Metabolic engineering of microorganisms for the production of multifunctional non-protein amino acids: γ-aminobutyric acid and δ-aminolevulinic acid

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Summary
Gamma-aminobutyric acid (GABA) and delta-aminolevulinic acid (ALA), playing important roles in agriculture, medicine and other fields, are multifunctional non-protein amino acids with similar and comparable properties and biosynthesis pathways. Recently, microbial synthesis has become an inevitable trend to produce GABA and ALA due to its green and sustainable characteristics. In addition, the development of metabolic engineering and synthetic biology has continuously accelerated and increased the GABA and ALA yield in microorganisms. Here, focusing on the current trends in metabolic engineering strategies for microbial synthesis of GABA and ALA, we analysed and compared the efficiency of various metabolic strategies in detail. Moreover, we provide the insights to meet challenges of realizing industrially competitive strains and highlight the future perspectives of GABA and ALA production.

Introduction
Belonging to the non-protein amino acid, both gamma-aminobutyric acid (GABA) and delta-aminolevulinic acid (ALA) are not coded by DNA, but have great similar and important multifunction in overall metabolism of plants, animals and even humans (Sasaki, 2002; Kang et al., 2012; Rashmi et al., 2018). In plants, GABA is an endogenous signalling molecule involved in various physiological and biochemical processes that plays an important role in the promotion of plant growth and development, and relates to the plant metabolism in response to adverse environmental conditions (Fait et al., 2008; Li et al., 2016b). Similarly, ALA as the first product of tetrapyrrole biosynthesis can effectively control the biosynthesis of chlorophyll and plays vital functions in plant photosynthesis and cellular energy metabolism (Wu et al., 2018). Based on these characters, GABA and ALA have been used as safe, environmentally compatible and biodegradable novel plant growth regulator (PGR) in agriculture (Meng et al., 2016; Li et al., 2019). In animals and humans, GABA as an important inhibitory neurotransmitter in mammalian nervous systems has great potential to be used as an anti-diabetic, anti-hypertensive, relaxation and immunity enhancing molecule (Yuan and Alper, 2019). Likewise, ALA as the second generation of photosensitizer with good curative effects and less side-effects has been widely applied as photodynamic medicine for cancer therapy and tumour localizing (Thunshelle et al., 2016; Inoue, 2017). In addition, GABA and ALA are also used as widely available additives in food, feed, fertilizer and cosmetics (Kang et al., 2017; Diez-Gutierrez et al., 2020).

Due to their wide function and significant application, GABA and ALA have become popular value-added products with increasing demand, and their anticipated global market size will be increased up to 50 million USD (https://www.qyresearch.com/index/detail/2135154/gamma-aminobutyric-acid-market) and 222.1 million USD (https://www.qyresearch.com/index/detail/1932502/delta-aminolevulinic-acid-market) by 2026 respectively.
However, the current international market of GABA and ALA still relies heavily on their chemical synthesis, which is a complicated procedure with pollution, high price, low production, and potential unsafety to animals and humans (Noh et al., 2017; Kim et al., 2018). To conquer those problems, microbial syntheses of GABA and ALA have become an inevitable trend based on their resource-conserving, environment-friendly and economically sustainable characteristics. Moreover, the rapid development of synthetic biology and metabolic engineering has accelerated the overproduction of GABA and ALA by microbes to constantly satisfy the demands of the growing global market.

Therefore, in view of the fact that GABA and ALA have some common characteristics, this paper reviewed the recent progress of metabolic engineering of microorganisms for their production, especially focusing on the advances of metabolic engineering strategies of microbes in GABA and ALA syntheses. Furthermore, the challenges and prospects of industrial production of GABA and ALA were also analysed with highlighting their potential application as novel PGR in agriculture.

**Biosynthesis pathways of GABA and ALA**

GABA and ALA are 4-carbon and 5-carbon non-protein amino acids respectively. And the only difference in their chemical structures is that ALA has one more carbonyl group (C = O) than GABA. Both GABA and ALA have two alternative biosynthesis pathways, and their overall biosynthesis pathways are shown in Fig. 1.

GABA is naturally biosynthesized via glutamic acid decarboxylation (GAD) pathway (Choi et al., 2015) or putrescine (Puu) pathway (Jorge et al., 2016). In GAD pathway, GABA is synthesized by decarboxylation of glutamate via glutamate decarboxylase (GAD) (Fig. 1). GAD is a pyridoxal 5'-phosphate (PLP)-dependent enzyme composed of six identical subunits, which have strict substrate specificity towards glutamate and is the only key rate-limiting enzymes during the GABA biosynthesis (Yu et al., 2019). In some bacteria, GABA has two isoforms named GadA and GadB (Wu et al., 2017; Lyu et al., 2018). Additionally, the gadC gene encodes the GABA antiporter (Soma et al., 2017). GAD pathway is common and usually exists in Lactobacillus spp., Escherichia coli and Listeria monocytogenes (Diez-Gutierrez et al., 2020). In Puu pathway, the precursor putrescine can be converted into GABA in two sequential reactions catalysed by putrescine transaminase (PatA) and γ-aminobutyraldehyde dehydrogenase (PatD) (Jorge et al., 2016) (Fig. 1). Puu pathway is not common and has been reported in E. coli (Cha et al., 2014) and Aspergillus oryzae (Akasaka et al., 2018). Lastly, GABA is decomposed to succinic semialdehyde (SSA) by GABA aminotransferase (GabT) and succinate there after by SSA dehydrogenase (GabD). Additionally, the gabF and gabC genes encode the GABA-specific importer and antiporter respectively (Fig. 1) (Shi et al., 2017).

