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Metabolic engineering of strains: from industrial-scale to lab-scale chemical production

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Abstract A plethora of successful metabolic engineering case studies have been published over the past several decades. Here, we highlight a collection of microbially produced chemicals using a historical framework, starting with titers ranging from industrial scale (more than 50 g/L), to medium-scale (5–50 g/L), and lab-scale (0–5 g/L). Although engineered *Escherichia coli* and *Saccharomyces cerevisiae* emerge as prominent hosts in the literature as a result of well-developed genetic engineering tools, several novel native-producing strains are gaining attention. This review catalogs the current progress of metabolic engineering towards production of compounds such as acids, alcohols, amino acids, natural organic compounds, and others.

Introduction

Microorganisms have been at the center of daily life even since ancient times where early biotechnology got its start in food and beverage production. The capacity of organisms for industrial-scale microbial fermentation was realized by Pfizer in 1919 through a process to produce citric acid using *Aspergillus niger* [99]. Next, the emergence of genetic engineering allowed great advances in engineering capabilities, leading to the formalization of the field of "metabolic engineering" in 1991 with the goal of "the improvement of cellular activities with the use of recombinant DNA technology" [16]. This early era of metabolic engineering was marked by the use of gene modifications, pathway complementation, and the classical strategy of inverse metabolic engineering [17]. Through this work, several lab strains: *Escherichia coli* and *Saccharomyces cerevisiae* gained popularity as engineering hosts. Meanwhile, several forces allowed the field to move beyond the early molecules of insulin, lysine, and ethanol. First, the development of many powerful tools for metabolic engineering along with the emergence of novel fields, such as genetic engineering, systems biology, and synthetic biology have expanded the reach of strain engineering efforts. Second, the diversity of compounds produced from engineered cells has been broadened to include pharmaceuticals, fuels, polymers, and commodity chemicals with titers spanning the gamut from milligrams to hundreds of grams per liter. Last, the catabolism of sustainable substrates has been developed as an environmentally friendly and renewable process. As an example, ethanol has been produced from monomer sugars (glucose, xylose, or arabinose) and even raw materials with higher polymerization degrees (raw corn starch, cellulose, ground rice, xylan, etc.).

In this review, we highlight and discuss the progress of metabolic engineering in producing a variety of compound classes with a particular emphasis on the titer (g/L) of bioprocesses from industrial-scale to lab-scale. We additionally highlight the pairing of microorganisms with their most highly produced compounds, as informed from publications. In such a fashion, the compounds which have been successfully studied for decades and well-developed in industrial-scale production will be presented first, while the compounds which have recently achieved lab-scale production via the

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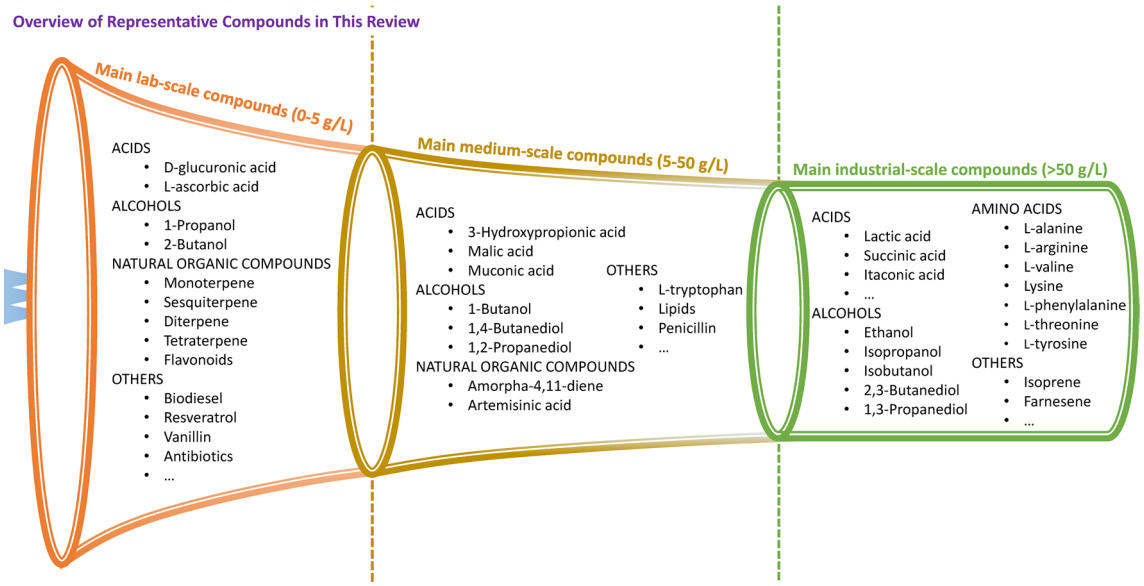

Fig. 1 Overview of current progress in metabolic engineering. The compounds are organized into three tiers: industrial- (*red*), medium- (*orange*), lab-scale (*green*). For each scale, the main compounds discussed in this review were categorized. It should be noted that reported titer (one of many metrics) was used to classify each of these molecules. While titer is not the only determining factor for scale-of-production, it provides a meaningful baseline to compare these molecules (color figure online)

aid of newly emerging metabolic engineering technologies will be presented last. Figure 1 provides an overview of all the major classes of compounds discussed here.

