Thermodynamic approach to estimating reactions and stoichiometric coefficients of anaerobic glucose and hydrogen utilization

Arini Wреста1,2,3 | Rani Widyarani3 | Ramaraj Boopathy4 | Tjandra Setiadi1,5

1Department of Chemical Engineering, Institut Teknologi Bandung (ITB), Bandung, Indonesia
2Research Center for Electrical Power and Mechatronics, Indonesian Institute of Sciences (LIPI), Bandung, Indonesia
3Research Unit for Clean Technology, Indonesian Institute of Sciences (LIPI), Bandung, Indonesia
4Department of Biological Sciences, Nicholls State University, Thibodaux, Louisiana, USA
5Centre for Environmental Studies (PSLH), Institut Teknologi Bandung, Bandung, Indonesia

Correspondence
Arini Wреста, Department of Chemical Engineering, Institut Teknologi Bandung (ITB), Jl. Ganesa 10, Bandung 40132, Indonesia.
Email: awреста@email.com

Funding information
Program Pendukung Beasiswa Saintek, Kementerian Riset dan Teknologi, Republik Indonesia; The World Class Professor of Ministry of Research, Technology, and Higher Education, Republic of Indonesia, Grant/Award Number: T/82/D2.3/KK.04.05/2019

Abstract
Anaerobic digestion plays an important role in the gastrointestinal tract and in organic waste treatment. Thermodynamic analysis based on the reaction Gibbs free energy can be used to predict the favorability of some reactions occurring during anaerobic digestion. In this study, we used a thermodynamic approach to evaluating reactions and stoichiometric coefficients of the anaerobic process of in vitro rumen microbiota. The favorability of glucose, butyrate, propionate, and hydrogen utilizations was analyzed by calculating the Gibbs free energy change of each reaction. A previously published Gibbs free energy dissipation method was also used to calculate stoichiometric coefficients of the total metabolism reaction of glucose and hydrogen utilization. For glucose utilization in which the metabolism follows several different pathways, the fraction of glucose following each pathway is estimated by considering the number of electron transfer attributed throughout the catabolism reaction. Glucose utilization always occurs in the system, and the syntrophic correlation among butyrate, propionate, and hydrogen utilizations run well with propionate utilization following the alternative pathway that yields lower hydrogen. The approach applied in this research significantly reduces the stoichiometric coefficients that must be predicted in kinetic modeling. To verify the calculation result, the yield coefficients obtained were then applied in the previous mechanistic model of in vitro rumen microbiota, and the results were compared to the experimental data from literature.

KEYWORDS
anaerobic process, reactions, stoichiometric coefficients, thermodynamic

1 | INTRODUCTION

Anaerobic digestion is a biological process, which plays an important role in the gastrointestinal tract. It is also widely used to produce energy via biohydrogen or biogas production from organic waste and to determine ruminal diets. The
anaerobic process mainly involves hydrolysis, acidogenesis, acetogenesis, and methanogenesis,\textsuperscript{1,2} which are being carried out by different groups of anaerobic microorganisms with different metabolic activities.

The complex anaerobic process has implications in the complexity of kinetic modeling of anaerobic digestion. The application of the general anaerobic digestion model, Anaerobic Digestion Model No. 1/ADM1,\textsuperscript{3} needs many parameters analysis (e.g., concentration of various substrates and microorganisms involved in the process). Simplifying the process mechanism for the ease of solving and implementation is vital in anaerobic process modeling. Thermodynamic analysis based on the reaction Gibbs free energy can be used to predict the favorability of some reactions occurring during anaerobic digestion. A positive Gibbs free energy change ($\Delta G$) indicates that the reaction is not favorable without external energy input\textsuperscript{4} and can be neglected, whereas negative $\Delta G$ means that the reaction is favorable. McCarty predicted the possibility of propionate and butyrate utilizations under certain conditions\textsuperscript{5} and obtained negative $\Delta G$ values for those reactions. Yuan \textit{et al.}\textsuperscript{6} analyzed the Gibbs free energy of long-chain fatty acid, valerate, and butyrate utilization in anaerobic digestion of blue alga and obtained positive $\Delta G$ values for those reactions.

Stoichiometric coefficients are important parameters in biological process modeling, including anaerobic digestion. The empirical determination of yield coefficients makes the model-solving more complex, thus requiring the prediction of many parameters. Researchers use the thermodynamic approach to estimating stoichiometric coefficients of biological reactions.\textsuperscript{7,8} Further application of this thermodynamic approach into kinetic modeling simplifies the model-solving, that is, it decreases the number of parameters to be predicted or process parameters to be analyzed, including anaerobic digestion with many biochemical reactions in series and in parallel.

Researchers use different thermodynamic approaches to estimating stoichiometric coefficients of anaerobic processes. For example, Plavostatis and Giraldo-Gomez\textsuperscript{9} and Speece\textsuperscript{5} used the McCarty approach\textsuperscript{10} to estimating the stoichiometric coefficient of acetate, propionate, and hydrogen utilization. Many of them, including Xiao and VanBriesen\textsuperscript{11} and McCarty,\textsuperscript{12} expanded and modified the McCarty approach; this method is called the thermodynamic electron equivalent model (TEEM). Heijnen \textit{et al.}\textsuperscript{13} proposed a Gibbs free energy dissipation method to estimate stoichiometric coefficients of biological reactions. Rodríguez \textit{et al.}\textsuperscript{14} and González-Cabaleiro \textit{et al.}\textsuperscript{15} continuously developed and improved the gray box model based on adenosine triphosphate (ATP) yield, however this method requires rather detailed biochemical knowledge that in many experimental systems are not available.\textsuperscript{4}

The TEEM\textsuperscript{10,12} and the Gibbs free energy dissipation\textsuperscript{13} methods were developed based on black box model, requiring only the identification of carbon and nitrogen source and the electron donor-acceptor couple in the catabolic reaction equation.\textsuperscript{4} These methods calculate the biomass yield by simultaneously solving the electron and energy balances of the metabolism process.\textsuperscript{11} For the TEEM method, three half-reaction equations need to be solved: electron donor reaction, electron acceptor reaction for energy generation and cell biomass reaction. For the Gibbs free energy dissipation method,\textsuperscript{11} four half-reaction equations need to be solved: oxidation of biomass, oxidation of the carbon source, electron donor reaction, and electron acceptor reaction. The Gibbs free energy of each reaction is calculated based on the Gibbs free energy per mole electron in the half-reaction of the component involved in the reaction ($\Delta G_r$), stated as kilojoules per electron equivalent.

Kleerebezem and van Loosdrecht\textsuperscript{4} improved the Gibbs free energy dissipation method proposed by Heijnen \textit{et al.}\textsuperscript{13} in order to use it generally, not just for components present in the biological thermodynamic table. The reason is that the calculation of the Gibbs free energy of each reaction is based on the Gibbs free energy per mole component, $\Delta G$ (kJ/mol), not the Gibbs free energy per electron equivalent. This method also defines the energy generation (catabolism) and cell formation (anabolism) reactions as an individual couple of the oxidation–reduction (electron donor and acceptor) reaction, simplifying the step to solve the metabolism reaction equation. The electron and energy balances can be solved separately. The electron balance is solved first by balancing the oxidation–reduction reaction equations of each catabolism and anabolism reaction in order to obtain the stoichiometric equation of these two reactions. The metabolism reaction equation is then estimated by solving the energy balance between catabolism and anabolism reaction equations. Once the catabolism and anabolism reaction equations and the environmental conditions are known, the Gibbs free energy dissipation and the Gibbs free energy of catabolism and anabolism reactions can be calculated, and the metabolism reaction equation and biomass yield can be estimated. A few studies were conducted on the application of this method in anaerobic digestion, for example, the estimation of the stoichiometric coefficients of lactate, format and hydrogen utilization,\textsuperscript{16} the stoichiometric coefficients of lactate, format, acetate, and hydrogen utilization\textsuperscript{17} and the stoichiometric coefficients of butyrate, ethanol, and acetate utilization.\textsuperscript{18}

Since many anaerobic processes occur in different systems and under different environmental conditions, $\Delta G$ will also be different, in addition to reactions which occur. The stoichiometric coefficients of the metabolism reaction also depend on the $\Delta G$ values. Since an anaerobic process often runs very close to thermodynamic equilibrium,\textsuperscript{19} the
favorability and stoichiometric coefficients of reactions need to be analyzed when the process is carried out in the different system.

