Steps towards “drop-in” biofuels: focusing on metabolic pathways

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Abstract

The past decade has witnessed rapid advance in microbial production of “drop-in” biofuels from renewable resources. Various biosynthetic pathways have been constructed to produce biofuels with diverse structures, and multiple metabolic engineering strategies have been developed to increase biofuel titers, yields, productivities and system robustness. In this review, we intend to give a brief but comprehensive overview of the most recent progresses on four essential pathways leading to “drop-in” biofuel production, with an emphasis on the metabolic pathway efficiencies and biofuel structures. Furthermore, we also provide an insightful discussion on optimization strategies to improve the robustness of the microbial platforms for biofuel production.

Introduction

Since bioethanol and biodiesel were commercialized as the first-generation biofuels, the biofuel field has achieved tremendous advances on various scales from benchtop to large-scale reactors. To produce "drop-in" biofuels on an industrial scale, several efforts have been made: The feedstock choices have been expanded from food crops (e.g., corns and oilseeds) to abundant and low-cost non-food biomass (e.g., corn stover and switchgrass) [1], solid wastes (e.g., industrial wastes and sewage sludge) [2], natural resources (syngas, methanol and methane) [3], and carbon dioxide [4]. Meanwhile, the fermentation host choices have expanded beyond the traditional model microorganisms such as *Escherichia coli* and *Saccharomyces cerevisiae* to a wide variety of non-model organisms. These new hosts variously feature unique capabilities in assimilating target feedstock, adapting to specialized fermentation conditions, and producing target biofuels in high efficiencies.

Meanwhile, by engineering different biosynthetic pathways, significant progress has been made in diversifying the structures of the bioproduced fuels, so that their physiochemical and combustion properties match with conventional engines and transportation infrastructures [5]. Most of these "drop-in" biofuels are derived from five landmark pathways, including the α-keto acid pathway, the non-decarboxylative Claisen condensation pathway, the fatty acid pathway, the isoprenoid pathway, and the polyketides pathway. Compared to conventional bioethanol and biodiesel, biofuels produced from these pathways often have properties closer to those of petroleum-based fuels, and they are compatible with spark-ignition, compression-ignition, and gas-turbine engines [6]. Because the polyketides pathway has been reviewed thoroughly

elsewhere [7], this review will focus on progress from the other four pathways within the past three years, with an emphasis on the structures of pathway products and strategies to improve pathway efficiencies.

α-keto acid pathway

Short-chain alcohols (including isobutanol, 3-methyl-1-butanol, and 2-methyl-1-butanol) were produced from the α-keto acid pathway by decarboxylation and reduction of cellular short-chain α-keto acids, intermediates of the branched-chain amino acid biosynthesis, using an α-keto acid decarboxylase and an alcohol dehydrogenase [8](Figure 1, light blue). Furthermore, by altering the substrate specificity of LeuABCD enzymes, the acyl chain length of α-keto acids can be further increased by recursively condensing with acetyl-CoA and releasing CO_2. Using this strategy, alcohols with medium chain-lengths (up to C8) or with branched/aromatic terminals have been produced [9] (Figure 1, green). Meanwhile, α-keto acids or their derived alcohols can also be converted to short-chain alkanes [10] and to acetate-based esters [11] for biofuel applications.

One advantage of the α-keto acid pathway is its ability to add one carbon atom at a time to the acyl-chain, allowing precise control over chain-length. However, this chain elongation process sacrifices carbon efficiency and accumulates the reducing cofactor NADH (Table 1), which might be the reason for the lower titer of medium-chain alcohols (C6-C8) compared to that of isobutanol.

