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# An improved Kinetic Model for Acetone-Butanol-Ethanol Pathway of *Clostridium acetobutylicum* and Model-Based Perturbation Analysis

Ru-Dong Li[1,2,§] Yuan-Yuan Li[1,2,§] Ling-Yi Lu[1,2] Cong Ren[3] Yi-Xue Li[1,2] Lei Liu[1,2*]

1 Key Laboratory of Systems Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai, China
2 Shanghai Center for Bioinformatics Technology, Shanghai, China
3 Institutes of Plant Physiology and Ecology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai, China
§ These authors contributed equally
* Corresponding author

**Abstract** Kinetic models help to understand microbial metabolism and optimize biomass production. Up to date, only one kinetic model for *Clostridium acetobutylicum* acetone-butanol-ethanol (ABE) process has been reported. In the present work, we developed an improved kinetic ABE model, whose simulation results were more consistent with experimental observations, especially in terms of reflecting butyryl-phosphate and butyrate kinetics. Based on our model, we find that butyrate kinase has positive influence on butanol production while CoA transferase has negative effect, indicating that the path through butyrate kinase is preferred by the bacteria for converting butyrate to butanol. Analyses of the predictions may provide insight in the regulatory mechanisms of ABE process.

**Keywords** Kinetic model; ABE process; Butyryl-phosphate; Butyrate kinase; Butanol

## 1 Introduction

System modeling for microbial metabolism can reveal relevant factors about high yield of target products. Based on such models, we can develop operation strategies or optimize cultivation processes [1-4]. *C. acetobutylicum* is an extensively studied organism used for industrial production of acetone and butanol through acetone-butanol-ethanol (ABE) pathway (additional file 8) [5, 6], which comprises two branches as acidogenesis and solventogenesis. During acidogenesis, cells grow exponentially and vigorously produce acetate and butyrate, while solvents (butanol, acetone and ethanol) are not obviously generated. When shifting to solventogenesis, cells arrest their growth at stationary phase, solvents are produced and acids are reassimilated [5]. Multiple models based on metabolic flux analysis (MFA) and flux-balance analysis (FBA) have been built to simulate ABE pathway so far [5, 7-10]. Although stoichiometric models can simulate overall flux distributions with limited data by using physicochemical constraints, they cannot properly reflect the

dynamics. In contrast, kinetics models are more efficient in modeling dynamics. By perturbation tests, system states deviating from normal can be simulated and it is possible to find the reactions with potential impacts on target product production. To date, many experiments have explored kinetic features in ABE process and a kinetic model was recently developed by H. Shinto *et al.* [11]. However, as most current models did, this model did not integrate metabolic regulatory effects of transcriptional control and other complex factors [12-14]. Moreover, it did not include some key metabolites, e.g. butyryl-phoshate (BuP), which was proved to be important in solventogensis [13-16].

In this work, we developed an improved kinetic model by integrating experimental information and knowledge not included in Shinto's model. Our model incorporated BuP, described regulatory effects of transcriptional control and other complex factors with a time division pattern, and quantified them with enzyme activity coefficient (EAC). The simulation results were much more consistent with published experiments than those of Shinto's model. Furthermore, the results of perturbation tests may provide insights in the regulatory mechanisms of ABE process.

# 2 Results

All results were based on our new model (Equation (1), Method section). We did dynamical simulation with respect to the condition in Zhao *et al*'s work and compared the results with their observations. We then did perturbation analyses to detect which reactions had large impacts on butanol production.

## 2.1 Dynamical simulation

The initial value was set according to the condition in Zhao *et al.*'s experiment (2005) [13] and simulated kinetic profiles of metabolites were shown in Figure 1A and 1C. These results were quite consistent with experimental observations (Figure 1B and 1D). The metric units in Figure 2A and 2B are different (Figure 1A: mM; Figure 1B: pmol/gDW), since the measurement of BuP in Zhao *et al*'s experiment accepts the unit of pmol/gDW. It's impossible to know the exact conversion between mM and pmol/gDW because there is no such relation in SI metric unit system. But the quantity scale can be approximated given the size of ordinary *C. acetobutylicum* cell, and this scale is consistent with that in our simulation.

