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Central metabolic nodes for diverse biochemical production

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Central carbon metabolism is conserved among all organisms for cellular function and energy generation. The connectivity of this metabolic map gives rises to key metabolite nodes. Five of these nodes in particular, pyruvate, citric acid, tyrosine and aspartate, acetyl-CoA, serve as critical starting points for the generation of a broad class of relevant chemical molecules with ranging applications from fuels, pharmaceuticals and polymer precursors. This review highlights recent progress in converting these metabolite nodes into valuable products. In particular, acetyl-CoA, the most well-connected node, serves as the building block for several classes of molecules including fatty acids and terpenes. Systematic metabolic engineering efforts focused on these metabolic building blocks has enabled the production of industrially-relevant, biobased compounds.

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Introduction

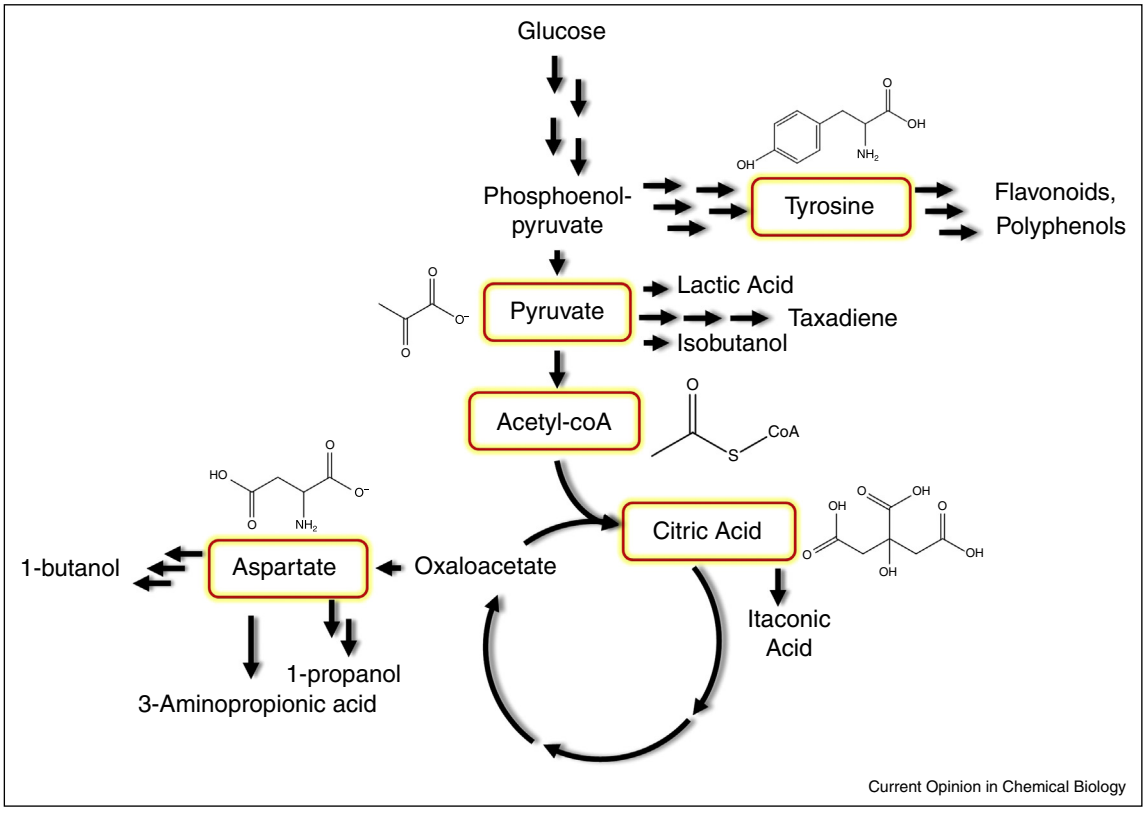
At first glance, the biochemical diversity of metabolism across all living species is immense and complex. Yet, most heterotrophic cellular systems are linked by a common thread — the ability to assimilate glucose into biological building blocks and energy. As a result, organisms have a highly conserved central carbon metabolism that embodies optimal pathways for energy and precursor generation. Toward this end, extensive network analysis of metabolism in *Escherichia coli* demonstrates a high interconnectivity of metabolites with minimum walks between key biomass precursors [1,2]. These interconnected metabolites provide the chemistry of life, but also enable routes to important biobased chemicals — including most in the 2004 US Department of Energy report of 'top value added chemicals from biomass' [3,4••]. The intersection of this list with key metabolites reveal pyruvate, citric acid, tyrosine, aspartate, and acetyl-CoA as critical metabolic nodes for bioproducts (Figure 1). Among these, the acetyl-CoA node has a central role in many biosynthetic pathways of study. This role is not surprisingly given that this molecule serves as a bridge between glycolysis and the tricarboxylic acid (TCA) cycle. Certainly each of these nodes have been the subject of systematic engineering for improved production of fuels, specialty chemical, and commodity chemicals [5•]. In this review, we seek to contextualize the myriad of biobased molecules and demonstrate how a select few precursors (specifically, pyruvate, citric acid tyrosine, aspartate and acetyl-CoA) can give rise to a wide variety of industrially-relevant, biobased compounds for fuels and biochemicals applications (Table 1). Specifically, we highlight a few important chemicals derived from each of these metabolites with a special focus on acetyl-CoA as it serves as the most fruitful node for producing important industrial biochemicals.

