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Biosystem design of *Corynebacterium glutamicum* for bioproduction

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Corynebacterium glutamicum, a natural glutamate-producing bacterium adopted for industrial production of amino acids, has been extensively explored recently for high-level biosynthesis of amino acid derivatives, bulk chemicals such as organic acids and short-chain alcohols, aromatics, and natural products, including polyphenols and terpenoids. Here, we review the recent advances with a focus on biosystem design principles, metabolic characterization and modeling, omics analysis, utilization of nonmodel feedstock, emerging CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) tools for *Corynebacterium* strain engineering, biosensors, and novel strains of *C. glutamicum*. Future research directions for developing *C. glutamicum* cell factories are also discussed.

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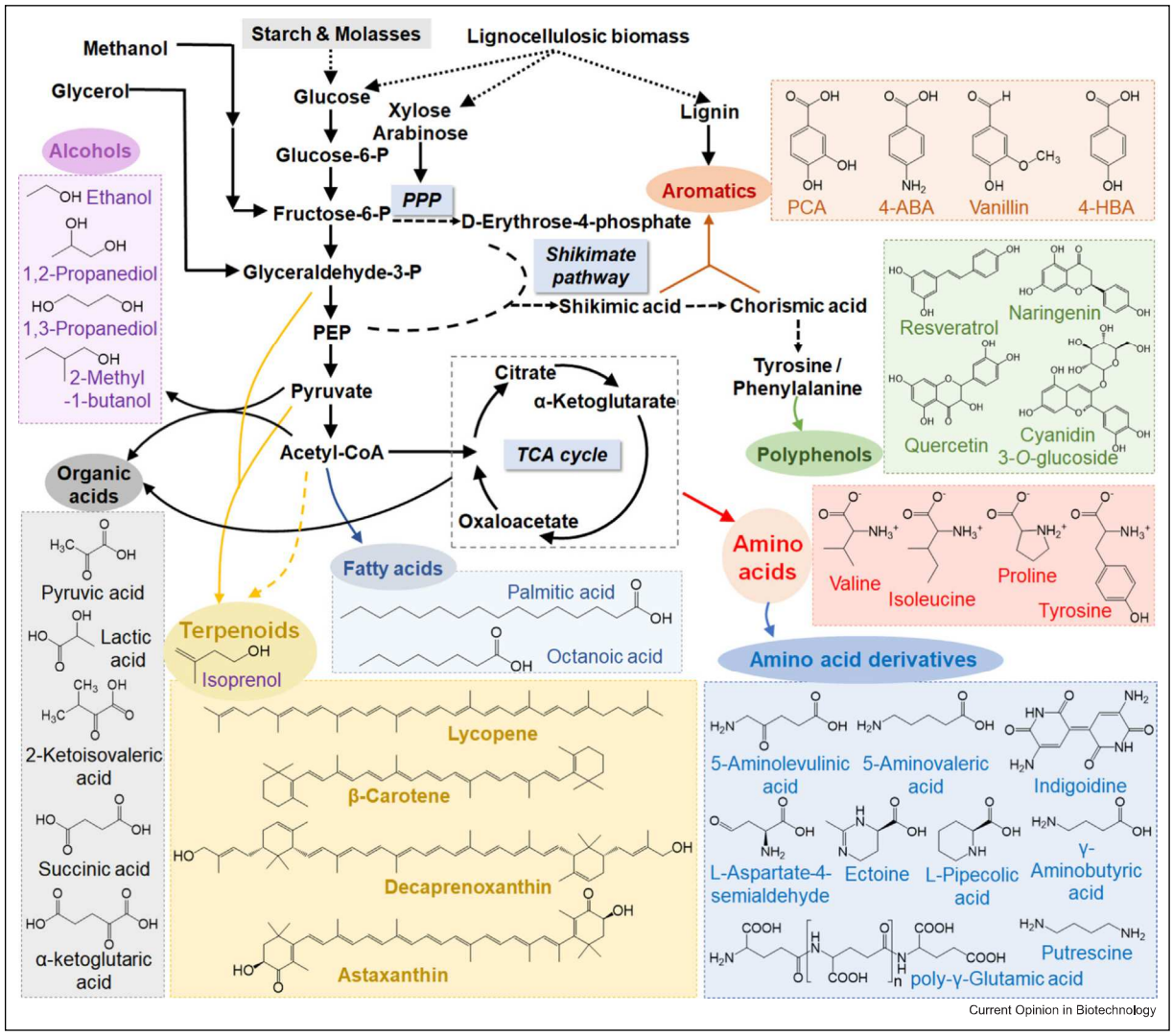
Introduction

