![frontiers in Microbiology]

EMUlator: An Elementary Metabolite Unit (EMU) Based Isotope Simulator Enabled by Adjacency Matrix

Chao Wu[1], Chia-hsin Chen[2], Jonathan Lo[1], William Michener[1], PinChing Maness[1] and Wei Xiong[1]*

[1] National Renewable Energy Laboratory, Golden, CO, United States, [2] Institute of Nuclear Energy Research, Taoyuan, Taiwan

Stable isotope based metabolic flux analysis is currently the unique methodology that allows the experimental study of the integrated responses of metabolic networks. This method primarily relies on isotope labeling and modeling, which could be a challenge in both experimental and computational biology. In particular, the algorithm implementation for isotope simulation is a critical step, limiting extensive usage of this powerful approach. Here, we introduce *EMUlator* a Python-based isotope simulator which is developed on Elementary Metabolite Unit (EMU) algorithm, an efficient and powerful algorithm for isotope modeling. We propose a novel adjacency matrix method to implement EMU modeling and exemplify it stepwise. This method is intuitively straightforward and can be conveniently mastered for various customized purposes. We apply this arithmetic pipeline to understand the phosphoketolase flux in the metabolic network of an industrial microbe *Clostridium acetobutylicum*. The resulting design enables a high-throughput and non-invasive approach for estimating phosphoketolase flux *in vivo*. Our computational insights allow the systematic design and prediction of isotope-based metabolic models and yield a comprehensive understanding of their limitations and potentials.

Keywords: adjacency matrix, elementary metabolite unit (EMU), fractional labeling (FL), *Clostridium acetobutylicum*, phosphoketolase

INTRODUCTION

^{13}C metabolic flux analysis (MFA) is currently the only experimental methodology to quantitatively understand intracellular biochemical networks by means of stable isotope tracing, labeling pattern analysis and indispensably, metabolic modeling which is based on mass and isotope balancing (Stephanopoulos, 1999; Sauer, 2006). Importantly, isotope modeling can provide additional key information that further defines the system, enabling quantification of the fluxes in parallel or cyclic pathways that cannot be estimated reliably by mass balance only. A variety of mathematical models were developed to establish relationship between isotope distribution and metabolic flux, including isotopomers (Schmidt et al., 1997), cumomers (Wiechert et al., 1999), and bondomers (Van Winden et al., 2002). However, previous methods suffer from the computational challenge in resolving realistic and large-scale metabolic network, as a large number of isotopomer equations need to be solved.

To address this limitation, a creative computational framework based on Elementary Metabolite Unit (EMU) was proposed (Antoniewicz et al., 2007a). The EMUs of a metabolite are defined as the non-empty subsets of all that compound's atoms (usually carbon atom). EMU can be cataloged by size, i.e., the number of atoms in it. With atom transition map, this framework will trace and identify the minimal relevant metabolic information needed to simulate isotope patterns and solve the optimization problem, and therefore greatly reduces the number of balance equations and computation burden, i.e., 95% reduction of the variables needed for the simulation in an *E. coli* model without any loss of information (Antoniewicz et al., 2007b). To date, EMU algorithm has garnered increasing attention in metabolic analysis (Quek et al., 2009; Sokol et al., 2012; Weitzel et al., 2013; Kajihata et al., 2014; Shupletsov et al., 2014; Young, 2014). Better leveraging this approach in understanding cell metabolism will require the development of novel computational packages which are easy to program and can fit new and broad application scenarios. However, to date, computational approaches that can straightforwardly and efficiently implement the EMU algorithm are still insufficient.

Here, we develop a new computational toolbox for steady state metabolic modeling analysis, the *EMUlator*, which accomplishes EMU modeling through an adjacency matrix-based approach. In graph theory, an adjacency matrix is used to quantitatively represent a graph, of which the elements indicate the connectivity of vertex pairs in rows and columns. Essentially, metabolic network is a directed graph with branches, thus it can be transformed into adjacency matrix for the ease of programming. Utilizing adjacency matrix, the *EMUlator* can efficiently simulate isotope distributions for ^{13}C-MFA. To demonstrate its functionality, we decompose the EMUs for isotope simulation in a tricarboxylic acid (TCA) cycle which represents a realistic metabolic network. Furthermore, we applied this newly developed software in modeling and analyzing the flux of phosphoketolase pathway in *Clostridium acetobutylicum* xylose catabolism. By decomposing the network and simulating metabolite isotopomer patterns, we found a good correlation between phosphoketolase flux and the fractional labeling of acetate, which has never been characterized in an isotope tracer experiment. Coupled with GC-MS analysis of acetate, this EMUlator-enabled analysis leads to a novel and high-throughput methodology for quantitatively understanding the phosphoketolase pathway in response to environmental and genetic perturbation. As exemplified, *EMUlator* aims to be a universal and powerful tool for isotope tracer modeling and for gaining quantitative understanding of cell metabolism. The software and its instruction are available at **Supplementary Information**.

RESULTS

Overview of the *EMUlator* Pipeline

The *EMUlator* pipeline is designed in Python, capable of performing a complete isotope simulation and prediction of a metabolic network for ^{13}C-MFA. Previous tools, such as Metran (Antoniewicz et al., 2006, 2007a), OpenFlux (Quek

et al., 2009; Shupletsov et al., 2014) and INCA (Young, 2014), are able to perform such modeling, however based on Matlab platform which is not an open and free computing environment. In addition, previous EMU modeling was substantially less transparent than the one we present here. A key distinguishing feature of *EMUlator* is the usage of adjacency matrix. This ensures a graphic expression of the algorithm which can be understood intuitively and implemented iteratively. In particular, *EMUlator* provides a more detailed and principled procedure of EMU modeling, which decomposes metabolic network into EMU reactions, sets up EMU balances and simulates labeling distribution. Most importantly, the EMU deconstruction results in the reduction of the metabolic network model leading to a smaller set of EMU reactions which preserves all the information contained in it but decreases running time significantly.

EMUlator Transforms Metabolic Network Into Adjacency Matrix

To illustrate algorithm of the program comparably, we implement TCA cycle model as an example, as this representative metabolic network was also used in the original EMU work (Antoniewicz et al., 2007a). In this network, aspartate and acetyl coenzyme A (AcCoA) are the substrates, while CO_2 and glutamate are final products. Reactions with carbon atom transitions are listed in **Figure 1**. As a directed graph, any metabolic network with branches (due to cleavage and condensation reactions) can be transformed into a metabolite adjacency matrix (MAM). All metabolites are grouped in both row and column coordinates, thus forming a square matrix. Row metabolites appear as reactants while column metabolites are products of each reactions. Elements determined by row and column coordinates are connecting reactions for reactants and products. Reaction in element may not be unique because identical reactant and product could be involved in different reactions. As such, inputs and outputs of the network are easy to identify. Herein, columns without element are identified as substrates since they have no precursors (i.e., columns for AcCoA and Aspartate in dashed red boxes, **Figure 2**), while rows without element are identified as final products (i.e., rows for CO_2 and glutamate in dashed green boxes, **Figure 2**). Overall, MAM reflects the connectivity of metabolites in a network.

