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Bio-based production of monomers and polymers by metabolically engineered microorganisms

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Recent metabolic engineering strategies for bio-based production of monomers and polymers are reviewed. In the case of monomers, we describe strategies for producing polyamide precursors, namely diamines (putrescine, cadaverine, 1,6-diaminohexane), dicarboxylic acids (succinic, glutaric, adipic, and sebacic acids), and ω-amino acids (γ-aminobutyric, 5-aminovaleric, and 6-aminocaproic acids). Also, strategies for producing diols (monoethylene glycol, 1,3-propanediol, and 1,4-butanediol) and hydroxy acids (3-hydroxypropionic and 4-hydroxybutyric acids) used for polyesters are reviewed. Furthermore, we review strategies for producing aromatic monomers, including styrene, *p*-hydroxystyrene, *p*-hydroxybenzoic acid, and phenol, and propose pathways to aromatic polyurethane precursors. Finally, *in vivo* production of polyhydroxyalkanoates and recombinant structural proteins having interesting applications are showcased.

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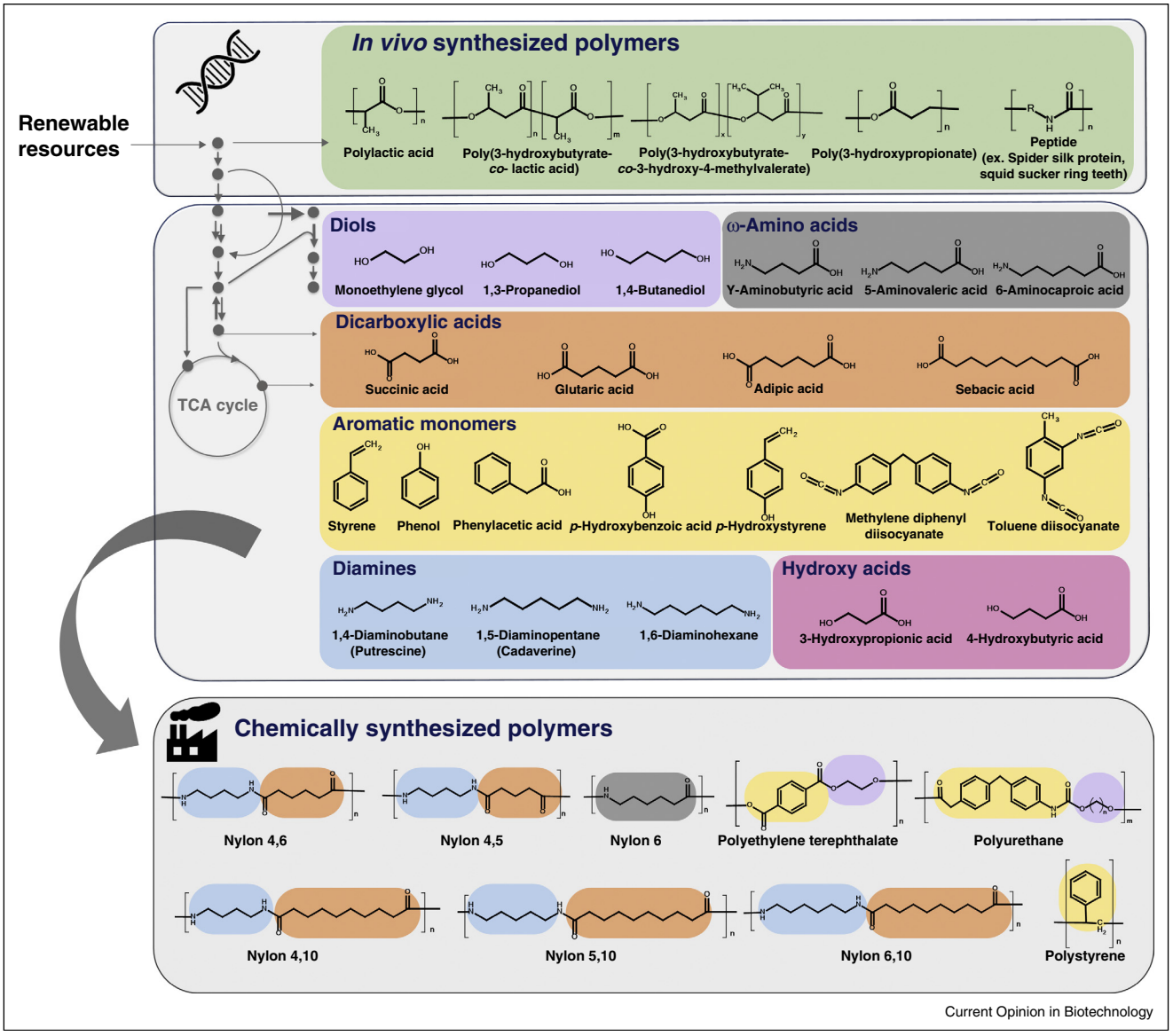
Introduction

Plastics are polymeric materials widely used in our daily lives in the forms of various goods, including electronics, coffee cups, clothes, and adhesives. Furthermore, advanced engineering plastic materials find uses as structural components in many applications, due to their remarkable strength, low weight, and ease of manufacture. However, the monomers required for synthesis of these polymers have traditionally been derived from non-renewable petroleum. According to European Plastics, more than 280 million metric ton of plastics were produced from non-renewable resources in 2012 [1]. Due to concerns over the impact of petroleum utilization on climate change, a switch to renewable alternatives is highly desirable. One such alternative is the conversion of renewable biomass into chemicals of interest in so-called biorefineries, analogous to the traditional petroleum-refinery process. In these, metabolically engineered microorganisms convert renewable starting materials into valuable products, through redirecting their metabolic fluxes toward the desired compounds. Development of diverse microbial cell factories for biorefineries has been thriving, supported by advances in metabolic engineering, that together with synthetic biology tools have enabled the construction of highly efficient microbial cell factories for conversion of renewable resources to industrially valuable chemicals. This is indicated by the growing number of bio-based chemicals produced in commercial scale [2].

In this review, recent metabolic engineering strategies applied to microbial production of important plastic monomers are discussed. Focus is given on the production of polyamide (PA, also known as nylon) precursors, including diamines (putrescine, cadaverine, 1,6-diaminohexane), dicarboxylic acids (succinic acid, glutaric acid, adipic acid, and sebacic acid), and ω-amino acids (γ-aminobutyric acid, GABA; 5-aminovaleric acid, 5AVA; and 6-aminocaproic acid, 6ACA). Furthermore, we discuss the production of diols (1,3-propanediol, 1,3-PDO; 1,4-butanediol, 1,4-BDO; and monoethylene glycol, MEG) and hydroxy acids (3-hydroxypropionic acid, 3-HP; and 4-hydroxybutyric acid, 4-HB), which are used as monomers in polyester synthesis. We also review the production of aromatic monomers (styrene, *p*-hydroxystyrene, *p*-hydroxybenzoic acid, and phenol), and precursors for the important aromatic diisocyanates toluene diisocyanate (TDI) and methylene diphenyl diisocyanate (MDI). In addition, advances in direct *in vivo* polymer synthesis, exemplified by polyhydroxyalkanoates (PHAs), and structural proteins (spider silk protein, squid sucker ring teeth protein, elastin, and mussel adhesive protein) produced directly from renewable resources are highlighted. These products are summarized in Figure 1, and their respective production pathways are shown in Figure 2. Additionally, the legend for Figure 2 lists a key to all gene abbreviations used throughout the text.