The growing concerns on climate change and energy supply have driven fast development of microbial manufacturing of diverse bioproducts from renewable resources [1–3]. One of the most commonly used industrial microbes is *Corynebacterium glutamicum*, a Gram-positive and nonpathogenic bacterium adopted industrially for the production of amino acids. *C. glutamicum* demonstrates several physiological properties advantageous to fermentative production, such as high rates of sugar consumption under either aerobic or anaerobic conditions, regardless of cell density, high tolerance to osmotic pressure and various chemicals (including the final products), and capability of simultaneously utilizing mixtures of sugars without carbon catabolite repression [4]. Recently, the product portfolio of this host platform has been expanded substantially to cover organic acids, short-chain alcohols, phenolics, and plant natural products (Figure 1), attributed to the elucidation of more physiological information, the establishment of genome-scale models, and the development of sophisticated genetic manipulation tools. In this review, we summarize the latest progress on the engineering of *C. glutamicum*, with a focus on biomanufacturing, utilization of various substrates, emerging approaches of gene editing and metabolic regulation, metabolic modeling and omics analysis, and novel strains of *C. glutamicum*.

Production of primary metabolites, amino acids, and amino acid derivatives

C. glutamicum has been applied industrially to produce 17 natural amino acids (except glycine, methionine, and aspartate [5–8]) as well as amino acid derivatives such as 5-aminovalerate and polyglutamic acid (Table 1) [9–11]. The general principles of strain engineering include (1) introduction of the biosynthetic pathway consisting of heterologous genes, (2) balancing of the amino acid biosynthetic pathway and the downstream pathway, and (3) deletion or suppression of competing pathways. For example, the heterologous pathway involving gene *davTBA* responsible for aminovaleramide formation from lysine was overexpressed in a lysine-producing *C. glutamicum* strain, followed by expression of various aldehyde reductase orthologs for the generation of 5-hydroxyvaleric acid. The resulting strain achieved a titer of 52 g/L in fed-batch fermentation [12]. Another example is the production of glutaric acid. The L-lysine catabolic pathway from *P. putida* was expressed in *C. glutamicum*, converting L-lysine to glutaric acid, with a titer of 105 g/L [13]. Moreover, *C. glutamicum* metabolism has been studied by ^{13}C-metabolic flux analysis (MFA). The metabolic knowledge

Figure 1



The portfolio of typical chemicals produced by engineered *C. glutamicum*. The chemicals include amino acids, their derivatives, organic acids, short-chain alcohols, fatty acids, aromatics, terpenoids, and polyphenols. The carbon sources for *C. glutamicum* include molasses and starch (common industrial fermentation media), hemicellulosic hydrolysates, xylose, methanol, glycerol, aromatics, and so on.

led to heterologous expression of transhydrogenase and site-directed mutagenesis of pentose phosphate pathway enzymes to promote cofactor balance and L-methionine production [14]. In addition to amino acids and their derivatives, *C. glutamicum* is an excellent host to synthesize various organic acids (i.e. lactate, succinate, pyruvate, and α-ketoglutarate) [15,16] and short-chain alcohols (Table 1) [17].

Biosynthesis of natural products

C. glutamicum is a generally regarded as safe microbe that can produce pharmaceuticals and nutraceuticals. It has a strong shikimate pathway for the synthesis of phenylalanine and tyrosine, which are primary building blocks for polyphenol biosynthesis. Polyphenols usually exhibit antimicrobial properties. *C. glutamicum* is naturally more resistant to polyphenols than *E. coli*, and can even metabolize polyphenols as carbon sources under certain conditions. As a consequence, *C. glutamicum* has been recently engineered to produce diverse subgroups of flavonoid compounds, including naringenin, kaempferol, eriodictyol, and cyanidin-3-*O*-glucoside [18,19]. Moreover, *C. glutamicum* has been employed to produce aromatics, such as indole, protocatechuate, 4-hydroxybenzoate, and 4-aminobenzoate (Figure 1) [4]. *C. glutamicum* has also been used to synthesize various terpenoids, including astaxanthin, valencene, and lycopene [20]. However, its performance for the biosynthesis of natural products is generally lower than those obtained in *E. coli*, *S. cerevisiae*, or *Y. lipolytica* [21]. One

Table 1

Recent achievements in *C. glutamicum*-based biosynthesis of compounds.

