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A New Approach to Bioconversion Reaction Kinetic Parameter Identification

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The commonly used methods for bioconversion kinetic parameter identification are linear plotting and nonlinear regression. However, linear plotting methods generally require considerable experimentation, and nonlinear regression can lead to "local optimization" because the obtained parameters depend heavily on given initial values. In this article, a new and reliable nonlinear regression-based approach to bioconversion kinetic parameter estimation is reported. By obtaining preliminary values of kinetic parameters on a step-by-step basis, the number of estimated parameters in each step can be reduced to 3 or 4. These preliminary values can then be used as initial guesses for the final parameter estimation via nonlinear regression. Compared with the linear plotting method, the proposed approach can significantly reduce the number of experiments required for kinetic parameter estimation. The transketolase catalyzed synthesis of 1,3-dihydroxypentan-2-one from propionaldehyde and β-hydroxypyruvate is used as an experimental example to illustrate the approach. © 2008 American Institute of Chemical Engineers *AIChE J,* 54: 2155–2163, 2008
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Introduction

Bioconversion reaction kinetics, which characterize the dynamic relationship between substrate and product concentrations in bioconversion reactions, are essential for bioconversion process development. In addition, they are required to evaluate opportunities for process integration and operational optimization, as well as providing guidance for biocatalyst improvement. The kinetic models can be derived based on reaction mechanisms—the reactions understood to be occurring between an enzyme and its substrates—resulting in a set of differential equations. Although the determination of the correct mechanism for a bioconversion reaction is an important step in itself, in this article, we have assumed that the mechanism has previously been determined and we address the subsequent problem of kinetic parameter identification.

In general, bioconversion kinetic parameter identification methods fall into two categories—linear plotting and nonlinear regression. Linear plotting methods are based on algebraic expressions of simplified kinetic differential equations at initial rate conditions to give a series of straight line equations. To achieve this, initial rate data under a range of different starting substrate concentrations are required. The most commonly used linear plotting methods are Lineweaver-Burk[1] (also known as the double-reciprocal plot), Hanes,[2] Eadie-Hofstee,[3–5] the parametric plot (i.e., direct linear

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plot),[6] and the Dixon plot[7] (which is used in particular for determining enzyme inhibition constants).

A number of published reports[8–11] have reviewed and compared these linear plotting methods. It was found that linear plotting methods are (1) theoretically undesirable because they distort the error structure after linearization and (2) have large errors in initial rate determination. Furthermore, initial rates are normally obtained from progress curves, each of which can only generate a single initial rate datum, thus not making use of all the available experimental data. Therefore, using linear plots for kinetic parameter determination requires considerable experimentation. This is both time consuming and requires a significant quantity of often scarcely available materials.

Nonlinear regression provides an alternative method. This uses optimization algorithms to search kinetic parameters by regressing (e.g., using least squares) data in the form of progress curves (i.e., concentrations of substrate and product as a function of time), and is nowadays widely used.[12,13] The advantage of using such a method is that it is theoretically correct, programmable, and consequently faster. In addition, it requires fewer experiments than the linear plotting method.[14] However, there is a danger when applying nonlinear regression that, because the estimated parameter values depend heavily on the estimated initial values, local optimization may give unreliable results.[15,16] Biocatalytic processes are now increasingly used in the synthesis of complex molecules, using 2 or more substrates, via condensation or other synthetic reactions. In such cases, the task of bioconversion kinetic parameter estimation based on nonlinear regression needs to identify six or more parameters in a single differential equation.

In this work, we have developed a new approach to obtain bioconversion kinetic parameters which retains the advantages, and avoids the disadvantages, of linear and nonlinear methods. It also requires less experimentation than using a linear method. In this article, we will first give a brief summary of the general characteristics of bioconversion reaction kinetics, in particular substrate and product inhibition, followed by construction of the experimental framework. The transketolase mediated synthesis of 1,3-dihydroxypentan-2-one from propionaldehyde and β-hydroxypyruvate is then used as an example to illustrate the approach.

General Features of Enzyme Kinetics

Compared with chemical catalytic reactions, enzymatic reactions have three typical features. The first feature is the reaction selectivity. Most chemical catalytic reactions are not selective (i.e., they will catalyze similar reactions involving many different kinds of substrates). Although a few enzymes have been characterized that are not very selective, the vast majority will catalyze only one reaction involving only certain substrates. Therefore, unlike the use of differential equations to describe chemical catalytic reaction networks, bioconversion kinetic models can normally be expressed by a single rate equation.

The second distinguishing feature of bioconversion syntheses is that enzymes quite often require cofactors, which can be for example be metal ions or a coenzyme (e.g., nicotinamide adenine dinucleotide (NAD^+), or thiamine pyrophosphate (TPP)). This results in complex kinetics under nonsaturating conditions, which are beyond the scope of this article.

The third and most significant difference between chemical and enzymatic reactions is the frequent existence of substrate and product inhibition of enzymatic activity. This is a vital element of bioconversion reaction rate expressions and makes enzymatic reaction kinetic models more difficult to determine since they require the estimation of many parameters in a single differential equation.

