

1 **An efficient process for co-production of γ -aminobutyric acid and probiotic**

2 ***Bacillus subtilis* cells**

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28 **Abstract** This study was to establish an integrated process for the co-
29 production of γ -aminobutyric acid (GABA) and live probiotics. Six probiotic
30 bacteria were screened and *Bacillus subtilis* ATCC 6051 showed the highest
31 GABA-producing capacity. The optimal temperature and initial pH value for
32 GABA production in *B. subtilis* were found to be 30 °C and 8.0, respectively. A
33 variety of carbon and nitrogen sources were tested, and potato starch and peptone
34 were the preferred carbon and nitrogen sources for GABA production, respectively.
35 The concentrations of carbon source, nitrogen source and substrate (sodium L-
36 glutamate) were then optimized using the response surface methodology. The
37 GABA titer and concentration of viable cells of *B. subtilis* reached 19.74 g/L and
38 6.0×10^8 cfu/mL at 120 h. The GABA titer represents the highest production of
39 GABA in *B. subtilis*. This work thus demonstrates a highly efficient co-production
40 process for GABA and probiotic *B. subtilis* cells.

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42 **Keywords** γ -Aminobutyric acid • *Bacillus subtilis* ATCC 605 • Viable cells •
43 Optimization • Response surface methodology

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50 **Introduction**

51 γ -Aminobutyric acid (GABA), a four-carbon non-proteinogenic amino acid, is well-
52 known as its diverse biological functions such as anxiety inhibition, sleep
53 promotion, blood pressure reduction, diabetes treatment, and immune enhancement
54 (Diana et al., 2014; Park and Oh, 2006; Pham et al., 2015). In addition, GABA has
55 also been applied extensively in agriculture for fruit/vegetable cultivation,
56 fruit/vegetable preservation and animal feeds. GABA can alleviate the low-light
57 induced stress via adjusting the antioxidant defense system and improving
58 photochemical efficiency in pepper seedlings (Li et al., 2017). Exogenous GABA
59 treatment may be an effective method to promote growth and production yield of
60 higher plants under soil salinity conditions (Li et al., 2015). GABA can reduce
61 chilling injury in tomato seedlings at low temperatures (Malekzadeh et al., 2014),
62 and it has shown protective effects in preventing freezing damage and maintaining
63 banana fruit quality (Wang et al., 2014b). GABA treatment can decrease the loss of
64 citrate and some important amino acids and thus becomes a very promising way to
65 maintain postharvest quality (Sheng et al., 2017). On the other hand, diets
66 containing GABA for Wenchang chicken can reduce heat stress induced injuries
67 and improve the growth performance under heat stress conditions (Chen et al.,
68 2015). Similarly, GABA as supplements for dairy cows can reduce heat stress by
69 alleviating rectal temperature, increase feed intake and improve the milk production
70 and nutritional quality (Cheng et al., 2014). GABA is also used as a feed additive
71 to improve the productivity and egg quality in layers (Park and Kim, 2016).

72 In addition to small molecules such as GABA, probiotics, including *Bacillus*
73 *subtilis*, are also widely used in plant protection and animal production to produce
74 safe and healthy food. *B. subtilis* can produce novel antifungal lipopeptide antibiotic.

75 Therefore, *B. subtilis* has been used to control fruit postharvest disease (Janisiewicz
76 and Korsten, 2002). *B. subtilis* as a multifunctional probiotic bacterium, has the
77 potential capacity used in functional feeds for aquaculture. *B. subtilis* can be used
78 in pig farming to improve the growth performance and lipid metabolism in
79 subcutaneous fat (Olmos and Paniagua-Michel, 2014).

80 Therefore, it is desirable to integrate the biological activities of GABA and
81 probiotics in one production process. The aim of this study was to establish a co-
82 production process of *B. subtilis* and GABA, and the resulting product will have
83 great potential in agriculture for green and healthy plant or animal production.

84 **Materials and methods**

85 **Microbial strains and cultivation conditions**

86 *Lactobacillus bulgaricus* ATCC 11842, *Streptococcus thermophilus* ATCC 19258,
87 *Lactobacillus casei* ATCC 393, *Lactobacillus casei* NRRL B-441, *B. subtilis* ATCC
88 6051 and *Bacillus* sp. NRRL B-14911 were obtained from the American Type
89 Culture Center (ATCC) or Agricultural Research Service Culture Collection
90 (NRRL). *L. bulgaricus* ATCC 11842, *S. thermophilus* ATCC 19258, *L. casei* ATCC
91 393 and *L. casei* NRRL B-441 were cultured in 50-mL centrifuge tube containing
92 40 mL of MRS medium at 30 °C without shaking for 5 days (three replicates). *B.*
93 *subtilis* ATCC 6051 and *Bacillus* sp. NRRL B-14911 were grown in 250-mL
94 flasks containing 100 mL of LB medium at 30 °C on a rotary shaker at 200 rpm for
95 5 days (three replicates). Sodium L-glutamate was added as substrate to the cultures
96 at a final concentration of 5 g/L at 48 h for GABA production.