Chemicals derived from key nodes in metabolism

Pyruvate-derived molecules

Situated at the end of glycolysis, pyruvate is one of the most connected metabolites — it serves as a precursor to critical amino acids (valine, leucine, and isoleucine) and can interconvert in either direction of the TCA cycle. A long-standing bioproduct derived from this node is lactic acid, generated through dehydrogenation using redox equivalents similar to ethanol production. Polymerization of lactic acid into the bioplastic polylactic acid has growing interest especially for applications in 3D printing [6]. Alternatively, lactic acid can be polymerization to poly(lactate-*co*-glycolate), a biodegradable and nontoxic polymer for biomedical applications, which has been achieved through dual uptake of glucose and xylose in *Escherichia coli* [7]. High level production of lactic acid was achieved in *Saccharomyces cerevisiae* by introducing lactate dehydrogenase in addition to cytosolic modulation of NADH redox to obtain 117 g/L in a 2 L bioreactor [8]. For further improved titers, immobilization of *Rhizopus oryzae* in a sponge-like cubic particle led to 231 g/L lactic acid from glucose [9]. Pyruvate also serves as the gateway to the isoprenoid and terpene pathways via condensation with the glycolysis metabolite glyceraldehyde-3-phosphate. In a recent example, optimization of the isoprene pathway led to production of 1020 mg/L of taxadiene, an important intermediate to the anticancer

Figure 1



Central nodes in carbon metabolism as precursors to relevant biochemical. A simplified schematic of metabolism is provided with an emphasis on the core metabolites discussed in this review. Each arrow corresponds to two enzymatic steps when more than one step occurs.

drug known as Taxol or paclitaxel [10]. Likewise, the biofuel isobutanol has been produced from pyruvate as part of the valine pathway with titers of 22 g/L achieved in *E. coli* [11]. Finally, in conjunction with acetyl-CoA (see section below), pyruvate can serve as a precursor backbone for short and medium chain fatty acid ester production. As examples, isobutyl acetate titers in *E. coli* of 19.7 g/L were obtained in a recent study with further demonstrations that the longer chain isobutyl butyrate was also possible with a similar reaction, albeit with a lower initial titer (27.1 mg/L) [12,13]. Utilizing these pathways, longer chain esters (from C12–C18 fatty acid backbones) were obtained with titers of 25.4 mg/L in *S. cerevisiae* and just over 1 g/L in *E. coli* [14,15]. High level production from the pyruvate branch highlights the importance of this central metabolite for bioproducts.