In general, there are three types of reversible interaction between substrate, enzyme, and inhibitor in a single substrate reaction leading to competitive, uncompetitive, and noncompetitive inhibition. The kinetics for competitive, uncompetitive, and noncompetitive inhibition can be represented as[17–20]:

$$\begin{aligned}
&\text{competitive inhibition:} \quad -\frac{d[\mathrm{S}]}{dt} = \frac{V_{\max}[\mathrm{S}]}{\alpha K_\mathrm{s} + [\mathrm{S}]} \\
&\text{uncompetive inhibition:} \quad -\frac{d[\mathrm{S}]}{dt} = \frac{(V_{\max}/\alpha)[\mathrm{S}]}{K_\mathrm{s}/\alpha + [\mathrm{S}]} \\
&\text{noncompetitive inhibition:} \quad -\frac{d[\mathrm{S}]}{dt} = \frac{(V_{\max}/\alpha)[\mathrm{S}]}{K_\mathrm{s} + [\mathrm{S}]} \\
&\text{where} \quad \alpha = 1 + \frac{[\mathrm{I}]}{K_\mathrm{i}}
\end{aligned} \tag{1}$$

where the inhibitor is also acting as a substrate (i.e., in a two-substrate bioconversion with interactive substrate inhibition) Equation set 1 can be modified to give Equation set 2, although the complete form of the kinetic model depends on other factors such as product inhibition and substrate and product binding mechanisms.

$$\begin{aligned}
&\text{competitive inhibition:} \quad -\frac{d[\mathrm{S}]}{dt} = \frac{V_{\max}[\mathrm{S}][\mathrm{I}]}{\alpha K_\mathrm{s}[\mathrm{I}] + K_\mathrm{I}[\mathrm{S}]} \\
&\text{uncompetive inhibition:} \quad -\frac{d[\mathrm{S}]}{dt} = \frac{(V_{\max}/\alpha)[\mathrm{S}][\mathrm{I}]}{K_\mathrm{s}[\mathrm{I}]/\alpha + K_\mathrm{I}[\mathrm{S}]} \\
&\text{noncompetitive inhibition:} \quad -\frac{d[\mathrm{S}]}{dt} = \frac{(V_{\max}/\alpha)[\mathrm{S}][\mathrm{I}]}{K_\mathrm{s}[\mathrm{I}] + K_\mathrm{I}[\mathrm{S}]} \\
&\text{where} \quad \alpha = 1 + \frac{[\mathrm{I}]}{K_\mathrm{i}}
\end{aligned} \tag{2}$$

and I and S are different substrates. I is the inhibitory substrate.

Plots of initial rate changes with the inhibitory substrate concentration for each of these inhibition mechanisms are given in Figures 1a–c. Figure 1d is for noninteractive inhibition in a two-substrate system (i.e., the inhibition by the two substrates act independently). The shapes of the curves vary for different inhibition types, with initial rate increasing rapidly at low substrate concentrations, but then slowing down or even declining as the concentration of the inhibitory substrate increases toward and above the K_i value.

Proposed Approach

The main obstacle to applying the nonlinear regression method for bioconversion kinetic parameter identification is the risk of local optimization. There are three possible ways

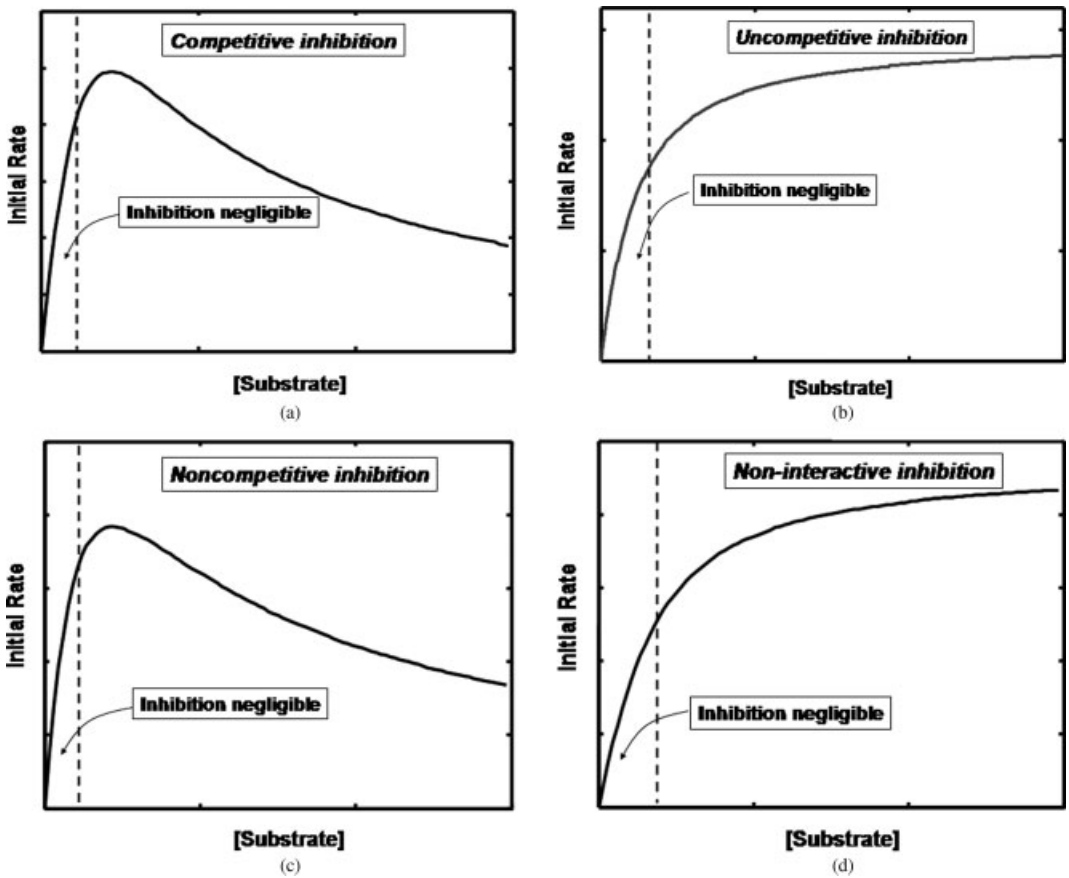

Figure 1. Different types of substrate inhibition mechanism plots based initial rate as a function of inhibitory substrate concentration.

