

Medium optimization for production of gamma-aminobutyric acid by *Lactobacillus brevis* NCL912

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Abstract Production of gamma-aminobutyric acid (GABA) was carried out in Erlenmeyer flasks by *Lactobacillus brevis* NCL912. Traditional methods were first adopted to select the key factors that impact the GABA production to preliminarily determine the suitable concentration ranges of the key factors. It was found that glucose, soya peptone, Tween-80 and $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ were the key factors affecting GABA production. Then, response surface methodology was applied to analyze the optimum contents of the four key factors in the medium, and the production of GABA was predicted as 349.69 mM under the optimized conditions with this model. Afterward, the experiment was performed under the optimized conditions, and the yield of GABA reached 345.83 mM, which was 130% higher than the initial medium. The results showed that experimental yield and predicted values of GABA yield were in good agreement.

Keywords Gamma-aminobutyric acid · *Lactobacillus brevis* NCL912 · Medium optimization · Response surface methodology

Introduction

Gamma-aminobutyric acid (GABA) is a non-protein amino acid that is widely distributed in nature (Manyam et al. 1981). It is the major inhibitory neurotransmitter in the mammalian brain. GABA has several well-known physiological functions, such as neurotransmission, induction of

hypotensive, diuretic effects, and tranquilizer effects (Jakobs et al. 1993; Wong et al. 2003). GABA is also involved in the regulation of cardiovascular functions, such as blood pressure and heart rate, and plays a role in the sensations of pain and anxiety (Mody et al. 1994). A recent study showed that GABA is a strong secretagogue of insulin from the pancreas (Adeghate and Ponery 2002) and effectively prevents diabetic conditions (Hagiwara et al. 2004).

Gamma-aminobutyric acid can be produced by microorganisms including bacteria (Maras et al. 1992; Smith et al. 1992), fungi (Kono and Himeno 2000) and yeasts (Hao and Schmit 1993). In recent years, many studies have focused on the GABA-producing lactic acid bacteria (LAB) because LAB possess special physiological activities and commercial potential as starters in fermented foods, such as dairy products, bread, fermented vegetables, meats and fish, etc. Some fermented food such as yogurt and cheese containing GABA (Nomura et al. 1998; Park and Oh 2007; Skeie and Ardo 2000) have been developed with GABA-producing LAB as starters.

Several GABA-producing LAB species have been reported in recent decades, including *Lactobacillus brevis* (Huang et al. 2007b; Li et al. 2008; Park and Oh 2007; Siragusa et al. 2007; Ueno et al. 1997; Yokoyama et al. 2002), *Lactococcus lactis* (Nomura et al. 1998; Siragusa et al. 2007), *Lactobacillus paracasei* (Komatsuzaki et al. 2005; Siragusa et al. 2007), *Lactobacillus delbrueckii* subsp. *bulgaricus* (Siragusa et al. 2007), *Lactobacillus buchneri* (Park and Oh 2006; Cho et al. 2007) and *Lactobacillus plantarum* (Siragusa et al. 2007). Glutamate decarboxylase (GAD; EC 4.1.1.15) is the key enzyme for the bioconversion of GABA. GAD has been isolated from several LAB and its biochemical properties have been characterized (Huang et al. 2007a; Komatsuzaki et al. 2008; Nomura et al. 1998; Ueno et al. 1997).

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Gamma-aminobutyric acid-producing ability of LAB widely varies among the strains (Huang et al. 2007b; Komatsuzaki et al. 2005; Park and Oh 2007; Yokoyama et al. 2002) and is affected significantly by culture conditions, such as medium. It is important to optimize the medium for enhancing GABA production during fermentation. Traditional optimizing methods involve changing one independent variable while fixing the others at given levels. This single-dimensional search technique is simple, but often fails to yield optimized conditions because it does not consider possible interactions among factors. Response surface methodology (RSM) is an efficient experimental strategy to seek optimal conditions for multivariable system. RSM has been successfully employed for optimizing the medium composition (Lee and Chen 1997). Based on the key factors influencing the GABA production opted with one independent variable, RSM was applied to optimize the factors of medium for enhancing the GABA production.