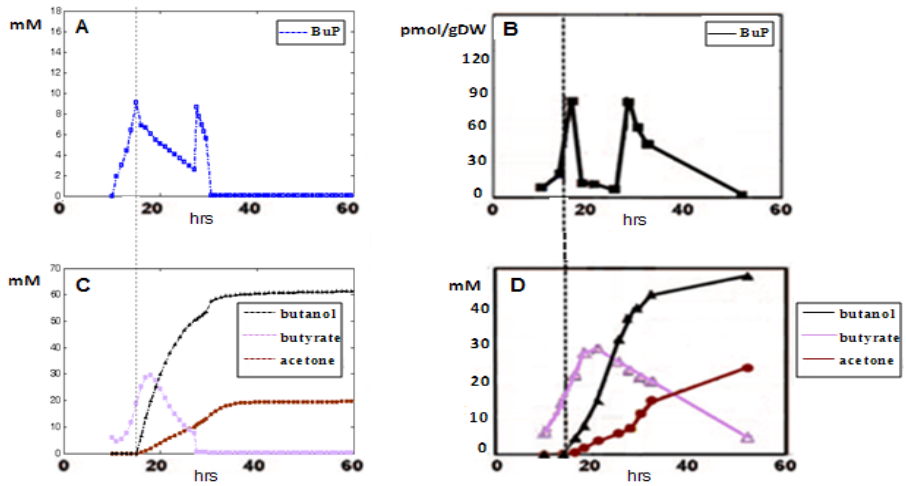


**Figure 1. Comparison of simulation results with Zhao *et al.'s* experimental observations.** A is simulated BuP kinetics; B is experimental observation of BuP [13]; C is the simulated kinetics of butanol, butyrate and acetone; D is experimental observations of butanol, butyrate and acetone [13]. The figure shows that the simulated curves are consistent with experimental ones both in quantity scale and shape.

In our results, the first peak of BuP was shown to coincide with the onset of solvent production (Figure 1A, 1C). This was a phenomenon reported in experimental studies and had biological implications [13-16]. Besides BuP, we also demonstrated that we had a more precise simulation on butyrate, a crucial product in cell growth and solvent production [5] (Figure 2). In Shinto's model, when substituting in Zhao *et al*'s condition, the quantity scale of butyrate curve didn't resemble precisely with Zhao *et al*'s experimental curve (Figure 2B). This further demonstrated that our model had more capability in approaching real biological events.

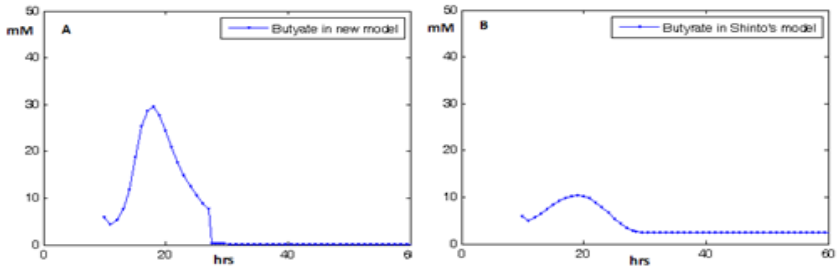


**Figure 2. Comparison of simualted butyrate kinetics with that of Shinto's model under Zhao *et al*'s condition.** A is simulated butyrate kinetics based on our model. B is simulated butyrate kinetics based on Shinto's model. As shown, A is more consistent with Zhao *et al*'s experimental observation.

## 2.2 Perturbation analysis

Butanol was most valuable among the solvents produced in ABE fermentation as it had advantageous properties (e.g. better value for the heat of combustion) [6]. Thus we did a series of perturbation analyses to assess which enzymes/reactions had large impacts on butanol production. We used *Rd* values to measure the impacts (see Methods section for *Rd*'s definition). We did perturbations with magnitude of ±5% on both single parameters and double parameter pairs. We traversed the entire parameter set and the result set of single, double parameter perturbations included

100 and 4900 entries respectively (additional files 1, 2; additional files 3-6). Among all results, there were several interesting ones that might provide insights in ABE process. It could be intuitively concluded that BK was important in solventogensis as it linked two crucial intermediate But and BuP. And based on our analyses, we indeed found that shifting BK's *Vmax* resulted in relatively large influences on butanol production (additional files 1-6). Actually, BK activity had positive correlation with butanol production and the butanol change caused by shifting BK's *Vmax* ranked the 5th in the profile of single parameter shifts (additional files 1 and 2). This indicated that BK, which coupled PTB to generate butyrate as well as catalyzing its reassimilation, was important in butanol production as compared with other enzymes such as AAD (the enzyme produced ethanol). Besides, BK activity's impact was greater than that of AK (additional files 1, 2), indicating butyrate reassimilation had more influence on butanol production than acetate reassimilation. Our computation also showed that CoAT, which also received butyrate as substrate, had negative correlation with butanol production as up-shifting its capacity (increasing *Vmax* or decreasing *Km*) decreased butanol production (additional files 1, 2). As BK and CoAT both accepted butyrate as substrate, up-shifting BK's capacity or down-shifting CoAT's capacity caused more butyrate received by BK and the reverse operations led to the contrary case, we concluded that if more butyrate was received by BK, butanol production increased; and if more butyrate was received by CoAT, the situation was on the contrary (Figure 3). Therefore we further deduced that BK had more efficiency than CoAT during solventogenesis and butyrate reassimilation relied more on BK rather than CoAT.