Citric acid-derived molecules

Citric acid, an important organic acid in the TCA cycle, serves as a critical node where acetyl-CoA enters for energy generation. Citric acid itself also has applications as a preservative used in cosmetics, food and beverage industries. This compound has historically been produced through submerged fermentation of *Aspergillus niger*; however, recent efforts have created an alternative with the oleaginous yeast *Yarrowia lipolytica*. Specifically, introduction of an inulinase enzyme to degrade inulin as a feedstock led to the production of nearly 78 g/L of citric acid in shake flasks [16]. Due to its metabolic positioning, citric acid can be converted through short pathways to important precursors and monomers such as itaconic acid [17••]. In a two-step reaction, itaconic acid can be generated from citric acid through a cis-aconitic acid decarboxylase resulting in a *Y. lipolytica* strain producing 4.6 g/L [18]. These examples demonstrate the importance and variety of industries that make use of the citric acid node.

Tyrosine-derived molecules

The aromatic amino acids (specifically those linked with tyrosine) serve as a rich branch of chemistry with the functionality of the aromatic ring. These molecules are produced via the shikimate pathway by combining erythrose-4-phosphate and phosphoenolpyruvate. After seven enzymatic conversions, chorismate serves as the branch point between tryptophan, phenylalanine and tyrosine. Chorismate is also a metabolite with one of the shortest mean path lengths upon network analysis with downstream pathways leading to the bioproduction

Table 1

List of products generated through microbial conversion of core metabolic precursors (N/A: not applicable, indicates precursor molecule is also the product)

