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Mathematical model for kinetics of enzymatic conversion of benzaldehyde and pyruvate to (*R*)-phenylacetylcarbinol

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Abstract

A mathematical model for the enzymatic biotransformation of benzaldehyde and pyruvate to *R*-phenylacetylcarbinol (PAC) and its associated by-products has been developed using a schematic method devised by King and Altman [E.L. King, C. Altman, A schematic method of deriving the rate laws for enzyme catalysed reactions, J. Phys. Chem. 60 (1956) 1375–1378] for deriving the rate equations for a complex enzyme-catalysed reaction. PAC is the commercial intermediate for the production of ephedrine and pseudoephedrine. A combinatorial theorem was applied using Visual Basic to create all of the possible reaction patterns for a simplified form of the pyruvate decarboxylase (PDC) biotransformation mechanism. The rate equations for substrates, product, and by-products have been derived from the patterns for yeast PDC and combined with a deactivation model for PDC from *Candida utilis*. The batch biotransformation profile generated by the model validated previously for a data set at initial substrate concentrations 50–150 mM benzaldehyde and 60–180 mM pyruvate, provided an acceptable fit for published data at initial concentrations of 400 mM benzaldehyde and 600 mM pyruvate.

Keywords: Biotransformations; (*R*)-phenylacetylcarbinol; Biokinetics; Enzyme biocatalysis; King and Altman procedure; Modelling

1. Introduction

R-phenylacetylcarbinol (PAC) is the commercial precursor for the synthesis of the anti-asthmatic and nasal decongestants, ephedrine and pseudoephedrine. PAC is produced from benzaldehyde and pyruvate catalysed by the enzyme pyruvate decarboxylase (PDC). Three by-products, acetaldehyde, acetoin, and carbon dioxide, are generated also from pyruvate by PDC. The overall reactions are illustrated in Fig. 1. The non-oxidative decarboxylation of pyruvate to acetaldehyde requires thiamine pyrophosphate (TPP) [2] and Mg^{2+} as cofactors. Fig. 2 shows the decarboxylation mechanism on the active site of PDC.

The biotransformation of benzaldehyde and pyruvate to PAC and three by-products is a complex enzymatic process involving up to eight enzyme species including free enzyme, as well as binary and ternary enzyme complexes of enzyme-bound substrates, by-products, and PAC. An alternative schematic method of deriving rate laws for complex enzyme-catalysed reactions developed previously by King and Altman [1] has been employed in the present study to establish the requisite rate equations for the PAC biotransformation process. This set of non-linear differential equations provides a comprehensive mathematical model for the reactions. The model developed in the present study includes also an equation describing the deactivation of PDC by benzaldehyde, which is tested experimentally [3]. The combined rate equations can then be used to generate a profile of the batch biotransformation kinetics for PAC production.

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Nomenclature

A	pyruvate
B	benzaldehyde
C	carbon dioxide
E	free pyruvate decarboxylase (PDC) enzyme
EA	binary enzyme complex between PDC and pyruvate
E_0	initial activity of PDC enzyme (U carboligase activity ml^{-1})
EP	binary enzyme complex between PDC and PAC
EQ	binary enzyme complex between PDC and 'active acetaldehyde'
EQB	ternary enzyme complex between PDC, 'active acetaldehyde', and benzaldehyde
EQC	ternary enzyme complex between PDC, 'active acetaldehyde', and CO_2
EQQ	ternary enzyme complex between PDC, 'active acetaldehyde', and acetaldehyde
ER	binary enzyme complex between PDC and acetoin
h	exponent for benzaldehyde with similar functionality to Hill coefficient (no unit)
i	iteration loop identifier of each species to be used in numerical integration
k_n	rate constant for forward reactions; n ranges from 1 to 10
$k_{(-n)}$	rate constant for backward reactions; n ranges from 1 to 10
k_{d1}	first order reaction time deactivation constant (h^{-1})
k_{d2}	first order benzaldehyde deactivation coefficient ($mM^{-1}\,h^{-1}$)
K_b	intrinsic binding constant for benzaldehyde ($mM^{-1}\,h^{-1}$)
K_{ma}	affinity constant for pyruvate (mM)
K_{mb}	affinity constant for benzaldehyde (mM)
K_r	rate constant product for the simplified model; r ranges from 1 to 19
P	PAC
Q	acetaldehyde
R	acetoin
t	time (h)
t_{lag}	lag time (h)
V_p	overall rate constant for the formation of PAC ($\mu mol\,h^{-1}\,U^{-1}$)
V_q	overall rate constant for the formation of acetaldehyde ($ml\,h^{-1}\,U^{-1}$)
V_r	overall rate constant for the formation of acetoin ($l^2\,h^{-1}\,U^{-1}\,mol^{-1}$)