Table 1 The carbon efficiencies, cofactor balances, and highest titers produced from four pathways

Pathways	Products	Carbon recovery from glucose	Reducing cofactor imbalance	Highest titer in *E. coli*
α-keto acid pathway	1-butanol	44.4%	+ 6 NADH	1 g/L [12]
	isobutanol	66.7%	+ 1 NADH, - 1 NADPH	22 g/L [8]
	3-methyl-1-butanol	55.6%	+ 4 NADH, - 1 NADPH	4.4 g/L [13]
	2-methyl-1-butanol	83.3%	+ 1 NADH, - 3 NADPH	1.25 g/L [14]
	1-pentanol	41.7%	+ 8 NADH	4.3 g/L [15]
	1-hexanol	40.0%	+ 10 NADH	302 mg/L [9]
	1-heptanol	38.9%	+ 12 NADH	80 mg/L [9]
	1-octanol	38.1%	+ 14 NADH	2.0 mg/L [9]
Fatty acid pathway	Fatty acids (C14-C18)	66.7%	+ 14 NADH, - 12 NADPH (C14)	21.5 g/L [16]
	Fatty alcohols (C14-C18)	66.7%	+ 12 NADH, - 12 NADPH (C14)	3.82 g/L [17]
	Alkanes (C9–C13)	60-61.9%	+ 13 NADH, - 12 NADPH (C13)	580 mg/L [18]
Non-decarboxylative Claisen condensation	1-butanol	66.7%	0	30 g/L [19]
	Fatty acids (C6-C10)	66.7%	+ 2 NADH (C8)	3.8 g/L [20]
MVA pathway	Limonene	55.6%	+ 8 NADH	1.35 g/L [21]
MEP pathway	Limonene	66.4%	0	35.8 mg/L [22]

Fatty Acid Pathway

Because oilseed-derived biodiesel suffers from inferior low-temperature performance and competes with food supply, engineering efforts have been made to overproduce microbial-based fatty acid-derived fuels. Fatty acids (FAs) are biosynthesized *in vivo* by either the discrete fatty acid synthase complex FASII (in most bacteria) or a multifunctional FASI synthase (in eukaryotic cells). Initiated by the condensation of acetyl-CoA with malonyl-ACP, the four-carbon acyl chain product is elongated on acyl carrier proteins (ACPs) through repeated cycles of reactions to yield straight long-chain acyl-ACPs (C14-C18) for lipid synthesis. Medium-chain FAs (C8-

C12), which are preferred for biodiesel, can be produced in *E. coli* by overexpressing a medium-chain-specific thioesterase [23] (Figure 2, Chain Length Control Module). Similarly, the *S. cerevisiae* FASI has been engineered to produce medium-chain FAs at a titer of 464 mg/L using a rational protein engineering strategy [24]. These medium-chain FAs can then be derived using engineered conversion pathways to alkanes [18], alcohols [25], and esters [26] for various biofuel applications (Figure 2, α-Terminal Structure Control Module).

Biofuels derived from straight-chain fatty acids (SCFAs) have relatively high freezing points and viscosities compared to their branched-chain isomers, thus limiting their low-temperature operability—an essential feature for diesel and jet fuels. Such limitation promoted the engineering of branched-chain fatty acid (BCFA) production. In engineered *E. coli*, the BCFA pathway contains a branched-chain α-keto acid dehydrogenase (BKD) that converts branched-chain α-keto acids to branched-chain acyl-CoAs, a branched-chain-specific FabH that converts branched-chain acyl-CoAs to branched-chain acyl-ACPs, and a thioesterase that hydrolyzes elongated branched-chain acyl-ACPs to BCFAs [27-29] (Figure 2, ω-Terminal Structure Control Module). Due to competition with the natural FA pathway, the initially engineered strains produced only a small amount of BCFAs, but co-produced a large amount of SCFAs [27,29]. To increase BCFA titers and percentages, several strategies were developed, including the dynamic regulation of branched-chain specific FabH [27] and deletion of the *E. coli fabH* gene [29]. In addition, overexpression of the rate-limiting enzyme complex BKD was found to deplete the protein lipoylation capacity of the host cell and

inhibit cell growth [28]. This BKD-caused toxicity was effectively mitigated by engineering a synthetic protein lipoylation pathway, increasing the BCFA titer to 276 mg/L [28]. Because α-keto acids eventually become the ω-terminal groups of BCFAs, the branch structure can be controlled by regulating the cellular pool of each α-keto acid. Anteiso, even-chain-iso, and odd-chain-iso BCFAs have been separately produced to 77%, 61%, and 77% of the total free FAs, respectively [28]. The ability to control FA chain length and α-functional group, together with the ability to engineer branches at the ω-terminal of FAs significantly expands the repertoire of chemicals derived from the FA pathway and opens several avenues for producing "drop-in" biofuels.

