Green Chemistry

Accepted Manuscript

This is an *Accepted Manuscript*, which has been through the Royal Society of Chemistry peer review process and has been accepted for publication.

Accepted Manuscripts are published online shortly after acceptance, before technical editing, formatting and proof reading. Using this free service, authors can make their results available to the community, in citable form, before we publish the edited article. We will replace this *Accepted Manuscript* with the edited and formatted *Advance Article* as soon as it is available.

You can find more information about *Accepted Manuscripts* in the Information for Authors.

Please note that technical editing may introduce minor changes to the text and/or graphics, which may alter content. The journal's standard Terms & Conditions and the Ethical guidelines still apply. In no event shall the Royal Society of Chemistry be held responsible for any errors or omissions in this *Accepted Manuscript* or any consequences arising from the use of any information it contains.

www.rsc.org/greenchem

1 **Efficient one-step Preparation of γ-Aminobutyric Acid from Glucose**
2 **without Exogenous Cofactor by the Designed *Corynebacterium***
3 ***glutamicum***

4 Rongzhen Zhang[1,2], Taowei Yang[1], Zhiming Rao[1,2]*, Hongmei Sun[1], Meijuan
5 Xu[1], Xian Zhang[1], Zhenghong Xu[3], Shangtian Yang[4]
6 [1] The Key Laboratory of Industrial Biotechnology, Ministry of Education, School
7 of Biotechnology, Jiangnan University, Wuxi, 214122, P. R. China
8 [2] National Laboratory for Food Science, Jiangnan University, Wuxi 214122, P.
9 R. China
10 [3] School of Medicine and Pharmaceuticals, Jiangnan University, Wuxi, 214122,
11 P. R. China
12 [4] Department of Chemical Engineering, Ohio State University, 140 West 19th
13 Avenue, Columbus, OH 43210, USA
14
15 *Author for correspondence;
16 Zhiming Rao
17 Tel: +86-510-85916881；
18 *E-mail:* raozhm@jiangnan.edu.cn
19 Present address: School of Biotechnology, Jiangnan University, 1800 Lihu
20 Avenue, Wuxi, Jiangsu, 214122, P. R. China

Green Chemistry Accepted Manuscript

21 **Abstract**

22 *Lactobacillus plantarum* CCTCC M209102 efficiently produces γ-aminobutyric
23 acid (GABA) from L-glutamate, in which glutamate decarboxylase and
24 pyridoxal kinase are involved in the transformation. Pyridoxal kinase catalyzes
25 ATP-dependent phosphorylation of pyridoxal to produce
26 pyridoxal-5'-phosphate, which is the cofactor required for glutamate
27 decarboxylase to biotransform GABA from L-glutamate. *Corynebacterium*
28 *glutamicum* G01 is a good producer of L-glutamate from glucose. However, it
29 can not yield GABA from L-glutamate due to the absence of glutamate
30 decarboxylase and pyridoxal kinase. In this work, to realize the efficient
31 one-step preparation of GABA from glucose without exogenous
32 pyridoxal-5'-phosphate, the metabolic module from L-glutamate to GABA
33 basing on glutamate decarboxylase and pyridoxal kinase in *L. plantarum* was
34 grafted into *C. glutamicum*. To further improve the GABA production, the
35 pathways to by-product pools of L-arginine, L-proline and L-lysine were
36 blocked using insertional mutation technique. The engineered *C. glutamicum*
37 APLGGP carrying *arg*B::*tacgad*, *pro*B::*tacgad* and *dapA*::*tacplk* could
38 efficiently convert glucose into GABA in one-step without exogenous co-factor.
39 In fed-batch cultures, the recombinant *C. glutamicum* APLGGP produced 70.6
40 $g.L^{-1}$ GABA at 30°C and 70 h through a two-stage pH control strategy. To our
41 knowledge, this is the highest reported GABA production using glucose as
42 substrate, and this designed *C. glutamicum* should be an excellent candidate

Green Chemistry Accepted Manuscript

43 for producing GABA on an industrial scale. This work is hoped to pave the way

44 to redesign the bioreactor for efficient one-step biosynthesis of GABA from

45 glucose without exogenous co-factor.

46

47 **Keywords**: *Corynebacterium glutamicum*; glucose; γ-aminobutyric acid;

48 efficient one-step preparation; two-stage pH control strategy; reactor design

49 **Abbreviations:** *argB*, N-acetyl-L-glutamate kinase gene; *dap*A,

50 dihydropicolinate synthase gene; DHDPS, dihydropicolinate synthase; GABA,

51 γ-aminobutyric acid; GAD, glutamate decarboxylase; *gad*, glutamate

52 decarboxylase gene; GAK, glutamate kinase; NAGK, N-acetyl-L-glutamate

53 kinase; PLK, pyridoxal kinase; *plk*, pyridoxal kinase gene; *proB*, glutamate

54 kinase gene.

55

Green Chemistry Accepted Manuscript

56 ## Introduction

57 Gamma-aminobutyric acid (GABA), a non-protein amino acid, functions as a
58 major inhibitory neurotransmitter in animals, and it has many physiological
59 properties related to anti-anxiety and hypotension as tranquilizers, diuretics,
60 and analgesics [1]. GABA can be used as a bioactive component in the food,
61 feed, and pharmaceutical fields. Although there have been many attempts for
62 the GABA synthesis chemically or biologically, recent special attention is
63 focused on GABA preparation by employing "green" and efficient bioprocesses
64 since they have a simple reaction procedure, high catalytic efficiency, mild
65 reaction condition and environmental compatibility [2].

66 In eukaryotes and prokaryotes, including bacteria, fungi and yeasts,
67 glutamate decarboxylase (GAD, EC 4.1.1.15) catalyzes the conversion of
68 L-glutamate to GABA [3]. However, GAD is a pyridoxal-5'-phosphate-dependent
69 enzyme that catalyzes irreversible α-decarboxylation of L-glutamate to GABA
70 with pyridoxal-5'-phosphate as cofactor [4]. Very recently, Rowley and
71 Whisstock's group have done excellent jobs about GAD structure
72 determination and kinetic studies for regulation of GABA production [5].

73 Using lactic acid bacteria harboring GAD to produce GABA-enriched food
74 has attracted great attention since they are safe and recognized as probiotics [6].
75 During the GABA production by lactic acid bacteria, glutamate is added as a
76 precursor into the fermentation medium, and some expensive nitrogen sources
77 are supplemented for microorganism cultivation [7, 8]. The fermentation method

Green Chemistry Accepted Manuscript

78 is not cost-effective, so, a new approach is required for the sustainable
79 industrial application [9]. Kook *et al*. reported the maximum GABA production of
80 27.3 $g.L^{-1}$ by expressing *Lactobacillus plantarum* GAD gene in *Lactobacillus*
81 *Sakei* [10]. *Corynebacterium glutamicum* is an important industrial producer of
82 various amino acids with great potential. Takahashi *et al*. successfully
83 produced GABA from glucose in one-step using the recombinant *C.*
84 *glutamicum* expressing *Escherichia coli* GadB [11]. As known, the enzyme GAD
85 is the key enzyme for the reaction from L-glutamate to GABA, the introduction
86 of one extra copy will promote the GABA production improvement. *C.*
87 *glutamicum* is an important industrial producer of various amino acids with
88 great potential for the production of other metabolites. Shi *et al*. improved
89 GABA production using the recombinant *C. glutamicum* coexpressing two
90 GAD genes *gadB*1 and *gadB*2 from *Lactobacillus brevis* Lb85 [12]. Through the
91 fermentation condition optimization, they further increased GABA production to
92 a maximum level of 27.13 $g.L^{-1}$ after 120-h flask cultivation and 26.32 $g.L^{-1}$
93 after 60-h fed-batch fermentation [13]. However, the expensive pyridoxal
94 5′-phosphate is required for GABA production. The reported methods and
95 productivity of GABA were summarized in Table 1.