97 **Determination of the titers of GABA**

98 The GABA titer in the fermentation broths were measured using a colorimetric
99 method. Briefly, one milliliter of fermentation broth of each sample was taken and
100 centrifuged at $9,391 \times g$ for 10 min. Three hundred microliters of supernatant was
101 collected in a test tube, into which 0.2 mL of 0.2 M borate buffer (pH 9.0), 1 mL of
102 6% phenol and 0.8 mL of 5% sodium hypochlorite were added. The test tube was
103 oscillated intensively and put in a boiling water bath for 10 min, and then placed
104 immediately into an ice bath for 10 min. The tube was shaken vigorously until the
105 blue color appeared. Finally, the reaction mixture was diluted with 2 mL of 60%
106 ethanol, and the optical density of the sample was recorded at 645 nm on a UV-Vis
107 spectrometer (Zhang et al., 2014).

108 **Selection of carbon and nitrogen sources for GABA production**

109 To find out what carbon source works best for GABA production, the broth was
110 inoculated and cultured using the above-described method. A modified LB broth
111 (10 g tryptone/L, 5 g yeast extract/L, 5 g NaCl /L and 2.5 g K_2HPO_4 /L) served as
112 the control medium. The medium composition of the experimental groups
113 contained 10 g tryptone/L, 5 g yeast extract/L, 5 g NaCl /L, 2.5 g K_2HPO_4 /L and
114 the selected carbon source at a final concentration of 2.5 g/L. Nine different carbon
115 sources were tested, including glucose, lactose, sucrose, fructose, glycerol, dextrin,
116 potato starch, soluble starch, and malt extract.

117 A similar approach was used to identify the best carbon source for GABA
118 production. A modified LB broth (10 g tryptone/L, 5 g yeast extract/L, 5 g NaCl /L,
119 2.5 g K_2HPO_4 /L and 2.5 g potato starch/L) was used as the control. Tryptone (10
120 g/L) and yeast extract (5 g/L) in LB medium served as the control nitrogen source.
121 The medium composition of the experimental groups contained 5 g NaCl /L, 2.5 g
122 K_2HPO_4 /L, 2.5 g potato starch/L and the selected nitrogen source at a final

123 concentration of 15 g/L. Seven nitrogen sources were tested, including NaNO₃,
124 (NH₄)₂HPO₄, tryptone, peptone, milk power, yeast extract, and soy flour.

125 **Determination of the concentrations of viable cells of *B. subtilis***

126 To determine the concentrations of viable cells of *B. subtilis* in the cultures, five
127 serial dilutions were prepared with fresh LB medium (three replicates). The diluted
128 cultures were spread on LB agar plates, and incubated at 30 °C for 24 h before
129 enumeration (Wang et al., 2014a).

130 **Optimization of the culture medium using the response surface methodology**

131 Response surface methodology (RSM) has been widely used as a statistical tool in
132 the investigation and optimization of several complex processes (Filotheou et al.,
133 2010). In the RSM design, the Box–Behnken experimental design (BBD) needs the
134 fewest runs in the experimental design (Ay et al., 2009). In this work, BBD was
135 used to optimize the culture medium for GABA production. All experiments were
136 performed in triplicate, and the averages of the GABA production were used as the
137 responsive values. ANOVA evaluated the significant variation in GABA production
138 in different culture media (Wang et al., 2015). A second-order polynomial
139 regression model was calculated using BBD analysis. The optimal medium
140 composition for the production of GABA was obtained through Design Expert
141 version 10.

142 **Statistical method**

143 Statistical differences were calculated using a paired Student's t-test. A paired two-
144 sided Student's t-test was used to determine the statistical significance of differences
145 in GABA production. A two-tailed *p* value of <0.05 was considered to be significant.