Likewise, ALA is naturally biosynthesized via C4 or C5 pathway (Li et al., 2016a). In the C4 pathway (the Sheinin pathway), ALA is produced through one-step catalysis of ALA synthase (ALAS, encoded by hemA or ALAS gene) under the presence of essential cofactor PLP from the condensation of succinyl-CoA and glycine (Fig. 1). In this pathway, ALAS is the only rate-limiting key enzyme occurring in mammalian, fungi (like yeasts) (Hara et al., 2019) and purple non-sulfur photosynthetic bacteria (like Rhodobacter sphaeroides) (Tangprasisittipap et al., 2007).

In the C5 pathway, ALA is produced through catalysis by three enzymes, including glutamyl-tRNA synthetase (GluRS, encoded by gltX), a NADPH-dependent glutamyl-tRNA reductase (GluTR, encoded by hemA) and glutamate-1-semialdehyde aminotransferase (GSA-AT, encoded by hemL) (Fig. 1). In this pathway, glutamate-1-semialdehyde is an unstable intermediate that is quickly converted to ALA by the action of GSA-AT, and the GluTR and GSA-AT have a synergistic effect in this procedure. Moreover, GluTR is the rate-limiting enzyme in the C5 pathway and is strictly regulated by feedback inhibition of haem. C5 pathway exists in higher plants, algae and various bacteria (like E. coli and Corynebacterium glutamicum) (Yu et al., 2015; Noh et al., 2017), while, in very few microorganisms, like Euglena gracilis and Arthrobacter globiformis (Yang and Hooper, 1995), the C4 and C5 pathways coexist. In addition, intracellular membrane transport protein RhtA for threonine and

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**Fig. 1.** Overall metabolic pathways for ALA and GABA biosynthesis and transformation/degradation (drawn based on references). Dark green is Puu pathway, and light green is GAD pathway for GABA synthesis. Red is C5 pathway, and orange is C4 pathway for ALA synthesis. Genes abbreviated are as follows: zwf, glucose-6-phosphate dehydrogenase; pgk, phosphoglycerate kinase; idhA, L-lactate dehydrogenase; PDH, pyruvate dehydrogenase complex (E1, E2 and E3 components encoded by aceE, aceF and ηd genes respectively); pta, phosphate acetyltransferase; acsA, acetyl-CoA synthetase; ackA, acetate kinase; ppc, phosphoenolpyruvate carboxylase; pyr, pyruvate carboxylase; pck, phosphoenolpyruvate carboxykinase; gltA, citrate synthase; sucr, α-ketoglutarate dehydrogenase; succCD, succinyl-CoA synthetase; sdh, succinate dehydrogenase; mdh, malate dehydrogenase; gdh, glutamate dehydrogenase; hemA, glutamyl-tRNA reductase (C5 pathway); hemA, ALA synthase (C4 pathway); hemL, glutamate-1-semialdehyde aminotransferase; hemB, δ-aminolevulinic acid dehydratase; rdhA, inner membrane transporter for L-threonine; Gad (gadA, gadB), glutamate decarboxylase; gabT, γ-aminobutyric acid transaminase; gadB, succinate semialdehyde dehydrogenase; gabC, Glu/ GABA antiporter; speC, L-ornithine decarboxylase; patA, putrescine transaminase; patD, γ-aminobutyraldehyde dehydrogenase; gabP, GABA-specific importer.

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homoserine exporting also approved the ALA efflux (Kang et al., 2011), and two molecules of ALA are condensed into one molecule of porphobilinogen (PBG) by ALA dehydratase (ALAD, encoded by hemB) that was further converted into other haem compounds (Fig. 1) (Su et al., 2019).

Accordingly, GAD and C4 pathways are related to one key enzyme with common cofactor PLP, while Puu and C5 pathways are related to multiple key enzymes. On the other hand, GAD and C5 pathways share the common metabolic routes at the early stage (from glycolysis to TCA) and common precursor glutamate. Since GABA and ALA have common metabolic pathway, their metabolic engineering strategies are comparable and can inspire each other.