96 **Insert Table 1**

97 The enzyme GAD is active to produce GABA with pyridoxal 5′-phosphate as
98 cofactor. Pyridoxal kinase (PLK) was reported to catalyze the ATP-dependent
99 phosphorylation reaction of pyridoxal to produce pyridoxal 5′-phosphate [4].

Green Chemistry Accepted Manuscript

100 Although the fermentation medium, such as corn steep liquor and yeast extract,
101 contains pyridoxal, the precursor of pyridoxal 5′-phosphate, it is not enough to
102 support the quick biotransformation of GABA. So the introduction of the
103 enzyme PLK involved in the formation of pyridoxal 5′-phosphate will be
104 beneficial to the GABA biosynthesis.

105 In our previous work, we reported that a modified strain through
106 multi-mutagenesis, *L. plantarum* GB 01-21, showed high GAD activity, and the
107 engineered *E. coli* BL21 harboring *L. plantarum* GAD gene (*gad*, accession No:
108 JN248358) yielded 204.5 $g.L^{-1}$ GABA from L-glutamate at 24 h. The mole
109 conversion rate of the substrate reached 97.9% [14]. By analysis of the
110 metabolic pathways, L-arginine and L-proline are the main by-products during
111 the biotransformation of GABA from L-glutamate in *L. plantarum* GB 01-21
112 (Figure 1). We also isolated a GRAS (generally regarded as safe) strain, *C.*
113 *glutamicum* G01, which could accumulate about 106 $g.L^{-1}$ L-glutamate from
114 glucose [13]. L-lysine is the main by-product during the biosynthesis of
115 L-glutamate from glucose in *C. glutamicum* G01 (Figure 1).

116 **Insert Figure 1**

117 In this work, to realize the efficient production of GABA from glucose in
118 one-step without exogenous pyridoxal-5'-phosphate, we grafted the metabolic
119 modular from L-glutamate to GABA basing on GAD and PLK in *L. plantarum*
120 GB 01-21 to *C. glutamicum* G01. To further improve the GABA production, we
121 not only knocked out the genes involved the by-products pathways of

Green Chemistry Accepted Manuscript

122 L-arginine, L-proline and L-lysine through insertional inactivation, but also
123 introduced two copies of GAD genes into the engineered *C. glutamicum*.
124 Finally, GABA production reached 70.6 g.L^{-1} after 70 h of fermentation under a
125 two-stage pH control strategy.

126 Results and discussion

127 Construction of the engineered *C. glutamicum*

128 *Lactobacillus plantarum* GB 01-21 catalyzes the biotransformation of
129 γ-aminobutyric acid (GABA) with an excellent performance. The glutamate
130 decarboxylase (GAD) and pyridoxal kinase (PLK) are the key enzymes
131 involved in the conversion of L-glutamate to GABA. The enzyme GAD
132 catalyzes L-glutamate to GABA with pyridoxal-5'-phosphate as cofactor.
133 Pyridoxal kinase catalyzes ATP-dependent phosphorylation of pyridoxal to
134 produce pyridoxal-5'-phosphate, which is required by GAD [4]. Although
135 *Corynebacterium glutamicum* G01 is an excellent producer of L-glutamate
136 from glucose, it can not yield GABA due to the absence of GAD and PLK. In
137 order to produce GABA from glucose in one-step by *C. glutamicum*, the coding
138 genes of GAD and PLK from *L. plantarum* were cloned into *C. glutamicum.* The
139 GAD gene (*gad*) from the *L. plantarum* was inserted into the plasmid pDXW-10
140 to generate pDXW-*gad*. In the same way, the PLK gene (*plk*) from *L. plantarum*
141 was cloned into the plasmid pDXW-10 to create pDXW-*plk*. The recombinant
142 plasmids were verified by DNA sequencing.

143 Since L-arginine and L-proline are the main by-products during the

Green Chemistry Accepted Manuscript

144 biotransformation of GABA from L-glutamate by *C. glutamicum* G01, the
145 pathway to by-product pools need to be blocked to increase the carbon flux
146 from glucose to L-glutamate. Lazcano's group has reported that
147 N-acetyl-L-glutamate kinase (NAGK) is involved in the arginine biosynthesis
148 pathway, and the three enzymes, glutamate kinase (GAK),
149 glutamate-γ-semialdehyde dehydrogenase and pyrroline-5-carboxylate
150 reductase catalyzes the proline synthesis in bacteria [15]. L-lysine is the other
151 by-product during the production of L-glutamate from glucose in *C. glutamicum*
152 G01. Dihydropicolinate synthase (DHDPS) is a key enzyme for lysine
153 biosynthesis [15]. As described in Experimental, the recombination plasmids
154 pMD-R*arg*B, pMD-*R*proB and pMD-R*dap*A containing flanks of *argB* (R*argB*),
155 *proB* (R*proB*) and dapA (R*dapA*) were constructed [16]. So, by homologous
156 recombination technique, the coding genes of NAGK, GAK and DHDPS (*argB*,
157 *proB* and *dap*A) were expected to be knocked out.

158 The *gad* gene with the *tac* promoter from the plasmid pDXW-*gad* was
159 inserted into pMD-R*argB* and pMD-R*proB* to create the recombinant
160 pMD-R*arg*B::tacgad and pMD-R*pro*B::tacgad, respectively. The gene *plk* with
161 the *tac* promoter from pDXW-*plk* was cloned into the pMD-R*dap*A to create the
162 plamid pMD-*dap*A::tacplk. Then the R*arg*B::tacgad, R*pro*B::tacgad and
163 dapA::tacplk fragments were inserted into the suicide plasmid pK18*mobsac*B,
164 respectively, to construct the recombinant plasmids pK18-R*arg*B::tacgad,
165 pK18-R*pro*B::tacgad and pK18-*dap*A::tacplk. After verified by restriction

Green Chemistry Accepted Manuscript

166 enzymes analysis and DNA sequencing, the recombinant plasmids
167 pK18-R*arg*B::tacgad, pK18-R*pro*B::tacgad and pK18-*dap*A::tacplk were
168 introduced into *C. glutamicum* G01 one by one using electroporation method [17].
169 The recombinant *C. glutamicum* AG carrying pK18-R*arg*B::tacgad, *C.*
170 *glutamicum* APGG carrying pK18-R*arg*B::tacgad and pK18-R*proB*::tacgad, *C.*
171 *glutamicum* APLGGP carrying pK18-R*arg*B::tacgad, pK18-R*proB*::tacgad and
172 pK18-*dap*A::tacplk were constructed. In *C. glutamicum* AG, the *gad* gene was
173 introduced and the gene *argB* was deleted. In *C. glutamicum* APGG, the *gad*
174 gene with two copies was introduced and the *argB* and *proB* genes were
175 knocked out. In *C. glutamicum* APLGGP, the targets genes *gad* and *plk*
176 involved in the biotransformation of GABA from L-glutamate were introduced,
177 and the genes *argB*, *proB* and *dapA* involved in the by-product pathways of
178 L-arg, L-pro and L-lys were knocked out. More important, glutamate
179 decarboxylase gene was integrated into *C. glutamicum* APLGGP genome with
180 two copies.

