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Advanced Biotechnology: Metabolically Engineered Cells for the Bio-Based Production of Chemicals and Fuels, Materials, and Health-Care Products

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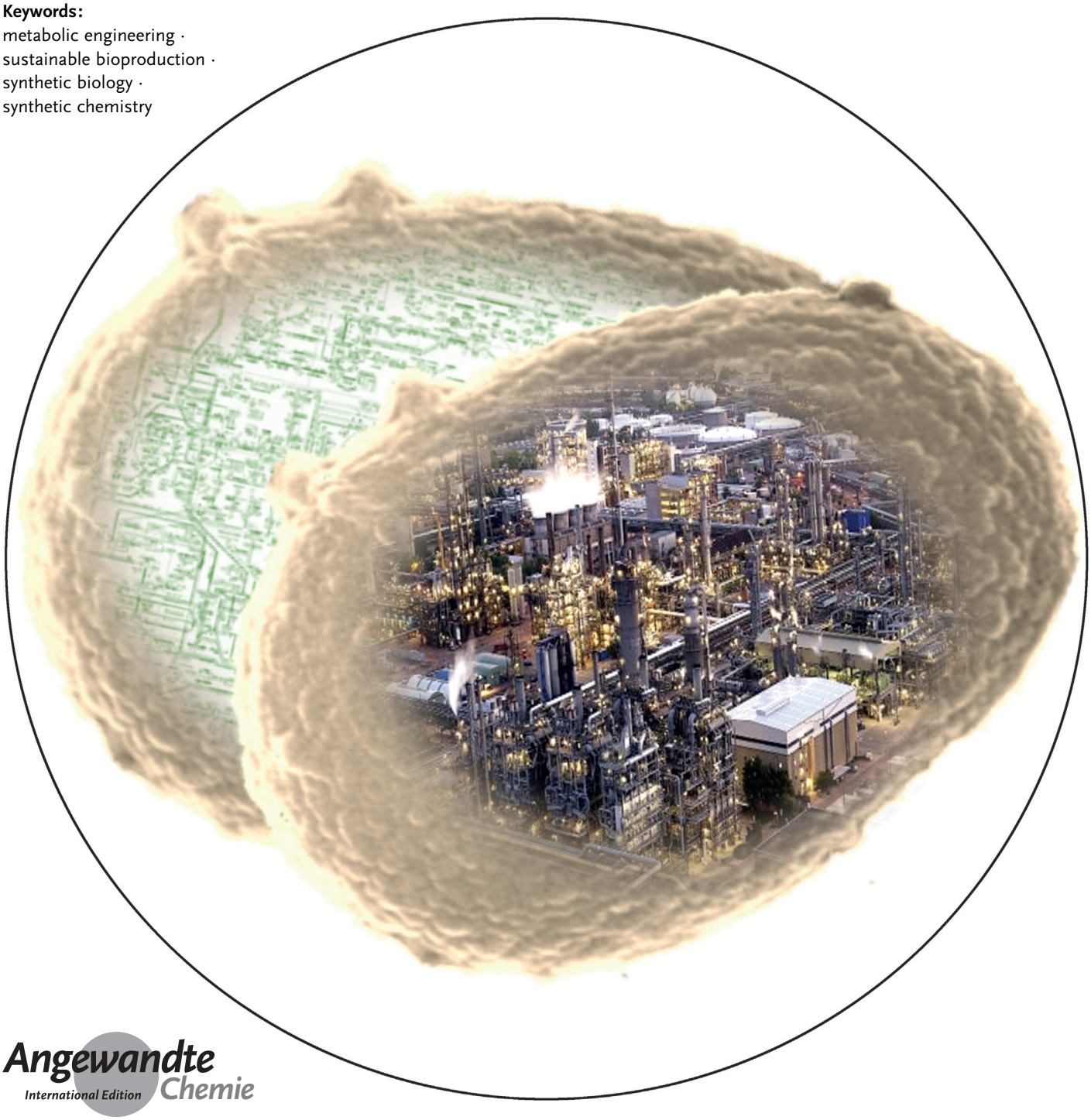
metabolic engineering · sustainable bioproduction · synthetic biology · synthetic chemistry



Corynebacterium glutamicum, Escherichia coli, and Saccharomyces cerevisiae in particular, have become established as important industrial workhorses in biotechnology. Recent years have seen tremendous progress in their advance into tailor-made producers, driven by the upcoming demand for sustainable processes and renewable raw materials. Here, the diversity and complexity of nature is simultaneously a challenge and a benefit. Harnessing biodiversity in the right manner through synergistic progress in systems metabolic engineering and chemical synthesis promises a future innovative bio-economy.

From the Contents

1. Introduction

Throughout the past century industrialization has established a close-meshed network between our everyday needs and the petrochemical industry. Industrial chemistry uses a variety of simple chemical units, mainly derived from fossil resources, to build more-complex compounds that are applied as solvents, fuels, polymers, textiles, nutrient, flavors, and pharmaceuticals. Concerns over environmental pollution, climate change through the effect of greenhouse gases, and the shortage of raw oil, however, have driven the search for novel and sustainable sources to feed into the synthetic chemistry pipeline.[1] Living systems often provide unique solutions for the chemo-, regio-, and stereoselective "bio"-synthesis of chemical units from inexpensive and renewable carbon feedstocks.[2] Advances in recombinant DNA technology and synthetic biology strongly accelerated the development of novel routes for the biotechnological production of natural products and beyond. Embedding synthetic biology and synthetic chemistry in a comprehensive concept thus promises streamlined biorefinery applications for sustainable production. Biotechnology—known since ancient times through baking and brewing—entered a completely new era after the establishment of production processes based on fermentation. Primarily used for the industrial-scale production of antibiotics,[3] L-amino acids,[4] and related compounds,[5] the range of products produced by biotechnology has been substantially extended during the last 150 years.[1b,6] The biotechnology business has in the meantime grown into a billion dollar market.[1c] Nature itself supports a diversity of organisms, enzymes, pathways, and metabolic reactions that enable simple carbon sources to be converted into complex and chemically nonrelated molecules. For many decades, the natural pathway set of a specific organism defined the limits of complexity and variability. However, recombinant DNA technology, in combination with systems and synthetic metabolic engineering, has crossed these natural boundaries, and now enable the reconstruction of complete synthetic metabolic pathways in heterologous—in general microbial—production hosts. The limitations and drawbacks, such as slow growth, high nutritional requirements, pathogenicity, stress sensitivity, and low product yields and concentrations, of the previous native producers can thereby be circumvented.[7] Furthermore, these strategies give reasonable access to metabolic compounds of a large number of unculturable organisms, and to those of higher plants or animals.[8] In addition to the diversity of products, research has more recently focused on the range of fermentable substrates. Classically, biotechnological production processes are based on complex raw materials such as corn or beet molasses as well as hydrolysates from starch or cassava, which are rich in the hexose glucose. Novel strategies to achieve sustainability and added value now tend to utilize carbon feedstocks from so far unused industrial waste streams from the agriculture and forestry, paper, dairy, and biodiesel industries.[1c,9] Their composition strongly differs from the conventional feedstocks, thus directing attention to alternative carbon sources such as the pentose sugars xylose and arabinose as well as polymers thereof, the polyalcohol glycerol, and the organic acids lactate and acetate. In the most successful cases, systems and synthetic metabolic engineering have been applied to the major industrial production organisms *Corynebacterium glutamicum*, *Escherichia coli*, and *Saccharomyces cerevisiae*, thereby breeding highly versatile cell factories. Such synthetic biology approaches aim at tailored producer strains for the economic bioproduction of fine, platform, and commodity chemicals, a selected set of which is presented in Figure 1. This will pave the way for a novel sustainable and (bio)synthetic chemistry based on renewable resources by combining biotechnology and petrochemistry.

This Review gives an overview of recent achievements in the field of bio-based production, and highlights advances in metabolic engineering for the de novo biosynthesis of chemicals, materials, and fuels through fermentation, including the value-added chemicals derived from biomass, as highlighted by the US Department of Energy.[10] It thus covers innovations for both industrial products and novel compounds that

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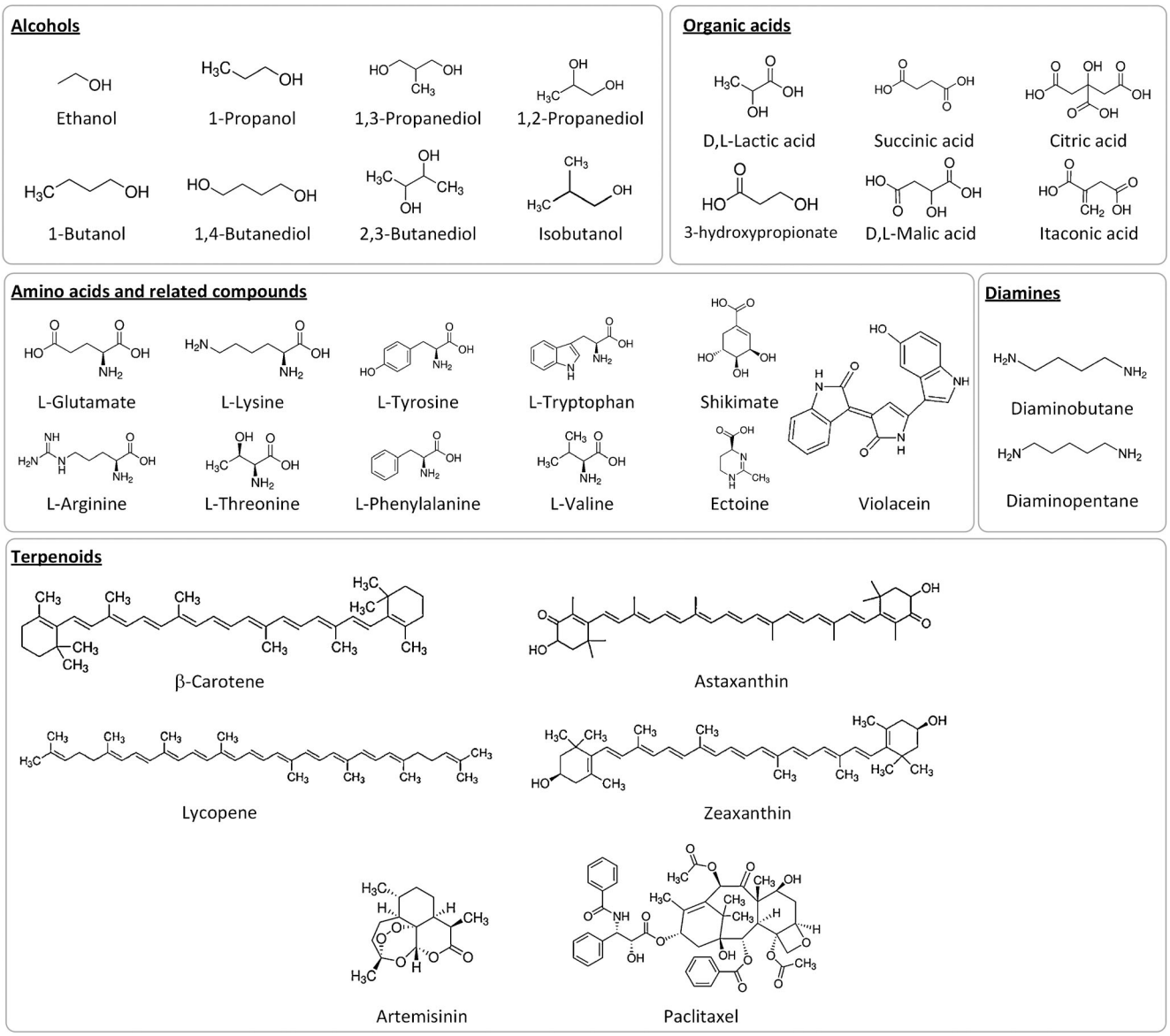

Figure 1. Selected set of the versatile bio-based product spectrum obtainable by microbial fermentation.

