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INVITED REVIEW PAPER

Recent progress in metabolic engineering of *Corynebacterium glutamicum* for the production of C4, C5, and C6 chemicals

Kei-Anne Baritugo*,‡, Jina Son*,‡, Yu Jung Sohn*,‡, Hee Taek Kim, Jeong Chan Joo***, Jong-il Choi****, and Si Jae Park*,†**

*Department of Chemical Engineering and Materials Science, Graduate Program in System Health and Engineering, Ewha Womans University, Seoul 03760, Korea
**Department of Food Science and Technology, College of Agriculture and Life Sciences, Chungnam National University, Daejeon 34134, Korea
***Department of Biotechnology, The Catholic University of Korea, Bucheon-si, Gyeonggi-do 03760, Korea
****Department of Biotechnology and Bioengineering, Chonnam National University, Gwangju 61186, Korea
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Abstract−Recent environmental problems caused by petroleum-based production of chemicals have accelerated the development of biorefineries for bio-based production of chemicals. Non-pathogenic microorganism, *Corynebacterium glutamicum*, has extensively been engineered and used as an industrial platform host strain for the commercial production of amino acids, such as L-lysine and L-glutamate. However, only recently has it been developed beyond its use for amino acid production. Recent advances in multiomics approaches, synthetic biology tools and metabolic engineering strategies have enabled the development of recombinant *C. glutamicum* into a versatile microbial cell factory for bio-based production of value-added platform chemicals and polymers by utilization of a broad range of biomass-derived sugars. In this review, we discuss the recent development of synthetic biology tools and techniques used for the enhancement of *C. glutamicum*'s ability to utilize renewable resources, specifically lignocellulosic biomass, for the production of platform chemicals with C4-C6 carbon backbone such as C4-isobutanol, 2,3-butanediol, C5-itaconic acid, 3-methyl-1-butanol, 2-methyl-1-butanol and C6-muconic acid.

Keywords: *Corynebacterium glutamicum*, Synthetic Biology, Metabolic Engineering, Biorefinery, Platform Chemicals

INTRODUCTION

Recent concerns regarding the ongoing climate crisis, environmental problems and fossil fuel depletion related to the petroleum-based mass production of platform chemicals have increased our interest towards greener alternatives such as biorefinery processes for the sustainable bio-based production of platform chemicals and polymers [1-8]. Biorefineries have extensively utilized renewable resources such as lignocellulosic biomass and even alternative carbon sources such as plastic wastes and C1 gases by using engineered microbial strains for the fermentative production of value-added platform chemicals, biofuel, and biopolymers [8-21]. Recent progress in multiomics approaches, synthetic biology tools and metabolic engineering strategies have improved the metabolic capacity of engineered host strains for the conversion of complex biomass feedstocks into a broad range of value-added products. For example, recombinant *Escherichia coli*, *Saccharomyces cerevisiae* and *Corynebacterium glutamicum* strains have been developed as efficient microbial cell factories and used as host strains for sustainable biorefinery systems [3,12-24]. However, biorefineries that use *E. coli* and *S. cerevisiae* are often limited by carbon catabolite repression (CCR) when the carbon sources are mixed sugars from complex biomass. Moreover, the protein-secretion system in both strains has been limited by the solubility of the target product, which often leads to its retention in periplasm or aggregation as inclusion bodies. On the other hand, *C. glutamicum* displays weak catabolite repression, which allows the efficient utilization of mixed carbon sources and has minimal protease activity, which allows secretion of properly folded and functional proteins [24-27].

C. glutamicum is a non-pathogenic and facultative anaerobic microbe that is mainly used for the industrial production of amino acids, including L-lysine and L-glutamate. Compared to *E. coli* and *S. cerevisiae*, the physical and genetic properties of *C. glutamicum* strains have been comprehensively studied and several metabolic engineering strategies and synthetic biology tools (Fig. 1) have been developed for the construction of a superior microbial cell factories for the conversion of a broad range of biomass into a wide variety of value-added platform chemical production [8,13,14,22]. Biomass-derived substrates that *C. glutamicum* naturally utilizes include fructose, glucose and sucrose, and the enhancement of substrate uptake has increased the yield of biochemical products [24]. Moreover, several *C. glutamicum* strains have been developed in order to utilize unconventional lignocellulosic biomass-derived carbon sources such as cellulose, xylan, glucans, cellulose, D-xylose, and L-arabinose

†To whom correspondence should be addressed.
E-mail: parksj93@ewha.ac.kr
‡Kei-Anne Baritugo, Jina Son and Yu Jung Sohn contributed equally to this work.

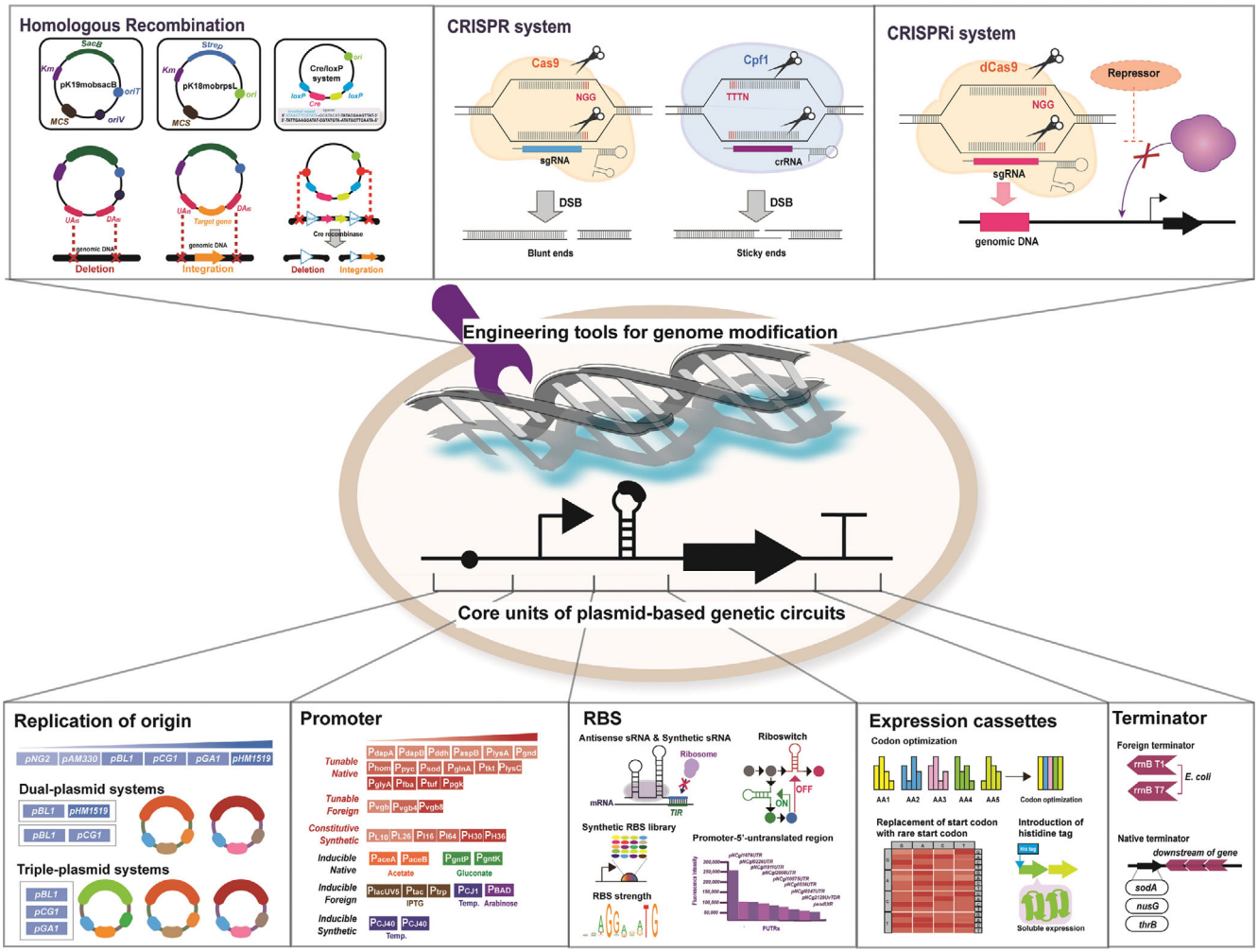

Fig. 1. Schematic overview of plasmid-based synthetic biological circuits and genomic engineering tools techniques for engineering *C. glutamicum*.

(Fig. 2) [13,14,22]. However, even though *C. glutamicum* naturally shows insignificant catabolite repression towards mixed carbon sources, it cannot directly utilize complex polysaccharide biomass such as cellulose, xylan and pectin. In this regard, several tools and techniques such as consolidated bioprocesses have been developed for the enrichment of a range for substrate utilization by recombinant *C. glutamicum* strains [22]. In addition to the development of multi-carbon co-utilization systems, systematic engineering of *C. glutamicum* has improved the production of native amino acids and enabled the enhanced production of platform chemicals such as diamines (cadaverine, putrescine), amino-carboxylic acids (gamma-amino-butyric acid, 5-aminovaleric acid), and carboxylic acids (succinic acid, glutaric acid and 5-hydroxyvaleric acid). Numerous excellent publications have already presented a detailed description of metabolic engineering strategies used to develop recombinant *C. glutamicum* strains for the production of aforementioned platform chemicals [28-40]. In this paper, we focus on discussing metabolic engineering strategies for developing and enhancing the production of platform chemicals with C4-C6 carbon backbone such as C4-isobutanol, 2,3-butanediol, C5-itaconic acid, 3-methyl-1-butanol, 2-methyl-1-butanol and C6-cis, cis-muconic acid (Fig. 3) [8]. In this review, we focus on the recent advances in synthetic biology and metabolic engineering strategies and tools used for the development of engineered *C. glutamicum* strains for bio-based production of C4-C6 platform chemicals from renewable lignocellulosic biomass.

1. Synthetic Biological Circuits and Genome Editing for Metabolic Engineering

In engineering *C. glutamicum* strains for biomass utilization and platform chemical production, synthetic biological circuits based on plasmid systems are used to implement and/or reinforce the designed pathways by further evaluation and optimization. These synthetic biological circuits based on plasmids are composed of synthetic DNA parts such as replication origin, promoters, ribosome binding sites, expression cassettes and terminators. Each of these parts can be customized for specific modulation of gene expression levels and thus optimization of designed pathways [8,14,41].