146 **Results and discussion**

147 **Screening of an efficient GABA-producing strain from six probiotic bacteria**

148 Probiotic bacteria such as lactic acid bacteria are widely used to produce GABA
149 because its well-known ability to produce this compound (Dhakal et al., 2012).
150 Some *Bacillus* strains were also reported to produce GABA using solid-state
151 fermentation (Suwanmanon and Hsieh, 2014b; Torino et al., 2013). However,
152 there were few studies on GABA production through liquid fermentation. In this
153 study, a total of 4 lactic acid bacteria (*Lactobacillus bulgaricus* ATCC 11842,
154 *Streptococcus thermophilus* ATCC 19258, *Lactobacillus casei* ATCC 393, and
155 *Lactobacillus casei* NRRL B-441) and 2 *Bacillus* strains (*B. subtilis* ATCC 6051
156 and *Bacillus* sp. NRRL B-14911) were evaluated for their GABA-producing ability.
157 All of them were able to produce GABA (Fig. 1(A)). Among these six strains, the
158 two *Bacillus* strains showed better ability to produce GABA than the four lactic
159 acid bacteria strains. *B. subtilis* ATCC 6051 showed the highest GABA production
160 titer (7.40 ± 0.17 g/L). Moreover, *B. subtilis* ATCC 6051 is a food grade probiotic.
161 It was previously used to produce fermented edible seeds containing high levels of
162 bioactive components and *B. subtilis* cells through solid state fermentation (Gan et
163 al., 2017; Torino et al., 2013). The same strain was also used for microbial
164 biotransformation of a synthetic glucocorticoid named dexamethasone, yielding
165 three metabolites including 6-hydroxydexamethasone, 17-oxodexamethasone, and
166 6-hydroxy-17-oxodexamethasone. It may be used as an *in vitro* model to
167 understand the metabolism of similar glucocorticoids (Pervaiz et al., 2015). *B.*
168 *subtilis* ATCC 6051 was previously reported to have a weak capacity to produce
169 GABA (2.69 mg/g after 96-h fermentation) with solid state fermentation (Limón et
170 al., 2015). By contrast, our results indicated that it produces a higher amount of

171 GABA in liquid fermentation. Therefore, it will be of interest to combine the
172 benefits of GABA and *B. subtilis* for agricultural applications.

173 **Fig. 1**

174 **Effect of culture temperature and initial pH on GABA production in *B. subtilis***
175 **ATCC 6051**

176 The effect of the fermentation temperature on GABA production by *B. subtilis*
177 ATCC 6051 in LB broth was tested. Four different temperatures were tested,
178 including 25, 30, 35 and 40 °C. Fig. 1(B) shows that the titers of GABA were 6.12
179 ± 0.18 , 7.50 ± 0.24 , 7.30 ± 0.26 and 5.56 ± 0.21 g/L at these temperatures,
180 respectively, and 30 °C showed the best titer among the four tested temperatures.
181 This is consistent with a previous report in which Ghasemi and Ahmadzadeh found
182 that *B. subtilis* UTB96 grew better at 30 °C (Ghasemi and Ahmadzadeh, 2013). The
183 effect of initial pH of the culture medium on GABA production was then examined.
184 Five pH values (5, 6, 7, 8 and 9) were tested. As shown in Fig. 1(C), the titer of
185 GABA increases with increasing initial pH in the range of pH 5-8. At pH 8, the titer
186 of GABA reached 7.55 ± 0.29 g/L. The titer decreased when the initial pH was 9
187 and was determined to be 6.01 ± 0.42 g/L. Therefore, the optimal initial pH was
188 found to be 8 for GABA production in *B. subtilis* ATCC 6051. In contrast,
189 Suwanmanon et al. found that the optimal pH value for GABA production was 7.0
190 when using a *B. subtilis* strain isolated from rice straw (Suwanmanon and Hsieh,
191 2014a), suggesting that different *B. subtilis* strains may have different preferences
192 to the initial pH value of the fermentation medium. The pH change of the
193 fermentation broths during the 120-h period was measured. As shown in Fig. 1(D),
194 although the five cultures started at different initial pH values, after 24 h of cell
195 growth, the pH of all the broths changed to about 8 and slightly increased to

196 approximately 9 in a similar pattern during the remaining period of fermentation.
197 Thus, the main pH difference among the five cultures (with initial pH of 5, 6, 7, 8
198 or 9) was mainly shown in the first 24 h, which might have affected the initial
199 growth rate of the cells.