Figure 1



Microbial cell factory for the production of various monomers (diamines, dicarboxylic acids, hydroxy acids, ω-amino acids, diols, and aromatic chemicals) and *in vivo* synthesized polymers (PHAs and recombinant proteins). Nylon, polyethylene terephthalate, polyurethane, and polystyrene monomers are produced through biological routes, then chemically synthesized (gray arrow) into polymers. The colored circular box of each polymer represents specific functional group(s): purple for diols, gray for ω-amino acids, orange for dicarboxylic acids, yellow for aromatic monomers, blue for diamines, and pink for hydroxy acids.

Production of aliphatic monomers

In this section, recent works describing metabolic engineering strategies for production of aliphatic monomers important for the synthesis of PA's and polyesters are discussed. These have been divided into diamines, dicarboxylic acids, hydroxy acids, ω-amino acids, and diols, as shown in Figure 1. Together, these diverse monomers constitute a basis for renewable production of a variety of important PA's (PA-4,6; PA-4,10; PA-5; PA-5,10; PA-6; PA-6,4; PA-6,5; PA-6,6; and PA-6,10), polyesters, and polyurethanes.

Diamines

Diamines are mainly used for PA production, and the most important diamines that are being investigated for microbial production are putrescine (1,4-diaminobutane), cadaverine (1,5-diaminopentane), and 1,6-hexanediamine. Putrescine and cadaverine are both well-known microbial products that have been produced through metabolic engineering. Companies, including BASF (Ludwigshafen, Germany), CJ CheilJedang (Seoul, Korea), and DSM (Heerlen, Netherlands), have been actively involved in research and development for microbial production of

these diamines that are useful for PA synthesis. For example, polymerization of putrescine with adipic acid, putrescine with sebacic acid, cadaverine with succinic acid, and cadaverine with sebacic acid yields PA-4,6; PA-4,10; PA-5,4; and PA-5,10; respectively. Furthermore, cadaverine has also been highlighted as an important industrial platform chemical with a variety of applications [3,4].

Microbial production of putrescine uses either the ornithine decarboxylase pathway or the arginine decarboxylase pathway. The highest putrescine titer of 24.2 g/L reported in literature was achieved using engineered *Escherichia coli* (Figure 2, gray highlight) [5]. This engineered strain was developed by overexpression of *speC* (encoding ornithine decarboxylase) as well as arginine biosynthetic genes (encoded by *argECBH*), deletion of putrescine degradation genes (encoded by *speE*, *speG*, *argI*, and *puuA*), deletion of *puuP* (encoding putrescine importer) and deletion of *rpoS* (encoding the global stress response regulator σ38). In addition, *Corynebacterium glutamicum* has been explored as a production organism for putrescine. Recently, modifying the promoter strength, translation start codon, and ribosome binding site of *argF* (encoding ornithine transcarbamoylase) from a previously developed strain resulted in production of 19 g/L of putrescine with productivity of 0.55 g/L/h [6], and it has been known that close to 100 g/L of putrescine is being produced in the industry. Furthermore, ornithine (the metabolic precursor for putrescine) was also produced recently using engineered *C. glutamicum* [7]. The titer and productivity of ornithine was 51.5 g/L and 1.3 g/L/h, respectively, demonstrating the potential of this host for the production of putrescine and ornithine.

Cadaverine production has been achieved using the L-lysine biosynthetic pathway with L-lysine decarboxylase. Production of cadaverine was also reported by employing engineered *E. coli*, with a cadaverine titer of 9.6 g/L (Figure 2, light purple highlight) [8]. This strain was designed by overexpression of biosynthetic genes (encoded by *cadA* and *dapA*) and deletion of genes responsible for cadaverine degradation (encoded by *speE*, *speG*, *ygjG*, and *puuPA*). Based on the engineered strain, recently developed synthetic sRNA technology was applied to repress *murE* (encoding UDP-*N*-acetylmuramoylalanyl-D-glutamate 2,6-diaminopimelate ligase), resulting in production of 12.6 g/L of cadaverine [9]. As an obvious alternative approach, *C. glutamicum* has also been explored for cadaverine production, considering the strain's capability to produce L-lysine to a very high level. For example, integration of *ldcC* (encoding lysine decarboxylase) into the *bioD* locus, deletion of *lysE* (encoding the L-lysine exporter) and overexpression of *cg2893* (encoding the major facilitator permease), resulted in production of cadaverine to a titer of 88 g/L in an L-lysine producing *C. glutamicum* strain [10••]. Through optimization of the downstream extraction process, 99% pure cadaverine was obtained, and subsequently polymerized *in vitro* with sebacic acid to synthesize PA-5,10 [10••]. This was the first report that showed integration of the biological production of a diamine and its chemical polymerization to PA, paving the way toward bio-based PA production. Such bio-based PA's are increasingly used in industry and recent examples include the use of Cathay Industrial Biotech (Shanghai, China)'s Terryl, DuPont (Wilmington, Delaware)'s Zytel, and DSM's EcoPaXX (PA-4,10) for manufacturing of automotive parts for Ferrari (Maranello, Italy) and Mercedes Benz (Stuttgart, Germany), and also in electric vehicles such as those developed by SIM-Drive (Tokyo, Japan).

1,6-Diaminohexane is the diamine with highest market demand since it is used as monomer for PA-6,6 and PA-6,10, two of the most widely used PA's. However, development of engineered microbes for 1,6-diaminohexane production has not yet been demonstrated. The main bottleneck for renewable production of 1,6-diaminohexane is the lack of a naturally occurring metabolic pathway. To overcome this problem, a number of non-natural pathways have been designed by several companies, including Genomatica (San Diego, California) [11]. However, the validation of these pathways and metabolic engineering strategies remains in order to achieve commercial production of bio-based 1,6-diaminohexane.