(a) Competitive inhibition; (b) uncompetitive inhibition; (c) noncompetitive inhibition; (d) noninteractive inhibition.

to reduce the chance of incurring local optimization: (1) using an alternative optimization algorithm which is better able to find the global optimal solution; (2) reducing the problem dimension (i.e., decreasing the number of search variables (parameters) for optimization); and (3) providing better initial values for optimization to start parameter searching.

Details of the approach

Accordingly, we first chose a better algorithm—the mesh adaptive pattern search algorithm in Matlab® (MathWorks, Natick, MA, USA) known as: "The Genetic Algorithm and Direct Search Toolbox." This is more likely to achieve global optimization than gradient-based methods. Second, we have made use of the kinetic characteristics, especially inhibition, to mean that fewer parameters need to be obtained under given conditions. This in turn means that fewer parameters need to be obtained at any given point in the procedure.

The new approach we are proposing to identify kinetic parameters is schematically represented in Figure 2. There are five steps in the approach (as indicated in the "PROCEDURES" column in Figure 2). We have labeled the procedural steps respectively by letters (A–E) and the experimental steps by numbers (1–3) for easy reference. Thus the first procedural step (A) is to determine the linear range of initial reaction rate changes at different enzyme concentrations using the data from the first experimental step (1). It is quite common that increasing the enzyme concentration in a reaction will not increase the overall activity in a linear manner. Thus the region of proportionality between enzyme concentration and reaction rate needs to be established to ensure that any increase in enzyme concentration contributes fully to the measured kinetics. All subsequent enzyme concentrations used for kinetic parameter identification will need to be carried out within the defined linear region.

A further preliminary experimental step (2) is required to determine the inhibition behavior of substrates and products. As shown in Figure 1, when the concentration of a reagent in the bioconversion increases, inhibition by that reagent increases and then eventually dominates. However, at low concentrations of inhibitor (it can be substrate or product), the inhibition exists but is negligible and can therefore be ignored.

In the case of a low concentration of inhibitor, bioconversion kinetics can be simplified by ignoring the inhibition constants (i.e., the simplified kinetic model only requires rate and Michaelis constants to be obtained). For example, the kinetic model for a reaction with two substrates (A and B), producing two products (P and Q) can be derived as Eq. 3 if

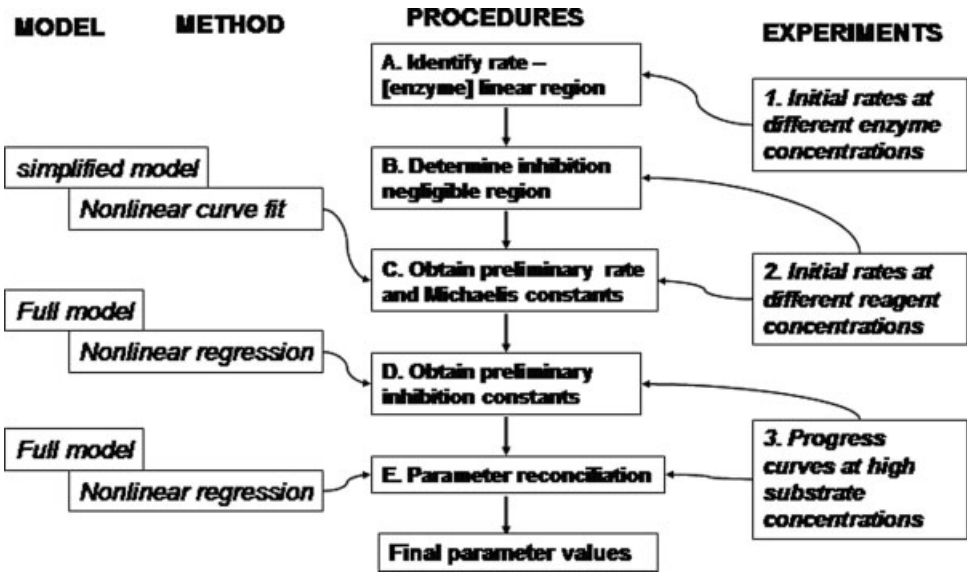

Figure 2. The flow sheet of the new approach for kinetic parameter identification.

it follows a ping-pong bi-bi mechanism and reagent inhibitions are noninteractive.

$$\frac{d[\mathrm{Q}]}{dt} = \frac{V_\mathrm{f}V_\mathrm{r}([\mathrm{A}]\mathrm{B}] - [\mathrm{P}][\mathrm{Q}]/K_\mathrm{eq})}{\mathrm{Const}}$$

$$\mathrm{Const} = V_\mathrm{r}K_\mathrm{b}[\mathrm{A}] + V_\mathrm{r}K_\mathrm{a}[\mathrm{B}] + V_\mathrm{r}[\mathrm{A}][\mathrm{B}] + \frac{V_\mathrm{f}K_\mathrm{q}[\mathrm{P}]}{K_\mathrm{eq}} + \frac{V_\mathrm{f}K_\mathrm{p}[\mathrm{Q}]}{K_\mathrm{eq}} + \frac{V_\mathrm{f}K_\mathrm{q}[\mathrm{A}][\mathrm{P}]}{K_\mathrm{eq}K_\mathrm{ia}} + \frac{V_\mathrm{f}[\mathrm{P}][\mathrm{Q}]}{K_\mathrm{eq}} + \frac{V_\mathrm{r}K_\mathrm{a}[\mathrm{B}][\mathrm{Q}]}{K_\mathrm{iq}}$$

