzyme are widely distributed in *Lactobacillus brevis*,

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Lactobacillus plantarum, *Lactobacillus fermentum*, *Lactobacillus reuteri*, *Streptococcus thermophilus*, *Lactococcus lactis subsp. cremoris*, *Lactococcus lactis subsp. lactis* and some *Bifidobacterium* species (Wu et al., 2016). Because of its beneficial effects in health, it has become a popular constituent of pharmaceuticals as well as nutraceuticals (Grewal and Khare, 2016). Now a days the current research is focused on biosynthesis of GABA due to its high catalytic activity, simple reaction procedure and environmental compatibility (Dhakal et al., 2012). Several attempts have been made to develop functional foods enriched with GABA such as fermented milk products, cheese, yoghurt, Kimchi, tempeh, brown rice, tea, wheat bran, lactic acid fermented foods etc (Dikshit and Tallapragada, 2015; Liao et al., 2013).

Soybean milk is a water extract from soybeans rich in unsaturated fatty acids, proteins mainly it contains all essential amino acids, oligosaccharides and iso-flavones which are required for biological fermentation (Valle et al., 2014). For improving the yield of GABA biologically, several nutritional and physical factors are optimized. Response surface methodology (RSM) and Artificial Neural Network (ANN) are the effective approach for screening and optimizing the fermentation conditions (Dikshit and Tallapragada, 2015). RSM is an intelligent model for understanding response and achieving optimized process conditions.

ANN has the ability to model any linear or nonlinear relationship between the input and output data points (Zheng et al., 2011). ANN models are designed in a way in which human brain process information. ANNs acquire their information by detecting the patterns and relationships in data and learn (or are trained) through experience, not from programming. ANN is one of the most effective ways to understand any nonlinear relationships in data, given the historical input and output (Zheng et al., 2011). ANN models mimic the way how neurons in the human brain processes the information. Hence the information they require to identify the underlying patterns is very huge, where people tend to use traditional statistical or current machine learning based approaches where no need to provide that much of data. These ANN models are widely used in economics, geology, hydrology, sociology etc. Recently these models are applied to food processing applications such as quality control, food freezing, thermal processing and drying applications (Zheng et al., 2013). It is a non-statistical machine learning computational model formed by several single units, artificial neurons, connected with coefficients (weights) which constitutes the neural structure called processing elements (PE). ANN network is an interconnected neurons organized in three layers, input, output and middle hidden layers, where input layer represents the data which is being fed and output layer represents the final expected output, and there can be any number of hidden layers where other than the input nodes, each node is a neuron that uses a nonlinear activation function (Ripley, 1996). The output data produced by ANN model have particular weights which are used as analytical tool for prediction of new set of input data (Agatonovic-Kustrin and Beresford, 2000).

The present study is focused on production of GABA from *Lb. fermentum* isolated from palm wine using soybean milk. Statistical and computational models were developed for optimizing of GABA yield. The results were authenticated statistically through RSM and computational analytical tool i.e ANN.

2. Materials and methods

2.1. Isolation of bacterial strains from palm wine

Ten palm wine samples were collected from various Toddy palm trees from different places of Vishakhapatnam, Andhra Pradesh, India. The samples were preserved in an ice box and transferred to lab. The samples were serially diluted and plated on Man-Rogosa Sharp (MRS) media and incubated at 37 °C for 48 h (Okolie et al., 2013). The pure cultures of the isolates were obtained on MRS media and screened for GABA production.

2.2. Screening of the isolates and conformation of GABA production by mass spectrometry analysis

To screen for the production of GABA by LAB, the strains were allowed to grow in MRS broth supplemented with 1% of MSG (mono sodium glutamate) and incubated. The cultures were centrifuged at 8000 g for 10 min and 5 µL of supernatant was spotted on thin layer chromatography (TLC) (Silica gel 60F254 aluminum sheets from Merck, Darmstadt, Germany) using n-butanol: acetic acid: water (5:3:2) as mobile phase. The presence of GABA compound in the sample was observed by red color spots on plate after addition of 0.2% of ninhydrine (Villegas et al., 2016). 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 flow rate was set to 1 mL/min and detected at 235 nm (Panatda et al., 2010).

2.3. Colorimetric estimation of GABA

The culture supernatants were spotted on TLC plates. The spots obtained on TLC were scraped and added to the reaction solution containing 3 mL of borate buffer and 0.5 mL of ninhydrine solution (2% w/v, dissolved in acetone). 3 mL of borate buffer without addition of sample was considered as blank. The analysis mixture was incubated in water bath at 80 ± 5 °C for 20 min. After color development the tubes were allowed to cool down and absorbance was read at 570 nm after addition of 50% ethanol (Dikshit and Tallapragada, 2015). Standard graph of GABA was obtained with 10 µg/mL to 10 mg/mL using the protocol as given by Hosseinimehr et al. (2010).

2.4. Preparation of soybean milk for GABA production

Soybean milk was prepared according to Ko et al. (2013) with some minor modifications. Soybeans (*Glycine max*) was procured from local market Bangalore, India. Moisture, ash, protein, fat and dry weight content was analyzed for soybeans using Association of Official Analytical chemists (AOAC, 1998) official methods. Washed and overnight soaked beans were cooked at 100 °C for 50 min. The cooked beans were homogenized and the milk was extracted through filtration using double layered cheese cloth. The milk was sterilized at 121 °C for 15 min and used as fermentation medium for bacterial strain.

2.5. Medium formulation for increasing GABA production using soybean milk as basal medium

2.5.1. Effect of various carbon and nitrogen sources

Different types of carbon sources like glucose, sucrose, maltose and lactose at 1% concentration was added to soybean milk. Nitrogen sources like beef extract, yeast extract, peptone and MSG at 1% was added to soybean milk and inoculated with 24 h old culture of *Lb. fermentum* with initial count of CFU/mL. Bacterial count, GABA concentration and pH was determined after fermentation for 48 h at 37 °C. Viable cell count in soybean milk was determined by colony forming unit (CFU)/mL by serial dilution and plating on MRS media. pH was determined by using pH meter.