200 **Effect of carbon and nitrogen sources on the GABA production in *B. subtilis***
201 **ATCC 6051**

202 The effect of various carbon and nitrogen sources on GABA production in *B.*
203 *subtilis* ATCC 6051 were studied. The GABA titer for each tested carbon source is
204 shown in Table 1. Table 1 shows that the titers of GABA for above different carbon
205 sources were 8.14 ± 0.50 , 7.97 ± 0.50 , 8.71 ± 0.45 , 8.89 ± 0.44 , 8.15 ± 0.29 , $8.77 \pm$
206 0.34 , 9.40 ± 0.49 , 8.59 ± 0.43 and 9.15 ± 0.42 g/L, respectively, all of which were
207 higher than 7.84 ± 0.51 g/L in the control. Potato starch showed the best titer among
208 the nine tested carbon sources. The results indicated that the carbon source
209 significantly affects GABA production. A comparison of the titers indicated that the
210 slow-acting carbon sources (potato starch, soluble starch and malt extract) have
211 overall higher GABA production titers than quick-acting carbon sources (glucose,
212 lactose, sucrose and fructose). Among the quick-acting carbon sources, fructose is
213 relatively more conducive to the production of GABA and the titer was 8.89 ± 0.44
214 g/L. Stuke et al. previously reported that glucose was the most preferred source for
215 carbon and energy for *B. subtilis* (Stülke and Hillen, 2000). Suwanmanon et al.
216 found that fructose is a better carbon source for GABA production (Suwanmanon
217 and Hsieh, 2014a). This is consistent with our result. However, potato starch
218 showed an even better effect on GABA production, and the titer reached 9.40 ± 0.49
219 g/L. Potato starch is cheaper than glucose and fructose. Considering the cost and
220 productivity, potato starch was chosen as the carbon source for the following

221 optimization studies in this work.

222 The effect of nitrogen source on the production of GABA was also examined.
223 NaNO_3 , $(\text{NH}_4)_2\text{HPO}_4$, tryptone, peptone, milk power, soy flour, and yeast extract
224 were respectively provided as the nitrogen source in the culture medium. A
225 modified LB broth (10 g tryptone/L, 5 g yeast extract/L, 5 g NaCl/L, 2.5 g K_2HPO_4
226 /L and 2.5 g potato starch/L) as the control. Tryptone (10 g/L) and yeast extract (5
227 g/L) in LB medium served as the control nitrogen source. The experimental groups
228 contained 5 g NaCl/L, 2.5 g K_2HPO_4 /L, 2.5 g potato starch/L and the selected
229 nitrogen source at a final concentration of 15 g/L. The GABA production for each
230 nitrogen source is shown in Table 1. Among the eight tested nitrogen sources,
231 peptone gave the highest titer of GABA (9.86 ± 0.48 g/L). Yeast extract also showed
232 a great effect on GABA production (9.51 ± 0.28 g/L). Similarly, Suwanmanon et al.
233 reported that yeast extract was the most promising nitrogen source for GABA
234 production (Suwanmanon and Hsieh, 2014a). Moreover, organic nitrogen sources
235 shown much higher production than inorganic nitrogen sources.

236 **Table 1**

237 **Optimization of the culture medium and substrate concentration using the**
238 **response surface methodology**

239 Carbon and nitrogen sources are two essential nutrients in the culture media. Based
240 on the above results, potato starch and peptone were chosen as the carbon and
241 nitrogen sources, respectively, in the subsequent experiments. GABA is produced
242 from L-glutamine through decarboxylation. Thus, its concentration will affect the
243 production titer of GABA. Three major factors, including potato starch
244 concentration, peptone concentration and sodium L-glutamate concentration, were

245 then used for optimization. Through single-factor experiments, the appropriate
246 ranges for these three factors were determined: 20 to 60 g/L for peptone
247 concentration, 5 to 20 g/L for potato starch concentration, and 5 to 20 g/L for sodium
248 L-glutamate concentration. The data obtained from the BBD (Table 2) presents the
249 design matrix. The GABA titer represented the response. By using Design Expert
250 version 10, quadratic model (Equation 1) and their subsequent ANOVA (Table 2)
251 were found to be the best model to explain the correlation between the GABA titer
252 and three variables.

$$253 \quad [GABA] \text{ (g/L)} = 16.1+2.93A-0.71B+0.83C+0.32AB-1.44AC-1.76BC+0.92A^2- \\ 254 \quad 0.86B^2-2.06C^2 \quad (1)$$

255 Where A is peptone concentration, B is potato starch concentration and C is sodium
256 L-glutamate concentration.

257 **Table 2**

258 The model *p*-value of 0.0002 and “lack of fit” *p*-value of 0.2208 from the
259 analysis of ANOVA (Table 3) showed that equation (1) was highly significant to
260 describe the actual relationship between the GABA titer and three factors. The *p*-
261 value of component tests were used to determine the significance of each coefficient.
262 The smaller *p*-value indicates a higher significance for the corresponding
263 coefficient (Zhang et al., 2017). The corresponding *p*-values of each coefficient
264 indicated that peptone concentration (*p*-value<0.0001), potato starch concentration
265 (*p*-value =0.0346), and sodium L-glutamate concentration (*p*-value = 0.0189) can
266 significantly affect the production of GABA (Table 3). Moreover, the peptone
267 concentration with F-value of 115.36 and *p*-value of <0.0001 is one of the most
268 important factor for the GABA production (Table 3).