Overall, the highly efficient natural FAS pathways make FA an attractive intermediate for biofuel production. On the other hand, the essentiality of FA presents challenges to engineering: 1) incorporating non-native FAs into the cell membrane may change the membrane fluidity, inhibiting cell growth, 2) expressing native FA biosynthetic genes is often growth-associated, limiting the production of FA-derived fuels to the growth phase, and 3) interaction between natural FAS enzymes may prevent short- and medium-chain acyl-ACPs from interacting with desired heterologous enzymes in biofuel pathways.

Non-decarboxylative Claisen condensation pathway

Non-decarboxylative Claisen condensation pathway was first discovered in nature from *Clostridium kluyveri* [30](Figure 1, purple). In this pathway, two molecules of acetyl-CoA are condensed to acetoacetyl-CoA, which is then reduced to butyrate by β-reduction reactions. In contrast to the FA biosynthetic pathway that grows the carbon

chain on ACP, the non-decarboxylative Claisen condensation pathway grows the carbon chain on CoA, and uses acetyl-CoA or other short-chain acyl-CoAs as primers. Dellomonaco *et al.* engineered a synthetic non-decarboxylative Claisen condensation pathway to elongate carbon chains by reversing β-oxidation pathway [31]. The elongation cycle can be terminated by an acyl-CoA reductase and an alcohol dehydrogenase to produce straight-chain alcohols, or by an acyl-CoA thioesterase to produce carboxylic acids. By switching thiolases and β-reduction enzymes, researchers further expanded the primer from acetyl-CoA to six other ω-functionalized primers, and incorporated three different extender units [32] (Figure 1, grey structures). This platform has greatly enlarged the structural diversity of biosynthesized molecules, yielding a series of novel bioproducts, such as 2-methylpentanoic acid and 2,3-dihydroxybutyric acid [32].

Compared to the fatty acid pathway and the α-keto acid pathway, the non-decarboxylative Claisen condensation pathway has two distinctive advantages: the ability to incorporate internal functional groups (including branch structures) into the product, and the ability to orthogonally control the structures of both primers and extenders. This pathway will become more versatile as new enzymes are discovered to incorporate more extenders and new strategies are developed to precisely control the number and order of elongation cycles.

Isoprenoid pathway

Terpenes and terpenoids generated from the isoprenoid pathway can also be used as fuel replacements. The isoprenoid pathway utilizes C5 isopentenyl pyrophosphate

(IPP) and dimethylallyl pyrophosphate (DMAPP) as building blocks, which are produced from either the mevalonate (MVA) or methylerythritol-4-phosphate (MEP) pathway (Figure 3). The carbon chain is elongated by iterative assembly of IPP or DMAPP, generating polyisoprenoid diphosphates which are then converted to terpenes or terpenoids by terpene synthases or polyisoprenoid diphosphate modifying enzymes such as hydrolases or esterases.

Since monoterpenes (C10), sesquiterpenes (C15) and their derivatives have been identified as diesel and jet fuel substitutes, a variety of terpenes and terpenoids with biofuel potential have been produced by branching the pathway at geranyl pyrophosphate (GPP) and farnesyl pyrophosphate (FPP) (Figure 3). Among them, pinene and limonene have properties similar to jet fuels, while the hydrogenated products of farnesene and bisabolene have properties similar to those of diesel fuels [6]. Novel products including linalool [33], 1,8-cineole [33], and geraniol [34] have also been biosynthesized and proposed as fuel candidates.