1-1. Engineering Replication Origin for Tunable Plasmid Copy Number and Multiple Plasmid Systems

Plasmids have a replication origin (ori), which is an important part for maintenance and reproduction of plasmids in host strains. Replication origins for the construction of plasmids used in *C. glutamicum* have been from low copy plasmid, pNG2, the endoge-

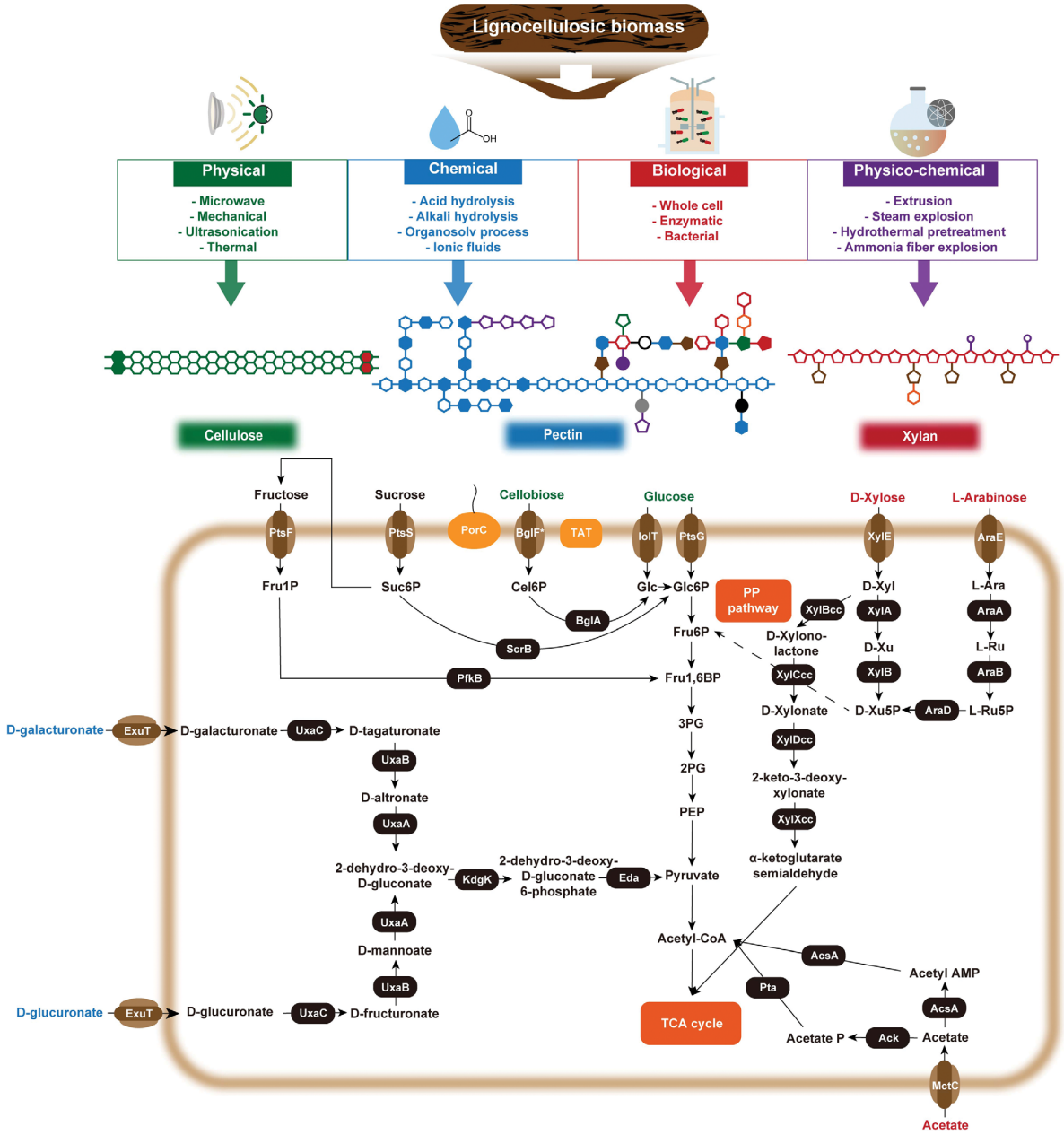

Fig. 2. Schematic overview of consolidated bioprocesses and pathways for utilization of lignocellulosic biomass derived substrates (Cellulose, Xylan, Pectin). Purple circle indicates acetic acid, brown pentagon indicates arabinose, red pentagon indicates D-xylose, green filled hexagon indicates glucose, orange hexagon indicates AHG, blue hexagon indicates D-Galacturonic acid, blue filled hexagon indicates L-rhamnose, purple pentagon indicates L-arabinose, brown filled pentagon indicates D-apiose, red hexagon indicates L-aceric, black circle indicates D-galactose, purple filled circle indicates L-arabinopyranose, brown filled pentagon indicates L-arabinofuranose, green pentagon indicates D-fucose-(2-O-methyl, 3-O-acetyl), green filled pentagon indicates L-fucose, orange hexagon indicates D-glucuronic acid, red pentagon indicates D-xylose-(2-O-methyl), gray filled circle indicates DHa, black filled circle indicates KDo.Fru1P, fructose 1-phosphate; Suc6P, sucrose 6-phosphate; Cel6P, cellulose-6-phosphate; Glc, glucose; Glc6P, glucose 6-phosphate; Fru6P, fructose 6-phosphate; Fru1,6BP, fructose-1,6- biphosphate; 3PG, 3-phosphoglycerate; 2PG, 2-phosphoglycerate; PEP, phosphoenolpyruvate; D-Xyl, D-xylose; D-Xu, D-xylulose; D-Xu5P, D-xylulose 5-phosphate; L-Ara, L-arabinose; L-Ru, L-ribulose; L-Ru5P, L-ribulose 5-phosphate; Acetyl AMP, Acetyl adenylate; Acetyl P, acetyl phosphate; PtsF, fructose-specific EIIABC permease; PfkB, fructose-1-phosphate kinase; PtsS, sucrose-specific phosphotransferase system; ScrB, sucrose-6-phosphate hydrolase; PorC, porin protein; BglF*, cellobiose specific PTS; BglA, β glucosidase A; TAT, twin-arginine translocation signal protein; IolT, inositol transporter; PtsG, glucose specific PTS; XylE, D-xylose transporter; XylA, xylose isomerase; XylB, xylulokinase; XylD, Xylonate dehydratase; XylX, 2-dehydro-3-deoxy-D-xylonate dehydratase; AraE, L-arabinose specific transporter; AraA, L-arabinose isomerase; AraB, ribulokinase; AraD, L-ribulose-5-phosphate 4-epimerase; MctC, monocarboxylic acid transporter; AcsA, acetyl-CoA synthetase; Ack, Pta, phosphotransacetylase; ExuT, transmembrane protein; UxaC, uronate isomerase; UxaB, mannonate oxidoreductase; UxaA, urinate isomerase; KdgK, 3-deoxygluconokinase; Eda, 2-keto3-deoxygluconate-6-phosphate aldolase.

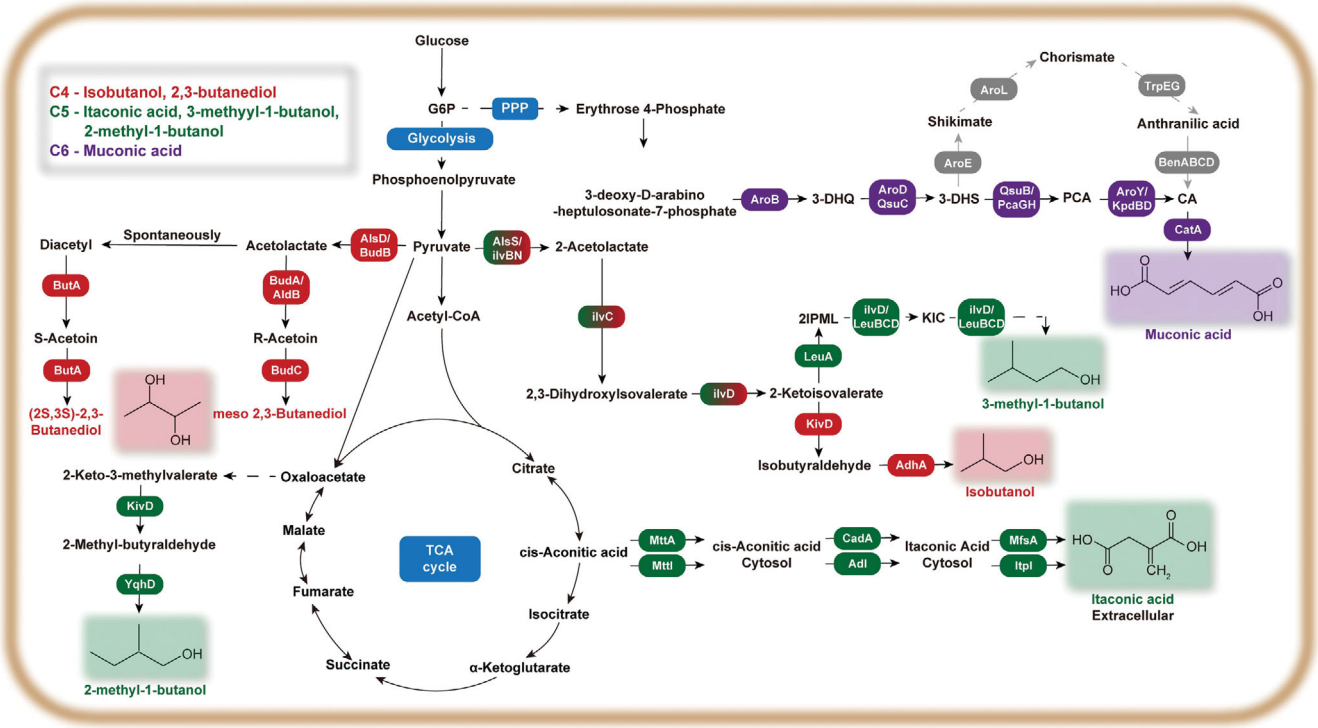

Fig. 3. General pathway for the production of C4-C6 platform chemicals in engineered *C. glutamicum* strains G6P, glucose-6-phosphate; 3-DHQ, 3-dehydroquinate; 3-DHS, 3-dehydroshikimate; PCA, protocatechuic acid; CA, catechol; 2IPML, 2-Isopropylmalate; KIC, 2-ketoisocaproate; AroB, DHQ synthase; AroD, 3-dehydroquinate dehydratase; QsuC, 3-dehydroquinate dehydratase; PcaGH, PCA dioxygenase alpha/beta subunit genes; AroY, protocatechuate decarboxylase; KpdBD, PCA decarboxylase subunit; CatA, catechol 1,2-dioxygenase; AroZ, 3-dehydroshikimate dehydratase; AroE, shikimate dehydrogenases; AroL, shikimate kinase; TrpEG, anthranilate synthase; BenABC, benzoate 1,2-dioxygenase; BenD, benzoate diol dehydrogenase; AlsS, acetolactate synthase; ilvBN, acetohydroxyacid synthase; ilvC, acetohydroxyacid isomeroreductase; ilvD, dihydroxyacid dehydratase; LeuA, 2-isopropylmalate synthase; LeuB, 3-isopropylmalate dehydrogenase; LeuCD, 3-isopropylmalate dehydratase subunit; KivD, 2-ketoisovalerate decarboxylase; AdhA, alcohol dehydrogenase; MttA, putative mitochondrial tricarboxylic transporter; MttI, mitochondrial TCA transporter; CadA, cis-aconitate decarboxylase; AdI, cytosolic aconitate-Δ-isomerase; MfsA, a major facilitator superfamily transporter; ItpI, itaconate transport protein; AlsD, Alpha-acetolactate decarboxylase; BudB, Acetolactate synthase; BudC, Diacetyl reductase; ButA, Diacetyl reductase; YqhD, Alcohol dehydrogenase.