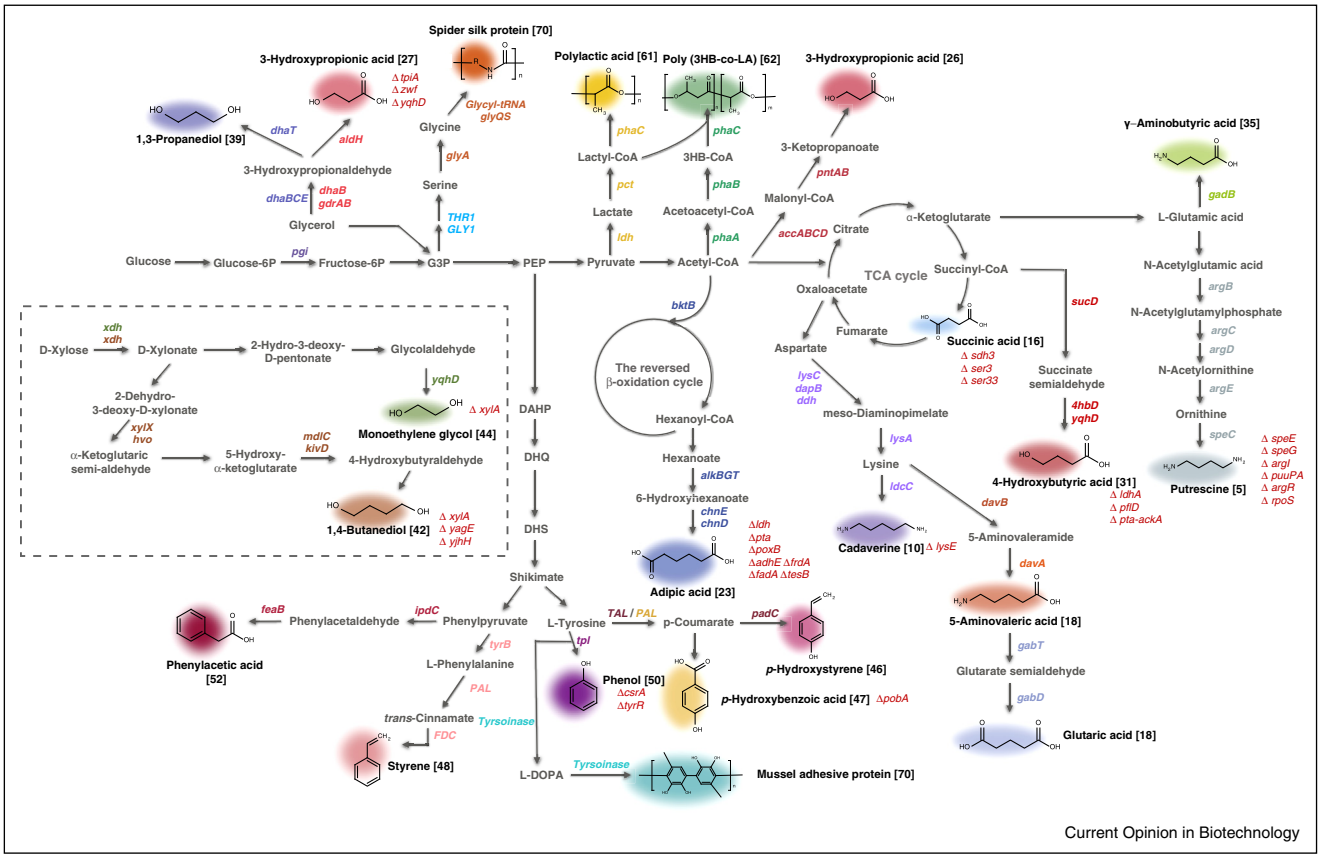
Dicarboxylic acids

Dicarboxylic acids are important chemicals that can be used for production of polyesters, polyurethanes, and PA's. Here, we describe the recent progress in production of succinic acid, glutaric acid, adipic acid, and sebacic acid.

Succinic acid has been a popular metabolic engineering target, as it was identified as one of the top 12 renewable platform chemicals by the US Department of Energy (DOE) in 2004 [12]. Traditionally, succinic acid has been produced by chemical conversion from petroleum feedstock, including hydrogenation of maleic acid, oxidation of 1,4-butanediol, and carbonylation of MEG. A number of microorganisms that naturally produce succinic acid are known, including *Actinobacillus succinogenes* [13] and *Mannheimia succiniciproducens* [14], both of which have been metabolically engineered to produce succinic acid with very high productivity and yield. In addition, an engineered *C. glutamicum* strain, with deletion of *ldhA* (encoding lactate dehydrogenase) and overexpression of *pyc* (encoding pyruvate carboxylase), was able to produce 146 g/L of succinic acid [15], which is the highest titer reported in literature so far. Due to the advanced state of succinic acid producing microorganisms, several companies have now commercialized bio-based production of succinic acid [2].

More recently, a succinic acid producing *Saccharomyces cerevisiae* strain was developed by a combination of *in silico*