181 **Identification of correct insertional mutation by enzyme**
182 **assays**

183 *C. glutamicum* is an important industrial producer of various amino acids with
184 great potential for the production of other metabolites. In order to identify if the
185 insertion mutation was correctly integrated into the *C. glutamicum* genome, the
186 specific activities of enzymes were determined using cell-free extracts of the
187 recombinant strain *C. glutamicum* AG, APGG and APLGGP. As shown in Table

Green Chemistry Accepted Manuscript

188 2, in the recombinant strain *C. glutamicum* AG, NAGK activity was observed,
189 while in the parent strain *C. glutamicum* G01, GAD activity was 2.13 U.mg^{-1},
190 but no any NAGK activity was detected. The result suggested the enzyme
191 GAD was expressed, while NAGK involved in the L-arg biosynthesis was
192 successfully knocked out in the recombinant *C. glutamicum* AG strain. In the
193 engineered *C. glutamicum* APGG, the activities of NAGK and GAK were not
194 observed, while the GAD activity was almost improved 2-fold (Table 2) due to
195 the introduction of an extra copy of *gad* and the deletion of NAGK and GAK
196 genes involved in the synthesis of L-arg and L-pro. In the recombinant *C.*
197 *glutamicum* APLGGP, GAD and PLK were active, but NAGK, GAK, and
198 DHDPS involved in the by-product pathways of L-arg, L-pro and L-lys were
199 inactive. More important, *C. glutamicum* APLGGP showed 4.2-fold PLK activity
200 higher than the other strains: wild-type, *C. glutamicum* AG and *C. glutamicum*
201 APGG, making it possible biotransformation of GABA without
202 pyridoxal-5'-phosphate. These above results indicated that the key enzymes,
203 GAD and PLK catalyzing L-glutamate to GABA were expressed, while the
204 NAGK, GAK, and DHDPS involved in the by-products L-arg, L-pro and L-lys
205 were successfully knocked out.
206 **Insert Table 2**

207 The insertional mutation had little effect on cell growth

208 Since L-arg, L-pro and L-lys have separate and distinct roles in the
209 microbial metabolism, the deletion of genes involved in their synthesis maybe

Green Chemistry Accepted Manuscript

210 affect on cell growth of the recombinant *C. glutamicum*. Meanwhile, the effects
211 of introduction of GAD and PLK into *C. glutamicum* on the cell-growth were
212 investigated. The fermentation characteristics of the recombinants had been
213 compared with the data from flask experiments under the same conditions.
214 Cell growth was determined by measuring the turbidity of the culture at OD_{560}
215 using a UV–visible spectroscopy system. Based on the three-stage division of
216 cell growth curves (Figure 2), the engineered *C. glutamicum* strains AG, APGG,
217 and APLGGP grew at a similar rate as compared to the parent strain. The
218 results suggested that the knock out of genes involved in L-arg, L-pro and L-lys
219 biosynthesis and introduction of the key genes of *gad* and *plk* related to the
220 biotransformation of GABA had nearly no effect on cell growth properties. The
221 insertion mutation in *C. glutamicum* did not inhibit cell growth. The insertion
222 mutation system based on the five enzymes, GAD, PLK, NAGK, GAK, and
223 DHDPS would be preferable for further studies of bioconversion of GABA from
224 glucose without exogenous cofactor.
225 **Insert Figure 2**

226 One-step preparation of GABA from glucose without 227 exogenous cofactor was realized by *C. glutamicum* APLGGP

228 By fermentation with glucose, GABA production was compared between the
229 engineered *C. glutamicum* and parental strain. The results are summarized in
230 Table 3. The strain *C. glutamicum* AG accumulated 45.8 g.L^{-1} GABA with a
231 productivity of 0.48 g·L^{-1}·h^{-1} in the fermented broth, while the wild-type *C.*

Green Chemistry Accepted Manuscript

232 *glutamicum* did not produce GABA after 80 h of fermentation (Table 3).
233 However, the strain *C. glutamicum* AG did not secrete L-arg into the
234 fermentation liquid, while the parental strain *C. glutamicum* G01 did (Table 3).
235 These results further confirmed that *L. plantarum* GAD was active, but NAGK
236 was inactive in the recombinant *C. glutamicum* AG. These above results
237 demonstrated that GAD expression is a key factor for enhancing the GABA
238 production, which is similar with the other reports [12]. More interesting, in the *C.*
239 *glutamicum* AG harboring one copy of GAD coding gene, 40.7 $g.L^{-1}$
240 L-glutamate was detected in the broth, suggesting that much L-glutamate was
241 not transformed to GABA.
242 Since the GAD enzyme is a key factor for biotransformation of GABA from
243 glucose, one extra copy of GAD gene was introduced into the recombinant *C.*
244 *glutamicum* APGG. In this recombinant strain, the by-product pathways of
245 L-arg and L-pro were also blocked to further improve GABA yield. As shown in
246 Table 3, the production of GABA reached 69.9 $g.L^{-1}$ in the broth of *C.*
247 *glutamicum* APGG after 80-h fed-batch cultivation. Compared to *C.*
248 *glutamicum* AG, the GABA production was further improved from 45.8 $g.L^{-1}$ to
249 69.9 $g.L^{-1}$, and L-glutamate was almost consumed by *C. glutamicum* APGG.
250 Meanwhile, L-arg and L-pro were not detected. The enhanced GABA yield is
251 due to the two reasons. First, the introduction of an extra copy of GAD induced
252 the higher GAD activity for the transformation of GABA from L-glutamate.
253 Second, the inactive NAGK and GAK by insertional mutation redistributed

Green Chemistry Accepted Manuscript

254 carbon flux of glucose to the GABA pathway. Shi *et al*. also improved GABA
255 production (27.13 g.L^{-1} at 120 h) by co-expressing *gadB*1 and *gadB*2 in *C.*
256 *glutamicum* ATCC13032 [13].

257 As the GAD requires pyridoxal-5'-phosphate as a cofactor for its activity [4],
258 PLK involved in the generation of pyridoxal-5'-phosphate and two copies of
259 GAD were introduced into the recombinant *C. glutamicum* APLGGP, in which
260 the relative genes involved in by-product pathways of L-arg, L-pro and L-lys
261 were knocked out. The fermentation results showed that *C. glutamicum*
262 APLGGP produced 70.6 g.L^{-1} GABA from glucose without addition of
263 pyridoxal-5'-phosphate. Compared with the fermentation results of *C.*
264 *glutamicum* APGG with addition of pyridoxal-5'-phosphate, *C. glutamicum*
265 APLGGP slightly improved GABA production without exogenous cofactor.
266 Thus, the one-step biotransformation of GABA from glucose was realized by
267 the recombinant strain *C. glutamicum* APLGGP.

268 **Insert Table 3**

269 Enhanced production of GABA through a two-stage pH control 270 strategy

271 During the biotransformation of GABA from glucose, L-glutamate is the
272 precursor of GABA. It is very important that maintain the neutral conditions to
273 steadily biosynthesize GABA from L-glutamate. Ammonia not only can take on
274 this responsibility, but also can be used as the nitrogen source to promote the
275 GABA production. Moreover, the L-glutamate dehydrogenase, which is critical

Green Chemistry Accepted Manuscript

276 for L-glutamate biosynthesis, exhibits the highest activity at pH 7.5, whereas at
277 pH 6.5, half of its activity is lost [18]. So ammonia supplementation has a great
278 influence on the biosynthesis of GABA from glucose. In this work, since
279 L-glutamate is rapidly synthesized within 48 h of fermentation, ammonia was
280 supplemented during 0–48 h of fermentation to remain the pH values at 7.3. As
281 shown in Figure 3A, it was found that time profiles of GABA production could
282 be divided into two stages. At the first 48 h, pH values were constant at about
283 7.3, and L-glutamate was quickly accumulated, while GABA was produced
284 very slowly. When ammonia supplement was stopped, the pH value drop
285 rapidly to about 5.4, GABA began to secret to the fermentation broth.