$$K_\mathrm{eq} = \frac{V_\mathrm{f}K_\mathrm{q}K_\mathrm{ip}}{V_\mathrm{r}K_\mathrm{ia}K_\mathrm{b}} = \frac{V_\mathrm{f}K_\mathrm{p}K_\mathrm{iq}}{V_\mathrm{r}K_\mathrm{ib}K_\mathrm{a}} = \frac{K_\mathrm{ip}K_\mathrm{iq}}{K_\mathrm{ia}K_\mathrm{ib}} = \left(\frac{V_\mathrm{f}}{V_\mathrm{r}}\right)^2\frac{K_\mathrm{p}K_\mathrm{q}}{K_\mathrm{a}K_\mathrm{b}}$$

$$V_\mathrm{f} = E_\mathrm{i}K_\mathrm{f} \quad V_\mathrm{r} = E_\mathrm{i}K_\mathrm{r} \tag{3}$$

where [A], [B], [P], and [Q] are the concentrations of substrates A and B, and products P and Q, respectively; K_f and K_r are the rate constant of the forward and reverse reactions, respectively; K_a, K_b, K_p, and K_q are the Michaelis constants for A, B, P, and Q, respectively; K_ia, K_ib, K_ip, and K_iq are the inhibition constants of A, B, P, and Q, respectively; E_i is the concentration of enzyme used in the reaction.

The total number of parameters in this kinetic expression is 10. Although the form of Eq. 3 will be modified by adding the α term defined in Eq. 2 according to the inhibition mechanism, the number of kinetic parameters remains the same.

When the reaction is carried out at low concentrations of substrates A and B and the initial rate data are used, Eq. 3 can be simplified to Eq. 4 since the concentrations of products are low and therefore product inhibition and the reverse reaction is negligible. Nonlinear regression can be applied to get initial estimates of V_f, K_a, and K_b using initial rate data obtained in the second step. Alternatively, Eq. 4 can be linearized (to give Eq. 4a) and a linear plotting method can be applied. Using the initial rate data in the second step, V_f, K_a, and K_b can be obtained according to slopes and intercepts by plotting $1/\upsilon$ against 1/[A] and 1/[B]. Therefore, both linear plotting and nonlinear curve fitting can be used to obtain V_f, K_a, and K_b in this stage.

$$\upsilon = \frac{d[\mathrm{Q}]}{dt} = \frac{V_\mathrm{f}[\mathrm{A}][\mathrm{B}]}{K_\mathrm{b}[\mathrm{A}] + K_\mathrm{a}[\mathrm{B}] + [\mathrm{A}][\mathrm{B}]} \tag{4}$$

$$\frac{1}{\upsilon} = \frac{K_\mathrm{a}}{V_\mathrm{f}}\frac{1}{[\mathrm{A}]} + \frac{K_\mathrm{b}}{V_\mathrm{f}}\frac{1}{[\mathrm{B}]} + \frac{1}{V_\mathrm{f}} \tag{4a}$$

Similarly, V_r, K_p, and K_q, can be obtained (step C) when P and Q are used as the substrate for the reverse reaction.[21]

Using the initial estimated parameter values of V_f, K_a, K_b, V_r, K_p, and K_q obtained in step C, the rest of the four inhibition parameters, the initial estimate values for K_ia, K_ib, K_ip, and K_iq, can be identified by applying nonlinear regression with progress curves (experimental step 3) at high concentrations of A and B (inhibition dominating region) and the full kinetic model (Eq. 3). Under these circumstances, global optimization is much easier to obtain because only four parameters need to be estimated, and the results of parameter identification will generally not be influenced by given initial values for optimization (i.e., they are stable with respect to any given initial value).

Generally, the initial estimate parameter values gained in steps C and D are quite close to the "true" values. However, because rate constants and Michaelis constants of substrates and products are obtained using the data in the preliminary experiments with the simplified kinetic model, the values of parameters obtained are inaccurate. Such an error will be propagated to the inhibition parameter identification and result in imprecise values for inhibition parameters. Therefore, it is necessary to reconcile the parameters to correct for such an effect. In step E, the values of the parameters obtained in steps C and D are used as initial values for optimization to identify the "true" values of all the kinetic parameters using the full model with progress curves of a wider range of concentrations of substrates and biocatalyst. In this step, the progress curve used for the previous step can be

reused and extra progress curves at low substrate concentrations may be required. Because the initial values are close to the "true" parameter values (i.e., the initial state is close to the global optimal state), global optimization can be guaranteed.

Experiments required

The experiments required (1–3) for this approach (as shown in the "EXPERIMENTS" column in Figure 2) can be summarized as follows:

1. Preliminary experiments to find the region in which initial rate changes linearly with enzyme concentration by measuring initial rates at different enzyme concentrations in the bioconversion under constant substrate concentration;
2. Measure initial rates at different substrate and product concentrations to locate the regions in which substrate inhibition can be ignored;
3. Produce progress curves, i.e., concentrations of substrate and product as a function of time, at high substrate concentrations and relatively high enzyme concentrations in order to complete progress curves within hours to avoid possible time-dependent effects such as reagent degradation.

An Example: Transketolase Mediated Synthesis of 1,3-Dihydroxypentan-2-one

To exemplify the methodology, the reaction kinetics of the transketolase (TK) mediated 1,3-dihydroxypentan-2-one (DHP) synthesis from β-hydroxypyruvate (HPA) and propionaldehyde (PROP) are used as an illustration.

Materials and methods

The strain used to obtain the lysate for bioconversion studies was XL10-gold *Escherichia coli* hosting the pQR711 plasmid which expresses the *E. coli* transketolase mutant D469T from its own promoter. Because the expression of transketolase is constitutive, no inducer was required. Cells were cultured using modified Luria-Bertani (LB) medium containing glycerol (10 g L^{-1}) and ampicillin (100 mg L^{-1}) at 300 rpm, 37°C for 8–12 h.