Figure 2



Metabolic pathways for the microbial production of various monomers and polymers. Metabolic pathways for the biosynthesis of chemicals have been established in various microorganisms. Each chemical is highlighted in the same color as the genes that have been overexpressed to form its production pathway. In addition, reported gene deletions are listed in red next to their corresponding chemical. The following gene abbreviations have been used, listed in alphabetical order: *accABCD*, acetyl-CoA decarboxylase; *ackA*, acetate kinase; *adhE*, aldehyde-alcohol dehydrogenase; *aldH*, aldehyde dehydrogenase; *alkBGT*, alkane monooxygenase operon; *argB*, acetylglutamate kinase; *argC*, N-acetyl-gamma-glutamyl-phosphate reductase; *argD*, acetylornithine transaminase; *argF*, ornithine carbamoyltransferase chain F-monomer; *argI*, ornithine carbamoyltransferase chain I-monomer; *argR*, arginine repressor; *bktB*, acetyl-CoA C-acetyltransferase; *chnD*, 6-hydroxyhexanoate dehydrogenase; *chnE*, 6-oxohexanoate dehydrogenase; *csrA*, carbon storage regulator; *dapB*, 4-hydroxy-tetrahydrodipicolinate reductase; *davA*, delta-aminovaleramidase; *davB*, L-lysine 2-monooxygenase; *ddh*, meso-diaminopimelate D-dehydrogenase; *dhaB*, glycerol dehydratase; *dhaT*, 1,3-PDO oxidoreductase; *fadA*, acetyl-CoA acetyltransferase; *FDC*, ferulic acid decarboxylase; *feaB*, phenylacetaldehyde dehydrogenase; *4hbD*, NADH-dependent 4-hydroxybutyrate dehydrogenase; *frdA*, fumarate reductase; *gabD*, glutarate-semialdehyde dehydrogenase; *gabT*, 5-AVA/GABA aminotransferase; *gadB*, glutamate decarboxylase; *gdrAB*, glycerol dehydratase reactivase; *glyA*, serine hydroxymethyltransferase; *GLY1*, L-threonine aldolase; *glyQS*, glycine-tRNA ligase; *hvo*, 2-dehydro-3-deoxy-D-xylonate dehydratase; *kivD*, keto-acid decarboxylase; *ipdC*, indolepyruvate decarboxylase/phenylpyruvate decarboxylase; *ldcC*, L-lysine decarboxylase; *lysC*, L-lysine-sensitive aspartokinase; *ldh*, L-lactate dehydrogenase; *ldhA*, D-lactate dehydrogenase; *lysA*, diaminopimelate decarboxylase; *lysE*, L-lysine exporter; *mdlC*, keto-acid decarboxylase; *padC*, phenolic acid decarboxylase; *PAL*, L-phenylalanine ammonia-lyase; *pct*, propionyl-CoA transferase; *pflD*, formate acetyltransferase 2; *pgi*, glucose-6-phosphate isomerase; *phaA*, 3-ketothiolase; *phaB*, acetoacetyl-CoA reductase; *phaC*, polyhydroxyalkanoate synthase; *pntAB*, nicotinamide nucleotide transhydrogenase; *pobA*, *p*-hydroxybenzoate hydroxylase; *poxB*, pyruvate dehydrogenase; *pta*, phosphotransacetylase; *puuA*, glutamate-putrescine ligase; *puuP*, putrescine importer; *rpoS*, RNA polymerase sigma factor RpoS; *sdh3*, succinate dehydrogenase complex subunit; *ser3*/*ser33*, 3-phosphoglycerate dehydrogenase (isozymes); *speC*, ornithine decarboxylase; *speE*, spermidine synthase; *speG*, spermidine N-acetyltransferase; *sucD*, succinate-semialdehyde dehydrogenase; *TAL*, tyrosine ammonia-lyase; *tesB*, acyl-CoA thioesterase; *THR1*, homoserine kinase; *tkt*, transketolase; *tpiA*, triosephosphate isomerase; *tpl*, tyrosine phenol-lyase; *tyrR*, transcriptional regulatory protein involved in transcriptional regulation of aromatic amino acid biosynthesis and transport; *xdh*, D-xylose dehyrdogenase; *xylA*, xylose isomerase; *xylX*, 2-dehydro-3-deoxy-D-xylonate dehydratase; *yagE* and *yjhH*, 2-dehydro-3-deoxy-D-xylonate aldolases; *yqhD*, alcohol dehydrogenase; *zwf*, glucose-6-phosphate-1-dehydrogenase. In addition, the following gene abbreviations appear in the manuscript but are not included in this figure: *adh*/*adhE*, alcohol dehydrogenase; *argE*, acetylornithine deacetylase; *bioD*, dethiobiotin synthetase; *BTE*, acyl-ACP thioesterase; *buk1*, butyryl kinase; *cadA*, L-lysine decarboxylase; *cg2893*, major facilitator permease; *crt*, enoyl-CoA hydratase; *dapA*, dihydrodipicolinate synthase; *dhaBX*, dehydratase reactivation factor; *dte*, D-tagatose 3-epimerase; *ech*, enoyl-CoA hydratase; *fadAx*, acetyl-CoA acetyltransferase; *fadB*/*fadB2x*, 3-hydroxyacyl-CoA dehydrogenase; *fucA*, D-ribulose-phosphate aldolase; *fucK*, D-ribulokinase; *fucO*, glycolaldehyde reductase, *gltA*, citrate synthase; *gpd1*, glycerol-3-phosphate dehydrogenase; *gpp2*, glycerol-3-P phosphatase; *hadA*, 2-hydroxy-4-methylvalerate-CoA transferase; *hadBC*, 2-hydroxy-4-methylvalerate-CoA dehydratase; *hadI*, activator of 2-hydroxy-4-methylvalerate-CoA dehydratase; *hbd*, 3-hydroxybutyryl-CoA dehydrogenase; *iclR*, acetate operon repressor; *lpdA*, dihydrolipoyl dehydrogenase; *mdh*, malate dehydrogenase; *murE*, UDP-N-acetylmuramoylalanyl-D-glutamate

modeling-assisted gene deletion, identifying *sdh3* (encoding the cytochrome b subunit of the succinate dehydrogenase complex) and *ser3*/*ser33* (encoding 3-phosphoglycerate dehydrogenase isozymes) as targets. A second round of gene deletions, guided by transcriptome analysis, resulted in a strain with 30-fold improved succinic acid production compared to the wild-type, nicely demonstrating the power of integrating *in silico* modeling and systems biology tools in metabolic engineering efforts (Figure 2, light blue highlight) [16•].

Glutaric acid, the five carbon dicarboxylic acid, can be chemically produced by the ring-opening of butyrolactone. In addition, there are two reports utilizing the L-lysine catabolic pathway for the production of glutaric acid in *E. coli* [17,18]. The first demonstration of glutaric acid production in *E. coli* was coupled with the production of 5AVA [18]. The *gabT* (encoding 5AVA aminotransferase) and *gabD* (encoding glutarate semialdehyde dehydrogenase) genes were used to convert 5AVA into glutaric acid. The resulting strain produced 1.7 g/L of glutaric acid from 10 g/L of L-lysine with supplementation of α-ketoglutarate (Figure 2, light gray highlight) [18].

Adipic acid is primarily used as the precursor of PA-6,6, and it is one of the most important bulk chemicals, with a production volume of 2.6 million tons [19]. Currently, it is produced from a mixture of cyclohexanol and cyclohexanone, which is oxidized with nitric acid and further processed into adipic acid via multiple processes. Due to its importance and large production demand, microbial production of this compound has gained much interest. For instance leading biotech-companies, including DSM, Genomatica and Verdezyne (Carlsbad, California), are aiming for its large-scale production [20,21]. The first adipic acid production from glucose in *E. coli* was demonstrated through the synthesis of 3-keto-adipyl-CoA by amplifying *paaJ* (encoding β-ketoadipyl-CoA thiolase), which resulted in condensation of acetyl-CoA and succinyl-CoA. This was subsequently converted to adipyl-CoA through reduction of 3-keto-adipyl-CoA (by *hbd* encoding 3-hydroxybutyryl-CoA dehydrogenase or *paaH1* encoding 3-hydroxy-acyl-CoA reductase), dehydration of 3-hydroxyadipyl-CoA by *crt* (encoding crotonase) or *ech* (encoding putative enoyl-CoA hydratase) and reduction of 2,3-dehydroadipyl-CoA by *ter* (encoding *trans*-enoyl-CoA reductase). Finally, overexpression of *ptb* (encoding phosphate butyryltransferase) and *buk1* (encoding butyryl kinase) resulted in the removal of CoA from adipyl-CoA. This, together with knocking out *ptsG* (encoding the glucose-specific phosphotransferase system transporter), *poxB* (encoding pyruvate dehydrogenase), *pta* (encoding phosphotransacetylase), *sdhA* (encoding succinate dehydrogenase subunit), and *iclR* (encoding the isocitrate lyase regulator) to increase the succinyl-CoA pool, resulted in production of 639 μg/L of adipic acid [22].