Classification	Chemicals	Titer	Culture conditions	Reference
Amino acids and derivatives	L-Leucine	40 g/L	Fermenter	[69]
	5-Hydroxyvaleric acid	52 g/L	Fermenter	[12]
	5-Aminolevulinic acid	16.3 g/L	Fermenter	[70]
	Poly-γ-glutamic acid	21.3 g/L	Fermenter	[71]
	Ectoine	65.3 g/L	Fermenter	[72]
	Putrescine	12.5 g/L	Fermenter	[56]
	Indigoidine	49.3 g/L	Fermenter	[73]
	Spider silk protein	0.56 g/L	Fermenter	[74]
Aromatics	Dipicolinic acid	2.5 g/L	Shake flask	[75]
	Protocatechuate	16 g/L	Fermenter	[76]
	Vanillin	0.31 g/L	Shake flask	[77]
Alcohols	1,3-Propanediol	98 g/L	Fermenter	[78]
	4-Amino-1-butanol	24 g/L	Fermenter	[79]
	Isoprenol (3-methyl-3-buten-1-ol)	1.25 g/L	Shake flask	[80]
	Isobutanol	20.75 g/L	Shake flask	[81]
Organic acids	Succinate	94 g/L	Fermenter	[15]
	Muconic acid	85 g/L	Fermenter	[82]
	Adipic acid	35 μg/L	Shake flask	[83]
Terpenoids	Astaxanthin	22 mg/L	Shake flask	[84]
	CoQ10	0.4 mg/L	Shake flask	[85]
Polyphenols	Cyanidin-3-*O*-glucoside	40 mg/L	Shake flask	[18]
	Naringenin	37 mg/L	Shake flask	[86]
	Resveratrol	158 mg/L	Shake flask	[86]
	Salidroside	9.7 g/L	Fermenter	[87]

possible reason is that enzyme expression in *C. glutamicum* leads to insoluble inclusion bodies. To improve the expression of heterologous proteins, the fusion of a soluble peptide tag has been shown to be an effective approach [18].

Utilization of cellulosic sugars and nonmodel feedstock

C. glutamicum can use glucose, sucrose, and fructose but not pentoses [22,23]. Recent research to expand the spectrum of *C. glutamicum* carbon sources targets methanol, chitin, pentoses (xylose and arabinose) from hemicellulosic hydrolysates, galactose and lactose that are abundant in whey-based fermentation media, and glycerol that is a major by-product from the biodiesel industry [24] (Figure 1). The relevant strategies for strain engineering toward sugar utilization contain adaptive evolution, introduction of sugar transporters from other microbes, activation of cryptic transporters, and expression of sugar pathway genes for subsequent catabolism [25]. *C. glutamicum* contains an endogenous yet silent glycerol-catabolizing pathway. Earlier attempts regarding glycerol utilization in this bacterium involved activation of the endogenous pathway or introduction of heterologous pathways; however, these methods only led to limited success [26]. A recent study optimized the expression of the heterologous genes involving *glpF* (encoding aquaglyceroporin), *dhaD* (encoding glycerol dehydrogenase), and *dhaK* (encoding ATP(Adenosine triphosphate)-dependent dihydroxyacetone kinase). The best strain achieved a glycerol utilization rate of 1.34 g/g DCW/h and the maximum specific growth rate of 0.37 h^{-1} with glycerol as the sole carbon source [26].