Product	Precursor molecule	Molecule classification	Quantity produced	Organism used	Reference
Lactic acid	Pyruvate	Commodity chemical	117 g/L	*S. cerevisiae*	[8]
Lactic acid	Pyruvate	Commodity chemical	231 g/L	*Rhizopus oryzae*	[9]
Taxadiene	Pyruvate	Specialty chemical	1.02 g/L	*E. coli*	[10]
Isobutanol	Pyruvate & AcCoA	Fuel	22 g/L	*E. coli*	[11]
Isobutyl acetate	Pyruvate & AcCoA	Commodity chemical	19.7 g/L	*E. coli*	[12]
Isobutyl butyrate	Pyruvate & AcCoA	Commodity chemical	27.1 mg/L	*E. coli*	[13]
Fatty acid short-chain esters	Pyruvate & AcCoA	Fuel	25.4 mg/L	*S. cerevisiae*	[14]
Fatty acid short-chain esters	Pyruvate & AcCoA	Fuel	1007 mg/L	*E. coli*	[15]
Citric acid	N/A	Commodity chemical	77.9 g/L	*Y. lipolytica*	[16]
Citric acid	N/A	Commodity chemical	12.0 g/L	*Y. lipolytica*	[18]
Itaconic acid	Citric acid	Commodity chemical	4.6 g/L	*Y. lipolytica*	[18]
P-coumaric acid	Tyrosine	Specialty chemical	1.93 g/L	*S. cerevisiae*	[21]
Naringenin	Tyrosine	Specialty chemical	100.6 mg/L	*S. cerevisiae*	[19•]
Naringenin	Tyrosine	Specialty chemical	29 mg/L	*E. coli*	[20]
Flavan-3-ol	Tyrosine	Specialty chemical	40.7 mg/L	*E. coli*	[22]
Kaempferol 3-O-rhamnoside	Tyrosine	Specialty chemical	57 mg/L	*E. coli*	[23]
Propanol	Aspartate	Fuel	1 g/L	*E. coli*	[26]
Butanol	Aspartate	Fuel	1 g/L	*E. coli*	[26]
3-Aminopropionic acid	Aspartate	Commodity chemical	32.3 g/L	*E. coli*	[27•]
Fatty acid	Acetyl-CoA, malonyl-CoA	Fuel	8.6 g/L	*E. coli*	[29]
Fatty acid	Acetyl-CoA, malonyl-CoA	Fuel	39.1 g/L	*Y. lipolytica*	[30]
Fatty acid	Acetyl-CoA, malonyl-CoA	Fuel	85 g/L	*Y. lipolytica*	[31]
Omega fatty acid	Acetyl-CoA, malonyl-CoA	Specialty chemical	15% dry cell weight	*Y. lipolytica*	[32]
Pentane	Acetyl-CoA, malonyl-CoA	Fuel	4.98 mg/L	*Y. lipolytica*	[33]
Pentane	Acetyl-CoA, malonyl-CoA	Fuel	2.8 mg/L	*E. coli*	[34•]
Heptane	Acetyl-CoA, malonyl-CoA	Fuel	4.3 mg/L	*E. coli*	[34•]
Dodecanol/tetradecanol	Acetyl-CoA, malonyl-CoA	Specialty chemical	1.55 g/L	*E. coli*	[35]
Butanol	Acetyl-CoA	Fuel	16 g/L	*Clostridium tyrobutyricum*	[38]
Isopentenol	Acetyl-CoA	Fuel	1.0 g/L	*E. coli*	[39]
Triacetic acid lactone	Acetyl-CoA, malonyl-CoA	Specialty chemical	1.6 g/L	*S. cerevisiae*	[40]
Triacetic acid lactone	Acetyl-CoA, malonyl-CoA	Specialty chemical	5.2 g/L	*S. cerevisiae*	[41]
Thailanstatin	Acetyl-CoA	Specialty chemical	2.5 g/L	*Burkholderia* sp. *FERM* BP-3421	[42]
Artimisinic acid	Acetyl-CoA	Specialty chemical	25 g/L	*E. coli*	[43]
Amophadiene	Acetyl-CoA	Specialty chemical	40 g/L	*S. cerevisiae*	[44]
Proptopanaxadiol	Acetyl-CoA	Specialty chemical	1.12 g/L	*S. cerevisiae*	[45]
Dammarenediol	Acetyl-CoA	Specialty chemical	1.55 g/L	*S. cerevisiae*	[45]
Miltiradiene	Acetyl-CoA	Specialty chemical	365 mg/L	*S. cerevisiae*	[46]

of specialty chemicals including flavonoids and polyphenols [2]. These plant-derived aromatic compounds exhibit pharmaceutical properties such as antioxidative, anti-inflammatory, antiviral, and anticancer [19•,20]. Tyrosine-derived flavonoid p-coumaric acid and naringenin have been extensively produced recombinantly. Nearly 2 g/L of p-coumaric acid was produced in *S. cerevisiae* through introducing a heterologous shikimate pathway and deleting by-product forming enzymes [21]. Naringenin has been optimized through approaches including pathway balancing and modular optimization to result in *E. coli* production ranging from 29 to 100 mg/L [19•,20]. Additional efforts in *E. coli* have produced the flavonoids flavan-3-ol and kaempferol 3-O-rhamnoside at 40.7 and 57 mg/L, respectively [22,23]. The tyrosine branch has long-served as an important source for active pharmaceuticals including precursors for Tamiflu [24]. More recently, this pathway has been utilized in yeast to enable the complete synthesis of thebaine and hydrocodone in yeast [25••]. Thus, this node serves as an important, interconnected pathway prime for diversification.