269 **Table 3**

270 Figs. 2(A), 2(B) and 2(C) showed the effect of GABA production for each

271 pair of factors. The graphs depicted the effects of various factors on GABA
272 production. As shown in this figure, two pair of the factors (peptone
273 concentration/sodium L-glutamate concentration and potato starch
274 concentration/sodium L-glutamate concentration) exerted a great effect on GABA
275 production.

276 **Fig. 2**

277 The optimal composition for GABA production obtained from the
278 maximum point of the model. The optimal conditions for the highest GABA
279 production (20.0 g/L) were obtained with 60 g peptone/L concentration, 11.5 g
280 potato starch/L, and 11.8 g sodium L-glutamate/L.

281 **Co-production of GABA and probiotic *B. subtilis* with the optimized medium** 282 **composition**

283 The bacterial growth profile and GABA production from 0 to 168 h were then
284 monitored in the optimized culture medium. The medium without optimization was
285 used as the control. As shown in Fig. 3(A), viable cells in the control medium
286 increased rapidly during the first 48 h of incubation and then entered the stationary
287 phase for about 36 h. The maximum concentration of viable cells reached 6.8×10^8
288 cfu/mL at 60 h. However, in the optimized medium, after about 72 h, the growth of
289 the strain entered the stationary phase, and the highest concentration of viable cells
290 reached 9.9×10^8 cfu/mL at 84 h. The rich nutrients in the optimized medium could
291 support the strain growth for a longer period. However, the stationary phase of the
292 strains in the control and optimized groups just lasted about 36 h and 24 h,
293 respectively, and then the concentration of viable cells rapidly declined. High
294 concentration of GABA may inhibit the growth of the strain and accelerate the aging

295 of the strain.

296 **Fig. 3**

297 Time course analysis of GABA production (Fig. 3(B)) revealed that the
298 GABA titer in the control group increased rapidly during the first 96 h, and then
299 slowed down and maintained a relative stable level. By contrast, the optimized
300 group had a longer period of active production of GABA and the titer has been
301 increasing steadily in the first 132 h. Accumulation of GABA, death of *B. subtilis*
302 ATCC 6051 and consumption of the nutrients may contribute to the decreased rate
303 of GABA production in the late stage of the fermentation (Li and Cao, 2010;
304 Tajabadi et al., 2015).

305 For this co-production process of GABA and live cells of *B. subtilis*, the
306 GABA titer and concentrations of viable cells in the control group reached 7.89 g/L
307 and 2.3×10^8 cfu/mL at 96 h, respectively. The GABA titer and concentration of
308 viable cells in the optimized group reached 19.74 g/L and 6.0×10^8 cfu/mL at 120
309 h, respectively. The GABA titer of 19.74 g/L was very close to the predicted value
310 of 20 g/L in the model, indicating that this model is appropriate for optimization of
311 GABA production in *B. subtilis*. Suwanmanon et al. screened a strain of *B. subtilis*
312 from rice straw, and the titer of GABA reached 15.4 g/L in liquid fermentation
313 (Suwanmanon and Hsieh, 2014a). The GABA titer obtained in this study is higher
314 than any other reported production titer by *B. subtilis*. Optimization of the
315 fermentation conditions increased the GABA production and viable cell
316 concentration by 150.19% and 165.92%, respectively.

317 In summary, six bacterial strains were tested for GABA production in this
318 work and *B. subtilis* ATCC 6051 showed the best production ability. The optimal
319 temperature and initial pH value for the biosynthesis of GABA in *B. subtilis* ATCC
320 6051 were 30 °C and 8.0, respectively. The optimal medium components for GABA

321 production in *B. subtilis* ATCC 6051 were 11.481 g potato starch/L, 60 g peptone/L,
322 5 g NaCl/L, and 2.5 g K₂HPO₄/L. The optimal concentration of sodium L-glutamate
323 was determined to be 11.825 g/L, which was added into the medium after 48 h.
324 Under the optimized conditions, the GABA titer and concentration of viable cells
325 reached 19.74 g/L and 6.0 × 10⁸ cfu/mL at 120 h, respectively. To conclude, by
326 screening several probiotic strains, our work shows that *B. subtilis* ATCC 6051 is
327 valuable for producing GABA-rich foods. After rationally optimizing the culture
328 conditions, this research provides a highly efficient co-production process for
329 GABA and probiotic *B. subtilis* cells. The resulting product may be used in
330 agriculture as health-benefiting plant or animal feed.