Subsequently, cells were harvested by centrifugation (4000 rpm, 10 min) and pellets resuspended in sodium phosphate buffer (5 mM, pH 7.0) to give a final concentration of 0.1 g wet cell paste per mL. After cell disruption by sonication, cell debris was removed by centrifugation and the lysate stored at −20°C. A protein densitometry method[22] was used to determine the enzyme concentration of the lysate.

The bioconversion experiments were carried out in triplicate essentially as described originally by Hobbs.[23] Enzyme was incubated with cofactors for 20 min in opened 5 mL vials and covered as soon as the substrates were added (bioconversion started). Experimental step 1 was carried out with a reaction volume of 500 μL at fixed (30 mM) equimolar concentrations of the two substrates and samples were taken at a range of reaction times, between 5 and 20 min. All experiments were carried out at 25°C.

For Experimental step 2, bioconversions were carried out with a reaction volume of 150 μL. Two sets of experimental protocols were established: (1) PROP was kept constant at 50 mM and the initial rate variation with HPA concentrations was studied up to 160 mM; (2) HPA concentration was maintained at 30 mM and PROP concentration was varied between 10 and 600 mM.

Table 1. Substrate Concentrations Used for the Experiments to Produce Progress Curves

	Substrate Concentrations for Kinetic Experiments								
[HPA] (mM)	140	150	120	200	200	200	300	300	320
[PROP] (mM)	120	140	140	220	180	200	300	280	300
E_i (g L^{-1})	1.15				1.44			1.73	

In each progress curve, samples were taken each 30 min for a total of 360 min.

The reaction progress curves used for kinetic parameter identification and refinement as described in Experimental step 3 were obtained with a reaction volume of 800 μL and 13 samples were withdrawn over time for each reaction condition, i.e., for each set of substrate and enzyme concentration described in Table 1.

From all the above experiments samples (20 μL) were taken and diluted with 180 μL 0.1% trifluoroacetic acid (TFA) solution. A Bio-Rad Amines HPX-87H Reverse Phase column (300 × 7.8 mm) was used to separate HPA, PROP, and DHP by HPLC (Dionex, Camberley, Surrey, UK). The column was maintained at 60°C and the resulting peaks measured by UV detection. The mobile phase was a 0.1% TFA solution with a flowrate of 0.6 mL min^{-1} giving retention times of 8.5, 15, and 26 min, respectively, for HPA, DHP, and PROP.

Reaction kinetic model

As shown in Figure 3, using HPA as the ketol donor, the enzyme catalyses the transfer of a 2-carbon ketol group from HPA to an aldehyde acceptor (PROP) and requires Mg^{2+} and TPP as cofactors, in which the decarboxylation renders the reaction irreversible.[24] We have assumed the reaction kinetics of this TK mediated DHP synthesis follow the ping-pong bi–bi mechanism[25] with competitive inhibition for both substrates, as is well known for this type of reaction. On this basis, the kinetic model can be written as:

$$\frac{d[\mathrm{Q}]}{dt}=\frac{k_{\mathrm{cat}}E_{\mathrm{i}}[\mathrm{A}][\mathrm{B}]}{K_{\mathrm{b}}[\mathrm{A}]\left(1+\frac{[\mathrm{A}]}{K_{\mathrm{ia}}}\right)+K_{\mathrm{a}}[\mathrm{B}]\left(1+\frac{[\mathrm{B}]}{K_{\mathrm{ib}}}\right)+[\mathrm{A}][\mathrm{B}]+\frac{K_{\mathrm{a}}}{K_{\mathrm{iq}}}[\mathrm{B}][\mathrm{Q}]+\frac{K_{\mathrm{a}}K_{\mathrm{ib}}}{K_{\mathrm{iq}}}[\mathrm{Q}]} \tag{5}$$

where [A], [B], and [Q] represent the concentrations of HPA, PROP, and DHP, respectively. k_{cat} is the reaction rate constant; K_a and K_b are the Michaelis constants of A and B, respectively; K_{ia}, K_{ib}, and K_{iq} are the inhibition constants of A, B, and Q, respectively; E_i is the enzyme concentration in the bioconversion.

Using the linear plotting method

To date, there is no reported kinetic study for this system, but a similar case of TK-mediated L-erythrulose synthesis using HPA and GA by Gyamerah and Willetts[25] can be used for the purpose of comparison because we have assumed the same kinetic mechanism and therefore the number of parameters that need to be estimated will be the same. Table 2 summaries the experiments carried out by Gyamerah and

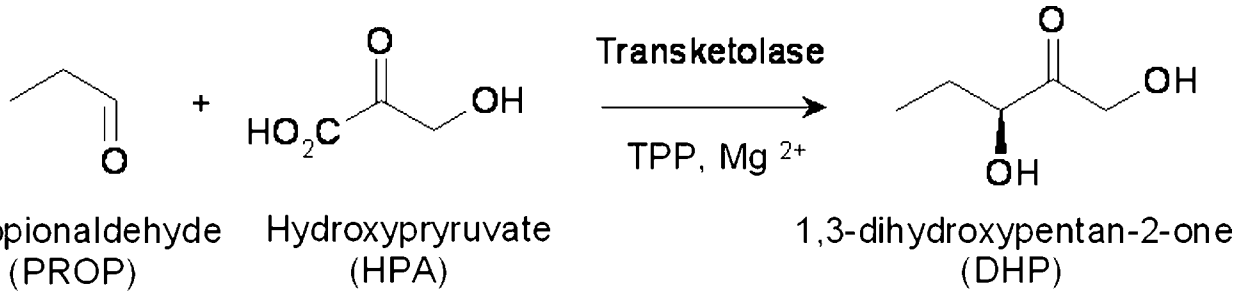

Figure 3. Reaction scheme of transketolase mediated 1,3-dihydroxypentan-2-one synthesis from β-hydroxypyruvate and propionaldehyde.