EMUlator Decomposes MAM Into EMU Adjacency Matrix (EAM)

EMU decomposition of a metabolic network can start at the size of EMU(s) that need to be simulated. In this example, we simulate the Mass Distribution Vector (MDV, the fractional abundance of each isotopolog normalized to the sum of all possible isotopologues.) (Nanchen et al., 2007) of glutamate Glu_{12345} (size 5). All EMU reactions that are needed for this simulation can be identified iteratively via MAM. Glu_{12345} as a product can be found in the column of EAM (size 5) (see **Figure 3A**), its precursor AKG_{12345} is illustrated in the corresponding row through the reaction V_3. Similarly, for the product AKG_{12345}, we can locate its precursor Cit_{12345} via v_2. Lastly for the product (in column) Cit_{12345}, we identify reaction
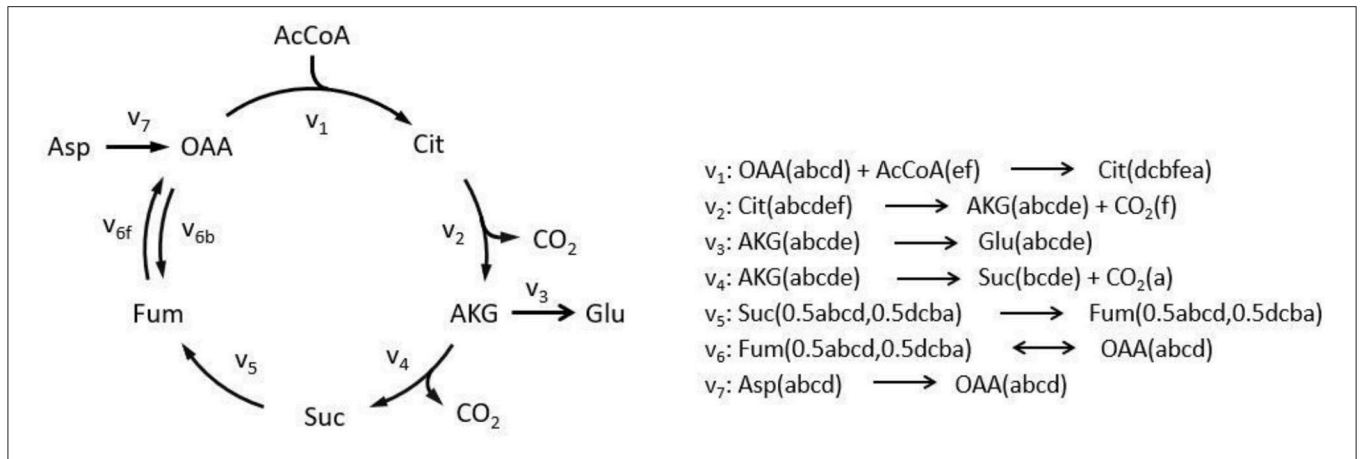
FIGURE 1 | Simplified tricarboxylic acid (TCA) cycle to illustrate adjacency matrix-based EMU decomposition. Reactions involved in the metabolic model are listed on the right. Lowercase letters in brackets demonstrate atom transition in each reaction. Decimals indicate EMU equivalents due to rotation axis within molecule. AcCoA, acetyl coenzyme A; AKG, α-ketoglutarate; Asp, aspartate; Cit, citrate; Fum, fumarate; Glu, glutamate; OAA, oxaloacetate; Suc, succinate; subscript f, forward reaction; subscript b, backward reaction.
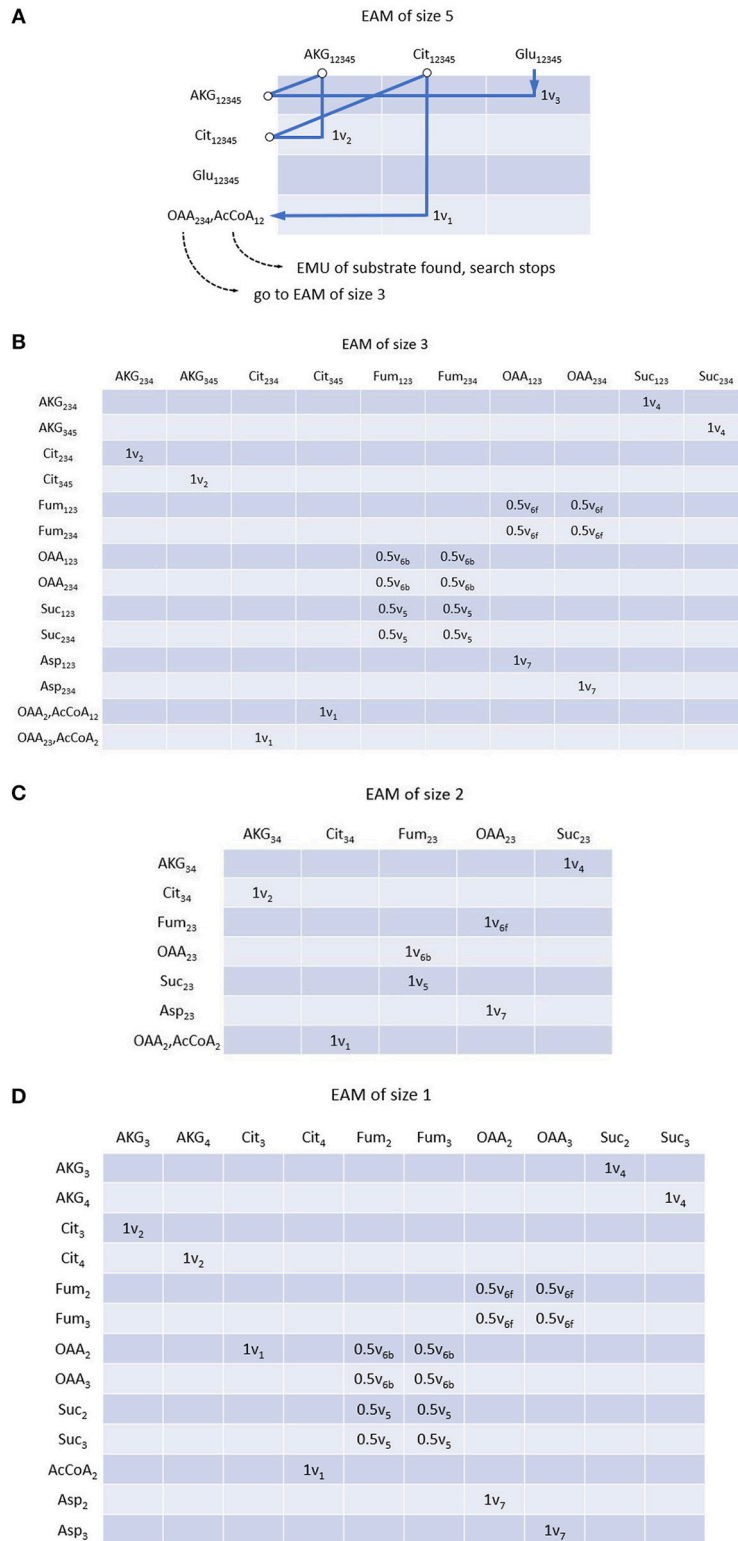

FIGURE 2 | Metabolite adjacency matrix (MAM) of the TCA cycle network. MAM is a square matrix with all involved metabolites on both rows and columns. Each element indicates reaction(s) through which reactant (row metabolite) is converted into product (column metabolite). Metabolites with no element in column are identified as initial substrates (dashed red box); metabolites with no element in row are identified as final products (dashed green box).

v_1, in which both OAA_{234} and $AcCoA_{12}$ are the reactants. Since EMUs of smaller size are identified in condensation reactions, they will be used as new start points for searching. Therefore, we can follow the OAA_{234} ($AcCoA_{12}$ is identified as an EMU of the substrate, and thus the searching stops), and search all other EMUs at size 3 (**Figure 3B**). All precursor EMUs for multiple precursors [e.g., due to equivalent EMUs (Antoniewicz et al., 2007a)], can be identified through breadth-first search. As such, adjacent matrix provides a straightforward and iterative path allowing us to trace back EMUs of smaller sizes until the EMUs of network substrates are identified (i.e., Aspartate and AcCoA in this example). Once all set of the EMUs are obtained, EMUs

can be arranged into different EAMs per size. Similar to MAM, row and column coordinates of EAM correspond to reactants and products of each reaction, respectively, with the difference that EMUs subject to convolution also appear in rows of EAM, and the coefficients of an element equal to the stoichiometric coefficients of corresponding reactant and product. Complete EAMs after EMU decomposition of the example are shown in **Figure 3**.