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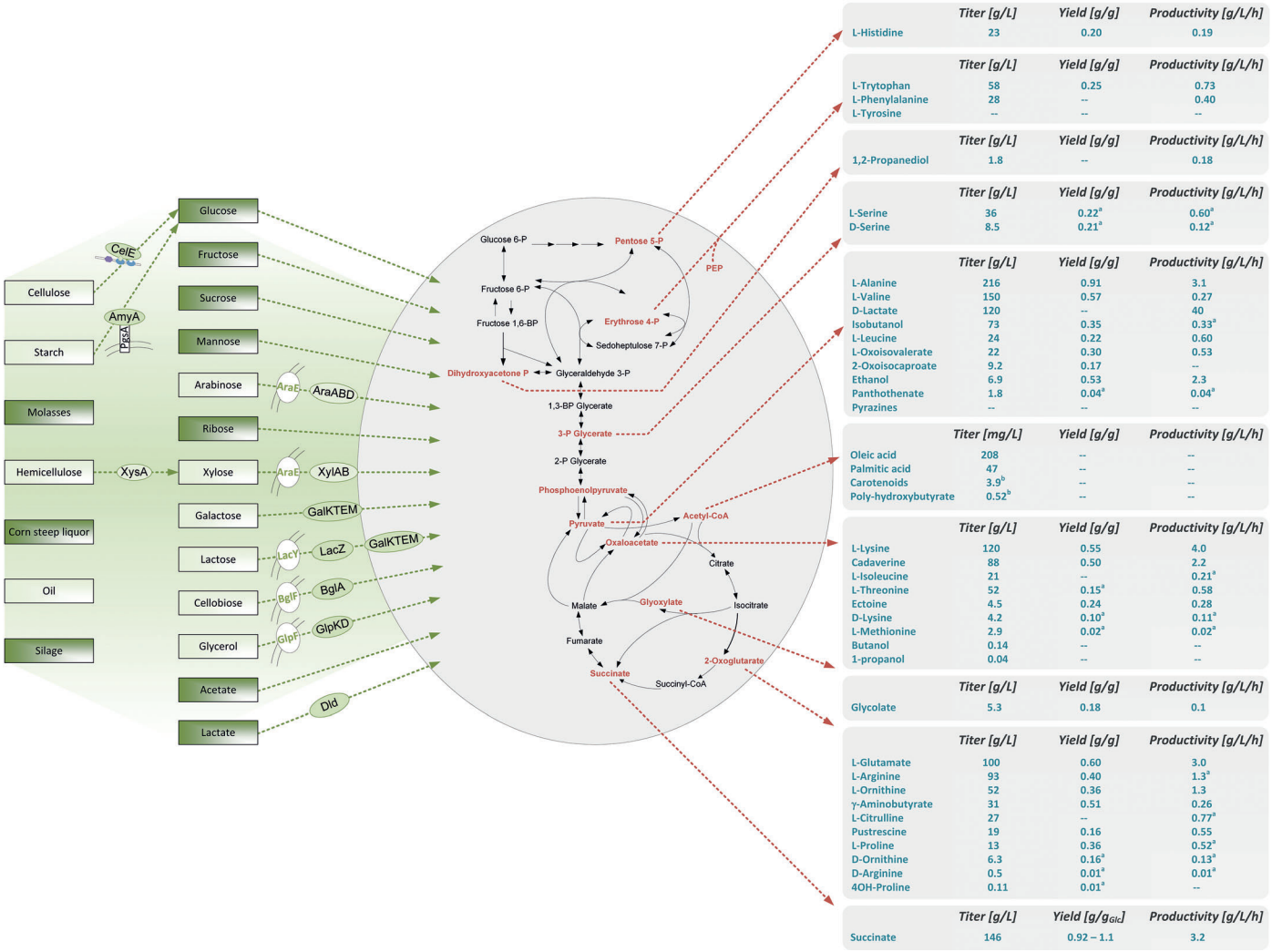
have not yet matured to the market place. For a more detailed view on single- and multistep biotransformations which require specific precursors as the starting compounds for product formation, the reader is addressed to other recent reviews.[2b,11]

2. Industrial Cell Factories

2.1. Corynebacterium glutamicum

The Gram-positive soil bacterium *Corynebacterium glutamicum* has been applied as an industrial producer, mainly for L-amino acids, for almost six decades.[4,12] Besides these traditional products, advances in recombinant DNA technology enabled specialized cell factories to be developed for the production of various natural and non-natural compounds such as D-amino acids,[13] vitamins,[14] pyrazines,[15] diamines,[16] organic acids,[17] alcohols,[18] pyrimidines,[7b] and polymers[19] (Figure 2). Classical aerobic processes have been complemented with two-phase aerobic/anaerobic processes to allow the production of typical anaerobic products. Homologous and heterologous genetic engineering—comprising native and non-native genes, respectively—of substrate assimilation routes further leveraged the integration of *C. glutamicum* in the rising era of biorefinery application.[1c] Today, its product spectrum has the potential to serve consumer markets for health, nutrition, textiles, housing, energy, and agriculture.[1b]



	Titer [g/L]	Yield [g/g]	Productivity [g/L/h]
L-Histidine	23	0.20	0.19
L-Trytophan	58	0.25	0.73
L-Phenylalanine	28	--	0.40
L-Tyrosine	--	--	--
1,2-Propanediol	1.8	--	0.18
L-Serine	36	0.22[a]	0.60[a]
D-Serine	8.5	0.21[a]	0.12[a]
L-Alanine	216	0.91	3.1
L-Valine	150	0.57	0.27
D-Lactate	120	--	40
Isobutanol	73	0.35	0.33[a]
L-Leucine	24	0.22	0.60
L-Oxoisovalerate	22	0.30	0.53
2-Oxoisocaproate	9.2	0.17	--
Ethanol	6.9	0.53	2.3
Panthothenate	1.8	0.04[a]	0.04[a]
Pyrazines	--	--	--

	Titer [mg/L]	Yield [g/g]	Productivity [g/L/h]
Oleic acid	208	--	--
Palmitic acid	47	--	--
Carotenoids	3.9[b]	--	--
Poly-hydroxybutyrate	0.52[b]	--	--

	Titer [g/L]	Yield [g/g]	Productivity [g/L/h]
L-Lysine	120	0.55	4.0
Cadaverine	88	0.50	2.2
L-Isoleucine	21	--	0.21[a]
L-Threonine	52	0.15[a]	0.58
Ectoine	4.5	0.24	0.28
D-Lysine	4.2	0.10[a]	0.11[a]
L-Methionine	2.9	0.02[a]	0.02[a]
Butanol	0.14	--	--
1-propanol	0.04	--	--
Glycolate	5.3	0.18	0.1
L-Glutamate	100	0.60	3.0
L-Arginine	93	0.40	1.3[a]
L-Ornithine	52	0.36	1.3
γ-Aminobutyrate	31	0.51	0.26
L-Citrulline	27	--	0.77[a]
Pustrescine	19	0.16	0.55
L-Proline	13	0.36	0.52[a]
D-Ornithine	6.3	0.16[a]	0.13[a]
D-Arginine	0.5	0.01[a]	0.01[a]
4OH-Proline	0.11	0.01[a]	--

	Titer [g/L]	Yield [g/g_{Glc}]	Productivity [g/L/h]
Succinate	146	0.92 – 1.1	3.2

Figure 2. Bio-based production of chemicals, fuels, and materials accessible by the fermentation of genetically streamlined *C. glutamicum* cell factories. Natural (dark green) and non-natural (light green) carbon sources are metabolized via central intermediates (red) to desired products. P = phosphate, BP = bisphosphate. Data on production performance are taken from the literature.[5,7b,13a,b,d,14,18,20] If not specified elsewise, yields are given in $g\,g_{Glc}^{-1}$ (glc = glucose). Key studies include the production of L-lysine,[13a] L-valine,[20k] L-arginine,[20y] and cadaverine.[20a] AmyA: α-amylase; AraABD: L-ribulokinase, L-arabinose isomerase, L-ribulose-5-phosphate 4-epimerase; AraE: arabinose transporter; BglA: phospho-β-glucosidase; BglF: β-glucoside-specific enzyme IIBCA component (PTS); CelE: endoglucanase; Dld: D-lactate dehydrogenase; GalKTEM: galactokinase, galactose-1-phosphate uridylyltransferase, UDP-glucose 4-epimerase, aldose 1-epimerase; GlpF, aquaglyceroporin (glycerol uptake facilitator protein); GlpKD: glycerol kinase, glycerol 3-phosphate dehydrogenase; LacY: lactose permease; LacZ: β-galactosidase; PgsA: anchor protein; XylAB: xylose isomerase, xylulokinase; XysA: xylanase. [a] Estimated from reference. [b] Given as $mg\,g_{CDM}^{-1}$. CDM = cell dry mass.

2.2. Escherichia coli

Escherichia coli is probably the best-studied organism of all the microbes because of its function as a model organism for Gram-negative bacteria as well as being a potent microbial cell factory for industrial application. *E. coli* strains can naturally grow on a variety of monomeric carbon sources including sugars, sugar alcohols, and organic acids under aerobic or anaerobic conditions. The molecular toolbox for this organism, which enables genetic engineering and the study of regulation and gene expression, is presumably the largest that exists for one particular organism.[21] This has strongly driven the generation of versatile *E. coli* based producers. The product portfolio of *E. coli* ranges from classical products such as amino acids[12b] to biofuels[22] and innovative compounds used as building blocks for the chemical synthesis of chemicals,[6a] polymers,[23] and pharmaceuticals[24] (Figure 3).