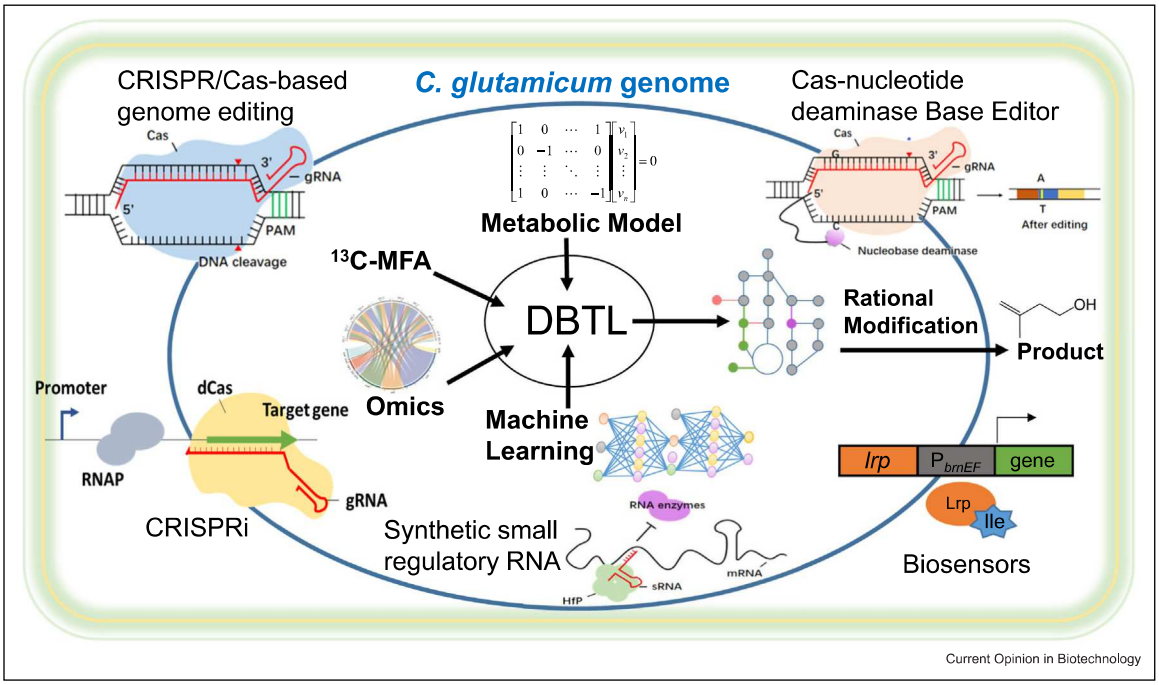
A consolidated process using starch as the feedstock has been achieved in *C. glutamicum* that lacks hydrolases to decompose starch. Surface display of α-amylase from *Streptococcus bovis* enabled the engineered *C. glutamicum* to degrade starch into glucose, which is then metabolized to produce lysine [27,28]. On the other hand, a coculture approach has been applied. Through the division of labor [29], the partner strain (α-amylase-producing *E. coli*) is designed to digest starch into glucose, whereas *C. glutamicum* uses glucose to produce value-added chemicals [30].

Recently, new methods have been developed to depolymerize lignin [31]. While a range of molecules can be released from lignin, aromatic molecules such as *para*-coumarate and ferulate are natively catabolized by *C. glutamicum* [32,33]. Therefore, lignocellulosic biomass could release both monomeric sugars and aromatics as feedstock for this organism.

New tools to engineer *C. glutamicum*

Traditional gene knockout or knock-in in *C. glutamicum* uses allelic exchange plasmids, which is a multistep and overall inefficient process. Better gene modifications can be achieved by CRISPR/Cas9 (CRISPR-associated protein 9) in conjunction with ssDNA-binding repair protein RecT from *E. coli* (Figure 2) [34]. Adapting these techniques to *C. glutamicum* has required some optimization:

Figure 2



The new genetic tools and models developed for metabolic engineering of *C. glutamicum*.

expressing Cas9 alone can generate double-strand breaks that are highly toxic to the cell, thus leading to a low genome editing efficiency, especially when Cas9 is expressed constitutively. In contrast, Cas12a (Cpf1) from *Francisella novicida* is nontoxic and highly efficient in nucleotide modifications with the aid of single-stranded DNA [35]. Inspired by this, similar toolboxes have been developed for *C. glutamicum* genome editing through optimized expression of guide RNA and Cas9 and coexpression of recombinases [36]. Another newly developed tool is the adenine/cytosine base editor. In this system, the catalytically dead Cas9 is fused to a cytosine deaminase (CDA) or adenine deaminase (AID), which enables base pair transition from C:G to T:A or from A:T to G:C. Expression of the guide RNA and the fusion construct Cas9-CDA or Cas9-AID triggers precise base editing in either the genome or the plasmid [37]. By applying this tool to tune the sequences of ribosome-binding sites or promoter regions, the pathway genes can be regulated in parallel and their expression levels can be controlled in a large range [37]. Moreover, the genome-targeting scope of such base editors has been expanded by using the Cas9 variants, thus providing 3.9-fold more target loci for *C. glutamicum* gene modifications [36].