EMUlator Significantly Reduces the Size of EAMs

EMUlator can also reduce the scale of EAM. In steady state isotope modeling, labeling pattern can only be modified by

FIGURE 3 | EMU adjacency matrix (EAM) of size 5 **(A)**, size 3 **(B)**, size 2 **(C)** and size 1 **(D)**. Each element indicates reaction(s) through which reactant (row metabolite) is converted into product (column metabolite) as well as reaction coefficients. Construction of EAM starts from the EMU to be simulated, i.e., Glu_{12345} in this example. All precursors of corresponding EMU can be found with MAM and atom transition using breadth-first search. Precursor searching continues until initial substrates. If condensation reactions are encountered, precursors of smaller size are considered as new start points, respectively. EMUs of same size are located in the same EAM.

convolution of two or more metabolites and unimolecular reactions may be lumped without affecting simulations which helps to reduce the scale of EAM. Unimolecular reactions can be easily identified in EAM as those with solo element in a column, which means its corresponding product only has a single source. Those columns will be deleted with identical metabolites in rows all renamed by their precursors. For example, in EAM of size 1, after AKG_3 column is eliminated, AKG_3 in row will be renamed with Cit_3 which produces AKG_3 by reaction v_2 (**Figure 4**). Moreover, since Cit_3 is still from a unimolecular reaction (v_1), we eliminate the column as well and Cit_3 in row will be renamed by its precursor OAA_2 via v_1. The deletion of the column and renaming of corresponding row continue until there is no solo-element column in EAM. Finally, multiple rows with identical metabolite name will be combined, indicating the identical reactant and product are connected by different reactions.

EMUlator Identifies and Combines Equivalent EMUs

Rotationally symmetric molecules (i.e., fumaric acid and succinic acid) will give rise to equivalent EMUs (Antoniewicz et al., 2007a), which are undistinguishable for enzyme, and react identically in the reactions. EMUlator can combine those EMUs as they will have the same probability to get certain labeling pattern. Metabolites which could generate equivalent EMUs are indicated with fractional carbon atoms in **Figure 1**. Fum_2 and Fum_3 are equivalent EMUs of size 1 in this example (**Figure 5**). Element coefficients of row equivalent EMUs are combined, while coefficients of column equivalent EMUs are combined and divided by the number of equivalents. Eventually, EMU variables were reduced from 24 of initial EMU model to 9 after lumping unimolecular reactions and combining equivalent EMU, yielding the same results as the original EMU work (Antoniewicz et al., 2007a).

EMUlator Establishes EMU Balances From EAMs

To simulate the labeling pattern of a given metabolite, EMU balances are established from EMU reaction subnetworks of different size, and MDVs can be calculated according to:

$$X_i = A_i^{-1} \cdot B_i \cdot Y_i$$

Where A_i and B_i are matrix function of flux variable of size i. A_i is square matrix whose shape is dependent on the number of EMU balance m of current size. B_i's shape is m × n where n is the number of available EMU variables (Antoniewicz et al., 2007a). Simulation starts from size 1, and Y_1 are MDVs of network substrates. Other MDVs are calculated and then used in Y of larger size. A_i and B_i can be easily deduced from EAM as demonstrated in **Figure 6**. Diagonal elements of EAM are first set to be the negative sum of other elements of current column. Then the transposed upper square submatrix will be A_1, and the lower submatrix will be B_1 after transposition and all elements negated. The transformation is made according to the isotopomer balance which states that the sum of all influx to an EMU multiplied by its MDV ($\sum v_i \cdot MDV$) is equal to the sum of the individual

product of each influx v_i multiplied by MDV_i $\left(\sum (v_i \cdot MDV_i) \right)$. Apparently, diagonal element represents the total influx of corresponding EMU. Multiplied by MDV of balanced EMU, the product is equal to the sum of all labeling pattern sources, including those unknown (X_i) and known (Y_i) EMU variables. EMU balances of larger size can be established likewise until the Glu_{12345} are eventually simulated. EMU balances of all sizes are shown below:

EMU balance of size 1

$$\begin{bmatrix} -v_{6b} - v_5 & 0.5v_{6b} + 0.5v_5 & 0.5v_{6b} \\ v_{6f} & -v_{6f} - v_7 & 0 \\ v_{6f} & 0 & -v_{6f} - v_7 \end{bmatrix} \cdot \begin{bmatrix} MDV_{Fum_2} \\ MDV_{OAA_2} \\ MDV_{OAA_3} \end{bmatrix}$$

$$= \begin{bmatrix} 0 & 0 & -0.5v_5 \\ -v_7 & 0 & 0 \\ 0 & -v_7 & 0 \end{bmatrix} \cdot \begin{bmatrix} MDV_{Asp_2} \\ MDV_{Asp_3} \\ MDV_{AcCoA_2} \end{bmatrix}$$

EMU balance of size 2

$$\begin{bmatrix} -v_{6b} - v_5 & v_{6b} \\ v_{6f} & -v_{6f} - v_7 \end{bmatrix} \cdot \begin{bmatrix} MDV_{Fum_{23}} \\ MDV_{OAA_{23}} \end{bmatrix} = \begin{bmatrix} 0 & v_5 \\ -v_7 & 0 \end{bmatrix}$$

$$\cdot \begin{bmatrix} MDV_{Asp_{23}} \\ MDV_{OAA_2} \times MDV_{AcCoA_2} \end{bmatrix}$$

EMU balance of size 3

$$\begin{bmatrix} -v_{6b} - v_5 & 0.5v_{6b} & 0.5v_{6b} \\ v_{6f} & -v_{6f} - v_7 & 0 \\ v_{6f} & 0 & -v_{6f} - v_7 \end{bmatrix} \cdot \begin{bmatrix} MDV_{Fum_{123}} \\ MDV_{OAA_{123}} \\ MDV_{OAA_{234}} \end{bmatrix} =$$

$$\begin{bmatrix} -0.5v_5 & -0.5v_5 & 0 & 0 \\ 0 & 0 & -v_7 & 0 \\ 0 & 0 & 0 & -v_7 \end{bmatrix} \cdot \begin{bmatrix} MDV_{OAA_2} \times MDV_{AcCoA_{12}} \\ MDV_{OAA_{23}} \times MDV_{AcCoA_2} \\ MDV_{Asp_{123}} \\ MDV_{Asp_{234}} \end{bmatrix}$$
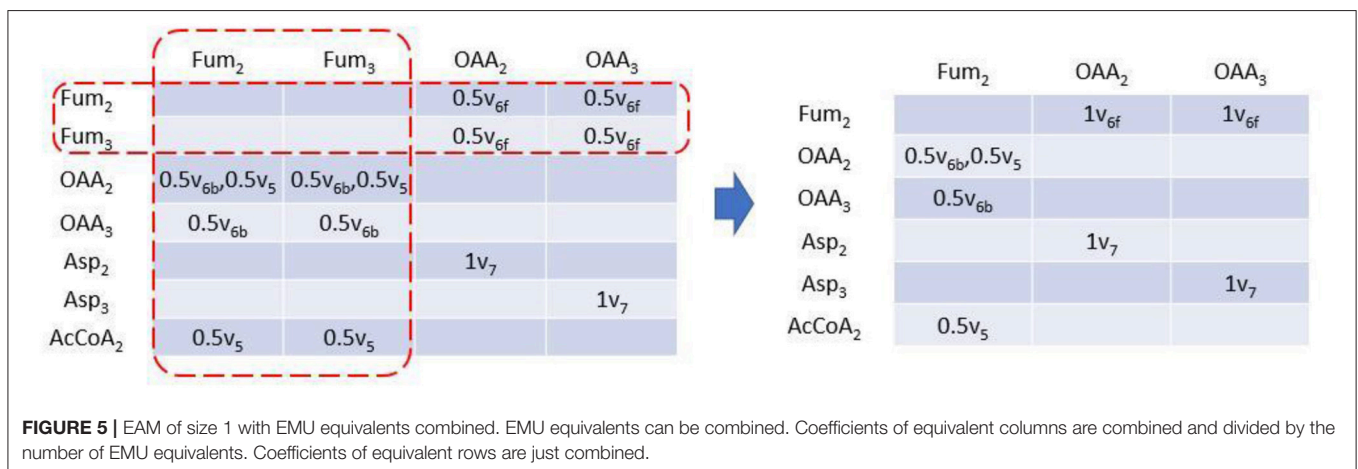
EMU balance of size 4

$$[-v_3] \cdot [MDV_{Glu_{12345}}] = [-v_3] \cdot [MDV_{OAA_{123}} \times MDV_{AcCoA_{12}}]$$

EMUlator Designs Optimal ^{13}C-Tracer Experiment for Quantifying Metabolic Flux of Interest

To demonstrate the performance of *EMUlator* in a large-scale setting that reflects the complexity of realistic cell metabolism, we next performed simulation of isotope distributions in a larger network model of *C. acetobutylicum*. *C. acetobutylicum* is a solventogenic clostridium and represents a promising chassis microbe capable of utilizing lignocellulose-derived pentose sugar (i.e., xylose) for biofuels production (Mitchell, 1998; Gu et al., 2011). In this case, pentose catabolism through phosphoketolase pathway (Grimmler et al., 2010; Servinsky et al., 2010; Liu et al., 2012) is of special interest in that this pathway has been recognized as a key target for constructing synthetic pathways (e.g., Non-oxidative glycolysis) that bypass CO_2 loss via pyruvate decarboxylase and thus enhance carbon yield in final products

FIGURE 4 | Reduced EAM of size 1. Column metabolites with only one element can be lumped because it has solo influx. These columns can be eliminated therefore, and their corresponding row metabolites will be replaced by its precursor. Elimination and replacement occur iteratively until no solo element column exists. Rows with identical metabolite will be combined.

FIGURE 5 | EAM of size 1 with EMU equivalents combined. EMU equivalents can be combined. Coefficients of equivalent columns are combined and divided by the number of EMU equivalents. Coefficients of equivalent rows are just combined.
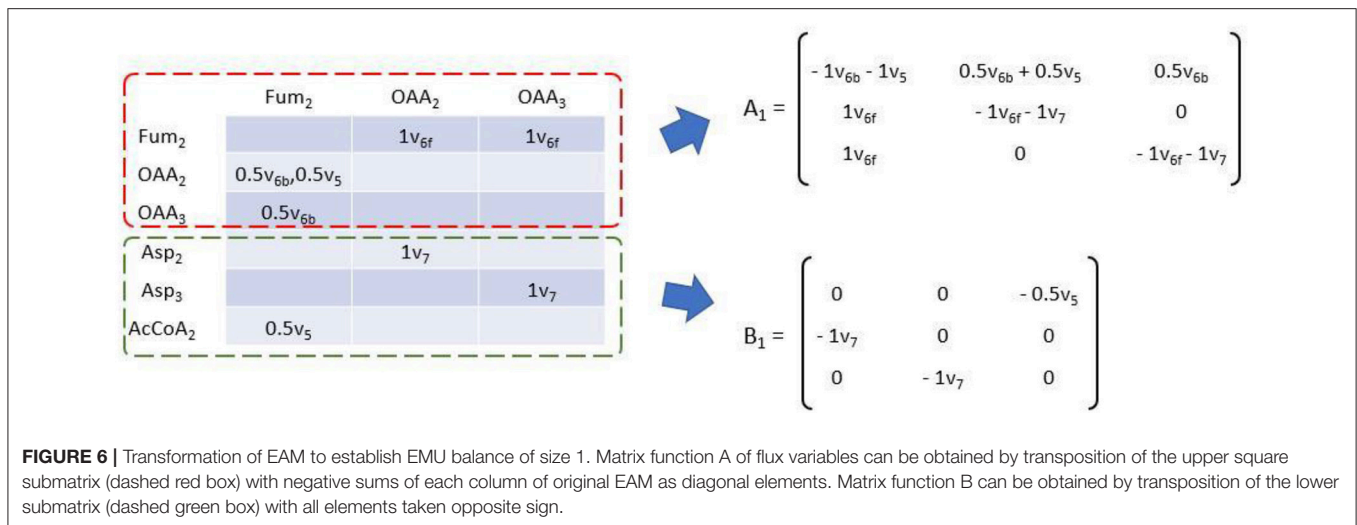
(Bogorad et al., 2013). Here we used the adjacency matrix-based *EMUlator* to simulate labeling patterns of metabolites and show how it facilitates the selection of best isotope substrates and readouts quantifying the *in vivo* phosphoketolase activity.

First, a biochemical network for xylose metabolism of *C. acetobutylicum* was constructed, based on the genome information (Nölling et al., 2001; Bao et al., 2011). As shown in **Figure 7A**, after phosphorylation, xylose can be metabolized either through the non-oxidative pentose phosphate pathway or cleaved by phosphoketolase to form acetyl-phosphate and glyceraldehyde-3-phosphate. Acetyl-phosphate is further directed to generate extracellular fermentative products (i.e., acetate, ethanol, acetone, butanol, butyrate) and glyceraldehyde-3-phosphate can enter pentose phosphate pathway in which reactions are highly reversible due to the nature of isomerase, epimerase, transketolase, and transaldolase. The oxidative

pentose phosphate pathway is not considered which was verified to be inactive in *C. acetobutylicum* (Au et al., 2014). The TCA cycle is not included as it does not influence the labeling patterns of the upstream metabolites.