Aspartate-derived molecules

Aspartate, a negatively charged amino acid, is another well-connected molecule in metabolism serving as a precursor to threonine biosynthesis and 3-aminopropionic acid as well as its connection with the TCA cycle (through fumarate and oxaloacetate) [2]. Recent studies have imported two enzymes from *Lactococus lactis* and *S. cerevisiae* into *E. coli* to overproduce 2-ketobutyrate from aspartate [26]. Additionally, higher-energy density alcohols for fuels, including propanol and butanol were produced at levels of 1 g/L, and 1 g/L respectively [26]. A single step enzymatic conversion of aspartate into 3-aminopropionic acid, a platform chemical in the synthesis of acrylamide, acrylonitrile, and nylon-3, has been

demonstrated in *E. coli* with high titers of 32 g/L [27•]. As demonstrated, aspartate is a unique node leading to high production of fuels and chemicals.

Acetyl-CoA-derived molecules

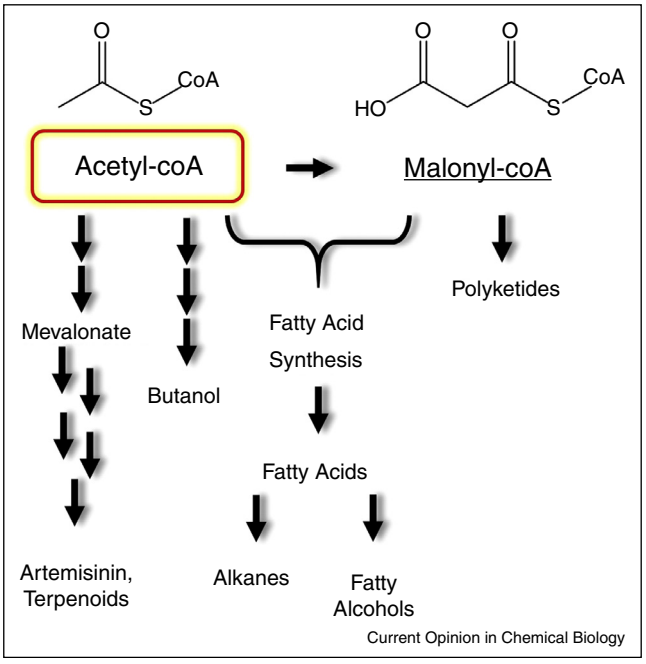
Among all the metabolites in the cell, acetyl-CoA is by far the most important due to placement between glycolysis and the TCA cycle. Not surprising, this molecule is one of the most well connected metabolites and has been the focus of many metabolic engineering strategies [2] (Figure 2). The unique combination of a thioester bond and acetic acid serves as a gateway to a variety of bioproducts. Moreover, the conversion of acetyl-CoA into malonyl-CoA via carboxylation establishes an important intermediate for fatty acid and terpene biosynthesis. A recent review on the production and consumption pathways of acetyl-CoA in eukaryotes and prokaryotes highlights the metabolic engineering efforts of this node [28••].

The pathways of fatty acid synthesis and beta-oxidation utilize acetyl-CoA and malonyl-CoA intermediates. Due to the incorporation of several molecules of malonyl-CoA into one longer chain fatty acid molecule, optimal production of these precursors is crucial [29]. Modular pathway engineering of three distinct portions of the fatty acid biosynthesis pathway resulted in high lipid titers of 8.6 g/L [29] in *E. coli*. By utilizing an alternative host, such as *Y. lipolytica*, significantly increased titers can be achieved, ultimately exceeding 85 g/L, with 40 g/L in minimal media [30,31]. Modified fatty acids through the incorporation of desaturase and elongase enzymes can enable the high-level production of heart-healthy polyunsaturated fatty acids which have been linked to heart health. This trait has been demonstrated by DuPont with the production of eicosapenanoic acid at 15% of dry cell weight [32].