2.3. Saccharomyces cerevisiae

S. cerevisiae has the longest tradition in biotechnology from its ancient application in baking, brewing, and wine pressing. It is the best-characterized eukaryote and its biotechnological application has enlarged tremendously beyond foods and beverages. Properties such as high robustness, its "generally regarded as safe" status, the capability to aerobically and anaerobically grow on diverse carbon sources, its compatibility with a wide pH range, and fermentation

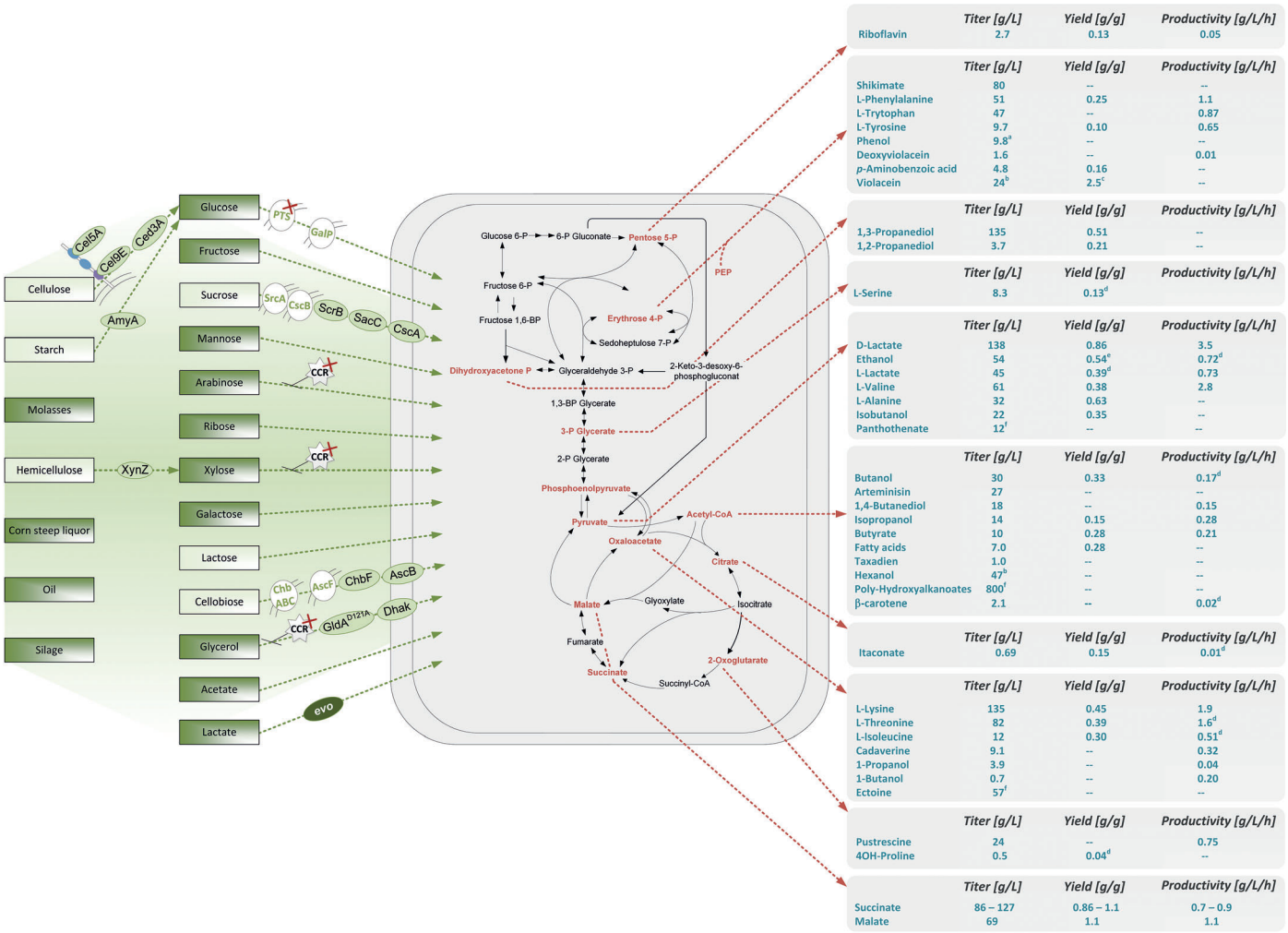


Figure 3. Bio-based production of chemicals, fuels, and materials accessible by the fermentation of genetically streamlined *E. coli* cell factories. Natural (dark green) and non-natural (light green) carbon sources are metabolized via central intermediates (red) to desired products. Data on production performance are taken from the literature.[7c, 12b, 20e, 23, 24b, 25] If not specified elsewise, yields are given in $g\,g_{Glc}^{-1}$. Key studies include the production of L-lysine,[25x] L-threonine,[25ad] butanol,[25u] and taxadien.[24a] AscB: phospho-β-glucosidase; AscF: cellobiose/arbutin/salicin transporter; CcGH: β-glucosidase; CCR: carbon catabolite repression; Ced3A: cellodextrinase; Cel5A: endoglucanase; Cel9E: exoglucanase; ChbABC: PEP-dependent transporter; ChbF: glucosidase; ChbG: protein of unknown function; ChbR: dual activator/regulator; CscB: sucrose H^+ symporter; CscK: fructokinase; CscA: sucrose 6-phosphate hydrolase; DHAK: dihydroxyacetone kinase; Evo: evolutionary adaptation; GalP: galactose permease; GldA: glycerol dehydrogenase; GlpD: glycerol 3-phosphate dehydrogenase; GlpK: glycerol kinase; PHB: polyhydroxybutyrate; PTS: phosphotransferase system; SacC: β-fructofuranosidase; ScrB: sucrose 6-phosphate hydrolase; ScrA: sucrose transporter; XynZ: xylanase. [a] Concentration in extraction phase. [b] Given as $mg\,L^{-1}$. [c] Given as $mg\,g^{-1}$. [d] Estimated from reference. [e] Higher than theoretical maximum because of complex nutrients. [f] Given as $mg\,g_{CDM}^{-1}$.

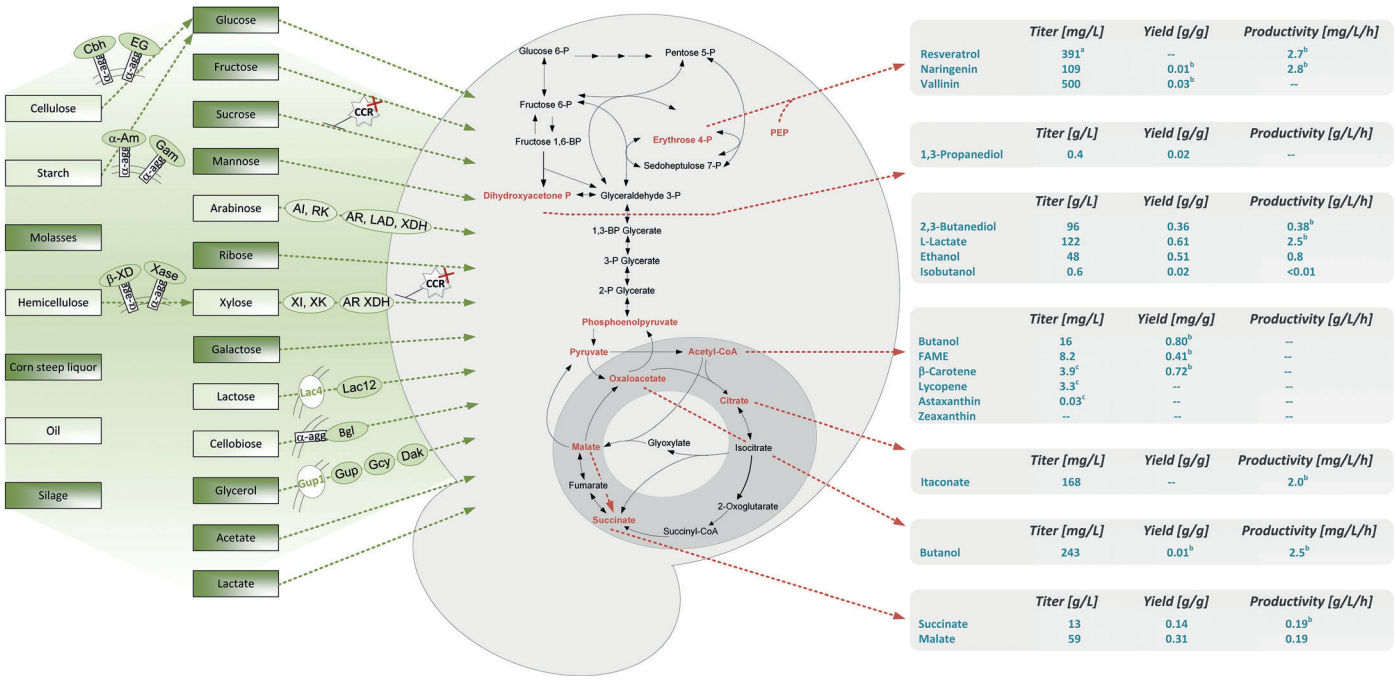


Figure 4. Bio-based production of chemicals, fuels, and materials accessible by fermentation of genetically streamlined *S. cerevisiae* cell factories. Natural (dark green) and non-natural (light green) carbon sources are metabolized via central intermediates (red) to desired products. Data on production performance are taken from the literature.[30] If not specified elsewise, yields are given in $g g_{Glc}^{-1}$. Key studies include the production of naringenin,[30c] lactate,[30f] and malate.[30t] α-agg: α-agglutinin; AI: arabinose isomerase; α-Am: α-amylase; AR: aldose reductase; BGL: β-glucosidase; CBH: cellobiosehydrolase; Dak: dihydroxyacetone kinase; EG: endoglucanase; Gam: glucoamylase; Gcy: glycerol dehydrogenase; Gup1: glycerol uptake protein; LAC4: β-galactosidase; LAC12: lactose permease; LAD: arabitol dehydrogenase; RK: ribulokinase; Xase: xylanase; β-Xd: β-xylosidase; XDH: xylitol dehydrogensase; XI: xylose isomerase; XK: xylulokinase; ALX: xylulose reductase. [a] Synthesized from coumaric acid. [b] Estimated from reference. [c] Given as $mg g_{CDM}^{-1}$.

scales up to 100 m^3 favored the rise of *S. cerevisiae* as a producer platform for biofuels,[26] chemicals,[6b] materials,[27] and products applied for health and nutrition[28] (Figure 4).[29]

2.4. Aspergillus spp.

The fungi of the species *Aspergillus* have gained industrial interest because of their efficient production of organic acids and proteins.[31] The most prominent products are citric[32] and itaconic acid,[33] the latter being one of the top value-added bio-based chemicals.[10] Aerobic fermentations are equally carried out as surface or submerse cultures, mostly batchwise, with reactor volumes up to 900 m^3.[31a] Strain morphology has a substantial impact on the production efficiency, and is thus a major parameter that needs to be regulated for optimal production.[32,34]

2.5. Bacillus subtilis

Comparable to *E. coli*, *B. subtilis* is the Gram-positive model for studies of cellular processes, partly with a clinical focus because of its pathogenic relatives *Bacillus anthra*cis, *Clostridium perfringens*, and *Staphylococcus aureus*.[35] Most recently, an integrative systems-biology study provided novel insights into osmoregulation from a multiomics perspective.[36] From an industrial viewpoint, *B. subtilis* is highly attractive as a potent producer of proteins, antibiotics, insecticides, some flavor enhancers, and the vitamin riboflavin.[37] As *B. subtilis* is free of exo- und endotoxins, its products are considered as generally recognised as safe (GRAS) and thus applicable for human health and nutrition.

2.6. Ashbya gossypii.

The mold *Ashbya gossypii* was first isolated in 1929 as a plant pathogen that causes stigmatomycosis of cotton and thus is a major threat to cotton production in some subtropical regions.[38] Soon after the discovery of its natural potential to efficiently synthesize the vitamin riboflavin,[39] it joined the collection of production strains for biotechnology.[40] The unraveling of its rather small and haploid genome[41] in combination with the development of molecular tools for targeted genome engineering[42] has led to genetically modified strains with improved synthesis performance being bred.[43] Today, riboflavin production with *A. gossypii* accounts for more than 4000 tons per year, which represents about 50 % of the global market volume.[44]

3. Systems and Synthetic Metabolic Engineering

Despite microbial metabolism involving all kinds of desirable molecules, it has not evolved to serve the practical

use of society, which means that the natural ambition of microbes to produce and secrete these compounds in appropriate amounts is rather limited and typically necessitates engineering their metabolism.[45] Initially, this was addressed by iterative application of UV light and chemical mutagens to generate mutated strains with stepwise-improved and finally remarkable properties.[40,46] However, this untargeted approach entailed substantial metabolic burden through inherent accumulation of, in the best case, non-useful or silent mutations, and in the worst case, detrimental ones. A producing strain obtained through multiple rounds of random mutation and selection may carry up to several thousands of mutations, of which only a small subset is probably beneficial for production. Often, such strains grow slowly and exhibit low stress tolerance and increased nutrient demand. Enabled by recombinant DNA technology, metabolic engineering has turned strain breeding into a more knowledge-based and targeted strategy, which provided the next level of production strains.[47] For existing industrial processes, it appeared, however, not easy to reach the high performance of the classical strains. Clearly, the rather local concepts of metabolic engineering fail to achieve comprehensive engineering on a global scale with full consideration of the complex interactions throughout the entire pathway network.[12b] Systems and synthetic metabolic engineering has meanwhile upgraded strain engineering to optimization on a global scale, and thereby advanced and variegated the field (Figure 5). Driven by the tremendous conceptual and technological achievements in quantitative, systems-wide analysis of cellular components, pathway activities, metabolic and regulatory network modeling, protein engineering, as well as targeted genomic modification, strain engineering has in the meanwhile evolved into a design science.[12b,45,48] Systems and synthetic metabolic engineering leads to tailor-made cell factories with designed properties, and promises significant impact on the economic success of industrial bioproduction.