We selected acetate (AC), ethanol, 3-phosphoglycerate, erythrose-4-phosphate and ribose-5-phosphate as candidate readouts for reflecting phosphoketolase activity since MDVs of these metabolites can be experimentally obtained either from direct determination or derivation from amino acid MDVs (Nanchen et al., 2007). Meanwhile, we tested all commercially available xylose tracers: $1^{-13}C$ xylose, $2^{-13}C$ xylose, $3^{-13}C$ xylose, $4^{-13}C$ xylose, $5^{-13}C$ xylose, $1,2^{-13}C$ xylose, U-^{13}C xylose. MDVs were simulated using *EMUlator* in all possible combinations of these candidate readouts and tracers. Goodness of correlation between Fractional Labeling (FL) (defined in Materials and Methods) and flux ratio through phosphoketolase (i.e., v_2/v_1 in
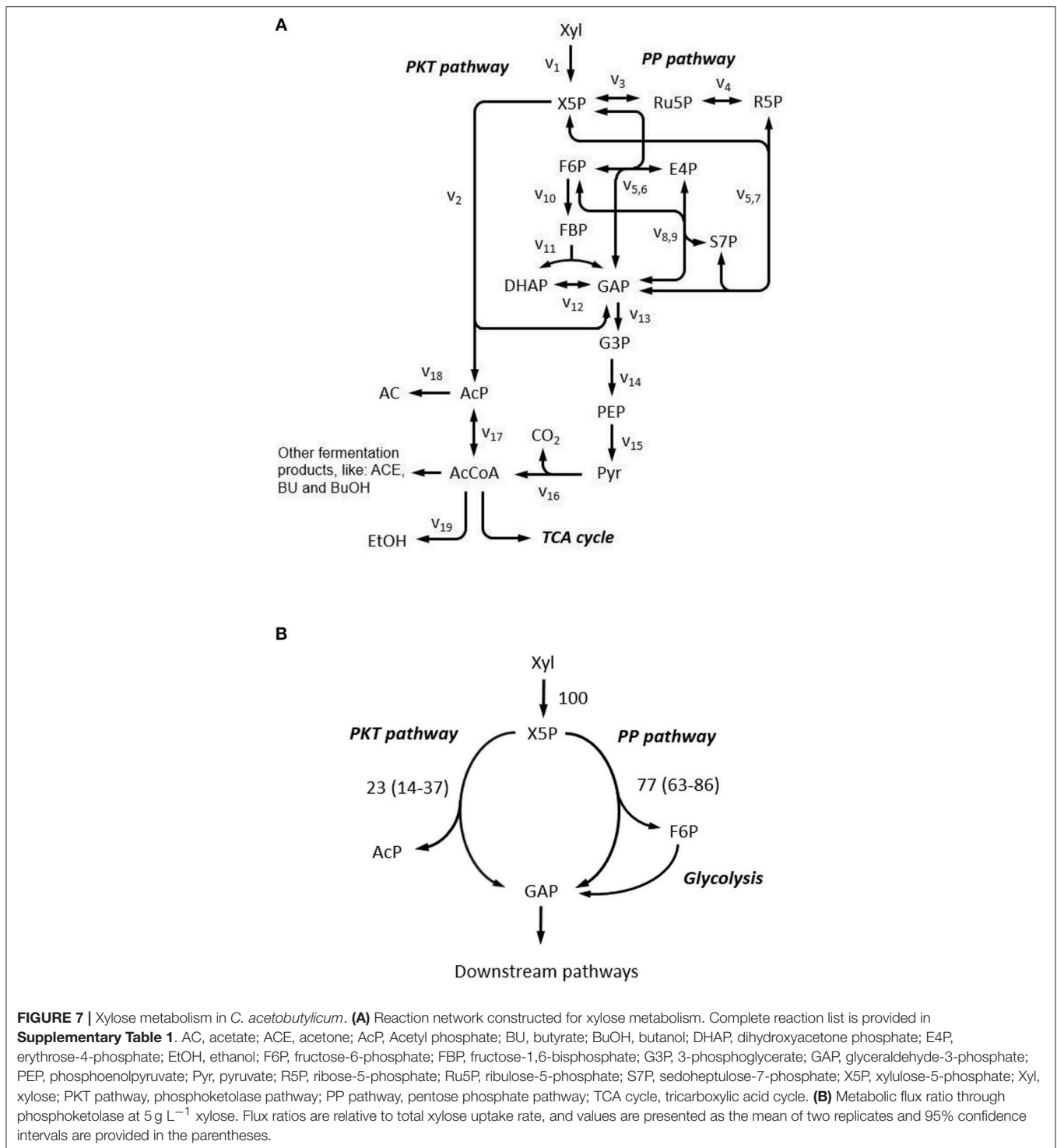
FIGURE 6 | Transformation of EAM to establish EMU balance of size 1. Matrix function A of flux variables can be obtained by transposition of the upper square submatrix (dashed red box) with negative sums of each column of original EAM as diagonal elements. Matrix function B can be obtained by transposition of the lower submatrix (dashed green box) with all elements taken opposite sign.

Figure 7A) and range of effective FL are used as the selection criteria. The modeling results are shown in **Figure 8**.

Among various xylose tracers, 1,2-^{13}C xylose yields EMU AC_{12} with best correlation between its FL and phosphoketolase flux ratio (Spearman correlation coefficient = 0.7). The widest effective range of FL (slope of regression line = 0.43) indicates a good sensitivity on the flux ratio of phosphoketolase (**Figure 8A**). EMU AC_1 and AC_2 show identical correlationship between FL and flux ratio with that of AC_{12} (**Figures 8B,C**) because the carbons of acetate originate from C2 and C1 of xylose which are both labeled (probably also from C4 and C5 of xylose converted from AcCoA) and behave equivalently in the atom transition. FL of $G3P_{123}$ could have a good correlation with the phosphoketolase flux ratio, which is, however, disturbed by many randomly distributed points (**Figure 8E**). This is probably due to the reversibility of the reactions in glycolytic and pentose phosphate pathways. $EtOH_{12}$, $E4P_{1234}$ and $R5P_{12345}$ all show poor correlation, and thus cannot be used as the indicators for phosphoketolase flux (**Figures 8D,F,G**). As for other tracers, 1-^{13}C xylose and 2-^{13}C xylose labeling result in poor correlation in AC_1 and AC_2, respectively (**Supplementary Figures 1, 2**). AC_{12} and $EtOH_{12}$ will be totally unlabeled using 3-^{13}C xylose tracer as no C3 of xylose will fractionate into these metabolites. $G3P_{123}$ shows a good correlation with phosphoketolase flux while the range of effective FL is too small to determine phosphoketolase activity (**Supplementary Figure 3**). If 4-^{13}C xylose and 5-^{13}C xylose are used as substrate, correlation between FL and flux ratio are inverted for most of the EMUs. In addition, AC_2 and AC_1 are totally unlabeled per 4-^{13}C xylose and 5-^{13}C xylose, respectively. (**Supplementary Figures 4, 5**). As a control, FLs of all MDVs are constant when fed with a mixture of U-^{13}C labeled and unlabeled xylose (**Supplementary Figure 6**), which is, therefore excluded from the isotope tracer selection. In comparison of all tracer/readout combination, 1,2-^{13}C-xylose/ AC_{12} performed the best and this selection paves the way to the experimental measurement of flux in phosphoketolase pathway.

Guided by the above simulations, *C. acetobutylicum* was thereafter cultivated in 5 g L^{-1} 1,2-^{13}C xylose in following experiment. Fermentation kinetics including cell growth and the production of cell products over time are as shown in **Supplementary Figure 7**. The flux ratio from phosphoketolase pathway was then quantified by harvesting the supernatant and determining the isotope pattern of AC with GC-MS. MDVs of AC_1 and AC_{12} were measured, and MDVs of AC_2 can be deduced. Accordingly, FL of AC_1, AC_2 and AC_{12} were calculated to be 0.462 ± 0.018, 0.469 ± 0.012 and 0.465 ± 0.015, respectively, which is consistent with our prediction that FLs of all fragments' EMUs should be identical. A distribution of phosphoketolase flux ratio was obtained from simulated sample points with corresponding FL of AC_1, AC_2 and AC_{12} falling into the measured ranges. The average and 95% confidence interval of the flux ratio through phosphoketolase were estimated as 22.8% and 14.3–36.6%, respectively under current conditions (**Figure 7B**).

