Examples:
Colorimetric/enzymatic
Biosensor
Growth/single cell

Examples:
Linear construction
Sample points
Branchpoint separation

1 **Screening and modular pathway design for metabolic pathway optimization**

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Abstract

Biological conversion of substrate sugars to a variety of products ~~such as acids and alcohols~~ is an increasingly popular option for chemical transformation due to its ~~generation of a variety of products with~~ high specificity and because of significant interest in the use of renewable feedstocks. However, pathway optimization through metabolic engineering is often needed to make such molecules economically at a relevant scale. Employing effective methods to search and narrow the immense pathway parameter space is essential in order to meet performance metrics such as high titer, yield and productivity with efficiency. This review focuses on two practices that increase the likelihood of finding a more advantageous pathway solution: implementing a screen to identify high producers and utilizing modular pathway design to streamline engineering efforts. While screens seek to couple product titer with a high-throughput measurement output, modular design aims to rationally construct pathways to allow parallel optimization of various units. Both of these methodologies have proven widely successful in metabolic engineering, with combinations of them resulting in synergistic enhancements to pathway optimization. This review will particularly highlight their utility for microbially derived acid and alcohol products, which are of interest as fuels and value added products.

Keywords

Metabolic engineering, high-throughput screening, modular pathway design, free fatty acid synthesis, microbial alcohol production, biosensors

1 Introduction

2 Biologically-derived alcohols and acids have a variety of uses, including as
3 biofuels, precursors for polymeric materials, food additives, cosmetics and fragrances.
4 The production scale of these molecules is currently highly varied [1], making them a
5 great class of compounds to be tackled by metabolic engineers, especially by looking
6 through past successes and failures. Moving products from laboratory-scale to
7 industrial-scale production requires pathway optimizations enabled by high-throughput
8 titer determination, identification of pathway bottlenecks, rational focusing of libraries,
9 and redesign of natural pathways. Screening technologies permit the user to sample a
10 larger parameter space by coupling a high-throughput readout (i.e. spectrophotometric,
11 fluorescence, cell sorting) with the final titer linked to the underlying genotype. Similarly,
12 modular design similarly can focus parameter space through rational design of
13 pathways to minimize the number of pathway architectures to be tested as well as to
14 optimize modules individually that may be later combined into a full pathway. As these
15 two techniques have become widely utilized, they can be adopted simultaneously to
16 further enhance the metabolic engineering process by allowing the exploration of
17 multiple modular orientations when a high-throughput screen is available or, conversely,
18 by focusing on pathway designs with a higher likelihood of success when screening is
19 limited (**Figure 1**).

20 Screens

22 The final number of pathway combinations or library members that can be feasibly
23 tested, or screened, limits the scope of an optimization study. Direct measurement of
24 product titer by gas or liquid chromatography analysis is low throughput (less than 10^4
25 members) and falls at the bottom of the screening/selection ladder [2]. Higher
26 throughput screens in multiple micro-titer plates, on agar plates or by flow cytometry
27 require proper pairing of reporter molecules [3] and diversity generation [4] with
28 throughput size (**Figure 1**). It is imperative that the screen is coupled as closely with
29 desired metabolic output as possible, to avoid false-positives or cross-talk with other
30 endogenous cellular pathways. Additionally, throughput size should correlate with the
31 parameter space to be searched to avoid generically large screens that might not focus

on the molecule of interest. In this section, we highlight screens for the biosynthesis of acids (**Table 1**) and alcohols (**Table 2**) that span the throughput spectrum. This review will mostly focus on screens that have led to increased acid or alcohol titers; however, these methods have also been applied to many other biologically produced molecules, which are thoroughly reviewed elsewhere [1,2].