Greek letters

$\sum$	sum of kappa products from all enzyme species for simplified model
v	rate of R-phenylacetylcarbinol (PAC) formation ($mM\,h^{-1}$)

2. Methods

2.1. Model development

2.1.1. Proposed reaction mechanism

The proposed model for the enzymatic biotransformation of pyruvate and benzaldehyde to PAC and associated by-products (acetaldehyde, acetoin, and CO_2) is shown in Fig. 3. It consists of twenty composite reactions relating free PDC enzyme and its possible binary and ternary complexes. The transition mechanisms between binary (EA, EQ, ER, and EP) and ternary complexes (EQB, EQC, and EQQ) were expanded from the simple reaction mechanism involving two substrates and/or products proposed by Cornish-Bowden [4].

The model shows the interaction of the substrate pyruvate (A) with the free PDC enzyme (E) to generate the enzyme complex EA. The decarboxylation function of the enzyme is illustrated via the formation of the ternary complex EQC from EA and its subsequent conversion to EQ with release of carbon dioxide (C). Also reverse reactions are considered including the possibility of EQ formation from free enzyme (E) and acetaldehyde (Q).

Following decarboxylation of pyruvate, three fates are possible for EQ:

(i) Release of acetaldehyde from EQ as in the usual function of PDC for ethanol production. The free acetaldehyde is a by-product that may be used in the formation of acetoin.
(ii) Binding of free acetaldehyde to EQ to create the ternary complex EQQ. Carboligation results in enzyme-bound

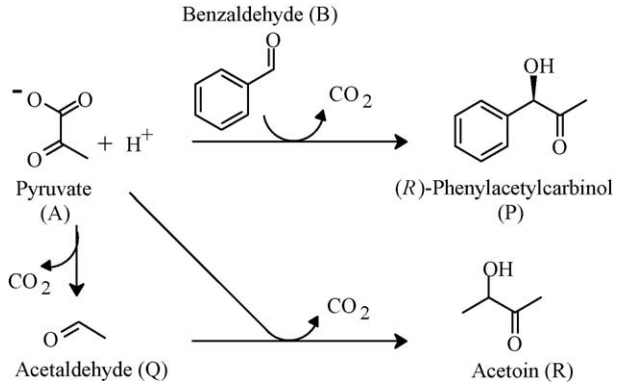

Fig. 1. Formation of PAC and by-products by pyruvate decarboxylase.