2.5.2. Effect of initial medium pH and temperature

Soybean milk containing 1% of glucose and MSG with different ranges of pH 4, 5, 6 & 7 was inoculated with *Lb. fermentum* and incubated for 48 h at 37 °C. To determine the effect of temperature, soybean milk was incubated at various temperatures 25, 30, 35 & 40 °C supplemented with 1% of glucose and MSG. Bacterial count, GABA and pH was determined as above described. Soybean milk with neutral pH at 37 °C was considered as control.

2.5.3. Effect of inoculum volume and incubation period

Different volumes 1, 2, 3 & 4% of inoculum was inoculated in the

soybean milk supplemented with 1% of glucose and MSG and incubated for 48 h at 37 °C. To determine the effect of incubation period the culture media was incubated for 24, 48, 72 & 96 h. As described above for each parameter the bacterial count, pH and GABA concentration was estimated.

2.6. Experimental design by response surface methodology

A three variables set of 20 experiments was performed with each variable at five levels (Table 1). Glucose (X1), MSG (X2) and Incubation period (IP) (X3) were considered as independent variables and GABA as dependent variable. The relations of actual values, coded values, independent variables and responses were calculated according to the second order quadratic model (Table 2). MATLAB software was used to analyze the regression, graphical analysis of experimental data. The interaction of two variables on the response were examined in three dimensional (3D) contour plots.

2.7. Time course synthesis of GABA during fermentation

To determine the time course of GABA production, the optimized conditions were maintained in one set of soybean milk flask containing 1% of glucose, MSG and inoculum volume and incubated for 72 h at 37 °C. For every 4 h of regular time intervals, bacterial cell count, pH and GABA was estimated. The unoptimized soybean milk flask was considered as control.

2.8. Deep learning {Artificial Neural Network (ANN)}

ANNs are the mathematical model commonly called as multilayered perceptron (MLP). It is a feed forward ANN consisting of three or more layers of neurons, with the first layer representing the independent variable as inputs. Each of the neuron is connected to one or more hidden layers and finally connected to output layer i.e GABA yield. In this set up the data always flows in forward direction i.e from input to output and during training process the coefficients (weights) were get updated by the technique called back propagation. First the elementary neurons introduces the input data to hidden layers via weights (coefficients) and these are considered as connections between the two neurons. First they sum up the weighed inputs to neurons including bias as shown in the following equation (Eq (1)).

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

where w_i (i = 1, n) are the connection weights, n is called bias and x_i is the input parameter.

The number of neurons required in the hidden layer was determined by trial and error to minimize the deviation of predictions from experimental results. The weighted output is then passed through an activation function. The activation function shifts the space in nonlinearity of input data. The logistic output function is used in this work, shown by the following equation (Eq (2)).

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

A total of 14 (70%) of experimental results were used to train the network, with the remaining results split evenly between network validation and testing data (Pilkington et al., 2014). Training an ANN is an iterative process where this pre-specified error function is minimized by adjusting the weights appropriately. The commonly used error function root mean square error (RMSE) in this work is defined as equation (Eq (3)).

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

where N refers to the number of patterns used in the training; M denotes the number output nodes; i denotes the index of the input pattern (vector) and yi and byi are the desired (target) and predicted outputs of the nth output node, respectively.

Table 1
Experimental range and levels of independent variables.

		Actual factor level at coded factor levels at				
Variables with designate	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 with codes for GABA yield in fermented soya milk.

S. No	Glucose (X1)	MSG (X2)	IP (X3)	Experimental Values	RSM Predicted values	ANN predicted values
				GABA (g/L)	GABA (g/L)	GABA (g/L)
1	−1	−1	−1	2.51	2.3766	2.4177
2	1	−1	−1	2.47	2.4966	2.2946
3	−1	1	−1	2.73	2.5041	2.4188
4	1	1	−1	4.05	3.4541	3.8651
5	−1	−1	1	2.09	2.1041	2.1203
6	1	−1	1	1.96	1.6041	1.9261
7	−1	1	1	4.37	3.7616	4.0518
8	1	1	1	4.54	4.0916	4.3781
9	−2	0	0	1.77	1.9559	2.0321
10	2	0	0	2.01	2.4059	2.4151
11	0	−2	0	2.3	2.2334	2.2912
12	0	2	0	4.2	4.8484	4.7022
13	0	0	−2	1.47	1.6434	1.8765
14	0	0	2	1.6	2.0084	2.6092
15	0	0	0	4.11	3.6486	2.2576
16	0	0	0	3.09	3.6486	3.0137
17	0	0	0	3.66	3.6486	3.6371
18	0	0	0	3.96	3.6486	3.0137
19	0	0	0	3.13	3.6486	3.0137
20	0	0	0	3.36	3.6486	3.4771

3. Results

3.1. Isolation of bacterial strain from palm wine

After 72 h of incubation many colonies were observed on MRS media. Most predominant 14 colonies were isolated and identified morphologically and biochemically. Morphologically the colonies showed gram positive, rod shaped, non-sporing bacteria. The cultures were Indole, MR-VP and catalase negative. Further these colonies were screened for GABA production.

3.2. Screening of GABA production by bacterial strains using soybean milk as culture media

The bacterial strains were screened for GABA production by observing the red color spots on TLC plate (Fig. 1). Among all fourteen bacterial strains for GABA production, three strains showed same retention value (R_f value = 0.54) with standard GABA. Quantitatively GABA production by bacterial strains were estimated by calorimetrically using ninhydrine method. The quantity of GABA produced by bacterial strains ranged from 0.26 to 3.07 g/L. Among three strains, W1 strain produced maximum GABA of 3.07 g/L in soybean milk. The bacterial strain W1 was identified as *Lb. fermentum* using 16s rRNA sequencing method and the NCBI accession No is **MF611624**. Conformation of

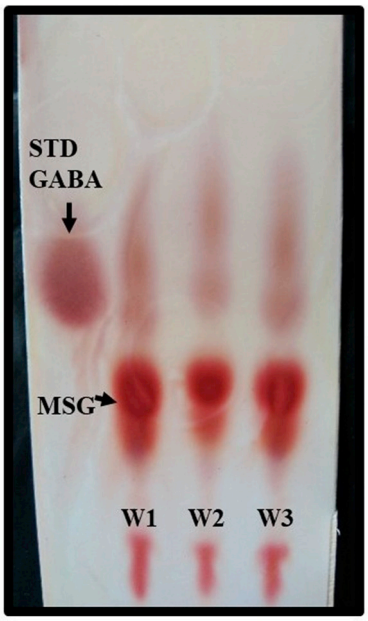

Fig. 1. TLC plate with red color spots conforming the presence of GABA. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

GABA was detected by mass spectral analysis. GABA has an elemental composition $C_4H_9NO_2$ (parent ion M^+ 103) and this was analyzed by liquid chromatography-MS results revealed the *m/z* for GABA to be 104. The calculated value of mass by charge for GABA ($M^+ + H^+$) was 104.95 (Fig. 2).