In this study, the anaerobic process of in vitro rumen microbiota was analyzed thermodynamically. Kinetic modeling by Muñoz-Tamayo et al.\textsuperscript{20} neglects butyrate and propionate utilization by hydrogen-producing acetogens. Butyrate and propionate utilization reactions play an important role in ensuring the favorability of hydrogen utilization by hydrogenotrophic methanogens. Therefore, besides glucose and hydrogen utilizations, we also evaluate butyrate and propionate utilization reactions to understand the favorability of these reactions occurring in the system. To define the yield coefficients of the reaction in kinetic modeling in order to reduce the number of kinetic parameters which need to be predicted, Muñoz-Tamayo et al.\textsuperscript{20} used stoichiometric equations of catabolism and anabolism reactions for glucose and hydrogen utilizations. However, by using this method, kinetic modeling still needs empirical prediction of the cell biomass yield ($Y_{X/S}$), which links the amount of new cell biomass formed with the amount of substrate consumed. To complete the utilization of these two half metabolism reaction equations, we also estimated the stoichiometric coefficients of the total metabolism reaction equation using the Gibbs free energy dissipation method.\textsuperscript{4}

Some researches implemented the integration of thermodynamic approach based on product yields into kinetic modeling. Rodríguez et al.\textsuperscript{21} implemented the variable product stoichiometry from glucose fermentation based on mixed culture fermentation model\textsuperscript{14} in the ADM1 prediction. Heijnen and Kleerebezem\textsuperscript{22} explained in detail how to integrate the thermodynamic and kinetic modeling, by first calculating the maximum specific substrate consumption rate constant and yield coefficients of components based on dissipation method and then applied the result into the kinetic modeling, giving an example by simulating the metabolism reaction of sulfate reduction with lactate as electron donor. González-Cabaleiro et al.\textsuperscript{23} implemented the same approach as Heijnen and Kleerebezem,\textsuperscript{22} improved it by considering the biomass decay rate and the hydrolysis of the dead biomass and simulated this approach for anaerobic glucose fermentation. González-Cabaleiro et al.\textsuperscript{23} implemented the same integration concept as References 22,23 for glucose fermentation; however, the product distribution was calculated based on the gray box model. Recent improvement by Delattre et al.\textsuperscript{24} integrated the black box model into kinetic modeling of sulfate reduction and methanogenesis by considering the pH dynamic and liquid–gas mass transfer of gas products. The model was built based on the experiment of synthetic microbial communities using Na-lactate as the carbon source. While the gray box model has been implemented in both single main substrate model\textsuperscript{14,15} and complete kinetic model of ADM1,\textsuperscript{21} the black box model was still applied in the single main substrate only.\textsuperscript{22-24}

González-Cabaleiro et al.\textsuperscript{23} simulated the glucose utilization through several pathways, each of which was carried out by different microorganisms. This approach is more suitable with the real condition, however differentiating the microorganisms are not always possible. As Muñoz-Tamayo et al.\textsuperscript{20} assumed that all pathways of glucose utilization were carried out by one virtual microorganism, we estimated the fraction of glucose following each pathway by considering the number of electron transfer attributed throughout the catabolism reaction. We improved the implementation of black box model by integrating the stoichiometric coefficients of glucose and hydrogen utilizations in previous completely mechanistic model of in vitro rumen microbiota and verifying the result with the original experimental data from the literature. This model was built based on the utilization of complex substrate with inoculum from diet-fed-goat.\textsuperscript{25} The substrate utilization involved inoculum from the real process consisting many species of anaerobic microorganisms. Such system is widely found in the real anaerobic processes, for example, in the formation of biofuels from organic waste such as biogas and biohydrogen or in the ruminants-diets. The model also already considered the biomass decay rate, hydrolysis of the dead biomass, liquid–gas mass transfer, and pH dynamic.\textsuperscript{20} Applying the thermodynamic into a completely anaerobic model of a complex system similar to the real condition and verifying the integration model with experimental data is important from the standpoint of scientific knowledge to ensure the applicability of the model in the real anaerobic process.

2 MATERIALS AND METHODS

The favorability of glucose, butyrate, propionate and hydrogen utilization was analyzed by calculating the Gibbs free energy change of each reaction. Stoichiometric coefficients of glucose and hydrogen utilization were estimated using the Gibbs free energy dissipation method.\textsuperscript{4} The fraction of glucose following different pathways in glucose utilization was estimated by calculating the maximum specific substrate utilization rate constant of each pathway based on the number of electron transfer throughout the catabolism reaction. We obtained the biomass yield coefficients and the stoichiometric equations of the total metabolism reaction of glucose and hydrogen utilization (detail calculation can be seen in Supplementary file [Microsoft Excel]). To verify the calculated results, the yield coefficients obtained was then included in the
previous mechanistic kinetic model built by Reference 20 and the simulation result was compared with the experimental value from Serment et al.\textsuperscript{25}

### 2.1 Gibbs free energy change of reaction

The Gibbs free energy change of reaction was calculated based on general stoichiometric equation for the reaction as follow:

$$\text{Re} : -Y^\text{Re}_S \cdot S + \ldots + Y^\text{Re}_P \cdot P = 0$$ \hspace{1cm} (1)

where $\text{Re}$ is reaction, $-Y^\text{Re}_S$ is stoichiometric coefficient of substrate, $S$ is substrate, $Y^\text{Re}_P$ is stoichiometric coefficient of product, and $P$ is product. To determine the favorability of a reaction in the system, we calculated the actual Gibbs free energy change using the Gibbs–Helmholtz equation\textsuperscript{4} as follows:

$$\Delta G^1_T = \Delta G^1_{T_S} \cdot \frac{T}{T_s} + \Delta H^0_{T_S} \cdot \frac{T_s - T}{T_s}$$ \hspace{1cm} (2)

where $\Delta G^1_T$ is the actual Gibbs free energy change of reaction at temperature $T$ (kJ/reaction), $\Delta G^1_{T_S}$ is the actual Gibbs free energy change of reaction at standard temperature ($T_s = 298.15$ K) (kJ/reaction), $\Delta H^0_{T_S}$ is the enthalpy change of reaction at standard temperature (kJ/reaction), and $T$ is the reaction temperature (K). $\Delta G^1_{T_S}$ can be calculated as:

$$\Delta G^1_{T_S} = \Delta G^0_{T_S} \cdot \frac{T}{T_s} + R \cdot T_s \cdot \sum_{i=1}^{n} Y^\text{Re}_{S_i} \cdot \ln a_{S_i},$$ \hspace{1cm} (3)

where $\Delta G^0_{T_S}$ is the standard Gibbs free energy change of reaction (kJ/reaction), $R$ is the ideal gas constant (8.314 J/mol K), $Y^\text{Re}_{S_i}$ is the stoichiometric coefficient of component $S_i$ in reaction Re, $n$ is the number of component involved in reaction Re, and $a_{S_i}$ is the actual activities of component $S_i$, which is in dilute system equal to the molar concentration (mol/l) and for gas components are represented by partial pressure (atm). It should be noted that $Y^\text{Re}_{S_i}$ is negative for reactant (substrate) and positive for product.

### 2.2 Stoichiometric coefficients of the metabolism reaction

The Gibbs free energy dissipation method\textsuperscript{4} was used to estimate the stoichiometric coefficient of metabolism reaction of glucose and hydrogen utilization. This method was proposed based on the simplification of metabolism process as a coupling of catabolism and anabolism reaction and neglected the energy consumed for maintenance. Substrate ($S$) is converted to product ($P$) in the catabolism process by releasing energy (in the form of ATP), that is then consumed during the anabolism process, that convert the carbon ($S_x$) and nitrogen ($N_x$) sources to a biomass cell ($X$). The Gibbs free energy released from the catabolism process ($\Delta G_{\text{cat}}$) is used to form biomass in the anabolism process; however, since some work is needed to produce biomass, significant energy is dissipated during cell biomass formation, and the total energy needed by the anabolism process is the sum of the Gibbs free energy change of the anabolism reaction ($\Delta G_{\text{an}}$) and Gibbs free energy dissipation ($\Delta G_{\text{dis}}$) to form 1 C-mol biomass.

To clarify this concept, we write the general stoichiometric equation for the catabolism and anabolism reactions as a function of substrate, product, and biomass cell. In many cases, the substrate for the catabolism ($S$) is same as the substrate (carbon source ($S_x$)) for the anabolism,\textsuperscript{26} so that both of the substrate and the carbon source are symbolized as $S$. The stoichiometric equation is arranged on the basis of the formation of 1 C-mol biomass. These two equations are then combined with the metabolism reaction equation\textsuperscript{6} as follows:

$$\text{Cat} : -Y^\text{Cat}_S \cdot S + \ldots + Y^\text{Cat}_P \cdot P = 0$$ \hspace{1cm} (4)

$$\text{An} : -Y^\text{An}_S \cdot S - Y^\text{An}_{N_x} \cdot N_x + \ldots + CH_{1,8}O_{0,5}N_{0,2} = 0$$ \hspace{1cm} (5)

$$\text{Met} : \lambda_{\text{Cat,An}}$$ \hspace{1cm} (6)
where \( \text{Cat} \) is the catabolism reaction equation, \( \text{An} \) is the anabolism reaction equation, \( \text{Met} \) is the metabolism reaction equation, \(-Y_{\text{Cat}}^S\) is the stoichiometric coefficient of the substrate in the catabolism reaction, \( Y_{\text{Cat}}^P \) is the stoichiometric coefficient of the product in the catabolism reaction, \(-Y_{\text{An}}^S\) is the stoichiometric coefficient of the carbon source (substrate) in the anabolism reaction, \(-Y_{\text{An}}^N_X\) is the stoichiometric coefficient of the nitrogen source in the anabolism reaction, and \( \lambda_{\text{Cat}} \) is the multiplication factor of the catabolism reaction equation so that the summation of Gibbs free energy change of catabolism and anabolism process is zero. \( \lambda_{\text{Cat}} \) can be calculated as follows:

\[
\lambda_{\text{Cat}} = \frac{\Delta G_{\text{An}} + \Delta G_{\text{Dis}}}{-\Delta G_{\text{Cat}}} \tag{7}
\]