Due to the diverse functionality of terpene synthases, the most outstanding feature of the isoprenoid pathway is its capability to generate structurally diverse products. So far, thousands of different terpene structures have been identified [35], and over hundreds of terpene synthases have been sequenced [36]. However, most terpene synthases have not been characterized, and the links between most terpene synthases to their corresponding terpene products are not established. New methods for high throughput characterization of new terpene synthases could facilitate the discovery of new biosynthetic routes to better biofuels.

Strategies for Pathway Optimization

Besides novel biosynthetic pathways and biofuel candidates, new strategies are developed to optimize production pathways and to increase product titers, yields, and productivities. Conventional concept of metabolic engineering is to drive the metabolic flux into the production pathway. Various methods have been developed to facilitate this step, including upregulating pathway enzymes [37], deleting or downregulating competing pathways [38], and adjusting the global metabolism by engineering transcriptional factors [39,40]. Meanwhile, an increasing number of strategies have been developed to balance metabolic pathways and to engineer host cells by increasing their robustness, enhancing cell viability, and regulating cell populations [41].

Metabolite overproduction often competes with natural metabolism for limited cellular resources and disrupts the host's homeostasis for some metabolites or cofactors [42], leading to impaired cell growth [43]. Therefore, to increase the system robustness and thus to obtain high efficient production, engineered metabolic pathway must be balanced by eliminating pathway bottlenecks while preventing the accumulation of excess proteins or intermediates. A metabolic pathway can be balanced statically via engineering promoters [44] or RBSs [45], or dynamically via a metabolite biosensor to adjust flux in response to a rate-limiting or a toxic metabolite by regulating its production and/or consumption [46,47]. An alternative strategy to avoid adverse effects on cell growth is to separate the production process from cell growth phase. Several strategies were developed to induce the transition from growth phase to production, including stationary phase signals [48], quorum sensing [49], and depletion of a nutrient

[50] that is not needed for metabolite production. For example, based on the Crabtree effect, a GAL regulatory circuit was engineered in *S. cerevisiae* to autosense glucose depletion and simultaneously induce the producing pathway [34,51].

Furthermore, the accumulation of some products may trigger a variety of detrimental effects on the host cells, mainly physiological changes of cell membranes. By altering the composition of cell membranes FA (e.g. chain length, degree of saturation, and abundance of cyclic rings) [52] and the distribution of phospholipid head groups [53], the engineered hosts could demonstrate enhanced tolerance to various toxic compounds (such as alcohols, carboxylic acids and aromatic compounds) and adverse industrial conditions (such as low pH and high temperature) [52].

In addition, the heterogeneity of producing cells strongly affects the overall production of the whole culture [54,55]. A recently developed strategy named PopQC positively links metabolite production with cell growth, enabling the enrichment of high-producing variants from a heterologous culture and leading to several folds enhancement in overall product titers [16]. Selecting non-genetic variants is a promising new path to increase the production yield, with a strong potential for novel development based on the system.

Conclusion

We review the most recent development of four major metabolic pathways for the bioproduction of “drop-in” fuels. Although in the field of metabolic engineering, increasing attentions were paid to the synthesis of high-value products (pharmaceuticals, fine chemicals, etc.), significant progresses were still made towards better biofuels in

the past a few years. We expect new strategies will be continuously developed in the near future to increase titers, yields, productivities, and robustness in large-scale production, making microbial production of "drop-in" fuels more and more economically competitive.

Acknowledgements

This work was supported by the Defense Advanced Research Projects Agency (D13AP00038), the Natural Science Foundation (MCB1453147), and the Department of Energy (DESC0018324).