3.3. Determination of the optimum culture conditions for GABA production in soybean milk by Lb. fermentum

Fig. 3 (A-F) shows the effect of carbon, nitrogen, temperature, pH, incubation period and inoculum volume on GABA production. Based on the single variable test among sucrose, maltose and lactose, *Lb. fermentum* yielded maximum GABA (4.56 g/L) in soybean milk supplemented with glucose. Low yield of GABA (3.89 g/L) was observed at 0.5% of glucose. There is a significant increase in GABA yield (4.86 g/L) and cell count (7.3 CFU/mL) at 1% of glucose by *Lb. fermentum*. In soybean milk the GABA yield was decreased as concentration of glucose was increased from 1 to 1.5% (4.2 g/L) and 2% (0.77 g/L). Reduction in cell count was observed with increasing the concentration of glucose and the pH (4.4) was constant. Increased concentration of GABA 4.45 g/L was observed by *Lb. fermentum* in soybean milk supplemented with 1% of MSG. Even maximum cell growth (7.6 CFU/mL) was observed at 1% of MSG. In soybean milk by increasing the concentration of MSG from 1 to 4%, reduction in GABA (4.14 g/L) yield and cell growth (4.8 CFU/mL) was observed. Constant pH (4.4) was observed in every MSG concentration. It was apparent that extra high concentration of MSG and glucose was harmful to growth of the strain and leads to decrease in GABA yield.

The optimum temperature, pH and inoculum volume for the GABA production in soybean milk by *Lb. fermentum* is at 37 °C, pH 5, 1% inoculum volume (v/v) and incubation period of 48 h. Maximum GABA yield (3.95 g/L) and bacterial growth was observed at 37 °C temperature. Lower concentrations of GABA and bacterial population was observed at below and above 37 °C temperatures. Highest GABA was observed at pH 5 when compared with different ranges of initial pH (4–7) in the soybean milk. No growth and production of GABA was observed by *Lb. fermentum* at pH 4.1% of bacterial culture is the significant volume for the maximum yield of GABA. Further increase in inoculum concentration to till 4%, no significant increase in GABA concentration in soybean milk but resulted in lower concentration of

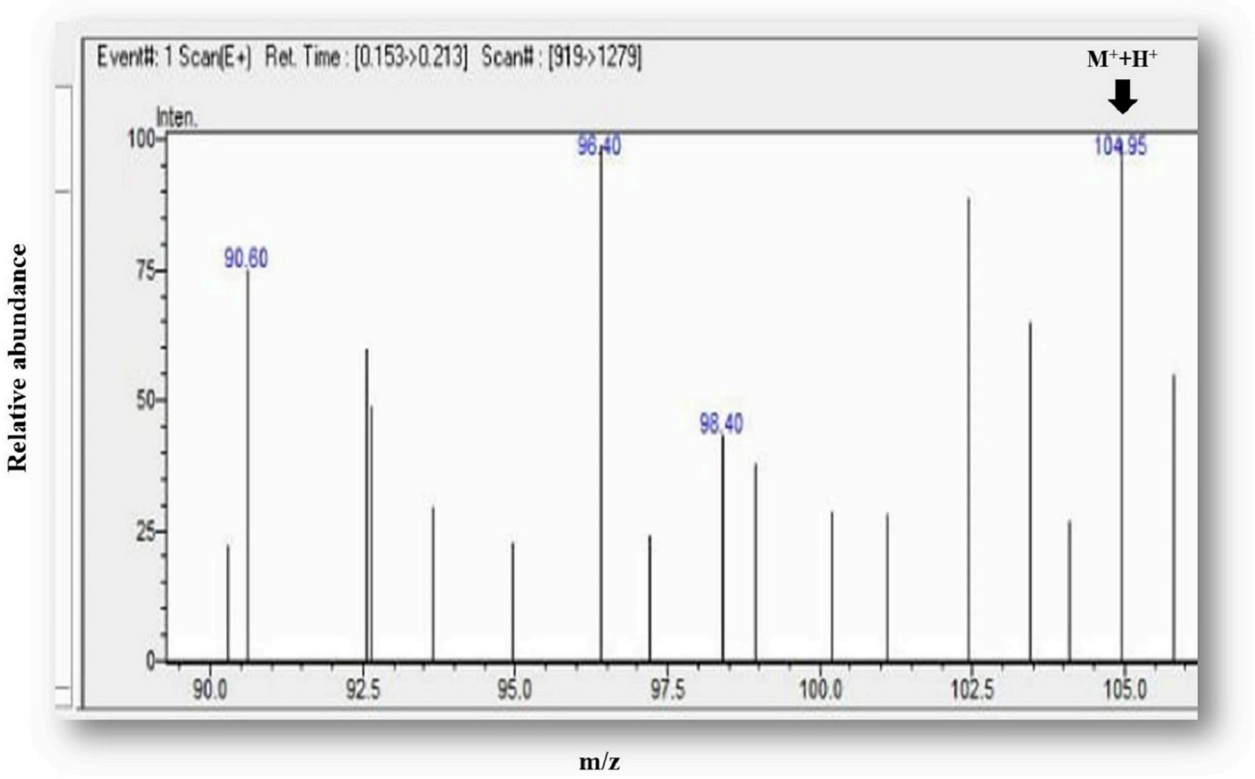

Fig. 2. Chromatogram for mass spectral analysis (m/z) for conformation of GABA.