286 It was reported that the enzyme GAD shows activity in a relatively acid
287 condition[4, 13]. Ma *et al*. observed that GAD exhibited a stringent pH
288 dependence for substrate transport and functions at acidic pH, without activity
289 above pH 6.5 [12]. Seo *et al*. found that the optimal pH of GAD activity was 5.2 [19].
290 We further determined the effects on GABA conversion in the range of pH
291 4.0-5.8. As shown in Figure 4, GAD showed the highest activity was at about
292 pH 4.8, and the conversion rate of GABA was highest at pH 4.6-5.0. Basing on
293 the above pH preference, a two-stage control strategy was proposed in favour
294 of GABA formation. At the first 48 h, the pH value was controlled at about 7.3
295 for L-glutamate formation, and then pH was adjusted to 4.8 to promote GABA
296 accumulation. As expected, through the strategy, the GABA production was up
297 to 70.6 $g \cdot L^{-1}$ after 70 h fed-batch cultivation, and the GABA formation rate was

Green Chemistry Accepted Manuscript

298 up to 1.04 $g{\cdot}L^{-1}{\cdot}h^{-1}$ (Figure 3B), which is the highest production report of GABA
299 with glucose as substrate in one-step. Thus, an excellent GABA producer from
300 glucose was redesigned through combining the metabolic modules (from
301 L-glutamate to GABA) in *L. plantarum* and *C. glutamicum* (from glucose to
302 L-glutamate), and blocking the by-product pathways of L-arg, L-pro and L-lys.
303 The engineered *C. glutamicum* directly conversed glucose to GABA without
304 exogenous cofactor through a two-stage pH control strategy.

305 **Insert Figure 3**

306 **Insert Figure 4**

307 ## Conclusions

308 An excellent producer of GABA was successfully redesigned by grafting the
309 metabolic modular of L-glutamate from glucose in *L. plantarum* into *C.*
310 *glutamicum*, making the engineered *C. glutamicum* highly yield GABA from
311 glucose in one-step. Furthermore, the by-product pathways of L-arg, L-pro and
312 L-lys were blocked to redistribute the metabolic flux to GABA, and a two-stage
313 pH control strategy was proposed basing on the pH preference of the key
314 enzymes involved in the GABA synthesis. The production of GABA reached up
315 to 70.6 $g{\cdot}L^{-1}$ after 70 h. More important, *C. glutamicum* is a GRAS
316 microorganism and it engineered strain can grow in no-selective medium. So
317 the newly designed *C. glutamicum* system is an excellent GABA producer from
318 glucose in food and pharmaceutical industry. This work is hoped to pave the
319 way to redesign the bioreactor for efficient one-step preparation of GABA from

Green Chemistry Accepted Manuscript

320 glucose.

321 Experimental

322 General information

323 All chemicals, *i.e.*, glutamate, N-Acetyl-L-glutamate and pyridoxal *et al.* were
324 purchased from the Sigma-Aldrich Chemical Co. Inc (Shanghai, China), and
325 used without further purification. The precise determination of the amino acids
326 was carried out on an automatic amino acid analyzer by high-performance
327 liquid chromatography.

328 Organisms and growth conditions

329 *C. glutamicum* G01 has been deposited in the China Center for Type Culture
330 Collection (CCTCC) under collection number CCTCC M2013418. *E. coli*
331 JM109 strain was grown at 37 °C in Luria-Bertani (LB) medium with ampicillin
332 (50 $\mu g{\cdot}mL^{-1}$) and/or kanamycin (50 $\mu g{\cdot}mL^{-1}$). *C. glutamicum* was grown in LBG
333 medium (LB medium supplemented with 5 $g{\cdot}L^{-1}$ glucose) at 30°C with 30
334 $\mu g{\cdot}mL^{-1}$ kanamycin if necessary. The competent cells of *C. glutamicum* were
335 cultured in the LB medium with 30 $g{\cdot}L^{-1}$ glycine and 1 $g{\cdot}L^{-1}$ Tween 80.

336 Culture conditions for L-glutamate and GABA fermentation from glucose

337 *C. glutamicum* strains were subcultured weekly and stored at 4 °C or frozen at
338 −70 °C in 15 % (v/v) glycerol. Stock and inoculum cultures were grown at 30°C
339 with shaking at 160 rpm in seed medium (25 $g{\cdot}L^{-1}$ glucose, 20 $g{\cdot}L^{-1}$ corn steep
340 liquor, 10 $g.L^{-1}$ yeast extract, 6 $g.L^{-1}$ urea, 1.5 $g.L^{-1}$ $K_2HPO_4{\cdot}3H_2O$, 0.4 $g.L^{-1}$
341 $MgSO_4{\cdot}7H_2O$, pH 7.5) for 12 h. The incubated seed culture was then

Green Chemistry Accepted Manuscript

342 inoculated (4% v/v) into the fermentation medium (160 $g.L^{-1}$ glucose, 30 $g.L^{-1}$
343 corn steep liquor, 10 $g.L^{-1}$ yeast extract, 5.5 $g.L^{-1}$ urea, 1.5 $g.L^{-1}$ $K_2HPO_4{\cdot}3H_2O$,
344 0.8 $g.L^{-1}$ $MgSO_4{\cdot}7H_2O$, 0.02 $g.L^{-1}$ $MnSO_4{\cdot}4H_2O$, 0.02 $g.L^{-1}$ $FeSO_4{\cdot}7H_2O$, 200
345 $\mu g.L^{-1}$ vitamin B_1, 0.1 $mmol.L^{-1}$ pyridoxal-5′-phosphate, pH 7.5). Only for the
346 recombinants carrying pyridoxal kinase gene, 0.1 $mmol.L^{-1}$
347 pyridoxal-5′-phosphate was not added into the fermentation medium. For all
348 the recombinant strains, 1 $\mu mol.L^{-1}$ Isopropyl β-D-1-thiogalactopyranoside was
349 added to fermentation medium to induce GAD expression. Fed-batch
350 fermentation was carried out in a 5-L bioreactor (BIOTECH-2002, Baoxing
351 Biological Equipment Co., Shanghai, China) containing 2.0-L initial medium at
352 30 °C, 350 rpm and airflow rate 0.66 vvm. Ammonia (NH_4OH) was added
353 during the first 48 h of fermentation to maintain the alkaline environment. And
354 then, the addition of ammonia was stopped to form an acid fermentation
355 environment, which was suitable for the GABA production. Glucose at 80 %
356 (w/v) was fed into the bioreactor to maintain the glucose concentration
357 between 20 and 30 $g.L^{-1}$ from 16–44 h.

358 **Plasmid construction and gene deletion**

359 The glutamate decarboxylase gene (*gad*) from *L. plantarum* GB 01-21 was
360 amplified by PCR using primers gadP1 and gadP2. The amplified *gad* gene
361 was inserted into the *Nhe* I and *Bgl* II sites of the pDXW-10 plasmid to create
362 pDXW-*gad*. For deletion of *argB* and *proB* in *C. glutamicum* G01 by
363 homologous recombination, the recombination flanks of *argB* (R*argB*) and