Then, the metabolism reaction equation can be written as follows:

\[
\text{Met} : - (\lambda_{\text{Cat}}Y_{\text{Cat}}^S + Y_{\text{An}}^S) . S - Y_{\text{An}}^N_X . N_X + ... + \lambda_{\text{Cat}}Y_{\text{Cat}}^P . P + CH_{1.8}O_{0.5}N_{0.2} = 0 \tag{8}
\]

The yield coefficients of this reaction can be calculated based on the stoichiometric coefficients of each component involved in the metabolism reaction as follows:

\[
Y_{\text{Met}}_{X/S} = \frac{Y_{\text{Met}}^X}{Y_{\text{Met}}^S} = \frac{1}{(Y_{\text{An}}^S + \lambda_{\text{Cat}}Y_{\text{Cat}}^S)} \tag{9}
\]

\[
Y_{\text{Met}}_{P/S} = \frac{Y_{\text{Met}}^P}{Y_{\text{Met}}^S} = \frac{\lambda_{\text{Cat}}Y_{\text{Cat}}^P}{(Y_{\text{An}}^S + \lambda_{\text{Cat}}Y_{\text{Cat}}^S)} \tag{10}
\]

where \( Y_{\text{Met}}_{X/S} \) also written as \( Y_X/S \), is the yield coefficient which links the amount of new cell biomass formed with the amount of substrate consumed in the metabolism reaction, whereas \( Y_{\text{Met}}_{P/S} \) or \( Y_P/S \) is the yield coefficient which links the amount of product formed with the amount of substrate consumed. In the case when the substrate of catabolism is different from the carbon source, it is easy to practically suit the calculation method by multiplying the catabolism reaction equation with \( \lambda_{\text{Cat}} \) and summing the equation obtained with the anabolism reaction equation, of course by writing the substrate of catabolism as different component from the carbon source of anabolism. After obtaining the metabolism equation, the yield coefficients can be calculated based on the stoichiometric coefficients of each component involved in the reaction.

2.3 Stoichiometric reaction equations

Some well-known reactions in anaerobic digestion were used to analyze the favorability of reactions occurring in batch anaerobic process of in vitro rumen microbiota. For glucose utilization, three pathways were used, which followed the three pathways used for kinetic modeling by,\(^{20}\) respectively:

\[
\text{C}_6\text{H}_{12}\text{O}_6 + 2\text{H}_2\text{O} \rightarrow 2\text{CH}_3\text{COOH} + 2\text{CO}_2 + 4\text{H}_2 \tag{11}
\]

\[
\text{C}_6\text{H}_{12}\text{O}_6 \rightarrow \frac{2}{3}\text{CH}_3\text{COOH} + \frac{4}{3}\text{CH}_3\text{CH}_2\text{COOH} + \frac{2}{3}\text{CO}_2 + \frac{2}{3}\text{H}_2\text{O} \tag{12}
\]

\[
\text{C}_6\text{H}_{12}\text{O}_6 \rightarrow \text{CH}_3\text{CH}_2\text{CH}_2\text{COOH} + 2\text{CO}_2 + 2\text{H}_2 \tag{13}
\]

For butyrate utilization the following pathway was used:\(^5\):

\[
\text{CH}_3\text{CH}_2\text{COOH} + 2\text{H}_2\text{O} \rightarrow 2\text{CH}_3\text{COOH} + 2\text{H}_2 \tag{14}
\]

For propionate utilization, since it was often a critical step in anaerobic process,\(^{27}\) the classical pathway\(^3\) and the alternative route via Smithella pathway that may improve the precarious stability of propionate degradation\(^{27}\) were studied:

\[
\text{CH}_3\text{CH}_2\text{COOH} + 2\text{H}_2\text{O} \rightarrow \text{CH}_3\text{COOH} + 3\text{H}_2 + \text{CO}_2. \tag{15}
\]
\[
\text{CH}_3\text{CH}_2\text{COOH} + \text{H}_2\text{O} \rightarrow 1.5\text{CH}_3\text{COOH} + \text{H}_2
\] (16)

For hydrogen utilization, the reaction was as follows:
\[
4\text{H}_2 + \text{CO}_2 \rightarrow \text{CH}_4 + 2\text{H}_2\text{O}.
\] (17)

Since rumen microbiota performs metabolism reactions with very few acetotrophic methanogens, the metabolism reaction to convert volatile fatty acids to methane is neglected.

For stoichiometric coefficients estimation, the anabolism reaction equations were arranged based on the method of Reference 4. For glucose utilization, the carbon and nitrogen source are C\text{6}H\text{12}O\text{6} and NH\text{4}\text{+}, respectively. The half reaction of biomass formation is written as follow:
\[
0.167\text{C}_6\text{H}_{12}\text{O}_6 + 0.2\text{NH}_4^+ + 0.2e^{-} \rightarrow \text{CH}_{1.8}\text{O}_{0.5}\text{N}_{0.2} + 0.5\text{H}_2\text{O}
\] (18)

Because the half reaction of biomass formation is an electron-accepting reaction, an electron donor (C\text{6}H\text{12}O\text{6}) is needed to supply the electron. The electron-donor reaction is:
\[
\text{C}_6\text{H}_{12}\text{O}_6 + 12\text{H}_2\text{O} \rightarrow 6\text{HCO}_3^- + 30\text{H}^+ + 24e^{-}
\] (19)

By multiplying the electron-donor reaction equation by 0.008 and summing it with the half reaction of biomass formation, the anabolism reaction equation is obtained as follows:
\[
0.175\text{C}_6\text{H}_{12}\text{O}_6 + 0.2\text{NH}_4^+ \rightarrow 0.05\text{HCO}_3^- + 0.25\text{H}^+ + \text{CH}_{1.8}\text{O}_{0.5}\text{N}_{0.2} + 0.4\text{H}_2\text{O}
\] (20)

For hydrogen utilization, the carbon and nitrogen sources are CO\text{2} and NH\text{4}\text{+}, whereas the electron donor is hydrogen. By using the same way, the anabolism reaction equation for hydrogen utilization is obtained as follows:
\[
2.1\text{H}_2 + \text{CO}_2 + 0.2\text{NH}_4^+ \rightarrow \text{CH}_{1.8}\text{O}_{0.5}\text{N}_{0.2} + 1.5\text{H}_2\text{O} + 0.2\text{H}^+
\] (21)

### 2.4 Fraction of glucose following different pathway in glucose utilization

The catabolism reaction equations for glucose utilization occur through three different pathways (pathway1, pathway2, and pathway3) following Equations (11)–(13), respectively. We called Equations (11)–(13) as catabolism1, catabolism2, and catabolism3, then the metabolism reaction equation of each pathway was called as metabolism1, metabolism2, and metabolism3. Muñoz-Tamayo et al.\textsuperscript{20} defined the fraction of glucose that followed pathway1, pathway2, and pathway3 as \(\lambda_1\), \(\lambda_2\), and \(\lambda_3\); and predicted the values through kinetic modeling. \(\lambda_1\), \(\lambda_2\), and \(\lambda_3\) represent the differences of process rate of each pathways that are influenced by kinetic parameters, such as: maximum specific substrate utilization rate constant (\(k_m\), [mol substrate/mol cell]/h), half saturation constant (\(K_s\), mol/L), and biomass concentration (mol biomass cell/L). By assuming the same \(K_s\) value for all pathways\textsuperscript{23} and neglecting the microbial diversity of glucose utilizations,\textsuperscript{14} the value of \(\lambda_1\), \(\lambda_2\), and \(\lambda_3\) will only depend on the value of maximum specific substrate utilization rate constant of each microbial reaction, which are: \(\lambda_1 = k_m / (k_m + k_{m2} + k_{m3})\), \(\lambda_2 = k_{m2} / (k_{m1} + k_{m2} + k_{m3})\), and \(\lambda_3 = 1 - (\lambda_1 + \lambda_2)\). The value of \(k_{m1}\), \(k_{m2}\), and \(k_{m3}\) are calculated based on Reference 23, which is: \(k_m = 3/N_e\), with \(N_e\) is the number of electron transfers attributed throughout a catabolism per mol substrate (mol electron/mol substrate). Based on \(N_e\) values for catabolism1 and catabolism3 of 8 and 12\textsuperscript{23} and for catabolism2 of 10.67, it was obtained the value of \(\lambda_1\), \(\lambda_2\), and \(\lambda_3\) of 0.41, 0.31 and 0.28, respectively. This obtained \(\lambda_1\), \(\lambda_2\), and \(\lambda_3\) values were then used to estimate the total metabolism reaction equation of glucose utilization before it was applied in kinetic model.

### 2.5 The required data

To calculate the Gibbs free energy change of reactions, the data of \(\Delta G_f\), \(\Delta H_f\), concentration of each component and partial pressure of gas components are needed. For stoichiometric coefficients estimation, the data of Gibbs
free energy dissipation of the carbon source is also required. The data of $\Delta G_f$, $\Delta H_f$, and $\Delta G_{\text{Dis}}$ were obtained from Reference 4 except $\Delta G_f$ and $\Delta H_f$ of acetate, propionate, and butyrate that were obtained from Reference 28. The data of concentration and partial pressure of components were obtained from Reference 20 simulation based on batch experiment of anaerobic process of in vitro rumen microbiota for a low-concentration substrate with inoculum from low-diet-fed goat at 39°C.25 The data of $\Delta G_f$, $\Delta H_f$, and $\Delta G_{\text{Dis}}$ can be seen in Table S1 (Supplementary file), and the data of concentration and partial pressure of each component are presented in Table S2 (Supplementary file).