There were some places where our model's predictions differed from those of Shinto's. For example, our results showed that PTS had positive influence on butanol production, while by Shinto's model, PTS's influence was negative. Given the fact that PTS acted in nutrient uptake and many processes related to ABE pathway were subjected to nutrient induction, our prediction was more consistent with intuitive knowledge [17].

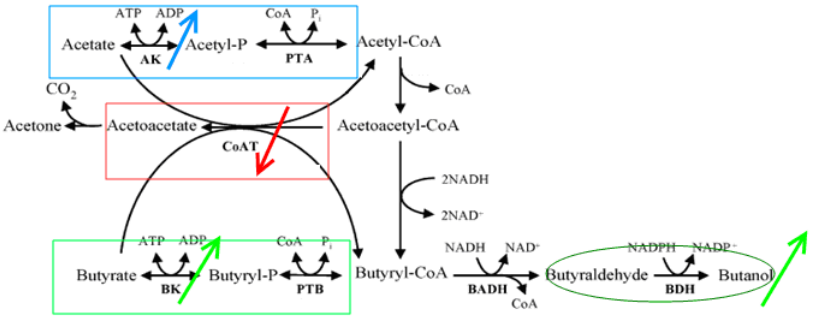


**Figure 3. Illustration of BK, CoAT and AK's influences on butanol production.** BK's influence is positive correlation with butanol production shown by the green arrow. CoAT's influence is negative correlation shown by the red arrow. AK's influence is positive correlation shown by the blue arrow, indicating smaller magnitude than BK.

# 3 Discussion

Rational system modeling can help understanding biological mechanisms. We can retrieve quantitative knowledge from dynamical simulation and use it in in-lab

experiments [2-4]. As many studies related to *C. acetobutylicum* ABE pathway have been reported, kinetic modeling becomes feasible and enables us to make computational predictions. Nevertheless, the previous model (Shinto *et al*, 2007) has several drawbacks as described in earlier context. To overcome these drawbacks, we have established a new model with improved properties and superior results.

## 3.1 Model improvements

First, BuP is key metabolite and we incorporate it into model to reflect relevant biological events that are specific to ABE kinetics [12-15]. The correspondence between BuP concentration climax and solventogenesis onset is not merely a natural consequence that BuP is the intermediate between BCoA and But. There are implications on the genetic level as stated in Zhao *et al*'s study [13-16]. Many important solventogenic genes, such as *adh*E1 (CAP0162), *adh*E2 (CAP0035 ), *ctf*A (CAP0163), *ctf*B (CAP0164), *adc* (CAP0165), *bdh*A (CAC3298), *bdh*B (CAC3299), etc. have expression profiles that strictly correlated with BuP kinetics [13]. Although the detailed mechanism of how BuP acts to regulate ABE process has not been very clear yet, its functional importance has been experimentally confirmed [13-16]. Our model accounts for this knowledge and is successful in representing the phenomenon. Second, as suggested by experimental studies [12, 18-20], we assume that enzymes are regulated by complex factors (e.g. transcription control) to exhibit different activities to fulfill conditional requirements of different periods. In kinetic models based on classical biochemical system theory (BST), enzyme activity levels are constant by default and they are only regulated by substrates/products, as in Shinto's model, the shut-downs of enzymes are due to glucose insufficiency only. But various evidences indicate shutting-down of acidogenic enzymes in solventogenic phase despite glucose sufficiency and inactivation of solventogenic enzymes at the beginning of acidogenesis. Thus it is suggested BST can not properly simulate *in vivo* conditions. Therefore, to maintain the appropriateness for *in vivo* consitions, we describe the regulatory effects of complex factors using a time division pattern, where time is divided into several periods according to enzyme activity variations, allowing enzyme activities to vary. As shown by our simulation, this pattern can better reflect the 2-phase mode of ABE process, in which acids are vigorously generated during the earlier phase and solvents are produced during the latter one. Our definition of the time division pattern is amount to extending BST's application to *in vivo* conditions. Third, we introduce "enzyme activity coefficient (EAC)" to quantify enzyme activity variations (see Method section for EAC's definition and calculation). To approximate enzyme activity curves, numerical interpolation (Lagrange, Legendre, etc.) should have been employed as to obtain fully continuous functions. But measurements in activity assays are usually not precise, and interpolation results in huge mistakes and distorts the original curve profile when errors are large. On the contrary, calculating the average as in EAC's definition leaves the error just as the original, and the curve at least will not be distorted when measurements are not precise. Moreover, our design of EAC is calculating a ratio, not the particular value at each time instance. This practice allows the error to be divided by a denominator, further lowering the error level and enhancing the reliability of EAC values.