References

1. Frederix M, Mingardon F, Hu M, Sun N, Pray T, Singh S, Simmons BA, Keasling JD, Mukhopadhyay A: **Development of an E. coli strain for one-pot biofuel production from ionic liquid pretreated cellulose and switchgrass**. *Green Chemistry* 2016, **18**:4189-4197.
2. Gaeta-Bernardi A, Parente V: **Organic municipal solid waste (MSW) as feedstock for biodiesel production: A financial feasibility analysis**. *Renewable Energy* 2016, **86**:1422-1432.
3. Whitaker WB, Jones JA, Bennett RK, Gonzalez JE, Vernacchio VR, Collins SM, Palmer MA, Schmidt S, Antoniewicz MR, Koffas MA, Papoutsakis ET: **Engineering the biological conversion of methanol to specialty chemicals in Escherichia coli**. *Metabolic Engineering* 2017, **39**:49-59.
4. Oliver NJ, Rabinovitch-Deere CA, Carroll AL, Nozzi NE, Case AE, Atsumi S: **Cyanobacterial metabolic engineering for biofuel and chemical production**. *Current Opinion in Chemical Biology* 2016, **35**:43-50.
5. Peralta-Yahya PP, Zhang F, del Cardayre SB, Keasling JD: **Microbial engineering for the production of advanced biofuels**. *Nature* 2012, **488**:320-328.
6. Zargar A, Bailey CB, Haushalter RW, Eiben CB, Katz L, Keasling JD: **Leveraging microbial biosynthetic pathways for the generation of 'drop-in' biofuels**. *Curr Opin Biotechnol* 2017, **45**:156-163.
7. Cai W, Zhang W: **Engineering modular polyketide synthases for production of biofuels and industrial chemicals**. *Curr Opin Biotechnol* 2018, **50**:32-38.
8. Atsumi S, Hanai T, Liao JC: **Non-fermentative pathways for synthesis of branched-chain higher alcohols as biofuels**. *Nature* 2008, **451**:86-U13.
9. Marcheschi RJ, Li H, Zhang KC, Noey EL, Kim S, Chaubey A, Houk KN, Liao JC: **A Synthetic Recursive "+1" Pathway for Carbon Chain Elongation**. *Acs Chemical Biology* 2012, **7**:689-697.
10. Zhang L, Liang Y, Wu W, Tan X, Lu X: **Microbial synthesis of propane by engineering valine pathway and aldehyde-deformylating oxygenase**. *Biotechnology for Biofuels* 2016, **9**:80.
11. Rodriguez GM, Tashiro Y, Atsumi S: **Expanding ester biosynthesis in Escherichia coli**. *Nature Chemical Biology* 2014, **10**:259-265.
12. Shen CR, Liao JC: **Metabolic engineering of Escherichia coli for 1-butanol and 1-propanol**

production via the keto-acid pathways. *Metabolic Engineering* 2008, **10**:312-320.