Materials and methods

Strain, media and culture conditions

A GABA-producing LAB strain *Lb. brevis* NCL912 was isolated from paocai, a Chinese traditional fermented vegetable (Li et al. 2008). The initial medium used for GABA production was composed of (g L⁻¹): glucose, 25; yeast extract, 6.25; soya peptone, 6.25; MgSO₄·7H₂O, 0.2; MnSO₄·4H₂O, 0.05; sodium L-glutamate, 500 mM; and Tween 80, 2 mL L⁻¹. The pH was adjusted to 5.0 before autoclaving. Nitrogen sources, sodium L-glutamate and the other compositions were autoclaved separately at 121°C for 20 min and mixed together prior to inoculation. Unless otherwise stated, the strain was kept in static cultivation at 34°C for 48 h in 250-mL flasks containing 100-mL medium.

GABA assay

The GABA concentrations in the culture broths were determined by pre-staining paper chromatography (Li et al. 2009).

Response surface methodology

Box–Behnken design using three coded levels was employed for determining the optimal conditions of the four screened factors in the single-factor experiments. The behavior of the system was explained by the following quadratic equation:

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ij} X_i X_j + \sum \beta_{ii} X_i^2$$

where Y is the predicted response, β_0 the offset term, β_i the linear offset, β_{ii} the squared offset, β_{ij} the interaction effect, and x_i is the dimensionless coded value of X_i . Various assemblies used in the design are shown in Table 1.

Results and discussion

Evaluation of the key factors

Effects of carbon sources on GABA production

In a previous work, we found that the carbohydrates that could be fermented by *Lb. brevis* NCL912 were L-arabinose, ribose, D-xylose, galactose, glucose, fructose, maltose, melibiose, α -methyl D-glucoside, N-acetyl D-glucosamine and gluconate (Li et al. 2008). From these carbohydrates, only glucose and maltose were tested in the present study because the others were expensive or unavailable.

Two concentrations (1.25 and 2.5%, w/v) were tested for glucose and maltose, respectively, while fixing the other composition levels of the basal medium. The results showed that glucose was a better carbon source than maltose for GABA production (Fig. 1). It was also an important factor affecting GABA production since a higher GABA yield was observed at a concentration of 2.5%. Therefore, 2.5% glucose was used in the following tests.

Effects of nitrogen sources on GABA production

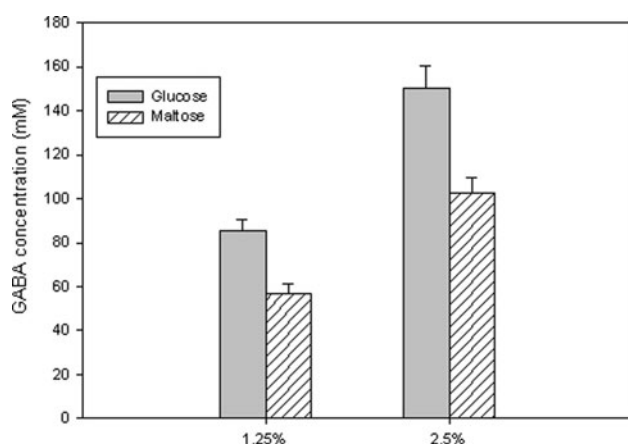
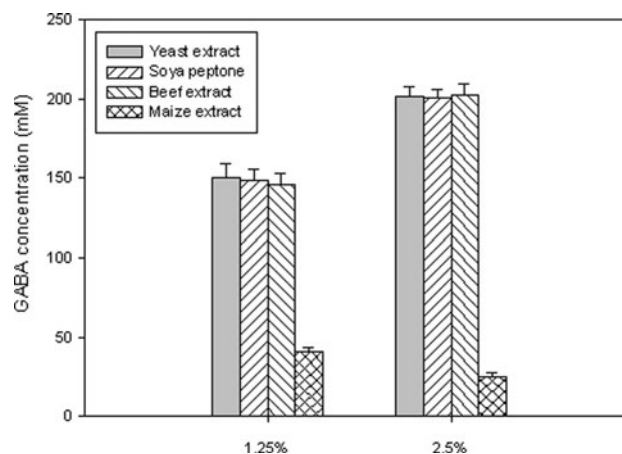
The effects of nitrogen sources on the GABA production were investigated when 2.5% glucose was used as the carbon source. Figure 2 showed that yeast extract, soya peptone and beef extract were the best nitrogen sources with high GABA concentrations. A low production of GABA was observed when maize extract was used as the nitrogen source. On the other hand, yeast extract, soya peptone and beef extract are all important factors affecting GABA production, because significant GABA concentration differences were obtained between 1.25 and 2.5% concentrations of the three nitrogen sources, respectively. Since soya peptone is cheaper and more available than yeast extract and beef extract, a 2.5% concentration of soya peptone was used in the subsequent single-factor experiments.