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338 **Compliance with ethical standards**

339 **Conflict of interest.** The authors declare no conflicts of interest.

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448 **Figure Captions**

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450 **Fig. 1** Comparison of the production of GABA by six bacterial strains and the
451 effects of temperature and pH on GABA production. **(A)** Evaluation of GABA
452 production by six different bacterial strains at 30 °C for 5 days. **(B)** Effect of culture
453 temperature on GABA production in *B. subtilis* ATCC 6051. **(C)** Effect of initial
454 pH on GABA production in *B. subtilis* ATCC 6051. **(D)** pH changes of the broths
455 during the 120-h fermentation period

456 **Fig. 2** Three-dimensional response surface plots for GABA production in *B. subtilis*
457 ATCC 6051. **(A)** Effect of the concentrations of peptone and potato starch on GABA
458 production. **(B)** Effect of the concentrations of peptone and sodium L-glutamate on
459 GABA production. **(C)** Effect of the concentrations of potato starch and sodium L-
460 glutamate on GABA production

461 **Fig. 3** A comparison of the concentration of viable cells of *B. subtilis* ATCC 6051
462 and GABA titer before and after optimization. **(A)** The concentration of viable cells
463 of *B. subtilis* ATCC 6051 at different time points. **(B)** The titers of GABA in batch
464 cultures at different time points

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471 **Table 1** The effects of various carbon and nitrogen sources on GABA
 472 production in *B. subtilis* ATCC 6051

	Nutritional components	Titer of GABA (g/L)
	Control	7.84±0.51
	Glucose	8.14±0.50 ^{ns}
	Lactose	7.97±0.50 ^{ns}
	Sucrose	8.71±0.45 [*]
	Fructose	8.89±0.44 [*]
Carbon source	Glycerol	8.15±0.29 ^{ns}
	Dextrin	8.77±0.34 [*]
	Potato starch	9.40±0.49 ^{**}
	Soluble starch	8.59±0.43 ^{ns}
	Malt extract	9.15±0.42 [*]
	Control	8.95±0.30
	NaNO ₃	2.24±0.15 ^{***}
	(NH ₄) ₂ HPO ₄	1.25±0.11 ^{***}
Nitrogen source	Tryptone	8.52±0.13 [*]
	Peptone	9.86±0.48 ^{**}
	Milk power	3.69±0.17 ^{***}
	Soy flour	7.10±0.40 ^{**}

Yeast extract

9.51±0.28*

473 *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ns: $p > 0.05$.

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497 **Table 2** Box–Behnken experimental design for GABA production in *B.*
 498 *subtilis* ATCC 6051
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Run	Factor A	Factor B	Factor C	Titer of GABA	
	Peptone (g/L)	Potato starch (g/L)	Sodium L- glutamate (g/L)	Observed value (g/L)	Predicted value (g/L)
1	40	20	5	13.12±0.22	13.38
2	40	5	20	16.73±1.01	16.47
3	20	12.5	5	10.50±0.37	9.76
4	40	12.5	12.5	15.59±1.10	16.10
5	40	12.5	12.5	16.48±0.73	16.10
6	60	12.5	20	16.53±1.06	17.27
7	40	12.5	12.5	15.28±1.06	16.10
8	20	20	12.5	11.71±1.01	12.19
9	40	5	5	11.13±0.53	11.29
10	60	20	12.5	19.27±1.75	18.69
11	20	5	12.5	13.68±0.88	14.26
12	60	12.5	5	18.18±1.79	18.50
13	40	12.5	12.5	16.45±1.11	16.10
14	60	5	12.5	19.96±1.26	19.48
15	40	20	20	11.69±0.38	11.53
16	40	12.5	12.5	16.68±1.29	16.10
17	20	12.5	20	14.61±0.31	14.29

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503 **Table 3** Analysis of variance (ANOVA) for the regression

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Source	SS	DF	MS	<i>F</i> value	Prob> <i>F</i>
Model	123.70	9	13.74	23.09	0.0002*
A	68.68	1	68.68	115.36	<0.0001
B	4.08	1	4.08	6.85	0.0346
C	5.49	1	5.49	9.23	0.0189
AB	0.41	1	0.41	0.69	0.4342
AC	8.29	1	8.29	13.93	0.0073
BC	12.36	1	12.36	20.75	0.0026
A ²	3.59	1	3.59	6.03	0.0438
B ²	3.14	1	3.14	5.28	0.0551
C ²	17.94	1	17.94	30.14	0.0009
Residual	4.17	7	0.60		
Lack of Fit	2.63	3	0.88	2.28	0.2208**
Pure Error	1.54	4	0.38		
Cor Total	127.86	16			

505 $R^2=0.967$ and adjusted $R^2=0.926$, * significant, ** not significant.