3 | RESULTS AND DISCUSSIONS

3.1 Favorability of glucose, butyrate, propionate, and hydrogen utilization

To evaluate the favorability of glucose, butyrate, propionate, and hydrogen utilizations, we used stoichiometric reaction in Equations (11)–(17), and calculated the Gibbs free energy change of each reaction using Equations (2) and (3). For this study, we call Equations (11)–(13) as glucose utilization1, glucose utilization2, and glucose utilization3, and Equations (15) and (16) as propionate utilization1 and propionate utilization2, respectively. Figure 1 presents the actual Gibbs free energy change for each reaction during the batch anaerobic process of in vitro rumen microbiota calculated based on data from20 simulation.

The actual Gibbs free energy changes ($\Delta G_f^1$) of the three glucose utilization pathways were significantly negative (ranging from $-265.10$ to $-290.35$ kJ/reaction), indicating that these reactions always occur in the system. For butyrate and hydrogen utilizations, too, $\Delta G_f^1$ were negative; however, the negative values were low. For butyrate utilization, $\Delta G_f^1$ ranged from $-19.96$ to $-17.96$ kJ/reaction, whereas for hydrogen utilization, $\Delta G_f^1$ ranged from $-48.43$ to $-55.15$ kJ/reaction. For propionate utilization, through pathway1 (propionate utilization1), $\Delta G_f^1$ was positive in the first 6-h then became negative until the end of the process, $\Delta G_f^1$ ranged from $-2.51$ to $+2.29$ kJ/reaction. Through pathway2 (propionate utilization2), the $\Delta G_f^1$ was negative during the process, ranging from $-4.80$ to $-5.89$ kJ/reaction, the negative value was lower than $\Delta G_f^1$ of butyrate and hydrogen utilizations.

Butyrate, propionate, and hydrogen utilization reactions yielded low Gibbs free energy change, $<100$ kJ/reaction; therefore, some changes in conditions, such as a different concentration of each component and a different partial pressure of hydrogen ($P_{H_2}$), probably lead to a positive $\Delta G_f^1$ for one of these reactions, and these processes could not occur simultaneously. Propionate utilization often become the critical step,27 since it produced the lowest Gibbs free energy.

Hydrogen-producing acetogens and hydrogenotrophic methanogens are in syntrophic correlation,30 and the critical balance between both affects substrate utilization reactions.31 These substrate utilization reactions only occur under a specific condition, that is, when $\Delta G_f^1$ for both the acetogenesis and methanogenesis reactions is negative. The acetogens can supply hydrogen to be used as a substrate for hydrogenotrophic methanogens. Hydrogen plays an important role as an intermediate product, and $P_{H_2}$ significantly affects the favorability of these reactions.

**FIGURE 1** Actual Gibbs free energy change for glucose, butyrate, propionate and hydrogen utilization, calculated based on data from Reference 20 simulation. Glucose utilisation1: $C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H_2$; glucose utilisation2: $C_6H_{12}O_6 \rightarrow \frac{3}{2}CH_3COOH + \frac{3}{2}CH_3CH_2COOH + \frac{3}{2}CO_2 + \frac{3}{2}H_2O$; glucose utilisation3: $C_6H_{12}O_6 \rightarrow CH_3CH_2CH_2COOH + 2CO_2 + 2H_2$; butyrate utilisation: $CH_3CH_2CH_2COOH + 2H_2O \rightarrow 2CH_3COOH + 2H_2$; propionate utilisation1: $CH_3CH_2COOH + 2H_2O \rightarrow CH_3COOH + 3H_2 + CO_2$; propionate utilisation2: $CH_3CH_2COOH + H_2O \rightarrow 1.5CH_3COOH + H_2$; hydrogen utilisation: $4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$.
Figure 2 presents the correlation between $\Delta G^1_T$ of butyrate, propionate, and hydrogen utilization and $P_{H_2}$ during the process. $P_{H_2}$ ranged from 0.00041 to 0.00074 atm. In this condition, $\Delta G^1_T$ of butyrate and hydrogen utilization was negative, ranging from $-17.96$ to $-55.15$ kJ/reaction. For propionate utilization, through the classical pathway (propionate utilization1), initially, the $\Delta G^1_T$ was positive then became negative when $P_{H_2} < 0.00056$ ($\Delta G^1_T$ ranged from $-2.51$ to $+2.29$ kJ/reaction). The positive $\Delta G^1_T$ indicated that inhibition might occur due to the high $P_{H_2}$ value more than 0.00056. As a response to hydrogen consumption by methanogens, $P_{H_2}$ decreased below the limiting value, then the reaction became favorable. Through this pathway, propionate utilization was at a critical point, that inhibition could occur any time as the increase of $P_{H_2}$. However, through the alternative pathway (propionate utilization2), $\Delta G^1_T$ was always negative, although the negative value was lower than butyrate and hydrogen utilization. The $\Delta G^1_T$ ranged from $-4.80$ to $-5.89$ kJ/reaction; however, it was not very sensitive to $P_{H_2}$ change. As stated by Reference 27, the higher hydrogen yield for the classical pathway make it more sensitive to $P_{H_2}$ change, which make the opportunity of the reaction to be exergonic was lower. The propionate utilization1 produces 3 mol hydrogen per mol propionate consumed whereas the propionate utilization2 produces only 1 mol hydrogen per mol propionate consumed. Since it yielded lower hydrogen, the opportunity of propionate utilization2 to be exergonic was higher. Giving attention to the standard Gibbs free energy change of propionate utilization1 and 2 of 62 and 17.7 kJ/reaction, this result was in agreement with Reference 29 that propionate utilization1 which can produce the higher energy and yield higher hydrogen gets inhibited at a lower $P_{H_2}$ than propionate utilization2. The different opportunity of reactions to be exergonic then brings to the sensitivity to $P_{H_2}$ change, as it was illustrated as a syntrophic correlation in Figure 3, based on the mean process condition.

The sensitivity of the reaction with the change of hydrogen partial pressure can be observed from the slope of the graph correlating $\Delta G^1_T$ and log $P_{H_2}$ in Figure 3. The higher slope showed the higher effect of $P_{H_2}$ change on the favorability of the reaction ($\Delta G^1_T$). Among butyrate, propionate utilization1 and propionate utilization2, the highest slope was observed at propionate utilization1 (slope = 17.93), so propionate utilization1 was the most sensitive to the increase of $P_{H_2}$ (confirmed by the positive $\Delta G^1_T$ at the first 6-h process). Syntrophic cooperation between propionate utilization1 and

**FIGURE 2** Actual Gibbs free energy change for syntrophic acetogenesis and hydrogenotrophic methanogenesis. Butyrate utilization: $\text{CH}_3\text{CH}_2\text{CH}_2\text{COOH} + 2\text{H}_2\text{O} \rightarrow 2\text{CH}_3\text{COOH} + 2\text{H}_2$; propionate utilization1: $\text{CH}_3\text{CH}_2\text{COOH} + 2\text{H}_2\text{O} \rightarrow 1.5\text{CH}_3\text{COOH} + \text{H}_2 + \text{CO}_2$; propionate utilization2: $\text{CH}_3\text{CH}_2\text{COOH} + \text{H}_2\text{O} \rightarrow 1.5\text{CH}_3\text{COOH} + \text{H}_2$; hydrogen utilization: $4\text{H}_2 + \text{CO}_2 \rightarrow \text{CH}_4 + 2\text{H}_2\text{O}$

**FIGURE 3** Syntrophic correlation among butyrate, propionate, and hydrogen utilizations. The area within the green-dashed rectangle: butyrate and hydrogen utilization occur simultaneously; within violet-dashed dotted line: butyrate, propionate utilization2, and hydrogen utilization occur simultaneously, within yellow-dotted line: all propionate, butyrate, and hydrogen utilization occur simultaneously; within blue solid box: the area of batch anaerobic process of in vitro rumen microbiota.
hydrogen utilization occurs in the small range of hydrogen partial pressure (area within the yellow-dotted rectangle: log $P_{H_2}$ ranged from $-3.25$ to $-5.42$; $\Delta G_f^1$ for propionate utilization1 was $0$ to $-38.91$ kJ/reaction; and for hydrogen utilization was $0$ to $-51.88$ kJ/reaction).

As the consequence of the lower slope (slope = 5.98), the opportunity of propionate utilization2 to be exergonic was higher (than propionate utilization1) even though it produced a low Gibbs free energy. Syntrophic cooperation between propionate utilization2 and hydrogen utilization occurs in a larger range of hydrogen partial pressure. The area within the violet-dash dotted rectangle was where propionate utilization2 and hydrogen utilization could occur simultaneously. Log $P_{H_2}$ ranged from $-2.37$ to $-5.42$. $\Delta G_f^1$ for propionate utilization2 was $0$ to $-18.24$ kJ/reaction and for hydrogen utilization was $0$ to $-72.95$ kJ/reaction.

The slope of butyrate utilization (slope = 11.96) was higher than propionate utilization2, however the favorable range of $P_{H_2}$ was larger. This is because butyrate utilization produced a higher Gibbs free energy change than propionate utilization2 and the graph log $P_{H_2}$ versus $\Delta G_f^1$ never intersect propionate utilization2 at the negative $\Delta G_f^1$ values. Compared to propionate utilization1 and 2, butyrate utilization provided the largest range of $P_{H_2}$ to be exergonic. The area within the green-dashed rectangle (Figure 3) was where butyrate and hydrogen utilization could occur simultaneously. Log $P_{H_2}$ ranged from $-1.68$ to $-5.42$. $\Delta G_f^1$ of butyrate, and hydrogen utilization was negative: butyrate utilization ranged from $0$ to $-44.75$ kJ/reaction, and hydrogen utilization ranged from $0$ to $-89.49$ kJ/reaction.