Willetts[25] in order to obtain the six kinetic parameters. The number of pairs in each designed experiment is the full combination for each concentration (e.g., in the first experiment (experiments for obtaining k_{cat}, K_a, K_b), the experimental pairs are [GA]:[HPA] = 30:10, 30:20, 30:30, 30:40, 30:50, ... 10:200, 10:300, 10:400, ... 200:10, 200:20, 200:30, 200:40, 200:50, ...). In this way, the total number of experiments for kinetic parameter identification is 130.

Using the nonlinear method

All nine progress curves obtained in the experiments described in Experimental step 3 were used to obtain kinetic parameters by nonlinear regression via the "pattern search" optimization algorithm in Matlab® with maximum likelihood[26,27] using the objective function defined in Eq. 6.

$$\Phi = -\frac{1}{2}\sum_{j=1}^{M}\left[N_j\left(\ln\left(2\pi\right)+1\right)+N_j\ln\left[\left(\frac{1}{N_j}\right)\sum_{i=1}^{N_j}\left(y_{i,j}-\hat{y}_{i,j}\right)^2\right]\right] \tag{6}$$

where y and $\hat{y}$ are experimental results and model predicted values, respectively; $y_{i,j}$ is the experimental result of jth response variable in the ith experiment; N_j is the number of observations of the jth response variable; M is the number of variables.

The lower and upper bounds for the "pattern search" were set at 1 and 2000, respectively. Table 3 lists the estimated parameters using nonlinear regression with different initial values (10, 50, 100, and 200) for all six parameters. The results show that different parameter values are obtained dependent on the given initial values, although the squared sum of the residuals was similar. We can conclude therefore, that the nonlinear regression method for parameter identification is not reliable in this case.

Table 2. Experiments for Obtaining Kinetic Parameters for Transketolase Mediated L-Erythrulose Synthesis from β-Hydroxypyruvate and Glycolaldehyde Using the Linear Plot Method (Based on the Work of Gyamerah and Willetts[25])

Designed Experiments	Experiment Conditions	Number of Experiments
Experiments for obtaining k_{cat}, K_a, K_b	[GA] = 30, 50, 100 mM [HPA] = 10, 20, 30, 40, 50 mM	15
Experiments for obtaining K_{ia}	[GA] = 10, 20, 30, 40, 50 mM [HPA] = 200, 300, 400 mM	15
Experiments for obtaining K_{ib}	[GA] = 200, 300, 400, 500 mM [HPA] = 10, 20, 30, 40, 50 mM	20
Experiments for obtaining K_{iq}	[ERY] = 0, 20, 40, 60, 80 mM [GA] = 20, 30, 50, 50 mM [HPA] = 10, 20, 30, 40 mM	80
Total		130

Using the proposed approach

First (step A), a preliminary experiment (Experimental step 1) was carried out to determine the linear region of the relationship between reaction rate and enzyme concentration. Figure 4 shows the result of such an experiment, indicating that the linear relationship can be maintained up to an enzyme concentration of 6 g L^{-1} in this bioconversion, setting the upper limit for all subsequent experiments.

Secondly (step B), an experiment (Experimental step 2) was carried out to determine the behavior of substrate inhibition such that a "region of negligible inhibition" can be defined using the plot of initial rate against substrate concentration. In this approach, the accurate initial rate values, which normally need to be derived from the progress curves, are not necessary because the parameters obtained using these data will be reconciled later. Instead, samples taken as close as possible to the beginning of the reactions are considered to determine the initial rates. Here, the sampling time

Table 3. Values of Estimated Parameters Based on Different Initial Values for Nonlinear Regression

	Initial Values Used for Optimization			
Kinetic Parameters	10	50	100	200
Rate constant: k_{cat} (min^{-1})	470	474	1029	512
Michaelis constant for HPA: K_a (mM)	12	8	6	14
Michaelis constant for PROP: K_b (mM)	106	98	174	101
Inhibition constant for HPA: K_{ia} (mM)	52	46	32	43
Inhibition constant for PROP: K_{ib} (mM)	61	325	155	639
Inhibition constant for DHP: K_{iq} (mM)	9	293	473	655
Squared sum of residual	211	167	195	168

In the column of "Initial values used for optimization," the value of 10 means 10 is used as initial value for all six parameters.

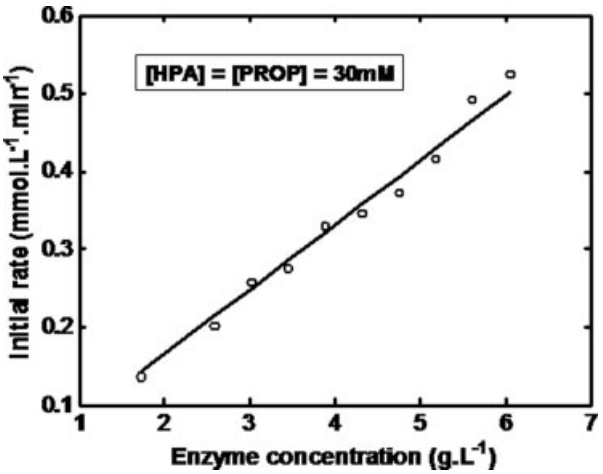

Figure 4. Experimental result of initial rate as a function of enzyme concentration.