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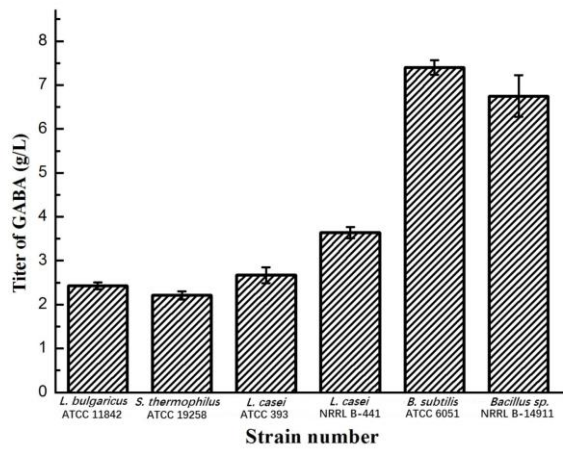
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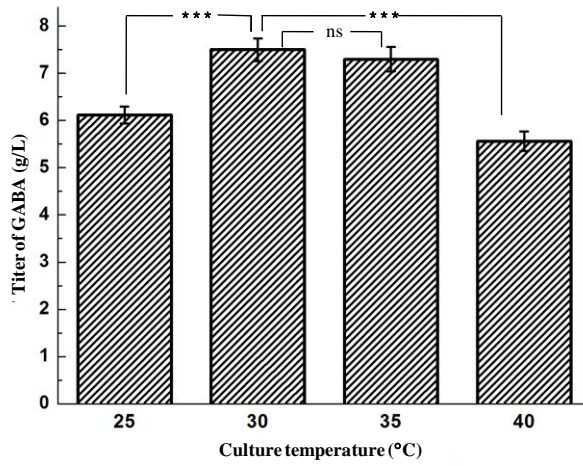
513 **Fig. 1**

514 (A)



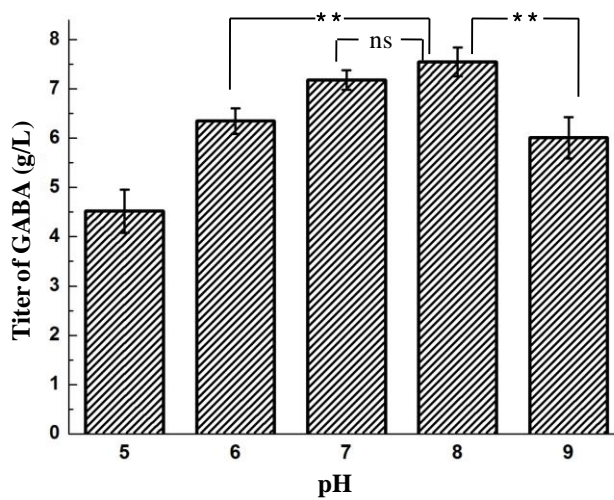
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516 (B)



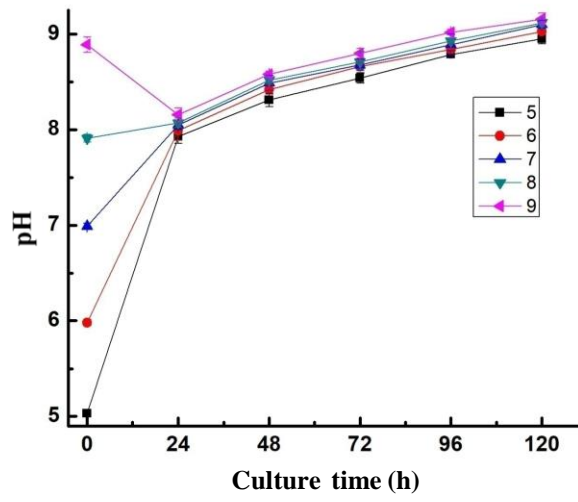
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520 (D)