DISCUSSION

Although equivalent to isotopomer models, EMU method is able to significantly reduce the variables needed to simulate labeling patterns of metabolites (90% reduction in our *C. acetobutylicum* case) without any loss of information, which therefore greatly facilitates ^{13}C flux modeling of realistic and large-scaled metabolic network and dynamic systems (Young et al., 2008). Here we introduce a computational tool *EMUlator* to the metabolic research community. This engine utilizes adjacency matrix-based approach which is intuitively straightforward and easy to program. Metabolic network can be decomposed into EAMs of different size which can be further reduced by lumping unimolecular reactions and combining equivalent EMUs. For MDV simulation, matrix multiplication starts from EAM of the smallest size, and iteratively continues to larger size until the required EMU is simulated. Overall, the computation time for

FIGURE 7 | Xylose metabolism in *C. acetobutylicum*. **(A)** Reaction network constructed for xylose metabolism. Complete reaction list is provided in **Supplementary Table 1**. AC, acetate; ACE, acetone; AcP, Acetyl phosphate; BU, butyrate; BuOH, butanol; DHAP, dihydroxyacetone phosphate; E4P, erythrose-4-phosphate; EtOH, ethanol; F6P, fructose-6-phosphate; FBP, fructose-1,6-bisphosphate; G3P, 3-phosphoglycerate; GAP, glyceraldehyde-3-phosphate; PEP, phosphoenolpyruvate; Pyr, pyruvate; R5P, ribose-5-phosphate; Ru5P, ribulose-5-phosphate; S7P, sedoheptulose-7-phosphate; X5P, xylulose-5-phosphate; Xyl, xylose; PKT pathway, phosphoketolase pathway; PP pathway, pentose phosphate pathway; TCA cycle, tricarboxylic acid cycle. **(B)** Metabolic flux ratio through phosphoketolase at 5 g L^{-1} xylose. Flux ratios are relative to total xylose uptake rate, and values are presented as the mean of two replicates and 95% confidence intervals are provided in the parentheses.
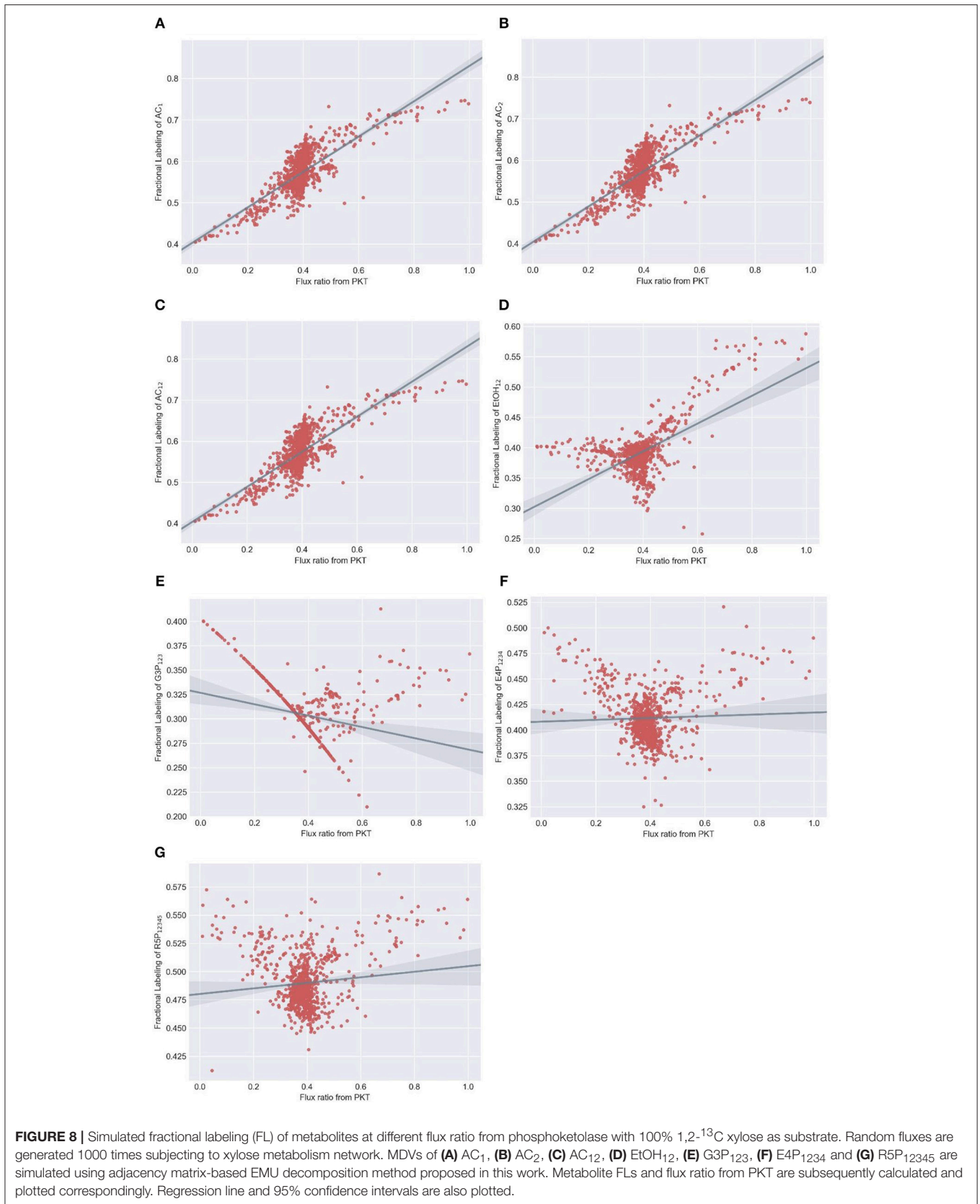
EMUlator to perform MDV simulation depends on the network complexity, or connectivity which can be represented by the number of non-zero elements in EAMs. For a realistic and moderate-sized network shown in the above examples, the time complexity is roughly $O(n^3)$, where n is the number of EMUs.

The *EMUlator* allowed large-scale and efficient isotope modeling. To exemplify its capability, we applied it in

quantitative understanding of the phosphoketolase pathway in the central carbon metabolism of an industrial model microbe (*C. acetobutylicum*). We simulated labeling pattern of both intracellular and secretory metabolites using different xylose tracers, and identified the best tracer/readout combination reflecting phosphoketolase activity. ^{13}C-flux measurement of the phosphoketolase pathway was enabled recently (Liu et al.,

FIGURE 8 | Simulated fractional labeling (FL) of metabolites at different flux ratio from phosphoketolase with 100% 1,2-^{13}C xylose as substrate. Random fluxes are generated 1000 times subjecting to xylose metabolism network. MDVs of **(A)** AC_1, **(B)** AC_2, **(C)** AC_{12}, **(D)** $EtOH_{12}$, **(E)** $G3P_{123}$, **(F)** $E4P_{1234}$ and **(G)** $R5P_{12345}$ are simulated using adjacency matrix-based EMU decomposition method proposed in this work. Metabolite FLs and flux ratio from PKT are subsequently calculated and plotted correspondingly. Regression line and 95% confidence intervals are also plotted.