Compounds synthesized through metabolic engineering

Metabolic engineering boasts a growing panel of chemical products including amino acids, antibiotics, biofuels, biopolymers, chemical compounds, precursors, and pharmaceuticals. Several key performance metrics to evaluate routes to synthesize bioproducts include titer (g product/L), yield (g product per g substrate), and productivity (g product/L/h). Among them, titer can significantly affect downstream separation cost when it is a high fraction of total cost of goods sold (COGS). Depending on the amount of engineering required and the ease of interfacing with host strains, titers achieved differ dramatically. For compounds achieving high titers, it is usually the case that the host possesses innate tolerance to high concentrations of the compound, the pathway to synthesize the compound requires minimal (or at least known) engineering, a highly preferred carbon source was used, and/or the host already natively produces the compound. For low titer compounds, the opposite is generally the case. As a result, compounds with high titer are produced at industrial scales while for others, more advanced technologies are required. We organize our discussion below on the engineering timeline of microbially produced compounds on the basis of titers from industrial- (greater than 50 g/L), to medium- (5–50 g/L), and lab-scale (0–5 g/L), knowing that titer is only one important metric (in addition to rate and yield) to judge the success of a bioprocess. It is important to keep in mind that the definition of "industrial-scale" is more than just titer and must also take into account rate, yield, and market demand.

Compounds of industrial-scale production

The production of compounds with reported titers over 50 g/L (which could be considered a minimum for industrial-scale production) is discussed in this section. In contrast to lab-scale and medium-scale production, industrial-scale production relies on well-developed downstream processes as well as optimized fermentation conditions. These success stories strongly illustrate the power of metabolic engineering to produce profitable quantities of the desired compound.

Acids

Lactic acid and its derivatives are the building blocks of polylactic acid, which is an exciting renewable and biodegradable plastic. In 2005, Fong et al. [51] used the OptKnock framework to identify an optimal genotype for lactic acid production in *E. coli* and used adaptive evolution to further improve production. Later, deletions of pyruvate formatelyase, acetate kinase, alcohol dehydrogenase,

lactate dehydrogenase, fumarate reductase, and integration of L-(+)-lactate dehydrogenase from *Pediococcus acidilactici* resulted in the production of optically pure L-(+)-lactate from glucose or xylose in *E. coli* [173]. Additionally, a catabolite repression mutant combined with high expression of xylose isomerase demonstrated 57.1 g/L L-lactic acid production [43]. An alternative strategy deleted the methylglyoxal synthase gene, thus removing the native pathway to produce precursors for both L(+)- and D(-)-lactate. Next, a native gene and additional copy of lactate dehydrogenase from *P. acidilactici* could enable *E. coli* to produce D(+)-lactate and L(+)-lactate at 118 and 116 g/L, respectively [56]. A combinatorial strain engineering approach was also applied to engineer *E. coli* towards production of D-lactate, achieving 125 g/L in a bioreactor [172]. Yeast have not widely served as a platform for lactate production as an engineered strain could only achieve 8.5 g/L L-lactic acid from xylose [73].

Succinic acid is an important chemical building block in the synthesis of 1,4-butanediol, tetrahydrofuran, and 1,4-diaminobutane. Engineered *S. cerevisiae* has been shown to produce succinic acid at a titer of 3.62 g/L [115]. Another fungus, *Yarrowia lipolytica*, was also engineered by introduction of mutations to succinate dehydrogenase. A strain undergoing additional selection was then obtained that produced succinate at 45 g/L from glycerol [161]. A promising bacterium, *Mannheimia succiniciproducens* MBEL55E, was isolated from cow's rumen. The complete genome sequence enabled the identification of several critical enzymes involved in synthesizing succinic acid. By eliminating several competing by-product formation pathways, these bacteria were rewired to produce 52.4 g/L succinic acid [77]. In the commonly used bacterium *E. coli*, a much higher titer of 87 g/L was achieved by removing pathways for side-product synthesis [63], and upwards of 99.2 g/L was achieved through optimization of fermentation conditions [141]. However, the highest reported titers come from another important microorganism, *Corynebacterium glutamicum*, where similar modifications led to a final titer of 146 g/L [106].

Itaconic acid, a precursor to several widely used polymers, has been recognized by the Department of Energy as one of the top ten targets for bio-based chemical production [150]. In yeast, expression of the *cis*-aconitic acid decarboxylase gene from *Aspergillus terreus* along with additional strain engineering is sufficient to enable itaconic acid production at 168 mg/L, albeit at much lower levels than in *A. terreus* [24]. As part of a proof-of-principle approach for an RNAi-based rapid strain prototyping method, Crook et al. [37] identified several strain-gene knockdown combinations which resulted in increased itaconic acid production in yeast. In this experiment, the knockdown of *ADE3* in the Sigma strain background was identified as the most promising background for further engineering efforts in yeast enabling a baseline production of 70 mg/L. However, the production of itaconic acid in engineered yeasts like *S. cerevisiae* to date is miniscule compared to the native producer (*A. terreus*) from which more than 85 g/L itaconic acid was recorded in industrial-scale production [105].