Green Chemistry Accepted Manuscript

364 *proB* (R*proB*) were constructed and individually cloned into pMD18-T vector
365 (Takara Co., Dalian, China), resulting in the vectors pMD-RargB and
366 pMD-*R*proB. Recombination flanks of argB and proB were individually
367 amplified from the *C. glutamicum* G01 chromosome by PCR as follows: flank A
368 (445 bp) of *argB* and flank A (646 bp) of *proB* were generated with the primer
369 pairs argBP1/argBP2 and proBP1/proBP2, respectively; flank B (529 bp) of
370 *argB* and flank B (613 bp) of *proB* were created with the primer pairs
371 argBP3/argBP4 and proB3/proBP4, respectively; the recombination flanks
372 were then constructed by splicing of the corresponding flanks A and B. The *Bgl*
373 II restriction site was introduced into the splicing site of flanks A and B. The *gad*
374 gene, containing the *tac* promoter from the pDXW-*gad* plasmid, was then
375 PCR-amplified using the primers tacgadP and gadP2. The amplified tac-*gad*
376 gene was inserted into the pMD-R*argB* and pMD-R*proB* plasmids to create the
377 pMD-R*arg*B::tacgad and pMD-RproB::tacgad plasmids, respectively. The
378 pyridoxal kinase gene (*plk*) from *L. plantarum* GB 01-21 was amplified using
379 primers plkP1 and plkP2. The amplified *plk* gene was inserted into the *Pst* I
380 and Hind III sites of the pDXW-10 plasmid to create the pDXW-*plk* plasmid.
381 The *plk* gene, containing the *tac* promoter from the pDXW-*plk* plasmid, was
382 then amplified using the primers tacplkP and plkP2. The dihydropicolinate
383 synthase gene (*dapA*) from *C. glutamicum* G01 was amplified using primers
384 dapAP1 and dapAP2. The amplified *dap*A gene was cloned into pMD18-T
385 vector to create pMD-dapA. The amplified tac-*plk* gene was inserted into the

Green Chemistry Accepted Manuscript

386 pMD-dapA plasmid to create the plasmid pMD-dapA::tacplk.

387 The pK18-R*arg*B::tacgad, pK18-RproB::tacgad and pK18-*dap*A::tacplk
388 vectors were created by inserting a R*arg*B::tacgad, RproB::tacgad and
389 dapA::tacplk fragments of pMD-R*arg*B::tacgad digested by *Xba* I and *Hind* III,
390 pMD- RproB::tacgad digested by *Xba* I and *Hind* III and dapA::tacplk digested
391 by *Xba* I and Sal I (agarose-purified, Gel DNA Purification Kit, Takara) into
392 same restriction enzymes linearized pK18*mobsac*B, respectively. The deletion
393 of *argB*, *proB* and *dapA* and introduction of *gad* and *plk* genes in *C.*
394 *glutamicum* were preformed by homologous recombination [20]. The genetic
395 modifications were verified by enzyme assays and gene sequencing. The
396 primers (restriction sites were underlined) used in this work are listed in Table
397 S1 in the Supporting information. All the oligonucleotide primers were
398 synthesized at Takara Co. (Dalian, China).

399 **Construction of the recombinant *C. glutamicum* strains**

400 By electroporation, the plasmid pK18-R*arg*B::tacgad was introduced into the
401 competent cells of *C. glutamicum* G01 to generate the strain *C. glutamicum* AG,
402 in which the *gad* gene was introduced and *arg*B was knocked out. The
403 recombinant pK18-R*arg*B::tacgad and pK18-R*pro*B::tacgad were both
404 introduced into *C. glutamicum* G01 to construct *C. glutamicum* APGG, in which
405 two copies of *gad* genes were introduced, and *arg*B and *pro*B were deleted.
406 The recombinant pK18-R*arg*B::tacgad, pK18-R*pro*B::tacgad and
407 pK18-*dap*A::tacplk were simultaneously introduced into *C. glutamicum* G01 to

Green Chemistry Accepted Manuscript

408 create *C. glutamicum* APLGGP, in which *plk* gene and two copies of *gad* genes
409 were introduced, and *arg*B, *pro*B and *dap*A were knocked out.

410 **Enzyme Assays**

411 The activities of GAD, NAGK, GAK, PLK and DHDPS were assayed according
412 to previously reported methods [21, 22]. All mesurements were repeated at least
413 three times.

414

415 ## Acknowledgements

416 This work was supported by the Program for New Century Excellent Talents in
417 University (NCET-10-0459, NCET-13-0833), the National Basic Research
418 Program of China (973 Program) (2012CB725202), the National Natural
419 Science Foundation of China (21276110 and 31370100), the Research Project
420 of Chinese Ministry of Education (113033A), the Fundamental Research Funds
421 for the Central Universities (JUSRP51306A and JUSRP21121), the 111 Project
422 (111-2-06) and a Project Funded by the Priority Academic Program
423 Development of Jiangsu Higher Education Institution.

424

425 **Notes and references**

426 1 (*a*) I. Mody, Y. De Koninck, T. Otis and I. Soltesz, *Trends Neurosci.*, 1994,
427 **17**, 517-525; (*b*) C. Wong, T. Bottiglieri and O. Snead, *Ann. Neurol.*, 2003,
428 **54**, S3–S12; (*c*) Y. Cao and H. Li, *Amino Acids*, 2010, **29**, 1107-1116.
429 2 (*a*) S. I. Choi, J. W. Lee, S. M. Park, M. Y. Lee, G. J.I, M. S. Park and T. R.

Green Chemistry Accepted Manuscript

430 Heo, *J. Microbiol. Biotechnol.*, 2006, **16**, 562-568; (*b*) J. Huang, L. Mei, H.
431 Wu and D. Lin, *World J. Microbiol. Biotechnol.*, 2007, **23**, 865-871; (*c*) K.
432 Inoue, T. Shirai, H. Ochiai, M. Kasao, K. Hayakawa, M. Kimura and H.
433 Sansawa, *Eur. J. Clin. Nutr.*, 2003, **57**, 490-495; (*d*) H. Ueno, *J. Mol. Catal.*
434 *B: Enzym.*, 2000, **10**, 67-79.
435 3 (*a*) G. Capitani, D. De Biase, C. Aurizi, H. Gut, F. Bossa and G. Gruetter,
436 *EMBO J.*, 2003, **22**, 4027-4037; (*b*) Y. Su, J. Wang, T. Lin and T. Pan, *J. Ind.*
437 *Microbiol. Biotechnol.*, 2003, **30**, 41-46; (*c*) K. Masuda, X.-f. Guo, N. Uryu, T.
438 Hagiwara and S. Watabe, *Biosci, Biotech. Bioch.*, 2008, **72**, 3265-3272.
439 4 (*a*) G. Battaglioli, H. Liu and D. Martin, *J. Neurochem.*, 2003, **86**, 879-887;
440 (*b*) P. Cheung, C. Fong, K. Ng, W. Lam, Y. Leung, C. Tsang, M. Yang and M.
441 Wong, *J. Biochem.*, 2003, **134**, 731-738.
442 5 G. Fenalti, R. H. P. Law, A. M. Buckle, C. Langendorf, K. Tuck, C. J. Rosado,
443 N. G. Faux, K. Mahmood, C. S. Hampe, J. P. Banga, M. Wilce, J.
444 Schmidberger, J. Rossjohn, O. El-Kabbani, R. N. Pike, A. I. Smith, I. R.
445 Mackay, M. J. Rowley and J. C. Whisstock, *Nat. Struct. Mol. Biol.*, 2007, **14**,
446 280–286.
447 6 S. Siragusa, M. De Angelis, R. Di Cagno, C. Rizzello, R. Coda and M.
448 Gobbetti, *Appl. Environ. Microbiol.,* 2007, **73**, 7283-7290.
449 7 H. Li, T. Qiu, D. Gao and Y. Cao, *Amino Acids*, 2010a, **38**, 1439-1445.
450 8 (*a*) K. Park and S. Oh, *Bioresource Technol.*, 2007, **98**, 1675-1679; (*b*)
451 H. Li, T. Qiu, D. Gao and Y. Cao, *Amino Acids*, 2010a, **38**, 1439-1445; (*c*) Y.