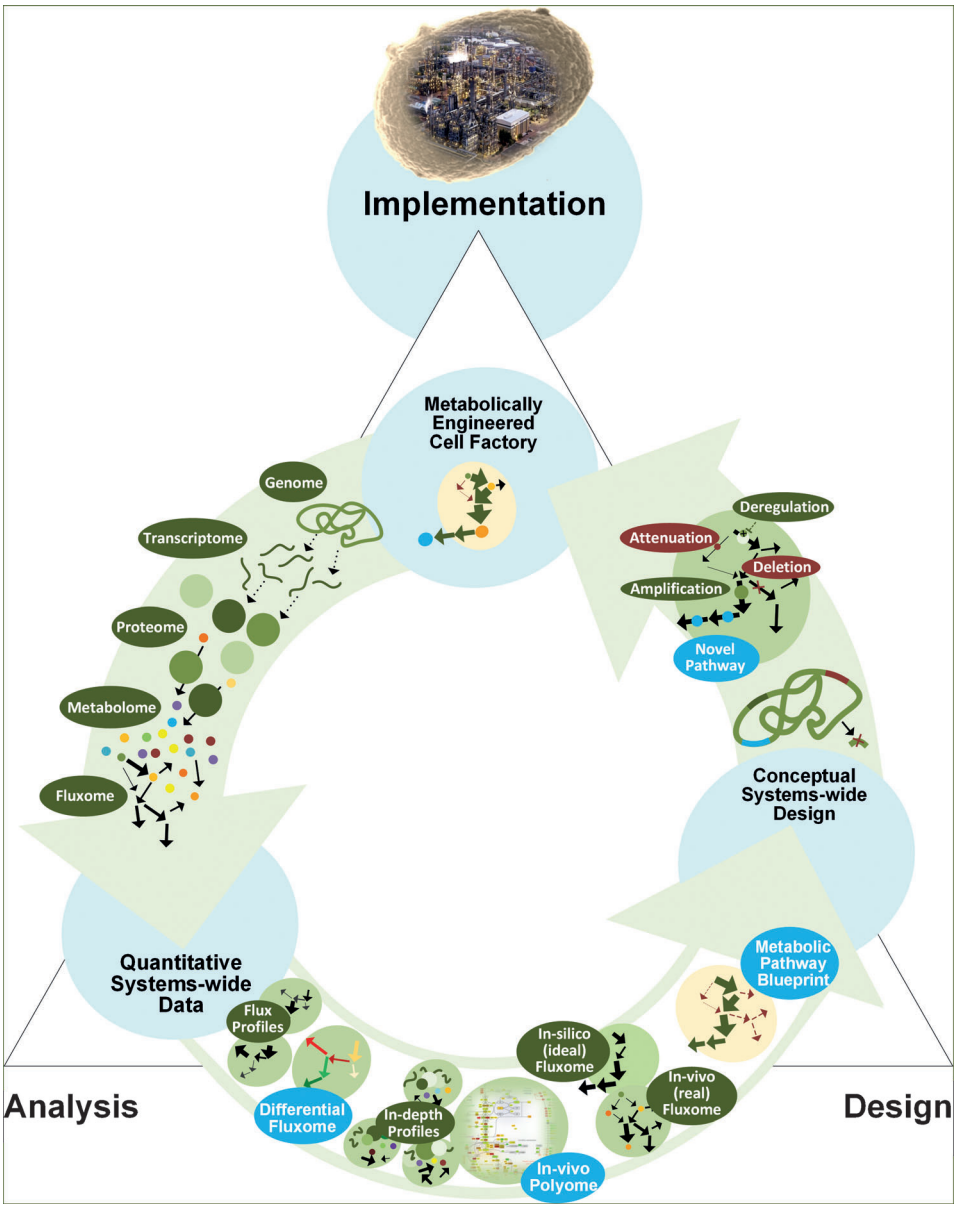


Figure 5. Systems metabolic engineering for the design and creation of optimized industrial producers. The iterative approach is based on systems-biology analysis, which recruits modern omics technologies for deciphering the genome, transcriptome, proteome, metabolome, and fluxome, applied as a single or multiomics study. Comprehensive analysis and evaluation of the data combined with computational modeling at the systems level then provides a holistic metabolic design, which is taken further into a genetic blueprint for targeted genomic modification to create a metabolically engineered cell factory for production.

3.1. Systems Biology Analysis

A fundamental understanding of cellular physiology and its regulation forms the knowledge base for rational strain engineering. So-called "omics" platform technologies provide what is needed: comprehensive and quantitative data at the

various layers of genes, transcripts, proteins, metabolites, and pathway fluxes, which disclose the best strategy for the genetic engineers to change the organism. The genome bears the entire genetic repertoire. Today, a microbial genome may be sequenced within a few days and at a continuously decreasing cost. In recent years, the advanced technology massively increased the number of sequenced genomes and supported the understanding of microbes through the identification of biosynthetic pathways and the discovery of evolutional traits.[41,49] In particular, in silico modeling strongly benefited from the vast accumulation of genome sequences.[50] Genome sequences further facilitated genetic engineering workflows, as well as transcriptome and proteome profiling. Comparative sequence analysis of nonproducing and classically derived strains, and of wild-type and evolutionary adapted strains, even directly unravels genetic targets that encode desired phenotypes. As an example, this has proven valuable through "genome breeding" to create minimally mutated *C. glutamicum* strains for the efficient production of L-lysine.[51]

On the basis of genome data, DNA microarrays for systems-wide profiling of the transcriptome were developed for determining dynamic gene expression differences between different strains, cultivation conditions, and growth phases. These included studies on physiological aspects of carbon,[52] nitrogen,[53] and sulfur[54] metabolism. Transcriptomics is now widely applied to decipher global regulatory networks in microorganisms,[55] and also directly guided rational strain design. To mention a few examples, it was applied to improve the *C. glutamicum* based production of L-lysine,[56] diaminopentane,[57] and L-valine,[58] *E. coli* based production of L-valine[59] and L-threonine,[25ad] riboflavin production with *B. subtilis*,[60] and xylose assimilation by engineered *S. cerevisiae*.[61] Today, advanced RNA sequencing even allows absolute quantification of RNA transcripts with increased information content and precision compared to DNA microarrays.[62] This technology provided novel insights into transcriptional organization and regulatory functions of small RNA molecules, thus promising the future discovery of global transcription networks.[63]

Proteome analysis builds on the efficient electrophoretic or chromatographic separation of proteins or proteolytic peptides obtained therefrom, combined with their straightforward identification using mass spectrometry and database searches.[64] In the context of systems biology, proteomics gave insights into the cellular response of bacteria to environmental changes,[36,65] including starvation[36] and nutrient availability.[66] Furthermore, the technique allowed gene-function analyses, the dissection of metabolic pathways, or the curation of misannotations of genes with a potential impact for industrial applications.[67]

Metabolome analysis, which aims at the analysis of cellular metabolites, is one of the most challenging "omics" approaches. Appropriate analysis quests for efficient quenching that immediately freezes the metabolic state of the cell without biasing the metabolome through leakage effects,[68] and also demands for powerful analytical platforms, which can handle the high number of the often low concentration chemically quite diverse and partly unstable metabolites in a small amount of sample. Current research continuously focuses on improved experimental workflows and analytics for generating metabolome data sets with relevant biological meaning.[68a,69] When carefully applied, metabolomics is a powerful tool for identifying metabolic bottlenecks, so far unassigned pathways, and biochemical reactions towards strain design.[70] The fluxome, that is, the in vivo activity of biochemical reactions and pathways, is closest to the physiological phenotype of an organism. It displays the integrated output of genes, transcripts, proteins, and metabolites and links all these components to cellular functions. Thus, the fluxome appears indispensable for understanding cellular systems.[48a] Fluxome analysis has proven valuable to decipher pathway function, discover novel pathways, and understand network rigidity and robustness.[48a] The most advanced fluxomics of *C. glutamicum* have systematically investigated different strains,[71] environmental conditions,[72] and production phases.[73] This approach has substantially guided strain improvement with an impact on the industrial production of commercial goods.[7b,74]

3.2. Metabolic Design

Clearly, a strain suitable for industrial production demands a global redirection of the flux, which needs a well-balanced combination of beneficial modifications, typically located at rather distant parts of the metabolism. The art at this point for beneficial cellular engineering is to extract truly relevant information from the large and complex data sets, often generated from systems biology experiments. Models, statistical approaches, and databases help to address this multitarget challenge. Most directly applied to data sets, statistical methods, combined with software packages for data processing and visualization, can be used to extract and integrate biologically meaningful and significant correlations and conclusions.[36,75] Single omics data already provide important insights into the cellular metabolism. The knowledge gain and information content is even broadened, when multiomics data sets are comprehensively integrated. In particular, transcriptome and proteome analysis are readily combined as the most conveniently applied techniques, mainly to unravel regulation levels of expression control.[48a,76] The integration of metabolites and fluxes is still in its infancy, although pioneering studies revealed their particular importance and value to bridge cellular components with network function and regulation.[36,48a,73a] Of value in this context are in silico reconstructions of metabolic networks from genome sequences. They serve as informative bibliographies to map experimental data on individual cellular components into global biological contexts, and furthermore enable theoretical investigations of network behavior, robustness, and natural boundaries.[50] Such network models were successfully applied to elucidate relevant aspects for the production of L-methionine,[77] L-lysine,[13a,50b,78] riboflavin,[79] succinic acid,[80] and diverse alcohols,[79b,81] including the prediction of optimal yields, optimal pathways, and genetic targets solely from simulations. Among the most advanced approaches at present is the model-based design of cells, which integrates in vivo

and in silico flux data, and thereby predicts full sets of targets across the network to achieve the desired pathway fluxes. Recently, such advanced flux design was successfully applied to breed optimized cell factories for producing commercially interesting goods.[13a,30b,50b,79b,82]

3.3. Genome Manipulation

Typically, genetic engineers working in the field are faced with a challenging wish list. The metabolic blueprint, predicted from systems-wide analysis, has to be translated into a suitable genetic blueprint to allow precise and tunable control of pathway flux. Fortunately, the genetics toolbox has continuously advanced throughout the past decades, thereby providing polymerase chain reactions for the targeted amplification of DNA fragments,[83] restriction enzymes[84] and ligases[85] as molecular scissors and glues for DNA, and sophisticated techniques for chemical gene synthesis,[86] as well as in vitro and in vivo DNA assembly,[87] which even allow the construction of artificial genomes.[88] Taken together, a rich set of tools are available for the highly precise and targeted manipulation of DNA from the level of single-point mutations up to the synthetic construction of whole pathways and control circuits that span thousands of base pairs.