Furthermore, the detailed impact of $P_{H_2}$ can be observed based on the reduction of Gibbs free energy available by $P_{H_2}$ gradient, as illustrated clearly in Table 1. Our study showed that propionate utilization1 was the most sensitive to $P_{H_2}$ change as indicated by the high free energy available reduction in the low $P_{H_2}$ increase (Table 1). Based on the

| Reaction                     | $P_{H_2}$, atm | $\Delta G_f^1$, kJ/reaction | Energy reduction, % |
|------------------------------|----------------|-----------------------------|---------------------|
| Propionate utilization1      | $4.10 \times 10^{-4}$ | $-2.42$                     | –                   |
|                              | $4.50 \times 10^{-4}$ | $-1.68$                     | 30.62               |
|                              | $4.90 \times 10^{-4}$ | $-1.00$                     | 58.58               |
|                              | $5.10 \times 10^{-4}$ | $-0.72$                     | 70.13               |
|                              | $5.30 \times 10^{-4}$ | $-0.42$                     | 82.48               |
|                              | $5.60 \times 10^{-4}$ | $0.00$                      | 100.00              |
| Propionate utilization2      | $4.10 \times 10^{-4}$ | $-6.07$                     | –                   |
|                              | $5.60 \times 10^{-4}$ | $-5.27$                     | 13.29               |
|                              | $7.40 \times 10^{-4}$ | $-4.54$                     | 25.22               |
|                              | $1.40 \times 10^{-5}$ | $-2.89$                     | 52.45               |
|                              | $2.50 \times 10^{-5}$ | $-1.38$                     | 77.22               |
|                              | $4.26 \times 10^{-5}$ | $0.00$                      | 100.00              |
| Butyrate utilization         | $4.10 \times 10^{-4}$ | $-20.42$                    | –                   |
|                              | $5.60 \times 10^{-4}$ | $-18.81$                    | 7.91                |
|                              | $7.40 \times 10^{-4}$ | $-17.36$                    | 15.01               |
|                              | $4.10 \times 10^{-5}$ | $-8.47$                     | 58.52               |
|                              | $8.50 \times 10^{-5}$ | $-4.69$                     | 77.05               |
|                              | $2.10 \times 10^{-2}$ | $0.00$                      | 100.00              |
| Hydrogen utilization         | $7.40 \times 10^{-4}$ | $-54.78$                    | –                   |
|                              | $5.60 \times 10^{-4}$ | $-51.88$                    | 5.29                |
|                              | $3.00 \times 10^{-4}$ | $-45.40$                    | 17.11               |
|                              | $7.40 \times 10^{-5}$ | $-30.87$                    | 43.64               |
|                              | $7.40 \times 10^{-6}$ | $-6.97$                     | 87.27               |
|                              | $3.78 \times 10^{-6}$ | $0.00$                      | 100.00              |
lowest $P_{H_2}$ value from Reference 20 simulation of 0.00041 atm, the increase of $P_{H_2}$ approx. 10% (to $P_{H_2}$ 0.00045 atm), reduced the available energy from 2.42 to 1.68 kJ/reaction or it reduced 0.74 kJ/mol propionate, approx. 30.6% of the available energy. When $P_{H_2}$ was 1.37 times (0.00056 atm), the available energy reduction was 100%

Propionate utilization2 was more tolerant to $P_{H_2}$ change. When propionate utilization1 reduced 100% of the available energy, the increase of $P_{H_2}$ to 0.00056 atm only reduced 13.29% of the available energy (it reduced from 6.08 to 5.27 kJ/reaction or it reduced 0.81 kJ/mol propionate). The 100% energy reduction occurs when $P_{H_2}$ was 10.39 times higher (0.0043 atm); then the available energy was 0.

Butyrate utilization was the most tolerant to $P_{H_2}$ change. Based on the lowest $P_{H_2}$ value of 0.00041 atm, the increase of $P_{H_2}$ to 0.00056 atm, reduced the available energy from 20.42 to 18.81 kJ/reaction. It reduced 1.62 kJ/mol butyrate, only 7.91% of the available energy, at the condition when the reduction of propionate utilization1 and propionate utilization2 was 100% and 13.29% of the available energy. When $P_{H_2}$ increased 10 times (0.0041 atm), the available energy reduction of butyrate utilization was 58.52%; and 100% energy reduction occur at $P_{H_2}$ 0.021 atm, a much higher value than propionate utilization1 and propionate utilization2.

For hydrogen utilization, based on the highest $P_{H_2}$ value from Reference 20 simulation of 0.00074 atm, the decrease of $P_{H_2}$ to 0.00056 reduced the available energy from 54.78 to 51.87 kJ/reaction or from 13.69 to 12.97 kJ/mol H$_2$, equivalent to the reduction of 0.72 kJ/mol H$_2$, approx. 5.29% of the available energy. When $P_{H_2}$ was one-tenth of the highest value (0.000074 atm), the available energy reduction was 43.64%. A 100% energy reduction occurs at $P_{H_2}$ 3.78 $\times$ 10$^{-6}$ atm, a very low value of hydrogen partial pressure. Based on Reference 20 simulation, $P_{H_2}$ of 0.00074 atm was equivalent to H$_2$ concentration approx. of 4.81 $\times$ 10$^{-6}$ mol/L, and $K_s$ for hydrogenotrophic methanogens was 5.84 $\times$ 10$^{-6}$ mol/L. In the viewpoint of process rate, for hydrogen utilization, the decrease of hydrogen concentration of approx. 25% decreased the energy flux of approx. 15%.

To support the syntrophic cooperation, $P_{H_2}$ should be maintained at sufficiently low value, ranging from 3.78 $\times$ 10$^{-6}$ to 0.021 atm (area within the green-dashed rectangle in Figure 3, log $P_{H_2}$ ranged from $-1.68$ to $-5.42$), when the methanogenesis and one of the acetogenesis reaction were favorable. Outside the green-dashed rectangle, syntrophic cooperation among butyrate, propionate, and hydrogen utilizations could not occur. At a $P_{H_2}$ higher than 0.021 atm, it could lead to a positive $\Delta G^1_T$ for all the acetogenesis reactions (log $P_{H_2}$ $> -1.68$; $\Delta G^2_T$ for butyrate utilization $>0$ kJ/reaction, for propionate utilization1 $>28.21$ kJ/reaction, and for propionate utilization2 $>4.13$ kJ/reaction); none of those reactions was energy yielding, meaning that no substrate for hydrogen-consuming methanogens was produced. In contrast, at a $P_{H_2}$ lower than 3.78 $\times$ 10$^{-6}$ atm, it could lead to a positive $\Delta G^2_T$ for hydrogen utilization (log $P_{H_2}$ $<-5.42$; $\Delta G^2_T$ $>0$ kJ/reaction), then the methanogenesis was not favorable.

Syntrophic cooperation among butyrate, hydrogen, and both pathways of propionate utilizations occur in the area within the yellow-dotted rectangle (Figure 3), where the methanogenesis and all the acetogenesis were favorable. Log $P_{H_2}$ ranged from $-3.25$ to $-5.42$. $\Delta G^1_T$ for butyrate utilization was $-18.81$ to $-44.75$ kJ/reaction; for propionate utilization1 was 0 to $-38.91$ kJ/reaction; for propionate utilization2 was $-5.27$ to $-18.24$ kJ/reaction; and for hydrogen utilization was 0 to $-51.88$ kJ/reaction. The range of log $P_{H_2}$ obtained in this study was different but still within the same order of magnitude compared to the value reported by Reference 3 (approx. $-4.$ to $-5.7$). The different value might be due to the different environmental conditions affected the actual Gibbs free energy change of reaction.

Overall, syntrophic cooperation among butyrate, propionate, and hydrogen utilization could run well during the anaerobic process of in vitro rumen microbiota (the area within the blue solid box in Figure 3) with propionate utilization following the alternative pathway (propionate utilization2) that yielded lower hydrogen. Since butyrate and propionate utilizations were thermodynamically favorable in the system, we suggested to include these two reactions in kinetic modeling.

### 3.2 Stoichiometric equation of metabolism reactions

As defined in the previous section, the catabolism reaction of glucose utilization following pathways in Equations (11)–(13) was called as catabolism1, catabolism2, and catabolism3, respectively, whereas the anabolism reaction followed Equation (20). For hydrogen utilization, the catabolism reaction followed Equation (17), and the anabolism reaction followed Equation (21). Table 2 presents the coupling of catabolism and anabolism reaction equations for glucose and hydrogen utilizations.
### TABLE 2 Catabolism and anabolism reaction equations

| Process             | Reaction                        | Stoichiometric equation |
|---------------------|---------------------------------|-------------------------|
| Glucose utilization | Catabolism 1                    | C₆H₁₂O₆ + 2H₂O → 2CH₃COOH + 2CO₂ + 4H₂ |
|                     | Catabolism 2                    | C₆H₁₂O₆ → 0.67CH₃COOH + 1.33CH₂CH₂COOH + 0.67CO₂ + 0.67H₂O |
|                     | Catabolism 3                    | C₆H₁₂O₆ → CH₃CH₂CH₂COOH + 2CO₂ + 2H₂ |
|                     | Anabolism                       | 0.175C₆H₁₂O₆ + 0.2NH₄⁺ → 0.05HCO₃⁻ + 0.25H⁺ + CH₁₈O₅.5N₀.₂ + 0.4H₂O |
| Hydrogen utilization| Catabolism                      | 4H₂ + CO₂ → CH₄ + 2H₂O |
|                     | Anabolism                       | 2.1H₂ + CO₂ + 0.2NH₄⁺ → CH₁₈O₅.5N₀.₂ + 1.5H₂O + 0.2H⁺ |

Note: Catabolism reaction equations were modified from Reference 20, whereas anabolism reaction equations were arranged based on Reference 4.