**Comparative analysis of metabolic engineering strategies for GABA and ALA biosyntheses**

Microbial fermentation has obvious advantages of low cost, no chemical residue and high yield, and is an ideal way to produce value-added compounds (Yuan and Alper, 2019). GABA and ALA are emerging value-added non-protein amino acids with great significance to realize their high yield by microbial fermentation. Initially, efforts for increasing the yields focused on natural producers and usually performed through random mutagenesis and optimizing fermentation conditions. Recently, remarkable efforts have been made to improve the yield of biosynthetic GABA and ALA, through natural or engineered strains (Choi et al., 2015; Cui et al., 2019; Zhang et al., 2020). We screened the development in recent five years from the representative and authoritative journals, and summarized their metabolic engineering strategies in detail, which include every metabolic engineering step and its effectiveness, as well as the synthetic pathway, substrate, final titre, biomass, fermentation time, form and scale, as well as the calculated yields based on the substrate (Tables 1 and 2).

To further analyse the metabolic engineering strategies and its effectiveness of GABA and ALA biosyntheses, the proportion and distribution of host strains were counted (Fig. 2A), and the metabolic engineering strategy-related genes were summarized and analysed (Fig. 2B). Moreover, the improved titre times of each metabolic strategy in different host strains were calculated and compared (Fig. 2C).

**Diversity of GABA- and ALA-producing microbes**

For GABA biosynthesis, *Lactobacillus* spp. are the most common and efficient natural producers (Table 1 and Fig. 2A up), which are usually isolated from traditional fermented products and present food safety character; hence, their GABA product has better application prospect and market value (Tajabadi et al., 2015; Cui et al., 2020). Among the *Lactobacillus* spp., *L. brevis* is the most frequently reported producer with naturally high GABA productivity (Lyu et al., 2017; Lyu et al., 2018; Gong et al., 2019). However, due to the lack of effective genetic manipulation in *Lactobacillus* spp., *E. coli* and *C. glutamicum* are still accounted for a large proportion in the current studies on metabolic engineering (Table 1 and Fig. 2A up). As for ALA biosynthesis, relatively few kinds of host strains have been researched (Table 2 and Fig. 2A), which may be due to the fact that natural ALA high-yielding microbes are rarely discovered. Similar to the GABA production, *E. coli* and *C. glutamicum* still are the most studied chassis in ALA production (Fig. 2A) due to their well-characterized genetics and advanced metabolic engineering tools.

**Metabolic engineering strategies for improving the GABA and ALA production**

Figure 2B, C obviously showed that the biggest increase in gene manipulation is amplifying the key pathway genes. For GABA synthesis, GAD pathway is common in the producing strains, in which GAD or gadB gene plays a key role and this gene usually from *Lactobacillus* spp. has been used for metabolic engineering. On the other hand, Jorge et al. (2016) first reported that the heterologous expression of *patA* and *patD* from *E. coli* in a putrescine producer *C. glutamicum* enabled it to produce GABA via the Puu pathway. Then, they further developed the Puu route to synthesis GABA in *C. glutamicum* and obtained up to the titre of 63.2 g l⁻¹, reached the highest volumetric productivity for fermentative GABA production (1.34 g l⁻¹ h⁻¹) by the time of the report (Jorge et al., 2017). And this glucose-based GABA production via Puu route presented a higher volumetric productivity than that via the GAD pathway. For ALA synthesis, ALAS (hemA) usually from *R. sphaeroides* and *Rhodopseudomonas palustris* in C4 pathway, and hemA usually from the mutated *Sazlmonella arizonae* and hemL from *E. coli* in C5 pathway have been employed for metabolic engineering. In addition, the optimization of the metabolic flux was also achieved by releasing the feedback regulation of key enzymes (gadT and gadD for GABA; hemB, hemD, hemF for ALA); increasing export of target amino acids out of the cells (gadC for GABA; rhaT for ALA); and common reducing metabolic fluxes of TCA cycle (*sucA, sucCD*) to down-regulate competitive pathway. Besides, the upstream common key genes (*pyc* and *gapA*) also related their overproduction. In this case, promoter and RBS engineering strategies are most applied to balance and regulate gene expression levels. Moreover, from Tables 1
**Table 1. Metabolic engineering of microorganisms for the production of GABA.**