Gene deletion, that is, elimination of the corresponding biochemical reaction from the network, is a common method to avoid carbon consumption by undesired and competing pathways. In particular, anaerobic fermentation processes often suffer from high by-product formation fluxes and thus strongly rely on deletion mutants to avoid mixed fermentation.[17,18,89] Although these modifications mainly refer to the periphery of the metabolic network, the elimination of reactions within the carbon core metabolism is also considered, often to address the supply of carbon building blocks. Prominent examples comprise lysine, diaminopentane, and glutamate production with *C. glutamicum*,[70d,90] as well as the production of aromatic compounds, threonine, and valine with *E. coli*.[25ad,46b,59,91] Point mutations, although only affecting a single nucleotide within the whole genome, are impressively powerful for enzyme engineering and expression control. Implemented at the regulatory control site or the substrate binding site, point mutations can be used to release enzymes from feedback inhibition and metabolic control,[92] to modify the catalytic efficiency,[51c] and to change the substrate and cofactor specificity[93] for improving production. Even silent point mutations, namely, mutations without any effect on the protein sequence itself, can help to modify the final enzyme activity within the cell at the level of translational efficiency.[94] This is the artifice of codon optimization for improving heterologous expression of a donor gene in a foreign host.[7b,16b,20v,95] Vice versa, variation of the start codon is helpful for the gradual attenuation of competing pathways without crude elimination through gene deletion,[96] the latter often entailing undesired site effects.[97] The codon strategy gives access to enzymes and pathways that are rather sensitive towards genetic modifications and, of crucial importance, for growth and viability.

Practically all engineering strategies involve a type of modification for overexpression of the genes that encode production-relevant proteins. A straightforward method to achieve amplified expression is the use of episomal, autonomously replicating plasmids, which increase the copy number of the gene within the cell.[16b,25k,98] However, the gene dosage is barely adjustable by such plasmids, and the choice of low-, medium-, or high-copy plasmids only allows a vague and imprecise overexpression, sometimes together with the formation of inclusion bodies and thus loss of enzyme function.[23a] Implementation of additional gene copies directly into the genome can here be of benefit to more precisely adjust the expression levels.[13a] More convenient and practical is, however, the use of alternative promoters. Research over the last few decades has provided a rich set of constitutive and inducible promoters.[98b,99] Besides these naturally occurring promoters, synthetic promoter libraries display a breakthrough for systems metabolic engineering. They have substantially increased the palette of promoters covering manifold strengths for an individually fine-tuned gene expression.[100] In addition, the expression of foreign genes allows the natural genetic boundaries to be overcome: foreign genes from selected donor organisms can be heterologously implemented into the chosen production host, thus conferring a novel metabolic function. In particular, this provides the possibility to produce complex molecules from higher organisms, such as plants and mammals, in microorganisms. This has substantially extended the product and substrate portfolio of industrial microbial producers.[1b,c,30h,45] Classical cloning techniques, which leave the gene sequence of the donor unmodified, are increasingly replaced by approaches that recruit synthetic genes, gene clusters, and operons. Artificial gene synthesis benefits from the possibility of the aforementioned codon optimization, for example, the adjustment of the gene sequence to the expression machinery of the host, thereby dramatically increasing the likelihood of high protein expression.[101] This appears particularly relevant when expressing genes from higher plants and animals in microbial systems.

4. Bio-Based Chemicals

4.1. Propanediol

Propanediols (PDOs) are platform chemicals with three carbon atoms that are currently produced chemically from nonrenewable resources. The application of 1,2-PDO ranges from being used as a deicer, antifreeze, moisturizer in cosmetics, or food additive, whereas 1,3-PDO predominantly serves as a polymerization building block. Metabolic engineering for 1,2-PDO production has basically been built on the methylglyoxal route by recruiting methylglyoxal synthase and glycerol dehydrogenase. In *E. coli*, overexpression of the native genes already enable production, whereas in *S. cerevisiae* and *C. glutamicum* production requires heterologous gene expression.[18c,102] *E. coli* and *S. cerevisiae* were further optimized beyond a basic proof-of-concept, both benefiting from deletion of triosephosphate isomerase.[25e,102b] The best

production of 3.7 $g L^{-1}$ 1,2-PDO was obtained by a combined approach of rational and evolutional engineering of *E. coli*.[25e] Glycerol is the preferred carbon source for the production of 1,3-PDO, as only two enzymes—glycerol dehydratase and 1,3-PDO oxidoreductase, alternatively 1,3-PDO dehydrogenase—are needed to convert glycerol into 1,3-PDO.[25e] However, *E. coli* and *S. cerevisiae* were also engineered to produce 1,3-PDO from glucose via glycerol as a biosynthetic intermediate.[25f,30d] For *E. coli*, this even resulted in commercialized biotechnological production by DuPont and Genencor International, Inc.[103] Their strain was substantially engineered by:

1) expression of the *S. cerevisiae* glycerol pathway (glycerol 3-phosphate dehydrogenase and glycerol 3-phosphate phosphatase),
2) expression of glycerol dehydratase from *Klebsiella pneumoniae*,
3) endogenous overexpression of 1,3-PDO dehydrogenase with improved error-prone PCR-derived catalytic efficiency,
4) inactivation of the glycerol assimilation pathway,
5) down-regulation of glyceraldehyde 3-phosphate dehydrogenase,
6) replacement of the phosphotransferase system by PEP-independent glucose uptake by galactose permease and glucokinase.[25f,103]

The optimized producer accumulated 135 $g L^{-1}$ 1,3-PDO in a a yield of 0.51 $g g^{-1}$ (= g product per gram substrate).[25f]

4.2. Butanediol

Similar to propanediols, the two butanediols (BDOs) 1,4-butanediol and 2,3-butanediol are broadly used as platform chemicals to manufacture drugs, cosmetics, solvents, and polymers with a market exceeding 2.5 million tons per year.[30g,104] 2,3-BDO is a natural fermentation product of *S. cerevisiae* that is readily detected in top-fermented beers. Production was optimized by elimination of competing pathways, overexpression of the biosynthetic route, and further evolution towards accelerated glucose consumption.[30g] The substrate spectrum for 2,3-BDO production was extended to xylose and cellobiose.[105] The glucose-based production, however, yielded the highest titer of 96 $g L^{-1}$.[30g] In *E. coli*, 2,3-BDO production was enabled by implementing genes for acetolactate decarboxylase (*budA*), acetolactate synthase (*budB*), and butanediol dehydogenase (*budC*) from *K. pneumoniae*.[106] The maximum production titers of approximately 1 $g L^{-1}$ are, however, far from being competitive to yeast.[107] *E. coli* was the first organism used for the biocatalytic conversion of renewable feedstocks into 1,4-BDO, with a considerable reported production of 18 $g L^{-1}$.[104] Further advances were made in the commercial-scale production of BDO within a joint campaign by Genomatica and DuPont Tate & Lyle Bio Products Company, which produced over 2000 metric tons of BDO by direct fermentation.

4.3. Succinic Acid

Succinic acid has received increasing interest during the last few decades as one of the "top 12 candidates" for producing bio-based value-added chemicals.[10] The rising popularity of the "bio" label for succinic acid has driven the development of fermentation processes. Industrial scale production has now been reached by different companies, including Myriant (with ThyssenKrupp Uhde), BioAmber (joint venture with Mitsui & Co.), and Succinity GmbH (joint venture of BASF SE and Corbion Purac) by using proprietary organisms such as metabolically engineered *E. coli*, yeast, and *Basfia succiniciproducens*, respectively.[6a,89a] Academic research nicely illustrates that engineering towards improved production greatly profited from elimination of by-product formation by deleting genes leading to alternative fermentation products, including lactate, acetate, formiate, and ethanol.[20x,25z,aa,108] For *E. coli* and *C. glutamicum*, high-level production of up to 127 $g L^{-1}$ and 146 $g L^{-1}$, respectively, were achieved (Figures 2 and 3), with uncoupled growth and production phases for *C. glutamicum*.[20x,25y] Supplementing the production medium with a high concentration of carbonate for CO_2 fixation by anaplerosis allowed yields surpassing 1.0 $g g^{-1}$.[108a] Aerobic succinate production in *C. glutamicum* was realized by elimination of by-product formation, disruption of the TCA cycle downstream of succinate, and overexpression of anaplerotic carboxylation.[109] Further improvement was achieved by acetate recycling and increased flux of the oxidative TCA cycle through overexpression of citrate synthase.[110] Production, however, did not reach the performance of the anaerobic processes. *S. cerevisiae* based succinate production is still in its infancy, but is of high interest because of the ability of yeast to grow at low pH values, which facilitates downstream processing.[30r] Both aerobic and anaerobic production is currently evaluated,[29,30r,80,111] whereby a mixed oxidative/reductive route appears most advantageous.[112]

4.4. Malic Acid

Bio-based malic acid production benefits over chemical synthesis by providing pure stereospecific L-malic acid. Similarly to succinic acid, the pathways for malic acid biosynthesis in microorganisms comprise the reductive route, oxidative route, and glyoxylate shunt. As succinate-producing *E. coli* already secretes malate as a by-product,[25aa] this was taken as the starting point for further engineering through deletion of malate-associated reactions catalyzed by fumarate reductase, fumarase, and malic enzyme.[113] Although the final concentration of 34 $g L^{-1}$ was substantially lower than the best achieved succinate titers, the yield was impressively high (1.1 $g g^{-1}$).[113] In the case of *S. cerevisiae*, engineering of pyruvate carboxylation, oxaloacetate reduction, and malate export provided a strain that produced 59 $g L^{-1}$ malic acid in a yield of 0.31 $g g^{-1}$.[30t]

4.5. Itaconic Acid

Itaconic acid—a C_5 dicarbonic acid—is widely used for the homopolymeric production of polyacrylates and rubber.[114] Metabolically related to citric acid, it can be synthesized via *cis*-aconitate and subsequent decarboxylation of this important intermediate of the tricarboxylic acid cycle.[33] Industrially, itaconic acid is produced by submerse fermentation of *Aspergillus* spp in an excellent production performance of 86 $g L^{-1}$ and yields of 0.62 $g g^{-1}$.[115] Product titers even reach the maximum solubility in water.[115,116] Recently, *S. cerevisiae* was engineered towards itaconate production by heterologous expression of the aconitate decarboxylase of *A. terreus*, although with low efficiency so far (Figure 4).[30q] A similar approach enabled the production of itaconic acid in *E. coli*. Titers of up to 690 $mg L^{-1}$ were achieved by introducing citrate synthase and aconitase from *Corynebacterium glutamicum* and by deleting the genes encoding phosphate acetyltransferase and lactate dehydrogenase.[25ae]

5. Materials

5.1. Diamines

The C_6 diamine diaminohexane is one of the most important building blocks in the polymer industry (more specifically in polyamide production) with an annual market size of 6.6 million tons.[20a] As diaminohexane is so far not accessible by fermentation, research on the bio-based production of diamines as an alternative for the petroleum-based route has focused on diaminopentane (DAP, cadaverine) and diaminobutane (putrescine).[1a,16c,23a] These compounds naturally occur as intermediates of the degradation pathways of L-lysine and L-ornithine, respectively.[117]