### TABLE 3 The calculation of \( \lambda_{\text{Cat}} \)

| Time   | Reaction   | \( \Delta G_{\text{f}, \text{kJ/reaction}} \) | \( \Delta G_{\text{Dis}, \text{kJ/C-mol}} \) | \( \Delta G_{\text{An}} + \Delta G_{\text{Dis}, \text{kJ/C-mol}} \) | \( \lambda_{\text{Cat}} \) |
|--------|------------|---------------------------------------------|---------------------------------------------|-------------------------------------------------|-----------------------------|
| 3 h    | Catabolism1| -287.29                                     |                                             |                                                 | 0.8331                      |
|        | Catabolism2| -290.35                                     |                                             |                                                 | 0.8243                      |
|        | Catabolism3| -269.33                                     |                                             |                                                 | 0.8886                      |
|        | Anabolism  | -21.76                                       | 261.1                                       | 239.34                                          |                             |
| 6 h    | Catabolism1| -287.62                                     |                                             |                                                 | 0.8326                      |
|        | Catabolism2| -288.13                                     |                                             |                                                 | 0.8311                      |
|        | Catabolism3| -268.87                                     |                                             |                                                 | 0.8907                      |
|        | Anabolism  | -21.63                                       | 261.1                                       | 239.47                                          |                             |
| 12 h   | Catabolism1| -286.75                                     |                                             |                                                 | 0.8363                      |
|        | Catabolism2| -285.14                                     |                                             |                                                 | 0.8410                      |
|        | Catabolism3| -267.37                                     |                                             |                                                 | 0.8969                      |
|        | Anabolism  | -21.30                                       | 261.1                                       | 239.80                                          |                             |
| 24 h   | Catabolism1| -285.06                                     |                                             |                                                 | 0.8430                      |
|        | Catabolism2| -281.72                                     |                                             |                                                 | 0.8530                      |
|        | Catabolism3| -265.10                                     |                                             |                                                 | 0.9064                      |
|        | Anabolism  | -20.80                                       | 261.1                                       | 240.30                                          |                             |

### TABLE 4 \( Y_{X/S} \) for each pathway

| Time   | Metabolism1 | Metabolism2 | Metabolism3 |
|--------|-------------|-------------|-------------|
| 3 h    | 0.9920      | 1.0007      | 0.9402      |
| 6 h    | 0.9925      | 0.9939      | 0.9384      |
| 12 h   | 0.9889      | 0.9843      | 0.9329      |
| 24 h   | 0.9824      | 0.9728      | 0.9247      |

### 3.2.1 Stoichiometric coefficients of glucose utilization

On the basis of the three pathways of the catabolism and anabolism reaction equations for glucose utilization (Table 2), \( \lambda_{\text{Cat}} \) and \( Y_{X/S} \) were calculated using Equations (7) and (9), respectively. The calculation result is presented in Table 3 (\( \lambda_{\text{Cat}} \)) and Table 4 (\( Y_{X/S} \)).

The biomass yield of each pathway was not significantly different during the process (\( Y_{X/S} \) changed less than 3% for all the pathways), so in kinetic modeling \( Y_{X/S} \) could be assumed to be constant. Note that the value of biomass
yield could be higher than 1 because it was stated as C-mol biomass/mol substrate, and also because beside the carbon source, the nitrogen source was also needed for biomass formation. From the mean \( \lambda_{\text{Cat}} \) value for each pathway, the metabolism reaction equation for pathway1, pathway2, and pathway3 was written in Equations (22)–(24), respectively.

\[
1.01C_6H_{12}O_6 + 0.2NH_4^+ + 1.27H_2O \rightarrow CH_{1.8}O_{0.5}N_{0.2} + 1.67CH_3COOH + 1.67CO_2
+ 3.34H_2 + 0.05HCO_3^- + 0.25H^+ \quad (22)
\]

\[
1.01C_6H_{12}O_6 + 0.2NH_4^+ \rightarrow CH_{1.8}O_{0.5}N_{0.2} + 0.56CH_3COOH + 1.12CH_3CH_2COOH
+ 0.56CO_2 + 0.05HCO_3^- + 0.25H^+ + 0.96H_2O \quad (23)
\]

\[
1.07C_6H_{12}O_6 + 0.2NH_4^+ \rightarrow CH_{1.8}O_{0.5}N_{0.2} + 0.90CH_3CH_2CH_2COOH + 1.79CO_2
+ 1.79H_2 + 0.05HCO_3^- + 0.25H^+ + 0.4H_2O \quad (24)
\]

The total \( Y_{X/S} \) for glucose utilization depends on the values of \( \lambda_1, \lambda_2, \) and \( \lambda_3 \). Based on \( \lambda_1, \lambda_2, \) and \( \lambda_3 \) obtained from this study (0.41, 0.31 and 0.28, respectively), the total metabolism reaction equation of glucose utilization was written as follow:

\[
1.03C_6H_{12}O_6 + 0.2NH_4^+ + 0.13H_2O \rightarrow CH_{1.8}O_{0.5}N_{0.2} + 0.88CH_3COOH + 0.35CH_3CH_2COOH
+ 0.24CH_3CH_2CH_2COOH + 1.35CO_2 + 1.88H_2 + 0.05HCO_3^- + 0.25H^+ \quad (25)
\]

The values of \( \lambda_1, \lambda_2, \) and \( \lambda_3 \) obtained from this thermodynamic approach were slightly different from the value of Reference 20 simulation, which was 0.46, 0.32, and 0.22, respectively. By using \( \lambda_1, \lambda_2, \) and \( \lambda_3 \) from Reference 20, the total metabolism reaction equation was obtained as follows:

\[
1.02C_6H_{12}O_6 + 0.2NH_4^+ + 0.2H_2O \rightarrow CH_{1.8}O_{0.5}N_{0.2} + 0.96CH_3COOH + 0.36CH_3CH_2COOH
+ 0.19CH_3CH_2CH_2COOH + 1.34CO_2 + 1.94H_2 + 0.05HCO_3^- + 0.25H^+ \quad (26)
\]

The yield coefficients of substrates, products, and microbes were calculated based on the metabolism reaction equation. Notations \( Y_{IN/\text{su}}, Y_{X_{\text{su}}/\text{su}}, Y_{ac/\text{su}}, Y_{pro/\text{su}}, Y_{bu/\text{su}}, Y_{IC/\text{su}}, \) and \( Y_{H_{\text{su}}/\text{su}} \) were respectively attributed to the yield coefficients for inorganic nitrogen, biomass cell, acetate, propionate, butyrate, inorganic carbon, and hydrogen per mol glucose consumed. The yield coefficients obtained based on \( \lambda_1, \lambda_2, \) and \( \lambda_3 \) from the thermodynamic approach were 0.1947, 0.9734, 0.8555, 0.3423, 0.2308, 1.3657, and 1.8303 mol/mol respectively. Based on \( \lambda_1, \lambda_2, \) and \( \lambda_3 \) from Reference 20 simulation, the yield coefficients were 0.1953, 0.9765, 0.9372, 0.3529, 0.1840, 1.3541, and 1.8897 mol/mol, respectively. \( Y_{X/S} \) values obtained from this calculation (0.9734 C-mol biomass/mol glucose when \( \lambda_1, \lambda_2, \) and \( \lambda_3 \) were estimated thermodynamically and 0.9765 C-mol biomass/mol glucose when using \( \lambda_1, \lambda_2, \) and \( \lambda_3 \) from Reference 20) was not significantly different from the original value obtained by Reference 20 (0.950 C-mol biomass/mol glucose). However, verification was still required (see the next subsection) since the value of \( \lambda_1, \lambda_2, \) and \( \lambda_3 \) were different. We could compare the value of the yield coefficients above based on the two thermodynamic approaches. Significant differences were observed for yield coefficient of acetate, butyrate, and hydrogen, which were: by estimating \( \lambda_1, \lambda_2, \) and \( \lambda_3 \) thermodynamically the yield coefficients for acetate and hydrogen were lower and for butyrate was higher than the value when using \( \lambda_1, \lambda_2, \) and \( \lambda_3 \) from Reference 20. By using thermodynamically estimated \( \lambda_1, \lambda_2, \) and \( \lambda_3 \), it was found that the propionate yield coefficient is lower, albeit slightly. These different values might influence the calculation result of component concentrations in the reactor.

### 3.2.2 Stoichiometric coefficients of hydrogen utilization

On the basis of catabolism and anabolism reaction equations for hydrogen utilization (Table 2), \( \lambda_{\text{Cat}} \) and \( Y_{X/S} \) were calculated using Equations (7) and (9), respectively (Table 5).