| Host strain | Metabolic engineering strategies | Titre (g l⁻¹) | Pathway | Fermentation results | Yield (g g⁻¹ substrate⁻¹) | Substrate | References |
|-------------|----------------------------------|---------------|---------|----------------------|--------------------------|-----------|------------|
| C. glutamicum | E. coli wild-type gadB³ with strong synthetic P₁₅₄ promoter E. coli gadB mutant (GluB9Gln/Δ452-466) with strong synthetic P₁₅₄ promoter | 0.34 | GAD | 38.6 g l⁻¹; 0.54 g l⁻¹ h⁻¹; OD:76.6; 72 h; 5 l | 0.40 | Glucose | Choi et al (2015) |
| L. plantarum | Optimization of GABA production conditions (WT) | 0.74 | GAD | 1.14 g l⁻¹; 60 h SF | 0.02 | MSG | Tajabadi et al (2015) |
| C. glutamicum | | 1.14 | | | | | |
| B. methanolicus | | | | | | | |
| C. glutamicum | ΔargF, ΔargR, spec, argF₂₁, patA (E. coli), patD (E. coli) | 5.3 | Puu | 8.0 g l⁻¹; DCW:15 g l⁻¹; 26 h; SF | 0.20 | Glucose | Jorge et al (2016) |
| | ΔcgmA | 5.1 | | | | | |
| | ΔsnaA | 5.7 | | | | | |
| | Modified CGXII medium | 8.0 | | | | | |
| | ΔgabTDP operon | | | | | | |
| | gadB¹ (E. coli); gadA¹ | 0.03; 0.03 | GAD | 9.0 g l⁻¹; DCW: 47.5 g l⁻¹; 31 h; 3 l | - | Methanol | Irla et al (2017) |
| | (S. thermosulfidoxidans) gadB¹, 50 for 10–12 h and 37 to 24 h | | | | | | |
| | gadA¹, pH shift from 6.5 to 4.6 after 27 h | 9.0 | | | | | |
| E. coli | TA3000 (gdhA¹, gadB¹), TA4024 (gadhA¹, gadB¹, gadC¹) | 1.8 | GAD | 4.8 g l⁻¹; 32 h SF | 0.28 | Glucose | Soma et al (2017) |
| | TA4024, TA4076 (gadhA¹, gadB¹, gadC¹, sucA control unit) | 1.54; 1.54; 3.86 | | | | | |
| | TA4077 (gadhA¹, gadB¹, gadC¹, sucA control unit, pyc¹) | | | | | | |
| | TA4053 combined a sucA aceE control unit in TA4077 | 4.66 | | | | | |
| | IPTG was added at 6 h | | | | | | |
| | | 4.8 | | | | | |
| L. brevis | Wild type gadA¹ | 41.49 | GAD | 43.65 g l⁻¹; OD:6.5; 48 h; 5 l | 0.69 | MSG | Lyu et al (2017) |
| | F₆,F₁-ATPase-defective mutants | 43.65 | | | | | |
| E. coli | Opt gadB³ (L. lactis) Removed the C-plug of gadC Molecular chaperones Gro7 | 300.65; 307.12; 307.4 | GAD | 308.26 g l⁻¹; 308.26 g l⁻¹; 308.26 g l⁻¹ | 0.70 | Glutamate | Yang et al (2018) |
| L. brevis | Native strain L. brevis CK gadB¹, gadC¹, Fed-batch fermentation (0-24 h: pH 5.2, 35; 35; 24-102 h: pH 4.4, 40) | 82.47; 104.38 | GAD | 104.38 g l⁻¹; OD:6.5; 72 h; 5 l | 0.56 | MSG | Lyu et al (2018) |
| L. brevis | Mutant strain with much higher expression level of gadR pH-controlled, mixed-fed fermentation | 9.65; 11.62 | GAD | 177.74 g l⁻¹; OD:13; 36 h; 3 l | 1.19 | MSG | Gong et al (2019) |
and 2, we can also see that the fermentation time of E. coli is shorter than that of C. glutamicum, but Fig. 2C showed the gene operation of C. glutamicum is more obviously effective than that of E. coli for improving the yield, whatever in GABA and ALA synthesis.

### Optimization of GABA and ALA fermentation conditions

Up to now, the highest titre of GABA production by metabolic engineering is 308.26 g l\(^{-1}\) in E. coli through heterologously expressed gadB gene from L. lactis via GAD pathway (Yang et al., 2018), while the highest titre of ALA bioproduction is 18.5 g l\(^{-1}\) in C. glutamicum through heterologously expressed ALAS (hemA) gene from R. palustris via C4 pathway (Chen et al., 2020). However, these significant achievements realized by combining whole-cell biocatalysts using 3 M glutamate as substrate and 4 g l\(^{-1}\) glycine supply respectively. In fact, most of the GABA and ALA production contributed to the complex medium in complicated cultivation process and continual feeding of the precursors (Feng et al., 2016; Yang et al., 2016; Li et al., 2016a; Hara et al., 2019; Zhu et al., 2019; Chen et al., 2020).

On the one hand, whole-cell biocatalysts indeed remarkably enhance production, but depending on the amount of substrate and the number of cells uses. On the other hand, comparing with the C4 pathway, an obvious advantage of the C5 pathway is that it can achieve de novo synthesis of ALA just from glucose, and the C5 pathway is usually more efficient than the C4 pathway with the exogenous glycine or succinic acid supplement. Furthermore, we conclude that the two-stage pH and temperature control with substrate-feeding strategy is mostly applied in current fermentation optimization. However, even a good fermentation optimization in fermenter can only improve the yield by 3–4 times (Tables 1 and 2, Fig. 2C), and these strategies are undesirable for economical and sustainable industrial GABA and ALA production. Hence, higher titre still depends on the initial strain engineering transformation and it is still rather attractive to directly generate GABA and ALA from glucose. Indeed, the application of metabolic engineering strategies is the inexhaustible driving force for GABA and ALA sustained high yield. Meanwhile, the metabolic engineering can save production more costs than the complex fermentation processes do.