Effects of Tween-80 on the production of GABA

Tween-80 is a growth-stimulating factor for most of LAB. The effect of Tween-80 on GABA production was tested in a media containing 2.5% glucose and 2.5% soya peptone. As much as 2.0 mL L⁻¹ of Tween-80 was added to the

Table 1 Box–Behnken experimental design matrix with experimental and predicted values of GABA production

Trials	Coded values and actual values				GABA (mM)	
	Glucose (g L ⁻¹)	Soya peptone (g L ⁻¹)	Tween-80 (mL L ⁻¹)	MnSO ₄ ·4H ₂ O (g L ⁻¹)	Experimental	Predicted
1	-1.00 (25)	-1.00 (10)	0.00 (2)	0.00 (0.01)	110.09	105.31
2	1.00 (75)	-1.00 (10)	0.00 (2)	0.00 (0.01)	230.5	229.30
3	-1.00 (25)	1.00 (40)	0.00 (2)	0.00 (0.01)	220.37	221.72
4	1.00 (75)	1.00 (40)	0.00 (2)	0.00 (0.01)	345.18	350.10
5	0.00 (50)	0.00 (25)	-1.00 (1)	-1.00 (0.005)	310.78	316.40
6	0.00 (50)	0.00 (25)	1.00 (3)	-1.00 (0.005)	312.64	314.45
7	0.00 (50)	0.00 (25)	-1.00 (1)	1.00 (0.015)	315.82	314.16
8	0.00 (50)	0.00 (25)	1.00 (3)	1.00 (0.015)	330.89	325.41
9	-1.00 (25)	0.00 (25)	0.00 (2)	-1.00 (0.005)	208.42	206.62
10	1.00 (75)	0.00 (25)	0.00 (2)	-1.00 (0.005)	338.68	335.94
11	-1.00 (25)	0.00 (25)	0.00 (2)	1.00 (0.015)	205.45	214.11
12	1.00 (75)	0.00 (25)	0.00 (2)	1.00 (0.015)	329.44	337.16
13	0.00 (50)	-1.00 (10)	-1.00 (1)	0.00 (0.01)	206.37	209.68
14	0.00 (50)	1.00 (40)	-1.00 (1)	0.00 (0.01)	325.7	327.18
15	0.00 (50)	-1.00 (10)	1.00 (3)	0.00 (0.01)	208.79	213.23
16	0.00 (50)	1.00 (40)	1.00 (3)	0.00 (0.01)	330.32	332.93
17	-1.00 (25)	0.00 (25)	-1.00 (1)	0.00 (0.01)	210.11	207.05
18	1.00 (75)	0.00 (25)	-1.00 (1)	0.00 (0.01)	334.9	329.20
19	-1.00 (25)	0.00 (25)	1.00 (3)	0.00 (0.01)	208.03	207.66
20	1.00 (75)	0.00 (25)	1.00 (3)	0.00 (0.01)	340.89	337.89
21	0.00 (50)	-1.00 (10)	0.00 (2)	-1.00 (0.005)	210.07	210.78
22	0.00 (50)	1.00 (40)	0.00 (2)	-1.00 (0.005)	335.98	332.39
23	0.00 (50)	-1.00 (10)	0.00 (2)	1.00 (0.015)	220.61	218.14
24	0.00 (50)	1.00 (40)	0.00 (2)	1.00 (0.015)	340.51	333.74
25	0.00 (50)	0.00 (25)	0.00 (2)	0.00 (0.01)	324.83	322.69
26	0.00 (50)	0.00 (25)	0.00 (2)	0.00 (0.01)	318.79	322.69
27	0.00 (50)	0.00 (25)	0.00 (2)	0.00 (0.01)	325.01	322.69
28	0.00 (50)	0.00 (25)	0.00 (2)	0.00 (0.01)	320.98	322.69
29	0.00 (50)	0.00 (25)	0.00 (2)	0.00 (0.01)	323.82	322.69

**Fig. 1** The effect of carbon sources on GABA production**Fig. 2** The effect of nitrogen sources on GABA production

medium, and medium without Tween-80 was the control. The results showed that NCL912 could hardly convert glutamate to GABA when Tween-80 was not added, while the GABA concentration reached 208.50 mM in the medium with Tween-80.

Effects of other factors on the production of GABA

The other substances, such as $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, CaCl_2 , $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, V_{B_6} , V_{C} and nicotinic acid that may possibly affect the production of GABA were determined experimentally. The effects of these factors on the GABA production were studied in the media containing 2.5% soya peptone as the nitrogen source, while the contents of other components were the same as the initial medium described in “Materials and methods”. The medium containing 2.5% glucose, 2.5% soya peptone, 500 mM glutamate and 2.0 ml L^{-1} Tween-80 was the experimental control. As shown in Table 2, addition of CaCl_2 , $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, V_{B_6} , V_{C} and nicotinic acid had no significant effect on GABA production. $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ was found to be essential for GABA production. GABA concentration reached 206.83 mM in the medium containing 0.1 g L^{-1} $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, which was higher than that of non- $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ by about 18%.

Determination of optimal concentration ranges of the key factors

In the above experiments, glucose, soya peptone, Tween-80 and $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ were determined as the key factors affecting GABA production by NCL912. To know the suitable concentration ranges of the four factors, a series of concentrations of these factors were selected for single-factor experiments in the following sections.

The effects of glucose concentration on GABA production

In this experiment, glucose concentrations were altered according to Table 3, and the other components in the medium were fixed as follows (g L^{-1}): soya peptone, 25; $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 0.05; sodium L-glutamate, 500 mM; and Tween 80, 2 ml L^{-1} . As shown in Table 3, GABA concentration increased with increase in glucose concentration from 0.5 to 5.0%. When the concentration of glucose was above 5.0%, the GABA concentration was almost stable with only a slight increase.

The effects of soya peptone concentration on GABA production

In this experiment, soya peptone concentrations were altered according to Table 4, and the other components in the medium were fixed as follows (g L^{-1}): glucose, 50; $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 0.05; sodium L-glutamate, 500 mM; and Tween 80, 2 ml L^{-1} . For soya peptone, GABA production significantly increased with concentration of soya peptone when the concentration of soya peptone was lower than 2.5%. GABA production was almost stable when the concentration of soya peptone was at or above 2.5%. Although a slight increase in GABA production was observed when soya peptone exceeded 2.5%, the higher the concentration of the soya peptone, the higher was the cost of the medium.

The effects of Tween-80 concentration on GABA production

The concentrations of Tween-80 used for testing are shown in Table 5. The other components in the medium were fixed as follows (g L^{-1}): glucose, 50; soya peptone, 25; $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 0.05; sodium L-glutamate, 500 mM. The results showed that almost no GABA could be detected in the absence of Tween-80, and the GABA concentration increased with increase in Tween-80 concentration from 0.05 to 2.0 ml L^{-1} . The GABA concentration was almost stable, although a slight increase was observed when Tween-80 concentration exceeded 2.0 ml L^{-1} .

The effects of $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ concentration on GABA production

The media used in this experiment contained (g L^{-1}): glucose, 50; soya peptone, 25; sodium L-glutamate, 500 mM; Tween-80, 2 ml L^{-1} ; and $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ concentrations tested were 0, 0.005, 0.01, 0.02, 0.03 g L^{-1} , respectively. GABA production increased with increase in $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ concentration from 0 to 0.01 g L^{-1} . In the absence of $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, there was a drastic reduction in the production of GABA (only 173.84 mM). GABA concentrations in 0.005, 0.01, 0.02 and 0.03 g L^{-1} $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ -containing media reached 216.45, 323.83, 325.62 and 325.80 mM, respectively. The data indicated that, although essential, increasing $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ concentrations above 0.01 g L^{-1} did not result in increased GABA production.

Table 2 The effects of some factors on the production of GABA

	Control	CaCl_2 (1 g L^{-1})	$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.2 g L^{-1})	$\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ (0.05 g L^{-1})	V_{B_6} (1 mg L^{-1})	V_{C} (1 mg L^{-1})	Nicotinic acid (1 mg L^{-1})
GABA (mM)	175.28 ± 8.45	175.82 ± 7.26	174.36 ± 10.19	206.83 ± 10.83	176.05 ± 8.87	176.61 ± 7.39	175.77 ± 6.71

Table 3 The effects of glucose concentration on GABA production

Glucose (g L ⁻¹)	5	25	50	75	100
GABA (mM)	44.38 ± 5.89	198.28 ± 8.85	322.67 ± 10.71	324.89 ± 9.78	331.73 ± 8.79

Table 4 The effects of soya peptone concentration on GABA production

Soya peptone (g L ⁻¹)	5	10	25	50	75
GABA (mM)	43.84 ± 6.90	193.45 ± 11.65	320.23 ± 14.62	326.60 ± 15.98	329.80 ± 13.89

Table 5 The effects of Tween-80 concentration on GABA production

Tween-80 (mL L ⁻¹)	0.1	0.5	1	2	3
GABA (mM)	235.84 ± 10.35	278.45 ± 12.12	307.23 ± 10.34	320.62 ± 9.78	322.80 ± 7.53

Further optimization of the key factors by RSM

Glucose, soya peptone, Tween-80 and MnSO₄·4H₂O were identified as the key factors that influenced GABA production from the above experiments, and their suitable concentration ranges were also preliminarily determined. In the following experiments, the four important components were optimized using RSM. Table 1 shows various combinations used and the corresponding GABA yield (experimental and predicted). Data were analyzed by linear multiple regression using the Design-Expert (Version 6.0.4.0; Stat-Ease, Inc.) and the following equation was obtained.

$$Y_{\text{GABA}} = 322.69 + 63.09X_1 + 59.30X_2 + 2.32X_3 + 2.18X_4 - 48.19X_1^2 - 47.89X_2^2 - 4.04X_3^2 - 1.04X_4^2 + 1.10X_1X_2 + 2.02X_1X_3 - 1.57X_1X_4 + 0.55X_2X_3 - 1.50X_2X_4 + 3.30X_3X_4$$

where X_1 , X_2 , X_3 and X_4 are the symbols of concentration of glucose, soya peptone, Tween-80 and MnSO₄·4H₂O, respectively.

To validate the regression coefficient, analysis of variance (ANOVA) for GABA production was performed (Table 6). Value of “Model Prob > F ” less than 0.0001 implies that the model is significant. In this case X_1 , X_2 , X_1^2 and X_2^2 are significant model terms. The lack of fit measures the failure of the model to represent data in the experimental domain at points that are not included in the regression (Rastogi and Rashmi 1999). The “Lack of Fit Prob > F value” of 0.0538 implies that the lack of fit is insignificant. Normally, a regression model having an R^2 value higher than 0.9 is considered as having a very high correlation. The R^2 value of this model is 0.9791, so it is reasonable to use the regression model to analyze the trends in the responses.

Table 6 ANOVA of RSA for GABA production

Source	Sum of squares	df	Mean square	F value	Prob > F
Model	117,400	14	8,382.94	261.53	<0.0001
A	47,769.22	1	47,769.22	1,490.30	<0.0001
B	42,201.44	1	42,201.44	1,316.60	<0.0001
C	64.77	1	64.77	2.02	0.1770
D	56.99	1	56.99	1.78	0.2037
A ²	15,064.25	1	15,064.25	469.97	<0.0001
B ²	14,874.94	1	14,874.94	464.07	<0.0001
C ²	106.07	1	106.07	3.31	0.0903
D ²	6.98	1	6.98	0.22	0.6479
AB	4.84	1	4.84	0.15	0.7034
AC	16.28	1	16.28	0.51	0.4877
AD	9.83	1	9.83	0.31	0.5885
BC	1.21	1	1.21	0.038	0.8487
BD	9.03	1	9.03	0.28	0.6039
CD	43.63	1	43.63	1.36	0.2628
Lack of fit	419.37	10	41.94	5.71	0.0538

The graphical representation provides a method to visualize the relationship between the response and experimental levels of each variable and the type of interactions between test variables to deduce the optimum conditions. In this design, the response surface curves for the four variables are shown in Fig. 3. The response surface representing GABA yield is a function of concentrations of the two medium components with another nutrient being at an optimal level.

From the study of plots, the maximum production of GABA was obtained when the concentration of glucose, soya peptone, MnSO₄·4H₂O and Tween-80 were at 55.25, 30.25, 0.0061 g L⁻¹ and 1.38 mL L⁻¹, respectively. The maximum value of GABA production predicted from this

