Tracing metabolism from lignocellulosic biomass and gaseous substrates to products with stable-isotopes

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Abstract

Bioengineered microbes offer a practical and sustainable alternative to traditional industrial approaches. To increase the economic feasibility of biological processes, microbial isolates are engineered to take up inexpensive feedstocks (including lignocellulosic biomass, syngas, methane, and carbon dioxide), and convert them into substrates of central metabolism and further into value-added products. To trace the metabolism of these feedstocks into products, isotopic tracers are applied together with isotopomer analysis techniques such as ^{13}C -metabolic flux analysis to provide a detailed picture of pathway utilization. Flux data is then integrated with kinetic models and constraint-based approaches to identify metabolic bottlenecks, propose novel metabolic engineering strategies, and improve process performance.

Introduction

The production of chemicals through bioconversion has received much attention over the past decade [1]. Focus is now shifting towards the utilization of cheap, renewable bio-feedstocks, ultimately leading to reduced operating costs, reduced reliance on non-renewable feedstocks and feedstocks that compete with food supply, and reduced environmental impact [2,3]. Renewable feedstocks include lignocellulosic biomass and gaseous substrates such as syngas, methane, hydrogen gas, and carbon dioxide. The primary renewable feedstock, lignocellulosic biomass, is composed of three main components, two of which, cellulose and hemicellulose, can be hydrolyzed to C6 and C5 sugar monomers and used as substrates in fermentations. Alternatively, biomass can be gasified to produce syngas, a mixture of carbon dioxide, carbon monoxide and hydrogen, for use in mixotrophic fermentations [4,5]. Moreover, biological processes such as anaerobic digesters produce large quantities of methane, yet another potential source of carbon

and energy. With the availability of these renewable feedstocks, metabolic engineering efforts are geared towards engineering organisms to utilize one or more of these substrates and produce value-added chemicals. These efforts have consisted of introducing new metabolic capabilities into model organisms such as *E. coli* and *S. cerevisiae*, and engineering promising new organisms capable of metabolizing these feedstocks at higher rates and yields [3,6].

With the wide range of potential substrates and products, it is of critical importance to evaluate the economic feasibility of bioprocesses, determine the capabilities of microbial systems, identify targets for improvements, and select ideal candidates for industrial implementation. Significant efforts have been directed at understanding cellular metabolism of microbes and identifying key regulatory mechanisms and potential kinetic limitations. A powerful method for characterizing *in vivo* metabolism is through the use of ^{13}C -labeled substrates (or ^{13}C -tracers). Tracing techniques allow quantitative evaluation of the flow of carbon from feedstocks to central metabolism and further into the desired products. Additionally, advanced techniques such as ^{13}C -metabolic flux analysis (^{13}C -MFA) can be applied to gain a fine-grained picture of native metabolism and metabolic changes that result from genetic manipulations. In this contribution, we provide an overview of the wide range of pathways that are being implemented to convert renewable substrates into value-added products, and discuss methods for elucidating metabolism through these pathways using advanced ^{13}C -tracing techniques.

Metabolism of lignocellulosic biomass

The major costs in biological processes are substrate-related [7]. Thus, organisms which can utilize a broad spectrum of renewable substrates offer a major competitive advantage in the

development of technologies for the production of next-generation fuels and chemicals. The primary renewable feedstock, lignocellulosic biomass, is composed of cellulose, hemicellulose, and lignin. Cellulose is a polymer of glucose, while hemicellulose is composed of both C6 (glucose, mannose, galactose) and C5 (xylose, arabinose) sugars. Biological conversion of these sugars has received much attention. Metabolism of sugars starts with transport of the sugar into the cells followed by activation (phosphorylation). The main mechanisms for sugar transport are active transport (via the PTS transport system, or ATP-dependent ABC transporters), and passive transport by permeases. For example, in *E. coli*, glucose is transported and phosphorylated simultaneously via the PTS system at the expense of one ATP equivalent, while two ATPs are expended when xylose serves as the carbon source, i.e. one for transport (high-affinity ABC transporter) and the second for activation (phosphorylation). Other sugars, e.g. lactose, are transported by permeases. The two classical pathways for sugar catabolism are glycolysis and pentose phosphate pathway (Figure 1). Hexoses feed directly into glycolysis at glucose 6-phosphate (G6P) or fructose 6-phosphate (F6P), while pentoses feed into the pentose phosphate pathway at xylulose 5-phosphate (X5P). Many organisms are capable of catabolizing more than one sugar; however, in most cases carbon catabolite repression (CCR) results in preferential utilization of certain sugars over others.