### Future prospects: challenges and insights

Recently, GABA and ALA as novel PGRs attracted great concern. By regulating photosynthesis, epigenetic modifications, nutrient distribution, and growth and development, GABA and ALA can break seed dormancy, improve drought tolerance and water use efficiency, enhance temperature tolerance and nitrogen use efficiency, promote shoot elongation and generation, increase shoot and root mass, and ameliorate the plants adapting to adverse environmental stress (Small and Degenhardt, 2018). They can also be widely used in field crops (corn, soya bean, wheat), fruits, vegetables, ornamental plants and lawns, and these huge planting areas are the future application market of GABA and ALA (Wu et al., 2018; Li et al., 2019). In addition, GABA and ALA application could avoid the drug resistance in the traditional insecticides, fungicides and herbicides, could reduce over-reliance on synthetic fertilizer and pesticides, and could improve the utilization rate of fertilizers. Moreover, the application dosage of GABA and ALA is low, safe and environmentally friendly. Therefore, the future increase in agricultural economy will benefit greatly from the application of GABA and ALA. However, the PGRs only occupy a proportion of 4–5% in the current international pesticide market. Furthermore, the application of GABA and ALA in medicine, food and feed requires the biosynthesis products. So, it is very important to develop a safe, high-activity, green and sustainable production strategy, and it is the time to develop and realize the industrial production of GABA and ALA by microorganisms.

Theoretically, the thermodynamic maximum yields of GABA and ALA produced from glucose were calculated according to Dugar and Stephanopoulos (2011) and are

### Table 1. (Continued)

| Host strain | Metabolic engineering strategies | Titre (g l\(^{-1}\)) | Pathway | Fermentation results | Yield (g g substrate\(^{-1}\)) | Substrate | References |
|-------------|----------------------------------|----------------------|---------|----------------------|-----------------------------|----------|------------|
| E. coli     | Native E. coli K12               | 0.11                 | GAD     | 19.79 g l\(^{-1}\); DCW: 0.86 g l\(^{-1}\); 33 h; 1 l SF | 0.57 MSG | Yu et al. (2019) |
| ΔgabT       | 0.32                             |                      |         |                      |                             |          |            |
| ΔgabT, ΔgabP| 0.55                             |                      |         |                      |                             |          |            |
| ΔgabT, ΔgabP, ΔpuuE| 0.73                   |                      |         |                      |                             |          |            |
| gadA\(^{∗}\), gadB\(^{∗}\), gadC\(^{∗}\)| 6.4                             |                      |         |                      |                             |          |            |
| gadA\(^{∗}\), gadB\(^{∗}\), gadC\(^{∗}\)| 6.4                             |                      |         |                      |                             |          |            |
| 1 l SF fermentation, adjust the pH to 4.2 | 19.79 | | | | | | |