13. Connor MR, Cann AF, Liao JC: **3-Methyl-1-butanol production in Escherichia coli: random mutagenesis and two-phase fermentation**. *Applied Microbiology and Biotechnology* 2010, **86**:1155-1164.
14. Cann AF, Liao JC: **Production of 2-methyl-1-butanol in engineered Escherichia coli**. *Applied Microbiology and Biotechnology* 2008, **81**:89-98.
15. Chen GS, Siao SW, Shen CR: **Saturated mutagenesis of ketoisovalerate decarboxylase V461 enabled specific synthesis of 1-pentanol via the ketoacid elongation cycle**. *Scientific Reports* 2017, **7**:11284.
16. Xiao Y, Bowen CH, Liu D, Zhang F: **Exploiting nongenetic cell-to-cell variation for enhanced biosynthesis**. *Nature Chemical Biology* 2016, **12**:339-344.
17. Haushalter RW, Groff D, Deutsch S, The L, Chavkin TA, Brunner SF, Katz L, Keasling JD: **Development of an orthogonal fatty acid biosynthesis system in E. coli for oleochemical production**. *Metabolic Engineering* 2015, **30**:1-6.
18. Choi YJ, Lee SY: **Microbial production of short-chain alkanes**. *Nature* 2013, **502**:571-+.
19. Shen CR, Lan EI, Dekishima Y, Baez A, Cho KM, Liao JC: **Driving Forces Enable High-Titer Anaerobic 1-Butanol Synthesis in Escherichia coli**. *Applied and Environmental Microbiology* 2011, **77**:2905-2915.
20. Wu J, Zhang X, Xia X, Dong M: **A systematic optimization of medium chain fatty acid biosynthesis via the reverse beta-oxidation cycle in Escherichia coli**. *Metabolic Engineering* 2017, **41**:115-124.
21. Willrodt C, David C, Cornelissen S, Bühler B, Julsing MK, Schmid A: **Engineering the productivity of recombinant Escherichia coli for limonene formation from glycerol in minimal media**. *Biotechnology Journal* 2014, **9**:1000-1012.
22. Du F-L, Yu H-L, Xu J-H, Li C-X: **Enhanced limonene production by optimizing the expression of limonene biosynthesis and MEP pathway genes in E. coli**. *Bioresources and Bioprocessing* 2014, **1**:10.
23. Torella JP, Ford TJ, Kim SN, Chen AM, Way JC, Silver PA: **Tailored fatty acid synthesis via dynamic control of fatty acid elongation**. *Proceedings of the National Academy of Sciences* 2013, **110**:11290-11295.
24. Gajewski J, Pavlovic R, Fischer M, Boles E, Grininger M: **Engineering fungal de novo fatty acid synthesis for short chain fatty acid production**. *Nature Communications* 2017, **8**:14650.
25. Youngquist JT, Schumacher MH, Rose JP, Raines TC, Politz MC, Copeland MF, Pfleger BF: **Production of medium chain length fatty alcohols from glucose in Escherichia coli**. *Metabolic Engineering* 2013, **20**:177-186.
26. Fan L, Liu J, Nie K, Liu L, Wang F, Tan T, Deng L: **Synthesis of medium chain length fatty acid ethyl esters in engineered Escherichia coli using endogenously produced medium chain fatty acids**. *Enzyme and Microbial Technology* 2013, **53**:128-133.
27. Haushalter RW, Kim W, Chavkin TA, The L, Garber ME, Nhan M, Adams PD, Petzold CJ, Katz L, Keasling JD: **Production of anteiso-branched fatty acids in Escherichia coli; next generation biofuels with improved cold-flow properties**. *Metabolic Engineering* 2014, **26**:111-118.
28. Bentley GJ, Jiang W, Guaman LP, Xiao Y, Zhang F: **Engineering Escherichia coli to produce branched-chain fatty acids in high percentages**. *Metabolic Engineering* 2016, **38**:148-158.
29. Jiang W, Jiang Y, Bentley GJ, Liu D, Xiao Y, Zhang F: **Enhanced production of branched-chain fatty**

acids by replacing -ketoacyl-(acyl-carrier-protein) synthase III (FabH). *Biotechnology and Bioengineering* 2015, **112**:1613-1622.