Various engineering strategies have been employed to eliminate CCR, often resulting in more efficient bioprocesses. A successful strategy has been knocking out components of the PTS system and applying adaptive evolution to improve sugar co-utilization. For example, Balderas-Hernandez et al. adaptively evolved *E. coli* lacking a functional PTS system under anaerobic conditions to achieve a glucose/xylose co-utilizing *E. coli* strain [8]. Adaptive evolution was also

successfully applied to generate an improved strain of the thermophile *Thermus thermophilus* that efficiently co-utilized glucose and xylose without CCR [9]. In another study, the *ptsG* gene was removed in *E. coli* to relieve CCR and allowed production of 3-hydroxypropionic acid from both glucose and xylose in fed-batch fermentations [10]. The model anaerobic organism *Clostridium acetobutylicum* was also evaluated for sugar co-utilization [11]. Analysis of CCR genes in *C. acetobutylicum* identified targets for engineering simultaneous utilization of glucose and xylose [12]. When glucose repression of xylose catabolism was removed, product titers from co-utilization of both substrates were comparable to wild-type titers [13].

Native and synthetic pathways for conversion of renewable feedstocks

Glycolysis and pentose phosphate pathway are the main routes for sugar catabolism in microbes; however, these pathways result in significant loss of carbon (CO₂ released), which reduces theoretical yields [5]. Recent metabolic engineering strategies have focused on integrating alternative catabolic pathways into host organisms with the goal of producing more efficient bioconversions (Table 1). Figure 2 provides an overview of the wide range of alternative metabolic pathways that are being considered for conversion of renewable feedstocks. A classic alternative to the glycolysis pathway is the Entner-Doudoroff pathway, which produces glyceraldehyde phosphate (GAP) and pyruvate, as well as the reducing cofactor NADPH. A less common variant of the ED pathway is the non-phosphorylated ED pathway which produces pyruvate and glyceraldehyde and does not involve phosphorylated intermediates. This pathway can connect to the classic ED pathway through the activity of 2-keto-3-deoxygluconate kinase, thus forming the semi-phosphorylated ED pathway. Studies have revealed presence of these alternative ED pathways in certain thermophilic and halophilic archaea [14].

Another route to bypass glycolysis and reach useful intermediates without carbon loss is by employing phosphoketolases [15]. Phosphoketolases are enzymes that are distantly related to transketolases and are found natively in certain *Lactobacillus*, *Bifidobacterium* and *Clostridium* species [11,16-18]. These promiscuous enzymes can cleave either F6P or X5P to acetyl-phosphate and a corresponding sugar phosphate, erythrose-4-phosphate (E4P) and GAP, respectively. The sugar phosphates can then be recycled back to F6P or X5P. It was demonstrated that by employing phosphoketolases, sugars can be converted to acetate and other products at near-theoretical yields, as first described in [19] and later in [20] (where it was rebranded “non-oxidative glycolysis”, NOG). Phosphoketolases have been successfully incorporated into *E. coli* and *S. cerevisiae* for acetone, ethanol and fatty acid production [21-23].

Other promising routes for sugar metabolism include the Weimberg and Dahms pathways and a new synthetic xylulose 1-phosphate pathway [24]. The Weimberg pathway bypasses glycolysis to reach the TCA cycle intermediate α -ketoglutarate, while the Dahms pathway bypasses glycolysis to produce pyruvate and glycoaldehyde. These conversions occur without phosphorylation of intermediates or carbon loss, which results in increased maximum theoretical yields of products such as itaconic acid, succinic acid, and glutamic acid. The Weimberg pathway was successfully introduced into *E. coli* and *C. glutamicum* for more efficient conversion of xylose and arabinose into various products [25-27]. This pathway is also being investigated for industrial purposes in solvent-tolerant organisms that natively express this pathway, such as *Pseudomonas taiwanensis* [28]. Recently, an alternative xylose degradation pathway was engineered, termed the xylulose 1-phosphate (X1P) pathway [24]. In X1P pathway,

the first two carbon atoms of xylose are converted to glycoaldehyde from which products such as ethylene glycol and glycolic acid can be produced [29,30]. Glycoladehyde can also enter the TCA cycle via the glyoxylate shunt after being converted to glyoxylate.