Colorimetric and enzyme assays

Many successful metabolic engineering screens have been applied to the production of colored compounds, such as carotenoids; however, neither acids nor alcohols can be measured as easily due to low wavelength absorption. As such, it is necessary to conjugate these compounds with dyes for visible or fluorescent detection. For instance, Nile Red dye has been used as a non-specific label for fatty acids to quantify their production [5,6]. When it is not possible to directly label the metabolic product, quantification of enzyme activity may serve as a proxy for final titer. Nitroblue tetrazolium was used to measure cellular NADH conversion to screen strain libraries for members with increased ethanol and *n*-butanol production [7]. Similarly, monitoring NADH or NADPH conversion enabled the engineering of a ketol-acid reductoisomerase and an alcohol dehydrogenase, the two enzymes that utilize a reduced cofactor in the pyruvate to isobutanol pathway [8,9]. Success in these enzyme-engineering approaches was the result of both rationally designed protein libraries and a lower-throughput screen [8,9]. In addition to enhancing enzyme activity and/or specificity, feedback inhibition can also be targeted. Insensitivity to CoA-SH was engineered into a thiolase enzyme, which condenses two acetyl-CoA molecules at the beginning of the fatty acid elongation pathway, by measuring NADPH consumption, resulting in an overall cellular increase in *n*-butanol titer [10] •.

Biosensors

Although direct measurement of product titer is ultimately a requirement of metabolic engineering studies, it is the lowest throughput measurement method. It is also difficult to find chemical probes to directly quantify *in vivo* production. Biosensors couple the detection of a desired product with the expression of a reporter gene [3]. Such screens

are advantageous because they allow for concentration-dependent, rapid, specific and intracellular measurement of compounds, including difficult-to-monitor pathway intermediates. Additionally, well-designed biosensors, after being used to screen production, can be incorporated as part of a metabolic control network for dynamic flux control. Many natural biosensors exist for exactly this reason and others are currently being developed to bind to compounds of interest in heterologous hosts [3].

Biosensors that act via transcriptional regulation are heavily utilized since they directly couple product sensing with gene transcription and thus have been the most successful biosensors to date. FadR is a naturally-occurring transcriptional regulatory protein that binds fatty acids. The FadR operator binding region was tuned to detect various concentrations of free fatty acids and was used to regulate their production by controlling the expression of downstream pathway enzymes [11]. FapR is a regulatory protein that interacts with malonyl-CoA, an intermediate in fatty acid synthesis. By monitoring the production of the malonyl-CoA intermediate, Xu and coworkers were able to toggle between synthesis of an upstream pathway intermediate and final free fatty acid product (**Figure 2**) [12] ••. Additionally, the binding kinetics of FapR have been modeled for future use in producing malonyl-CoA-derived compounds [13].

Natural sensors also exist for the detection of alcohols. The transcription factor BmoR binds *n*-butanol and was used to screen ribosome binding site (RBS) libraries, resulting in a 35% increase in *n*-butanol titer by controlling expression of pathway enzymes [14] •. Importantly, it was found that the BmoR protein does not bind ethanol, which is a major byproduct, resulting in low crosstalk *in vivo* [14] •. As mentioned earlier, monitoring NADH or NADPH consumption can be used to measure enzyme catalysis when a product is difficult to detect. The SoxR transcription factor binds NADPH and was used to report its intracellular concentration when an alcohol dehydrogenase was overexpressed [15]. While monitoring the reducing power of cells is useful since it is generally applicable to the optimization of numerous pathways, it is also subject to high crosstalk with other endogenous pathways that utilize NADH or NADPH [15].

It is worth noting that there are several other attractive biosensor designs that have yet to be applied to screening acid or alcohol production. RNA-based sensors are

94 especially interesting due to their modular design, our in-depth understanding of their
95 binding/folding properties, and their ease of implementation as transcriptional or
96 translational regulators. Two noteworthy studies include an RNA switch that measures
97 theophylline production [16], and a riboswitch that binds to **glucosamine 6-phosphate**
98 that can be diversified with other aptamers [17]. Rational modification of existing
99 transcription factors with new specificities is especially attractive and has already been
100 accomplished with an arabinose-binding transcription factor [18]. Lastly, little attention
101 has been paid to sensing without the need for a transcriptional reporter. Engineering
102 allosteric enzymes by transferring regulatory domains may make this possible [19] as
103 well as allow future pathway regulation at the enzyme level to eliminate wasteful and
104 unnecessary transcription and translation.

105 106 *Growth and single-cell screens*

107 **Growth-based screens have also been used for the optimization of engineered**
108 **pathways for acid and alcohol production.** These screens couple substrate utilization,
109 product formation and product/intermediate tolerance with growth rate, giving a
110 complete picture of how the organism will perform the desired conversion. Growth
111 screens are typically lower throughput than biosensor-based ones since each construct
112 is usually cultured separately in a micro-titer plate or on solid media. **Utilization of**
113 **cellobiose for the production of ethanol in yeast was enhanced by optimizing the cellular**
114 **uptake of the glucose polymer and by improving enzymatic conversion, resulting in**
115 **increased cell growth [20]. Improvements in xylose consumption and ethanol**
116 **production were achieved by a combinatorial engineering strategy that used**
117 **homologous enzymes and different yeast strains as part of a growth screen. [21].**
118 Large-scale genomic libraries have been assessed for roles in ethanol tolerance by
119 coupling them with a growth screen [22]. Similar gains in tolerance have been found by
120 transcription factor mutagenesis coupled with a growth screen in both ethanol and
121 glucose to simultaneously test substrate utilization and product tolerance [23]. Global
122 regulator engineering has also been attempted using acid shock screening to improve
123 low pH tolerance [24].

Whole-cell biosensors that couple an auxotrophic strain with synthesis of a molecule of interest are an attractive screen or selection due to their holistic consideration of cellular metabolism and high-throughput nature. One of the most successful uses of a whole-cell biosensor involved creation of an auxotrophic strain for two pathway intermediates, isopentenyl pyrophosphate or dimethylallyl pyrophosphate; only upon their supplementation through pathway addition could enhanced producers of mevalonate grow [25]. To determine appropriate knockouts for a pathway of interest, computational prediction of auxotrophic strains can be used [26]. In addition to rationally designed auxotrophic strains, others have isolated auxotrophic organisms for specific compounds of interest from environmental samples; for example, organisms that are auxotrophic for thiamine can be used to overproduce alpha-ketoglutaric acid [27]. Although using auxotrophs for metabolic engineering has proven effective in increasing product titers, single-cell measurements aim to greatly increase throughput and can be generally applied to many products. Monitoring the metabolism of single-cells in microfluidic devices has been used to screen for optimized lactate production and xylose consumption [28]. Automated measurement of pathway flux using ^{13}C labeling and NMR analysis could provide great detail for pathway optimization while being globally applicable with limited researcher time needed [29].

Modular design

Screening for metabolic product formation is a powerful tool in metabolic engineering, often leading to substantial increases in titer. However, many screens do not directly report the final titer for a molecule of interest leading to false-positive solutions, or they may be too difficult to implement (i.e. it may be more time consuming to design a biosensor for a pathway rather than to simply perform chromatographic analysis). For these reasons, it is useful to apply other constraints, such as modular design, when searching parameter space to increase the likelihood of obtaining an optimal solution. Modular pathway engineering gained attention in 2010 with its use in increasing taxol production [30] and was further described in the following review [31]. Modular design is generally defined as grouping pathway enzymes elements together into modules with the purpose of easing pathway construction and balancing pathway-enzyme expression

to control flux. To create a modular pathway, the enzymes are often grouped into expression cassettes under the control of different promoters and/or are separated on multiple plasmids. Additionally, depending on pathway design, modular construction can permit the independent optimization of pathway modules by directly feeding and sampling each unit (**Figure 1**). Other pathway optimization strategies that are often used in conjunction with modular design include: plasmid copy number control, promoter design, ribosome binding site tuning and post-translational modification [32]. This section of our review will focus on the modular construction of pathways for acid (**Table 1**) and alcohol (**Table 2**) production; an excellent and thorough review covering other ways of implementing modular construction and its role in producing alternative compounds is also available [33].

Microbially derived fatty acid chemicals have applications as biodiesel, surfactants and cosmetic products [34]. Pathway engineering has been explored in various scenarios including the deletion of the β -oxidation pathway (**Figure 2**) cycle [35] and then the implementation of the reverse β -oxidation cycle (**Figure 3**) [36]. In addition to high final titers for free fatty acids, specific control of chain length is desired to create a homogeneous product. Tseng and coworkers created a three module pathway (precursor, up and downstream) to produce short odd-chain fatty acids [37]. The functionality of each module was verified independently to focus on optimal designs for the final full pathway, and precursor supply was dictated to control final product length [37]. Another successful engineering approach also divided fatty acid synthesis into three modules and found that by varying plasmid copy number, followed by RBS tuning and bioreactor optimization, titers of fatty acids greater than 8.6 g/L could be obtained (**Figure 3a**) [6] ••. Combining modular design with a molecular sensor for an intermediate in fatty acid synthesis, Xu, Koffas and coworkers could oscillate between a source and sink for malonyl-CoA to slightly increase free fatty acid production (**Figure 2**) [12] ••. Similarly, the FadR transcription factor was used to control the final two modules of fatty acid synthesis so that these enzymes were only expressed when the intermediate pool was sufficiently high [11]. In another interesting study, fatty acid chain length was programmed through both targeted enzyme degradation and mutation to an auxiliary elongation enzyme [38]. The amount of the elongation enzyme FabB was

controlled by **targeted protein** degradation to limit the **chain length** of the resulting fatty acids [38].

In addition to fatty acids, production of alcohols has also taken advantage of modular pathway design (**Table 2**). Many of the C₄-C₅ chain alcohols currently produced from microbes are derived from amino acid synthesis [39], while longer chains are enabled by manipulation of the β -oxidation pathway [35] or use of the reverse β -oxidation cycle [36]. Extension of the carbon chain length using the cyclic beginning of the leucine biosynthesis pathway combined with rational enzyme mutagenesis enabled the production of longer-chained and branched alcohols (C₄-C₈) [40,41]; however, each iteration through the pathway is carbon wasteful and it does not generate a homogeneous product [41]. In a study by Sheppard and coworkers, they created a pathway for the specific production of isohexanol consisting of four modules, including a CoA-dependent **reverse** β -oxidation and amino acid precursor synthesis modules (**Figure 3b**) [42] ••. Modular design allowed for independent optimization of the four units of the pathway using homologous enzymes as well as permitted the exploration of specificity to reduce undesired **byproduct** formation [42] ••. In *Bacillus subtilis*, it was found that isobutanol production was increased by 60% using a native promoter in front of an upstream module and a strong promoter to control downstream enzyme expression [43]. Improved fatty alcohol yields were obtained by balancing the expression of a three-enzyme pathway as well as by using a solvent layer simultaneously to extract the alcohols [44]. In addition to increasing productivity, specific control of chain length for fatty alcohols was possible by adding different thioesterases to a downstream module [45]. Controlled chain length of dicarboxylic acids (C₆, C₈, and C₁₀) was obtained using different enzyme combinations in the iterative reverse β -oxidation cycle module (incorporated into the *E. coli* genome) and a downstream ω -oxidation module [46]. Using a β -oxidation knockout, it was possible to produce a variety of fatty acid short-chain esters through a combination of modules for fatty acid synthesis and 2-keto acid conversion to short chain alcohols [47].

Modular pathway design has proven its place in metabolic engineering; however, its potential is far from achieved as outlined in the following reviews [33,48].

Combination with other approaches to pathway engineering may enable additional

significant advances. Orthogonal T7 polymerases have been created which would permit high level production of multiple modules as well as separation of the sensing logic module from the functional module expression [49]. An autonomous controller module was developed using quorum sensing to successfully increase the production of 1,4-butanediol from a *de novo* designed pathway [50] •. Additionally, this work successfully decoupled cell growth from the production module by using two different carbon sources and introducing knockouts between the pentose phosphate pathway and glycolysis [50] •. Design of modular architecture has been limited to separating linear pathways at specific sampling or branch points. Using an unbiased network approach, it may be possible to rethink the network architecture used in modular design [51]. Additionally, systems biology can predict other non-canonical pathways to be included in modular design and whose crosstalk may affect specificity and yield [52]. Lastly, kinetic incorporation within modular pathway design must be considered beyond varying plasmid copy number and RBS tuning. Grouping enzymes into modules based on catalytic turnover is a logical next step [33]. Computational prediction of module outputs (i.e. through ensemble modeling) could further constrain modular pathway design to create constructs with the highest likelihood of success [53].