Additional acids have also been reported at industrial-scale titers. Pyruvate was produced in both *E. coli* and *S. cerevisiae* at titers of 90 g/L [175] and 135 g/L [139], respectively. The process to produce acetate in *E. coli* was developed by inactivating oxidative phosphorylation, disrupting the tricarboxylic acid pathway, and removing several native pathways [29]. Finally, the glycolytic flux was also increased, resulting in a titer of 51.8 g/L acetate. Another famous commercially produced compound is citric acid, which has been produced from various fungi (especially *Aspergillus* sp.) and yeasts (*Candida* sp. and *Yarrowia* sp.) [94]. Although, fungal submerged fermentation still plays a dominant role in industrial processes, continuous processes with yeasts also competitively reach titers of 200–250 g/L [6].

Alcohols

Biological production of ethanol (especially from cellulosic and lignocellulosic constituent sugars) has been the focus of metabolic engineering for decades [28]. In *E. coli*, metabolic networks were minimized by engineering six pathways, four of which were associated with converting pentose and hexose sugars into ethanol and two of which were related to anaerobic cell growth. The final resulting strain with alleviated catabolite repression could utilize glucose and xylose simultaneously and produce ethanol at a titer of 20 g/L [162]. The pyruvate decarboxylase and alcohol dehydrogenase from a native ethanol-producer, *Zymomonas mobilis*, were then integrated into the genome aided by selection under high levels of chloramphenicol. A strain with increased expression of heterologous genes could produce ethanol at 54.4 g/L from 10 % glucose and 41.6 g/L from 8 % xylose, respectively [104]. It should be noted that many additional strategies including the elimination of the oxidation of NADH [70], cell immobilization [171], and adaptive evolution [146] have been used, but do not explicitly report titers.

The most common host for ethanol production, *S. cerevisiae*, has also been engineered to increase its capacity to utilize lignocellulosic materials. Since *S. cerevisiae* does not harbor an endogenous xylose catabolic pathway, several heterologous pathways have been explored including the xylose reductase pathway from *Scheffersomyces stipitis* [64] and the xylose isomerase pathway from *Piromyces* sp. [74]. Lee et al. [78] undertook the directed evolution

of xylose isomerase from *Piromyces* sp. resulting in 77 % improvement to enzyme activity and a high ethanol yield of 0.5 g/g xylose. Adaptive evolution of the strain containing this mutant version as well as further pathway engineering recently enabled 0.45 g/g xylose in bioreactors and ethanol production rates upwards of 0.44 g ethanol g cell^{-1} h^{-1} [79]. For a more complex substrate such as phosphoric acid-swollen cellulose (PASC), additional pathway enzymes are needed to depolymerize the carbon source. The heterologous expression of secreted cellulose-degrading enzymes enabled *S. cerevisiae* to grow on medium with PASC as the sole carbon source [41]. Further incorporation of surface display technology involving a scaffoldin enhanced this synergy, resulting in higher ethanol titers [134, 149]. Other instances of lab-scale ethanol production in *S. cerevisiae* included the use of arabinose [19] and crude glycerol [160]. However, food-derived materials still remain the main source for industrial-scale production of ethanol in *S. cerevisiae*, such as raw corn starch resulting in a titer of 62 g/L [129] and unprocessed ground rice giving 70 g/L [155].

Isobutanol, a branched-chain higher alcohol, is considered as a next generation biofuel and has successfully reached an industrial level of production. In engineered *E. coli*, the keto-acid pathway followed by alcohol dehydrogenase directs flux from amino acid biosynthesis towards alcohol production. The overexpression of these pathways (*ilvIHCD*, *Kivd* and *Adh2*) resulted in the production of isobutanol at 22 g/L [12]. With in situ product removal by a gas stripping method, the titer could be improved to 50.8 g/L [15]. Using the same strategy, another promising alcohol, isopropanol, also reached industrial-scale production in *E. coli* (143 g/L) [60]. In 2013, Gevo published a patent reporting production of isobutanol at a titer of 111 g/L in engineered *S. cerevisiae* CEN.PK2 [50].

Two diols, 2,3-butanediol and 1,3-propanediol, are industrially important chemicals. Nakashima et al. [101] developed a xylose-inducible promoter, which avoids commonly used expensive inducers and demonstrated its utility to enable production of (*R,S*)-2,3-butanediol at a titer of 54 g/L. Recently, a combinatorial approach was applied to further improve the titer of 2,3-butanediol in *E. coli* to 73.8 g/L [154]. A strain of *S. cerevisiae* was also engineered to simultaneously utilize glucose and galactose and remove ethanol production [88]. In this strain, the expression of a heterologous 2,3-butanediol synthetic pathway enabled (*R,R*)-2,3-butanediol production to levels of more than 100 g/L. For 1,3-propanediol production, *E. coli* is the choice host organisms with maximal published titers of 135 g/L from glucose under fed-batch conditions [100] and nearly 104 g/L from the cheaper glycerol carbon source [133].