Renewable gases are also increasingly evaluated as potential co-substrates for the production of chemicals [31,32]. These gases include carbon dioxide, carbon monoxide, and hydrogen gas derived from biomass gasification, and methane derived from anaerobic digestion of organic wastes. Multiple avenues are implemented to increase the ability of microorganisms to utilize gaseous substrates. Two well studied carbon fixation pathways are the Calvin cycle and the Wood-Ljungdahl pathway [33]. The Calvin cycle fixes CO₂ to the C5 sugar ribulose biphosphate (RuBP) forming two molecules of 3-phosphoglycerate (3PG), catalyzed by ribulose1,5-bisphosphate carboxylase–oxygenase (RubisCO), an enzyme that is essential in photosynthesis. While the Calvin cycle is most prevalent in photosynthetic organisms, it is also found in some bacteria such as the *Sulfobacillus* and *Oscillochloris* species [34]. Compared to other carbon fixation pathways, however, the Calvin cycle is more energy demanding as it consumes seven ATP to form one molecule of pyruvate [33]. Regardless, there have been many reports of using autotrophic organisms to produce chemicals such as isopropanol, isoprene, and sugars from CO₂ [35]. There is also the potential to introduce this pathway into non-carbon fixing organisms, which could effectively increase product yields and decrease carbon loss [36-38]. In a recent study, it was demonstrated that expression of the Calvin cycle in *S. cerevisiae* resulted in higher ethanol yields and eliminated glycerol by-product formation [39].

In addition to the Calvin cycle, the Wood-Ljungdahl (WL) pathway is a widely studied carbon fixation route that can utilize both CO₂ and CO as substrates [4,40]. In WL pathway, one molecule of CO₂ is converted to CO by carbon monoxide dehydrogenase, and a separate cascade of steps forms a methyl group from CO₂. The enzyme acetyl-CoA synthase then brings the CO and methyl group together to form AcCoA. Depending on the carbon source, electrons can be obtained from H₂ or CO. The WL pathway is energetically more efficient than the Calvin cycle and is found exclusively in anaerobic organisms [5,41].

Methane is another potential substrate that can be converted into fuels and chemicals [42,43]. Methane is first converted to methanol and then to formaldehyde, which gives the cell access to several carbon assimilation pathways. Two of these pathways are depicted in Figure 2, the ribulose monophosphate (RuMP) pathway and the xylulose monophosphate (XuMP) pathway. The RuMP pathway uses two enzymes to fix formaldehyde to ribulose 5-phosphate (Ru5P) and enter central carbon metabolism as F6P. Similarly, the XuMP pathway attaches formaldehyde to a C5 sugar, X5P, and forms GAP and dihydroxyacetone phosphate (DHAP), which are intermediates of glycolysis. The RuMP pathway is found in methylotrophic bacteria such as *Mycobacterium gastri* and *Bacillus methanolicus*, while the XuMP pathway is found natively in methylotrophic yeasts. Methanol is an attractive substrate because it contains more electrons per carbon than sugars; thus, theoretical yields of reduced products, including biofuels, are higher with methanol as substrate. So far, it has been difficult to generate a non-native fully methylotrophic microbe [44], while engineering co-utilization of methanol and sugars has been more successful. For example, *C. glutamicum* was engineered to consume methanol and ribose and produce

cadaverine [45], and *Pichia pastoris* was shown to produce recombinant proteins from methanol and glucose [46].

Tracing metabolism with isotopic tracers

Tracing how substrates are utilized and converted into products is critical in assessing the efficiency of engineered pathways and to guide further metabolic engineering strategies. Stable-isotope labeling techniques (primarily with ^{13}C -tracers) are widely used in metabolic engineering to quantify carbon flux for this purpose [47,48]. In tracer experiments, an isotopically labeled substrate is added to the culture, e.g. $[\text{U-}^{13}\text{C}]$ glucose, resulting in the incorporation of ^{13}C atoms into intermediates of cellular metabolism and eventually into products. By quantifying fractional ^{13}C -labeling of various metabolites (products) the contribution of a particular substrate can be determined. Tracer experiments are especially useful when multiple substrates are present and when complex additives such as yeast extract are used in fermentations. By measuring ^{13}C -labeling over time it is also possible to elucidate relative rates of substrate utilization. For example, a constant isotopic labeling indicates that substrates are consumed simultaneously [5]. On the other hand, if labeling changes in time, then the rate at which the labeling changes can be translated into relative flux changes [49].