Future directions

Screens and modular pathway design can increase the likelihood of finding optimal metabolic solutions for the production of many compounds of interest. While modular design outlines general rules for how to construct pathways, screens and selections are often specific for only one molecule, thus requiring redesign before being applied to other pathways. Future studies will look to find global screens to limit the amount of optimization that must occur for a single pathway. For instance, Lee, Dueber and coworkers have created a general methodology for optimizing a pathway without the need for a high throughput screen [54] •. They used computational modeling to determine optimal expression levels for a three-enzyme pathway and verified their design by measuring the production of fluorescent proteins prior to testing their pathway with desired enzymes [54] •. The synergy of auxiliary pathways (i.e. generation of reducing equivalents, intermediate cofactors, ATP) with a pathway of interest must be

considered to achieve an overall balanced system [31,55]. This has led to the modularization of several of these pathways resulting in an increased yield of *n*-propanol [55] and could be easily applied to the synthesis of other molecules of interest.

As evidenced herein, pathway engineering combined with screening methodologies enables a narrowing of the parameter space to be searched to determine a more optimal pathway solution. Similarly, the expansive data sets available from next-generation sequencing technologies can provide great insight into pathway design, from enzyme choice to expression level, to further focus the explored parameter space. Genomic sequencing data along with the characterization of the fermentation products for yeast strains has been used to find the traits that matter most for ethanol production [56]. Similarly, RNAseq was performed on a variety of yeast strains to determine their response to different substrates, revealing a common set of differentially regulated genes [57]. The assembly of metagenomes from an environmental pool of organisms has identified species that overproduce ethanol as well as the pathways responsible for this trait [58]. Genomic sequencing and annotation of thermophilic organisms have led to discovery of enzymes that have or maintain increased activity at high temperatures. These enzymes were then used to enhance cellobiose conversion to *n*-butanol at elevated temperatures in a thermophilic bacterium [59]. Finding orthogonal pathways for the production of fatty acids and expression of these complexes in heterologous hosts has the potential to alter the specificity of production and could be more beneficial than mutating native machinery [60]. Thus, genome-scale techniques, enabled by high-throughput sequencing and data set comparisons, permit users to not only identify high titer producers, but also gain foundational insight into the biological traits and principles that lead to these favorable phenotypes.

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Annotated references

- [6] The authors used modular design to split a 15-enzyme pathway amongst three plasmids and found that varying copy numbers for the modules gave rise to a more optimal solution than placing all the enzymes on high copy plasmids.
- [10] This manuscript reports a clever extension of a NADPH consumption assay for thiolase activity by including the CoA-SH product to find variants that are feedback insensitive.
- [12] This manuscript describes an outstanding integration of a biosensor with modular pathway architecture. The authors used the sensing of a pathway intermediate to oscillate between an up and downstream module to create two production modes for the pathway.
- [14] The authors report an excellent use of a biosensor to find an optimal pathway solution from a large RBS library as well as a detailed exploration of crosstalk for non-desired molecules.
- [28] This manuscript reports a novel approach to a single-cell screening using a microfluidic device to measure substrate consumption and product generation in a high-throughput manner.
- [42] The manuscript describes an inventive combination of a portion of amino acid synthesis with the reverse β -oxidation pathway to produce a non-natural, branched alcohol. The authors used modular design to facilitate pathway construction and individually validate modules prior to assembling the entire pathway.
- [50] The authors used genetic knockouts to successfully decouple glucose consumption for cellular growth and xylose utilization for 1,4-butanediol production.
- [54] The authors report a modular strategy that can be adapted to numerous pathway architectures based on metabolic modeling and validated by fluorescent protein expression.

Figure legends

Figure 1

This schematic describes the necessary inputs to successfully implement a screen or design a modular pathway as well as the general outcomes of each. Screens are characterized by their high-throughput nature and can be divided into three classes: colorimetric/enzymatic, biosensors, or growth-based. Colorimetric/enzymatic screens result from the labeling of a final product for high throughput detection or by coupling an *in vitro* assay of enzyme activity with a product titer. Biosensors often act through transcriptional regulation, permitting the concentration-dependent expression of a reporter gene as a result of metabolic production. Growth screens couple the generation of product with growth rate to find strains with increased substrate uptake and conversion. Modular design is an attractive engineering choice when a high-throughput screen is not available by limiting the architectures tested using rational pathway construction. Modular design permits easy pathway assembly with the purpose of balancing enzyme production as well as enabling parallel optimization of modules through intermediate sampling/feeding to limit the number of final pathway designs to test. In the future, combining modular design with a high-throughput screen would allow for more pathway architectures to be explored with the purpose of finding general design rules to be applied to a variety of pathways of interest.

Figure 2

In [12], synthesis of free fatty acids from glucose was divided into two modules: an acetyl-CoA activation module (ACA) that resulted in the synthesis of malonyl-CoA from glucose (upstream module, green) and a free fatty acid synthesis module (FAS, pink) that converted malonyl-CoA into free fatty acids (downstream module). In addition to the overexpressed pathway enzymes, several endogenous enzymes are necessary to convert glucose to free fatty acids; these pathways are indicated by dotted lines. Natural β -oxidation was knocked out using a deletion of *fadD*. Expression of enzymes from the two modules was transcriptionally regulated by FapR, an intracellular transcription factor responsive to malonyl-CoA concentration. FapR can act as a repressor or activator depending on the promoters used and number of *fapO* sites present in gene expression cassettes. The upstream ACA module was activated by FapR while the downstream FAS module was repressed. Increasing concentrations of malonyl-CoA over culture time resulted in deactivation of the upstream module and de-repression of the downstream module, allowing the cell to toggle between the two modules to better utilize cellular resources.

Figure 3

A) In [6], conversion of glucose to free fatty acids was split into three modules, a glycolysis module (Gly, blue) that consisted of selected genes from glycolytic pathways, an acetyl-CoA activation module (ACA, green) that contained genes responsible for the

conversion of acetyl-CoA into malonyl-CoA, and a fatty acid synthesis module (FAS, pink) with genes to convert malonyl-CoA into free fatty acids. Endogenous pathways are indicated by dotted lines. For each module, the genes were present in a pseudo-operon where a promoter was placed directly before each gene but only a single terminator was present downstream. The pseudo-operons were expressed from plasmids that contained a high, medium, and/or low copy number origin of replication, permitting the exploration of module expression level on free fatty acid production. The optimal set of origins of replication is boxed. B) In [42], synthesis of isohexanol from glucose was implemented through a four-module pathway, which included valine synthesis (module 1, purple), acetyl-CoA activation (module 2, green), reverse β -oxidation cycle (module 3, pink), and acid reduction (module 4, red). An endogenous thioesterase was used to terminate the reverse β -oxidation cycle. Modular design permitted the parallel characterization of several of the pathway elements. Characterization of the substrate specificity of the carboxylic reductase (Car_{Ni}) in module 4 enabled the *de novo* construction of upstream pathway elements to synthesize the desired substrate, isohexanoate. By feeding the isobutyrate intermediate to cells that contained modules 2 and 3, combinations of homologous Ter and Phaj enzymes were tested to identify combinations that produced the highest isohexanoate titers (boxed). Modular design aided in separating different parts of the synthesis pathway so each module could be improved and tested separately, by feeding appropriate substrates and screening for corresponding products.

Table 1: Free fatty acid production

Fatty acid	Engineering approach	Titer ¹ (g/L)	Yield ²	Fold improvement ³	Library members screened (#)	Reference
Pentanoate	Modular design, precursor feeding	0.40	0.04 g/g _{gly}	n/a	37	[37]
Octanoate ⁴	Dynamic regulation	0.26	0.052 g/g _{gly}	1.32	6	[38]
C ₁₂ , C ₁₄ , C ₁₆ , C ₁₈ free fatty acids	Colorimetric screen, transposon library	0.89	0.223 g/g _{gly}	1.2	2786	[5]
C ₁₂ – C ₂₀ free fatty acids	Biosensor, modular design, promoter engineering	1.5	0.075 g/g _{gluc}	3.51	20	[11]
C ₁₄ , C ₁₆ , C ₁₈ free fatty acids ⁵	Modular design, plasmid copy number, RBS tuning	2.04	0.255 g/g _{gluc}	3.46 ⁶	17	[6] ●●
C ₁₄ , C ₁₆ , C ₁₈ free fatty acids ⁵	Biosensor, modular design	3.86	0.193 g/g _{gluc}	3.1	4	[12] ●●

¹ Titters reported from experiments conducted under screening/small-scale conditions. In some cases, higher titers were achieved by scaling-up to bioreactors.