30. Barker HA, Taha SM: **Clostridium kluyverii, an Organism Concerned in the Formation of Caproic Acid from Ethyl Alcohol**. *Journal of Bacteriology* 1942, **43**:347-363.
31. Dellomonaco C, Clomburg JM, Miller EN, Gonzalez R: **Engineered reversal of the beta-oxidation cycle for the synthesis of fuels and chemicals**. *Nature* 2011, **476**:355-U131.
32. Cheong S, Clomburg JM, 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.
33. Mendez-Perez D, Alonso-Gutierrez J, Hu Q, Molinas M, Baidoo EEK, Wang G, Chan LJG, Adams PD, Petzold CJ, Keasling JD, et al.: **Production of jet fuel precursor monoterpenoids from engineered Escherichia coli**. *Biotechnology and Bioengineering* 2017, **114**:1703-1712.
34. Jiang G-Z, Yao M-D, Wang Y, Zhou L, Song T-Q, Liu H, Xiao W-H, Yuan Y-J: **Manipulation of GES and ERG20 for geraniol overproduction in Saccharomyces cerevisiae**. *Metabolic Engineering* 2017, **41**:57-66.
35. Dickschat JS: **Bacterial terpene cyclases**. *Natural Product Reports* 2016, **33**:87-110.
36. Yamada Y, Kuzuyama T, Komatsu M, Shin-ya K, Omura S, Cane DE, Ikeda H: **Terpene synthases are widely distributed in bacteria**. *Proceedings of the National Academy of Sciences* 2015, **112**:857-862.
37. Jung J, Lim JH, Kim SY, Im D-K, Seok JY, Lee S-JV, Oh M-K, Jung GY: **Precise precursor rebalancing for isoprenoids production by fine control of gapA expression in Escherichia coli**. *Metabolic Engineering* 2016, **38**:401-408.
38. Rodriguez GM, Atsumi S: **Toward aldehyde and alkane production by removing aldehyde reductase activity in Escherichia coli**. *Metabolic Engineering* 2014, **25**:227-237.
39. Zhang F, Ouellet M, Batth TS, Adams PD, Petzold CJ, Mukhopadhyay A, Keasling JD: **Enhancing fatty acid production by the expression of the regulatory transcription factor FadR**. *Metabolic Engineering* 2012, **14**:653-660.
40. Ajjawi I, Verruto J, Aqui M, Soriaga LB, Coppersmith J, Kwok K, Peach L, Orchard E, Kalb R, Xu WD, et al.: **Lipid production in Nannochloropsis gaditana is doubled by decreasing expression of a single transcriptional regulator**. *Nature Biotechnology* 2017, **35**:647-+.
41. Lim JH, Jung GY: **A simple method to control glycolytic flux for the design of an optimal cell factory**. *Biotechnology for Biofuels* 2017, **10**.
42. Ohtake T, Pontrelli S, Laviña WA, Liao JC, Putri SP, Fukusaki E: **Metabolomics-driven approach to solving a CoA imbalance for improved 1-butanol production in Escherichia coli**. *Metabolic Engineering* 2017, **41**:135-143.
43. Ceroni F, Algar R, Stan G-B, Ellis T: **Quantifying cellular capacity identifies gene expression designs with reduced burden**. *Nat Meth* 2015, **12**:415-418.
44. Guo W, Sheng J, Zhao H, Feng X: **Metabolic engineering of Saccharomyces cerevisiae to produce 1-hexadecanol from xylose**. *Microbial Cell Factories* 2016, **15**:24.
45. George KW, Thompson MG, Kang A, Baidoo E, Wang G, Chan LJG, Adams PD, Petzold CJ, Keasling JD, Lee TS: **Metabolic engineering for the high-yield production of isoprenoid-based C5 alcohols in E. coli**. *Scientific Reports* 2015, **5**.
46. Liu D, Xiao Y, Evans BS, Zhang F: **Negative Feedback Regulation of Fatty Acid Production Based on a Malonyl-CoA Sensor-Actuator**. *ACS Synth Biol* 2015, **4**:132-140.

47. Xu P, Li LY, Zhang FM, Stephanopoulos G, Koffas M: **Improving fatty acids production by engineering dynamic pathway regulation and metabolic control**. *Proceedings of the National Academy of Sciences of the United States of America* 2014, **111**:11299-11304.
48. Yu X, Xu J, Liu X, Chu X, Wang P, Tian J, Wu N, Fan Y: **Identification of a highly efficient stationary phase promoter in Bacillus subtilis**. 2015, **5**:18405.
49. Gupta A, Reizman IMB, Reisch CR, Prather KLJ: **Dynamic regulation of metabolic flux in engineered bacteria using a pathway-independent quorum-sensing circuit**. *Nat Biotech* 2017, **35**:273-279.
50. Blazeck J, Hill A, Liu L, Knight R, Miller J, Pan A, Otoupal P, Alper HS: **Harnessing Yarrowia lipolytica lipogenesis to create a platform for lipid and biofuel production**. 2014, **5**:3131.
51. Kang M-K, Zhou YJ, Buijs NA, Nielsen J: **Functional screening of aldehyde decarbonylases for long-chain alkane production by Saccharomyces cerevisiae**. *Microbial Cell Factories* 2017, **16**:74.
52. Tan Z, Yoon JM, Nielsen DR, Shanks JV, Jarboe LR: **Membrane engineering via trans unsaturated fatty acids production improves Escherichia coli robustness and production of biorenewables**. *Metabolic Engineering* 2016, **35**:105-113.
53. Tan Z, Khakbaz P, Chen Y, Lombardo J, Yoon JM, Shanks JV, Klauda JB, Jarboe LR: **Engineering Escherichia coli membrane phospholipid head distribution improves tolerance and production of biorenewables**. *Metabolic Engineering* 2017, **44**:1-12.
54. Schmitz AC, Hartline CJ, Zhang F: **Engineering Microbial Metabolite Dynamics and Heterogeneity**. *Biotechnology Journal*:1700422-n/a.
55. Delvigne F, Zune Q, Lara AR, Al-Soud W, Sørensen SJ: **Metabolic variability in bioprocessing: implications of microbial phenotypic heterogeneity**. *Trends in Biotechnology* 2014, **32**:608-616.