Amino acids

Amino acids are the main components of protein and have been the target of engineering efforts for decades. Industrial production of several essential amino acids via microbial fermentation has been a major success story in the field [1, 7]. For L-valine, several typical metabolic engineering strategies were applied to achieve medium-scale production in *E. coli* [110]. Following these efforts, further metabolic network enhancements to provide more ATP and optimize the acetic acid feeding strategy improved titers to 32.3 g/L [109]. Further improvements (especially to tolerance) created a more efficient platform capable of producing 60.7 g/L [108]. This strain has been rewired to produce L-alanine, L-threonine, and L-tyrosine at titers of 114 g/L [165], 82.4 g/L [76], and ~50 g/L [54] (DuPont), respectively. An additional strategy consisting of selecting high-producing mutants of native producers of amino acids has also been reported. *C. glutamicum* has enabled high level production of other amino acids such as lysine, L-arginine, L-threonine, and L-phenylalanine at 120 g/L [20, 103], 92.5 g/L (5L bioreactor) [111], 58 g/L [62], and 28 g/L [7] respectively. Further process development of engineered *E. coli* strains harboring native pathways has been shown to enhance the production of L-threonine and L-phenylalanine to titers of 80 g/L [40] and 50 g/L [14], respectively.

Natural organic compounds

Terpenes and terpenoids are a large and diverse group of compounds produced by plants and have been the subject of extensive metabolic engineering efforts in recent years. Depending on the numbers of isoprene units, terpenes are classified into hemi-, mono-, sesqui-, di-, tri-, and tetraterpenes, etc., which correspond to an isoprene unit number of 1, 2, 3, 4, 6, and 8, respectively. The production of isoprene itself has reached industrial-scale production. Lindberg et al. [90] reported that the engineering of the cyanobacterium *Synechocystis* resulted in isoprene production from CO_2. In addition, the MEP pathway from *Bacillus subtilis*, along with isoprene synthase (*ispS*) from *Populus nigra* were expressed in *E. coli*, leading to an enhanced production of isoprene (314 mg/L) [169]. Further, the upper part of the MVA pathway which converts acetyl-CoA to mevalonate was introduced into *E. coli* and combined with site-directed mutagenesis to reach an isoprene titer of 6.3 g/L [156]. Genencor-DuPont and Goodyear have claimed engineered *E. coli* strain can produce isoprene at a titer of 60 g/L, through importing heterologous MVA pathway, introducing appropriate promoters, and efficient product removal [35]. Farnesene, belongs to sesquiterpenoid, exists in two isomers: α-farnesene and β-farnesene. These forms were biosynthesized in *E. coli* and *S. cerevisiae* by

introducing the corresponding farnesene synthase into a pre-engineered strain [121]. The titers of α-farnesene and β-farnesene produced by *E. coli* reached 400 and 1,100 mg/L, respectively [119]. In 2010, Amyris has engineered yeast strain to produce farnesene more than 100 g/L by scaling up bioprocesses [114].

Other compounds with industrial-scale production

E. coli has been exploited in other applications to efficiently produce compounds at industrial scale, such as biodegradable polymers (poly(3-hydroxybutyrate) at 141.6 g/L [33] and polyhydroxylalkaneoates at more than 100 g/L [80]) and sugars (mannitol at 66 g/L [69]). *B. subtilis* was found to produce acetoin (56.7 g/L) [101, 167] at industrial-scale. Engineered *C. glutamicum* could produce polymer building blocks at 88 g/L for a novel bio-nylon material [71].

Compounds of medium-scale production

A promising collection of chemicals have been able to reach medium-scale production (titers of 5–50 g/L) in microbial systems, which suggest strong potential for industrial applications. In most cases, these improvements were made through a combination of bioreactor conditions and further metabolic engineering. We highlight several examples of these molecules in this section. Once again, it should be noted that the data and titers described here are based on published reports. It is understood that the actual, realized value of all the medium- and industrial-scale compounds may indeed be higher than reported here. Nevertheless, a relative comparison is presented here to summarize the efforts of metabolic engineering for the production of compounds at different levels.

Acids

3-Hydroxypropionic acid (3-HP), a monomer used for novel biodegradable polymer materials, was originally reported at a lab-scale production in 2008 [116]. The engineered *E. coli* strain heterologously expressed glycerol dehydratase (*DhaB*) from *Klebsiella pneumonia* and overexpressed aldehyde dehydrogenase (*AldH*). A titer of 0.58 g/L 3-HP was produced from glycerol. Later, the two genes were placed under the control of an inducible promoter along with glycerol dehydratase reactivase [117]. *AldH* was then swapped with α-ketoglutaric semialdehyde dehydrogenase. Under shake-flask conditions, the titer improved to 2.8 g/L. A fed-batch strategy feeding vitamin B_{12} (necessary for *DhaB* function) in a bioreactor further dramatically increased the titer to 38.7 g/L from glycerol. The same group also investigated the production of 3-HP from lignocellulosic sugar (glucose) in *E. coli*. However, the titer still remained at lab-scale (193 mg/L) [118]. Most recently, further engineering of the glycerol-utilizing pathway in *E. coli* improved the titer to 42.1 g/L [66]. In parallel, a more suitable host to produce 3-HP was reported, *Klebsiella pneumonia*, which natively synthesizes vitamin B_{12} [9]. By deleting two major oxidoreductases and overexpressing the NAD^+-dependent γ-glutamyl-γ-aminobutyraldehyde dehydrogenase, production of 3-HP could reach 28 g/L in a bioreactor setting, which might represent a more economical process