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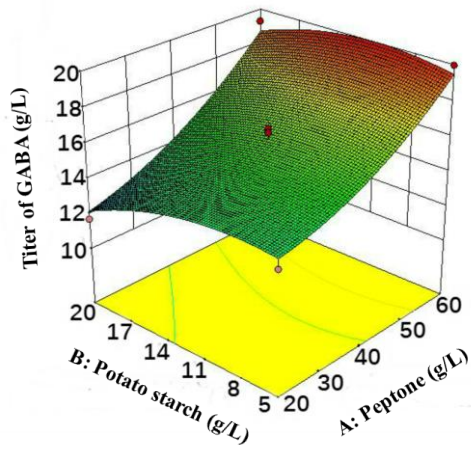
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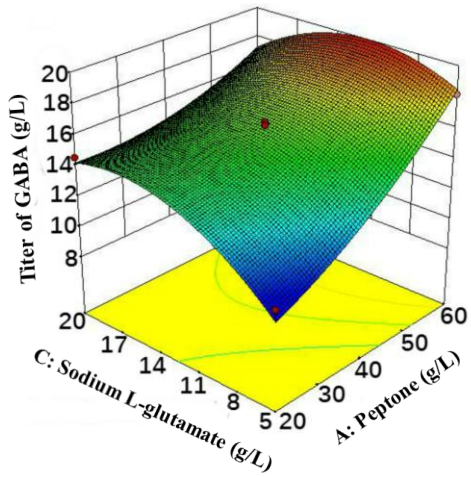
540 **Fig. 2**

541 (A)



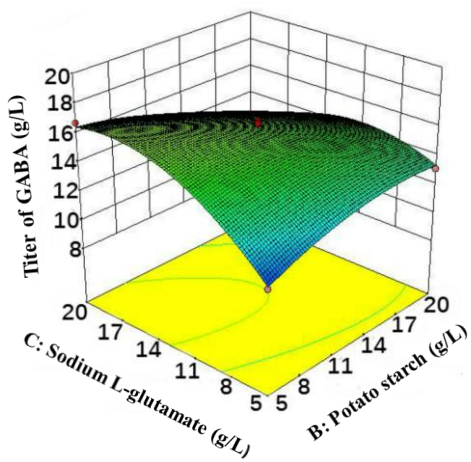
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543 (B)



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545 (C)



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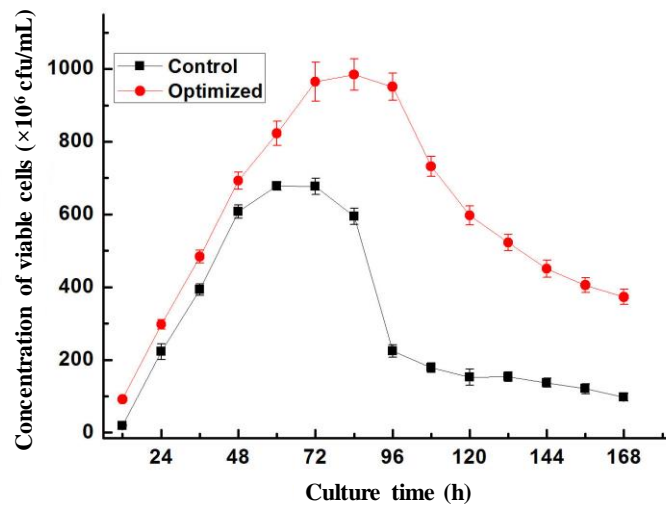
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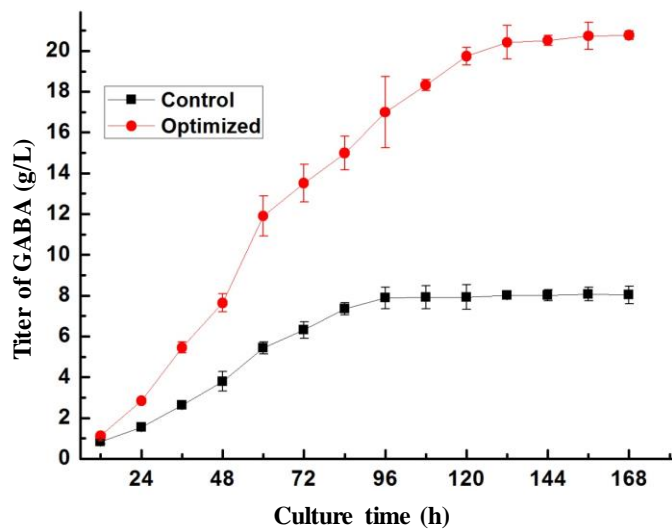
550 **Fig. 3**

551 **(A)**



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553 **(B)**



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