2012), in which 3-phosphoglycerate was used as the indicator for phosphoketolase flux measurement in a 1-^{13}C-xylose labeling experiment. This design is reasonable as the FL of 3-phosphoglycerate is monotonically reduced with the increased flux through phosphoketolase. The limitation is that 3-phosphoglycerate is not a direct product of phosphoketolase, therefore the flux estimation is largely dependent on the assumption that the labeling pattern of 3-phosphoglycerate and glyceraldehyde 3-phosphate, the product of phosphoketolase are identical. In our case, we compared all available xylose tracers and readouts with a more realistic metabolic network which also takes reversible reactions into account. Our results demonstrated that acetate (with EMUs of AC_1, AC_2, and AC_{12}) can be a better readout due to the strong correlations with phosphoketolase flux and a wide effective range of FL. Indeed, acetate can be exclusively derived from acetyl phosphate which is directly produced from phosphoketolase. More importantly, acetate is an extracellular metabolite and its isotope pattern can be easily measured by GC-MS without derivatization. The convenience of measurement without breaking the cells provides a high-throughput and non-invasive method for prompt ^{13}C-flux estimation, which, to our knowledge, was never developed previously. This estimation is based on the numerical relationship between fractional labeling of acetate and flux ratio through phosphoketolase which are positively correlated, even though it may not be strictly linear. It should be noticed that in the FL range 0.5-0.65, multiple flux ratio values are possible for a given value of FL. The jitter could be due to the reversibility of biochemical reactions and partial dependency of the EMU basis vectors (Crown and Antoniewicz, 2012), which cannot make all free fluxes absolutely solvable using only acetate labeling data. To further obtain a more precise prediction of phosphoketolase activity, advanced multiple regression method could be applied such as machine learning using FLs of other relevant EMUs as the training features.

We believe that the EMU modeling based on adjacency matrix approach opens a number of new possibilities in metabolic network analysis and ^{13}C-MFA. First, as exemplified, *EMUlator* can be used to select metabolites as readouts reflecting directly *in vivo* enzyme activities, and can also be used to do tracer simulations (Metallo et al., 2009; Young, 2014) which predict the labeling results of metabolic network models ahead of "wet" experiments, leading to further refinement. More promisingly, *EMUlator* is developed toward solving the inverse problem to estimate intracellular fluxes through an optimization search that minimize the sum-of-squared residuals between computationally simulated and experimentally determined measurements. With the EMU method, metabolic flux estimation can be further extended to computation-intensive scenarios as genome scale network (Gopalakrishnan and Maranas, 2015) and transient labeling process (Hendry et al., 2019). This task cannot be accomplished by other isotope modeling methods due to a tremendous computational burden. Currently, we are engaged in the development of an updated version focusing on the *de novo* and complete solution of ^{13}C-MFA, in which a global flux distribution can be estimated either from steady-state labeling or kinetic labeling experiments. It is our hope

that *EMUlator* will benefit the community and fuel metabolic research as the basis for innovative development of metabolic analysis tools.

MATERIALS AND METHODS

Implementation of EMU Algorithm in *EMUlator*

The TCA cycle example in the results illustrates the adjacency matrix approach for implementing EMU algorithm. The software and its instruction and are detailed in **Supplementary Information**.

Strain, Culture Conditions, and Medium

Clostridium acetobutylicum ATCC 824 was used in all experiments. For growth studies and biochemical characterization, *C. acetobutylicum* cells were grown anaerobically in 37°C in CTFUD defined medium (Olson and Lynd, 2012) which contains 3 g L^{-1} Na$_3$C$_6$H$_5$O$_7$·2H$_2$O, 1.3 g L^{-1} (NH$_4$)$_2$SO$_4$, 1.5 g L^{-1} KH$_2$PO$_4$, 0.13 g L^{-1} CaCl$_2$·2H$_2$O, 0.5 g L^{-1} L-Cysteine–HCl, 11.56 g L^{-1} MOPS sodium salt, 2.6 g L^{-1} MgCl$_2$·6H2O, 0.001 g L^{-1} FeSO$_4$·7H2O, 0.5 mL L^{-1} Resazurin 0.2% (w/v), supplemented with Wolfe's Vitamin solution (ATCC). D-xylose was supplied at concentration of 5 g L^{-1} as a carbon source. The cultures were started with the optical density at 600 nm (OD$_{600}$ = 0.05-0.08) and performed in mid-log-phase. For labeling experiments, 1,2-^{13}C-labeled xylose (99% pure; Cambridge Isotope Laboratories, Tewksbury, MA) was added to media at the concentration of 5 g L^{-1}. *C. acetobutylicum* strains were kept by freezing log-phase cultures at −80°C with 10% glycerol.

Quantitative Analysis of Fermentation Products

Cell growth was monitored by measuring the absorbance at OD$_{600}$ with a Spectronic 21D UV-Visible Spectrophotometer (Milton Roy, Houston, TX). To analyze extracellular metabolites, cell samples were harvested by centrifugation at 13,000 g for 10 min. After filtration with 0.2 μm filter, the supernatant was analyzed by Agilent 1200 high pressure liquid chromatography (HPLC) (Agilent Technologies, Santa Clara, CA) and injected into a Bio-Rad Aminex HPX-87H column with a Micro Guard Cation H Cartridge. 4 m M H$_2$SO$_4$ was used as mobile phase at a flow rate of 0.6 mL/min. The column temperature was set to 55°C. Metabolites were detected by refractive index detector and UV/VIS detector.

Isotope Analysis

Labeling pattern of proteinogenic amino acids from cell mass were analyzed by Gas Chomatograph-mass spectrometry (GC-MS) as detailed in Xiong et al. (2018). The labeling pattern of acetate in the supernatant was directly analyzed by GC-MS without derivatization. Analysis of samples was performed on an Agilent 6890N GC equipped with a 5973 MS Detector (Agilent Technologies, Palo Alto, CA). Samples were injected

at a volume of 1 uL in splitless mode, and the analyte of interest was separated on a Restek Stabilwax-DA column (Restek Corporation, Bellefonte, PA). A flow of $1\,mL\,min^{-1}$ was held constant throughout the run with the following temperature profile: $35°C$, hold for $3\,min$; ramped at $10\ °C\ min^{-1}$ to $225°C$, hold for $1\,min$; ramped at $15°C\ min^{-1}$ to $250°C$, hold for 5 min.

Isotope Modeling From the Metabolic Network of *C. acetobutylicum*

Simulations were repeated 1,000 times with metabolic fluxes randomly generated subjecting to mass balances determined by reactions listed in **Supplementary Table 1**. Fractional labeling (FL) was calculated to indicate labeling status of metabolites according to:

$$FL = \frac{\sum_{i=0}^{n} i \cdot m_i}{n}$$

where n is the number of carbon in a EMU, and m_i represents components of MDV (Nanchen et al., 2007).

AUTHOR CONTRIBUTIONS

CW developed the software and analyzed data. WX and CW designed the experiment. CC, JL, and WM conducted the experiment. WX proposed the idea and research questions, guided all stages of the research. CW and WX prepared the manuscript with editing from PM, JL, and CC.

ACKNOWLEDGMENTS

SUPPLEMENTARY MATERIAL

REFERENCES

Antoniewicz, M. R., Kelleher, J. K., and Stephanopoulos, G. (2006). Determination of confidence intervals of metabolic fluxes estimated from stable isotope measurements. *Metab. Eng.* 8, 324–337. doi: 10.1016/j.ymben.2006.01.004

Antoniewicz, M. R., Kelleher, J. K., and Stephanopoulos, G. (2007a). Elementary metabolite units (EMU): a novel framework for modeling isotopic distributions. *Metab. Eng.* 9, 68–86. doi: 10.1016/j.ymben.2006.09.001

Antoniewicz, M. R., Kraynie, D. F., Laffend, L. A., González-Lergier, J., Kelleher, J. K., and Stephanopoulos, G. (2007b). Metabolic flux analysis in a nonstationary system: fed-batch fermentation of a high yielding strain of *E. coli* producing 1,3-propanediol. *Metab. Eng.* 9, 277–292. doi: 10.1016/j.ymben.2007.01.003

