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Recent applications of metabolomics to advance microbial biofuel production

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Biofuel production from plant biomass is a promising source of renewable energy [1]. However, efficient biofuel production involves the complex task of engineering high-performance microorganisms, which requires detailed knowledge of metabolic function and regulation. This review highlights the potential of mass-spectrometry-based metabolomic analysis to guide rational engineering of biofuel-producing microbes. We discuss recent studies that apply knowledge gained from metabolomic analyses to increase the productivity of engineered pathways, characterize the metabolism of emerging biofuel producers, generate novel bioproducts, enable utilization of lignocellulosic feedstock, and improve the stress tolerance of biofuel producers.

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Introduction

Maximizing microbial biofuel production from plant biomass (i.e. lignocellulosic biomass or plant dry matter) requires reprogramming metabolism to ensure a seamless supply of carbon, energy (e.g. ATP), and reducing power (e.g. NAD(P)H) towards engineered biofuel pathways. This must be accomplished without eliciting unintended metabolic inefficiencies that frequently accompany engineering efforts. Furthermore, engineered microbes must be capable of catabolizing the diverse sugars (i.e. hexoses and pentoses) present in plant biomass and be tolerant to inhibitory chemicals generated during biomass pretreatment and fermentation (Figure 1). Over the last decade, advancements in chromatography, mass spectrometry, and computational analysis have enabled accurate systems-level quantification of intracellular metabolites and metabolic fluxes, helping to inform rational bioengineering by revealing the complex processes driving cellular metabolism [2,3]. This review highlights recent studies that have used knowledge gained from mass-spectrometry-based metabolomic analyses to improve the efficiency of engineered pathways, enable utilization of lignocellulosic sugars, and increase stress tolerance in biofuel-producing microbes (Table 1).

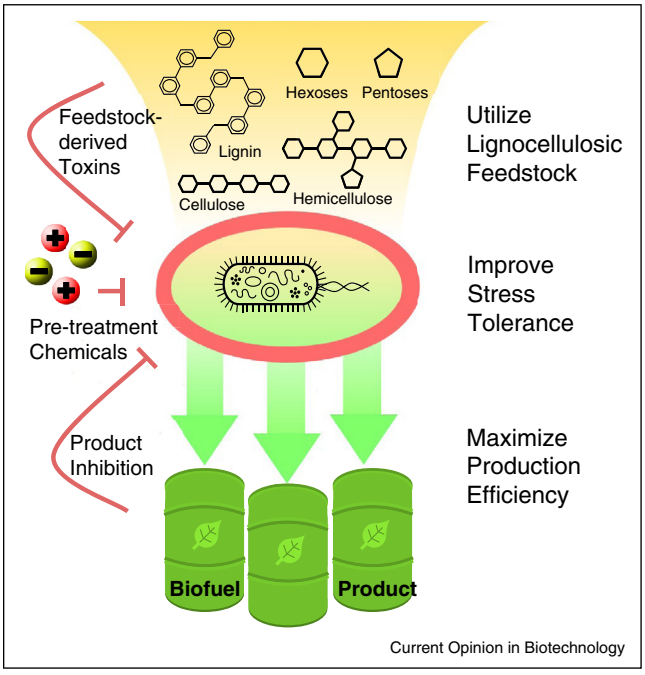
Improving activity of engineered pathways and increasing product yields

At the heart of efficient biofuel production lies the challenge of metabolic engineering [1]. Thanks to recent developments in synthetic biology and genetic engineering, manipulating heterologous enzyme expression to construct biofuel-producing pathways in a microbial host is no longer the roadblock it once was [4,5]. However, optimal pathway activity is rarely achieved by simple expression (or overexpression) of required enzymes; product formation can be affected by many other factors including consumption of substrates by competing pathways, energetic and redox imbalances caused by engineered pathway activity, and inhibition due to product accumulation. Metabolomic analyses can guide pathway optimization by identifying sources of metabolic inefficiency, revealing strategies to increase activity of engineered pathways.