For hydrogen utilization, \( Y_{X/S} \) (0.0118–0.0134 C-mol biomass/mol \( H_2 \)) mean value = 0.0125 C-mol biomass/mol \( H_2 \) was not significantly different during the process, so in kinetic modeling, \( Y_{X/S} \) could be assumed to be constant. From
TABLE 5 $\lambda_{\text{Cat}}$ and $Y_{X/S}$ for hydrogen utilization

| Time  | Reaction   | $\Delta G^1_T$ | $\Delta G_{\text{Dis}}$ of carbon source | $\Delta G_{\text{An}} + \Delta G_{\text{Dis}}$ | $\lambda_{\text{Cat}}$ | $Y_{X/S}$ |
|-------|------------|----------------|------------------------------------------|-----------------------------------------------|------------------------|---------|
| 3 h   | Catabolism | $-55.15$       |                                          |                                               | 18.0916               | 0.0134  |
|       | Anabolism  |                |                                          |                                               |                        |         |
| 6 h   | Catabolism | $-52.37$       |                                          |                                               | 19.0766               | 0.0128  |
|       | Anabolism  |                |                                          |                                               |                        |         |
| 12 h  | Catabolism | $-50.17$       |                                          |                                               | 19.9344               | 0.0122  |
|       | Anabolism  |                |                                          |                                               |                        |         |
| 24 h  | Catabolism | $-48.43$       |                                          |                                               | 20.6707               | 0.0118  |
|       | Anabolism  |                |                                          |                                               |                        |         |

the mean $\lambda_{\text{Cat}}$, the stoichiometric metabolism reaction equation was written as follows:

$$79.87H_2 + 20.44CO_2 + 0.2NH_4^+ \rightarrow CH_1.8O_0.5N_0.2 + 19.44CH_4 + 40.39H_2O + 0.2H^+$$  (27)

On the basis of the metabolism reaction equation, the value of $Y_{IC/H_2}$, $Y_{IN/H_2}$, $Y_{XH_2/H_2}$, and $Y_{CH_4/H_2}$ were calculated to be 0.2559, 0.0025, 0.0125, and 0.2434 mol/mol, respectively. $Y_{X/S}$ obtained in this study (0.0125 C-mol biomass/mol $H_2$) was higher than $Y_{X/S}$ obtained from kinetic modeling by Reference 20 (0.008 C-mol biomass/mol $H_2$), but lower than the value obtained using the Gibbs free energy dissipation method (0.0160 C-mol biomass/mol $H_2$) and using the McCarty method (0.02 C-mol biomass/mol $H_2$). Verification with experimental data was required to clarify the calculation result (see the next subsection).

### 3.2.3 Implementing the yield coefficients in kinetic model

To verify the calculated result, we implemented the yield coefficients of glucose and hydrogen utilization obtained from the thermodynamic approach into the in vitro rumen microbiota mechanistic model (rumen model) built by Muñoz-Tamayo et al. We studied two approaches to be applied in the model. The first approach was by including only the dissipation method to estimate the stoichiometric coefficient of each pathway and leaving $\lambda_1$ and $\lambda_2$ values as the values in the original model, which resulted in the metabolism reaction in Equation (26). For the second approach, besides the estimation of stoichiometric coefficients of each pathway, we also included the estimation of $\lambda_1$, $\lambda_2$, and $\lambda_3$ based on the thermodynamic estimation of kinetic parameters ($k_{m_1}$, $k_{m_2}$, and $k_{m_3}$), which resulted in metabolism reaction in Equation (25). Here, we could directly compare the value of butyrate, propionate, and acetate concentrations and also the amounts of gas products (CO$_2$, H$_2$, and CH$_4$) obtained to values of Reference 20 simulation and experimental data of Reference 25, as presented in Figure 4.

By applying the first approach into the rumen model (rumen model with thermodynamic1), the profile of butyrate, propionate, and acetate concentrations very much coincide with these profiles from the original rumen model of Reference 20. The same conditions occur for the profile of CO$_2$ and CH$_4$. The slight shift was observed for H$_2$ that was lower than the value of the original rumen model. It might be attributed to the lower hydrogen yield coefficient of glucose utilization due to the higher biomass yield estimated (compared to biomass yield obtained by Reference 20), since the other factors such as the higher biomass yield of hydrogen utilization did not lead to the lower H$_2$ in the gas phase. Since the yield coefficients estimation in this study were based on the simulation data from the original model, the good fitting of the rumen model with thermodynamic1 to the rumen model showed that the dissipation method estimated the yield coefficients accurately.

By using the second approach to be implemented in the rumen kinetic model (rumen model with thermodynamic2), some shifts were observed, especially for acetate, butyrate and hydrogen. As discussed earlier, the utilization of $\lambda_1$, $\lambda_2$, and $\lambda_3$ from the thermodynamic approach led to the lower acetate and hydrogen yield coefficients and higher butyrate yield coefficient. This was observed in Figure 4 that by using the second approach, acetate concentration and hydrogen in gas phase were lower than those from the rumen model and rumen model with thermodynamic1, while butyrate...
FIGURE 4 Applying the biomass yield from the thermodynamic approach into the rumen model built by Reference 20 and comparing the result to the original experimental data from Reference 25. Rumen model with thermodynamic1: including stoichiometric coefficients estimation of each pathway using dissipation method into rumen model; rumen model with thermodynamic2: including stoichiometric coefficients and $\lambda_1$, $\lambda_2$, and $\lambda_3$ estimation into rumen model was higher (Figure 4). The slightly lower values were also observed for propionate concentration and CH$_4$ in the gas phase. The lower propionate concentration might be attributed to a slightly lower propionate yield coefficient due to the utilization of $\lambda_1$, $\lambda_2$, and $\lambda_3$ values estimated based on thermodynamic approach. The lower CH$_4$ in gas phase might be caused by the lower hydrogen amount that consequently lead to the lower CH$_4$ formed. This difference does not affect the amount of CO$_2$ significantly since the order of magnitude of mol CO$_2$ ($10^{-3}$) was one higher than mol CH$_4$ ($10^{-4}$) and three higher than mol H$_2$ ($10^{-6}$). A significant shift in the amount of H$_2$ compared to the original model and rumen model with thermodynamic1 did not lead to a significant shift of CH$_4$ since the utilization of approx. 4 mol hydrogen only yielded 1 mol CH$_4$ (Equation (27)); moreover, CH$_4$ was two orders of magnitude higher than H$_2$.

The differences of component profile based on the second approach compared to the original rumen model and rumen model with thermodynamic1 might be as a result of the unrealistic assumption, in which the glucose utilization of each pathway was carried out by one virtual microorganism that was able to run all the pathways. The estimation also assumed the same $K_s$ values for all the pathways of glucose utilization. For reactions that are very favorable such as glucose, the substrate utilization rate is more limited by kinetics than thermodynamics.\textsuperscript{15} As a consequence of the assumption taken, there were no differences in biomass concentration running each pathway, whereas in the actual anaerobic system those pathways were carried out by different microorganisms. However, differentiating these microbes is not always possible and less applicable. Considering that the value shift of component concentrations (or mol component in gas phase) were not too large, we recommended to use $\lambda_1$, $\lambda_2$, and $\lambda_3$ obtained from the thermodynamic approach as the initial predictive values in kinetic modeling to facilitate the iteration of the model to obtain the desired values.

The first approach was more suitable to be applied in kinetic modeling since it resulted in a good fitting with the original rumen model and experimental data. By previously determining the yield coefficients of each pathway, the number of parameters that need to be predicted during the kinetic modeling can be reduced. In the case of glucose utilizations, without thermodynamic approach, all of the yield coefficients (at least 7 yield coefficients, see Equations (25) and (26)) must be predicted. By defining the catabolism and anabolism reaction equations as previously conducted by Reference 20, the coefficients (correlating with stoichiometric equations) to be predicted are significantly reduced. The parameters that
still must be predicted are $Y_{X/S}$, $\lambda_1$, and $\lambda_2$. Using the dissipation method which was conducted in this study, only $\lambda_1$ and $\lambda_2$ must be predicted. $\lambda_1$, $\lambda_2$, and $\lambda_3$ linked the three pathways of the glucose metabolism reaction equation, and $\lambda_3$ was automatically calculated based on the value of $\lambda_1$ and $\lambda_2$.

For hydrogen utilization, in a fully empirical kinetic modeling, at least 4 yield coefficients must be predicted. In the case of kinetic modeling by Reference 20, it only needs to predict the value of biomass yield, $Y_{X/S}$. The application of the dissipation method in this study provide all the yield coefficients needed in the kinetic modeling.

Compared to the experimental data from Reference 25 there were slight shifts of CO$_2$ and CH$_4$ observed in the original in vitro model, which were over prediction of CO$_2$ and under prediction of CH$_4$. The similar deviation was also observed in the rumen model with thermodynamic1, that might relate to the experimental device or the need to refine the model parameters; therefore, it needs a new set of data to identify. The larger deviations were resulted by the rumen model with thermodynamic2 that might be attributed to the unrealistic assumption taken for $\lambda_1$, $\lambda_2$, and $\lambda_3$ calculation, as previously explained. Since the data used for yield coefficient calculation in this study were based on simulation result and that the original rumen model might need a new set of data to identify some over and under prediction of components, future improvements can be done by calculating the yield coefficients based on a full experimental data, and then including the calculated results in kinetic modeling.

4 CONCLUSIONS
Glucose utilization always occurs in the system and the syntrophic cooperation among butyrate, propionate, and hydrogen utilizations run well with propionate utilization follows the alternative pathway yielding lower hydrogen. For glucose utilization, the biomass yield ($Y_{X/S}$) obtained in this study is close to $Y_{X/S}$ from the original in vitro rumen microbiota kinetic modeling, and for hydrogen utilization, $Y_{X/S}$ is in the range of previously reported values. Applying the Gibbs free energy dissipation method to estimate the yield coefficients of each pathway can reduce most of the yield coefficients that must be predicted in kinetic modeling. For hydrogen utilization, in which the reaction only followed one pathway, this approach provides all the stoichiometric coefficients, whereas for glucose utilization reaction that follows three pathways, it leaves $\lambda_1$ and $\lambda_2$ to be predicted in kinetic modeling, much less than 7 yield coefficients that are previously required. Implementing the yield coefficients of each pathway based on dissipation method into previous in vitro rumen microbiota model results in good fitting with the model and also with the experimental data. The values of $\lambda_1$, $\lambda_2$, and $\lambda_3$ obtained from the thermodynamic calculation are suggested to be used as the initial predictive values in kinetic modeling to ease obtaining the desired parameters. Integrating the thermodynamic approach into kinetic modeling based on fully experimental data is expected to improve this research.

ACKNOWLEDGMENTS
This study was supported by “Program Pendukung Beasiswa Saintek” and the World Class Professor (grant numbers T/82/D2.3/KK.04.05/2019) of Ministry of Research, Technology, and Higher Education, Republic of Indonesia. The authors would like to thank Rafael Muñoz-Tamayo (Institut National de la Recherche Agronomique) for sharing the code of the in vitro mechanistic model.

PEER REVIEW INFORMATION
Engineering Reports thanks the anonymous reviewers for their contribution to the peer review of this work.

DATA AVAILABILITY STATEMENT
Data sharing is not applicable to this article as no new data were created or analyzed in this study. Further material is available in the supplementary information of this article.

CONFLICT OF INTEREST
The authors declare no potential conflict of interest.

AUTHORS CONTRIBUTIONS
Arini Wresta contributed to the conceptualization, data analysis and writing of the paper; Rani Widyarani contributed to revising the paper critically for important intellectual content; Ramaraj Boopathy contributed to the comprehensive evaluation to the scientific value of the paper; Tjandra Setiadi contributed to the conceptualization and supervision.
REFERENCES

1. Abbasi T, Tauseef SM, Dan Abbasi SA. Anaerobic digestion for global warming control and energy generation—an overview. *Renew Sustain Energy Rev*. 2012;16:3228-3242. https://doi.org/10.1016/j.rser.2012.02.046.

2. Christy PM, Gopinath LR, Divya D. Microbial dynamics during anaerobic digestion of cow dung. *Int J Plant Animal Environ Sci*. 2014;4:86-94.

3. Batstone DJ, Keller J, Angelidaki I, et al. Anaerobic digestion model no. 1 (ADM1). *IWA Task Group for Mathematical Modelling of Anaerobic Wastewater Processes*. London, UK: IWA Publishing; 2002.

4. Kleerebezem R, van Loosdrecht MCM. A generalized method for thermodynamic state analysis of environmental systems. *Crit Rev Environ Sci Technol*. 2010;40:1-4. https://doi.org/10.1080/10408410902744530.

5. Speece RE. *Anaerobic Biotechnology for Industrial Wastewaters*. Nashville, TN: Archie Press; 1996;40–51.

6. Yuan XZ, Shi XS, Yuan CZ, et al. Modelling anaerobic digestion of blue algae: stoichiometric coefficients of amino acids acidogenesis and thermodynamic analysis. *Water Res*. 2014;49:113-123. https://doi.org/10.1016/j.watres.2013.11.015.

7. Brock AL, Kästner M, Trapp S. Microbial growth yield estimates from thermodynamics and its importance for degradation of pesticides and formation of biogenic non-extractable residues. *SAR QSAR Environ Res*. 2017;28(8):629-650. https://doi.org/10.1080/10643389.2017.1365762.

8. von Stockar U. Biothermodynamics: bridging thermodynamics with biochemical engineering. *Acta Sci Pharmaceut Sci*. 2019;3(7):121-129. https://doi.org/10.31080/ASPS.2019.03.032.

9. Plavostatis SG, Giraldo-Gomez E. Kinetic of anaerobic treatment: a critical review. *Crit Rev Environ Control*. 1991;21:411-490. https://doi.org/10.1080/10643389109388424.

10. McCarty PL. Energies of organic matter degradation. In: Mitchell R, ed. *Water Pollution Microbiology*. New York: Wiley Interscience; 1972:91-118.

11. Xiao J, VanBriesen JM. Expanded thermodynamic model for microbial true yield prediction. *Biotechnol Bioeng*. 2005;93:110-121. https://doi.org/10.1002/bit.20700.

12. McCarty PL. Thermodynamic electron equivalents model for bacterial yield prediction: modifications and comparative evaluations. *Biotechnol Bioeng*. 2006;97:377-388.

13. Heijnen JJ, Van Loosdrecht MCM, Tijhuis L. A black box mathematical model to calculate auto- and heterotrophic biomass yields based on Gibbs energy dissipation. *Biotechnol Bioeng*. 1992;40:1139-1154. https://doi.org/10.1002/bit.260401003.

14. Rodríguez J, Kleerebezem R, Lema JM, van Loosdrecht MCM. Modeling product formation in anaerobic mixed culture fermentations. *Biotechnol Bioeng*. 2005;93(3):592-606. https://doi.org/10.1002/bit.20765.

15. González-Cabaleiro R, Lema JM, Rodríguez J. Metabolic energy-based Modelling explains product yielding in anaerobic mixed culture fermentations. *PloS One*. 2015;10(5):e0126739. https://doi.org/10.1371/journal.pone.0126739.

16. Junicke H, Feldman H, Van Loosdrecht, M.C.M., & Kleerebezem, R. (2015b). Impact of the hydrogen partial pressure on lactate degradation in a coculture of Desulfovibrio sp. G11 and Methanobrevibacter arboriphilus DH1. *Appl Microbiol Biotechnol*, 99, 3599–3608. https://doi.org/10.1007/s00253-014-6241-2.

17. Heijnen JJ, Kleerebezem R, Lema JM, Rodríguez J. Kinetic and thermodynamic control of butyrate conversion in non-defined methanogenic communities. *Appl Microbiol Biotechnol*. 2016;100:915-925. https://doi.org/10.1007/s00253-015-6971-9.

18. Rodríguez J, Lema JM, Kleerebezem R. Energy-based models for environmental biotechnology. *Trends Biotechnol*. 2008;26(7):366-374. https://doi.org/10.1016/j.tibtech.2008.04.003.

19. Muñoz-Tamayo R, Giger-Reverdin S, Sauvant D. Mechanistic modelling of in vitro fermentation and methane production by rumen microbiota. *Anim Feed Sci Technol*. 2016;220:21-21. https://doi.org/10.1016/j.anifeedsci.2016.07.005.

20. Rodríguez J, Lema JM, van Loosdrecht MCM, Kleerebezem R. Variable stoichiometry with thermodynamic control in ADM1. *Water Sci Technol*. 2006;54(4):101-110. https://doi.org/10.2166/wst.2006.531.

21. Heijnen JJ, Kleerebezem R. *Bioenergetics of Microbial Growth*. Encyclopedia of Industrial Biotechnology: Bioprocess, Bioseparation, and Cell Technology: John Wiley and Sons, Inc.; 2010.

22. González-Cabaleiro R, Ofi¸teru ID, Lema JM, Rodríguez J. Microbial catabolic activities are naturally selected by metabolic energy harvest rate. *ISME J*. 2015;9:2630-2641. https://doi.org/10.1038/ismej.2015.69.

23. Delattre H, Chen J, Wade MJ, Soyer OS. Thermodynamic modelling of synthetic communities predicts minimum free energi requirements for sulfate reduction and methanogenesis. *J R Soc Interface*. 2020;17:20200053. https://doi.org/10.1098/rsif.2020.0053.

24. Serment A, Giger-Reverdin S, Schmidley P, Dhumez O, Broudiscou LP, Sauvant D. In vitro fermentation of total mixed diets differing in concentrate proportion: relative effects of inocula and substrates. *J Sci Food Agric*. 2016;96:160-168. https://doi.org/10.1002/jsfa.7076.

25. von Stockar U, Vojinovi ´cV, Maskow T, Liu J. Can microbial growth yield be estimated using simple thermodynamic analogies to technical processes? *Chem Eng Process*. 2008;47:980-990. https://doi.org/10.1016/j.cep.2007.02.016.

26. Dolfing J. Syntrophic propionate oxidation via butyrate: a novel window of opportunity under methanogenic conditions. *Appl Environ Microbiol*. 2013;79(4):4515-4516. https://doi.org/10.1128/AEM.00111-13.
SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section at the end of this article.

How to cite this article: Wresta A, Widyarani R, Boopathy R, Setiadi T. Thermodynamic approach to estimating reactions and stoichiometric coefficients of anaerobic glucose and hydrogen utilization. Engineering Reports. 2021;3:e12347. https://doi.org/10.1002/eng2.12347