In *E. coli*, putrescine production was achieved by disrupting the degradation of the C_4 diamine. Production of 24 $g L^{-1}$ was then realized by an improved supply of the precursor ornithine and increased expression of the diaminobutane-forming ornithine decarboxylase.[23a] Similarly, putrescine production with *C. glutamicum* depends on the ornithine decarboxylating activity, which, however, needs to be implemented heterologously.[16c] Uncoupling of pathway control from the arginine repressor ArgR and fine-tuned withdrawal of ornithine for arginine biosynthesis allowed the production of 19 $g L^{-1}$ putrescine in a yield of 0.16 $g g^{-1}$ (Figure 3).[16c,20j] Ongoing research in this field might further drive strain and process development.[118]

Diaminopentane, derived from lysine by decarboxylation, was successfully produced in engineered *C. glutamicum* and *E. coli* strains. For *E. coli*, the strategy was adapted from the production of putrescine, and entailed inactivation of diaminopentane degradation, improved supply of the building block lysine, and amplified expression of the DAP-forming lysine decarboxylase CadA (Figure 3). Engineering of *C. glutamicum*, however, went far beyond a basic proof-of-concept. Initial *C. glutamicum* DAP producers, which relied on the *E. coli* CadA variant of lysine decarboxylase,[16a] were soon superseded by production strains that recruited the LdcC enzyme variant of *E. coli*, the expression of which was additionally optimized by promoter variation and codon optimization.[16b] Further strain improvement involved amplified expression of biosynthetic pathway genes, elimination or down-regulation of competing reactions, deletion of genes responsible for by-product formation, improved product secretion, and increased supply of carbon building blocks and reducing power (Figure 6).[16b,20a,70d] The optimized *C. glutamicum* strain produced and secreted 88 $g L^{-1}$ DAP in a yield of 0.5 $g g^{-1}$ (Figure 2).[20a]

This strain was even integrated in a biorefinery concept for the bio-based production of materials with advanced properties. This comprehensive approach combined synthetic biology and synthetic chemistry to manufacture the novel 100% bio-based polyamide PA5.10.[20a] The process comprised the complete production workflow of upstream operations, fermentation with the streamlined DAP hyperproducing strain, product purification, and chemical polymerization with or without glass fiber reinforcement.[20a] Systematic material testing revealed excellent material properties in terms of the melting temperature and mechanical strength not being worse than petroleum-based PA6 and PA6.6. Its lower density even promises advantageous applications in energy-friendly transportation.[20a] A xylose- and hemicellulose-based process was established by additional implementation of the xylose assimilation pathway from *E. coli*.[98a] Accumulation of 60 $g L^{-1}$ nicely illustrates the feasibility of using alternative carbon feedstocks so far hardly considered in industrial biotechnology.[1c,98a]

5.2. Lactic and 3-Hydroxypropionic Acid

Lactic (LA) and 3-hydroxypropionic acid (3HP) are, as a result of their bifunctional biochemistry resulting from a hydroxy and a carboxyl group, particularly useful building blocks for the synthesis of the biodegradable polyesters polylactate (PLA) and poly(3-hydroxypropionate) (P3HP).[6a] 3HP is also a relevant raw material for the production of acrylic acid, which in turn serves as a platform chemical and polymer building block.

Current LA production plants are operated with classically optimized *Lactobacilli*. The major supplier in the field is NatureWorks, which is owned by Cargill Incorporated (USA), Purac (The Netherlands), Galactic (Belgium), and several Chinese companies.[119] PLA production has a comparably long tradition among the bio-based polymers, with the first large-scale plant being launched in 2002 by NatureWorks LLC, which to date holds the largest share of the PLA business.[119] In addition to the classical producers, *E. coli*, *C. glutamicum*, and *S. cerevisiae* have been trained for the production of lactic acid (Figures 2–4).[119] Several studies reported the production of D-lactate and L-lactate with engineered *E. coli*, by using either naturally or non-natural fermentable carbon sources.[25m,120] Key modifications comprised the elimination of by-product formation.[25m,p,120c] The best strain so far accumulated 138 $g L^{-1}$ lactate in a yield of 0.86 $g g^{-1}$ (Figure 2).[25p] Additional implementation of an evolved propionate CoA-transferase and polyhydroxyalka-

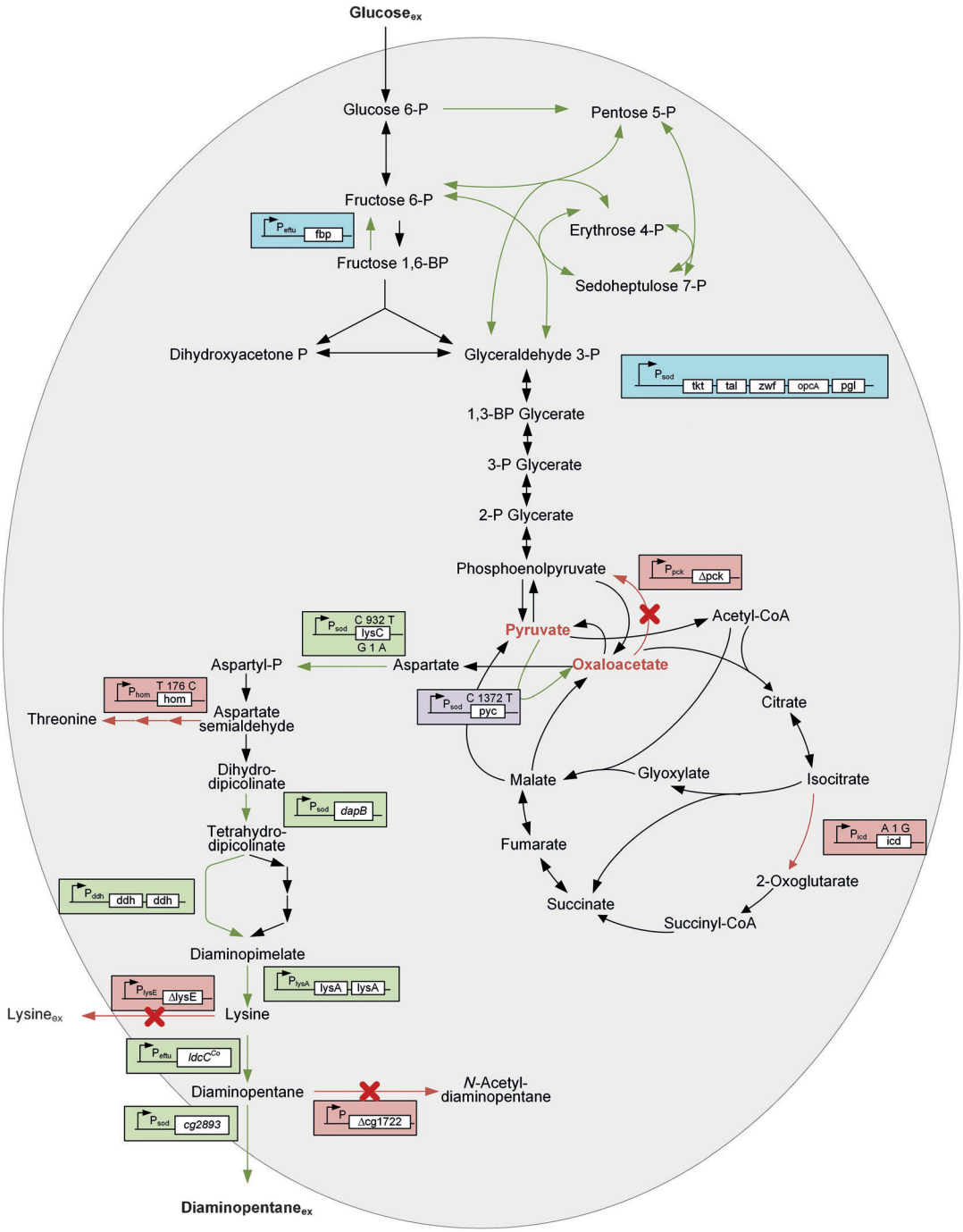


Figure 6. Systems-wide metabolic pathway engineering of *Corynebacterium glutamicum* for the production of diaminopentane.[20a] Green arrows indicate up-regulation, red arrows indicate down-regulation. Gene deletions are indicated by the red "X". The color code for genetic modifications is as follows: green: reactions and pathways involved in biosynthesis and export; red: pathways competing with the biosynthesis and precursor supply; purple: reactions involved in the supply of the carbon precursor; blue: pathways involved in the supply of NADPH. The enzymes encoded by the corresponding genes are: cg1722: *N*-acetyltransferase; cg2893: major facilitator permease; *dapB*: dihydrodipicolinate reductase; *ddh*: diaminopimelate dehydrogenase; *fbp*: fructose 1,6-bisphosphatase; *hom*: homoserine dehydrogenase; *icd*: isocitrate dehydrogenase; *ldcC*: lysine decarboxylase from *E. coli*; *lysA*: diaminopimelate decarboxylase; *lysE*: lysine exporter; *lysC*: aspartokinase; *pck*: phosphoenolpyruvate carboxykinase; *pgl*: 6-phosphogluconolactonase; *pycA*: pyruvate carboxylase; *tal*: transaldolase; *tkt*: transketolase; *zwf*: glucose 6-phosphate dehydrogenase.

noate (PHA) synthase allowed direct production of PLA and copolymers thereof up to 56 wt %.[121] L-lactic acid secretion is naturally induced in *C. glutamicum* as a response to oxygen limitation. This has successfully been demonstrated by using diverse substrates including glucose, arabinose, xylose, and cellobiose.[20u,122] In glucose-grown *C. glutamicum*, deletion of the intrinsic lactate dehydrogenase (LDH) and expression of the non-native D-LDH enabled the production of 120 g L^{-1} D-

lactate with a purity of 99.9%.[20u] As by-product formation was still present, further improvement can be expected by avoiding succinate and acetate secretion. The first attempts at the direct fermentative production of the polymer provided strains that produced P(LA-*co*-3HB) copolymers from lactate and 3-hydroxybutyrate with high LA fractions.[19a] Lactate production in *S. cerevisiae* relies on the heterologous expression of LDH.[30f,123] As the fermentative formation of ethanol is a major threat to the desired lactate secretion, this was tackled by deletion of selected genes of the ethanol pathway.[123,124] This enabled pure L-lactate to be obtained at a concentration of 122 $g L^{-1}$ (Figure 4).[30f]

From the reviewed organisms, reports on 3HP production are restricted to glycerol fermentation using engineered *E. coli*.[125] Indeed, this process resembles a bioconversion rather than a de novo synthesis, as only two enzymatic reactions are required.[6a,125] Interestingly, the number of studies on direct P3HP production—still requiring the supply of 3HP as a building block—is substantially higher. These even comprise different alternative strategies for 3HP synthesis using either glycerol (by the above-mentioned route) or glucose as carbon sources.[126] Approaches for glucose-based P3HP production comprise the β-alanine route and the malonyl-CoA route.[126a,c]

5.3. Polyhydroxyalkanoates

Polyhydroxyalkanoates (PHAs) are biodegradable and biocompatible polymers that are naturally synthesized by many organisms as storage compounds.[127] The material properties are highly versatile with regard to the numerous monomer variants that can be combined to form homo- and copolymers from short-chain-length (scl), medium-chain-length (mcl), and long-chain-length (lcl) building blocks.[127,128] *E. coli*, *C. glutamicum*, and *S. cerevisiae* are typically not used for the production of PHA, but some studies still report on successful strain engineering. These strains are generally equipped with heterologous genes of biosynthetic PHA operons from *Ralstonia eutropha*, *Bacillus cereus*, or *Pseudomonads*.[20v,27,129] Mostly, production is limited to scl-PHAs synthesized from the building blocks 3-hydroxybutyrate (3HB), 2-hydroxybutyrate (2HB), and 3-hydroxyvalerate (3HB). However, for *E. coli* and *S. cerevisiae*, the engineering of β oxidation increased the diversity and variability of the monomers by providing mcl units.[27,130]