The linear range of such a relationship can be maintained up to an enzyme concentration pf 6 g L^{-1}.

for initial rate measurements is 5 min, which was suggested by analyzing the results in Experimental step 1. Figure 5a shows plots of the initial rates as a function of concentration of substrate HPA (when the concentration of PROP is fixed at 50 mM), and Figure 5b is such a plot for PROP (when the HPA concentration is fixed). The "regions of negligible substrate inhibition" are beneath 20 and 50 mM for HPA and PROP, respectively. Therefore, the first four values of initial rates at the concentrations below 20 mM in Figure 5a and the first five below 50 mM in Figure 5b were used to determine the rate and Michaelis parameters by nonlinear regression with the simplified kinetic expression (Eq. 7) (Step C, Figure 2). The preliminary values of k_{cat}, K_a, and K_b can be obtained as 501 min^{-1}, 12, and 101 mM, respectively, by nonlinear curve fitting with arbitrary initial values. This also implies that nonlinear regression is reliable when the number of estimated parameters is 3 as in the situation here.

$$\frac{d[Q]}{dt} = \frac{k_{cat}E_i[A][B]}{K_b[A] + K_a[B] + [A][B]} \tag{7}$$

In the fourth step (D), nine progress curves with 13 sampling points in each progress curve generated in Experimental step 3 are used for preliminary K_{ia}, K_{ib}, and K_{iq} determination. Putting the values of k_{cat}, K_a, and K_b as 501 min^{-1},12, and 101 mM, respectively, into the full model (Eq. 5), the preliminary values of K_{ia}, K_{ib}, and K_{iq} can be identified as 44, 637, and 689 mM, respectively, by nonlinear regression using pattern search in Matlab® with "maximum likelihood" as the objective function. Here again lower and upper bounds were set as 1 and 2000, and the optimization was always found to converge to the same result for any given initial value within the bounds.

In the fifth step (E), the parameter values obtained previously are used as initial values for nonlinear regression, i.e., the initial values of k_{cat}, K_a, K_b, K_{ia}, K_{ib}, and K_{iq} are set as 501 min^{-1}, and 12, 101, 44, 637, and 689 mM, respectively, and lower and upper bounds are 1 and 2000 for optimization.

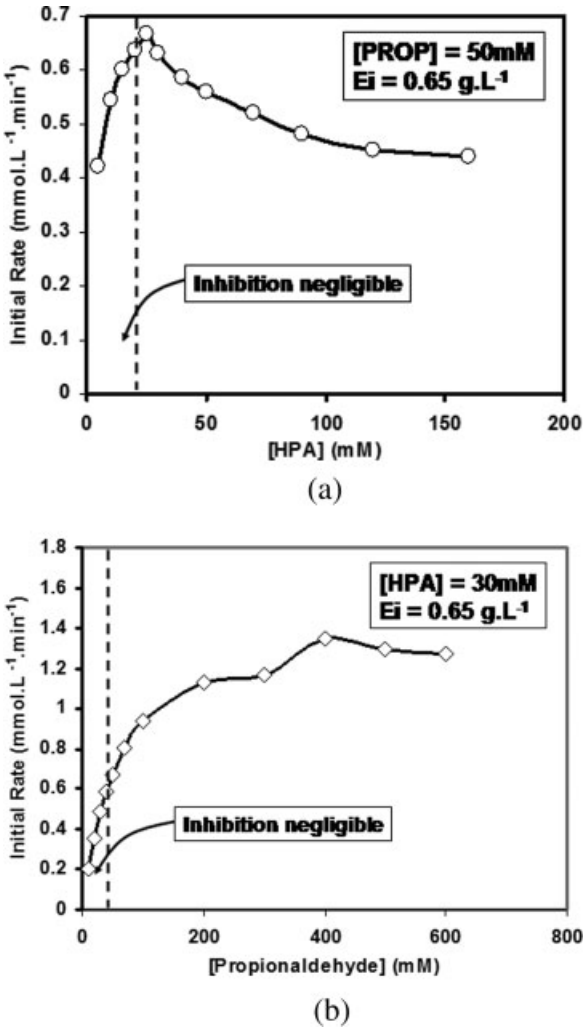

Figure 5. Initial rate against substrate concentrations plot to determine inhibition negligible regions.

(a) HPA inhibition plot indicates inhibition becomes dominant above 20 mM. (b) PROP inhibition plot indicates inhibition becomes dominant above 50 mM.

The final values of the kinetic parameters obtained in this way are shown in Table 4. Figures 6a,b illustrate the curve fits of the model and experimental data at two different starting substrate concentrations. The agreement between modeled and experimental values is excellent although this is not entirely unexpected as these data sets were used in establishing the kinetic parameters. To test the predictive power of

Table 4. Values of Kinetic Parameter Determined Using the Approach Developed in This Article

Kinetic Parameters	Value
Rate constant: K_{cat} (min^{-1})	501
Michaelis constant for HPA: K_a (mM)	12
Michaelis constant for PROP: K_b (mM)	98
Inhibition constant for HPA: K_{ia} (mM)	43
Inhibition constant for PROP: K_{ib} (mM)	625
Inhibition constant for DHP: K_{iq} (mM)	681

the full kinetic model, Figure 7 shows the comparison of the model predicted progress curves and a further set of experimental data obtained under conditions not previously used for parameter estimation ([Enzyme] = 1.44 g L^{-1}; [HPA] = 250 mM; [PROP] = 270 mM). Again there is an excellent agreement between model predictions and the measured values. This serves to verify both the approach outlined in Figure 2 and the kinetic model and constants described by Eq. 5 and in Table 4, respectively.

Discussion

The fundamental basis of linear plotting methods is that the kinetic expression can be simplified when initial rates at low substrate/product concentration are applied. Therefore, a large number of experiments with a wide range of reagent concentrations are required to determine kinetic parameters, especially for product inhibition constants as illustrated in Table 2. To get an accurate initial rate, values are normally calculated from progress curves (i.e., one initial rate value from one progress curve). This implies a large proportion of the data is redundant and not in fact directly involved in the parameter identification procedure. However, there is an advantage in using linear methods in that kinetic parameters are generally not far away from the "true" values. This implies that linear methods are to some extent reliable even though not as accurate, because estimated values of parameters are rather dependent on the way the initial rate was obtained.