² Yields were calculated from titers reported in this table and substrate amounts listed in the methods of each reference. The sugar feeds are abbreviated: glucose (gluc) and glycerol (gly).

³ Fold improvements were calculated using titers reported in this table and the base case/wild type reported in each reference. n/a - not applicable; first demonstration of the pathway.

⁴ A 92% pure octanoate product was achieved using dynamic regulation.

⁵ Some of the fatty acids produced contained a saturated bond.

⁶ A 2.37-fold increase was observed by varying plasmid copy number and an additional 1.46-fold increase was obtained through RBS tuning.

Table 2: Alcohol production

Alcohol	Engineering approach	Organism	Titer (g/L) ¹	Yield ²	Fold improvement ₃	Library members screened (#)	Reference
Ethanol	Growth screen	<i>S. cerevisiae</i>	34	0.42 g/g _{cel}	1.63 ⁵	10,000	[20]
	Cholorimetric enzyme assay, strain library	<i>C. acetobutylicum</i>	7.2	0.144 g/g _{gluc}	1.37	5150	[7]
<i>n</i> -butanol	Semi-rational design	<i>C. acetobutylicum</i>	12.4	0.196 g/g _{gluc}	1.23	1620	[10] •
	Biosensor growth screen, RBS library	<i>E. coli</i>	0.49	0.098 g/g _{gluc}	1.35	960	[14] •
	Rational design and directed evolution	<i>E. coli</i> (anaerobic)	13.4	0.157 g/g _{gluc}	9.5	130 (rational) 4000 (random)	[8]
Isobutanol	Modular design, promoter design	<i>B. subtilis</i>	3.8	- (fed batch)	1.6	7	[43]
	Thermostable enzyme prospecting	<i>G. thermoglucosidasi</i> <i>us</i>	3.3	0.092 g/g _{gluc}	n/a	8	[59]
Isopentanol	Synergistic design	<i>E. coli</i>	4.5	0.15 g/g _{gluc}	3.57	5	[55]
3-methyl-1-pentanol ⁴	Modular design, rational design	<i>E. coli</i>	0.79	0.040 g/g _{gluc}	132	14	[40]

Isohexanol	Modular design, <i>de novo</i> assembly	<i>E. coli</i>	0.192 ⁶	0.016 g/g _{gluc}	n/a	30	[42] ●●
<i>n</i> -heptanol ⁴	Modular design, rational design	<i>E. coli</i>	0.08	0.004 g/g _{gluc}	n/a	8	[41]
C ₁₁ , C ₁₃ , C ₁₅ fatty alcohols	Modular design, enzyme prospecting, promoter selection	<i>E. coli</i>	0.10 ⁷	0.003 g/g _{gly}	13.70	47	[45]
C ₁₂ , C ₁₄ , C ₁₆ , C ₁₈ fatty alcohols	Promoter selection, plasmid copy number, expression balance	<i>E. coli</i>	1.19 ⁸	0.097 g/g _{gluc}	4.23	22	[44]

¹ Titers reported from experiments conducted under screening/small-scale conditions. In some cases, higher titers were achieved by scaling-up to bioreactors.

² Yields were calculated from titers reported in this table and substrate amounts listed in the methods of each reference. The sugar feeds are abbreviated: glucose (gluc), glycerol (gly), and cellobiose (cel).

³ Fold improvements were calculated using titers reported in this table and the base case/wild type reported in each reference. n/a - not applicable; first demonstration of the pathway

⁴ Many other alcohol products were reported in these references; the ones reported herein are those the authors highlight in their abstract.

⁵ Fold improvement is calculated based on ethanol productivity (g/L-hr) at the beginning of the experiment; the final titers obtained were almost identical.

⁶ A more specific production for isohexanol is reported using a homologous alcohol dehydrogenase; however, with a decrease in titer to 0.092 g/L.

⁷ Titers were improved to 1.95 g/L upon scale-up.

⁸ Two modules were used in this reference: one was genomically-encoded and the second was plasmid-borne. Titers were improved to 1.65 g/L when a solvent was used to simultaneously extract the products.

Figure 1

Figure 1. Boock et. al. Current Op in Biotech

Figure 2

Figure 2. Boock et. al. Current Op in Biotech

A.

B.

Figure 3. Boock et. al. Current Op in Biotech