Muconic acid, another interesting polymer precursor molecule, is currently produced using unsustainable chemical procedures. Therefore, several groups have been investigating a more sustainable and environmentally friendly solution. Specifically, Niu et al. [102] have shown recombinant *E. coli* capable of producing 36.8 g/L of muconic acid after the expression of a composite, heterologous pathway composed of *Klebsiella pneumoniae* 3-dehydroshikimic acid dehydratase (*aroZ*) and protocatechuic acid decarboxylase (*aroY*) along with *Acinetobacter calcoaceticus* catechol 1,2-dioxygenase (*catA*). Translation of high muconic acid production levels to *S. cerevisiae* proved rather difficult. In this case, importing a similar pathway along with extensive remodeling of the *S. cerevisiae* aromatic amino acid production pathway to remove feedback inhibition and increase metabolic flux resulted in only 141 mg/L of muconic acid being produced [38].

Malic acid, a food additive and an intermediate in the synthesis of fine chemicals, is another attractive biological molecule. Moon et al. [97] engineered the phosphoenolpyruvate (PEP) carboxylation pathway in *E. coli* by introducing PEP carboxykinase from *Mannheimia succiniciproducens*, leading to a final production of 9.25 g/L malic acid. The salt form of malic acid, L-malate, was also produced using the succinate synthesis pathway in *E. coli*. The mutation of fumarate reductase was a critical step, leading to a titer of 34 g/L in two-stage fermentation [166].

Alcohols

Given its high energy content and low vapor pressure, 1-butanol is considered as an ideal gasoline substituent. The biological production of 1-butanol in *E. coli* has been reported from lab-scale to medium-scale. Atsumi et al. [11] assembled a synthetic 1-butanol pathway in *E. coli* based on several promising native producers. In rich media supplemented with glycerol, titer reached 552 mg/L. Similar strategies were applied to transfer the 1-butanol synthesis pathway from *Clostridium acetobutylicum* into *E. coli* [61]. Bond-Watts et al. [25] took this approach one step further by considering the reversibility of key enzymes in the pathway. By forcing certain steps to be irreversible, a titer of 4.7 g/L 1-butanol was achieved in *E. coli*. Next,

several NADH-consuming pathways were deleted in *E. coli* to increase the driving force for the NADH-dependent 1-butanol biosynthesis pathway, resulting in an increased titer of 30 g/L when coupled with in situ product removal [127]. Several native producers, *C. acetobutylicum* [75] and *Clostridium beijerinckii* [52], could also produce a moderate amount of 1-butanol. In contrast, the production of 1-butanol in *S. cerevisiae* is quite low, with only trace amounts of 1-butanol being reported [130].

Compounds related to 1-butanol, such as 3-methyl-1-butanol and 1,4-butanediol, were also produced using metabolic engineering. Using the keto-acid pathway described above, the branched-chain compound 3-methyl-1-butanol could be obtained through L-leucine biosynthesis. Connor et al. [36] developed an engineered *E. coli* strain to be selectively sensitive to 4-aza-D,L-leucine (a L-leucine analog), leading to direct production of 3-methyl-1-butanol at a titer of 9.5 g/L. 1,4-Butanediol cannot be natively synthesized by any known microorganism. Therefore, Yim et al. [157] elucidated all potential pathways using existing enzyme databases through the self-developed SimPheny Biopathway Predictor software and designed biosynthetic pathways for 1,4-butanediol production in *E. coli*. The most promising artificial route to synthesize 1,4-butanediol in *E. coli* was identified to direct flux through intermediate 4-hydroxybutyrate. Various strategies, such as promoter engineering, vector optimization, and codon optimization, were applied to increase the expression of critical genes in the pathway. The OptKnock framework was also utilized suggest strain modifications and led to 1,4-butanediol production from C6 and/or C5 sugars, resulting in a titer of over 18 g/L. Based on actual production of this molecule at industrial scale by Genomatica, it is known that this molecule can be easily transitioned to industrial scale production with further engineering and optimization.

Another common solvent, 1,2-propanediol, is widely used in detergents, cosmetics, and pharmaceuticals. The pathway to synthesize 1,2-propanediol in *E. coli* utilizes glycerol as a substrate [34]. The three native enzymes involved in the synthesis of the intermediate dihydroxyacetone phosphate were overexpressed while the other native PEP-dependent dihydroxyacetone kinase was swapped with an ATP-dependent enzyme from *Clostridium freundii*. The engineered *E. coli* could produce 1,2-propanediol at a titer of 5.6 g/L. On the other hand, *S. cerevisiae* is currently a less-than-ideal host to produce 1,2-propanediol as similar efforts can only produce less than 0.5 g/L [81].

Natural organic compounds

Terpenes and terpenoids are still among the most abundant group of compounds in this scale of production. An important sesquiterpene, artemisinin, was developed over several years for treating malaria. The production of two main precursors amorphadiene and artemisinic acid was firstly achieved in *S. cerevisiae* by combinatorial metabolic flux engineering [121], global transcriptional analysis [120], and controlled fermentation processes [83], resulting in lab-scale productions. A MVA pathway, comprised of genes from both *S. cerevisiae* and *Staphylococcus aureus*, was introduced to *E. coli*, leading to a titer of 27.4 g/L amorphadiene in fed-batch fermentation [135]. A similar pathway was also overexpressed in another popular strain of *S. cerevisiae* CEN.PK2, resulting in more than 40 g/L amorphadiene [151]. Meanwhile, artemisnic acid was also biosynthesized at medium-scale in *S. cerevisiae*, resulting in a titer of 25 g/L [107]. A more practical process to convert artemisinic acid into artemisinin was proposed which utilized a singlet oxygen source, providing a potential industrial route for artemisinin production [107].