In another recent report, glycerol was used to produce dicarboxylic acids through reversed β-oxidation by amplification of *bktB* (encoding acetyl-CoA C-acetyltransferase) and *ydiL* (encoding thioesterase), which were then converted into ω-hydroxy acids by amplification of the *alkBGT* operon (encoding the alkane monooxygenase complex). Two additional genes, *chnD* and *chnE* (encoding 6-hydroxyhexanoate dehydrogenase and 6-oxohexanoate dehydrogenase, respectively), were introduced into *E. coli*, and the final strain produced medium chain dicarboxylic acids, including adipic acid (170 mg/L) (Figure 2, blue highlight) [23••]. These results are promising for the future production of renewable adipic acid from simple carbohydrates. While Genomatica has applied for a proof-of-concept patent using a reversed adipic acid degradation pathway [20], Verdezyne is currently the only company that has actually produced adipic acid from renewable biomass at pilot scale, with their patent describing engineering of the β-oxidation and ω-oxidation pathways to produce 0.4 g/L adipic acid from vegetable oil using *Candida* sp. [21]. Although the concentration was rather low, the percentage of adipic acid produced was 98% of the produced diacids, suggesting the potential of this process for efficient adipic acid production when further improved.

Finally, the ten-carbon dicarboxylic acid, sebacic acid, has been receiving much industrial attention as it is an important monomer used for production of biobased PA's, such as DuPont's Zytel, and DSM's EcoPaXX. Sebacic acid is currently produced by chemical reaction from renewable castor oil. Recently, engineering strategies for enhanced production of sebacic acid using *C. tropicalis* have been proposed by Verdezyne [24]. In their pathway, long chain alkanes were converted to sebacic acid by engineered *C. tropicalis*, with a partially blocked β-oxidation pathway. As a result, 0.94 g/L of sebacic acid was produced from decane [24]. This route currently relies on petroleum-based hydrocarbons, but since short-chain alkane (such as nonane and dodecane) production from glucose was recently reported using metabolically engineered *E. coli* [25], a direct production route to sebacic acid from carbohydrates can be envisioned by combining these two pathways. In another recent work, *E. coli* was

(Figure 2 Legend Continued) 2,6-diaminopimelate ligase; *orfX*, dehydratase reactivation factor; *paaH1*, 3-hydroxyacyl-CoA dehydrogenase; *paaJ*, 3-oxoadipyl-CoA thiolase; *pduP*, propionaldehyde dehydrogenase; *pfl*, pyruvate-formate-lyase; *phaG*, 3-hydroxyacyl-CoA-acyl carrier protein transferase; *phaJ*/*phaJ3*, (R)-specific 2-enoyl-CoA hydratase; *pheA*: chorismate mutase/prephenate dehydratase; *PP2047*, 3-hydroxyacyl-CoA dehydrogenase; *PP2048*, acyl-CoA dehydrogenase; *ptb*, phosphate butyryltransferase; *ptsG*, phosphotransferase system (PTS) glucose-specific transporter; *pyc*; pyruvate carboxylase; *sdhA*, succinate dehydrogenase subunit; *ter*, *trans*-enoyl-CoA reductase; *tyrA*, chorismate mutase/prephenate dehydrogenase; *ydiL*, thioesterase; *ygjG*, putrescine/cadaverine aminotransferase.

engineered to produce mixed dicarboxylic acids from glycerol, including sebacic acid as one of the products [23••]. If the specificity of this pathway is improved by enzyme engineering, it could provide a second direct route to sebacic acid.

Hydroxy acids

3-HP, a three carbon hydroxy acid, can be used for production of biocompatible and biodegradable polyesters, making it popular for applications in the medical and industrial fields. In addition, it can be used to make acrylic acid, methacrylic acid, and acrylamide. Cargill (Minnetonka, Minnesota) has proposed seven possible biological pathways to produce 3-HP. Among them, Pathway II (using malonyl-CoA reductase) and Pathway III (through 3-oxopropanoate) enabled production of 3-HP, where Pathway II produced 49.0 g/L of 3-HP [26]. In another study, modifying the central metabolism of *E. coli* by overexpression of *dhaB* (encoding glycerol dehydratase) and *gdrAB* (encoding glycerol dehydratase reactivase) allowed dehydration of glycerol to 3-hydroxypropionaldehyde that, in combination with knocking out *tpiA* (encoding triosephosphate isomerase), *zwf* (encoding glucose-6-phosphate-1-dehydrogenase), and *yqhD* (encoding alcohol dehydrogenase), resulted in production of 4.3 g/L of 3-HP (Figure 2, pink highlight) [27]. Furthermore, dual synthetic pathways consisting of the ALDH and Pdu pathways were introduced into *E. coli*, which resulted in production of 5.1 g/L of 3-HP [28].

4-HB is a naturally synthesized metabolite in human as well as most animals. It is used as general anesthetic product, however, illegal in most western countries including Europe and the USA. Recently, it has been recognized as an important intermediate chemical in production of various C4 chemicals, such as 1,4-BDO [29]. It is also used for biosynthesis of various polyesters due to its biocompatibility and use as medical implant material [30]. Recently, engineered *M. succiniciproducens* was used to produce 4-HB through fermentation [31]. The succinic acid overproducing strain equipped with succinyl-CoA synthetase (encoded by *sucCD*), CoA-dependent succinate semialdehyde dehydrogenase (encoded by *sucD*), and either 4-hydroxybutyrate dehydrogenase (encoded by *4hbD*) or succinate semialdehyde reductase (encoded by *yqhD*) was able to produce 6.4 and 6.3 g/L of 4-HB, respectively (Figure 2, red highlight).

ω-Amino acids

ω-Amino acids are important chemicals for synthesis of polyamides, a well-known example being synthesis of PA-6 (for instance, DuPont's Zytel) using 6ACA. This non-natural ω-amino acid is also widely used as an industrially important chemical for automotive application, textile, bearings, firearms and gears. Alternative production of 6ACA can be performed by chemical modification of L-lysine, a well-established fermentation product with an annual production exceeding 3 million tons [32]. In addition, direct production of 6ACA from biomass is under development by DSM. In their proposed pathway, 6ACA is produced via 5-formylvaleric acid, by introduction of homocitrate synthase, 3-isopropylmalate dehydratase, isopropylmalate/isohomocitrate dehydrogenase and branched-chain α-ketoacid decarboxylase from several different species including methanogens [33].

Other ω-amino acids under production by engineered microbes are GABA and 5AVA. GABA can be used for production of 2-pyrrolidone and subsequently PA-4, which has excellent mechanical and thermal properties for use as an engineering material, as well as being biocompatible and biodegradable [34]. Production of GABA has mainly been studied using *C. glutamicum* strains, due to their capability of efficiently producing the precursor L-glutamic acid. For instance, metabolic engineering of *C. glutamicum* by introduction of an engineered *E. coli gadB* (encoding glutamate decarboxylase) with expanded pH range resulted in GABA production with titers up to 38.6 g/L from glucose (Figure 2, lime green highlight) [35].