Au, J., Choi, J., Jones, S. W., Venkataramanan, K. P., and Antoniewicz, M. R. (2014). Parallel labeling experiments validate *Clostridium acetobutylicum* metabolic network model for (13)C metabolic flux analysis. *Metab. Eng.* 26, 23–33. doi: 10.1016/j.ymben.2014.08.002

Bao, G., Wang, R., Zhu, Y., Dong, H., Mao, S., Zhang, Y., et al. (2011). Complete genome sequence of Clostridium acetobutylicum DSM 1731, a solvent-producing strain with multireplicon genome architecture. *J. Bacteriol.* 193, 5007–5008. doi: 10.1128/JB.05596-11

Bogorad, I. W., Lin, T. S., and Liao, J. C. (2013). Synthetic non-oxidative glycolysis enables complete carbon conservation. *Nature* 502, 693–697. doi: 10.1038/nature12575

Crown, S. B., and Antoniewicz, M. R. (2012). Selection of tracers for 13C-metabolic flux analysis using elementary metabolite units (EMU) basis vector methodology. *Metab. Eng.* 14, 150–161. doi: 10.1016/j.ymben.2011.12.005

Gopalakrishnan, S., and Maranas, C. D. (2015). 13C metabolic flux analysis at a genome-scale. *Metab. Eng.* 32, 12–22. doi: 10.1016/j.ymben.2015.08.006

Grimmler, C., Held, C., Liebl, W., and Ehrenreich, A. (2010). Transcriptional analysis of catabolite repression in Clostridium acetobutylicum growing on mixtures of D-glucose and D-xylose. *J. Biotechnol.* 150, 315–323. doi: 10.1016/j.jbiotec.2010.09.938

Gu, Y., Jiang, Y., Wu, H., Liu, X., Li, Z., Li, J., et al. (2011). Economical challenges to microbial producers of butanol: feedstock, butanol ratio and titer. *Biotechnol. J.* 6, 1348–1357. doi: 10.1002/biot.201100046

Hendry, J. I., Gopalakrishnan, S., Ungerer, J., Pakrasi, H. B., Tang, Y. J., and Maranas, C. D. (2019). Genome-scale fluxome of *Synechococcus elongatus*

UTEX 2973 Using Transient ^{13}C-labeling data. *Plant Physiol.* 179, 761–769. doi: 10.1104/pp.18.01357

Kajihata, S., Furusawa, C., Matsuda, F., and Shimizu, H. (2014). OpenMebius: an open source software for isotopically nonstationary 13C-based metabolic flux analysis. *Biomed. Res. Int.* 2014:627014. doi: 10.1155/2014/627014

Liu, L., Zhang, L., Tang, W., Gu, Y., Hua, Q., Yang, S., et al. (2012). Phosphoketolase pathway for xylose catabolism in Clostridium acetobutylicum revealed by 13C metabolic flux analysis. *J. Bacteriol.* 194, 5413–5422. doi: 10.1128/JB.00713-12

Metallo, C. M., Walther, J. L., and Stephanopoulos, G. (2009). Evaluation of 13C isotopic tracers for metabolic flux analysis in mammalian cells. *J. Biotechnol.* 144, 167–174. doi: 10.1016/j.jbiotec.2009.07.010

Mitchell, W. J. (1998). Physiology of carbohydrate to solvent conversion by clostridia. *Adv. Microb. Physiol.* 39, 31–130. doi: 10.1016/S0065-2911(08)60015-6

Nanchen, A., Fuhrer, T., and Sauer, U. (2007). Determination of metabolic flux ratios from 13C-experiments and gas chromatography-mass spectrometry data: protocol and principles. *Methods Mol. Biol.* 358, 177–197. doi: 10.1007/978-1-59745-244-1_11

Nölling, J., Breton, G., Omelchenko, M. V., Makarova, K. S., Zeng, Q., Gibson, R., et al. (2001). Genome sequence and comparative analysis of the solvent-producing bacterium Clostridium acetobutylicum. *J. Bacteriol.* 183, 4823–4838. doi: 10.1128/JB.183.16.4823-4838.2001

Olson, D. G., and Lynd, L. R. (2012). Transformation of *Clostridium thermocellum* by electroporation. *Methods Enzymol.* 510, 317–330. doi: 10.1016/B978-0-12-415931-0.00017-3

Quek, L. E., Wittmann, C., Nielsen, L. K., and Krömer, J. O. (2009). OpenFLUX: efficient modelling software for 13C-based metabolic flux analysis. *Microb. Cell Fact.* 8, 25. doi: 10.1186/1475-2859-8-25

Sauer, U. (2006). Metabolic networks in motion: 13C-based flux analysis. *Mol. Syst. Biol.* 2, 62. doi: 10.1038/msb4100109

Schmidt, K., Carlsen, M., Nielsen, J., and Villadsen, J. (1997). Modeling isotopomer distributions in biochemical networks using isotopomer mapping matrices. *Biotechnol. Bioeng.* 55, 831–840.

Servinsky, M. D., Kiel, J. T., Dupuy, N. F., and Sund, C. J. (2010). Transcriptional analysis of differential carbohydrate utilization by *Clostridium acetobutylicum*. *Microbiology* 156, 3478–3491. doi: 10.1099/mic.0.037085-0

Shupletsov, M. S., Golubeva, L. I., Rubina, S. S., Podvyaznikov, D. A., Iwatani, S., and Mashko, S. V. (2014). OpenFLUX2: (13)C-MFA

modeling software package adjusted for the comprehensive analysis of single and parallel labeling experiments. *Microb. Cell. Fact.* 13, 152. doi: 10.1186/PREACCEPT-1256381938128538

Sokol, S., Millard, P., and Portais, J. C. (2012). influx_s: increasing numerical stability and precision for metabolic flux analysis in isotope labelling experiments. *Bioinformatics* 28, 687–693. doi: 10.1093/bioinformatics/btr716

Stephanopoulos, G. (1999). Metabolic fluxes and metabolic engineering. *Metab Eng.* 1, 1–11.

Van Winden, W. A., Heijnen, J. J., and Verheijen, P. J. (2002). Cumulative bondomers: a new concept in flux analysis from 2D [13C,1H] COSY NMR data. *Biotechnol. Bioeng.* 80, 731–745. doi: 10.1002/bit.10429

Weitzel, M., Nöh, K., Dalman, T., Niedenführ, S., Stute, B., and Wiechert, W. (2013). 13CFLUX2–high-performance software suite for (13)C-metabolic flux analysis. *Bioinformatics* 29, 143–145. doi: 10.1093/bioinformatics/bts646

Wiechert, W., Möllney, M., Isermann, N., Wurzel, M., and De Graaf, A. A. (1999). Bidirectional reaction steps in metabolic networks: III. Explicit solution and analysis of isotopomer labeling systems. *Biotechnol. Bioeng.* 66, 69–85.

Xiong, W., Lo, J., Chou, K. J., Wu, C., Magnusson, L., Dong, T., et al. (2018). Isotope-assisted metabolite analysis sheds light on central carbon metabolism of a model cellulolytic bacterium clostridium thermocellum. *Front. Microbiol.* 9:1947. doi: 10.3389/fmicb.2018.01947

Young, J. D. (2014). INCA: a computational platform for isotopically non-stationary metabolic flux analysis. *Bioinformatics* 30, 1333–1335. doi: 10.1093/bioinformatics/btu015

Young, J. D., Walther, J. L., Antoniewicz, M. R., Yoo, H., and Stephanopoulos, G. (2008). An elementary metabolite unit (EMU) based method of isotopically nonstationary flux analysis. *Biotechnol. Bioeng.* 99, 686–699. doi: 10.1002/bit.21632