Other compounds with medium-scale production

Through metabolic engineering approaches, several additional important groups of compounds have been synthesized at the medium-scale. The amino acid L-tryptophan was produced at 13.3 g/L in a heavily engineered *E. coli* strain harboring site-directed mutations of feedback-resistant enzymes, transcriptional regulation of the trp repressor, elimination of the L-tryptophan-degrading pathway, and blocking of competing pathways [170]. *Y. lipolytica* was recently found to be an excellent host for producing high levels of lipids [22]. In this effort, native metabolism was rewired to achieve over 25 g/L lipids, which could be easily converted into fatty acid methyl esters. Although the chemical routes to synthesize antibiotics dominate the market, antibiotics have been produced engineered yeast strains and *Penicillium*, reaching trace levels (1 mg/L) [55] and more than 15 g/L [137] penicillin production, respectively. Other chemical compounds produced by microorganisms at medium scale, such as xylitol (8.52 g/L in *E. coli*) [3], isobutyl acetate (17.2 g/L in *E. coli*) [123], *p*-hydroxystyrene (17.6 g/L in *Pseudomonas putida*) [142], and hydroxybutyrate (9.4 g/L in *E. coli*) [87], may also have potential for industrial-scale production.

Compounds of lab-scale production

While this review highlights many metabolic engineering advances including industrial applications, most efforts and reported successes demonstrate low, lab-scale titers. Here, we define the production of compounds with equal to or below 5 g/L titer as lab-scale production and highlight several successes in the field. It is believed that more newly-emerging technologies will further improve the titers of these compounds, thus creating a promising pipeline for industrial-scale production.

Bulk chemicals

Acids A variety of acids have been synthesized at lab-scale by microorganisms. One value-added compound of interest is D-glucuronic acid, which may be used as a dietary supplement as a calcium salt form. Moon et al. [98] constructed a heterologous pathway in *E. coli* comprised of *myo*-inositol-1-phosphate synthase (*Ino*1) from *S. cerevisiae* and *myo*-inositol oxygenase (*MIOX*) from mice which resulted in 0.3 g/L glucuronic acid production. The titer was further improved to 1 g/L by introducing a third enzyme (urinate dehydrogenase from *Pseudomonas syringae*) to eliminate the accumulation of an intermediate (*myo*-inositol). Another compound, L-ascorbic acid (one form of vitamin C), is regarded as an important food additive for animals. Yeast possesses two endogenous enzymes (D-arabino-1,4-lactone oxidase and L-galactose dehydrogenase) which can be overexpressed to enabled the bioconversion of plant or animal intermediates into L-ascorbic acid with titers of 100 mg/L [124].

Alcohols Lab-scale production of alcohols, mainly as potential gasoline substitutes, consists of higher alcohols such as 1-propanol and 2-butanol. As an example, keto-acids derived from amino acid synthesis pathways were converted into equal ratios of 1-propanol and 1-butanol with a total titer of 2 g/L by optimizing metabolic pathways in *E. coli* [128]. This route was further developed by bypassing threonine biosynthesis, resulting in a shorter pathway [13]. To accomplish this, citramalate synthase (*CimA*) was evolved for enhanced activity over a wide range of temperatures resulting in an improvement in 1-propanol production to a titer of 3.5 g/L. In addition to using glucose as a carbon source, other sustainable materials such as cellobiose, cellulose, switchgrass, and corn stover have been used to produce 1-propanol. By adding a bi-functional butyraldehyde/alcohol dehydrogenase to *Thermobifida fusca* a titer of 0.48 g/L 1-propanol was achieved using switchgrass as a feedstock [42]. 2-Butanol, a chiral compound, is more preferable as a fuel to other butanols and an attractive precursor to fine chemicals. Engineered *E. coli* harboring a 2-butanol biosynthetic pathway and a heterologous alcohol dehydrogenase could produce 34 mg/L [26].

Alkanes, alkenes, fatty acid esters Biodiesel and various other hydrocarbons are attractive molecules for biosynthesis from renewable biomass. Kalscheuer et al. [67] expressed pyruvate decarboxylase from *Zymomonas mobilis* along with alcohol dehydrogenase and acyltransferase from *Acinetobacter baylyi* in *E. coli* to create a pathway for biodiesel. Using fed-batch fermentation with glucose and oleic acid supplementation, the production of fatty acid ethyl esters (FAEE, sometimes called microdiesel) reached 1.28 g/L. A similar approach produced a variety of compounds including FAEE, fatty alcohols, and waxes, in engineered *E. coli* strains directly from simple sugars with FAEE titers reaching 674 mg/L [131]. It was also possible to synthesize FAEEs from renewable plant biomass (hemicellulose) through the complementation of xylan-degrading enzymes, although the titer only reached 11.6 mg/L. A similar approach was taken in the yeast *S. cerevisiae* with glycerol as the carbon source where approximately, 0.52 g/L FAEE were produced and 85 % glycerol was consumed [159].