6. Biofuels

6.1. Ethanol

Ethanol is the classical fermentation product of *S. cerevisiae*, and together with *Zymomonas mobilis*, the yeast is the major producer of ethanol for both beverages and fuels.[6a,30h] Compared to *E. coli*, yeast has the distinct advantage of the limited formation of by-products combined with high tolerance to solvents.[30h] Ethanol production with *S. cerevisiae* often even comes close to the theoretical optimum of 0.51 $g g^{-1}$, with concentrations reaching 48 $g L^{-1}$.[30h,i] Glycerol is, however, readily detected in the yeast-based production of ethanol and considerable effort has been made to minimize or abolish by-product formation.[6b,131] However, glycerol null-mutants are unable to grow anaerobically, as the glycerol pathway serves as an essential electron sink for regeneration of nicotinamide adenine dinucleotide (NAD^+).[6b,131b] Alternative strategies aim at a modified redox metabolism with metabolic substitutes for electron balancing.[131a,132] Further engineering focused on broadening the substrate spectrum and improved tolerance.[30i,j,133] As a consequence of its natural wide substrate range, *E. coli* is a promising candidate for ethanol production. Indeed high-level ethanol production of 54.4 $g L^{-1}$ and 41.6 $g L^{-1}$ from glucose and xylose, respectively, can be achieved by expression of the ethanologenic pathway of *Z. mobilis*, combined with disruption of the *frd* gene responsible for succinate production.[25j] Another study reported the construction of a nontransgenic ethanol producer, that is, deriving the alcohol as a single product, by elimination of competing fermentative pathways through deletions of the genes encoding for fumarate reductase (*frdABCD*), lactate dehydrogenase (*ldhA*), acetate kinase (*ackA*), and pyruvate formate lyase (*pflB*). The obtained strain was additionally modified to anaerobically express pyruvate dehydrogenase for redox balancing, overall allowing the production of ethanol in 90% yield.[134] Mixed sugar co-fermentation was approached by deletion of the *mgsA* that takes control in sugar metabolism.[135] Similarly to *S. cerevisiae*, tolerance remains a key issue in the production of bioethanol with *E. coli*.[136] Ethanol fermentation in *C. glutamicum* strictly relies on heterologous genes. Engineered strains expressing pyruvate decarboxylase (*pdc*) and alcohol dehydrogenase (*adhB*) from *Z. mobilis*, and additionally deficient in lactate dehydrogenase and PEP carboxylase, produced 0.53 $g g^{-1}$ ethanol under oxygen deprivation.[18a] Low product titers and tolerance, however, demand for further engineering.

6.2. Higher Alcohols

As a consequence of their low vapor pressure, low hygroscopicity, and high energy density, alcohols with four or more carbon atoms are especially advantageous as biofuels.[25i] Isobutanol and 1-butanol are currently the best-studied examples. The isobutanol pathway is closely linked to the biosynthesis of the branched-chain amino acids; thus, engineering profited from the experience gained with traditional amino acid fermentation. Following the strategy towards valine overproduction, *C. glutamicum* was, for example, successfully re-engineered for isobutanol production.[18b] Inactivation of lactate dehydrogenase and pyruvate carboxylase increased the production to 4.9 $g L^{-1}$. Traces of other higher alcohols including 1-propanol, 2-methyl-1-butanol, 1-butanol, and 2-phenylethanol occurred as by-products. Increased production of up to 18 $g L^{-1}$ isobutanol was achieved by an approach that recruited a 2-ketovalerate production strain by inactivation of lactate and malate dehydrogenases, implementation of ketoacid decarboxylase

from *Lactococcus lactis*, alcohol dehydrogenase (ADH2) from *S. cerevisiae*, and expression of the *pntAB* transhydrogenase from *E. coli*.[137] Applying continuous solvent extraction during the fermentation of engineered *C. glutamicum* at high cell densities even allowed the accumulation of 73 $g L^{-1}$ isobutanol in the solvent.[20c] Similarly to *C. glutamicum*, isobutanol production in *E. coli* followed the branched-chain amino acids pathway. Overexpression of the genes *ilvIHCD* for 2-ketoisovalerate biosynthesis combined with implementation of 2-keto acid decarboxylase and alcohol dehydrogenase generated a basic producer. Elimination of by-product formation and expression of the *Bacillus subtilis alsS* gene shifted production to 22 $g L^{-1}$.[25i,138]

For *n*-butanol production, the complete native biosynthetic pathway from *Clostridium acetobutylicum*—branching off from the central intermediate acetyl-CoA—was implemented into *E. coli*.[138] Another strategy made use of a synthetic pathway that originated from threonine. Systematic improvement through pathway deregulation and by-product elimination allowed the simultaneous production of 2 $g L^{-1}$ 1-butanol and 1-propanol in a ratio of about 1:1.[139] However, the acetyl-CoA derived production turned out to be more efficient. Reconstruction of this pathway by selecting genes from different organisms resulted in superior production compared to solely using *C. acetobutylicum* genes.[25u,140] Overall, production was increased to 30 $g L^{-1}$.[25u] Further extension of the butanol pathway additionally enabled production of 1-hexanol in *E. coli*.[25r] Production of long-chain ($>C_4$) alcohols was demonstrated by the reverse β oxidation as a novel and promising platform for the advanced production of biofuel.[25s]

Isobutanol production in *S. cerevisiae* was first realized by endogenous overexpression of the valine pathway.[141] Production was, however, impeded by the cellular compartmentation. Thus, moving valine biosynthesis from the mitochondrium to the cytosol was beneficial for production.[30e] However, the strain performance (Figure 4) lagged behind that with *C. glutamicum* and *E. coli*.

Two different approaches have been carried out for the production of *n*-butanol. The heterologous strategy, originating from acetyl-CoA, generated producers that accumulated up to 16 $mg L^{-1}$ butanol.[30k] The endogenous strategy via threonine was, however, advantageous, as evident from the higher production of 243 $mg L^{-1}$.[30s]

6.3. Biodiesel

Biodiesel is mainly composed of fatty acid methyl esters (FAMEs), propyl esters, and ethyl esters. Their microbial production is highly desirable and has so far mainly been addressed by the engineering of *S. cerevisiae* and *E. coli*.[142] The first microbial biodiesel production with *E. coli* was achieved through transesterification of endogenous ethanol with supplemented oleic acid.[143] De novo synthesis of fatty acids was then achieved by amplified expression of fatty acid biosynthesis combined with inactivation of β oxidation.[142] The construction of a biodiesel producer in *S. cerevisiae* was initiated by inactivation of the synthesis of storage lipids. Heterologous expression of bifunctional wax ester synthase from *Acinetobacter calcoaceticus* then enabled the formation of FAMEs.[144] Further strain improvement included overexpression of acetyl-CoA carboxylase and the use of an alternative wax ester synthase from *Marinobacter hydrocarbonoclasticus* with favorable catalytic properties.[30m,145] Biodiesel production with *C. glutamicum* has so far only been approached by the de novo synthesis of fatty acids, which still require transesterification for the production of FAMEs.[20w]

7. Health and Nutrition

7.1. L-Glutamate

From the beginning until today, the flavor enhancer L-glutamate has held the largest market share of amino acid fermentation, with a current production of 2.5 million tons per year. Industrial L-glutamate processes rely on *C. glutamicum*, which was originally isolated for this purpose. Wild-type *C. glutamicum* secretes substantial amounts of L-glutamate in response to biotin-limitation, penicillin, or surfactant treatment.[146] However, improved production was achieved by carbon channeling through anaplerotic carboxylation[90c,147] and by attenuating carbon conversion by the competing enzyme 2-oxoglutarate dehydrogenase.[146a,b,148] So far, these rational approaches have not outcompeted the performance of classical producers, which reach product titers of 100 $g L^{-1}$ (Figure 2).[20l]

7.2. L-Lysine

The production of the essential feed amino acid L-lysine, with a market size of around 1.5 million tons per year, has become a highly competitive business, forcing the commercial suppliers to undergo continuous improvement. Production processes are equally common for *E. coli* and *C. glutamicum*, with company-specific patent coverage.[46a] Publicly accessible research studies, however, predominantly focus on the engineering of *C. glutamicum*, although a recent study describes an optimized fermentation process using engineered *E. coli*.[25x] In *C. glutamicum*, the close link of the lysine biosynthetic pathway to the central metabolic network[46a] stimulated local manipulation of the carbon core metabolism to eliminate or attenuate competing reactions,[90b,96] and to improve the supply of the building block oxaloacetate[90a,149] and of nicotinamide adenine dinucleotide phosphate (NADPH),[51c,92c,150] which provides the redox power to drive the biosynthesis. Unraveling of the genome sequence strongly supported the understanding of *C. glutamicum* and had a substantial industrial impact through revolutionizing genetic and metabolic engineering. It paved the way for global and systems-oriented engineering approaches such as genome breeding[51b] and comprehensive model-based metabolic strain design.[13a] The first global strategy for a minimally mutated L-lysine producer yielded a titer of 100 $g L^{-1}$ and a yield of 0.4 $g g^{-1}$.[51b] More recently,

this was exceeded by synthetic metabolic engineering of *C. glutamicum*. Twelve defined genome-based modifications converted the wild-type strain into an efficient producer, with a production of 120 $g L^{-1}$ L-lysine in a yield of 0.55 $g g^{-1}$ (Figure 2). Knowledge-based design and targeted genetic re-engineering even pushed this rationally created L-lysine hyperproducer beyond the performance of classical producers.[13a]

7.3. Aromatic L-Amino Acids and Related Compounds

For many years, the satisfying economic production of L-phenylalanine, L-tryptophan, and L-tyrosine was hampered by the complex network of metabolic inhibition, repression, and attenuation of the biosynthesis.[151] Targeted engineering of the terminal pathway, product secretion, and enhanced supply of the building blocks in combination with classical engineering circumvented these bottlenecks,[46b,74a,152] and industrially relevant production levels were achieved (Figures 2 and 3). Metabolic engineering has been carried out equally for *C. glutamicum* and *E. coli*, whereby slightly better L-tryptophan producers where described based on *C. glutamicum*, and better L-phenylalanine producers for *E. coli* (Figures 2 and 3). Further improvement can be expected from the current achievements in strain engineering.[63b,92b,153] The collected expertise has additionally driven the production of related aromatic compounds, mainly with *E. coli*.[91a] Shikimate, itself an intermediate of the aromatic amino acid metabolism, is probably the most popular and successful example thereof, with titers of 80 $g L^{-1}$ achieved.[25a] Other pathway intermediates are, for example, anthranilate and chorismate.[46b] Recent studies, however, went beyond the native biosynthetic pathways and produced derivatives of pathway intermediates including phenol,[25c] *p*-hydroxycinnamic acid, phenyllactic acid, phenylacetic acid, phenylethanol,[154] and the complex tryptophan-based derivatives violacein and deoxyviolacein, with the latter two being promising compounds for antiviral and antitumor treatment.[7c,d,25ac] Systems-wide pathway design and engineering combined with optimized process operation thereby surpassed the gram scale and produced 1.6 $g L^{-1}$ deoxyviolacein (Figure 3).[7c] As a consequence of its naturally high tolerance of yeast, *Saccharomyces cerevisiae* is also considered an interesting candidate for the production of aromatic compounds.[155] Pathway regulation is, however, even tighter than for *E. coli* and *C. glutamicum*. The better acceptance and tolerance of heterologous genes from plants, however, makes yeast an interesting candidate for the production of aromatic compounds. Promising examples comprise the production of resveratrol, naringenin, and other flavonoids derived from coumaric acid (Figure 4).[30h,156] A better understanding of the metabolism will further assist in developing strategies for improved de novo production.[157]

7.4. L-Threonine

Most engineering effort concerning the production of L-threonine has been done with *E. coli*, which is applied industrially for the annual production of around 250 000 tons. Initial studies used overexpression of the biosynthetic threonine pathway to improve the production of a basic threonine-producing mutant.[158] More recently, a genetically defined threonine producer was constructed by a synthetic biology approach based on systems-wide profiling by integrating data from genome, transcriptome, proteome, and in silico flux response analysis.[25ad] Very recently, uncoupling biosynthesis from feedback inhibition, and transcriptional attenuation in parallel to plasmid-based overexpression of the threonine pathway led to competing pathways being down-regulated, re-uptake of secreted threonine eliminated, anaplerotic carboxylation optimized, and the glyoxylate shunt regulator *iclR* deleted.[25ad] The final strain produced 82 $g L^{-1}$ L-threonine in a yield of 0.39 $g g^{-1}$ (Figure 3).