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| Host strain | Metabolic engineering strategies | Titré (g l⁻¹) | Pathway | Yield (g g⁻¹ substrate⁻¹) | Substrate | References |
|-------------|----------------------------------|---------------|---------|--------------------------|-----------|------------|
| C. glutamicum | Native hemA⁺, hemL⁺, hemA' (mutated Salmonella arizonae), hemC' (E. coli) | 0.07984 | C5 | 1.79 g l⁻¹; | Glucose | Yu et al. (2015) |
| | Reduced dissolved oxygen and Fe²⁺ concentrations | 0.42511 | OD:20; | 144 h; SF | | |
| | Add 0.3 mm maleic acid; add 0.1 mm of phthalic acid | 0.83 | | | | |
| | Add degradation ASV tag at the C-terminus of ALAD | 1.289; 1.507 | | | | |
| | | | | | | |
| E. coli | Opt hemT⁺ (S. cerevisiae) | 0.94 | C4 | 3.58 g l⁻¹; | Glycine, succinic acid, B. subtilis | Li et al. (2016) |
| | Opt hemT⁺ in various recombinant E. coli | 1.609 | C4, C5 | 4.8 h; SF | | |
| | Constructing T7 RNA polymerase gene on the plasmid | 2.013 | | | | |
| | ΔgusA ΔlofK and re-locating them to the plasmid | 1.725 | | | | |
| C. glutamicum | Auto-induction ( IPTG-free) system | 3.584 | C4 | 14.7 g l⁻¹; | Glucose | Yang et al. (2016) |
| | Native pC5 pathway | 0.0251 | OD:16; | SF | | |
| | SucCD⁺ | 0.09287 | | | | |
| | Opt hemA⁺ (R. capsulatus SB1003) | 7.6 | C5 | 11.5 g l⁻¹; | Glucose | Feng et al. (2016) |
| | Two-stage fermentation | 12.46 | | | | |
| C. glutamicum | Opt hemA⁺ (R. sphaeroides) with add 7.5 g l⁻¹ glucose | 1.44 | C4 | 7.53 g l⁻¹; | Glycine | Li et al. (2016) |
| | Δpco, Δpap, ΔackA, Δcat (acetate) and ΔadhA (lactate) | 1.92 | OD:140; | 33 h; | | |
| | ppc | 2.06 | | | | |
| | Δpbg1fs3 Δpbg1fsb Δpbg2b (HMW-pbps) | 2.35; 2.61; 2.53 | | | | |
| | rhaA (E. coli) | 3.14 | | | | |
| E. coli | Fed-batch culture | 7.53 | C5 | 3.4 g l⁻¹; | Glucose | Noh et al. (2017) |
| | Native hemA' (S. typhimurium), hemL⁺ | 0.01 | OD:18.5; | 18 h; SF | | |
| | ΔgusA | 0.74 | | | | |
| | gldA | 0.56 | | | | |
| | Varying the transcriptional strength of aceA | 0.37 | | | | |
| | Induction timing was delayed from 0.8 to 5.0 of OD600 | 1.09 | | | | |
| | hemA and hemL were integrated into chromosome with 98 copy number | 3.4 | | | | |
| | Optimization of fermentation conditions | 1.2 | | | | |
| | Add a degradation tag ssra to the C-terminus of ALAD | 1.7; 2.8 | | | | |
| | yaaA⁺; katG⁺ | 4.55 | | | | |
| | ΔrecA in MG136a (adaptive evolution of MG136) | | | | | |
| S. cerevisiae | Native hemT⁺ | 0.000058 | C4 | 0.00136 g l⁻¹; | Glucose | Hara et al. (2019) |
| | hemT⁺/Acu2⁺, add 5 mM glycine | 0.000022 | OD:48; 48 h; SF | | | |
| | | 0.00136 | | | | |
| E. coli | Assembled higher RBS of hemA and medium of hemL | 2.41 | C5 | 5.25 g l⁻¹; | Glucose | Zhang et al. (2019) |
| | ALA dehydratase was rationally regulated | 2.68 | OD:24; 0.16 g l⁻¹ h⁻¹; | 33 h; 3 l | | |
| | Cofactor PLP⁺ | 2.86 | OD:16; 0.16 g l⁻¹ h⁻¹; | 33 h; 3 l | | |
| | ΔrecA, hemA⁺ | 2.86 | OD:16; | 33 h; 3 l | | |
| | ΔrhaA, hemD⁺, hemF⁺ | 3.77 | OD:16; | 33 h; 3 l | | |
| E. coli | Carrying a pH two-stage strategy | 5.25 | C4 | 11.5 g l⁻¹; | Glucose | Zhu et al. (2019) |
| | E. coli bw24 | 5.3 | OD:55.3; 22 h; 5 l | | | |
| | Kale⁺ | 9.6 | 1.997 g l⁻¹; | | | |
| | SodB⁺ | 8.7 | OD:22; 42 h; 5 l | | | |
| | Kale⁺, sodB⁺ | 11.5 | SF | | | |
| E. coli | Chromosomal integration with 7 copies of hemA⁺ and hemL | 0.1696 | C5 | 0.03 g l⁻¹; | Glucose | Su et al. (2019) |
| | hemB⁺ | 0.862 | OD:22; 42 h; 5 l | | | |
| | Fed-batch fermentation with engineered strain | 1.997 | SF | | | |
| C. glutamicum | hemA⁺ (R. palustris ATCC 17001) | 3.8 | C4 | 18.5 g l⁻¹; | Cassava | Chen et al. (2020) |
| | Replace the original RBS with relatively high translational activities RBS | 4.4 | OD:177.2; 39 h; 5 l | | | |
| | Native ppc⁺ | 3.243 | | | | |
| | ppc expression was optimized using RBSs | 5.5 | | | | |
| | Fed-batch fermentation from glucose | 16.3 | | | | |
| | Fed-batch fermentation from cassava bagasse | 18.5 | | | | |

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Table 2. (Continued)

| Host strain | Metabolic engineering strategies | Titre (g l⁻¹) | Pathway | Fermentation | Yield (g g⁻¹ substrate⁻¹) | Substrate | References |
|-------------|----------------------------------|--------------|---------|--------------|---------------------------|-----------|------------|
| C. glutamicum | Native | 0.0053 | C5 | 3.16 g l⁻¹; Biomass: 9 g l⁻¹; 64 h; SF | 0.07 | Glucose | Zhang et al. (2020) |
| | Endogenous hemA⁺, hemL⁻ | 0.0299 | | | | | |
| | hemA⁺, hemL⁻ (mutated S. arizona), hemL⁻ (E. coli) | 0.51 | | | | | |
| | Constitutive overexpression of hemA⁺ and hemL⁻ | 0.62 | | | | | |
| | pyc¹ and ppc¹ under P₆₄₀ | 0.58; 0.7 | | | | | |
| | pckA_m, driven by P₆₄₀ (weak promoter) | 0.79 | | | | | |
| | Native icd driven by P₆₄₀, P₆₄₀ and P₆₄₀ | 1.01; 0.96; 0.92 | | | | | |
| | gap⁴ (C. glutamicum) was integrated into the genome driven P₆₄₀ | 1.28 | | | | | |
| | Add 5 mg l⁻¹ PLP | 1.61 | | | | | |
| | Cg0788-Cg0789 operon | 1.48 | | | | | |
| | (PLP synthase) under P₆₄₀ | 1.78 | | | | | |
| | ODHC activity was decreased by 68.5% | 1.93 | | | | | |
| | Two-stage fermentation | 2.38 | | | | | |
| | odcA driven by P₆₄₀ | 1.6; 1.8; 2.3 | | | | | |
| | rhtA⁺ driven by P₆₄₀, P₆₄₀ and P₆₄₀ | 2.95 | | | | | |
| | rhtA⁺ in an IPTG-inducible manner | 3.16 | | | | | |

: and; or; ↑: gene overexpression; ↓: gene knockdown; Δ: gene knockout; opt: codon-optimized; OD: OD₆₀₀; DCW: dry cell weight; SF: shake flask.

Fig. 2. Host strains, genes and efficiency of each metabolic strategy in different host strains for GABA (up) and ALA (down) production.
A. The proportion of host strain used for GABA and ALA production.
B. Word clouds of gene manipulation increased production, font size correlates with the frequency of occurrence, red means upregulation, and green means downregulation.
C. The improved titre times of each metabolic strategy in different host strains. Green means GABA production, and red means ALA production.

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shown in Table 3. The maximum yields of GABA and ALA and their precursor glutamate in mol/mol glucose and g g glucose−1 are nearby. However, from the data in Tables 1 and 2, the titre of GABA is generally higher about 10 times than that of ALA. What’s more, up to now, the highest yields of GABA and ALA are only 0.40 g g glucose−1. In this case, the microbial production of ALA and GABA still has more potential to be tapped.

Novel host strains with native high tolerance

First, the low yields of GABA and ALA may be caused by the low intrinsic tolerance of the microbes to these products. For GABA, it was reported that 113 g l−1 GABA caused a 50% decrease of the growth rate in C. glutamicum (Jorge et al., 2016). In Bacillus methanolicus, 7.2 g l−1 GABA caused a 50% decrease of the growth rate, and growth arrested completely at 16.5 g l−1 (Irla et al., 2017). For ALA, 15 g l−1 ALA caused a 36% decrease in initial specific growth rate of E. coli, and its growth was completely stopped when the ALA concentration reached 18 g l−1 (Zhu et al., 2019). Although Zhu et al. (2019) explained the cytotoxicity of ALA via generate reactive oxygen species (ROS). Attention also should be paid to that the ALA accumulation causes pH decrease, while GABA accumulation causes pH increase. Hence, high tolerance of acid–base stress microorganisms is needed for improving GABA and ALA production (Fig. 3), which is usually ignored and may be another breakthrough point to further increase the yield of GABA and ALA.

Hence, screening and selecting novel host strains with high tolerance and natural over productive features are a preferred way to improve GABA and ALA productions. While in fact, compared with traditional conventional microorganisms, extremophilic bacteria are better adapted to the rough conditions in industrial production, as well as greatly reducing the production cost (Chen and Liu, 2021). On the other hand, the non-conventional strains might have the capability to consume cheap

| Compounds | Chemical formula | MW g mol⁻¹ | Degree of reduction | Thermodynamic maximum yield g g glucose⁻¹ | Thermodynamic maximum yield g g glutamate⁻¹ |
|-----------|------------------|------------|---------------------|------------------------------------------|------------------------------------------|
| Glucose   | C₆H₁₂O₆          | 180        | 24                  | –                                        | –                                        |
| Glutamate | C₅H₈NO₄          | 147        | 18                  | 1.09                                     | –                                        |
| GABA      | C₅H₉NO₃          | 103        | 18                  | 0.76                                     | 1.4                                     |
| ALA       | C₄H₉NO₂          | 131        | 20                  | 0.87                                     | –                                        |

Fig. 3. Future insights for developing the production of GABA and ALA.
renewable bioresources (Fig. 3) including lignocelluloses, wastes, agro-industrial residues and C1 (methane, methanol, formic acid and carbon dioxide) compounds (Haldar and Purkait, 2020). However, it should also be noted that the genetic manipulation of wild-type strains is limited to stubborn bacterial issues, such as restricted modification systems, cell wall thickness and endophytic plasmids. Fortunately, the advances in high-throughput screening techniques and genome sequencing technologies have accelerated the isolation and engineering of non-conventional hosts (Li et al., 2020). Moreover, evolutionary engineering like adaptive laboratory evolution (ALE), chemically induced chromosomal evolution (CiChE) and genome shuffling (GS) are especially suitable for non-conventional strains which lack genetic tools (Fig. 3). Hence, now it is the adequate time to develop novel cell factories.

**Develop whole-cell biocatalysts to co-culture for GAD and C4 pathway**

Ignoring the cost, up to now, the highest titre GABA was realized by whole-cell biocatalysis through GAD pathway. Similarly, the C4 pathway is also catalysed by a key enzyme ALAS. Hence, the GAD and C4 pathways are more suitable for the whole-cell biocatalysts (Fig. 3). However, the yield heavily depends on the amount of precursor: glutamate, glycine and succinic acid. Among those precursors, glutamate is cheaper. Nonetheless, whole-cell transformations are not only retarded cell growth, but also unsustainable with high cost. Co-culture can diminish the metabolic burden on each microbial strain, so that the optimized metabolic pathway can be parallel constructed in a modular way to take the advantage of the favourable traits from each co-cultured organism (Zhang and Wang, 2016). Thus, co-culture strategy might be a resolve for the problem of high cost (Fig. 3). For example, co-culture of the succinic acid-producing engineering strain, glycine-producing engineering strain and expressing ALAS engineering strain could be used as a consortium for ALA production. Furthermore, use quorum sensing (QS) in co-culture regulation is a promising way.

**Develop DBTL for Puu and CS pathway**

The Puu and CS pathways are more suitable for metabolic engineering, based on the classical Design–Build–Test–Learn (DBTL) system to realize the sustainable production from glucose or cheap raw materials (Fig. 3). However, we have to admit that simply using metabolic engineering is a time-consuming and labour-intensive process. Luckily, with the development of artificial intelligence (AI) and the bio-big data, the metabolic simulation and prediction on genome-scale could guide experiments more accurately, reasonably and quickly (Fig. 3) (Ryu et al., 2019; Wytock and Motter, 2019). In addition, omics such as genomics, transcriptomics, proteomics, metabolomics and fluxomics have provided faster and comprehensive progress in metabolic engineering research (Fig. 3). What’s more, fine-tuning and balance could use the minor force winning energetically. On the one hand, it can be realized by RBS and promoter library, CRISPRi and point mutation strategy. On the other hand, regenerate cofactor (NADP+/NADPH) and energy (ATP/ADP) are necessary. In detail, NADP+/NADPH supply via the glycolytic pathway and the pentose phosphate pathway (Fig. 1), in which the related genes were gapA, aceEF, ldh, zwf and gdn; ATP regenerate-related genes were pgk, pyk and sucCD, need to be considered. Moreover, global regulation realizes growth-coupled production is also necessary for improving the production.

**Maximum utilization of fermentation**

Since GABA and ALA are extracellular secretion products, to maximize the use of microbial cell factories, complement each other or no compete intracellular products with extracellular simultaneous production might be considered. Besides, the most common aerobic problem in large-scale fermentation can be solved by overexpressing haemoglobin to increase aeration.

**Other strategies relate to plants and agriculture**

Since plants also have GABA and ALA synthesis pathways, introducing plants synthesis pathways or functional genes under rational design may bring surprising discovery. On the other hand, in agriculture, direct engineering the GABA- and ALA-producing microorganisms themselves can also be used as live therapeutics, that is to say as a novel plant growth-promoting rhizobacteria (PGPR) (Fig. 3). GABA- and ALA-producing strain can also be used as a novel species to add and enrich biofertilizer (Fig. 3). Furthermore, combine GABA-producing strain with ALA-producing strain may enhance their effectiveness in agriculture. Thus, modification of GABA- and ALA-producing strains or construction of stable recombinant strains that can breed in the soil should be a novel direction for future studies.

**Concluding remarks**

In summary, GABA and ALA have many common properties: (i) both belong to non-protein amino acids; (ii) both have multiple functions and are widely used in agriculture, medicine and other fields; (iii) both as novel
PGRs can increase plant resilience to abiotic stress and promote plants growth; (iv) they share a common synthesis system, with common precursor glutamate and common cofactor PLP; and (v) they have similar system for metabolic engineering strategies. Hence, putting GABA and ALA together to analyse and compare their metabolic engineering strategies will benefit to each other. Likewise, these strategies provide some ideas for microbial production research of other non-protein amino acids (such as the precursor of Puu pathway ornithine) or PGRs or value-added compounds. We believe that the increasing adoption of the strategies described here will allow development of strains capable of efficiently producing GABA and ALA on an industrial scale with reduced effort, time and cost. And their application and development can better promote the sustainable development of related industries.

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Conflict of interest

The authors declare no competing financial interest.

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