Initial progress has been made in alkane biosynthesis pathways where cyanobacteria pathways were expressed in *E. coli*, enabling upwards of 0.3 g/L of product consisting of C_{13} to C_{17} alkanes and alkenes [126]. A three-part metabolic engineering effort consisting of elimination of β-oxidation, overexpression of *AccABCD* acetyl-CoA carboxylase, and expression of a plant acyl–acyl carrier protein on plasmids resulted in a mixture of medium-chain fatty acids being produced which were decarboxylated into undecane at a total titer of 0.44 g/L [84]. Additionally, pentane was produced in an engineered strain of *Y. lipolytica* through the strong overexpression of a soybean lipoxygenase leading to a production of 4.98 mg/L [23].

Commodity chemicals

Amino acids Amino acid derivatives have also been explored in a variety of hosts. Vanillin, an important flavor compound, is synthesized from intermediates in aromatic amino acid biosynthesis and was initially produced at 45 mg/L in yeast [58]. This titer was improved using a technique called minimization of metabolic adjustment (MOMA) flux balance analysis (FBA) leading to two gene deletion targets that resulted in over a 10-fold increase in vanillin production (500 mg/L) [27]. Another commodity chemical branching from amino acids for biosynthesis, styrene, was produced in *E. coli* through the expression of phenylalanine ammonia lyase (*PAL2*) from *Arabidopsis thaliana* and trans-cinnamate decarboxylase (*FDC1*) from *S. cerevisiae* in an L-phenylalanine overproducing strain. These metabolic engineering efforts resulted in the production of 300 mg/L styrene directly from glucose.

Antibiotics Antibiotics and other active biologics play an important role in our ability to combat infection and disease. However, most natively producing hosts lack metabolic engineering tools or are poor candidates for bioprocessing, so pathways are commonly expressed in a heterologous fashion in more suitable host strains. An antibiotic with anti-tumor activities, echinomycin, was successfully produced in an engineered *E. coli* strain by expression of a 36-kb DNA cluster from *Streptomyces lasaliensis* [148]. The antituberculosis drug viomycin was also synthesized in trace

amounts in *Streptomyces lividans* [18]. In 2010, an antibacterial antibiotic, erythromycin A, was synthesized in *E. coli* by successfully transferring the native 55-kb gene cluster, with titers approaching 10 mg/L [163]. Due to the length of native antibiotic synthesis pathways, metabolic engineering of these molecules remains an obstacle. However, in the case of midecamycin production in *Streptomyces fradiae*, the host already possessed pathways for three major precursors used by polyketide synthases (PKSs). In this case, heterologous expression only required a small stretch of DNA corresponding to the PKS and production levels were able to reach 1 g/L [122].

Natural organic compounds With the exception of isoprene (discussed in industrial-scale production), most terpenes and terpenoids are still limited to lab-scale production. For monoterpenes, Dunlop et al. [45] generated a library of efflux pumps and expressed this library in *E. coli* with the goal of increasing monoterpene production. This strategy improved the production of limonene (a monoterpene) about 1.5-fold resulting in a titer of 60 mg/L.

Going from monoterpenes to sesquiterpenes, Peralta-Yahya et al. [113] identified bisabolane (a sesquiterpene) as an alternative advanced fuel. The precursor to this molecule, bisabolene, can be biosynthesized in both *E. coli* and *S. cerevisiae* to levels of more than 900 mg/L and converted chemically into bisabolane. Farhi et al. [48] increased the metabolic flux towards farnesyl diphosphate (an intermediate in this process) for the production of sesquiterpenes in yeast leading to 1.5 mg/L of valencene. Albertsen et al. [4] constructed a fusion protein of key enzymes (like farnesyl diphosphate synthase and patchoulol synthase) and enhanced the production of patchoulol (a sesquiterpene) by two-fold to 25 mg/L. Chang et al. [30] functionalized sesquiterpene derivatives through optimization of plant P450 expression in *E. coli,* resulting in the production of 105 mg/L 8-hydroxycadinene, a precursor to the antimalarial gossypol. Asadollahi et al. [8] discovered that altering the expression of HMG-CoA reductase could regulate the mevalonate pathway in *S. cerevisiae* and enabled the production of cubebol (a sesquiterpene alcohol) to a titer of 10 mg/L. The native methylerythritol phosphate pathway (MEP) in *E. coli* has been reported to have feedback inhibition and this was overcome through expression of the mevalonate (MVA) pathway from *S. cerevisiae* in *E. coli* [93]. The additional overexpression of enzyme *ispA* enabled the first direct production of farnesol in *E. coli* at a titer of 135.5 mg/L [144]. It should be noted that the titers of the majority of reported sesquiterpenes have been limited to lab-scale production. An important exception consists of those sesquiterpenes produced by Amyris, which has successfully produced them in titers of greater than 5 g/L.