7.5. Branched-Chain Amino Acids and Related Compounds

L-Valine, L-leucine, and L-isoleucine have a small but growing market as compounds in the pharmaceutical and agricultural industry. Improved *C. glutamicum* strains were mainly obtained by pathway deregulation, overexpression of terminal biosynthetic reactions, elimination of competing pathways, and improved supply of NADPH.[20f,k,s,159] This yielded strains with a remarkable production of up to 150 $g L^{-1}$ L-valine, 24 $g L^{-1}$ L-leucine, and 21 $g L^{-1}$ L-isoleucine (Figure 2). Recent achievements for *C. glutamicum* thereby exceeded the production performance of previously generated *E. coli* strains for L-valine and L-isoleucine (Figure 3).[25k,l,v] Related compounds of the branched chain amino acids have since attracted attention as therapeutic agents and aroma compounds. For example, pyrazines, naturally secreted by *C. glutamicum* and found in the headspace of a shaken flask, contribute to the aroma and flavor of coffee, pepper, and popcorn.[15] Further related metabolites which are interesting chemicals for pharmaceutical synthesis or feed additives are 2-oxoisovalerate,[20r] 2-oxoisocaproate,[20t] and the vitamin pantothenic acid.[14,25n,160] The expertise from pathway engineering towards L-valine, L-leucine, and L-isoleucine production might help to generate advanced producers thereof.

7.6. Other Amino Acids

Apart from the above-described amino acids, major targeted genetic engineering focused on *C. glutamicum*, more exactly on the C_3 amino acids L-alanine[20o,161] and L-serine.[20p] For L-arginine, however, a comprehensive strategy of random mutagenesis and metabolic engineering has recently proven to be highly beneficial. The generated producer strain allowed production of 92.5 $g L^{-1}$ in a 0.40 $g g^{-1}$ yield.[20y] Furthermore, *C. glutamicum* was further engineered for the production of a diverse set of nonpro-

teinogenic amino acids comprising pharmaceutically interesting D enantiomers,[13d] D/L-ornithine,[5,20h,z,162] γ-aminobutyric acid,[163] 4-hydroxyproline,[20e] and L-citrulline (Figure 2).[13b] For *E. coli*, basic producers for L-alanine, L-serine, and 4-hydroxyproline are described (Figure 3).[25o]

7.7. Riboflavin

Riboflavin is an essential vitamin, required to synthesize flavin mono- and dinucleotide, which function as cofactors for dehydrogenases and oxidoreductases.[40,43] Riboflavin deficiency in human and animal nutrition might cause dermatitis, diarrhea, and retarded growth. In addition, riboflavin is used as the food-coloring agent E-101.[40] Its production history nicely illustrates the race between chemistry and biotechnology for the development of a cost-efficient process.[40] Today, three microorganisms are used for bio-based production, namely *Bacillus subtilis*, *Ashbya gossypii*, and *Candida famanta*.[40] Industrial producers are in general the outcome of combined random mutagenesis and tailored metabolic engineering and achieve product titers of 15–20 $g\,L^{-1}$.[164] In *A. gossypii*, overexpression of riboflavin biosynthetic genes[165] and an improved supply of the precursor glycine by amplified expression of glycine-forming reactions[166] and elimination of glycine-consuming reactions[167] was found beneficial for production. Recently, disparity mutagenesis provided a strain that produced 14 $g\,L^{-1}$ riboflavin, thereby remarkably approaching the aforementioned titers of industrial producers.[44] Similarly, the expression efficiency and enzyme activity of the terminal riboflavin pathway, encoded by the *rib* operon, is of major importance for production in *B. subtilis*.[168] An improved energetic state of the cell and reduced cellular maintenance additionally supports production.[169] Modulation of the precursor metabolism through increased flux by purine biosynthesis,[92d] the pentose phosphate pathway,[170] and deregulated gluconeogenesis[171] further influenced riboflavin production.[25af] Ongoing research promises strains with even better performance.[172] Only recently, *E. coli* was engineered for the production of riboflavin. The metabolic design was closely related to that of *B. subtilis* and involved overexpression of biosynthesis and redirection of the flux towards the pentose phosphate pathway. The additional reduction of the acetate secretion and optimized fermenter operations enabled the production of 2.7 $g\,L^{-1}$ riboflavin.[25af]

7.8. Ectoine

The positive and stabilizing effect of the chemical chaperons ectoine and hydroxyectoine on biopolymers has stimulated their large-scale production for the pharmaceutical and wellness market.[7b] Currently, the annual production is carried out on a ton scale by a "bacterial milking" process, involving iterative operation cycles at high and low salinity, with the halophilic proteobacterium and native ectoine producer *Halomonas elangata*.[173] Attempts have, however, been made with heterologous production processes. In *E. coli*, intracellular accumulation of 57 $mg\,g_{CDM}^{-1}$ ectoine was achieved, but necessitated high salinity.[25w] Targeted synthetic reconstruction of the *Pseudomonas stutzeri* ectoine gene cluster recently also enabled the production of ectoine with *C. glutamicum*, thereby achieving reasonable titer (4.5 $g\,L^{-1}$), yield (0.24 $g\,g^{-1}$), and productivity (6.7 $g\,L^{-1}$ per day).[7b] Most favorably, this process is uncoupled from the harsh high-salinity conditions of the "bacterial milking" process, thus reducing corrosion and investment costs.

7.9. Citric Acid

The broad application field of citrate ranges from the food industry and housing to technical and medical applications. Its production by fermentation—which has now reached a scale of 1.5 million tons per year—was established in the 1930s with *Aspergillus niger*, which until now remains the major industrial producer.[31a,32] Overproduction requires a unique combination of nutrients and fermentation conditions. The finely tuned combination of excessive substrate concentrations, hydrogen ions, and dissolved oxygen, with suboptimal concentrations of specific trace metals and phosphate, are needed to synergistically influence the yield of citric acid.[32] Most processes recruit submerse fermentation, thereby reaching titers of 110–140 $g\,L^{-1}$ with yields of 70–90 %.[31a] The major microbial platform organisms were so far not in the focus as alternative production hosts.

7.10. Terpenoids

As a result of their red or yellow pigmentation, carotenoids have received considerable interest as coloring agents.[174] Moreover, some are competent quenching agents for oxygen radicals and UV radiation and thus said to detoxify oxidative stress and minimize cancer risks and cardiovascular diseases.[175] Their production, mainly based on organic extraction from natural plant sources, is however elaborate. Attempts towards microbial production have been mainly carried out by heterologous engineering of noncarotenogenic *E. coli* and *S. cerevisiae*,[174] which yield producers for β-carotene,[25t,176] lycopene,[30n,177] astaxanthin,[30l,178] and zeaxanthin.[30p,179] Although *C. glutamicum* naturally possesses a biosynthetic gene cluster for carotenoids, engineering towards overproduction has only started recently.[20i]

In addition to use as pigmentation, terpenoids are also interesting compounds for the pharmaceutical industry. Artemisinin, a sesquiterpene lactone isolated from the plant *Artemisia annua*, is recommended by the World Health Organization for treatment of malaria.[28] Price and availability, however, greatly fluctuate, thus favoring recombinant production, which promises to be more stable and controllable. Hereby, hetereologous expression of the mevalonate pathway in *E. coli* enabled fermentative production of the artemisinin precursor amorpha-4,11-diene.[24b] An optimized gene expression by the appropriate combination of genes from the donor organisms *S. cerevisiae* and *Staphylococcus aureus* led to improved process operation and allowed the

production of 27 g_L^{-1} amorpha-4,11-diene.[24b] This can be used in a subsequent chemical process to produce artemisinin. A similar semisynthetic approach for artemisinin production was established for *S. cerevisiae*.[180] The obtained fermentation product artemisinic acid, the precursor for chemical conversion into artemisinin, accumulated in up to 25 g_L^{-1}.[181]

The diterpenoid paclitaxel—commercialized as taxol—from yew tree bark is a potent cytostatic drug for cancer treatment.[182] Current semisynthetic production combines cell culture based Baccatin III production with subsequent chemical conversion. Both *S. cerevisiae* and *E. coli* have been used to generate early stage intermediates of the biosynthetic paclitaxel pathway.[8a,c,24a,182,183] Production now surpasses the gram scale, thus encouraging further engineering for process optimization.[8a]

The strategies for terpenoid production nicely illustrate how synthetic biology and synthetic chemistry might complement one another to establish alternative routes for bio-based production processes.

8. Concluding Remarks and Outlook

Driven by systems and synthetic metabolic engineering, the product portfolio of microbial industrial producers has grown substantially beyond their natural boundaries. Engineered cell factories provide novel and alternative routes for the synthesis of materials, fuels, commodity chemical compounds, and high-value specialty chemicals, food ingredients and therapeutics. This opens promising possibilities for the future sustainable use of renewable biomass to complement and partly replace petrochemistry of fossil fuels. The recently demonstrated novel bio-based nylon, largely derived with a metabolically engineered cell factory, sets a milestone in terms of the technological feasibility of bio-based routes.[20a] Admittedly, not all of the products mentioned here have reached the market place, although they provide valuable proof-of-concepts and need more attention. In this regard, recent years have shown that the sophisticated tools of systems biology analysis, modeling studies, and genetic engineering are most powerful when they are intricately integrated. The same holds for the interactive combination of experiment and model, which can create synergies for faster and more tailored progress in metabolic engineering. In addition, better integration of metabolic engineering with the upstream and downstream parts of the later value chain is something that seems important, as large-scale production processes have their own limitations. For example, limited mixing, high osmotic pressure, fluctuating concentrations and process parameters, and variation in raw material composition typically occur in the operational windows on a large scale and often cause stress for the producing cells. Although challenging, an early consideration of such aspects in cellular engineering might substantially improve the chance not to get lost in translation during later process development.[184] The combination of evolutive adaptation and reverse metabolic engineering appears straightforward to identify genetic traits that contribute to more tolerant and robust producers.

Taken together, we can expect the next level of strains to be generated upon integration of the present and newly emerging strategies into systems and synthetic metabolic engineering approaches. The growing toolbox for metabolic engineers will further speed up development times and extend product portfolios and increase the heartbeat and the outreach of industrial strain development.

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