## 3.2 Dynamical simulation

After estimating the newly introduced parameters (Method section), we compared our simulation with observations of a published experiment (Zhao *et al*, 2005), whose information are not utilized in parameter estimation. It turns out that our results are significantly consistent with the observations and superior over Shinto's model in reflecting the kinetics of BuP and But. This indicates that Shinto's model is well fitted for its own condition but may not be suited well for others. In contrast, our model can accommodate more conditions because of the improvements we have made.

Simulations of kinetic models can help develop in-lab strategies, thus facilitating the success of metabolic engineering. We have simulated thousands of perturbed states to detect and assess potential spots that largely influence butanol production. The magnitude of *in silico* perturbation should not be too large because real systems may exhibit alternative activations for other pathways when undergoing substantial fluctuations [21, 22]. It is fairly assumed that a perturbation of ±5% does not destroy the system's survival and the functional normality is not interrupted due to biological robustness [22-24]. In our computation, we find an interesting phenomenon that BK's activity exhibits positive influence on butanol production while CoAT has negative effects. And more convincingly, *Rd* decreases when increasing the *Vmax* values of BK and CoAT at the same time, which means CoAT's negative effect can balance BK's positive effect, confirming CoAT's counteraction to butanol production. Based on this discovery, we propose a possible scenario that BK plays the leading role in acid reassimilation. If more butyrate is received by BK as substrates, acid (butyrate) reassimilation efficiency is enhanceed and butanol production is benefited. And if more butyrate is received by CoAT, the situation is on the contrary. It may not seem economical for the bacteria to use the BK-PTB path to reassimilate butyrate since running through it consumes ATP. Therefore, the underlying mechanism is possibly that path BK-PTB generates ATP for bacteria growth during acidogenesis, while the bacteria doesn't need to grow in solventogenesis and ATP has surplus, and these surplus ATPs are utilized to proceed butyrate reassimilation. It's noteworthy that acids are severely poisonous to bacteria cells and it is a priority for the bacteria to convert acids to other forms. In addition, enhanced butanol production means more acids are converted. Therefore we can further deduce that the reason why the bacteria prefers path BK-PTB at the expense of ATP is because BK is efficient for responding to severe poison stress and the energetic basis for this process is the ATP surplus generated during acidogenesis.

## 3.3 Significance

Traditional kinetic models cannot accommodate complex metabolic regulatory effects (e.g. gene transcriptional control) and previous integrative bio-system models are mainly based on the FBA method, in which gene transcriptions are described by Boolean logic and the metabolic level is expressed by flux balance equations. Since FBA and Boolean logic cannot properly reflect system dynamics, we develop a new model to degenerate complex regulatory effects to a form that is compatible with kinetic models. In addition, our approach of representing complex regulatory effects with a time division pattern and EAC is extendable and can be generalized for

modeling other bio-processes. For instance, we can relate enzyme activities to gene transcriptional levels, building a formulism between them and including other factors such as stochasticity.

In the post-genomic era, massive information and experimental data have been accumulated. It is important to develop methods or tools that can make use of existed information/data and organize, manipulate and interpret them comprehensively [25, 26]. Our work attempts to serve that goal by integrating existed information from multiple aspects and describing them mathematically. Nevertheless, the usage of "net effects of regulatory factors" in our modeling doesn't seem to build direct links between genetic and metabolic levels. But if adequate information about regulatory factors is elucidated, better formulism can be built to link the two levels and further studies on the control of bacteria cellular systems can be conducted.