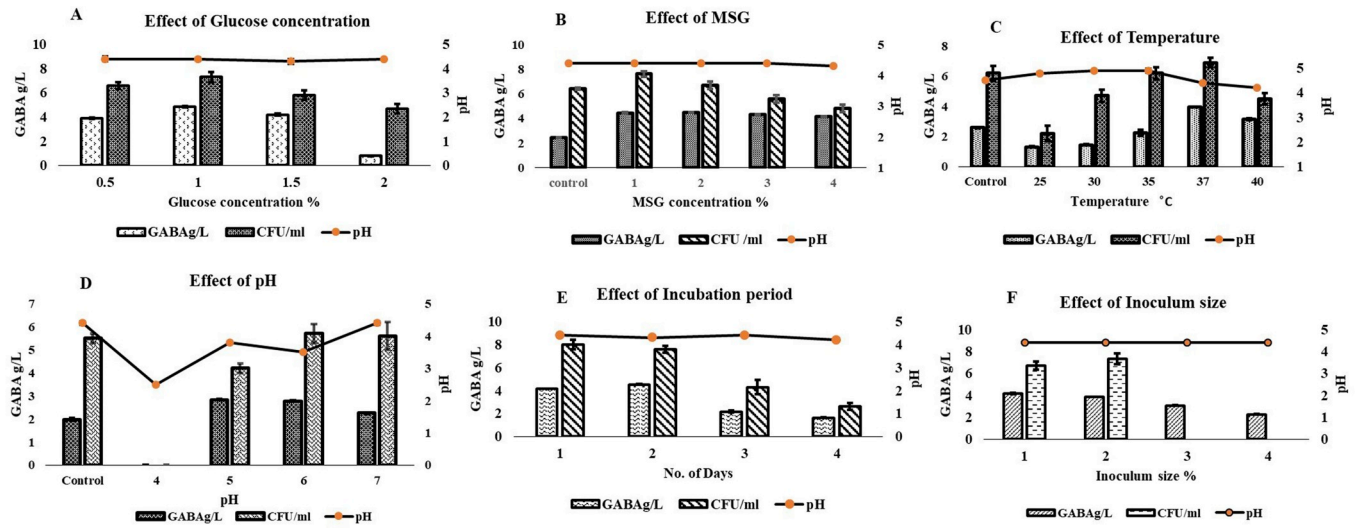

Fig. 3. (A–F) Changes in GABA concentration (g/L), bacterial cell viability (CFU/ml) and pH in fermented soya milk containing different glucose concentration (A), MSG concentration (B), temperature (C), pH (D), incubation period (E) and inoculum volume (F). The data was expressed in mean ± SD from triplicate experiments. Different letters on the top of the bars indicate significant difference ($p < 0.05$).

GABA (2.27 g/L) than that of 1% of inoculum (v/v). From the above study it was clear that appropriate bacterial growth is required for the GABA production. Incubation period (IP) strongly effects the growth of *Lb. fermentum* and GABA yield in the soybean milk. Maximum GABA (4.53 g/L) was observed after 48 h of incubation of *Lb. fermentum* in soybean milk. High bacterial population was observed in 24 h compared with 48 h of incubation. As increased in the incubation period to till 4 days, reduction in bacterial growth (2.6 CFU/mL) and GABA (1.6 g/L) yield was recorded.

3.4. Response surface methodology (RSM)

Based on the single variable factor test, three variables was applied in CCD (central composite design) Glucose concentration, MSG concentration and incubation period. Equation (4) explains the interaction effect of three independent variables (glucose, MSG, incubation period) for GABA yield by *Lb. fermentum* can be expressed as:

$$GABA\ yield\ \left(\frac{g}{L}\right) = -6414.09 + 11351.82 \times X1 - 5963.18 \times X2 + 235.56 \times X3 + 3320.0 \times X1 \times X2 - 51.67 \times X1 \times X3 + 127.5 \times X2 \times X3 - 5870.91 \times X_1^2 - 430.91 \times X_2^2 - 3.16 \times X_3^2 \quad \text{Eq. 4}$$

Table 2 shows the 30 various combination sets produced and the corresponding GABA yield for both experimental and from model (actual and predicted values). To study the interaction effect of variables on GABA yield (g/L) by plotting the 3D curves against two independent variables and keeping the third variable at its central level (0). Fig. 4 shows the dependency of GABA yield (g/L) on glucose and MSG, keeping the incubation period at its central level of 48 h. The graph predicted that low glucose concentration the GABA yield decreased with the increase in MSG concentration. Similarly for low MSG concentration the GABA yield first slightly increased and then sharply decreased with the increase in glucose concentration. The maximum GABA Yield (5.34 g/L) was observed with maximum MSG concentration (1.5% w/v) and mid glucose concentration (1.19% w/v).

The 3D plot depicting the interaction effects of glucose concentration and incubation period, keeping the MSG concentration at its central (0) level 1% w/v, had a typical bell curve was obtained which showed the peak yield at the centre of the plot (Fig. 5). The low GABA yield was

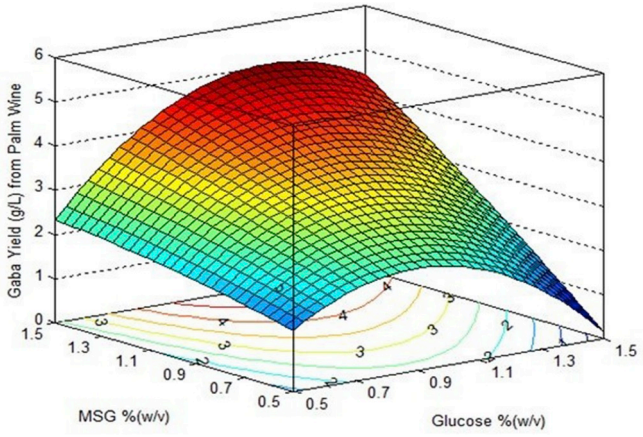

Fig. 4. 3D plot and contour plot for interaction of MSG and glucose, keeping incubation period at its central level.