The CRISPR system has been investigated in the interference of gene expression (CRISPRi) (Figure 2). By employing a catalytically dead Cas9 endonuclease that binds to one or several target sequences simultaneously with the aid of guide RNAs, the expression of the target gene(s) can therefore be repressed or, in some cases, activated [38]. For example, *C. glutamicum* was engineered for carotenoid production and CRISPRi tested 74 genes involved in its central metabolism, regulatory genes, and biosynthetic pathways. Such an effort led to the identification of new target genes for increased carotenoid bioproduction [39]. On the other hand, a synthetic small regulatory RNA (sRNA)-based gene knockdown strategy has been developed in *C. glutamicum* (Figure 2). This system contains an RNA chaperone Hfq from *E. coli* and a rationally designed sRNA consisting of the *E. coli* MicC (mRNA-interfering complementary OmpC) scaffold and a target-binding site. Upon expression in *C. glutamicum*, the sRNA binds to the mRNA of the target genes, represses translation and enzyme synthesis, and regulates the production of the target compounds [40].

Biosensors are useful in metabolic engineering. *C. glutamicum* contains many native transcription factors that respond to amino acids to trigger the expression of exporters. In addition, some endogenous regulatory proteins are responsive to native metabolites or natural products [41,42]. For example, multiple antibiotic resistance regulator-type regulator CrtR (The gene that encodes cytochrome p450 reductase), which represses the transcription of the promoter of the *crt* operon (PcrtE) and its own gene (PcrtR), can sense intracellular

geranylgeranyl pyrophosphate (GGPP), and the CrtR/PcrtE switch can be used to screen GGPP-overproducing strains for the production of carotenoids [42]. Recently, other biosensors have been discovered in *C. glutamicum* such as ShiR, NCgl0581, and CgmR, in addition to previously identified biosensors such as Lrp, GlxR, and LysG [43]. They can be applied in the screening of efficient producers or as a switch to modulate biosynthetic pathways in a dynamic manner. For instance, various dynamic pathway regulation tools have been reported, including quorum-sensing-based genetic circuits [44] and synthetic metabolic switches (responsive to cell growth [26] or effector molecules such as gluconate [45] and ferulic acid [46]).

Multiscale models and omics analysis to assist *C. glutamicum* engineering

A design–build–test–learn (DBTL) cycle for *C. glutamicum* engineering involves 1) *design* pathways, 2) *build* genetic constructs, 3) *test* strains for desired traits, and 4) *learn* new strategies for the next cycle of DBTL. In the design stage, metabolic modeling predicts strain metabolism and identifies biosynthesis bottlenecks. Several computational design tools, including models and algorithms, have been developed to greatly accelerate such a process. The recently updated genome-scale metabolic model of *C. glutamicum*, that is, model *i*CW773 established for strain ATCC 13032, consists of 773 genes, 950 metabolites, and 1207 reactions [47]. This model coupled with flux balance analysis and computational strain design could suggest the genetic interventions leading to hyaluronic acid overproduction. Engineering efforts following such predictions led to 28.7 g/L of hyaluronic acid (0.21–0.97 MDa) in fed-batch fermentation [48]. In another example, model-guided metabolic engineering reconstructed the TCA cycle, blocked product degradation, enhanced transport system, and improved gamma-aminobutyric acid production (achieving 23 g/L) [49]. Similarly, a pool influx kinetics (PIK) approach integrated dynamic ^{13}C labeling with model-based analysis, leading to the identification of key genes for improving L-histidine production in *C. glutamicum* [50]. Recently, an enzyme-constrained metabolic model was developed [51]. This model improved the prediction of *C. glutamicum* phenotypes and revealed the trade-off between biomass yield and enzyme usage efficiency, which could guide strain engineering for L-lysine production. In parallel to mechanistic models, data-driven approaches (such as AI (Artificial intelligence)) have been reported to facilitate successful DBTL cycles in other model organisms such as *E. coli* [52] and *S. cerevisiae* [53]. Moreover, the Automated Recommendation Tool for machine learning applications has been built to design synthetic biology components (such as promoters) [54]. The same machine learning approaches may enhance *C. glutamicum* strain development and biomanufacturing [55].