While 5AVA is not produced by the petroleum industry, it has potential for use in new applications such as renewable engineering plastics (PA-5 and PA-6,5) and as a precursor for valerolactam [36]. Metabolic engineering for production of 5AVA in *E. coli* has recently been demonstrated for the first time by overexpression of *davA* (encoding δ-aminovaleramidase) and *davB* (encoding L-lysine 2-monooxygenase) from *Pseudomonas putida* [17,18] in a previously engineered *E. coli* XQ56 strain overproducing cadaverine from glucose [8], yielding 5AVA titers of 0.5–0.9 g/L (Figure 2, orange highlight) [17,18]. Although improvement in this titer is needed for industrialization, this demonstrates the feasibility of direct fermentative 5AVA production from biomass-derived carbohydrates.

Diols

Short-chain diols are of importance as platform chemicals and as monomers for production of polyesters and polyurethanes [37]. The three diols 1,3-PDO, 1,4-BDO, and MEG have been receiving much attention for their bio-based production. 1,3-PDO is mainly used as a building block for the production of polymers, especially for the polyester polytrimethylene terephthalate (PTT), and can be produced through chemical synthesis by hydration of acrolein to form 3-hydroxypropionaldehyde, which is subsequently hydrogenated into 1,3-PDO. Alternatively, 1,3-PDO can also be produced through fermentation, a great example of successful metabolic engineering that led to commercialization [38]. A large number of bacteria are known to naturally produce 1,3-PDO in anaerobic growth on glycerol, where the glycerol undergoes rearrangement to 3-hydroxyproiponaldehyde followed by an NADH-dependent reduction to 1,3-PDO [38]. Using

glucose, DuPont (at that time Genencor) developed metabolically engineered *E. coli* capable of producing 1,3-PDO to a final titer of 135 g/L and a productivity of 3.5 g/L/h [38]. Their aerobic process using a glycerol producing *E. coli* strain utilizes glycerol 3-phosphate dehydrogenase (DAR1) and glycerol 3-phosphate phosphatase (GPP2) to provide glycerol from glucose [38]. With additional expression of glycerol dehydratase (encoded by *dhaB1*, *dhaB2*, and *dhaB3*) and reactivating factors (encoded by *dhaBX* and *orfX*), the engineered strain was able to produce 3-hydroxypropionaldehyde, which was further converted to 1,3-PDO through overexpression of *yqhD* (encoding oxidoreductase) (Figure 2, blue highlight) [39]. Although this process has been commercialized successfully, there are still new pathways under investigation for production of 1,3-PDO. For instance, a recent study reported the construction of a non-natural pathway for 1,3-PDO production from L-homoserine, by engineering wild-type glutamate dehydrogenase for increased deamination of L-homoserine to 4-hydroxy-2-ketobutyrate, which could subsequently be converted to 1,3-PDO by decarboxylation followed by reduction. While the production titer was low (51.5 mg/L), this study is a nice example of non-natural pathway design coupled with enzyme engineering [40••].

Another well-known diol for polymer synthesis is 1,4-BDO, which is used to manufacture polyesters and polyurethanes. Furthermore, dehydration of 1,4-BDO results in tetrahydrofuran, a main chemical for making elastomeric polyurethane fibers such as Spandex. Unlike 1,3-PDO, there are no known microbes that naturally produce 1,4-BDO, and it has solely been produced by hydrogenation of petroleum-based 1,4-butynediol. Nevertheless, the first direct production of 1,4-BDO using *E. coli* was achieved by Genomatica in 2011, initially capable of producing 18 g/L [29], providing a path to renewable 1,4-BDO. Their strain has since been engineered to produce 1,4-BDO to a titer of over 120 g/L, the currently highest reported production titer [41]. In order to achieve this feat, several candidate 1,4-BDO pathways were screened *in silico* using the SimPheny Biopathway Predictor. This, in combination with knockouts of *adhE*, *pfl*, and *mdh* (encoding aldehyde-alcohol dehydrogenase, pyruvate formate lyase, and malate dehydrogenase, respectively), and mutational engineering of *lpdA* (encoding dihydrolipoyl dehydrogenase) and *gltA* (encoding citrate synthase) resulted in the final production strain [29,41]. Production of 1,4-BDO provides another nice example of designing novel pathways for production of previously unattainable chemicals through bio-based route. As in the case of 1,3-PDO, a recent study has revealed a novel biosynthetic route to 1,4-BDO. In this pathway, carbon utilization for cell growth and product formation were separated, and product formation was dynamically controlled using an elegant combination of synthetic circuits and quorum-sensing [42•]. The separation of cell growth and 1,4-BDO production was achieved by introducing a pathway that is orthogonal to cellular central metabolism using different carbon sources, where D-xylose was utilized for production of 1,4-BDO and glucose was used for the central metabolism. This resulted in the production of 0.44 g/L of 1,4-BDO (Figure 2, brown highlight). While this titer is much lower than the one reported by Genomatica, this work exemplifies the feasibility of dynamic control of genetic devices for chemical production using microbes.

Finally, MEG is a large production volume diol that is used as a platform chemical for manufacturing polyols [poly(ethylene glycol)], polyesters, and polyurethanes. The traditional chemical route is derived from petroleum-based ethylene. As the bioethanol industry has grown significantly over the last decade, chemical production of ethylene from bioethanol has emerged as a renewable route to ethylene [43], and consequently to MEG. This process has been commercialized by a number of companies, including Braskem (São Paulo, Brazil), and ethanol-based MEG is used by for instance the Coca-Cola Company (Atlanta, Georgia) for production of green poly(ethylene terephthalate) (PET), trademarked as PlantBottle. Nevertheless, the current production volumes of bioethylene and MEG are far from meeting market demands due to the amount of bioethanol available for non-fuel usage and conversion efficiency. Further market growth of bio-based MEG can be expected when an efficient bioprocess for its direct fermentative production is developed as described below.

Recently, direct fermentative production of MEG from D-xylose using engineered *E. coli* was reported as an alternative to the bioethylene-based process currently in use [44••]. This was achieved through deletion of *xylA* (encoding xylose isomerase) to disrupt xylulose formation, combined with overexpression of *xdh* (encoding D-xylose dehydrogenase) from *Caulobacter crescentus* and the native NADPH-dependent aldehyde reductase encoded by *yqhD*. Together, these modifications directed the flux toward MEG, resulting in production of 11.7 g/L (Figure 2, dark green highlight) [44••]. In another study, 42 g/L of MEG was produced from simple sugar and glycerol by overexpressing the *fucK*, *fucA*, *fucO*, and *dte* genes [45]. Even though production of the above diols is close to commercialization, further process optimization is still necessary to reduce their production cost.