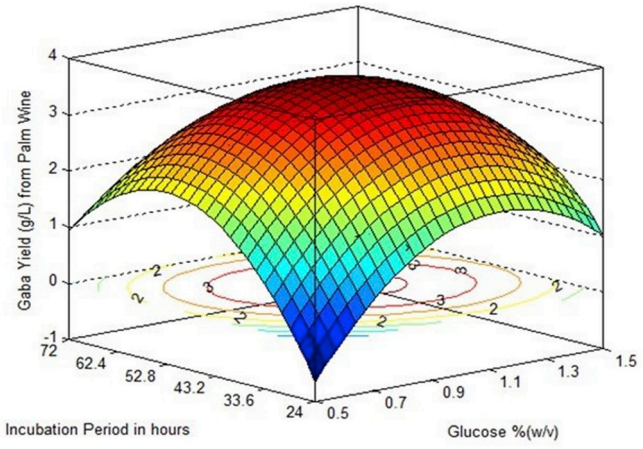

Fig. 5. 3D plot and contour plot showing the interaction of incubation period and glucose keeping MSG at its central level.

observed with lowest concentration of glucose and incubation period the yield being depicted as theoretically negative. With higher incubation period as well as glucose concentration also the yield was less as compared to the mid region. The maximum GABA Yield of 5.34 g/L was observed at the centre of the cube with glucose concentration of 1.05% (w/v) and incubation period of 48.83 h. Fig. 6 shows the interaction effects of MSG concentration and incubation period, keeping the glucose concentration at its central level (0) 1% w/v. Here the GABA yield at lower MSG concentration increased moderately with incubation period till some extent and then decreased sharply reaching to a theoretically negative value. The GABA yield however remained almost constant with increase in MSG concentration at lower incubation period. The maximum GABA Yield (5.25 g/L) was obtained at mid incubation period (58.8 h) and maximum MSG concentration (1.5% (w/v).

Table 3 shows the regression results from the data of the CCD experiments. Greater the magnitude of *t*-value and less *p*-value indicates the more significant corresponding coefficient (Subhagar et al., 2010). This indicates that the variables with most effect was the quadratic effect of glucose and incubation period. There is no significant difference was observed between glucose and incubation period. Furthermore, the interactive effect of MSG and incubation period, as well as the concentration of MSG was highly significant. This was followed by the quadratic effect of glucose, although the implication was adverse, as seen from the negative *t* value. In summary, the MSG and incubation period plays an important role in determining the GABA yield.

From the ANOVA (Table 4), the value of determination coefficient (R^2) was 0.8492, indicating that the experimental data fitted well to this model, which explains that 84.92% of the total variation in the observed response value could be explained by model or experimental parameter and their interactions. A small remainder (15.08%) of the total variations was not explained by the model (Table 4). The R^2_{adj} was 0.7136 which is close to R^2, conforming the model was highly significant. The coefficients estimates in the regression model for GABA synthesis has been presented in Table 3. Finally the coefficient was estimated by considering independent variables, quadratic term and interaction term.

Lb. fermentum was tested for time course of GABA production by maintaining all optimized conditions in soybean milk. Fig. 7 depicts that from 0–4th there was no increase in the bacterial growth and no change in pH. An exponential growth of *Lb. fermentum* observed from 8 to 24 h (7.1 CFU/mL) reached to log phase. The pH was dropped from 5 to 4.4 and the production of GABA (1.88 g/L) was observed from 24 h onwards. From 24 to 48 h there is no significant change in the bacterial growth was observed. Highest GABA production (5.24 g/L) was observed in 36–48 h in optimized soybean milk compared with unoptimized soybean milk (2.84 g/L). Decline phase was observed by reduction in bacterial growth (3.63 g/L) from 60 to 72 h. There was 1.7 fold increase in GABA production was observed in optimized soybean milk compared with unoptimized soybean milk.

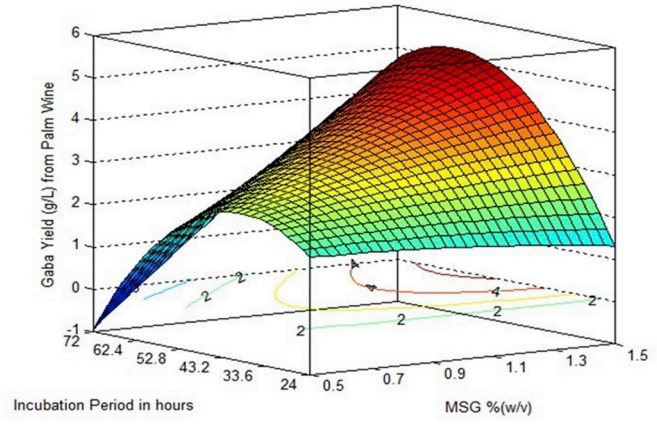

Fig. 6. 3D plot and contour plot showing the interaction of incubation period and MSG keeping glucose at its central level.

Table 3
The regression co-efficient results from the data of CCD for GABA production.

Component	Co-efficients	standard error	t-value	p-value
Constant	−6414.0909	6.3470	−1.0106	0.33605
Glucose (g)	11351.8182	5.5469	2.0465	0.06790
MSG (g)	−5963.1818	5.5469	−1.0751	0.30760
IP (days)	235.5587	0.1156	2.0384	0.06883
Glucose × MSG	3320.0000	3.0516	1.0880	0.30214
Glucose × IP	−51.6667	0.0636	−0.8127	0.43532
MSG × IP	127.5000	0.0636	2.0055	0.07272
Glucose × Glucose	−5870.9091	1.7213	−3.4107	0.00665
MSG × MSG	−430.9091	1.7213	−0.2503	0.80740
IP × IP	−3.1645	0.0007	−4.2356	0.00173

Note; MSG = Monosodium glutamate; IP = Incubation period.

Table 4
ANOVA (Analysis of variance) for response surface quadratic model for GABA production.