nous cryptic medium-copy plasmids, pBL1, pCG1 and pGA1 and even the engineered high-copy number ori, pCG1:$parB_{TGC\rightarrow TGA}$ [8,42,43]. However, low transformation efficiency and plasmid instability might occur if final plasmid size is more than 10 kb, and current plasmids are already large because it has two ori for plasmid maintenance in *C. glutamicum* and *E. coli* [8,44]. Thus, an alternative option for the expression of larger or multiple enzymes in the recombinant strain has been developed by dual- and triple-plasmid systems, in which the compatibility of ori is an important factor for allowance of the co-existence of plasmids [43]. To date, dual-plasmid systems such as CoryneBrick (pBL1) : pZ8 (pHM1519) and pVWEx1 (pCG1) : pEKEx3 (pBL1) and triple-plasmid systems such as pVWEx1 (pCG1) : pEKEx3 (pBL1) : pECXT99A (pGA1) have successfully been used in implementing pathways with three or four genes in recombinant *C. glutamicum* strains (Table 1) [37,45]. This allows higher gene transcription efficiency and better modulation of protein expression under different promoters [37,45].

1-2. Native and Synthetic Promoters for Tunable Modulation of Metabolic Pathways

Promoters, which are another tunable part of gene transcription in plasmids, are generally classified into inducible and constitutive groups. Inducible promoters used in *C. glutamicum* are either native (P_{aceA}, P_{aceB}, P_{gntB}, P_{gntK}), adapted from *E. coli* (P_{lacUV5}, P_{tacM}, P_{trp}, P_{araBAD}) or created from related *Corynebacterium ammoniagenes* (P_{CJ40}, P_{CJ10}) (Fig. 1) [8,46,47]. However, the use of constitutive promoters is preferred since inducible plasmids often require expensive inducers (isopropyl β-D-thiogalactopyranoside or IPTG), high concentration of toxic inducers (acetate, gluconate), additional expression of inducer's permease (L-arabinose) and alteration of culture conditions [8,47-49]. Strong constitutive promoters (P_{gapA}, P_{sod}, P_{tuf}) have been used for high-level of gene expression by replacement of weak native promoters in the chromosome for the enhanced production of target metabolites in recombinant *C. glutamicum*. Recently, a novel promoter of a putative protein coding gene CP_2454 (P_{CP_2454}) was identified and its expression level was comparable to that of strong constitutive promoters such as P_{sod} and P_{tuf} [50]. However, heterologous expression in recombinant strains often leads to a by-product accumulation and retarded growth rate; thus, recruited biosynthetic pathways need to be optimized for balanced target metabolite production and cell maintenance. For this pur-

Table 1. Plasmids and genomic modification systems for engineering *C. glutamicum* strains

System	Plasmids	Characteristics	Ref.
Plasmid-based protein expression system			
Dual Plasmid	pBbEB series	$pBL1_{ori}$, P_{trc}, lacUV5, IPTG-inducible, Cm^1	43,45
	pZ8-1	$pHM1519_{ori}$, P_{tac}, Constitutive expression, Km^1	
Triple Plasmid	pVWEx1	$pCG1_{ori}$, P_{tac}, IPTG-inducible, Km^1	43
	pEKEx3	$pBL1_{ori}$, P_{tac}, IPTG-inducible, Km^1	
	pECXT99A	$pGA1_{ori}$, P_{trc}, Constitutive expression, Tet^1	
Genomic manipulation systems			
Homologous Recombination 2-step verification	pK19mobsacB	oriV[2] pUC19 MCS, lacZα, Km^1	79
Cre/ lox	pCRB554	*loxP*, L-IR[3], R-IR[3], *lacZ*, Tn-Km[4], transposase of IS31831, Km^1, Sp^1	80
	pCRB555	*loxP*, L-IR[3], R-IR[3], Tn-Cm[4], *loxP* transposase of IS31831, Cm^1, Sp^1	
	pCRA419	*lox66* site, *lacP*:*lacZ*, $pMB1/M13_{ori}$ Sp^1	
Cre/mutant lox	pCRA406	$pBL1_{ori}$, $pMB1/M13_{ori}$, Cre recombinase, Cm^1	81
	pCRA418	*lox71* site, $pMB1/M13_{ori}$, Km^1	
	pCRA419	*lox66* site, *lacP*:*lacZ*, $pMB1/M13_{ori}$ Sp^1	
Homologous Recombination & Cre/lox site-specific recombination	pDTW109	oriE, rep_{TS}, Temperature sensitive, P_{tacM}:cat; P_{tac}:Cre, Cm^1, Km^1	82
	pDTW201	loxP-kan-loxP segment, Ap^1	
	pDTW202	loxL-kan-loxR segment, Ap^1	
RecET-Cre/loxP	PBS-Cre-Kan	Ptrc, theoE*-RBS[5]:Cre, T1 and T2 terminator, Ap^1, Km^1	83
	pEC-recE/recT	IPTG-inducible expression of RecET, Cm^1	
	PBS-crtB-L/R-Kan	Crtb-L-Kanamycin-Crtb-R cassette, Ap^1, Km^1	
Homologous Recombination 1-step verification	pk18mobrpsL	Str_R Host strain[6]; P_{tuf}, *rpsL*, Km^1	86
CRISPRi/dcas9	pZ8-Ptac	pZ8-1 derivative, IPTG inducible, $lacI^q$ .deactivated cas9, Km^1	88
	pAL-pgi-NT	pAL374 derivative, sgRNA targeting non-template strand of *pgi*, Sp^1	
CRISPR/Cas9	pFSC	$repA_{ori}$ IPTG inducible P_{trc}, trans-activating CRISPR RNA, 20 nt target sgRNA, CRISPR RNA, T1 T2 terminator, Km^1	88
	pFST	pBL1ori, pUCori, IPTG inducible P_{tac} SD sequence (AAAGGAGGA-CAACTA), Cas9 fron *S. pyogenes*, rrmB terminator, Cm^1	
Double plasmid based CRISPR/Cpf1	pJYS1Ptac	$pBL1_{ori}$, $pSC101_{ori}$, P_{tacM}:FnCpf1, P_{lac}:RecT, lacIq, Km^1	89
	pJYS1Peftu	$pBL1_{ori}$, $pSC101_{ori}$, P_{lacM}:FnCpf1, Petfu:RecT, Km^1	
	pJYS2_crtYf	rep_{ori}, $pMB1_{ori}$, P_{lacM}:FnCpf1, Pj23119: CRISPR RNA targeting crtYf, Sp^1	
All in one CRISPR/Cpf1	pJYS3_ΔcrtYf	$pBL1_{ori}$, $pSC101_{ori}$, P_{lacM}:FnCpf1 Pj23119: CRISPR RNA targeting crtYf, 1kb up- and down-stream homologous arms flanking 705 bp deletion fragment inside crtYF Km^1	
CRISPR/Cas9	pCas9	$pBL1_{ori}$, pUC_{ori},P_{tac} cas9, rrnB terminator,$lacI^q$ Cm^1	92
Genome deletion	pgRNA3	P_{11F}, guideRNA, trrnB terminator, $repA_{ori}$, upstream arm of target gene for deletion (TGD), Cas9-guide-RNA, downstream arm TGD, Km^1	
CRISPR/Cas9 Genome insertion	pgRNA4	P_{11F}, guideRNA, trrnB terminator, $repA_{ori}$, upstream arm TGD, Cas9-guide-RNA target gene for insertion (TGI), downstream arm TGD, Km^1	
ssDNA-directed recombineering CRISPR//Cas9+RecT	pgRNA5	P_{11F}, guideRNA, trrnB terminator, $repA_{ori}$, P_{prpD2}, RecT Km^1	
Double-locus Editing	pgRNA7	P_{11F}, guideRNA of TGI #1, trrnB terminator, P_{11F}, guideRNA of TGI #2, trrnB terminator, $repA_{ori}$, P_{prpD2}, RecT Km^1	

[1]Abbreviations for antibiotics used: Sp-Spectinomycin, Km-Kanamycin, Cm-Chloramphenicol, Tet-tetracycline, Ap-Ampicilin, Streptomycin
[2]oriV - origin of vegetative replication
[3]L-IR - inverted repeat; R-IR - Right inverted repeat
[4]Tn-Km - Kanamycin resistance cassette; Tn-Cm Chloramphenicol resistance cassette
[5]theoE*-RBS is Theophylline riboswitch E* for induction of Cre
[6]St_R - Steptomycin resistant host strain
- Information is not available from reference

pose, tunable modulation of gene expression using native, adapted from *Vitreoscilla* sp., and synthetic promoters has been examined. For tunable native promoters, sixteen native promoters with a wide range of strengths were characterized for precise gene modulation. High strength (P_{pgk}>P_{tuf}>P_{fba}>P_{glyA}>P_{lysC}>P_{tkt}>P_{glnA}>P_{sod}>P_{pyc}>P_{hom}) and low strength (P_{gnd}>P_{lysA}>P_{aspB}>P_{ddh}>P_{dapB}>P_{dapA}) promoters have been identified based on their expression of β-galactosidase [51]. In another study, the tandem repeats of the promoter from *Vitreoscilla* hemoglobin protein (P_{vgb}>P_{vgb4}>P_{vgb8}) allowed the tunable expression of key genes for gamma-aminobutyrate and 5-aminovalerate productions in recombinant *C. glutamicum* strains (Fig. 1) [35]. Recently, a library of synthetic promoters with leaderless transcripts was generated and green fluorescent protein was used as reporter enzyme to characterize them through fluorescent-activated cell sorting analysis (FACS). The resulting synthetic promoters (P_{H36}>P_{H30}>P_{I16}>P_{I51}>P_{L10}>P_{L26}) were able to constitutively express target genes for the high-level production of endoxylanase, antibody fragments, cadaverine, gamma-aminobutyrate, and 5-aminovalerate and even for secretory production of recombinant proteins when the synthetic promoters were combined with signal peptide sequence [32-36,52-57].