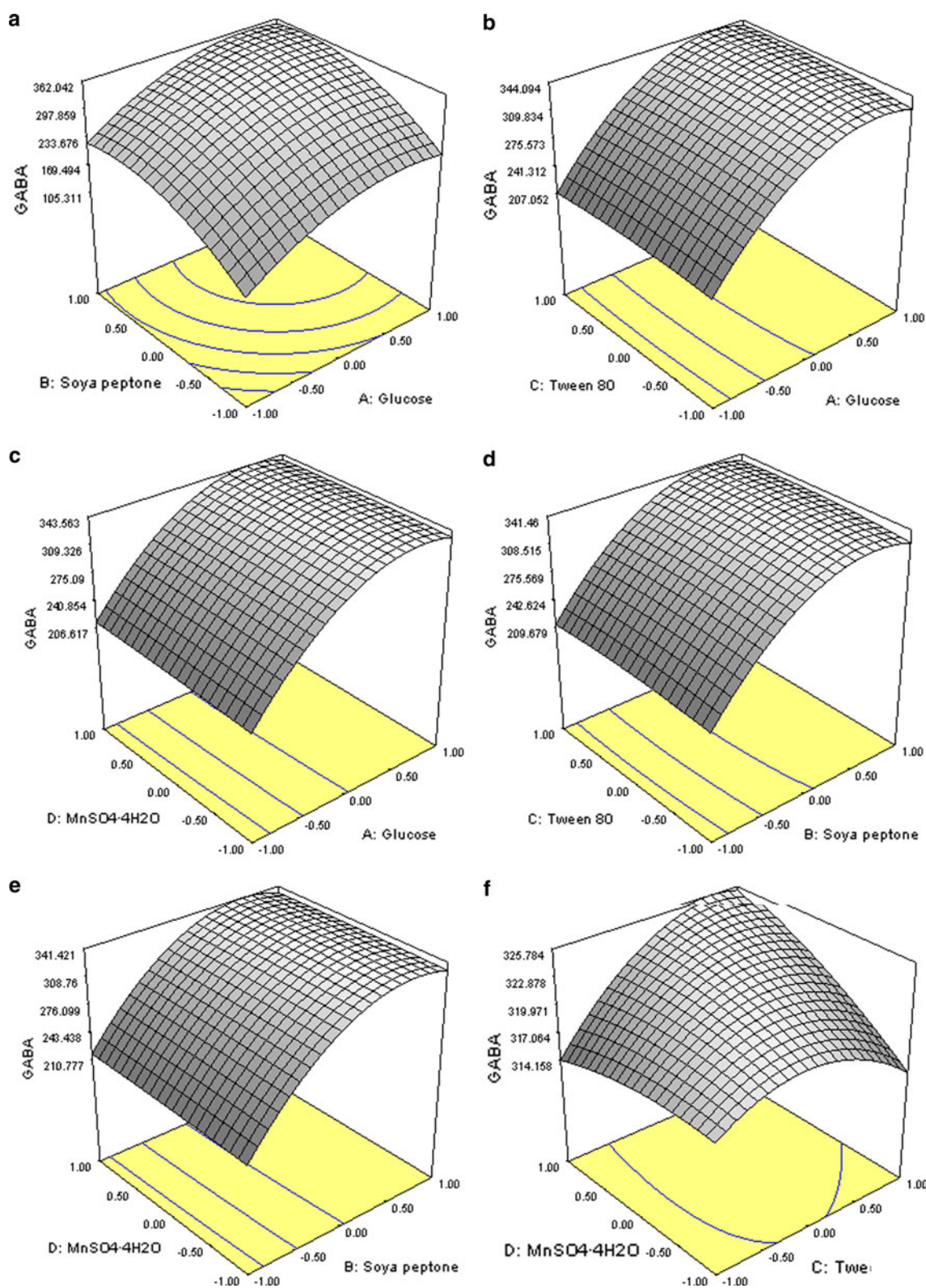


Fig. 3 Three-dimensional surface plots showing the effect of different variables on GABA production. **a** Effect of glucose (X_1) and soya peptone (X_2) on GABA production. **b** Effect of glucose (X_1) and Tween-80 (X_3) on GABA production. **c** Effect of glucose (X_1) and

$\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ (X_4) on GABA production. **d** Effect of soya peptone (X_2) and Tween-80 (X_3) on GABA production. **e** Effect of soya peptone (X_2) and $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ (X_4) on GABA production. **f** Effect of Tween-80 (X_3) and $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ (X_4) on GABA production

model was 349.69 mM. To verify the predicted results, the experiment was performed under the optimized nutrients levels, and the experimental value was 345.83 mM, which is 130% higher than the basal media, suggesting that experimental and predicted values of GABA yield were in good agreement. Therefore, the strain has great potential for industry applications.

Conventional process for optimization of conditions is time consuming and expensive, and fails to consider the interactions between different factors. The statistic method of Box–Behnken design that avoids the above problems has been proved to be useful in this optimization.

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