Lipids derived from acetyl-CoA and malonyl-CoA can also lead to other fuel and commodity chemicals. For example, the introduction of a soybean lipoxygenase led to nearly 5 mg/L of pentane in *Y. lipolytica* [33]. Additionally, Sheppard *et al.* activated reverse beta-oxidation for the production of shorter chain alkanes resulting in 2.8 mg/L pentane and 4.3 mg/L heptane in *E. coli* [34•]. Fatty alcohols useful as surfactants, detergents and other personal care products have been produced via overexpression of thioesterase, acyl-CoA reductase and aldehyde reductase to enable 1.55 g/L of dodecanol and tetradecanol production in *E. coli* [35]. Through gene tuning 1.95 g/L of odd-chain fatty alcohols was achieved in *E. coli* through fed-batch fermentation [36]. Similar efforts in *S. cerevisiae* resulted in 1.2 g/L hexadecanol using xylose as the substrate [37]. These varied applications of fatty acid and lipid molecules continue to be a fertile area of research.

Beyond lipids, acetyl-CoA can lead to important biomolecules such as butanol, isopentenol, polyketides, and terpenes. Several studies have imported the six-step fermentative pathway from acetyl-CoA to butanol. As an example, this pathway was utilized by Yu *et al.* in *Clostridium tyrobutyricum* in conjunction with deleting two genes responsible for the undesired products acetate and butyrate to lead to 16 g/L of L-butanol [38]. Likewise, 1 g/L of isopentenol has been produced using the conversion of acetyl-CoA to malonyl-CoA via a by-pass reaction which reduces the energy requirement and decreases the number of enzymes from 7 to 5 *in E. coli* [39]. Similar to fatty acid synthesis, polyketides are produced from acetyl-CoA and malonyl-CoA building blocks. Overproduction of the acetyl-CoA precursors and NADPH level to fuel the reactions increased production of triacetic acid lactone to 1.6 g/L in *S. cerevisiae* [40]. Further work achieved a maximum of 5.2 g/L with an industrial *S. cerevisiae* strain [41]. By introducing the P450 cytochrome fr6R in conjunction with media optimization, Eustaquio *et al.* enabled production of 2.5 g/L thailanstatin in the gram-negative bacteria *Burkholderia* sp. FERM BP-3421 [42]. In an alternative pathway, two molecules of acetyl-CoA can be condensed to establish terpenes. This mevalonate pathway, like fatty acid synthesis, enables a wide array of different molecules with pharmaceutical activity. Perhaps the most successful example is artimisinic acid, the chemical precursor of the antimalarial drug artemisinin, which has been produced at titers of at least 25 g/L with *E. coli* [43]. Another precursor in this pathway, amorphodiene has been produced at over

Figure 2



Connectivity of acetyl-CoA as a central node for production of several different classes of biomolecules. Underlined compounds are key precursors for synthesis to complex molecules.

40 g/L in *S. cerevisiae* [44]. Additional terpenes have been produced from this branch including the triterpenes protopanaxadiol and dammarenediol with metabolic engineering efforts leading to the production of over 1 g/L [45]. Efforts to produce the diterpenoid miltiradiene achieved 365 mg/L through systematic modification of active sites and a protein fusion [46]. As has been demonstrated, the acetyl-CoA node is a unique bridge between cellular metabolism and a myriad of important biochemical.

Conclusions and future prospects

The chemical palette within cells is rapidly expanding with an extensive list of unique bioproducts that can serve as new and replacement fuels, chemicals, and pharmaceuticals [47]. Much of these advances gravitate toward a small number of key metabolite nodes within cells. As shown in Figures 1 and 2 this highly-interconnected, small world of chemistry enables one to rapidly leverage the advances made for one product to create alternative ones. In this regard, Table 1 demonstrates great promise of biological production of industrially relevant chemicals from a select number of precursor molecules: pyruvate, citric acid, tyrosine, aspartate and acetyl-CoA. Thus, the production of biochemical base-strains is indeed becoming a possibility allowing chemical diversification to become more rapid and even 'plug-and-play'. In such a case, utilizing metabolic connectivity can identify rational targets for future engineering efforts leading to increased production of valuable bio-based molecules. Certainly the recent advances of rapid molecular biology and genomic editing tools such as Gibson cloning, CRISPR-Cas9 among others enables this approach to expand even beyond the canonical model host organisms going forward.

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