Green Chemistry Accepted Manuscript

452 Zhang, L. Song, Q. Gao, S. Yu, L. Li and N. Gao, *Appl. Microbiol.*
453 *Biotechnol.*, 2012, **94**, 1619-1627.
454 9 S. Yang, F. Lü, Z. Lu, X. Bie, Y. Jiao, L. Sun and B. Yu, *Amino Acids*, 2008,
455 **34**, 473-478.
456 10 M.-C. Kook, *J. Korean Appl. Biol. Chem.*, 2010, **53**, 816-820.
457 11 C. Takahashi, J. Shirakawa, T. Tsuchidate, N. Okai, K. Hatada, H.
458 Nakayama, T. Tateno, C. Ogino and A. Kondo, *Enzyme Microb. Tech.*, 2012,
459 **51**, 171-176.
460 12 F. Shi and Y. Li, *Biotech. Lett.*, 2011, **33**, 2469-2474.
461 13 F. Shi, J. Jiang, Y. Li and Y. Xie, *J. Ind. Microbiol. Biotechnol.*, 2013, DOI
462 10.1007/s10295-10013-11316-10290.
463 14 (*a*) T. Liu, T. Yang, S. Zhang, H. Xia and Z. Rao, *J. Food Sci. Biotechnol.*,
464 2010, **29**, 742-747; (*b*) L. Tian, M. Xu and Z. Rao, *Chin. J. Biotechnol.*,
465 2012, **1**, 65-75.
466 15 (*a*) V. Sakanyan, P. Petrosyan, M. Lecocq, A. Boyen, C. Legrain, M.
467 Demarez, J. N. Hallet and N. Glansdorff, *Microbiology*, 1996, **142**,
468 99-108;(*b*) E. Adams and L. Frank, *Ann. Rev. Biochem.*, 1980, **49**,
469 1005-1061; (*c*) A. M. Velasco, J. I. Leguina and A. Lazcano, *J. Mol. Evol.*,
470 2002, **55**, 445-449.
471 16 K. L. Heckman and L. R. Pease, *Nat. Protocols*, 2007, **2**, 924-932.
472 17 A. Schäfer, A. Tauch, W. Jäger, J. Kalinowski, G. Thierbachb and A. Pühler,
473 *Gene*, 1994, **145**, 69-73.

Green Chemistry Accepted Manuscript

474 18 (*a*) S. Delaunay, P. Gourdon, P. Lapujade, E. Mailly, E. Oriol and J.
475 Engasser, *Enzyme Microb. Technol.*, 1999, **25**, 762-768; (*b*) E. Bormann, B.
476 Eikmanns and H. Sahm, *Mol. Microbiol.*, 1992, **6**, 317-326.
477 19 M. J. Seo, Y. D. Nam, S. Y. Lee, S. L. Park, S. H. Yi and S. I. Lim, *Biosci.*
478 *Biotechnol. Biochem.*, 2013, **77**, 853-856.
479 20 (*a*). A. Schäfer, A. Tauch, W. Jäger, J. Kalinowski, G. Thierbach and A.
480 Pühler, *Gene*, 1994, **145**, 69-73; (*b*) J. Nešvera and M. Pátek, *Appl. Microb.*
481 *Biotech.*, 2011, **90**, 1641-1654.
482 21 D. McCormick, M. Gregory and E. Snell, *J. Biol. Chem.*, 1961, **236**,
483 2076-2084.
484 22 (*a*) S. Kitaoka and Y. Nakano, *J. Biochem.*, 1969, **66**, 87-94; (*b*) M. Xu, Z.
485 Rao, W. Dou, J. Yang, J. Jin and Z. Xu, *Amino Acids*, 2012, **43**, 255-266;
486 (*c*) C. Smith, A. Deutch and K. Rushlow, *J. Bacteriol.*, 1984, **157**, 545-551;
487 (*d*) C. Dereppe, G. Bold, O. Ghisalba, E. Ebert and H.-P. Schär, *Plant*
488 *Physiol.*, 1992, **98**, 813-821.
489

Green Chemistry Accepted Manuscript

490 **Figure captions**

491 **Figure 1** The strategy for designed *C. glutamicum* to produce GABA from
492 glucose in one-step.
493 *Lactobacillus plantarum* CCTCC M209102 is a good producer γ-aminobutyric
494 acid (GABA) from L-glutamate. The glutamate decarboxylase and pyridoxal
495 kinase are involved in the above biotransformation. Glutamate decarboxylase
496 biotransforms L-glutamate to GABA with pyridoxal-5'-phosphate as cofactor.
497 Pyridoxal catalyzes ATP-dependent phosphorylation of pyridoxal to generate
498 pyridoxal-5'-phosphate, which is supplied for the reaction by glutamate
499 decarboxylase. *Corynebacterium glutamicum* G01 is an excellent producer of
500 L-glutamate from glucose. However, it can not yield GABA from L-glutamate
501 due to the absence of glutamate decarboxylase and pyridoxal kinase. To
502 realize the efficient one-step production of GABA from glucose, the *C.*
503 *glutamicum* reactor is redesigned. The metabolic module from L-glutamate to
504 GABA in *L. plantarum* was grafted into *C. glutamicum*, making *C. glutamicum*
505 prepare GABA from glucose in one-step. FA, fragment of *argB::tacgad*; FB,
506 fragment of *proB::tacgad*; FC, fragment of *plk*::*tacdap*A.
507 **Figure 2** Growth curves of *C. glutamicum* G01, AG, APGG and APLGGP. The
508 engineered strains were cultivated at 37℃ in 2.5 L flask bottle with an initial
509 working volume of 1.0 L. Error bars represent standard deviations (n = 3)
510 **Figure 3** Fed-batch fermentation of GABA by the recombinant *C. glutamicum*
511 APLGGP. **(A)** pH value was controlled at 7.3 until 48 h, and then not controlled;

Green Chemistry Accepted Manuscript

512 **(B)** pH value was controlled at 7.3 until 48 h, and then controlled at 4.8. The
513 strains were cultured in 5-L bioreactor at 37 °C.
514 **Figure 4** Effects of pH on conversion rate of GABA
515
516 **Tables:**
517 **Table 1 The reported methods and productivity of GABA**
518 **Table 2** Crude enzyme activities in the recombinant strains
519 **Table 3** GABA production by the recombinant strains
520
521
522
523
524
525
526
527
528
529
530
531
532
533

Green Chemistry Accepted Manuscript

534 **Table 1** The reported methods and productivity of GABA

Strains	Engineered methods	Substrates	Fermentation durations (h)	GABA (g/l)	References
Lactobacillus brevis NCL912	Wild-type	Sodium L-glutamate	48	35.66	7
L. brevis TCCC13007	Wild-type	Monosodium glutamate	66	38	6
Corynebacterium glutamicum	Expressing GAD gene	L-glutamate	60	26.32	13
Lactobacillus Sakei	Expressing GAD gene	L-glutamate	48	27.3	10
C. glutamicum	Expressing GAD gene	Glucose	72	12.37	11
C. glutamicum ATCC 13032	Expressing GAD gene	Glucose	72	2.15	12
Escherichia coli	Expressing GAD gene	L-glutamate	24	204.5	14

535
536
537
538
539
540
541
542
543
544
545

Green Chemistry Accepted Manuscript

546 **Table 2** Crude enzyme activities in the recombinant strains

Strains	Specific activities (U/mg protein)				
	GAD	NAGK	GAK	DHDPS	PLK
G01	-	0.30 ± 0.02	0.22 ± 0.02	0.21 ± 0.01	0.11 ± 0.01
AG	2.13 ± 0.13	-	0.23 ± 0.02	0.20 ± 0.01	0.10 ± 0.01
APGG	4.20 ± 0.25	-	-	0.21 ± 0.01	0.10 ± 0.01
APLGGP	4.18 ± 0.23	-	-	-	0.43 ± 0.03

547 **Notes:** In the recombinant *C. glutamicum* AG, the NAGK gene was knocked out and GAD
548 gene was expressed. In the recombinant *C. glutamicum* APGG, the NAGK and GAK
549 genes were knocked out, and two copies of GAD genes were expressed. In the
550 recombinant *C. glutamicum* APLGGP, the genes of NAGK, GAK and DHDPS were
551 knocked out, and PLK and two copies of GAD were expressed.
552 GAD, Glutamate decarboxylase; NAGK, N-Acetyl-L-glutamate kinase; GAK, Glutamate
553 kinase; DHDPS, Dihydropicolinate synthase; PLK, Pyridoxal kinase;
554 -, non-detected.

Green Chemistry Accepted Manuscript

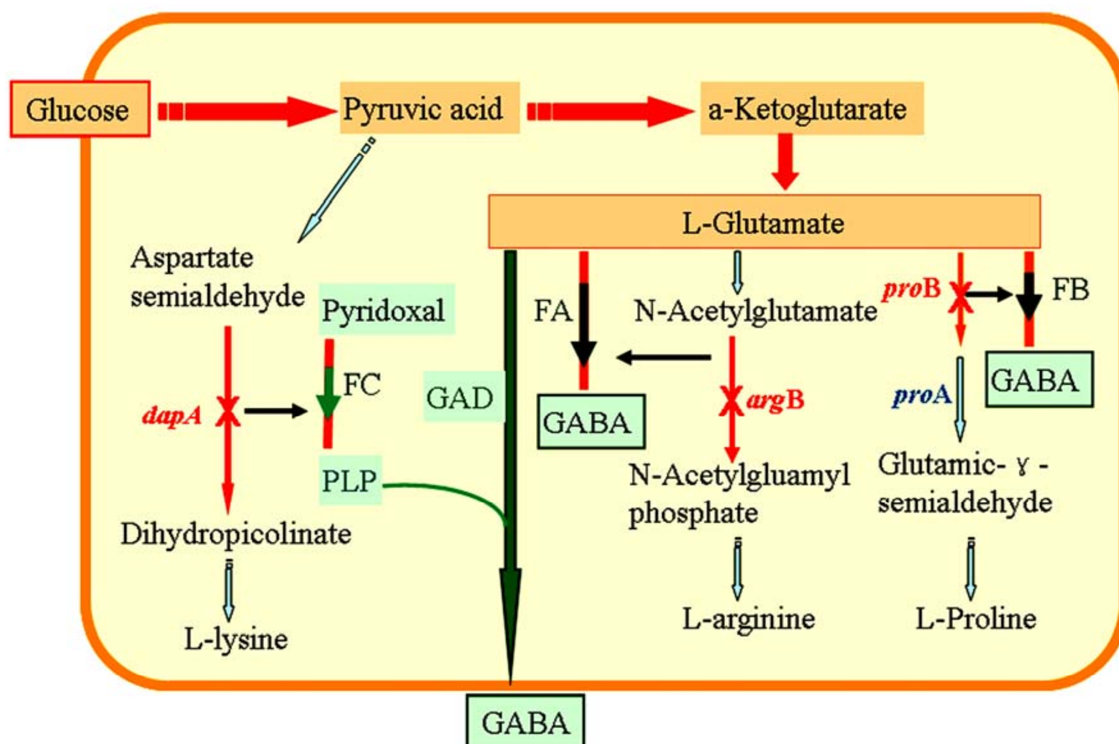
555 **Table 3** GABA production by the recombinant strains

Strains	L-glu (g/l)	L-arg (g/l)	L-pro (g/l)	L-lys (g/l)	GABA (g/l)
G01	106.3 ± 3.3	5.2 ± 0.3	4.3 ± 0.2	3.1 ± 0.2	-
AG	40.7 ± 1.2	-	4.5 ± 0.3	3.3 ± 0.2	45.8 ± 1.3
APGG	0.6 ± 0.1	-	-	3.2 ± 0.2	69.9 ± 1.9
APLGGP	0.5 ± 0.1	-	-	-	70.6 ± 2.1

556 **Notes:** The recombinant *C. glutamicum* APLGGP catalyzes the biotransformation without
557 addition of pyridoxal-5'-phosphate, while the other strains catalyzes the reaction with an
558 appropriate amount of pyridoxal-5'-phosphate.
559 **-**, non-detected.
560
561
562
563
564
565
566
567
568
569
570
571

Green Chemistry Accepted Manuscript

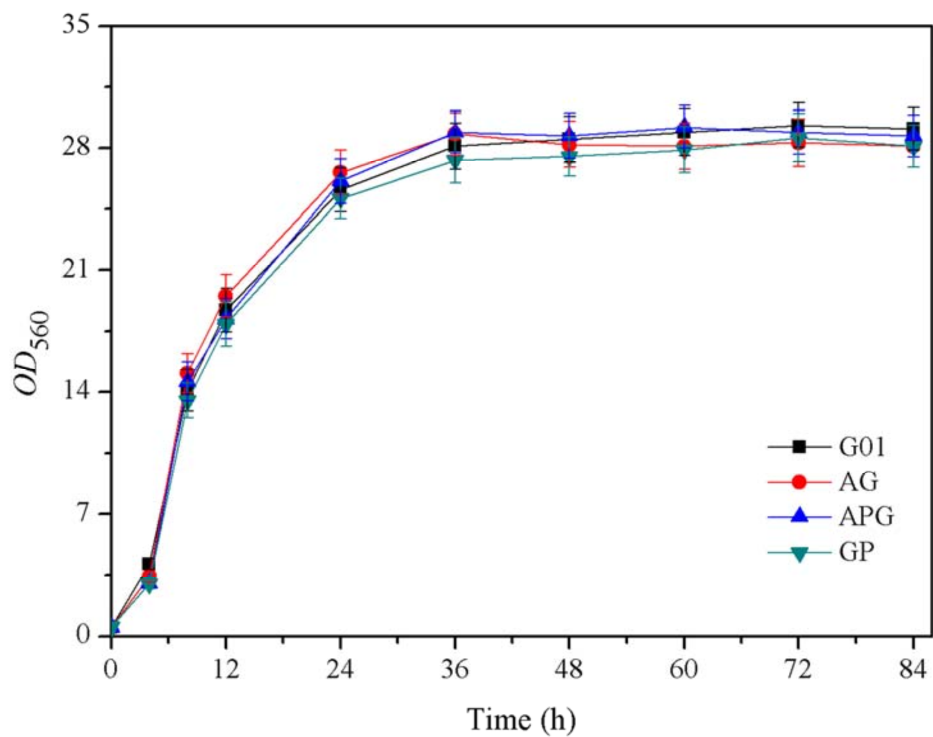
572 **Figure 1** The strategy for designed *C. glutamicum* to produce GABA from
573 glucose in one-step.



574
575
576
577
578
579
580
581
582
583
584
585

Green Chemistry Accepted Manuscript

586 **Figure 2** Growth curves of *C. glutamicum* G01, AG, APGG and APLGGP.