Tracing techniques require accurate and sensitive analytical approaches for measuring isotopic labeling. Currently, mass spectrometry (MS) based techniques are preferred due to their high sensitivity and the potential to obtain positional labeling information through tandem mass spectrometry [50]. MS-based approaches require a relatively small sample size and give access to intracellular metabolites. Figure 3 provides an overview of metabolites in central carbon

metabolism that can be measured with GC-MS and LC-MS/MS. While there is significant overlap in the range of metabolites that can be measured with these two approaches, LC-MS/MS is generally more suitable for measuring phosphorylated metabolites in the pentose phosphate pathway, nucleosides and other large molecules [51], while GC-MS is preferred for measuring labeling of amino acids, carbohydrates, and TCA cycle metabolites, given that GC-MS produces multiple fragments that yields additional labeling information [52,53]. Advanced GC-MS/MS approaches have also been developed for more complete analysis of labeling patterns [54]. Ultimately, the choice of the measurement technique depends on the information desired, the cost of performing labeling measurements, and available resources.

Measuring metabolism with ^{13}C metabolic flux analysis

In order for biological processes to be economically viable, substrates must be converted to products at near-theoretical yields [7]. This generally requires significant rewiring of cellular metabolism. Compared to native metabolism, which has evolved to optimize cell growth, optimal product formation will require a different balance of pathway activities to maximize the flow of carbons and electrons from feedstocks to products. ^{13}C -Metabolic flux analysis (^{13}C -MFA) is the best approach to gain quantitative insights into cellular metabolism [48]. Knowledge of metabolic fluxes is important to identify bottlenecks in metabolism and determine specific changes in pathway utilization resulting from genetic manipulations [55]. Experimentally validated fluxes are also used in constraint-based modeling approaches, and fluxes are critical for parameterizing kinetic models of metabolism [56], which are increasingly applied for analyzing metabolic pathways and predicting the outcomes of metabolic engineering interventions given the poor performance of traditional constraint-based approaches [57].

Methods for ^{13}C -MFA have advanced significantly in the past few years [48]. With current state-of-the-art approaches, it is possible to measure intracellular fluxes with a precision of about 1-3% [58]. The ability to generate high-resolution flux maps presents unprecedented opportunities to gain a much more detailed understanding of the regulation of metabolic pathways and *in vivo* enzyme kinetics. Current best approaches for flux estimation are based on the concept of parallel labeling experiments and integrated ^{13}C -MFA (Figure 4) [59]. A good example of the power of parallel labeling experiments is the study by Crown et al. [58], where 14 parallel labeling experiments were successfully integrated to generate a detailed flux map for *E. coli*. Parallel labeling experiments were also successfully applied for analysis of *Clostridium acetobutylicum* [60], *Geobacillus* spp. [61], and various mammalian systems [62-65].

The design of optimal labeling experiments is a critical step in ^{13}C -MFA, which until recently did not receive the attention it deserves. It is now well recognized that traditional tracers such as $[1\text{-}^{13}\text{C}]\text{glucose}$ and $[\text{U-}^{13}\text{C}]\text{glucose}$, although relatively cheap, are suboptimal for quantifying precise metabolic fluxes [66]. For example, it was demonstrated that doubly labeled tracers such as $[1,2\text{-}^{13}\text{C}]\text{glucose}$ and $[1,6\text{-}^{13}\text{C}]\text{glucose}$ are more optimal for ^{13}C -MFA, since these tracers produce fluxes with much smaller confidence intervals [67]. For xylose, optimal tracers include $[1,2\text{-}^{13}\text{C}]\text{xylose}$ and $[5\text{-}^{13}\text{C}]\text{xylose}$ [61].