1-3. Ribosome Binding Site Engineering for Fine Tuning Expression of Proteins

Ribosome binding site engineering via application of antisense small RNA (asRNA) and synthetic RBS libraries has enabled the modification of 5′ untranslated region (UTR) for the improved translational regulation of key genes for target metabolite production [8]. The use of asRNA modulates protein expression by binding to the target mRNA and consequently blocking gene translation from the mRNA. For example, glutamate production was enhanced in recombinant *C. glutamicum* strain by decreasing the specific activity of 2-oxoglutarate dehydrogenase complex through *odhA*-antisense RNA expression [58]. Recently discovered small RNAs (sRNAs) could also be useful for the construction of tailored DNA/RNA parts for engineering *C. glutamicum* [58]. Furthermore, tunable 5′ UTR libraries with conserved RBS (AGGA) and synthetic riboswitches have been used to synthesize tunable promoters and L-lysine riboswitch, by which L-lysine production in recombinant *C. glutamicum* strains was improved [56,60]. In another study, there was a significant improvement of enzyme expression levels after the replacement of conserved Shine-Delgarno (SD) sequence with *E. coli* SD; in addition, the introduction of a conserved SD sequence (GAAAGGCGA) with seven random mutations in the translation initiation region of target gene led to the enhanced enzyme activities of *tpi* (triosephosphate isomerase) and $nhaBAG_{Rr}$ (nitrile dehydratase) genes [61,62]. Recently, the optimization of RBS sequence by construction of RBS libraries for regulation of the RBS strength of *aroGBDE* genes resulted in the improvement of shikimic acid production using recombinant *C. glutamicum* [63]. Moreover, the replacement of internal RBS with strong RBS from *C. glutamicum* (GAAAGGAGGTTTGGACA) in decompressed *vio* operon (*vioABEDC*) from *Chromobacterium violaceum* led to the enhanced production of crude violacein using recombinant *C. glutamicum* strain [64]. In another study, the optimal RBS (GAAAGGAGA), aligned spacing (6 nucleotides) and promoter (P_{tacM}) for co-expression of glutamate decarboxylase genes (*gadB2* and $gadB1_{T171I/D294G/E312S/Q346H}$) from *Lactobacillus brevis* were identified for enhanced GABA production from glucose [65]. Recently, 90 native promoter-5′-untranslated region (PUTRs) were amplified and linked to a fluorescent reporter gene wherein 17 strong PUTRs were identified to be able to maintain stable expression strengths; the strongest fluorescent level was achieved under $P_{NCg1676}$UTR, which was five times higher than that of P_{sod}UTR [66].

1-4. Engineering Expression Cassettes of Plasmids for Enhanced Protein Expression

Another point to consider for the improvement of gene expression in the expression cassettes of plasmids includes several strategies such as codon optimization, modified start codons and addition of his-tag to target gene [8]. First, codon optimization improves protein expression by tailoring codon usage and GC content of the recruited heterologous gene for the target host strain. This strategy has been applied for the expression of recruited genes from *E. coli* and *P. putida* into recombinant *C. glutamicum* strains for the enhanced production of cadaverine and 5-aminovalerate, respectively [32,67]. Second, start codon engineering such as replacement of ATG start codon with the rare variants including GTG and TTG modulates gene expression by regulating transcription rate. For example, modification of start codons of the genes encoding pyruvate dehydrogenase and isocitrate dehydrogenase was found to increase L-lysine production [68,39]. Lastly, the addition of his_6-tag to N-terminus of target protein could enhance its expression [70] and the fusion of his_6-tag to N-terminus of codon-optimized *davA* gene encoding delta-aminovaleramidase resulted in the enhanced production of 5-aminovalerate using recombinant *C. glutamicum* [32].

1-5. Transcription Terminators for Efficient Termination of Metabolic Pathways

The last point to consider for optimal plasmids-based protein expression is a transcription terminator. The common terminators used in expression vectors for *C. glutamicum* are the rrnB T1 and T7 terminator from *E. coli* [71-73]. Native transcription terminators found in downstream regions of *thrB*, *sodA* and *nusG* genes were discovered to have palindromic structures [74]. Rho-independent functional transcription terminator fragments (185- and 127-bp) were identified from the downstream of homoserine kinase gene (*thrB*) [75]. A 19-nucleotide inverted repeat identified from the downstream of anti-terminator protein (*nusG*) was used for the promoter-probe vectors, pULCE0 [76]. The shuttle vector, pMM23, was equipped with transcriptional terminator found from 18 and 88 bp downstream of superoxide dismutase (*sodA*) in *Corynebacterium melassecola* [77]. Recently, the characterization of sRNAs in *C. glutamicum* ATCC 13032 via transcriptome sequencing has identified 69 sRNAs of Rho-independent terminators, which can be used for the construction of plasmids for *C. glutamicum* (Fig. 1) [59].

1-6. Genomic Modification of *C. glutamicum*

After optimization of engineered metabolic pathway for target metabolites production by identification of metabolic bottlenecks through the plasmid-based evaluation, genomic modification of the host strain is usually performed for the construction of stable industrial platform strains since plasmid instability and metabolic burden caused by plasmids-based expression system often lead to unwanted

by-product formation and poor cell growth [8]. Genomic engineering of host via gene integration, deletion, and replacement in chromosome allows the construction of a plasmid-free strain equipped with stable expression of synthetic pathway for target metabolites and block of competing pathways, thereby, enables the redirection of metabolic flux towards balanced target metabolite production and cell maintenance [78].

Traditional technologies for genomic engineering of *C. glutamicum* include homologous recombination method using pK19mobsacB and Cre-Lox systems, which use suicide vectors for gene disruption and/or insertion [79-81]. The pK19mobsacB system is based on SacB, which mediates sucrose hydrolyzation for the synthesis of levan, resulting in sucrose insensitivity of successful recombinant colonies [79]. Another dual-plasmid system for integration/amplification of gene in *C. glutamicum* is the Mu-transposition system which consists of a non-replicative integrative plasmid with transposing mini-Mu(LER) unit and the integration helper plasmids which express the transposition factor genes for MuA and MuB. This system allows consequent independent integration/amplification of target genes at high copy numbers [82]. Recently, an alternative and more rigorous one-step verification procedure using pK18mobrpsl-mediated genome editing method was developed by the use of counter-selectable marker identified as small ribosomal protein S12P (*rpsL*), which confers streptomycin resistance [83]. Another traditional method for genomic engineering is the Cre-loxP system, which relies on Cre recombinase which catalyzes specific recombination between two loxP sites. However, these methods are deemed laborious and inefficient because of numerous rounds for counter-selection and creation of false positives [80,81,84].

Recently, RecFACS and CRISPR-based technologies have been developed for rapid and precise genome editing of *C. glutamicum*. Genomic site saturation mutagenesis can be done using RecFACS system, which is a RecT-mediated single-strand recombination technology combined with Lrp-based biosensors for one-step generation and colony selection via fluorescence-activated cell sorting system (FACS) [84]. In addition, a RecET-Cre/loxP system for marker-less gene deletion was developed using exonuclease recombinase pair RecE and RecT for recombineering a designed self-excisable linear double strand DNA cassette equipped with Cre/loxP system [85,86]. Since it was found that *Cas* from *Streptococcus pyogenes* used in CRISPR/Cas9 is toxic to *C. glutamicum* strains, an alternative CRISPR/Cpf1 with *Cpf1* from *Francisella novicida* was developed for CRISPR-associated genome editing tools for *C. glutamicum* [87-90]. For instance, two RecET-assisted CRISPR/Cas9 genome editing systems were developed. The first one was a two plasmid system with pCas9 plasmid for expression of Cas9 under P_{tac} promoter and the pgRNA plasmid with RecT-RNA expression cassette, which would mediate precise introduction of single nucleotide changes and double-locus [91]. The other RecET assisted CRISPR/Cas9 strategy involved the genomic integration of inducible RecET-Cas9 components and the use of sgRNA-repair template plasmid [92]. Recently, MACBETH, a multiplex automated *C. glutamicum* base editing method using CRISPR/nCas9(D10A)-AID was developed that can edit three loci with 23.3% efficiency without foreign DNA template [93]. Multiplex engineering for combinatorial gene knockouts and repression were achieved using both CRISPR/Cas9 and CRISPR/Cpf1 systems [93-97]. These efficient genome editing tools enable the rapid development of robust industrial platform strains that can be used for bio-based production of value-added products (Fig. 1, Table 1) [8].

2. Metabolic Engineering of *C. glutamicum* for Utilization Lignocellulosic Biomass

2-1. Lignocellulosic Biomass

Lignocellulosic biomass is a non-food biomass mainly composed of 1) cellulose (35-50%), 2) hemicellulose (15-35%) and 3) lignin (15-25%) [13,22,98]. Lignocellulosic biomass pre-treatment is necessary for enhancing enzymatic hydrolysis of its components to release highly recoverable fermentable sugars. An ideal pre-treatment system should be cheap and efficient to easily overcome biomass recalcitrance with low generation of inhibitory products that might negatively affect enzymatic hydrolysis and microbial fermentation [2,99]. There are four types of biomass pre-treatment methods that can be applied to lignocellulosic biomass: 1) physical, 2) chemical, 3) physio-chemical, and 4) biological [100]. Physical pre-treatment exposes biomass to high pressure and/or high temperature in order to alter its physical structure (surface area, polymerization degree, size), while chemical pre-treatment involves subjecting biomass to chemicals (alkali, acid, sulfite, organosolv, ionic liquid) and high temperature in order to disrupt its chemical structure [2]. Physico-chemical pretreatments such as steam explosion (high pressure and high temperature), hydrothermal pre-treatment (high temperature and acids) and ammonia fiber explosion (high pressure; anhydrous liquid ammonia) processes result in treated hydrolysates with altered physical and chemical structure after combined exposure at physical and chemical stresses [101-118]. Lignocellulosic biomass is often pre-treated via physico-chemical process because the recalcitrant structure of raw biomass needs to be broken to make it more susceptible to chemical or enzymatic pre-treatment for further release of fermentable sugars. Acid hydrolysis of hemicellulose biomass, such as wheat/rice straw, corn cob and wheat bran with sulfuric acid at 105-134 °C, was reported to produce hydrolysates which contain D-xylose, L-arabinose acetate and glucose as major sugar components [118,119]. However, toxic inhibitors in hydrolysates need to be removed, for example, by treating activated charcoal before the usage of hydrolysates as carbon sources in microbial system [120]. Biological pre-treatment is rather mild compared to other methods since ligninolytic enzymes (laccase, lignin/manganese peroxidase, glyoxal/aryl alcohol oxidase) and/or microorganisms (white-, brown-, soft-rot fungi, actinomycetes) can efficiently degrade lignin with low energy input. Moreover, there is less by-production of toxic enzymatic hydrolysis and microbial fermentation inhibitors, such as 2-furaldehyde, 5-hydroxymethyl-2-furfural,4-hydroxybenzoic acid, vanillic acid, which are formed during chemical pretreatment under high temperature and acidic conditions. Thus, the use of biological pretreatment benefits the development of the biorefinery system that relies on pre-treated lignocellulosic hydrolysates as the substrates for the production of value-added chemicals and materials [114-117]. For example, enzymatic hydrolysis of oat spelts xylan and corns stalk using accelarase/accelarase XY and cellulolytic enzymes have released their related D-xylose, glucose and L-arabinose fractions, which can be used as

carbon sources for microbial fermentation [122]. In another study, *Miscanthus sacchariflorus*, an energy crop, was chopped, air-dried, then simultaneously hydrated and ground into a slurry, which was finally hydrolysed by Cellic CTec3 to produced glucose [33]. In another study, the combined use of ionic liquids and enzyme saccharification of sorghum biomass successfully released glucose components from cellulose [121]. Thus, the combination of biological pre-treatment strategies with physical and/or chemical methods has extensively been scrutinized to further develop efficient pre-treatment processes for lignocellulosic biomass and release its three main carbohydrate polymers--cellulose, xylan and pectin, which can be utilized as the carbon sources by microbial cell factories for platform chemical production [2,22].