Modeling central metabolism

Characterizing and modeling central carbon metabolism is a critical step in metabolic engineering as it gives a holistic picture of carbon, energy, and redox sources and sinks. Although the central metabolism of model organisms such as *Escherichia coli* and *Saccharomyces cerevisiae* has been extensively characterized, many organisms with less studied metabolism have emerged as promising alternatives to traditional microbes due to attractive qualities such as high heat tolerance, rapid carbon assimilation, or diverse substrate utilization [6–8]. Metabolomics data can be used to build computational models of carbon flux through metabolic networks in order to develop strategies for metabolically engineering new and promising microbes. Recently, ^{13}C Metabolic Flux Analysis (^{13}C MFA), a method that models flux based on steady-state isotopic labeling data, has been employed to model metabolism in emerging biofuel producers such as *Thermus thermophilus*, *Clostridium acetobutylicum*, and *Geobacillus* sp. [9•,10••,11••]. These studies verify genome annotations, characterize non-canonical pathways, and determine major sources and sinks of energy and reduced cofactors.

Figure 1



The major goals of metabolic engineering for microbial biofuel production are **(1)** to direct metabolic flux towards maximum biofuel generation, **(2)** to enable use of economical feedstock such as lignocellulose, and **(3)** to improve stress tolerance to inhibitors produced during pre-processing or biofuel production. The studies featured in this review apply knowledge gained from metabolomics-based methods to achieve these goals.

Although several organisms have already been identified as promising biofuel-producers, the vast diversity of microbial life has not yet been fully explored in the search for optimal organisms. A study by Hollinshead *et al.* develops a method to improve the rate at which central carbon metabolism can be mapped in novel organisms [12••]. This study describes a ^{13}C fingerprinting kit which allows for metabolic analysis of central pathways including both classical Embden–Meyerhof–Parnas (EMP) and Entner–Doudoroff (ED) glycolysis, the pentose phosphate (PP) pathway, and the TCA cycle via quantification of only a few amino acids. Low cost metabolic analysis such as this will greatly increase the rate at which new organisms with desirable native features can be characterized for further development as biofuel producers.

Central metabolism can be dramatically altered by genetic modifications aimed at increasing bioproduct yields. By quantifying the metabolic responses elicited by increased activity of biofuel-producing pathways, ^{13}C MFA of central metabolism can be used to propose new methods for supporting the carbon, energy and redox demands required for excessive biofuel production [13••,14•]. For example, He *et al.* used ^{13}C MFA to quantify the metabolic impact of increased fatty acid production on central metabolism in *E. coli* [13••]. Their study found that the PP pathway and TCA cycle were flexible nodes of central metabolism: flux through these pathways adjusted to accommodate carbon and redox demands from fatty acid overproduction, while ED glycolysis was a rigid node: flux through the ED pathway remained low despite its ability to increase supply of both NADPH and acetyl-coA for fatty acid synthesis. On the basis of these flux dynamics, He *et al.* identified upregulation of ED glycolysis as a potential strategy to further improve fatty acid production by *E. coli*.

Altering redox balancing to support biofuel production

Production of highly reduced fuels such as alcohols, fatty acids and hydrocarbons requires an adequate supply of reduced cofactors such as NAD(P)H. Since the availability of reduced cofactors is determined by the combined activity of many catabolic and biosynthetic pathways in primary and secondary metabolism, systems-level metabolic analysis can provide critical insight into redox regulation. Additionally, it has been shown that cellular responses to increased NAD(P)H demands can be metabolically rather than transcriptionally regulated, making metabolomics an important complement to transcriptomic analysis [15].

Recent studies have used metabolomics to identify changes in redox balance caused by metabolic engineering and evaluate the implications for biofuel production [13••,14•,15,16•]. Anasontzis *et al.* used systems-level metabolite quantification to determine the mechanism by which constitutive expression of phosphoglucomutase in upper glycolysis and transaldolase in the PP pathway resulted in increased ethanol production by *Fusarium oxyporum*. They found that increased expression of these two enzymes improved NADPH regeneration, leading to a shift in carbon flux away from acetate and towards ethanol production [16•]. He *et al.* employed ^{13}C MFA to show that flux through the oxidative PP pathway and conversion of NADH to NADPH by transhydrogenase increased to support NADPH demands from fatty acid overproduction [13••]. A complementary study by Wada *et al.* found that during overproduction of mevalonate, a bioproduct requiring less reducing power than fatty acids, PP pathway flux did not increase, and enhanced transhydrogenase activity was sufficient to support NADPH demands [14•]. Taken together, these studies reveal the potential for improving biofuel production by manipulating activity of the oxidative PP pathway and transhydrogenase reactions.

In addition to biofuel production, availability of reducing equivalents is essential for cellular maintenance and protein synthesis. The added energetic and redox demands required for heterologous enzyme expression often limits the efficiency of engineered pathways [17]. Nocon *et al.* used ^{13}C MFA to compare the flux profiles of