The choice of tracers becomes even more critical when multiple substrates are present. When studying glucose and xylose co-utilization, the combination of $[1\text{-}^{13}\text{C}]\text{glucose}$ and unlabeled xylose has been commonly used (e.g. to elucidate pentose phosphate pathway fluxes in a creA-

mutant of *Aspergillus nidulans* [68], and to study the effect of a modified redox pathway in *S. cerevisiae* [69]). A limitation of this tracer scheme is, however, that it only works well when the labeled substrate (in this case glucose) is the main carbon source. If xylose becomes the main carbon source, then ^{13}C -labeling becomes dramatically reduced and flux estimation fails. It is therefore advantageous to design labeling experiments that are less dependent on specific fluxes. Logically, it follows that both substrates should be labeled. A number of optimized tracer schemes have been proposed and successfully applied, including $[1,2-^{13}\text{C}]\text{glucose} + [1,2-^{13}\text{C}]\text{xylose}$ to elucidate glucose and xylose co-utilization in *E. coli* [70], and $[1,6-^{13}\text{C}]\text{glucose} + [5-^{13}\text{C}]\text{xylose}$ for flux analysis in *T. thermophilus* [9].

Metabolic fluxes from other lignocellulosic sugars have also been studied. For example, Fonseca et al. used $[2-^{13}\text{C}]\text{arabinose}$ to study arabitol production in arabinose-utilizing yeast strains [71], and Sund et al. used $[1-^{13}\text{C}]\text{glucose}$, $[1-^{13}\text{C}]\text{xylose}$, and $[1-^{13}\text{C}]\text{arabinose}$ to estimate phosphoketolase fluxes in *Clostridium acetobutylicum* [16]. Similarly, Fendt and Sauer used ^{13}C -glucose, ^{13}C -galactose, and ^{13}C -mannose tracers to elucidate sugar metabolism of *S. cerevisiae* [72]. Earlier studies with *Fibrobacter* also focused on elucidating catabolism of more complex substrates, including cellulose and cellobiose [73]. By examining the dilution of $[1-^{13}\text{C}]\text{glucose}$ and quantifying the labeling of products during growth on cellulose or cellobiose, the authors were able to determine how glucose was utilized in the presence of cellulose.

Conclusions

Combining isotopic tracers and ^{13}C -metabolic flux analysis is a powerful approach for studying metabolism of engineered microorganisms. This method is especially useful when exploring new pathways for the conversion of non-traditional feedstocks. As highlighted here, there have been extensive efforts in engineering both upstream and downstream metabolism for production of fuels and chemicals. Substrate utilization pathways have been targeted to bestow the ability to consume new carbon sources in industrially viable organisms, and new pathways have been engineered for production of value-added chemicals by bypassing conventional metabolic routes, reducing carbon loss, and improving yield. Given the demonstrated advantages of using both organic (sugars) and inorganic (CO_2 , CO , H_2 , CH_4) feedstocks, it is likely that co-utilization of mixed substrates by mixotrophy will remain an active area of research in the foreseeable future.

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TABLES

Table 1. Examples of native and synthetic pathways for bio-conversion of renewable feedstocks.

Metabolic pathway	<i>E. coli</i>	<i>S. cerevisiae</i>	Other organisms
Entner-Doudoroff pathway	Native	[74]	[75]
Wood-Ljungdahl pathway	-	-	[5]
Phosphoketolase pathways	[20,21]	[22,23]	[11,16,76-78]
Calvin cycle	[36-38]	[39]	[79]
Methylotrophic pathways	[44]	-	[46,80-82]
Weimberg and Dahms pathways	[25,26]	-	[27,28]
Other synthetic pathways	[24,83]	-	-

FIGURE LEGENDS

Figure 1. Conversion of lignocellulose-derived sugars into substrates of central carbon metabolism.

Figure 2. Native and synthetic metabolic pathways for conversion of renewable feedstocks into value-added products.

Figure 3. A) Example of an isotopic labeling experiment with multiple ^{13}C -tracers to quantify metabolism of glucose and xylose. B) Overview of mass spectrometry based measurements of metabolism in core metabolic pathways (here, glycolysis and pentose phosphate pathway) for analysis of isotopic labeling and ^{13}C -metabolic flux analysis.

Figure 4. Comparison of different designs of isotopic labeling experiments to quantify co-utilization of multiple substrates using classical ^{13}C -MFA (left), and integrated ^{13}C -MFA based on parallel labeling experiments (right).

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A Isotopic Labeling Experiments

B Isotopic Labeling Measurements

Traditional ^{13}C -MFA
(single tracer) or (multiple tracers)

vs. Parallel labeling experiments
(single tracers in parallel experiments)