Omics analyses are important tools to facilitate DBTL strain development. In a putrescine-producing *C. glutamicum* strain obtained via adaptive evolution, key engineering loci were identified at the genetic level using whole-genome sequencing and at the protein level using comparative proteomics analysis. Subsequent engineering efforts guided by the omics studies further increased the titer of putrescine by 30% [56]. In another study, transcriptomic and metabolomic data were analyzed to uncover the association between cellular metabolism and the amino acid-producing phenotype, suggesting that active pentose phosphate pathway and glyoxylate cycle are correlated with efficient production of branched-chain amino acids [57]. On the other hand, bioproduction scale-up from laboratory flasks to industrial fermenters requires multiscale process analyses and optimizations. Thereby, various process models have been built to predict *C. glutamicum* fermentations [58], to gain insights into cell metabolism under bioreactor conditions [59], and to quantify bioreactor mass transfer, hydromechanics, and power input [60]. Moreover, the integration of process models with intracellular omics analysis under scale-down conditions provides valuable perspectives on *C. glutamicum* physiologies inside inhomogeneous industrial fermenters [61].

Novel *C. glutamicum* strains for metabolic engineering applications

While genomic tools and computational model development have reached maturity for the ATCC 13032-type strain, differences between the type strain and other *C. glutamicum* isolates remain an untapped reservoir of potential metabolic capacity. A phylogenetic analysis of the 26 most common *C. glutamicum* isolates described in the literature identified 9 distinct groups with unique genomic islands and complex polymorphisms that may be related to their specific amino acid secretion phenotypes [62]. These *C. glutamicum* isolates can have differing potentials to produce desirable heterologous bioproducts. *N*-acetylglucosamine (GlcNAc) is a monosaccharide with potential applications in human health. Deng and coworkers introduced the *Caenorhabditis elegans GNA1* gene (encoding glucosamine-6-phosphate acetyltransferase) into different *C. glutamicum* isolates and detected GlcNAc titers at 3.0 g/L in the S9114 isolate. In contrast, ATCC 13032 produced 0.5 g/L GlcNAc. The authors were able to adapt standard *C. glutamicum* gene modification tools in the S9114 isolate to further boost titers in batch mode to 6.9 g/L in rich media [63]. Similarly, Banerjee and coworkers tested the production of a 5-gene isoprenol production pathway in a transformation-improved Δ*mrr* ATCC 13032 strain as well as in isolate BRC-JBEI 1.1.2, and found that isoprenol titers

were at the lower detection limit (15 mg/L) in the type strain but were twenty-fold higher in BRC-JBEI 1.1.2 [64]. Many (> 500) genes in these *C. glutamicum* isolates lack any functional characterization and have no known homologs in other species, and this trend will likely hold as more genomes from related *Corynebacteria* are identified from diverse microbiomes using high-quality metagenomic assembly approaches. Functional genomics approaches using parallel transposon-mutagenized mutant libraries that have been applied in other bacterial hosts will enable the comparison of gene function across these isolates, providing insights into the unknown genes harbored in these strains [65].

Conclusions and outlook for the industry

C. glutamicum has superior capability in the biosynthesis of diverse amino acids, organic acids, short-chain alcohols, and their derivatives, many of which are bulk chemicals. The fermentation facilities and bioseparation techniques for *C. glutamicum* factories have been established, facilitating the commercialization of other compounds beyond amino acids. Meanwhile, the development of omics analyses and high-throughput cultivation/screening [66] is momentously speeding strain characterization and development. Additionally, the existence of a natural aromatic-degrading pathway and the strong resistance to aromatic inhibitors in hemicellulosic hydrolysates suggest promising potentials of *C. glutamicum* for the utilization of lignocellulose to produce diverse chemicals [64]. On the other hand, it should be noted that *C. glutamicum* is not the best chassis organism for producing all compounds. For example, natural products are synthesized in this bacterium at low yields. To improve the functions of the plant-derived pathways in *C. glutamicum*, several approaches can be employed, including transporter engineering or cell wall remodeling to increase the efflux of the final products, enzyme modifications to enhance catalytic performances, and modular pathway engineering [67,68]. In addition, advanced metabolic modeling and emerging AI technologies may accelerate *C. glutamicum* engineering to synthesize various high-value products.

CRediT authorship contribution statement

Writing – original draft preparation: **JZ, ZZ**; Writing – review & editing: **ZX, TE, AM, MK, and YT**.

Conflict of interest statement

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

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