For diterpenoids, most reported compounds have been produced in *S. cerevisiae*, such as casbene (an antifungal), miltiradiene (precursor for bioactive class tanshionones), and taxadiene (precursor for an anticancer drug, paclitaxel), but with low titers (31 mg/L [72], 488 mg/L [39, 174], and 8.7 mg/L [47], respectively). When using *E. coli* as the engineering host, the production of taxadiene reached 1 g/L through feeding of glycerol [2]. In the case of sclareol which is a diterpenediol used for the production of odorants, genetically engineered *E. coli* were able to achieve a titer of 1.5 g/L through high-cell-density fermentation [125].

Most tetraterpenes have been most successfully produced in *E. coli*, including groups of carotenoids and isoprenoids. Astaxanthin, a value-added compound for many industrial applications, was produced in a β-carotene-producing *E. coli* strain by integrating heterologous astaxanthin biosynthetic genes [82]. The carotenoid lycopene has been the subject of multiple studies investigating different metabolic engineering strategies owing to the ease of its detection. In particular, three main strategies have been benchmarked using this compound as a test case: dynamic pathway regulation [49], unguided gene knockout/overexpression searches [65], and computational gene knockout/overexpression approaches [5, 32, 65]. The maximum lycopene titer obtained through any of these approaches was 283 mg/L through rational identification of overexpression targets [32]. The most studied isoprenoid compound is β-carotene, which is an antioxidant supplement in food, cosmetics, and nutrients. A carotenoid pathway from a native producer (*Xanthophyllomyces dendrorhous*) was integrated into the genome of *S. cerevisiae* along with the overexpression of several critical enzymes to achieve a final strain capable of producing appreciable amounts of β-carotene [143]. Finally, an *E. coli* strain harboring a complete MVA pathway with top portion from *Enterococcus faecalis* and bottom portion from *Streptococcus pneumonia* yielded 465 mg/L β-carotene from glycerol [158].

Another source of compounds rich with potential new drugs are plant-derived flavonoids. Leonard et al. engineered central flux towards the production of an intermediate (flavanone) and thus increased the production of eriodictyol, naringenin, and pinocembrin, and anthocyanins to 52 mg/L [85], 119 mg/L [85], 700 mg/L [86], and 113 mg/L [86], respectively. The production of naringenin was further enhanced by applying OptForce flux balance analysis, increasing the levels of the intermediate malonyl-CoA, resulting in a titer of 474 mg/L [112]. The flavonoid 7-*O*-methyl aromadendrin (an anti-inflammatory and anticancer compound derived from the naringenin pathway) could be synthesized at 30 mg/L through feeding naringenin [92]. A computational gene knockout approach was also applied to identify genotypes with enhanced cofactor

production, and enabled engineered *E. coli* to produce leucocyanidin and (+)-catechin (an antioxidant) [31].

In addition to the major groups of natural organic compounds discussed above, several other natural compounds have been reported at lab-scale production, such as indolylglucosinolate (anticancer drug) [95], magnoflorine (antibacterial agent) [96], anthraquinone (anticancer drug) [164], lovastatin (cholesterol level control drug) [10], simvastatin (cholesterol level control drug) [153], alkaloids (such as dihydrosanguinarine [53], scoulerine [96], and (*S*)-reticuline [59, 96]), hormones (such as glucagon [46], moth pheromone [44]), and various proteins (such as antibodies [57], antigens [140], viruses [91], and insulin-like growth factor 1 [136]). Resveratrol, a stilbenoid, has also gained much attention and can be synthesized in both *E. coli* [68, 89, 152], yeast [21, 132, 145, 147, 168], and mammalian cells [168]. It should be noted that several of these studies fed the strains with precursors, which might not be a cost-effective strategy for higher-scale production of resveratrol.

Other perspectives

Despite the myriad of compounds and organisms which have been involved in this impressive list of metabolic engineering success stories, the tool repertoire used by the investigators involved fall into a handful of categories. Fortunately, these tools are beginning to expand which allows for exploration of new hosts and new molecules. Beyond metabolic engineering efforts, there are further challenges involved in scale-up, including control of product quality and enhancement of microorganism performance [138].

It should be noted again that only published titers of compounds (from publications and patents) were discussed and compared in this review. However, various other important metrics including yield and productivity studies are important factors in determining lab and industrial scale differentiation. Unpublished titers recorded in research groups or much higher titers in industrial confidential documents are certainly demonstrating further successes in the field.

Conclusion and future prospects

In this review, we have described many successes in improving the type and breadth of molecules produced via metabolic engineering. In many cases, *E. coli* and *S. cerevisiae* dominate the metabolic engineering literature based on the number of laboratory tools available. Yet, for many industrial-scale productions, several alternative host strains which naturally overproduce the desired compound are more dominant. Thus, the development of molecular tools for engineering these alternative hosts presents a worthy challenge for the field which will enable significant gains to be realized. At present, the transition from laboratory scale to industrial scale indeed seems to be inversely proportional to the amount of heterologous enzymes that are added to the host. Fortunately, this trend is also beginning to reverse with successes and tools in synthetic biology. Nevertheless, the further development of high throughput techniques for engineering at the pathway scale is likely to be very important as we expand to more complex molecules that extend further away from central metabolism. To enable rapid design of bioprocesses with defined productivities, quantitative techniques must be developed and applied to systems of interest for metabolic engineers. Taken together, the work described above highlights the versatility and power of metabolic engineering while emphasizing its emergence as an engineering discipline and illustrating its promise for the future.

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