An ideal platform strain for lignocellulose-based biorefinery should be capable of direct and simultaneous utilization of multiple sugars in hydrolysed substrate. However, established industrial platform strains such as *E. coli* and *S. cerevisiae* display strong carbon catabolite repression (CCR) and preferably utilize glucose before other present carbon sources during fermentation. On the other hand, negligible CCR has been observed when *C. glutamicum* has been used in fermentation with mixed lignocellulose-derived sugars as carbon source and exhibits robust resistance to fermentation inhibitors often present in lignocellulosic hydrolysates [13,22]. Pre-treated lignocellulosic hydrolysates that have been used by *C. glutamicum* are summarized in Table 2, along with pre-treatment method and the related biomass-derived value-added products. Metabolic engineering strategies to enable direct utilization of these lignocellulosic carbohydrate polymers in a consolidated bioprocess using engineered *C. glutamicum* strains are described below according to the three main carbohydrate polymers and their related sugar monomers.

2-2. Cellulose

Cellulose is a polysaccharide that is made up of β-1,4-glycosidic bonded disaccharide cellobiose and monosaccharide glucose components. All *C. glutamicum* strains can naturally utilize glucose; however, cellobiose utilization is only observed in *C. glutamicum* R strain, which has a native mutant phosphotransferase permease subunit ($bglF_{V317M/A}$) for cellobiose degradation into its component glucose monomers (Fig. 2) [123]. To construct a cellulose degradation system in *C. glutamicum*, cellulolytic enzymes (cellobiohydrolase, β-endoglucanase, and β-glucosidase) from other microorganisms need to be recruited. The production of L-glutamate from cellulose was first demonstrated by recombinant *C. glutamicum* strain expressing endoglucanase gene (*celA*) from *Clostridium cellulovran* under *torA* signal sequence from *E. coli* for secretory production of endoglucanase into culture medium [124]. In another study, cellulose utilization was achieved by recombinant *C. glutamicum* with cell surface display system employing the twin-arginine translocation (TAT) signal protein for the secretion of endoglucanase (*XCC2387*) with porin protein (*porC*) as anchor for β-glucosidase from *Saccharophagus degradans* (*Sde1394*) (Fig. 2) [125]. Cellulose utilization by recombinant *C. glutamicum* strain was also observed when an endoglucanase E (CelE)-β glucosidase A (BglA) enzyme complex was anchored to cell surface using Msc mechanoselective channel [126]. Although secretion of cellulolytic enzymes enables direct degradation of cellulose, the secreted enzyme might be detached from the cell. Therefore, a cellulosome and cell surface display systems were modified for direct consumption of hydrolysed glucose [127]. Macromolecular constructs called cellulosomes can efficiently hydrolyse cellulose because they are a combi-

Table 2. Recent results on the development of *C. glutamicum* for utilization of lignocellulosic biomass-derived substrates

Biomass	Gene/Strategy	Product:	Titer (g/L)	Productivity (g/Lh)	Yield (g/g)	Ref.
Xylan	P_{I16}:*xylAB* from *E. coli*[1] P_{Cg1514}:*Cf1514*[2], *xlnA* from *S. coelicor*[1], P_{L10}:*xylE* from *E. coli*[1] CgR0949-*xynB* from *B. subtilis*[1]	L-lysine	1.12	-	-	55
Hemicellulose Fraction from Beech wood organosolv processing	Δ*pqo* Δ*ilvE* Δ*ldhA* Δ*mdh* CgLP4[3]: P_{tuf}:*xylA* from *X. campestris, xylB*[2] CgLP12[3]:P_{tuf}:*araBAD* from *E. coli*[1] *ilvBNCD*[2], *pntAB* from *E. coli*[1], *kivd* from *L. lactis*[1], *adhA*[2]	Isobutanol	0.53	-	0.31	71
Wheat bran and rice straw hydrolysate	*xylA* from *E. coli*[1] *araBAD* from *E. coli*[1]	L-lysine	6.1	0.085	0.25	119
Corn Cob dilute acid hydrolysate	Δ*ldhA*: *pta*-*ackA*[3]: P_{trc}:*xylAB* from *E. coli*[1]	Succinate	40.8	0.85	0.69	120
Corn Stalk Hydrolysate	Δ*ldh* Δ*ackA-pta* Δ*pqo* Δ*cat*: *xylAB* from *X. campestris*[1] *ldh*[3]:P_{tuf}:*araE* from *B. subtilis*[1] P_{trc}:*gltA, sucE*[2], P_{sod}:*pyc, ppc, tal, tkt*[2]	Succinate	98.6	4.29	0.81	121

[1]Heterologous expression of gene from specified microorganisms

[2]Overexpression of native genes under specified promoter

[3]*C. glutamicum* Loci with insertion of proceeding expression cassette

- Information is not available from reference

nation of several cellulolytic enzymes within a scaffolding, bound to dockerin domains of catalytic subunits. Thus, a recombinant *C. glutamicum* strain was developed by incorporation of several cellulases bound to scaffolding protein (CbpA), a carbohydrate binding module, two cohesion domains and chimeric *C. thermocellum* endoglucanase (*celE*) enzymes into the minicellulosome from *C. cellulovorans* [127]. In another study, a cell surface display system with β-glucosidase (*Sde1394*) from *S. degradans* with porin as an anchor protein enabled the resulting strain to produce 1.08 g/L of L-lysine from cellobiose (Table 2) [128].

2-3. Xylan

Xylans are polysaccharides made up of β-1,4-linked D-xylose with side branches of α-arabinofuranose and α-glucoronic acids [13]. To establish efficient systems for xylan degradation and pentose utilization in *C. glutamicum*, the heterologous expression of xylan hydrolases and several key enzymes for D-xylose and L-arabinose utilization is required [22]. During xylan degradation, the removal of L-arabinose, galactose and acetic acid side groups is catalyzed by xylanolytic enzymes, α-arabinofuranosidase, α-galactosidase and acetylxylan esterase. Additional expression of β-xylosidase separates xylooligosaccharide and xylobiose for production of D-xylose and L-arabinose [13,22]. Natural xylan catabolism has not been observed in most members of *Corynebacteriaceae*, except for *C. alkanolytiicum,* which can use xylooligosaccharide as a sole carbon source [129]. Xylan degradation and D-xylose utilization pathways were established in recombinant *C. glutamicum* strains by plasmid-based expression of β-xylosidase (*xylD*) and arabinoxylooligosaccharide transporter (*xylEFG*) from *C. alkanolyticum* and *xylAB* from *E. coli* [129]. The recombinant *C. glutamicum* strain with additional expression of endoxylanase (*xln*) from *Streptomyces coelicolor* A3 and xylosidase (*xynB*) from *Bacillus pumilus* with Cg1514 and CgR0949 as respective signal peptides could secrete these enzymes, which resulted in enhancement of the extracellular degradation of xylan [55]. Recently, the successful secretion of endoxylanase (*xynA*) from *S. coelicolor* A3 was reported by employing the porin protein (PorB) and Cg1514 from *C. glutamium* as signal peptides with endoxylanase under the promoters P_{porB} and P_{Cg1514} [52].

D-Xylose is the second most abundant sugar in lignocellulosic biomass and the engineered catabolism of D-xylose in *C. glutamicum* typically occurs either via the recruitment of D-xylose isomerase pathway (*E. coli*) or the Weimberg pathway (*Caulobacter crescentus*) [130]. In *E. coli*, D-xylose is converted into D-xylulose-5-phosphate via 2-step reaction using D-xylose isomerase (*xylA*) and xylulokinase (*xylB*) pathway and then D-xylulose-5-phosphate enters the pentose phosphate pathway (Fig. 2) [22]. For *C. glutamicum*, even though it has xylulokinase gene (*NCgl0111* or *xylB*), D-xylose cannot be utilized since native *xylB* has low activity and it lacks D-xylose isomerase for conversion of D-xylose to xylulose [129,130]. Therefore, the expression of *xylA* from *Xanthomonas campestris* was examined for better growth rate and higher biomass concentration (0.14/h, 3.37 gCDW/L) was achieved compared to the other recombinant strains with *xylA* from *E. coli* (0.09/h, 2.79 gCDW/L) [131]. In another study, the additional expression of *xylB* from *E. coli* was found to accelerate the growth rate (0.11± 0.004/h) [45]. In another study, D-xylose utilization rate through the D-xylose isomerase pathway was improved (0.13/h, 0.11/h> 0.07/h) compared to wild type by enhancement of the pentose phosphate pathway (PPP) by overexpression of transaldolase (*tal*) and 6-phosphogluconate dehydrogenase (*gnd*) from the pentose phosphate pathway [132]. In the alternative Weimberg pathway of *C. cresentus*, D-xylose is transformed into 2-oxoglutarate, an intermediate of the TCA cycle, via a 5-step reaction mediated by D-xylose dehydrogenase (*xylB*), xylonolactonase (*xylC*), D-xylonate dehydratase (*xylD*), 2-dehydro-3-deoxy-D-xylonate dehydratase (*xylX*), and α-ketoglutarate-semialdehyde dehydrogenase (*xylA*) (Fig. 2) [133]. In contrast to the recruited D-xylose isomerase pathway, when the Weimberg pathway from *C. cresentus* is expressed in recombinant C. *glutamicum* strains, it does not lead to a significant loss of carbon in the form of CO_2 and is more efficient for D-xylose utilization. However, growth rate was significantly reduced (0.07/h) due to the accumulation of toxic by-product, xylitol 5-phosphate [133]. Another study engineered *C. glutamicum* to uptake enhanced amounts of D-xylose by the de-repression of IolT1 gene for glucose myo-inositol permease. Introduction of D-xylose specific uptake system (XylE) or L-arabinose specific transporter (AraE) from *E. coli* or *B. subtilis* could also enhance D-xylose uptake and cell growth rate on D-xylose [55,122,135].