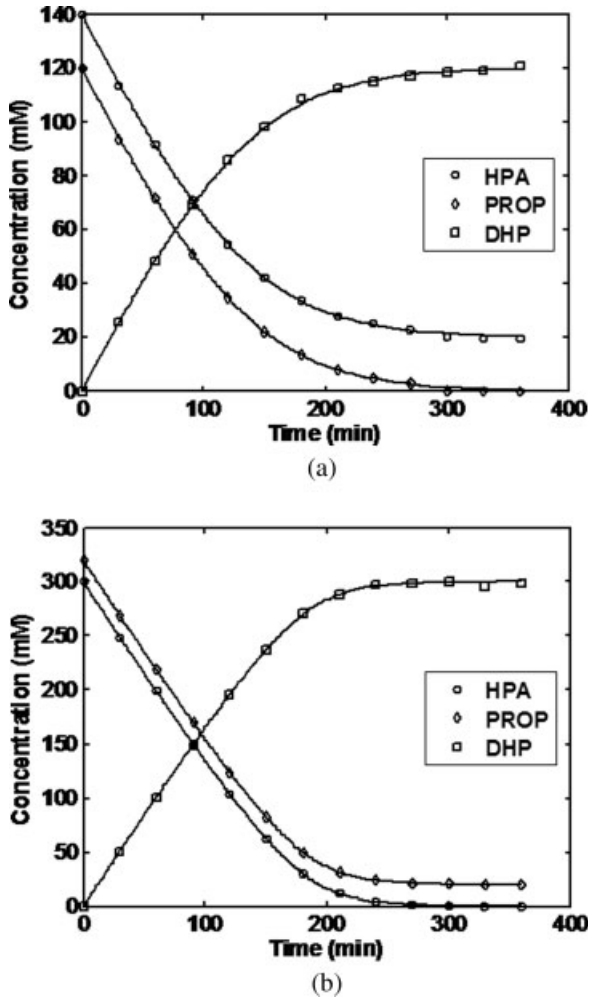

Figure 6. The curve fit figures of the model (solid line) and experimental data (symbols) at two different starting substrate concentrations of the nine progress curves.

It is generated in Experimental step 3 of the approach in this work, from which the model kinetic parameters were established.

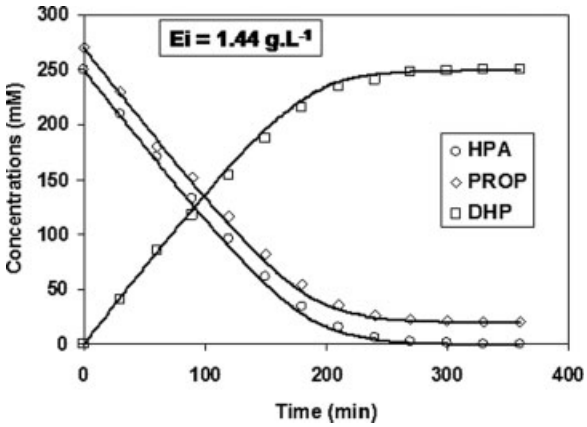

Figure 7. Verification of model predictions with an experimental data set (symbols) not used for kinetic parameter identification.

[Enzyme] = 1.44 g L^{-1}; [HPA] = 250 mM; [PROP] = 270 mM. Solid lines represent kinetic model predictions based on Eq. 5 and the parameters listed in Table 4.

The advantage of using nonlinear regression is that it makes use of all available information and thus needs fewer experiments compared to linear plotting methods. Nevertheless, the chance of ending in a local (rather than global) optimum arises when the number of identified parameters increases because of using numerical optimization algorithms. For bioconversion kinetic parameter estimation, such a number can be as small as six as the results shown in Table 3 illustrate. When faced with local optimization, the final result will depend on giving suitable initial values and the scenario of parameter structure will be very different from the "true" values as demonstrated in Table 4. In this sense, nonlinear regression is not reliable.

In the approach developed in this article, we make use of the advantages and address the disadvantages in both linear and nonlinear methods. Parameters are divided into groups to obtain preliminary values of parameters step-by-step. In this way, the number of estimated parameters in each step is reduced so that "local optimization" can be avoided. These values are then used as initial values when nonlinear regression is applied, to obtain the final values of the kinetic parameters.

Table 5 compares the bases, number of experiments required, and reliability of the three methods. It should be noted that 10 experiments in Experimental step 1 of this work were not taken into account in Table 5 because such a check should be performed when screening any bioconversion prior to establishment of kinetic parameters, regardless of the subsequent method used. Table 5 indicates that the method devel-

Table 5. Comparison of Linear Plotting and Nonlinear Regression with the Approach Developed in This Work

	Linear Plotting	Nonlinear Regression	This Work
Bases	Step-by-step parameter determination using initial rate	Nonline arregression using progress curves	Step-by-step parameter determination using initial rate and progress curves
Number of experiments required	130	9	33
Reliability	Reliable	Not reliable due to local optimisation	Reliable

oped in this work can significantly reduce the number of experiments compared with the linear method while retaining reliability, in contrast to nonlinear regression methods.

Conclusions

A new reliable nonlinear regression-based approach for bioconversion kinetic parameter estimation has been developed based on the inhibition characteristics of a two-substrate bioconversion reaction. Compared with linear plotting methods, the approach can significantly reduce the number of experiments required for kinetic parameter identification. By dividing kinetic parameters into groups and obtaining kinetic parameter estimates on a step-by-step basis, the approach can avoid local optimization in nonlinear regression and is therefore highly reliable.

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