Highlighted References

9. This study systematically engineered the elongation enzyme LeuA using a combination of modeling, structure-based protein engineering, and metabolic engineering, to preferentially select longer-chain substrates. (•)

16. This is the first study to quantify the effect of phenotypic heterogeneity on metabolite production, and developed a strategy to enrich high-producing variants, which led to several-fold enhancement in overall product yields. (••)

23. This study programmed the degradation of an essential ketoacyl synthase to slow down acyl-ACP elongation, thus redirecting flux from phospholipid synthesis to medium-chain fatty acid production. (••)

28. This work engineered a complimentary protein lipoylation pathway to mitigate BKD-caused depletion of protein lipoylation, and improved BCFA titer and percentage. (•)

32. This work demonstrated the capability of the Non-decarboxylative Claisen condensation pathway to incorporate multiple functional groups into the product and to orthogonally control the structure of both primer and extender. (••)

36. Using genome sequencing and bioinformatic analysis this study identified 262 distinct terpene synthases. (•)

47. This study used a malonyl-CoA biosensor to dynamically regulate the supply and consumption of malonyl-CoA, which resulted in an oscillatory malonyl-CoA pattern and a balanced metabolism between cell growth and product formation. (••)

52. This study engineered the cell membrane by altering the compositions and distributions of its components, and enhanced tolerance to various toxic compounds and adverse industrial conditions. (•)

Figure Legends

Figure 1. **Biofuels produced from the α-keto acid pathway (left) and non-decarboxylative Claisen condensation pathway (right)**. α-keto acids can be converted to aldehydes by an α-keto acid decarboxylase (Kivd). Aldehydes can then be converted to alcohols by an aldehyde dehydrogenase (AdhE) or to alkanes by an aldehyde decarbonylase (colored blue). α-keto acids can be elongated by a LeuABCD mutant to yield longer chain α-keto acids (colored green). The natural-occurring non-decarboxylative Claisen condensation pathway is colored purple. The engineered primers and extenders used in the non-decarboxylative Claisen condensation pathway are colored grey.

Figure 2. **Pathways for the production of fatty acid-derived biofuels.** Native *E. coli* fatty acid pathway is colored grey. The ω-terminal structure control module is colored red. The chain length control module is colored purple. The α-terminal structure control is colored blue. FabH: β-keto-acyl-ACP synthase III; FabB: β-keto-acyl-ACP synthase I; FabG: β-keto-acyl-ACP reductase; FabZ: β-hydroxyacyl-ACP dehydratase; FabI: enoyl-acyl-ACP reductase; FadD: acyl-CoA synthase; ACR: acyl-CoA reductase; AtfA: wax-ester synthase; AAR: acyl-ACP reductase; ADO: aldehyde decarbonylase; ADH: alcohol dehydrogenase; FAR: fatty acyl reductase.

Figure 3. **Biofuels from the isoprenoid pathways.** Mevalonate pathway is colored light blue. MEP pathway is colored purple. AA-CoA: acetoacetyl-CoA; MVA: mevalonate; MVAP: mevalonate-5-phosphate; MVAPP: mevalonate-diphosphate; MEP: 2-C-methylerythritol-4-phosphate; CDP-ME: 4-diphosphocytidyl-2-C-methylerythritol; CDP-MEP: 4-diphosphocytidyl-2-C-methyl-D-erythritol-2-phosphate; HMB-PP: (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate.