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Metabolic engineering strategies to bio-adipic acid production

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Adipic acid is the most industrially important dicarboxylic acid as it is a key monomer in the synthesis of nylon. Today, adipic acid is obtained via a chemical process that relies on petrochemical precursors and releases large quantities of greenhouse gases. In the last two years, significant progress has been made in engineering microbes for the production of adipic acid and its immediate precursors, muconic acid and glucaric acid. Not only have the microbial substrates expanded beyond glucose and glycerol to include lignin monomers and hemicellulose components, but the number of microbial chassis now goes further than *Escherichia coli* and *Saccharomyces cerevisiae* to include microbes proficient in aromatic degradation, cellulose secretion and degradation of multiple carbon sources. Here, we review the metabolic engineering and nascent protein engineering strategies undertaken in each of these chassis to convert different feedstocks to adipic, muconic and glucaric acid. We also highlight near term prospects and challenges for each of the metabolic routes discussed.

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Introduction

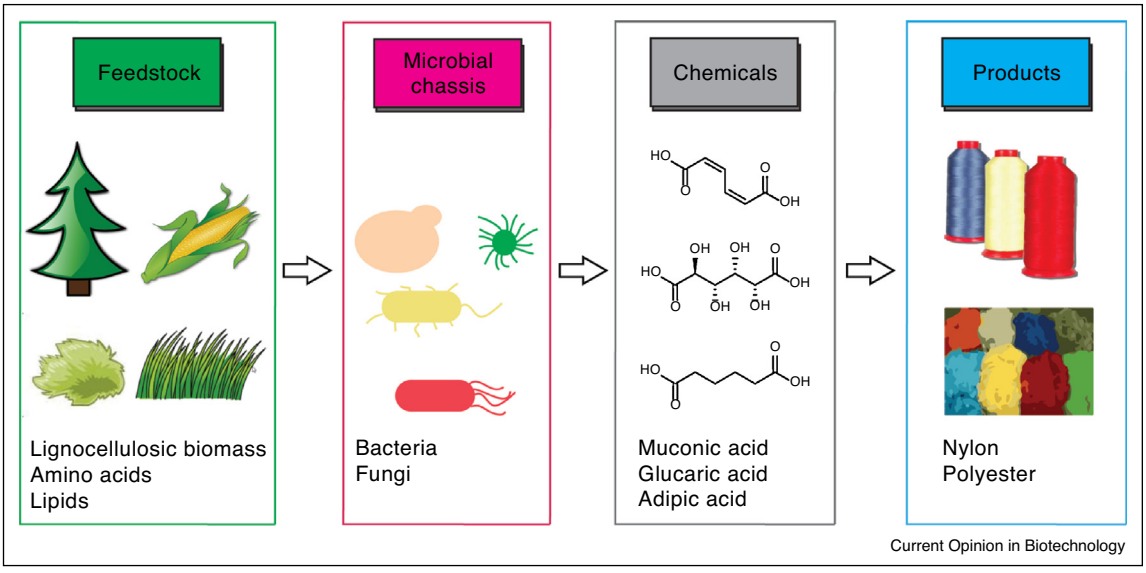
In 2010, global production of adipic acid was estimated at 2.6 million tons, with 65% going to the production of nylon-6,6 fibers [1,2]. Commercially, adipic acid is synthesized from petroleum-derived benzene, which is reduced to cyclohexane followed by oxidation to a mixture of cyclohexanol and cyclohexanone. This mixture is further oxidized using a vanadium or copper catalyst with nitric acid to produce adipic acid. The commercial synthesis of adipic acid not only generates ~10% of the world's man-made nitrous oxide [3], a greenhouse gas 300-times more potent than carbon dioxide, but it also uses benzene, a known carcinogen, as a starting material. Production of bio-adipic acid, wherein the carbons are derived from a renewable feedstock, has the potential to reduce greenhouse gas emissions and eliminate the need for fossil fuel precursors. Based on the 2.6 million tons of adipic acid produced annually from petroleum, renewable production of adipic acid could eliminate the use of benzene found in 6 billion barrels of crude oil.

Advances in metabolic engineering and synthetic biology now allow the engineering of microbes for the production of advanced biofuels [4], commodity chemicals and pharmaceuticals [5]. In the last two years, a number of different metabolic engineering strategies have been applied to the microbial synthesis of adipic acid and its immediate precursors muconic acid and glucaric acid (Figure 1, Table 1). In particular, there has been an expansion in the renewable feedstocks used as starting materials, as well as an increase in the number of metabolic pathways engineered for the microbial synthesis of these compounds. The number of chassis has also risen and now goes beyond the workhorse chassis of *Escherichia coli* and *Saccharomyces cerevisiae*, to include the naturally aromatic compound metabolizer *Pseudomonas putida* [6], the cellulolytic bacteria *Thermobifida fusca* [7], and *Klebsiella pneumonia* [8], which can metabolize a number of different carbon sources. In this review, we focus on these latest advances and direct the reader to recent comprehensive reviews on adipic acid and muconic acid production [2,9,10].

Metabolic pathways to muconic, glucaric, and adipic acid

A total of ten biosynthetic pathways from lignocellulosic biomass, lipids and amino acids have been leveraged for the production of adipic acid, muconic acid and glucaric acid, with pathways utilizing lignin, lipids and amino acids as precursors demonstrated since 2014 (Figure 2). In 1994, Frost and Draths developed the first production of bio-adipic acid by exploiting *E. coli* primary metabolism to convert glucose to 3-dehydroshikimic acid (DHS), and using three heterologous enzymes to produce protocatechuic acid (PCA), catechol and ultimately cis-cis muconic acid, which was in turn chemically hydrogenated to adipic acid [11]. More recent pathways to muconic acid

Figure 1



Bio-adipic acid production. In the last two years an increasing number of feedstocks and microbial chassis have been used for the production of adipic acid and its immediate precursors, muconic acid and glucaric acid. Adipic acid is one of the most industrially important dicarboxylic acids being used in the synthesis of nylon and polyesters.

include re-routing chorismate to anthranilic acid [12], 2,3 dihydroxybenzoic acid [13,14], salicylic acid [15,16], or *p*-hydroxybenzoic acid [17] to produce catechol, and a β-ketoadipate pathway modified to stop at PCA followed by a re-route to catechol [18••]. Glucaric acid has also been microbially produced from glucose via a synthetic five step pathway [19•]. Finally, adipic acid has been microbially synthesized directly from renewables by taking advantage of (1) the reversed adipate degradation pathway [20,21], (2) the reversed β-oxidation coupled to alkane degradation [22], and (3) the reversed phenylacetate degradation pathway coupled to reversed adipic acid degradation [23••].

Muconic acid from cellulose- and hemicellulose-derived feedstocks

Microbes can convert cellulose-derived glucose or hemicellulose-derived xylose to phosphoenolpyruvate (PEP) via glycolysis and erythrose-4-phosphate (E4P) via the pentose phosphate pathway. Aromatic amino acid biosynthesis takes PEP and E4P and eventually converts them to DHS, a key node in the biosynthesis of muconic acid. The concept of leveraging aromatic amino acid biosynthesis for muconic acid production is rooted in the successful engineering of *E. coli* for the production of aromatic amino acids at g/L levels [24].

In *E. coli*, DHS has been converted to muconic acid via PCA and catechol resulting in 36.8 g/L of muconic acid with a 22% yield from glucose [25•]. Key to achieving this yield was the introduction of a feedback resistant 3-deoxy-D-arabino-heptulosonic acid 7-phosphate (DAHP) synthase, which condenses PEP and E4P in the first committed step of muconic acid biosynthesis. Of note, in this strain the conversion of DHS to aromatic amino acids was blocked and cell growth required addition of aromatic amino acids and vitamins to the media. The same DHS to muconic acid pathway has been introduced in *S. cerevisiae* to produce 1.56 mg/L of muconic acid [26], and, in 2013, 141 mg/L of muconic acid with a 0.8% yield from glucose [27•]. Interestingly, the low mg/L titers were obtained despite successful implementation of similar metabolic engineering strategies as in *E. coli*, including relieving feedback inhibition of DAHP synthase, and deletion of glucose-6-phosphate dehydrogenase to force the carbon flux from glycolysis to the pentose phosphate pathway via the transketolase reaction, which is kinetically hindered *in vivo*, rather than glucose-6-phosphate dehydrogenase. More recently, the DHS to muconic acid pathway was introduced into a diploid *S. cerevisiae* strain carrying similar up- and down-regulations to increase the carbon flux to the shikimic acid pathway. Additionally, the diploid carried a 3-dehydroquinate dehydratase mutant (Aro: D1409A) that stopped conversion at DHS, reducing synthesis of the byproduct shikimate. When this strain was grown under anaerobic conditions to improve the activity of the oxygen sensitive protochatechuic decarboxylase, it produced 560 mg/L of muconic acid [28]. The *S. cerevisiae* results underscore the extensive regulation of aromatic amino acid biosynthesis in yeast, where limited pathway modifications do not result in the g/L titers seen in

Table 1

Yields for muconic acid, glucaric acid and adipic acid from glucose

Chassis	Pathway	Maximum theoretical yields [49]	Experimental percent of maximum theoretical yield[h,i]
E. coli	**DHS** → PCA → catechol → muconic acid	83%[a]	27% Niu *et al*. [25•][j] 7.81% Zhang *et al*. [48••][k]
E. coli	**Anthranilate** → catechol → muconic acid	50%	2.24% Sun *et al*. [29][k]
E. coli	**PHB** → PCA → catechol → muconic acid	52%	4.48% Sengupta *et al*. [17][k]
E. coli	**Isochorismate** → salicylate → catechol → muconic acid	53%	16.3% Lin *et al*. [15][k] 37.0% Noda *et al*. [16][k]
E. coli	**2,3-Dihydroxybenzoate** → catechol → muconic acid	67%	Wang and Zheng [13][l]
E. coli	**Glucose** → glucaric acid	99%[b]	9.79% Moon *et al*. [19•][k]
E. coli	**Fatty acids** → adipic acid	NC[c]	Clomburg *et al*. [22]
E. coli	**AcCoA** + **SuccCoA** → adipyl-CoA → adipic acid from glycerol	53%[d]	cCoA + 6.6% Cheong *et al*. [23••][k]
E. coli	**AcCoA** + **SuccCoA** → adipyl-CoA → adipyl-phosphate → adipic acid	92%[e]	<1% Yu *et al*. [44][k]
P. putida	Muconic acid from *p*-coumaric acid	76%	88% Vardon *et al*. [18••][j]
P. putida	Muconic acid from benzoate	40%[f]	93% Vardon *et al*. [18••][m]
S. cerevisiae	**DHS** → PCA → catechol → muconic acid	61%[g]	<1% Curran *et al*. [27•][k] <1% Weber *et al*. [26][k] 2.9% Suastegui *et al*. [28][k]

For all *E. coli* and *S. cerevisiae* calculations no reactions were removed from the model. For all *P. putida* calculations, PCA dioxygenase was replaced with PCA decarboxylase to allow for catechol synthesis; the muconic acid to β-ketoadipate conversion was not removed. Maximum theoretical yields from glucose unless specified. Genome scale models used for *E. coli P. putida and S. cerevisiae* were iAF1260b [50], iJN746 [51], iMM904 [52], respectively. Bold: last endogenous chemical in the model.

[a] Zhang *et al*. reported a theoretical maximum molar yield of 86% based on stoichiometry.

[b] The maximum theoretical yield (MTY) calculations did not account for cell growth. Practically, MTY will be lower as glucose will also need to be routed through glycolysis for cell growth.

[c] NC: not calculated. Without removing reactions from the *E. coli* model hexanoic acid is produced via fatty acid biosynthesis.

[d] All enzymes in this pathway are used in the reverse direction. The model does not take into account the kinetics of this process.

[e] One enzyme in this pathway is used in the reverse direction and the rest have specificity for C4 rather than C6 substrates. The model does not take these facts into account.

[f] Muconic acid to β-ketoadipate reaction could not be removed from the model and significant carbon is used for growth.

[g] Predicted MTY from glucose matches that from Curran *et al*., *Met Eng*, 2012.

[h] Experimenters use a variety of glucose and glycerol concentrations in both complex and minimal medias.

[i] Percent of theoretical yield calculated as $\frac{raw\ percent\ yield}{calculated\ maximum\ theoretical\ yield}$.

[j] Raw percent yield reported by the author, no details for carry out independent calculations provided.

[k] Raw percent yield calculated as $\frac{titer\ of\ product\ muconic,\ glucaric,\ or\ adipic\ acid)in\ moles}{total\ moles\ of\ fed\ carbon\ source\ glucose\ and\ glycerol)}$.

[l] No details for carry out independent calculations provided.

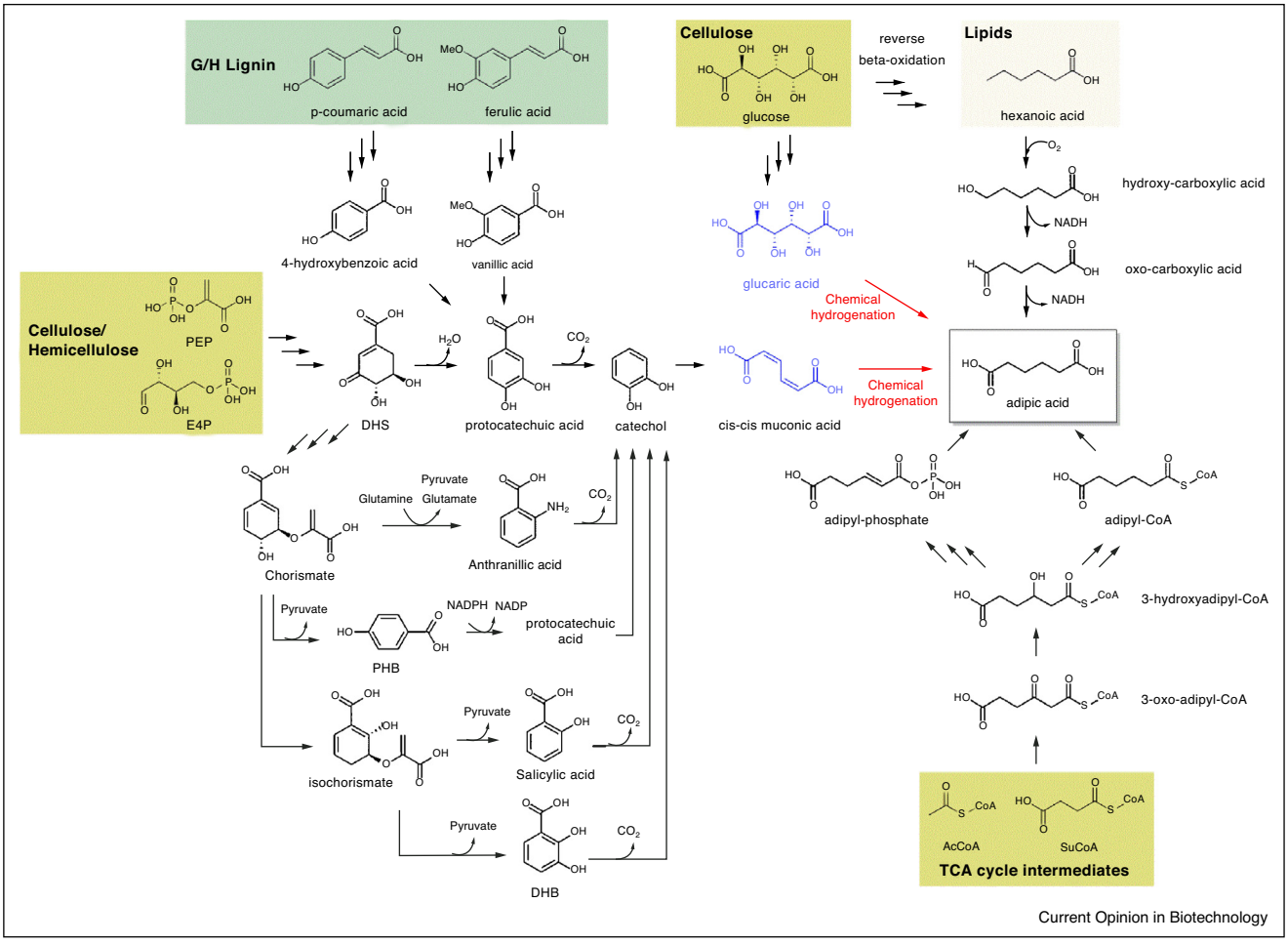
[m] Due to limitations in the modeling of adipic acid production from benzoate (see f), reported yield is the raw percent yield reported by the author.

E. coli. Improving muconic acid yields in *S. cerevisiae* will likely require global regulation of the transcription machinery, which will require a muconic acid high-throughput assay for identification.

Aromatic amino acid biosynthesis can take DHS to chorismate, which *E. coli* can convert to anthranilic acid or *p*-hydroxybenzoic acid (PHB), intermediates that can enter muconic acid biosynthesis at the level of catechol or PCA, respectively. *E. coli* production of muconic acid via anthranilic acid results in 390 mg/L of muconic acid from a glycerol/glucose mixture [29]. Crucial to this strategy was screening for an efficient heterologous anthranilate-1,2-dioxygenase to convert anthranilate to catechol, increasing the levels of chorismate, deleting the tryptophan biosynthetic pathway after anthranilate, and introducing a glutamine regeneration system as glutamine is depleted upon conversion of chorismate to anthranilic acid. Due to deletion of the tryptophan biosynthetic pathway, supplementation of the media with tryptophan was needed for cell growth. Production of muconic acid in *E. coli* via PHB resulted in 170 mg/L of muconic acid from glucose [10]. Key to this approach were, screening for an efficient heterologous PHB hydrolase to convert PHB to PCA, increasing PHB availability, knocking out genes predicted to increase carbon flux *in silico* by Robust-Knock [30], and overexpressing a transketolase to route carbon flux from glycolysis to pentose phosphate pathway. A drawback of this strategy is the use of NADPH by PHB hydrolase, potentially resulting in a redox imbalanced strain.

E. coli converts chorismate to isochorismate and subsequently salicylic acid via the siderophore biosynthetic pathway, or converts chorismate to isochorismate and subsequently 2,3-dihydrobenzoic acid (DHB) via the enterobactin biosynthetic pathway. Both salicylic acid and DHB enter muconic acid biosynthesis at the level

Figure 2



Metabolic engineering strategies to bio-adipic acid production. Shown, metabolic pathways highlighted in this review. Green box: lignin-derived substrates. Yellow box: cellulose- and hemicellulose-derived substrates. Grey box: lipid-derived substrates. Adipic acid, muconic acid and glucaric acid in blue. DHS: 3-dehydroshikimic acid; PHB: *p*-hydroxybenzoic acid; DHB: 2,3-dihydrobenzoic acid.

of catechol. Production of muconic acid in *E. coli* via salicylic acid results in 1.5 g/L of muconic acid from a glycerol/glucose mixture [15]. This work relied on previously identified efficient isochorismate synthase and isochrorismate pyruvate lyase [31] to produce salicylic acid, and a phenylalanine overproducing strain which could not synthesize phenylalanine or tyrosine due to deletions after chorismate. Further screening for an efficient heterologous salicylate-1-monooxygenase, and reducing the plasmid copy number for the expression of salicylate-1-monooxygease and catechol-1,2-dioxygenase resulted in the aforementioned yield. Due to deletions in the phenylalanine and tyrosine pathway, the production media needed to be supplemented with yeast extract for cell growth. *E. coli* production of muconic acid via DHB resulted in 480 mg/L of muconic acid from a glycerol/glucose mixture [14], and more recently in 605 mg/L of muconic acid (carbon source not specified) [13]. The earlier work identified a novel prokaryotic *Klebsiella pneumoniae* DHB decarboxylase to convert DHB into catechol, and upregulated DHB synthesis to achieve the mentioned yield. The latter work took advantage of the *K. pneumoniae* DHB decarboxylase and increased the extent of DHB synthesis upregulation.

Muconic acid from lignin-derived feedstock

Lignin-derived monomers such as *p*-coumaric acid, coniferyl alcohol and caffeic acid can serve as substrates to produce muconic acid via the PCA branch of a modified β-ketoadipate pathway. In the β-ketoadipate pathway PCA is ultimately converted to acetyl-CoA and succinyl-CoA. Stopping the β-ketoadipate pathway at PCA and re-routing PCA to catechol leads to the production of muconic acid. Deleting the transformation of muconic acid to β-ketoadipate results in muconic acid accumulation. The β-ketoadipate pathway is not present in *E. coli*

or *S. cerevisiae*, but it is endogenous of aromatic degrading organisms, such as *Pseudomonas putida*. Since 2015, *P. putida* has been extensively engineered to produce muconic acid not just from lignin-derived monomers, but also directly from pretreated biomass.

In 2015, *P. putida* was genomically engineered to route guaiacyl (G) units, derived from coniferyl alcohol, and *p*-hydroxyphenyl (H) units, derived from *p*-coumaric acid monomers, to convert G/H lignin into muconic acid [18••]. Specifically, PCA dioxygenase was replaced with PCA decarboxylase to convert PCA into catechol, and catB/catC were deleted to avoid degradation of muconic acid. This strain achieved 67% conversion of *p*-coumaric acid, 14% conversion of coniferyl alcohol, and 28% conversion of caffeic acid into muconic acid. It is worth highlighting that muconic acid is not the final product, but the immediate intermediate to adipic acid. Thus, any muconic acid production must be followed by a separation of muconic acid from the culture broth, purification and hydrogenated to produce the final adipic acid. The 2015 work in *P. putida* also tackled the downstream process of converting muconic acid into adipic acid. Specifically, by taking advantage of the different physical properties of the aromatic intermediates and the linear muconic acid, aromatics were removed from the culture media using activated carbon. Muconic acid was then crystalized from the media by lowering the pH and temperature resulting in the recovery of 74% of muconic acid with >97% purity. Crystallization of muconic acid from the media reduced the number of inhibitors from the biological process that could inactivate the catalyst, and hydrogenation of muconic acid over palladium and carbon resulted >97% conversion of muconic acid with 1% Pd/C loading after 35 min. Finally, alkaline pretreated biomass was converted to muconic acid with 67% molar yield. The most recent titers for muconic acid using benzoate as the substrate and glucose to support cell growth are 34.5 g/L [32]. Follow up work has focused on improving the chemical hydrogenation process, making nylon-6,6 using bio-adipic acid [33], and increasing the activity of PCA decarboxylase [34], a bottleneck in the conversion of PCA to muconic acid. Remaining challenges in the conversion of lignin to muconic acid include improving the enzymatic activity or removing the endogenous regulation of vanillate-*O*-methylase, which converts vanillate from G-lignin substrates into PCA. Though, this challenge can be circumvented by using a harsher biomass pretreatment processes that brings G/H-lignin substrates to phenol and guaiacol, which can be converted to catechol in a single step.

Key enzymes in microbial synthesis of muconic acid

Independent of the carbon source, the microbial synthesis of muconic acid depends on the activity of catechol-1,2-dioxygenase and, for pathways routing via PCA, PCA decarboxylase. Recently, a catechol-1,2-dioxygenase double mutant (Cat:L73F/P76A) was engineered using rational design to achieve a five-fold increase in turnover rate with an equivalent increase in K_M resulting in a comparable catalytic efficiency as the wild type enzyme [35]. Cat:L73F/P76A also showed improved expression when compared to wild type. When Cat:L73F/P76A was used in the microbial synthesis of muconic acid from glucose, it increased titers 22%. In separate work, catechol-1,2-dioxygenase was purposefully engineered for improved expression via codon optimization using a stationary phase codon optimization matrix compiled from the fifty most highly expressed proteins in *S. cerevisiae* after 3 days of growth [36]. The codon optimized catechol-1,2-dioxygenase had a two-fold higher conversion of catechol to muconic acid. To increase the *in vivo* PCA decarboxylase activity 14-fold over wild type in *E. coli*, *K. pneumonia* PCA decarboxylase was co-expressed with an upstream gene found in the same operon (KpdB) [37]. This strategy to increase PCA decarboxylase is chassis agnostic as it has also been successfully applied in *P. putida* [34].

Glucaric acid from cellulose-derived feedstock

Glucaric acid is one of the top value-added chemicals from biomass identified by the US Department of Energy in 2004 [38], and can be reduced to adipic acid. Mammals synthesize glucaric acid from glucose in 10-steps; however, a 5 step synthetic pathway has been engineered in *E. coli* to route glucose via myo-inositol to produce 1 g/L of glucaric acid [19•]. A bottleneck in the synthetic pathway is the conversion of myo-inositol to D-glucuronic acid by myo-inositol oxygenase (MIOX), the activity of which drops significantly after 24 hours. Using protein scaffolds to increase the myo-inositol concentration around MIOX increased MIOX specific activity and brought glucaric acid titers to 2.5 g/L [39]. Improved conversion of myo-inositol to glucaric acid has been achieved by increasing the solubility of MIOX using solubility tags, yielding an enzyme that retains almost 40% of its activity after 72 hours [40]. More recently, the use of a dynamic pathway regulator based on quorum sensing circuitry allowed for control of the timing of up- and down-regulation of the glycolysis flux control enzyme phosphofructokinase to route carbon from glycolysis to glucaric acid production. Using this strategy, a glucaric acid production of 0.7 g/L was achieved without the need for inducers [41].

Adipic acid production from lipids

Lipids, in the form of fatty acids, can be converted directly to adipic acid. Bacteria and some yeast degrade long chain fatty acids and alkanes via oxidation of the ω-carbon. Although biological systems produce long-chain fatty acids, microbes can be modified to produce medium-chain fatty acids, most successfully via reversal of the β-oxidation cycle. By bringing together reversed

β-oxidation and the ω-oxidation pathway, *E. coli* has been engineered to produce 170 mg/L of adipic acid from glycerol [22]. Key to this strategy has been the introduction of a thiolase that preferentially condenses acetyl-CoA and C4/C6 acyl CoAs and a thioesterase that favors hydrolysis of medium-chain fatty acyl-CoAs. With C6-C10 fatty acids in hand, introduction of *P. putida* ω-hydroxylase, which enables *P. putida* growth on C6-C16 alkanes, allowed the production of ω-hydroxy-carboxylic acids. Despite evidence of *P. putida* ω-hydroxylase multi-oxidizing fatty acid methyl esters to dicarboxylic acids [42], adipic acid production required expression of NAD^+-dependent alcohol and aldehyde dehydrogenases [22]. Insight for the choice of these dehydrogenases came from the degradation of cyclohexanol in *Acinetobacter*, which pointed at their activity on medium-chain ω-hydroxyacids [43]. Among the challenges to increasing adipic acid production via this strategy is the strain redox balance as two NADHs are produced per adipic acid generated. Further, as fatty acid hydroxylation requires molecular oxygen, using microaerobic conditions for cell growth to take advantage of oxidative phosphorylation to convert NADH into ATP is not an efficient strategy. Likely an NAD^+ recycling system or a hydroxylase that requires a metal rather than NAD^+ or $NADP^+$ to carry out the oxidation will be needed to further increase adipic acid titers.

Adipic acid production from tricarboxylic acid cycle intermediates

Glucose, xylose, lipids, and amino acids can be metabolized to the tricarboxylic acid (TCA) cycle intermediates acetyl-CoA (AcCoA) and succinyl-CoA (SuCoA). Inspired by the *Penicillium chrysogenum* adipic acid degradation pathway and the broad substrate specificity of *Clostridium acetobutylicum* butanol synthesis enzymes, a six step synthetic pathway to adipic acid from AcCoA and SuCoA has been engineered [44]. For this pathway, a thiolase which hydrolyzes 3-oxo-adipyl-CoA into AcCoA and SuCoA was used in reverse to generate 3-oxo-adipyl-CoA. Next followed reduction, dehydration, and hydrogenation of 3-oxo-adipyl-CoA by enzymes previously shown to convert 3-ketohexanoyl-CoA into hexanoyl-CoA [45] generated adipyl-CoA. Finally, adipyl-CoA was converted into adipyl-phosphate and ultimately into adipic acid using *C. acetobutylicum* enzymes with broad substrate specificity known to convert butyryl-CoA into butyrate. Introduction of the synthetic pathway in a succinic acid overproducing *E. coli* strain resulted in 639 μg/L of adipic acid from glucose. More recently, a second synthetic pathway to produce adipic acid from AcCoA and SuCoA in *E. coli* was engineered [23••]. This second synthetic pathway combines reversal of both the phenylacetate degradation pathway and the adipic acid degradation pathway in a mixed-acid fermentation-deficient *E. coli* that retained the reductive branch of the TCA cycle and overexpressed a CoA transferase for increased SuCoA availability to produce 2.5 g/L of adipic acid from glycerol in a bioreactor. Both adipic acid synthetic pathways rely on at least one enzyme used in reversed direction. For the butanol/butyrate inspired work, improving the thiolase reaction in the reversed direction, and optimizing the substrate specificity of the butanol/butyrate pathway enzymes will aid in increasing adipic acid titers. An eventual major concern will be the redox balance of the strain as 2 NADHs are needed per adipic acid produced, a similar problem to that faced in the production of butanol via the *C. acetobutylicum* pathway [46]. For the phenylacetate degradation inspired pathway, *all* the enzymes are used in the reversed direction and concerns about the thermodynamic and kinetic feasibility of this process need to be addressed.

Alternative chassis and co-cultures to produce adipic acid and its precursors

To date, production of adipic acid and its precursors has predominantly taken place in *E. coli*, *S. cerevisiae* and *P. putida*. Recently, alternative microbial chassis have been explored, including *T. fusca*, which has a reverse adipate degradation pathway, and *K. pneumoniae*, which has a catechol synthesis pathway. The pivotal work in *T. fusca* has been the identification of the reductase that converts 2,3-dehydroadipyl-CoA to adipyl-CoA as the flux control point in adipic acid biosynthesis [17]. Using *T. fusca* B6, in which the reductase is highly upregulated, resulted in 2.2 g/L of adipic acid from glucose. In *K. pneumoniae*, disruption of the aromatic amino acid biosynthetic pathway, introduction of *P. putida* catechol-1,2-dioxygenase, and deletion of endogenous enzymes downstream of muconic acid resulted in 2.1 g/L of muconic acid from glucose [19•]. Co-cultures from the same species have also been explored for the production of muconic acid. Specifically, production of muconic acid from simple sugars via DHS, PCA and catechol has been split into two different *E. coli* strains, the first specialized in DHS production, the second specialized in DHS to muconic acid conversion [47,48••]. Key to this strategy was DHS diffusion into the media from the first strain and using a DHS transporter to increase the DHS concentration in the second strain. This co-culture resulted in 2 g/L of muconic acid from glycerol. Introduction of all the enzymes in the same *E. coli* strain resulted in poor cell growth and significantly reduced muconic acid production. Further, by modifying the *E. coli* engineered to produce DHS to only utilize xylose and the *E.coli* engineered to convert DHS into muconic acid to only utilize glucose, the co-culture strategy circumvented the carbon catabolite repression and after media and reactor optimization the co-culture system produced 4.7 g/L of muconic acid from a glucose/xylose mixture.

Conclusion

Production of bio-adipic acid has the potential to eliminate the reliance on petroleum precursors and reduce

greenhouse emission from the chemical synthesis of adipic acid. Among the metabolic routes to produce muconic acid from cellulosic and hemicellulosic feedstock, the original pathway that routes DHS directly to PCA, catechol and ultimate muconic acid shows the highest theoretical yield (83%) and still has the highest muconic acid titers shown to date (36.8 g/L). However, if pre-treated lignin or lignin monomers are the desired substrate, *P. putida* with its endogenous β-ketoadipate pathway is the most promising chassis with 67% molar yield from *p*-coumarate and ferulate present in pretreated lignin. There is currently a single pathway engineered for the microbial synthesis of glucaric acid, and while this pathway has potentially a 99% theoretical yield from glucose, the great pull on glucose from glycolysis has resulted in a maximum titer of 2.5 g/L of glucaric acid or 9.8% of theoretical yield. Both muconic and glucaric acid require purification from the microbial broth and chemical hydrogenation to be converted to adipic acid and an exciting solution has been the development of synthetic pathways and the discovery of natural pathways for the direct microbial synthesis of adipic acid. Although the yields for the adipic acid pathways are currently relatively low, with the best adipic acid titer being 2.5 g/L, the removal of the chemical hydrogenation step has the potential to eliminate the need for precursor (muconic acid, glucaric acid) purification and a second reactor for chemical hydrogenation. Thus far, there has been limited engineering of enzymes in the muconic acid and adipic acid pathways. Increasing the enzymatic activity and/or disrupting the regulation of enzymes in the PCA branch of the β-ketoadipate, in particular vanillate-*O*-methylase, PCA decarboxylase and catechol-1,2-dioxygenase has the potential to increase muconic acid yields from lignin and cellulosic/hemicellulosic-derived substrates. With respect to adipic acid synthesis, engineering the substrate specificity of the ω-oxidation enzymes and reducing the dependence of the ω-oxidation enzymes on NAD^+ and molecular oxygen to carry out oxidations, will be necessary to improve yields. Additionally, increasing the activity of phenylacetate degradation enzymes in the reverse direction has the potential to increase adipic acid yields.

Conflict of interest

The authors declare no conflict of interests.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.copbio.2017.03.006.

References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. Merchant Research & Consulting: L. Adipic acid 2011 World Market Outlook and Forecast. (2011).
2. Polen T, Spelberg M, Bott M: **Toward biotechnological production of adipic acid and precursors from biorenewables**. *J Biotechnol* 2013, **167**:75-84.
3. Alini S, Basile F, Blasioli S, Rinaldi C, Vaccari A: **Development of new catalysts for N_2O-decomposition from adipic acid plant**. *Appl Catal B Environ* 2007, **70**:323-329.
4. Peralta-Yahya P, Zhang FZ, del Cardayre SB, Keasling JD: **Microbial engineering for the production of advanced biofuels**. *Nature* 2012, **488**:320-328.
5. Nielsen J, Keasling JD: **Engineering cellular metabolism**. *Cell* 2016, **164**:1185-1197.
6. Thompson B, Machas M, Nielsen DR: **Creating pathways towards aromatic building blocks and fine chemicals**. *Curr Opin Biotechnol* 2015, **36**:1-7.
7. Bugg TDH, Rahmanpour R: **Enzymatic conversion of lignin into renewable chemicals**. *Curr Opin Chem Biol* 2015, **29**:10-17.
8. Jung HM, Jung MY, Oh MK: **Metabolic engineering of *Klebsiella pneumoniae* for the production of muconic acid**. *Appl Microbiol Biotechnol* 2015, **99**:5217-5225.
9. Deng Y, Ma L, Mao Y: **Biological production of adipic acid from renewable substrates: current and future methods**. *Biochem Eng J* 2016, **105**:16-26.
10. Xie NZ, Liang H, Huang RB, Xu P: **Biotechnological production of muconic acid: current status and future prospects**. *Biotechnol Adv* 2014, **32**:615-622.
11. Draths KM, Frost JW: **Environmentally compatible synthesis of adipic acid from D-glucose**. *J Am Chem Soc* 1994, **116**:399-400.
12. Sprenger GA: **From scratch to value: engineering *Escherichia coli* wild type cells to the production of L-phenylalanine and other fine chemicals derived from chorismate**. *Appl Microbiol Biotechnol* 2007, **75**:739-749.
13. Wang J, Zheng P: **Muconic acid production from glucose using enterobactin precursors in *Escherichia coli***. *J Ind Microbiol Biotechnol* 2015, **42**:701-709.
14. Sun X, Lin Y, Yuan Q, Yan Y: **Biological production of muconic acid via a prokaryotic 2,3-dihydroxybenzoic acid decarboxylase**. *Chemsuschem* 2014, **7**:2478-2481.
15. Lin Y, Sun X, Yuan Q, Yan Y: **Extending shikimate pathway for the production of muconic acid and its precursor salicylic acid in *Escherichia coli***. *Metab Eng* 2014, **23**:62-69.
16. Noda S, Shirai T, Oyama S, Kondo A: **Metabolic design of a platform *Escherichia coli* strain producing various chorismate derivatives**. *Metab Eng* 2016, **33**:119-129.
17. Sengupta S, Jonnalagadda S, Goonewardena L, Juturu V: **Metabolic engineering of a novel muconic acid biosynthesis pathway via 4-hydroxybenzoic acid in *Escherichia coli***. *Appl Environ Microbiol* 2015, **81**:8037-8043.
18. •• Vardon DR, Franden MA, Johnson CW, Karp EM, Guarnieri MT, Linger JG, Salm MJ, Strathmann TJ, Beckham GT: **Adipic acid production from lignin**. *Energy Environ Sci* 2015, **8**:617-628.
The authors engineer *Pseudomonas putida* to produce muconic acid from model lignin monomers as well as from an alkaline pretreated biomass. The purification of muconic acid form the media and chemical hydrogenation of muconic acid to adipic acid is also demonstrated.
19. • Moon TS, Yoon SH, Lanza AM, Roy-Mayhew JD, Prather KLJ: **Production of glucaric acid from a synthetic pathway in recombinant *Escherichia coli***. *Appl Environ Microbiol* 2009, **75**:4660.
Seminal paper describing the engineering of a synthetic pathwya for the production of glucaric acid form glucose.

20. Yu JL, Xia XX, Zhong JJ, Qian ZG: **Direct biosynthesis of adipic acid from a synthetic pathway in recombinant *Escherichia coli***. *Biotechnol Bioeng* 2014, **111**:2580-2586.

21. Deng Y, Mao Y: **Production of adipic acid by the native-occurring pathway in *Thermobifida fusca* B6**. *J Appl Microbiol* 2015, **119**:1057-1063.

22. Clomburg JM, Blankschien MD, Vick JE, Chou A, Kim S, Gonzalez R: **Integrated engineering of beta-oxidation reversal and omega-oxidation pathways for the synthesis of medium chain omega-functionalized carboxylic acids**. *Metab Eng* 2015, **28**:202-212.

23. •• Cheong S, Clomburg J, Gonzalez R: **Energy- and carbon-efficient synthesis of functionalized small molecules in bacteria using non-decarboxylative Claisen condensation reactions**. *Nat Biotech* 2016, **34**:556-561.
The authors engineer a synthetic non-decarboxylative pathway for the production of various value-added chemicals. This route leads to the highest adipic acid titers from simple sugars to date

24. Rodriguez A, Martinez JA, Flores N, Escalante A, Gosset G, Bolivar F: **Engineering *Escherichia coli* to overproduce aromatic amino acids and derived compounds**. *Microb Cell Fact* 2014, **13**:126.

25. • Niu W, Draths KM, Frost JW: **Benzene-free synthesis of adipic acid**. *Biotechnol Progr* 2002, **18**:201-211.
This work routes glucose via 3-dehydroshikimic acid to protocatechuic acid, catechol and ultimate muconic acid in*E. coli* to produce the highest titers of muconic acid to date.

26. Weber C, Bruckner C, Weinreb S, Lehr C, Essl C, Boles E: **Biosynthesis of *cis,cis*-muconic acid and its aromatic precursors, catechol and protocatechuic acid, from renewable feedstocks by *Saccharomyces cerevisiae***. *Appl Environ Microbiol* 2012, **78**:8421-8430.

27. • Curran KA, Leavitt JM, Karim AS, Alper HS: **Metabolic engineering of muconic acid production in *Saccharomyces cerevisiae***. *Metab Eng* 2013, **15**:55-66.
Latests engineering of*S. cerevisiae* for the production of muconic acid. This work shows that extensive regulation of aromatic amino acid biosynthesis in this organism significantly hingers high muconic acid titers.

28. Suastegui M, Matthiesen JE, Carraher JM, Hernandez N, Quiroz NR, Okerlund A, Cochran EW, Shao ZY, Tessonnier JP: **Combining metabolic engineering and electrocatalysis: application to the production of polyamides from sugar**. *Angew Chem Int Ed* 2016, **55**:2368-2373.

29. Sun X, Lin Y, Qin H, Qipeng Y, Yan Y: **A novel muconic acid biosynthesis approach by shunting tryptophan biosynthesis via anthranilate**. *Appl Environ Microbiol* 2013, **79**:4024-4030.

30. Tepper N, Shlomi T: **Predicting metabolic engineering knockout strategies for chemical production: accounting for competing pathways**. *Bioinformatics* 2010, **26**:536-543.

31. Lin YH, Shen XL, Yuan QP, Yan YJ: **Microbial biosynthesis of the anticoagulant precursor 4-hydroxycoumarin**. *Nat Commun* 2013, **4**:2603.

32. Linger JG, Vardon DR, Guarnieri MT, Karp EM, Hunsinger GB, Franden MA, Johnson CW, Chupka G, Strathmann TJ, Pienkos PT *et al.*: **Lignin valorization through integrated biological funneling and chemical catalysis**. *Proc Natl Acad Sci U S A* 2014, **111**:12013-12018.

33. van Duuren JBJH, Wijte D, Karge B, Martins dos Santos VAP, Yang Y, Mars AE, Eggink G: **pH-stat fed-batch process to enhance the production of *cis,cis*-muconate from benzoate by *Pseudomonas putida* KT2440-JD1**. *Biotechnol Progr* 2012, **28**:85-92.

34. Johnson CW, Salvachúa D, Khanna P, Smith H, Peterson DJ, Beckham GT: **Enhancing muconic acid production from glucose and lignin-derived aromatic compounds via increased protocatechuate decarboxylase activity**. *Metab Eng Commun* 2016, **3**:111-119.

35. Han L, Liu P, Sun JX, Wu YQ, Zhang YY, Chen WJ, Lin JP, Wang QH, Ma YH: **Engineering catechol 1,2-dioxygenase by design for improving the performance of the *cis,cis*-muconic acid synthetic pathway in *Escherichia coli***. *Sci Rep* 2015, **5**:11.

36. Lanza AM, Curran KA, Rey LG, Alper HS: **A condition-specific codon optimization approach for improved heterologous gene expression in *Saccharomyces cerevisiae***. *BMC Syst Biol* 2014, **8**Artn 33.

37. Sonoki T, Morooka M, Sakamoto K, Otsuka Y, Nakamura M, Jellison J, Goodell B: **Enhancement of protocatechuate decarboxylase activity for the effective production of muconate from lignin-related aromatic compounds**. *J Biotechnol* 2014, **192**:71-77.

38. Werpy T, Peterse G: In: N.R.E.L. and P.N.N.L. **Top Value added chemicals from biomass**, vol I: Results of screenign for potential cadidates from sugars and syntehsis gas, 2004.

39. Moon TS, Dueber JE, Shiue E, Prather KLJ: **Use of modular, synthetic scaffolds for improved production of glucaric acid in engineered *E. coli***. *Metab Eng* 2010, **12**:298-305.

40. Shiue E, Prather KLJ: **Improving D-glucaric acid production from myo-inositol in *E. coli* by increasing MIOX stability and myo-inositol transport**. *Metab Eng* 2014, **22**:22-31.

41. Gupta A, Reizman IM, Reisch CR, Prather KL: **Dynamic regulation of metabolic flux in engineered bacteria using a pathway-independent quorum-sensing circuit**. *Nat Biotechnol* 2017, **35**:273-279.

42. Schrewe M, Julsing MK, Lange K, Czarnotta E, Schmid A, Buhler B: **Reaction and catalyst engineering to exploit kinetically controlled whole-cell multistep biocatalysis for terminal FAME oxyfunctionalization**. *Biotechnol Bioeng* 2014, **111**:1820-1830.

43. Cheng Q, Thomas SM, Kostichka K, Valentine JR, Nagarajan V: **Genetic analysis of a gene cluster for cyclohexanol oxidation in *Acinetobacter* sp. strain SE19 by in vitro transposition**. *J Bacteriol* 2000, **182**:4744-4751.

44. Yu JL, Xia XX, Zhong JJ, Qian ZG: **Direct biosynthesis of adipic acid from a synthetic pathway in recombinant *Escherichia coli***. *Biotechnol Bioeng* 2014, **111**:2580-2586.

45. Dekishima Y, Lan EI, Shen CR, Cho KM, Liao JC: **Extending carbon chain length of 1-butanol pathway for 1-hexanol synthesis from glucose by engineered *Escherichia coli***. *J Am Chem Soc* 2011, **133**:11399-11401.

46. Shen CR, Lan EI, Dekishima Y, Baez A, Cho KM, Liao JC: **Driving forces enable high-titer anaerobic 1-butanol synthesis in *Escherichia coli***. *Appl Environ Microb* 2011, **77**:2905-2915.

47. Zhang H, Li Z, Pereira B, Stephanopoulos G: **Engineering *E. coli–E. coli* cocultures for production of muconic acid from glycerol**. *Microb Cell Fact* 2015, **14**:134.

48. •• Zhang HR, Pereira B, Li ZJ, Stephanopoulos G: **Engineering *Escherichia coli* coculture systems for the production of biochemical products**. *Proc Natl Acad Sci U S A* 2015, **112**:8266-8271.
The authors use an *E. coli* co-culture strategy to split the muconic acid pathway to achieve not only high muconic acid titers, but also to enable the production of muconic acid form glucose and xylose simmultanously.

49. Schellenberger J, Que R, Fleming RMT, Thiele I, Orth JD, Feist AM, Zielinski DC, Bordbar A, Lewis NE, Rahmanian S, Kang J, Hyduke DR, Palsson BO: **Quantitative prediction of cellular metabolism with constraint-based models: the COBRA Toolbox v2.0**. *Nat Protoc* 2011, **6**:1290-1307.

50. Feist AM, Zielinski DC, Orth JD, Schellenberger J, Herrgard MJ, Palsson BO: **Model-driven evaluation of the production potential for growth-coupled products of *Escherichia coli***. *Metab Eng* 2010, **12**:173-186.

51. Nogales J, Palsson BO, Thiele I: **A genome-scale metabolic reconstruction of *Pseudomonas putida* KT2440: iJN746 as a cell factory**. *BMC Syst Biol* 2008, **2**Artn 79.

52. Mo ML, Palsson BO, Herrgard MJ: **Connecting extracellular metabolomic measurements to intracellular flux states in yeast**. *BMC Syst Biol* 2009, **3**Artn 37.