Sum of squares	Degree of freedom	*f*-value	*p*-value	Mean square error	R^2	Adj. R^2
2.9101	9	6.2591	0.00417	0.2910	0.8492	0.7136

3.5. Deep learning (ANN)

The accuracy of the RSM model is authenticated by comparing with ANN model. The feed forward back propagation neural network model illustrated in Fig. 8 was used to substantiate the results obtained by the RSM model. In ANN model the data was trained, tested and validated. The predicted values from ANN model was observed in Table 2. In ANN model while learning the data, error loss for variable and tested data was decreased and constant at 150 epoch (Fig. 9). Fig. 8 shows the final stage where the training of data was completed and reached the optimal solution as shown in the figure, where all orange dots are on one side and blue color dots are another side. In this stage all the individual weights for all inputs and intermediate hidden layers are saved for future purpose. Optimization of the weights is made by backward propagation of the error during training or learning phase. Linear fit results for the test data was observed in Fig. 10. The R^2 values for tested data from both RSM (0.18633) and ANN (0.16674) was almost same. The experimental data was well fitted to both RSM and ANN models. Fig. 11 shows the comparative plot for experimental values and predicted values from RSM and ANN models for GABA yield. The closeness of the RSM prediction to the ANN substantiates the accuracy of the RSM model.

4. Discussion

This is the first report on isolation and production of GABA using *Lb. fermentum* isolated from palm wine. Okolie et al. (2013) reported that the predominant microflora in palm wine was *Lb. plantarum*, *Leuconostoc mesenteroides* sp. *dextranicum, Leuconostoc lactis*, *Lb. casei* strain *zhang*, *Lactobacillus sp*. and *Pediococcus parvulus* identified by 16s rRNA sequencing method and are responsible for fermentation of palm wine. Physical, biochemical, nutritional and microbiological properties (viscosity, pH, total acidity and microflora) has been studied on two varieties of palm wine extracted from *Dura* and *Tenera* (Karamoko et al., 2016). Lin et al. (2017) isolated *Lb. fermentum* isolated from Chinese traditional pickled vegetable was used as source of GAB gene (gad B) and this gene was cloned and expressed in *Escherichia coli*. Estimation of GABA concentration in the sample through HPLC is quite incommodious and expensive. Colorimetric estimation using ninhydrine reagent is very precise, simple and cost effective method. GABA exists as zwitterions

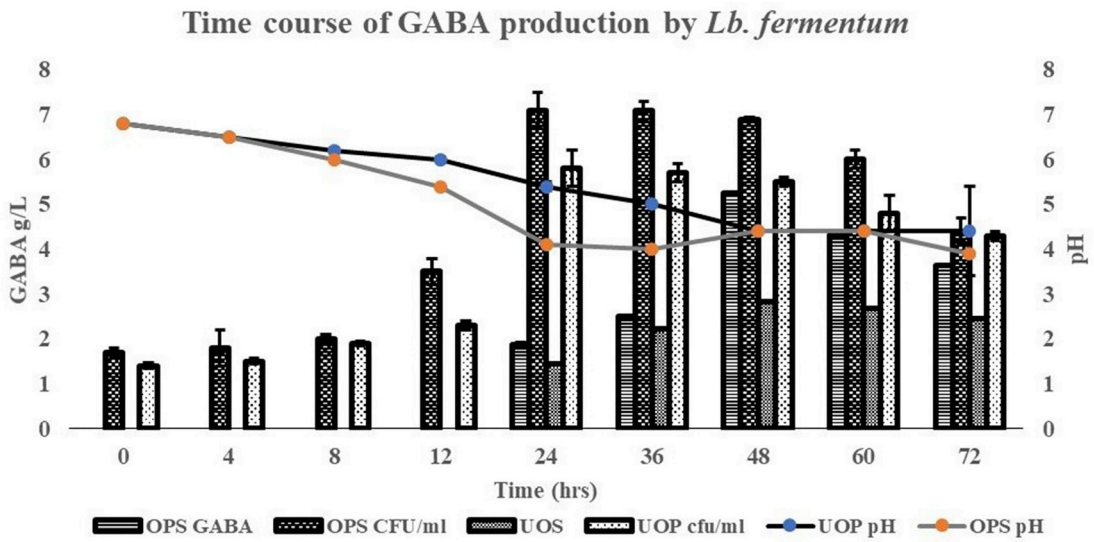

Fig. 7. Changes in *Lactobacillus fermentum* count, pH and GABA concentration during soya milk fermentation. OPS GABA - GABA in optimized soya milk; OPS – Bacterial count in optimized soya milk; OPS pH – pH in Optimized soya milk; UOS – GABA in unoptimized soya milk; UOP CFU/ml – Bacterial count in unoptimized soya milk; UOP pH – pH in unoptimized soya milk. Data expressed in mean ± SD from triplicate experiments.