Production of aromatic monomers

There have been several reports describing metabolic engineering efforts for production of important aromatic monomers used in synthesis of polymeric materials. Here, studies describing metabolic engineering of styrene and its analogues *p*-hydroxystyrene, *p*-hydroxybenzoic acid, and phenol are described. These are important chemicals for the production of various polymers including polystyrene and acrylonitrile butadiene styrene (ABS). Furthermore,

potential pathways for production of precursors to the important aromatic diisocyanates MDI and TDI (used as polyurethane monomers) are discussed.

Styrene and styrene analogues

Early metabolic engineering efforts enabled the production of styrene analogues such as *p*-hydroxystyrene [46] and *p*-hydroxybenzoic acid [47], through modification of the aromatic amino acid biosynthesis pathway. This was done by overexpression of *p*-coumaric acid decarboxylase and bi-functional phenylalanine/tyrosine ammonia-lyase (*PAL/TAL*), which allowed production of 0.4 g/L of *p*-hydroxystyrene in *E. coli* (Figure 2, pink highlight) [46]. In addition, overexpression of *PAL* and inactivation of *pobA* (encoding *p*-hydroxybenzoate hydroxylase) in a tyrosine overproducing *P. putida* strain resulted in production of 1.8 g/L of *p*-hydroxybenzoic acid (Figure 2, yellow highlight) [47].

More recently, McKenna and colleagues have developed a pathway for styrene production using engineered *E. coli* [48]. This was achieved by co-expression of *PAL2* (encoding L-phenylalanine ammonia-lyase 2) from *Arabidopsis thaliana* and *FDC1* (encoding ferulic acid decarboxylase 1) from *S. cerevisiae* in an L-phenylalanine overproducing *E. coli* strain, which resulted in production 0.26 g/L of styrene (Figure 2, light pink highlight) [48], proving the feasibility of microbial styrene production. Recent process engineering by coupling fermentation with *in situ* product removal by solvent extraction using *bis*(2-ethylhexyl)phthalate and by gas stripping to overcome limitation caused by styrene toxicity, enabled increasing the product titer to 860 mg/L and 560 mg/L, respectively [49].

Finally, a recent study showed direct production of phenol from glucose using engineered *E. coli* [50•]. This was achieved using synthetic sRNA technology to knock down the global carbon storage regulator *csrA* and the transcriptional regulator *tyrR*, together with overexpression of *tpl* (encoding tyrosine phenol-lyase), which allowed rapid screening of 18 *E. coli* strains for production of phenol. This screen identified a strain capable of producing 3.8 g/L of phenol through biphasic fed-batch fermentation (Figure 2, purple highlight) [50•].

Aromatic diisocyanates

The aromatic diisocyanates, MDI and TDI, are the two major diisocyanates needed for production of polyurethanes, which are used as elastomers, structural and insulating foams and plastics. Currently, neither of these compounds has a direct biological production route, but recent development shows promise for future biological production of their precursors. For example, Genomatica recently patented two metabolic pathways for production of aniline, a precursor of MDI [51]. These pathways both use chorismate as the aniline precursor, with the first pathway employing aminodeoxychorismate synthase, aminodeoxychorismate lyase, and 4-aminobenzoate carboxylase, while the second pathway uses anthranilate synthase and anthranilate decarboxylase [51]. The implementation of these pathways could enable production of aniline, and subsequently MDI, from renewable resources. Another recent report with relevance for renewable diisocyanate production is the construction of a phenylacetic acid producing *E. coli* strain [52]. This was derived from the shikimic acid pathway, by overexpression of *aroF* (encoding 3-deoxy-D-arabinoheptulosonate 7-phosphate synthase), *pheA* (encoding chorismate mutase/prephenate dehydratase), *feaB* (encoding phenylacetaldehyde dehydrogenase), combined with introduction of heterologous *ipdC* (encoding indol-3-pyruvate/phenylpyruvate decarboxylase) from *Azospirillum brasilense* and knock-out of *tyrA*. This engineered strain was able to produce 1.2 g/L of phenylacetic acid (Figure 2, plum highlight) [52]. Combining this result with the previously identified ability of the bacterium *Tolumonas auensis* to convert phenylacetic acid into toluene [53] could provide a route to renewable toluene for its further chemical conversion into TDI. Identification and heterologous expression of the genes involved in the *T. auensis* pathway will thus be an important future work.

In vivo polymer synthesis

The previous sections focused on metabolic engineering strategies for the bio-based production of monomers that can be used for polymer synthesis. An alternative is the direct *in vivo* synthesis of polymers, which is discussed in this section. We focus on PHAs and recombinant proteins having great material properties as examples of *in vivo* synthesized polymers with applications as renewable biomaterials.

Polyhydroxyalkanoates (PHAs)

The iconic, well-studied example of *in vivo* synthesized biopolymers is PHAs. PHAs are biodegradable and biocompatible polyesters composed of linear hydroxyalkanoates, accumulated by many microorganisms as carbon and energy storage compounds. PHAs can be produced as homo-polymers or co-polymers of diverse monomers, providing polymeric materials with a range of mechanical and thermal properties. Production of PHA with typical molecular weights in the range of 2×10^5 to 3×10^6 Da has been reported [54]. The most extensively studied PHA is poly(3-hydroxybutyrate) [P(3HB)], which has properties similar to polypropylene. P(3HB) biosynthesis starts with the condensation of two acetyl-CoA molecules into acetoacetyl-CoA by *phaA* (encoding 3-ketothiolase). Subsequently, acetoacetyl-CoA is reduced by *phaB* (encoding acetoacetyl-CoA reductase) to 3HB-CoA, which is polymerized into P(3HB) by *phaC* (encoding PHA synthase) (Figure 2, turquoise highlight) [55]. Recently, recombinant *E. coli* producing poly(3-hydroxypropionate), P(3HP), and its copolymer poly(3-hydroxybutyrate-*co*-3-hydroxypropionate) [P(3HB-*co*-3HP)] from glucose was developed.

This was achieved by overexpression of heterologous genes (encoded by *gpd1*, *gpp2*, *dhaB123*, *gdrAB*, and *pduP*) together with the genes involved in 3HB biosynthesis [56].

Medium chain-length (MCL) PHAs can be produced from fatty acids by modifying the β-oxidation pathway. Examples include production of a homo-polymer poly(3-hydroxyoctanoate) [P(3HO)] from sodium octanoate using *Pseudomonas mendocina*, which accumulated P(3HO) up to 31.4% of its dry cell weight [57]. In addition, C_{10} and C_{11} homopolymer PHAs have been produced in metabolically engineered *P. putida* KT2442, by deletion of six fatty acid degradation enzymes (encoded by *fadA*, *fadB*, *fadB2x*, *fadAx*, *PP2047*, and *PP2048*) and *phaG* (encoding 3-hydroxyacyl-CoA-acyl carrier protein transferase) [58], which resulted in the production of poly(3-hydroxydecanoate) [P(3HD)] homopolymer and P(3HD-*co*-84 mol% 3-hydroxydodecanoate) from decanoic and dodecanoic acids, respectively.

In another study, overexpression of *BTE* (acyl-ACP thioesterase), *phaJ3* (encoding enoyl-CoA hydratase), and *phaC2* (encoding PHA synthase) in *E. coli* resulted in production of MCL PHA to 15% of the dry cell weight [59]. Finally, production of PHAs containing branched chain subunits 3-hydroxy-4-methylvalerate (3H4MV) has been reported. This was achieved by overexpression of several leucine biosynthesis genes (encoded by *ldhA* and *hadAIBC*) together with PHA synthase, which resulted in production of P(3HB-*co*-3.0 mol% 3H4MV) from glucose [60].

Another important polyester that has been produced *in vivo* by *E. coli* is polylactic acid (PLA). PLA, being a non-natural polymer, could not previously be produced by one-step direct fermentation, and was thus produced by two-step processes consisting of fermentative lactic acid production followed by chemical ring-opening polymerization of its dimer lactide *in vitro*. More recently, metabolically engineered bacterial strains have been developed and used for *in vivo* production of PLA and lactate-containing polymers directly from glucose and other carbohydrates. Upon introduction of a key pathway consisting of an evolved pct_{cp} (encoding propionyl-CoA transferase) from *Clostridium propionicum* and an evolved $phaC1_{ps6\text{-}19}$ (encoding polyhydroxyalkanoate synthase 1) from *Pseudomonas* sp. MBEL 6-19, engineered *E. coli* strain could directly produce PLA and lactate-containing PHAs (Figure 2, yellow highlight) [61]. Further research has resulted in different strains capable of producing various lactate-containing copolymers, such as P(3HB-*co*-LA) [62] and P(2-hydroxybutyrate-*co*-3HB-*co*-LA) [63].

Proteins as materials

Despite the importance of the monomers and polymers that have been mentioned above, there are also naturally found polymers, which exhibit outstanding properties. Most of these polymers are composed of proteins and some structural proteins possess desirable material properties that are suitable for a wide range of industrial applications [64,65]. An early example is elastin possessing biologically interesting properties. Elastin is a protein with repetitive amino acids, where these repeats are an important component of the chemotactic properties for fibroblasts and monocytes [66]. Recently, silk elastin-like proteins (SELP) consisting of 5 repeats of silk consensus sequence (GAGAGS) and 9 repeats of elastin like sequence (VPAVG) was expressed in recombinant *E. coli*, resulting in 0.5 g/L in flask culture [67]. By further optimizing the fermentation processes including high pre-induction growth rates, lowering the concentration of the inducer, low oxygen concentration, and reducing post-induction feeding rates, 4.3 g/L of SELP could be produced [68].

Another example of a repetitive protein with interesting material properties is spider silk protein. This has attracted much attention due to its outstanding material characteristics, biodegradability, and biocompatibility, making it popular bio-inspired material in many fields [69]. However, recombinant spider silk proteins that yield natural spider silk-like properties could not be produced. One of the main reasons was found to be lower molecular weight of recombinant spider silk protein produced so far. Recently, native-sized, ultra-high molecular weight (285 kDa) spider silk protein was produced to 0.7 g/L by metabolically engineered *E. coli*. Fibers spun using this recombinant spider silk protein showed for the first time the ultra-high strength exhibited by natural spider silk. Here, a synthetic gene consisting of 96 repeats of silk protein monomer was expressed in an engineered *E. coli* capable of generating increased glycine-tRNA (Figure 2, orange highlight) [69,70].

An additional example is squid sucker ring teeth protein, which has been attracting much attention due to having material properties similar to several synthetic polymers, including poly(methyl methacrylate), poly(ether ether ketone), and polyamides [71••]. Ding and colleagues were the first to express and purify suckerins (nanoconfined β-sheet) up to 71 mg/L composed of codon optimized, poly-histidine-tagged suckerin and native, non-tagged suckerin. The protein was expressed in an inclusion body form, and the purification was optimized using a microfluidization process [71••]. Furthermore, mussel adhesive protein (MAP), a potential source of water-resistant bioadhesives, has been explored for production [72–74]. Through co-expression of a functional tyrosinase and recombinant MAP *in vivo*, soluble mussel adhesive protein was successfully produced, with 4-fold higher bulk-scale adhesive strength compared to *in vitro* tyrosinase-treated MAP (Figure 2, mint green highlight) [72]. Although only four example proteins were showcased here as *in vivo* polymer products, more examples of using recombinant proteins as advanced materials are bound to appear in the future.

Outlook

In this paper, we review the current state of metabolic engineering for microbial production of monomers and polymers, focusing on diamines, dicarboxylic acids, diols, ω-amino acids, hydroxy acids, aromatic monomers, and some *in vivo* synthesized polymers. While some of these have reached commercialization already (succinic acid, vegetable oil-based sebacic acid, 1,4-BDO; 1,3-PDO; and MEG from bioethanol), much work remains to be done before the current, petroleum-based processes can be fully replaced. A promising development is the on-going commercialization of cadaverine by Cathay Industrial Biotech and putrescine by CJ CheilJedang that, in combination with sebacic acid, opens the way to fully renewable production of the polyamides PA-4,10 and PA-5,10. However, commercial bio-based production of 1,6-diaminohexane, currently the most demanded diamine, as well as the ω-amino acids described in this review, remains to be achieved. If this can be demonstrated on top of putrescine and cadaverine, most relevant PA's will be accessible from renewable sources, opening up fully petroleum-free PA production. This makes 1,6-diaminohexane and the ω-amino acids described above highly prioritized future bioproduction targets. Another important development is production of MEG from bio-ethanol, and the potential future direct production from glucose, which has opened up a route to partially renewable polyesters, including the important PET. However, fully renewable PET requires also the availability of bio-based terephthalic acid, which has not yet been achieved and remains an invaluable future target. Bio-based production of aromatic monomers is rapidly progressing, with the production of both phenol and styrene being recent successes. These are both valuable monomers opening the route to a variety of polystyrene polymers, as well as polycarbonates through bisphenol A. For the realization of commercial production of such aromatic chemicals, further improvements in strain and process engineering are required in order to overcome the toxicity often associated with these products. Finally, the demonstration of the production of silk-elastin-like protein, spider silk protein, mussel adhesive protein, and squid suckerin are leading the way toward next-generation biomaterials accessible by fermentation. In addition, we did not cover curli fibers in depth in this article because not much work has been done from metabolic engineering perspective. A recent study has demonstrated a system using curli fibers for adhesion and protein immobilization, making it an attractive protein for overproduction [75]. The bio-based monomers and polymers reviewed in this paper, together with those suggested as important future target chemicals, will become increasingly important materials for sustainable manufacturing of polymers currently in use without our dependence on fossil resources. Since most of the currently commercialized processes still rely on starch-based or sugar-based biomass as a feedstock for their production, it will be vital to develop economically competitive biorefinery processes for the production of monomers and polymers from non-food, lignocellulosic biomass.

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