# 4 Methods

## 4.1 Incorporating butyryl-phosphate

BuP was key intermediate in conversions between But and BCoA. It was reported that BuP played a crucial role in solventogenesis, as the initial peak of its concentration marked the onset of solvent production [13]. Adding BuP means splitting the originally lumped reactions between But and BCoA (as in Shinto's model) so as to represent their intermediate BuP as a system component. Here we added two new reactions to denote the conversions from BuP to But and BCoA respectively. Hence, the butyrate formation/reassimilation branch was restructured and BuP appeared as another system component. Mathematically, we created rate equations for the new reactions and re-formulated the mass balance equations relating to But, BCoA and BuP. For details, see additional file 8.

## 4.2 Time division pattern

We assumed enzyme activity variations were net effects of transcriptional control and other complex factors. As experimental studies suggested enzyme activities varied with time [12, 18-20, 27], we developed a time division pattern to reflect the regulatory effects. We divided time into several intervals according to the enzymes' activity variation profiles [18, 19]. Here we only considered a subset of enzymes, which were either located on acid/solvent production reactions or directly associate to them. We adopted activity variations of the enzymes in consideration and regarded others' as constants. All enzyme activity profiles were collected from published experimental studies [18, 19] and the experiments were done under the identical culture conditions that our simulation based on [11, 13]. For details of constructing the time division pattern, see additional file 8.

## 4.3 Enzyme activity coefficient

We introduced EAC to quantify enzyme activity variations. EACs were formulated as time-dependent, piecewise linear functions. At each time instance, the EAC value was the ratio of the current enzyme activity to its maximum activity. Here we employed the divided intervals in the time division pattern (see the previous paragraph) as markers of time. And for computation simplicity, we approximated EAC with a set of $0^{th}$ splines with respect to these markers. In other word, the EAC value remained constant within a divided interval and it changed to another constant

when stepping into another interval. The constant was the ratio of the average activity level in this interval to the maximum activity. We calculated all EACs of the considered enzymes (additional file 8) and multiplied them to their corresponding rate equations to reflect activity variations. All data of enzyme activities were collected from literatures [18, 19]. For details of computing EAC, see additional file 8.

### 4.4 New model

The new model contained 21 rate equations and 17 differential equations, involving 50 kinetic parameters. The model was built by integrating ABE kinetic features identified so far. Except for those included in Shinto's model [11, 28-30], EACs were multiplied to rate equations. The model was expressed in the form of ordinary differential equation (ODE) system as in Equation (1):

$$\frac{d\mathbf{Y}}{dt} = \mathbf{A} \cdot \mathbf{E}(t) \cdot \tilde{\mathbf{R}}(\mathbf{Y}, \mathbf{P}) \tag{1}$$

where Y was the vector of metabolites' concentrations; A was the stoichiometric matrix of mass balance equations; E=diag{$EAC_1$,…,$EAC_{21}$} and EACs corresponding to enzymes with constant activities were set to 1; $\tilde{R}$ was the vector of rate equations without EACs; and P was the entire set of parameters. For details of the equations, symbols and abbreviations in the model, see additional file 8.

### 4.5 Parameter estimation

We applied Genetic Algorithm (GA) to *de novo* estimate unknown parameters introduced by new reactions (Section 4.1). We considered the experimental observations of 16 metabolites in Shinto's work to be valid, and assumed that the correct value assignment of the unknown parameters definitely reproduced these valid observations under Shinto's condition. Therefore the fitness function in optimization was formed by forcing the 16 metabolites' concentrations Y(1:16) to match Shinto's observations $Y_0$(1:16). We computed parameter values that minimized the fitness function and accepted them as numerical solutions. In addition, we didn't employ any qualitative or quantitative information of BuP or Zhao *et al.*'s experiment in this process. For parameter values, see additional file 7. And for details of parameter estimation, see additional file 8.

### 4.6 Perturbation analysis

We did perturbation analysis to assess enzymes/reactions' impacts on butanol production. By consecutively shifting the enzymes' *Vmax* and *Km* values and using the normal state as control, relative changes of *in silico* butanol production were computed. We defined the relative change in butanol production as *Rd* (a ratio expressed in Formula (2)):

$$Rd = \frac{\int_{t0}^{tf} y_p(t)dt - \int_{t0}^{tf} y_c(t)dt}{\int_{t0}^{tf} y_c(t)dt} \tag{2}$$

where $y_p$ was the instantaneous butanol concentration in perturbed state, and $y_c$ was that in normal state. For approximation, we discretized the integrals in Formula (2) with the trapezoid method. The results of perturbation analysis were in additional files 1-6, and for details of computation, see additional file 8.

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## Additional files

All additional files are at http://www.biosino.org/download/rdli/additional_files/

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