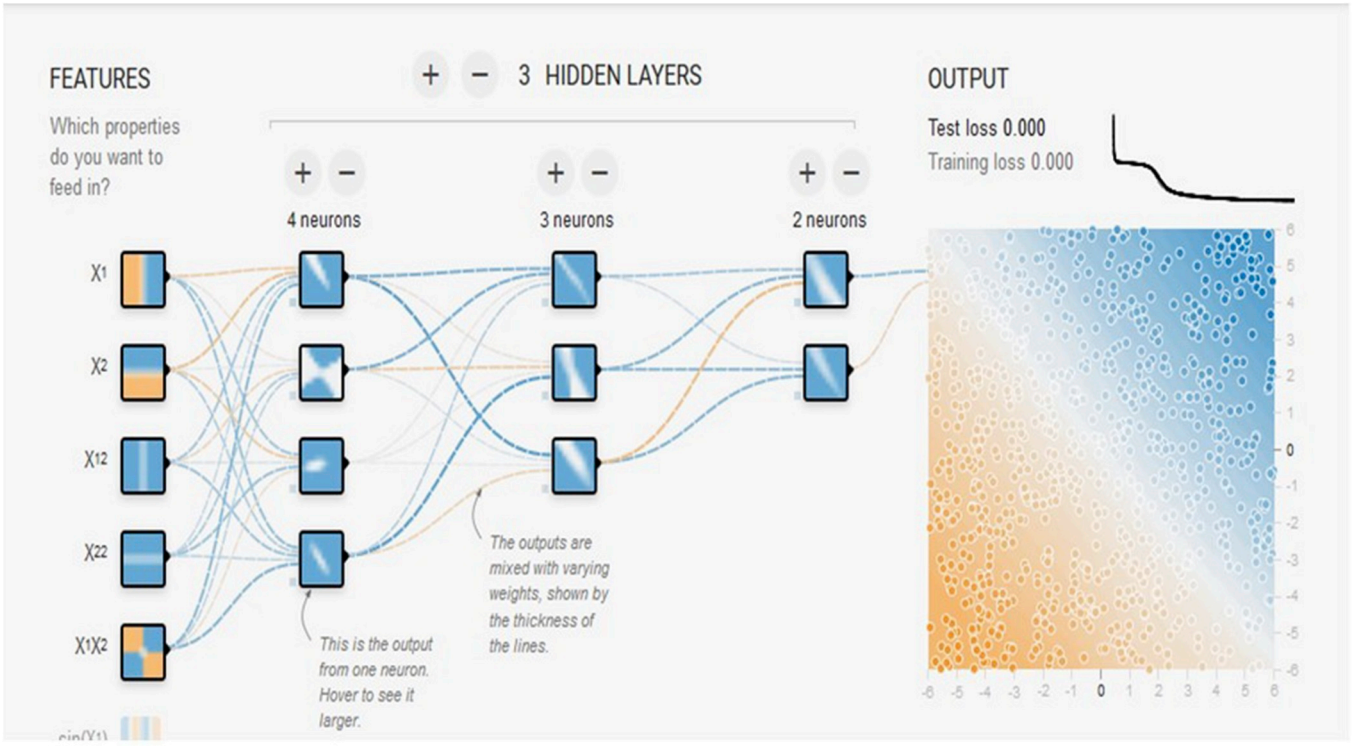

Fig. 8. Flow chart of feedforward back propagation of data in ANN model.

with a de-porotonated carboxyl group and protonated amino group. Ninhydrin reacts with amino group of GABA compound and forms Ruhemann's purple compound. The same protocol was applied by Hosseinimehr et al. (2010) and Bali and Guar (2011) for the estimation of baclofen and pregabalin which are the analogs for GABA.

Ko et al. (2013) observed the production of GABA (5.42 mg/mL) was observed by *Lactobacillus brevis* FPA 3709 using cooked black soybean milk. From the above results it is depicted that glucose concentration effects the GABA yield and growth of the *Lb. fermentum*. Similar results has been reported by Li et al. (2009) says that *Lactobacillus brevis* NCL912 produced higher GABA yielded (198 mM) at 2.5% of glucose. Cho et al. (2007) reported that *Lactobacillus buchneri* isolated from Kimchi yielded higher GABA (234 mM) by using 1% of glucose as carbon source. Baritugo et al. (2018) reported engineered *Corynebacterium glutamicum* H36GD1852 strain produced 35.47 g/L GABA from 90 g/L glucose and 10 g/L xylose in the EFB solution. The metabolic path way for the production of GABA is from Krebs cycle *via* glutamate. Malic acid from krebs cycle is converted into succinic acid, succinic semi-aldehyde and the end product is GABA. The enzymes involved in this GABA shunt is succinate dehydrogenase, succinate semialdehyde dehydrogenase and

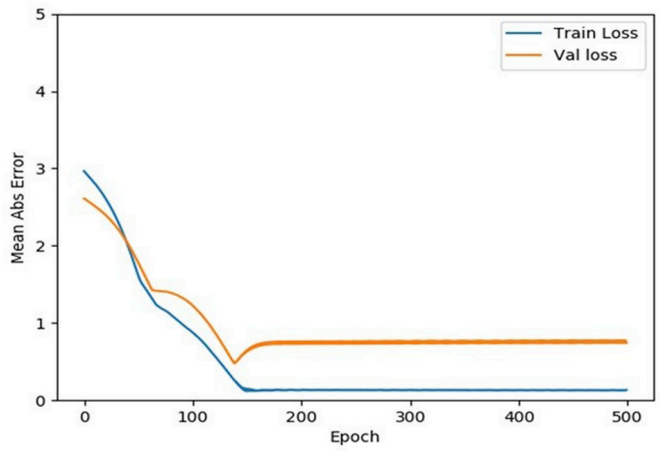

Fig. 9. Error loss as observed at 150 epoch in ANN.

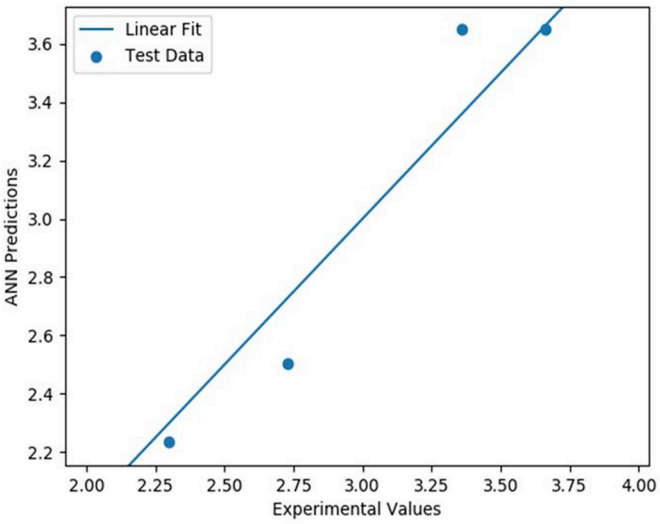

Fig. 10. Linear fit for the results from ANN model.

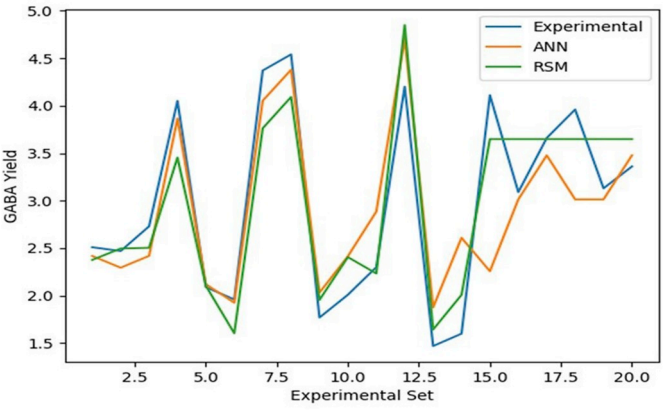

Fig. 11. Comparison of GABA yield obtained from experimental, RSM and ANN.

GABA aminotranseferase. These enzymes form domain-ligand interaction through the introduction of synthetic scaffolds (Pham et al., 2016). Glucose and MSG concentrations strongly influenced the GABA yield. No significant difference in GABA production was observed by *Lb. fermentum* in soybean milk supplemented with 1 and 2% of MSG. Ko et al. (2013) reported that *Lactobacillus brevis* FPA 3709 isolated from fish intestine could produce highest GABA (5.42 mg/mL) at 1% of MSG, brown sugar and 0.5% of peptone as the basal substrates in cooked black soybean bean milk. Similar results were reported by Lu et al. (2009) where *Lactococcus lactis* subsp. *lactis* isolated from Kimchi produced highest GABA 3.86 g/L in MRS broth supplemented with 1% MSG. Monosodium glutamate dissociates into Na^+ ions and glutamate. GABA is synthesized by decarboxylation of glutamate to GABA catalyzed by glutamate decarboxylase (GAD) and pyridoxyl pyrophosphate (PLP) as a cofactor. High concentration of glutamate activates GABA-Transaminase (GABA-T) leading to degradation of GABA and formation of succinic semi-aldehyde (SSA) by transamination of glutamate and α-ketoglutarate (Rowley et al., 2012). Temperature effects the bacterial growth and GABA production. In soybean milk *Lb. fermentum* could produces maximum GABA and bacterial growth was observed at 37 °C temperature. An elevated temperature increased the rate of reaction and influenced the final GABA yield. Higher the temperatures causes to enzyme inactivation and cell aging, leads to reduction in GABA yield. Similar results were reported by Li et al. (2016) where maximum GABA is produced by *Lactobacillus plantarum* at 37 °C in fermented chick pea milk. Higher or lower initial pH in media leads to inactivation of GAD enzyme and leads to decrease in GABA concentration. The results are in agreement with Li et al. (2010) where reported *Lactobacillus brevis* NCL912 produced maximum GABA at 35 °C temperature and pH 5. Bacterial strain utilizes the sugar molecules and releases low weight organic acids leads to increase in the acidic environment in the cell. To withstand the acidic stress the bacterial cell switches to GAD system and produce GABA by consuming the glutamate and one H^+ ions from the medium, resulting in reduction of acidic conditions (Li et al., 2010).

From the above results it is depicted that GABA production is not associated with growth of the *Lb. fermentum*. Maximum GABA production is observed in stationary phase of the *Lb. fermentum*. GABA was shown to be produced by LAB fermentation from many kinds of legume, including black soybean milk and lentil (Ko et al., 2013; Torino et al., 2013). The bacterial population in our study was similar to that of *Enterococcus faecium* strains, which had a cell density ranging from 8.06 to 8.96 CFU/mL after 16 h of fermentation in soybean milk (Crintina et al., 2012). Thus *Lb. fermentum* from wine is able to grow in soybean milk and can be used as good starter culture. GABA production in our results were in agreement with Li et al. (2016) reported *Lactobacillus plantarum* M-6 could produce maximum GABA of 537.23 mg/L at 48 h of fermentation in chick pea milk. In soybean milk fermentation by *Lb. fermentation*, the pH was dropped from 5 to 4.1. The reduction of pH in fermentation medium is due to production of low molecular weight organic acids produced by lactic acid bacteria (Schindler et al., 2012).

According to RSM results, the optimal cultural conditions for maximum GABA production (5.34 g/L) is 1% of MSG concentration and 1.19% of glucose concentration with 48 h of incubation. Supplementation of MSG to soybean milk fermentation increases the GABA production. Lu et al. (2008) reported that *Lactococcus lactis* subsp. *lactis* B produced maximum GABA (6.41 g/L) using brown rice juice, germinated soybean bean juice, skim milk in the ratio 33:58:9 (v: v: v). Similar results were reported by Tung et al. (2011) from RSM results *Lactobacillus plantarum* NTU 102 produced maximum GABA in fermented skim milk supplemented with 1% MSG. Soybeans are rich in proteins and amino acids. Glutamic acid is produced due to hydrolytic action during fermentation which is used as substrate for GABA production (Kim and Ji, 2015). Li et al. (2010) reported that from the plot study maximum GABA (349.69 mM) was produced with glucose, soy peptone, $MgSO_4$ (55.25, 30.25, 0.0061 g/L) and tween 80 (1.38 mL/L) respectively. In the present study the RSM predictions shows closeness to ANN model, which validates the accuracy of RSM model. The ANN model reads the input and output data points in the training data set and changes the value of the weighted links to reduce the difference between the predicted and target values. The error in prediction is minimized across

many training cycles until network reaches specified level of accuracy. This is the first report studying the RSM and ANN model for GABA production by *Lb. fermentum* in soybean milk. Dikshit and Tallapragada (2015) studied both RSM and ANN models for GABA production by *Monascus sanguineus* under solid state fermentation. Similarly Pathak et al. (2015) studied on both artificial intelligence and statistical modeling for optimizing the production of cholesterol oxidase (COD) by *Streptomyces Sp.* using soybean meal. Bajpai et al. (2015) reported that ANN-GA method is more suitable method for the production of co-enzyme Q10 in fermentation media using carrot juice as a precursor by *Pseudomonas diminuta* NCIM 2865 when compared to RSM based optimization method.

5. Conclusion

Lactobacillus sp have been primarily exploited for GABA production from different sources. To the author's knowledge this is the first report on production of GABA in soybean milk by *Lb. fermentum* isolated from palm wine. From the above results it is stated that *Lb. fermentum* can be a potential starter culture for the production of GABA. In this study, the soybean milk is considered as alternate economic basal substrate for GABA production instead of synthetic media. This statistical approach for optimizing the nutritional and physical parameters to enhance the GABA production and the results were authenticated by computation approach using ANN model was less studied. This encouraging outcome from this study indicates that this method can be applied for efficient GABA production from this experimental strains to be used in pharmaceutical and food industries.

6. Statistical analysis

Each experiment was carried out in triplicates and the mean values were calculated. ANOVA (Analysis of Variance) was used to determine the significant difference among means ($p < 0.05$) using SPSS software.

Declaration of competing interest

Authors declare that there is no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bcab.2019.101362.

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