L-Arabinose is the third major sugar in lignocellulosic biomass. Among the members of *Corynebacteriaceae*, only *C. glutamicum* ATCC 31831 can naturally utilize L-arabinose because it has functional genes for L-arabinose utilization operon composed of L-arabinose transporter (*araE*), L-arabinose isomerase (*araA*), ribulokinase (*araB*) and ribulose 5-phospate 4-epimerase (*araD*) [135-137]. Thus, several recombinant *C. glutamicum* strains were constructed by recruiting L-arabinose catabolism genes from *E. coli*, which resulted in the production of 5.44 g/L L-glutamate, 8.04 g/L L-lysine, 11.8 g/L L-ornithine and 4.36 g/L of L-arginine from L-arabinose [136]. Simultaneous utilization of glucose and L-arabinose was only observed when the concentration of L-arabinose in fermentation medium was up to 3.6 g/L. If there is more than 3.6 g/L of L-arabinose, there is significant CCR observed during fermentation with recombinant *C. glutamicum* ATCC 31831. To improve the simultaneous co-utilization of glucose and L-arabinose, pyruvate kinase (*pyk*) was overexpressed, and L-arabinose transcriptional regulator (*araR*) was deleted. There was no significant CCR observed when the resulting recombinant strain was cultured in medium containing L-arabinose up to 15 g/L (Fig. 2) [137].

2-4. Pectin

Pectin or pectic polysaccharides are commonly found in pulp- and peel-rich waste streams. Pectin is rich in hexuronic acidic sugars (HAS), D-galacturonate and D-glucoronate, which *C. glutamicum* cannot naturally utilize as the carbon source even though it has a putative urinate isomerase (*uxaC*) [22]. Therefore, D-galacturonate and D-glucoronate utilization system was established in *C. glutamicum* by the heterologous expression of genes related to HAS catabolism from *E. coli*. The recruited pathway from *E. coli* involves the uptake of both D-glucoronate and D-galacturonate catalyzed by the transmembrane protein (*exuT*) and then the HAS are degraded into 2-dehdro-3-deoxy-D-gluconate 6-phosphatase by sequential reaction catalyzed by urinate isomerase (*uxaC*), altronate oxidoreductase (*uxaB*), mannonate oxidoreductase (*uxuB*),

altronate dehydratase (*uxaA*), urinate isomerase (*uxuA*) and 2-keto-3-deoxygluconokinase (*kdgK*). Then, 2-dehdro-3-deoxy-D-gluconate 6-phosphatase is degraded into pyruvate and D-glyceraldehyde 3-phosphate by 2-dehdro-3-deoxy-D-gluconate 6-phosphatase aldolase (*eda*) (Fig. 2). The growth rates of the recombinant *C. glutamicum ΔcrtYeb* in D-galacturonate and D-glucoronate were 0.02±0.01/h and 0.04±0.01/h, respectively, while the recombinant ORN1 strain grew in both HAS at rates of 0.04±0.01/h [138].

3. Metabolic Engineering of *C. glutamicum* for the Production of Platform Chemicals

3-1. C4 - Isobutanol

Isobutanol is currently in high demand as a bio-based alternative fuel due to its low hygroscopic activity and high energy density [139]. Natural production of isobutanol has been observed in anaerobic *Clostridium* species. In engineered aerobic *E. coli* and *B. subtillis* strains, isobutanol could be produced by the conversion of 2-ketoisovalerate into isobutanal by consecutive action of 2-keto-acid decarboxylase and alcohol dehydrogenase [140,141]. For example, 50 g/L of isobutanol was produced from glucose by a recombinant *E. coli* JCL260 (pSA65/pSA69) strain with overexpression of 2-ketoisovalerate decarboxylase (*kivD*), alcohol dehydrogenase (*adhA*), acetolactate synthase (*alsS*), and ketol-acid reductoisomerase and dihydroxyacid dehydratase (*ilvCD*) genes and disruption of bifunctional acetaldehyde-CoA/alcohol dehydrogenase (*adhE*), fumarate reductase (*frdBC*), lactate dehydrogenase (*ldhA*), phosphate acetyl transferase (*pta*), and formate acetyltransferase (*pflB*) genes [142]. Since *C. glutamicum* can maintain metabolic activity under oxygen deprivation conditions, has higher tolerance for isobutanol along and can produce much higher concentration of 2-ketoisovalerate compared to *E. coli*, it was chosen as an excellent host for isobutanol production. For example, recombinant *C. glutamicum* was evaluated for isobutanol production after heterologous expression of acetolactate synthase (*alsS*) from *B. subtillis* and overexpression of *ilvCD* genes of *C. glutamicum*. When these genes were expressed in a pyruvate carboxylase and lactate dehydrogenase deficient host strain, isobutanol was produced to 4.9 g/L (Table 3, Fig. 3) [139].

Since 2-ketoisovalerate is an important direct precursor of isobutanol, the availability of 2-ketoisovalerate should be improved for subsequent enhancement of isobutanol production. Thus, intracellular pool of 2-ketoisovalerate was enhanced by attenuation of competing pathways through deletion of pyruvate dehydrogenase complex (*aceE*), pyruvate: quinone oxidoreductase (*pqo*), lactate dehydrogenase (*ldhA*), malate dehydrogenase (*mdh*) and transaminase B (*ilvE*). Further enhancement of metabolic flux towards 2-ketoisovalerate production was achieved by overexpression of acetohydroxyacid synthase, acetohydroxyacid isomeroreductase and dihydroxyacid dehydratase (*ilvBNCD*). Then, isobutanol production was achieved by heterologous expression of *kivD* from *L. lactis*, *adh2* from *S. cerevisiae* and transhydrogenase (*pntAB*) from *E. coli* (Fig. 3). The resulting strain was able to produce 13 g/L of isobutanol under optimized two-step fermentation process which divides aerobic 2-ketoisovalerate production and oxygen-deprived isobutanol production (Table 3) [141]. In another oxygen-deprived fermentation experiment, a recombinant *C. glutamicum* strain harboring alcohol dehydrogenases from *E. coli* (*adhP*) and *S. cerevisiae* (*adh2*) and *kivD* from *L. lactis* was able to produce 73 g/L of isobutanol by continuous extraction using oleyl alcohol [143]. In another study, since 2-ketoisovalerate is the main precursor of both L-valine and isobutanol, redox cofactor imbalance occurs during anaerobic fermentation. Thus, this imbalance was resolved by developing an artificial isobutanol production pathway wherein, 2-ketoisovalerate is subsequently reduced to isobutanol by using NAD-dependent mutant acetohydroxyacid isomeroreductase $ilvC_{TM}$ and NAD specific alcohol dehydrogenase (*adhA*). Then succinate by production was suppressed by deletion of phosphoenolpyruvate carboxykinase (*pckA*). However, the elevated intracellular NADH/NAD^+ ratio led to a decrease in glucose utilization. Therefore, limited glucose consumption was improved by recruiting four enzymes from the Entner-Doudoroff pathway of *Z. mobilis*, which involves the conversion of glucose-6-phosphate into 6-phospho-gluconate-1,5-lactone by glucose-6-phosphate dehydrogenase (*zwf*) and then its subsequent conversion to glycolysis intermediates, glyderaldehyde-3-phosphate and pyruvate, catalyzed by succeeding 6-phosphogluconolactonase (*devB*), 6-phosphogluconate dehydratase (*edd*) and 2-keto3-deoxy-6-phosphogluconate aldolase (*eda*). Isobutanol production was further improved by overexpression of glucose specific phosphotransferase system (*ptsIHG*) for enhanced glycolysis and inactivation of competing transaminase (*ilvE*). The resulting recombinant *C. glutamicum* strain was able to produce 20.8 g/L of isobutanol (Table 3) [144].

3-2. C4 - 2,3-Butanediol

2,3-Butanediol is a platform chemical used in wide range of applications from plasticizers and fumigants to anti-freezing agents. 2,3-Butanediol is also a precursor for 1-butadiene and 2-butanone, which are applied in synthetic rubber production and as fuel additives and resin solvent, respectively [145]. In microorganisms that naturally produce 2,3-butanediol such as *Klebsiella pneumoniae*, 2,3-butanediol is produced from pyruvate via three step reactions catalyzed by α-acetolactate synthase, aldehyde decarboxylase and 2,3-butanediol dehydrogenase (Fig. 3) [146]. Up to now, the highest titer of 111.3 g/L of 2,3-butanediol has been achieved after using engineered *K. pneumoniae* with productivity of up to 2.71 g/L·h during fed-batch fermentation [146]. However, *K. pneumoniae* is a pathogenic strain that cannot be easily employed as a host strain for bulk production of 2,3-butanediol. Thus, (2R, 3R)-2,3-butanediol production was examined in *C. glutamicum*, which is non-pathogenic, and naturally produces small amounts of 2,3-butanediol production due to the presence of native butanediol dehydrogenase [8]. To improve 2,3-butanediol production in *C. glutamicum*, the intracellular pool of important precursor, pyruvate was enhanced by deletion of acetate (E1 subunit of pyruvate dehydrogenase complex and pyruvate: quinone oxidoreductase, *aceE*, *pqo*), succinate (*mdh*) and lactate (*ldhA*) synthesis pathways, and then the pathway for 2,3-butanediol production was further enhanced by heterologous expression of *α*-acetolactate synthase (*als*), *α*-acetolactate decarboxylase (*aldB*) and butanediol dehydrogenase (*butA*) from *L. lactis* (Fig. 3). The resulting strain was able to produce 6.3 g/L of 2,3-butanediol when used in a two-stage fermentation process with initial aerobic stage for production of cell biomass (50 CDW/L) and a final stage for anaerobic conversion of glucose into 2,3-butanediol (Table 3) [145]. In another study, the

Table 3. Summary of C4-C6 platform chemical production by engineered strains of *C. glutamicum*

Product	Genes	Carbon source: Culture conditions	Titer (g/L)	Productivity (g/Lh)	Yield (g/g)	Ref.
Isobutanol	*Δpyc,Δldh:alsS* from *B. subtilis*[1] *kivd* from *L. lactis*[1], *ilvCD*[2], *adh*[2]	Glucose: Aerobic Flask	4.9	-	-	139
	ΔaceE Δpqo ΔilvE ΔldhA Δmdh : *ilvBNCD*[2], *kivd* from *L. lactis*[1], *adh2* from *S. cerevisiae*[1], *pntAB* from *E. coli*[1]	Glucose: Oxygen deprived fed-batch	13	0.33	0.2	141
	$\Delta ldhA\ \Delta ppc\ \Delta ilvC_{mut}$: P_{tac}:*pyk, gapA. pfk, gpi*[2] P_{gapA}:$ilvBNC^{TM2}$, P_{gapA}:*ilvD*[2] P_{ldhA}:*kivD* from *L. lactis*[1], P_{gapA}:*adhP* from *E. coli*[1],	Glucose: Oxygen deprived flask w/ continuous extraction	73	1.22	2.6	143
	ΔldhA Δppc gapA, pgk, tpi, pfkA, pgi[5]: $P_{lac(AA)}$: $ilvBNC_{mut}$[2], $P_{lac(GA)}$:*ilvD*[2], $P_{lac(GA)}$:*kivd* from *L. lactis*[1], $P_{lac(GA)}$:*adhA*[2]	Glucose: Oxygen deprived flask	20.8	-	-	144
2,3-Butanediol	*ΔaceE Δpqo ΔldhA Δmdh:* P_{trc} : *als, aldB, butA* from *L. lactis*[1]	Glucose: Oxygen-deprived Two-stage Fermentation	6.3	0.2	-	145
	P_{tac}:*budAB* from *K. pneumoniae*[1]	Glucose: Batch Glucose/Cassava powder: Batch	18.9 12.0	-	-	147
Itaconic acid	$icd_{ATG\rightarrow TTG}$: *CAD1* from *A. terreus*[1,4] fused to 3′ end of *malE* from *E. coli*[1]	Glucose: Flask	7.8	0.27	0.29	153
3-Methyl-1-butanol	*Δpyc, Δldh :alsS* from *B. subtilis*[1] *kivd* from *L. lactis*[1], *ilvCD*[2], *adh*[2]	Glucose: Aerobic Flask	0.43 ±0.01	-	-	139
	Δldh ΔaceE ΔilvE: P_{trc}: *kivd* from *L. lactis*[1], *adh3* from *Z. mobilis*[1]	Glucose: Test Tube	0.70	-	-	154
	$ilvE_{GTG}$, *ΔldhA ΔilvfA:* P_{tac}:*aro10* from *S. cerevisiae*[1], *yqhD* from *E. coli*[1]	Glucose: Flask	2.76	-	0.10	155
2-Methyyl-1-butanol	*Δpyc, Δldh :alsS* from *B. subtilis*[1] *kivd* from *L. lactis*[1], *ilvCD*[2], *adh*[2]	Glucose: Aerobic Flask	0.11 ±0.006	-	-	139
	$ilvE_{GTG}$, *ΔldhA*:P_{tac}:*kivD* from *L. lactis*[1], *yqhD* from *E. coli*[1]	Glucose: Flask	0.37	-	0.02	155
Muconic acid	*ΔcatB*: *catA*[2]	Catechol and glucose: Fed-batch	85	2.4	-	164
	ΔcatB, *ΔpcaGH*, *ΔaroE*: *aroY* and *kpdBD* from *K. pneumoniae*[1,4]	Glucose: Fed-batch	38-54	0	0	165
	ΔaroE ΔpcaG ΔpcaH ΔcatB ΔptsI ΔiolR: P_{sod}:*qsuB*[2], *aroY* and *kpdBD* from *K. pneumoniae*[1]	Glucose: Fed-Batch	4.5	-	-	166

[1]Heterologous expression of gene from specified microorganisms
[2]Overexpression of native gene
[3]Overexpression of specified gene by inserting another copy of it in a selected locus of host strain-specific islands that have been identified as dispensible for growth (136)
[4]Codon-optimized for *C. glutamicum*
- Information is not available from reference

heterologous expression of acetolactate decarboxylase and α-acetolactate synthase (*budAB*) from *K. pneumoniae* into resulting recombinant *C. glutamicum* SGSC102 strain resulted in the production of 12 g/L of 2,3-butanediol from 80 g/L of cassava powder (56.7 g/L glucose, 2.17 g/L fructose) during batch fermentation [147].

3-3. C5 - Itaconic Acid

Itaconic acid (IA) is an important C5 organic acid that can be used as building block for the production of chemicals polymers and fuels. IA is also considered as a feasible substitute for acrylic, maleic, methyrilic and fumaric acid and their derivatives. Initial industrial production of IA relied on the decarboxylation of aconitic acid, oxidation of isoprene and dry distillation of anhydride. Due to the complexity of the production process and low production rate, the chemical synthesis of IA is ineffective as compared to bio-based production process. IA was first discovered as a product of thermally decomposed citric acid and a metabolite produced by *Aspergillus itaconicus*. Bio-based production of itaconic acid has mainly been achieved by fermentation of *Aspergillus terreus* and

Ustilago maydis.

Fungi such as *Aspergillus* sp., *A. terreus*, *Ustiligo maydis* and *Pseudozyma antartica* Y-7808 naturally produce IA from broad range of substrates, in which IA is produced by the decarboxylation of cis-aconitic acid (CAA). It was reported that *A. terreus* NRRL 1960 can produce IA from broad range of substrates (sucrose, glucose, cellobiose, mannose, D-xylose, fructose, glycerol). Even though the highest concentration of IA can only be achieved by fermentative production employing fungus, it takes too much time for fermentation, which is one of disadvantages of fungal fermentation resulting in rather low productivity at the end of fermentation. For example, *A. terreus* XH86-8 produces 77.6 g/L IA by at least three days fermentation [148]. Therefore, *E. coli* and *C. glutamicum* have gained much interest as promising host strains for IA production because they could produce IA in shorter time with fewer by-products. Fermentation and whole cell bioconversion based on engineered *E. coli* could produce up to 0.15-41.6 g/L of itaconic acid [148-151]. For itaconic acid production from glucose, the recombinant *E. coli* ita23 strain was engineered as follows: (1) establishing itaconic acid production pathway by constitutive heterologous expression of codon optimized cis-aconitate decarboxylase (*cadA*) and citrate synthase (*gltA)* from *A. terreus* and *C. glutamicum*, respectively, (2) redirecting TCA cycle and glyoxylate cycle flux to itaconic acid production by deletion of isocitrate lyase (*aceA*) and succinate CoA ligase α- and β-subunits (*sucCD*), (3) reducing overflow of pyruvate production by deleting pyruvate kinase isoenzymes (*pykAF*), which converts PEP to pyruvate, (4) deletion of phosphate acetyl transferase (*pta*) to reduce acetate accumulation and (5) blocking high glutamate synthesis by downregulation of isocitrate dehydrogenase (*icd*) by replacing its native promoter with weak synthetic promoter BBa_J23115. Fed-batch fermentation of *E.coli* ita23, with glucose and glutamate supplementation, resulted in the production of 32 g/L of itaconic acid with 0.45 g/L of productivity [149]. In another study, 18 g/L, 20 g/L and 22 g/L of IA was produced during flask culture with glucose, D-xylose and glycerol as carbon source when a recombinant *E. coli* strain with the following characteristics was used as an IA producing strain: (1) codon optimized CAD from *A. terreus*, (2) deletion of isocitrate dehydrogenase (*icd*) and overexpression of aconitate hydratase B (*acnB*) and citrate synthase (*gltA*), phosphoenol pyruvate carboxylase (*ppc*) for enhanced intracellular pool of TCA cycle precursors for IA, (3) heterologous expression of *pyc* from *C. glutamicum* for enhanced oxaloacetate supply, and (4) high aeration during flask culture. Then, the same strain was able to produce 43 g/L of IA during fed-batch fermentation with glycerol as sole carbon source [143]. Recombinant *E. coli* has also been used for IA production via whole cell bioconversion of citrate. For example, the recombinant *E, coli* JY001, which has heterologous expression of *A. terreus's acn* and three gene copy of *cadA* via in two plasmid system, was able to convert 96 g/L of citrate into 41.6 g/L of IA in buffer- and cofactor-free conversion reaction [151]. In a follow-up study, the same strain was immobilized in barium alginate beads for the development of a reusable whole cell citrate bioconversion system. Under optimized reaction conditions, this system can still convert citrate to IA successfully even after four rounds of recycling [152]. However, the fermentative production of IA is highly toxic to *E. coli* thus a more tolerant strain such as *C. glutamicum* was evaluated for IA production. Heterologous expression of codon-optimized CAD from *A. terreus* in *C. glutamicum* ATCC 13032 led to the production of 0.2 g/L of IA. Then, CAD activity was improved by fusing CAD to C-terminus of maltose-binding protein (MalE) without its signal peptide, which allowed the resulting strain to produce 0.5±0.1 g/L of IA, with two-fold increase of CAD activity. Improvement of IA production up to 3.8 g/L was achieved under nitrogen-limited conditions to increase glucose availability for the production of IA instead of formation of biomass. IA production was further increased up to 7.8 g/L by enhancing cis-aconitate supply, IA precursor, through reduction of isocitrate dehydrogenase activity in the TCA cycle flux by exchanging the native ATG start codon to TTG (Table 3) [153].

3-4. C5 - 3-Methyyl-1-butanol and 2-Methyl-1-butanol

3-Methyl-1-butanol (3MB) and 2-methyl-1-butanol (2MB) are pentanol isomers that can potentially be applied as biofuels. Both pentanol isomers have been produced as by-products when *C. glutamicum* strains were engineered for isobutanol production. When a recombinant *C. glutamicum* has been engineered to overexpress the native dihydroxyacid dehydratase (*ilvCD*_and alcohol dehydrogenase (*adhA*) and heterologously express the acetolactate synthase (*alsS*) genes from *Bacillus subtilis* and *kivD* gene from *L. lactis*, the resulting strain could only produce 0.11±0.006 g/L of 2MB and 0.43±0.01 g/L of 3MB (Fig. 3, Table 3) [139]. In another study, 3MB production in *C. glutamicum* 13032 was first established by deletion of (*ldh*) and pyruvate dehydrogenase (*aceE*). Then, the resulting CG1 strain was mutated using diethyl sulfate and colonies resistant to a structural analog of L-leucine, Fmoc-3-4-thiazolyl-L-alanine, to isolate L-leucine over-producing mutant, was used to construct CG5 strain. Then, plasmid-based heterologous expression of 2-ketoisovalerate decarboxylase (*kivd*) from *L. lactis* and alcohol dehydrogenase (*adh3*) from *Z. mobilis* in mutant CG5 strain generated the CG7 strain that was able to produce 0.66 g/L of 3MB. Further, deletion of ilvE in CG7 strain resulted in CG13 strain that could produce 0.70 g/L of 3MB (Table 3) [154]. To improve titers of both 3MB and 2MB production, the plasmid-based expression of 2-keto acid decarboxylases (KDC) and alcohol dehydrogenase (ADH) was examined in recombinant *C. glutamicum* strains for overproduction of 2MB and 3MB from 2-keto-3-methylvalerate (KMV) and 2-ketoisocaproate (KIC), respectively. First, it was determined that high pentanol concentrations of 12 g/L can completely abolish cell growth although it was previously reported that 3 g/L of 3MB retarded growth [155]. Then, the KIC-overproducing host strain for 3MB production, was constructed based on L-leucine overproducing strain with mutated BCAA transaminase-encoding $ilvE_{GTG}$ to attenuate competing L-valine synthesis. For 2MB production, a KMV overproducing CB-KMVF1 strain was constructed based on *C. glutamicum* K2P55, a leucine-auxotrophic L-isoleucine producer with chromosomal $ilvE_{GTG}$ with exchanged start codon (Fig. 3). As a result, *C. glutamicum* MV-KICF1 and CB-KMVF1 were able to produce 3.55 g/L of KIC and 3.67 KMV, respectively. To avoid lactate accumulation during anaerobic cultivation with retarded growth, *ldhA* genes of both host strains were deleted. Additional deletion of *ilvA* in MV-KICF1 was done in order to minimize competing KMV production. Then, MV-KICF1 *ΔldhA ΔilvA* and CB-KMV-F1 *ΔldhA* strains were engineered by induc-

ible pEKEx2 plasmids carrying nine different possible combinations of KDC (*kivD* from *L. lactis*, *aro10* and KDC *Thi3* from *S. cerevisiae*) and ADH (*adh2* from *S. cerevisiae*, *adhA* from *C. glutamicum*, *yqhD* from *E. coli*). The titers of 2MB and 3MB obtained by the resulting recombinant strains with nine different combinations of KDC and ADH were 0.23-0.37 g/L of 2MB and 1.4-2.76 g/L of 3MB. However, an accumulation of 0.32-5.29 g/L isobutanol was also observed. For 2MB production, the recombinant *C. glutamicum* CB-KMVF1 ΔldhA (pEKEx2-*kivD-yqhD*) strain was able to produce the best titer of 0.37 g/L of 2MB, with yield of 0.02 g 2MB/g glucose for 2MB. Meanwhile, the best titer for 3MB was achieved by recombinant *C. glutamicum* MV-KICF1 *ΔldhA ΔilvfA* (pEKEx2-a*ro10-yqhD*), which was able to produce 2.76 g/L 3MB, with yield of 0.10 g 3MB/g glucose (Table 3) [155].

3-5. C6 - Muconic Acid

Cis, cis-Muconic acid (MA) is a valuable dicarboxylic acid, a building block for the production of adipic acid and terephthalic acid, which are eventually used for nylon and plastic synthesis. Current petroleum-based production of MA also produces carcinogenic benzene substances. Therefore, greener microbial production process for MA production has gained much attention. Production of MA in recombinant *E. coli* is achieved by establishing and re-engineering 3-dehydroshikimate (DHS) pathway via three routes: (1) MA production from DHS, (2) MA production from chorismite, and (3) MA production from aromatic amino acids (tyrosine, tryptophan and phenylalanine) [156-160]. An example of MA production from DHS is the one established in recombinant *E. coli* AB2834, which has leaky shikimate dehydrogenase (*aroE*) and thus accumulates DHS; and its MA biosynthesis pathway consists of DHS dehydratase (*aroZ*) and protocatechuate decarboxylase (*aroY*) from *K. pneumoniae* and catechol 1,2-dioxygenase (*catA*) from *Acinetobacter calcoaceticus*. The resulting strain produced 59.2 g/L of MA after further fermentation process optimization [161]. MA production from chorismate involves 2,3-dihydroxybenzoate (2,3-DHB) generation followed by subsequent conversion into MA [162]. First, isochorismate synthase (*entC*), isochorismatase A (*entB*) and isochorismate dehydrogenase (*entA*) genes were overexpressed in *E. glutamicum* to derive 2,3-DHB. Second, 2,3-DHB decarboxylase gene (*entX*) from *K. pneumoniae* and catechol 1,2-dioxygenase gene (*cat*) from *Pseudomonas putida* KT2440 were introduced for further conversion of 2,3-DHB into MA. Then, the final engineered *E. coli* strain with additional overexpression of DAHP synthase (*aroG*) and shikimate kinase (*aroL*) for the enhanced metabolic flux toward shikimate pathway could produce 0.61 g/L of MA [162]. In another pathway, MA can be produced via catechol from anthranilate, which is the first intermediate of tryptophan biosynthetic branch. Overexpression of anthranilate 1,2-dioxygenase (ADO) and catechol 1,2-dioxygenase for subsequent conversion of anthranilate to MA in anthranilate overproducing *E. coli* MA-4 strain, which has blocked competing tryptophan biosynthetic pathway and overexpressed key enzymes of shikimate pathway, has enabled the resultant strain to produce 0.39 g/L of MA [163].

In *C. glutamicum*, MA is naturally produced when aromatic compounds such as catechol is present as carbon source since native catechol 1,2-dioxygenase (*catA*) converts catechol to MA. However, intracellular MA is immediately converted to muconolactone by native uconate cycloisomerase (*catB*) in the catechol branch of *β*-ketoadipate. Thus, the deletion of *catB* gene in *C. glutamicum* host strain is necessary for it to naturally accumulate MA during fermentation with lignin-derived aromatic compounds (catechol, phenol, benzoic acid) and glucose as co-substrate. Furthermore, this MA biosynthesis pathway was optimized by constitutive overexpression of native catechol 1,2-dioxygenase (*catA*) in the resulting *C. glutamicum* MA-2 strain led to increase of the key enzymes activity and enhanced conversion of catechol to MA by tenfold while simultaneously lowering intracellular amount of catechol and lowering the systems overall toxicity (Fig. 3). Since catechol is toxic to *C. glutamicum*, fed-batch fermentation using *C. glutamicum* MA-2 strain with hourly feeding of glucose and catechol enabled it to produce of 85 g/L of MA from catechol after 60 h with productivity of 2.4 g/L·h (Table 3) [164].

In another study, the shikimate pathway of *C. glutamicum* was engineered for the production of MA from glucose. First, important MA precursors, DHS and protocatechuate (PCA), were generated by the removal of uconate cycloisomerase (*catB*), PCA dioxygenase alpha/beta subunit (*pcaGH*) and shikimate dehydrogenase (*aroE*) genes from *β*-ketoadipate pathway. Then, a downstream pathway for the conversion of PCA into catechol was completed by the heterologous expression of codon-optimized PCA decarboxylase (*aroY*) and PCA decarboxylase subunit (*kpdBD*) from *K. pneumoniae* (Fig. 3). The resulting strain was able to produce 38 g/L and 54 g/L of MA in 7-L and 50-L fed-batch fermentations, respectively (Table 3) [165]. Similarly, improved MA productivity was achieved by developing a recombinant strain with the following steps: (1) deletion of phosphoenolpyruvate protein phosphotransferase gene (*ptsI*) to develop a phosphoenol-pyruvate-independent phosphotransferase system for glucose consumption, that will not deplete intracellular concentration of PEP, an important precursor of the shikimate pathway gene, (2) enhanced glucose uptake by deletion of transcriptional regulator gene (*iolR*) and overexpression of glucose permease (*iolT1*) and phosphate/ATP dependent glucokinase (*ppgK*) genes, (3) overexpression of modified native protocatechuate dehydrogenase (*YBD*) for conversion of protocatechuate into catechol, (4) deletion of shikimate 5-dehydrogenase (*aroE*), protocatechuate dioxygenase *α*/*β* subunits (*pcaGH*), uconate cycloisomerase (*catB*) to induce the increased expression of glucose permease and phosphokinase genes and delete the competing pathway with shikimate *β*-ketoadipate pathways for the generation of MA and MA precursor, and (5) overexpression of phosphate isomerase (*qsuB*) gene for the enhanced production of MA precursor, protocatechuate [166]. The resulting *C. glutamicum* P30 strain was able to produce 4.5 g/L of MA, which was a 12% increase from starting strain (Table 3) [166].

CONCLUSION

C. glutamicum is mainly used for the commercial production of L-glutamate and L-lysine, with annual global production of 3.21 and 2.60 million metric tons, respectively [167]. Recent advances in multiomics approaches, synthetic biology tools and metabolic engineering strategies have accelerated the development of *C. glutamicum* beyond its traditional use for industrial amino acid pro-

duction and enabled it to produce a feasible amount of platform chemicals such as diamines, dicarboxylic and amino-carboxylic acids, pending further commercialization [8,22]. Compared to other established platform strains such as *E. coli* and *S. cerevisiae*, *C. glutamicum* has a broad range of customizable parts for plasmids and homologous recombination plasmid/systems such as pK19mobsacB and pK18mobrsL, Cre/Lox, RecET, CRISPR/Cas9 and CRISPR/Cpf1systems, which are readily available for rapid and accurate genome engineering and development of metabolically engineered *C. glutamicum* strains for tailored utilization of target biomass and value-added chemical production. Furthermore, a consolidated bioprocess for cellulose, xylan and pectin using recombinant *C. glutamicum* strains has already been validated. Once the consolidated bioprocess for lignin is fully established in *C. glutamicum*, it will become a powerhouse platform strain for lignocellulose-based biorefineries. Moreover, *C. glutamicum* can produce a broad range of platform chemicals such as isobutanol, 2,3-butanediol, itaconic acid, 3-methyl-1-butanol, 2-methyl-1-butanol and muconic acid. The recent progress and achievements described in this study suggest *C. glutamicum* as an ideal versatile strain for the sustainable production of value-added platform chemicals in biorefineries.

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Prof. Park is a professor at Department of Chemical Engineering and Materials Science, Ewha Womans University since 2017, Korea. Before joining Ewha Womans University, He worked for Department of Environmental Engineering and Energy, Myongji University as an assistant and associate professor (2012-2017), Chemical Biotechnology Research Center, Korea Research Institute of Chemical Technology (KRICT) as a senior research engineer (2009-2012), and Corporate R&D in LG Chem Research Park as a principal investigator of biomaterial research team (2003–2009). He received the BS. degree in chemical engineering from Seoul National University in 1997, and obtained the MS. and Ph.D degrees in chemical engineering from KAIST in 1999 and 2003, respectively. Currently, his research group focuses on the development of metabolically engineered microorganisms for the production of novel biopolymers, biochemicals and biofuels for biorefinery. As of April 2021, he has published over 100 scientific articles.