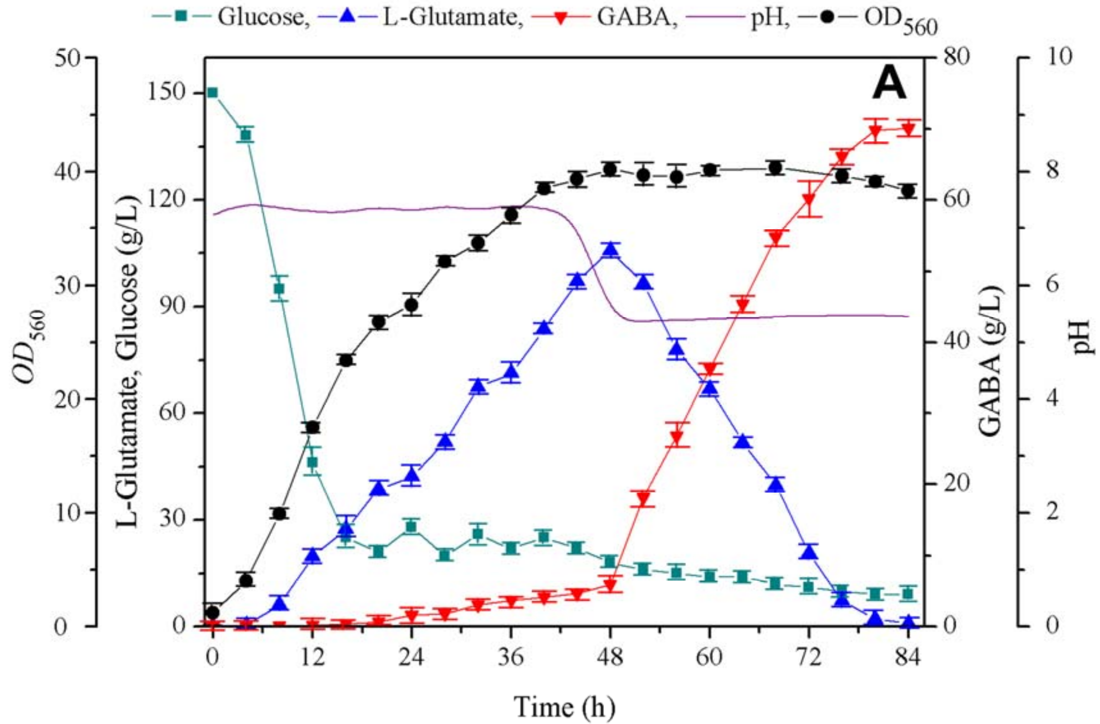


587
588
589
590
591
592
593
594
595
596
597
598

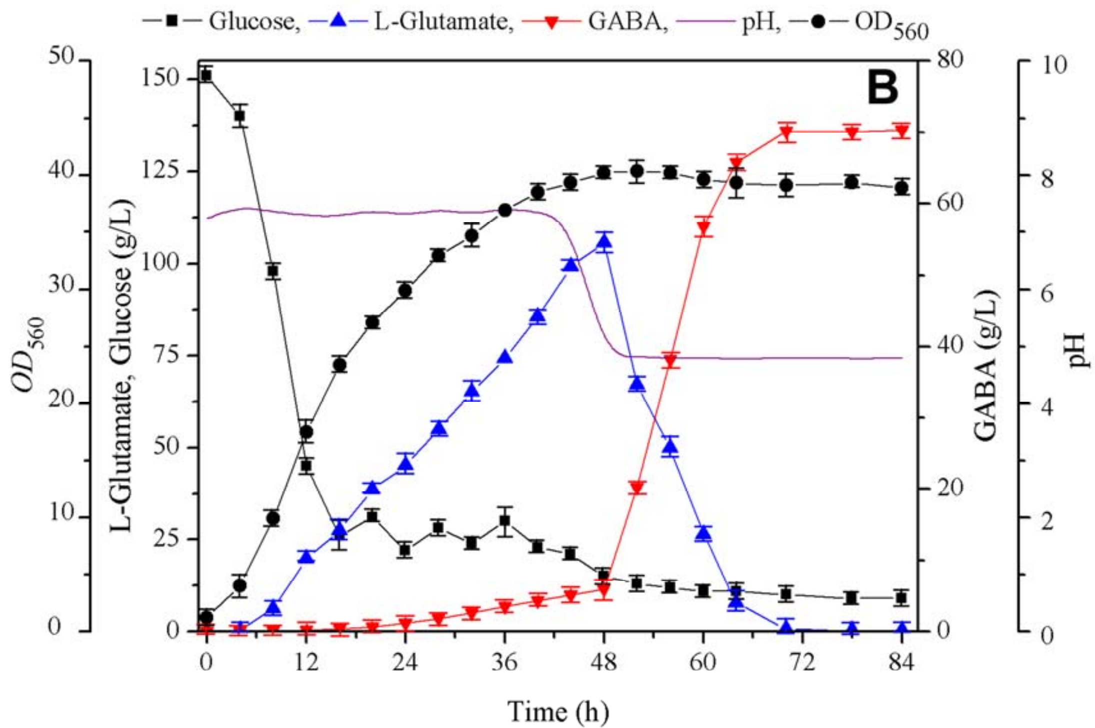
Green Chemistry Accepted Manuscript

599 **Figure 3** Fed-batch fermentation of GABA by the recombinant *C. glutamicum*

600 APLGGP.



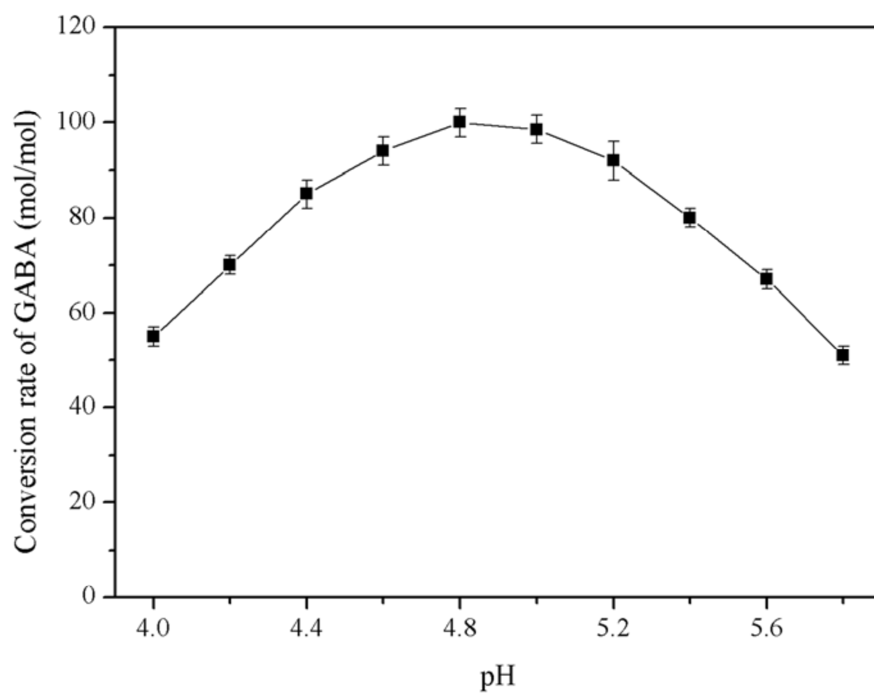
601



602

Green Chemistry Accepted Manuscript

603 **Figure 4** Effects of pH on conversion rate of GABA



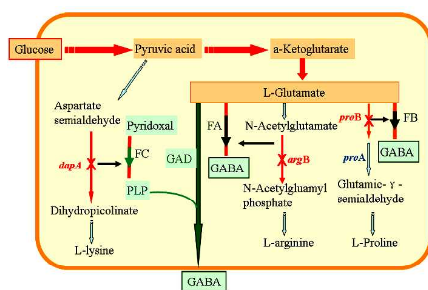
604

Green Chemistry Accepted Manuscript

Efficient one-step Preparation of γ-Aminobutyric Acid from Glucose without Exogenous Cofactor by the Designed *Corynebacterium glutamicum*

Rongzhen Zhang, Taowei Yang, Zhiming Rao*, Hongmei Sun, Meijuan Xu, Xian Zhang, Zhenghong Xu, Shangtian Yang

Text: Efficient one-step production of γ-aminobutyric acid from glucose without exogenous cofactor pyridoxal-5'-phosphate was realized by the designed Corynebacterium glutamicum.



Green Chemistry Accepted Manuscript