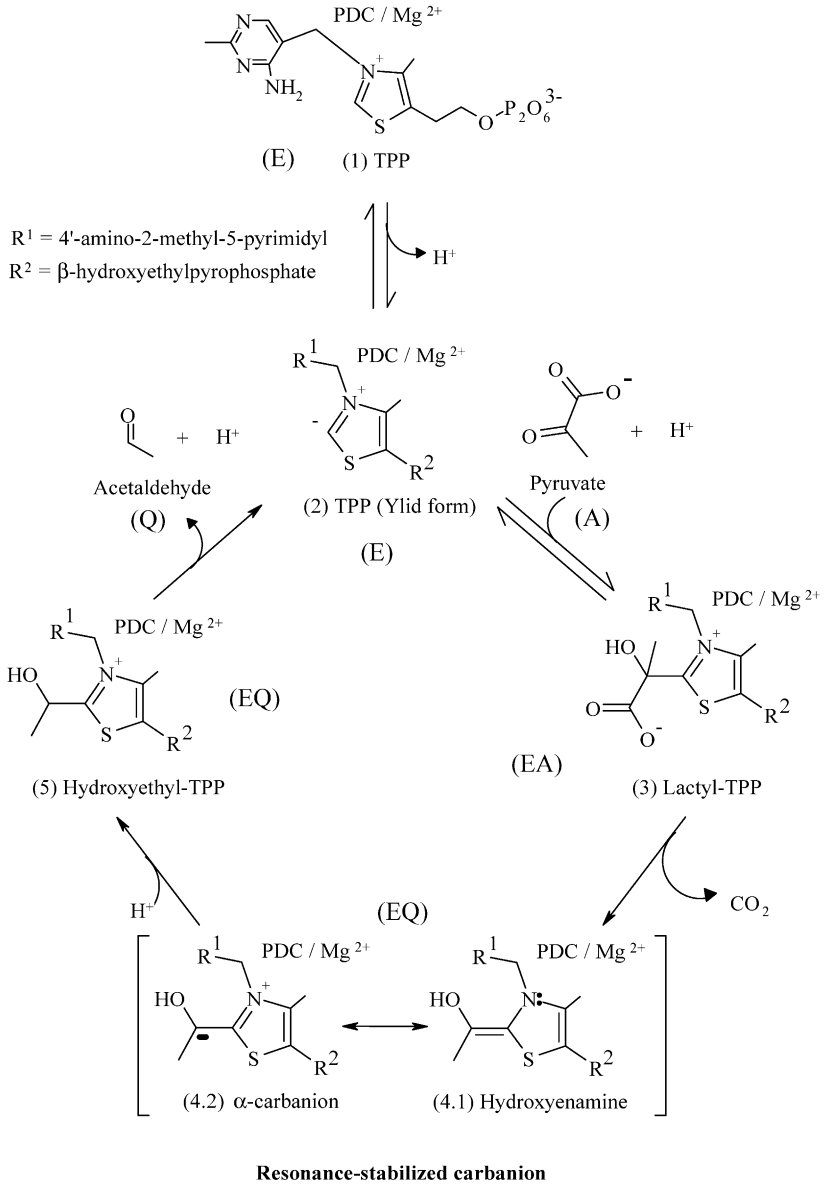

Fig. 2. The thiamine pyrophosphate (TPP) reaction mechanism on the active site of pyruvate decarboxylase (adapted from [38,39]).

by-product acetoin (ER). Acetoin (*R*) is released subsequently freeing PDC for further reaction.

(iii) Complexing of EQ with benzaldehyde resulting in the ternary complex EQB. Then PAC is formed through carboligation leading to the binary complex EP. PAC is later released and the enzyme again becomes available.

The structure of this reaction mechanism is consistent with the widely accepted concept of the formation of an 'active acetaldehyde' at the PDC active site [5–8]. This 'active acetaldehyde' or EQ represents two intermediates of the PDC associated TPP, which has a covalent bond to the C2 residue that originated from pyruvate (see Fig. 2), i.e.

(i) hydroxyenamine (stable, low energy state, and nonreactive intermediate);

(ii) α-carbanion (negatively charged, high energy state, highly reactive).

These two forms are resonance-stabilized mesomers. In [9,10], both forms or sometimes only one of the two forms have been referred to as 'active acetaldehyde' or hydroxyethyl-TPP also. Holzer and Beaucamp [11] showed that pyruvate decarboxylase was able to convert 2-^{14}C-labeled pyruvate to the appropriately labeled 2-([1-^{14}C]-1-hydroxyethyl-TPP). 'Active acetaldehyde' plays the central role in the carboligation reaction. According to Lobell and Crout [12], the nucleophilic attack of 'active acetaldehyde' on the carbonyl carbon atom of aldehydes such as acetaldehyde

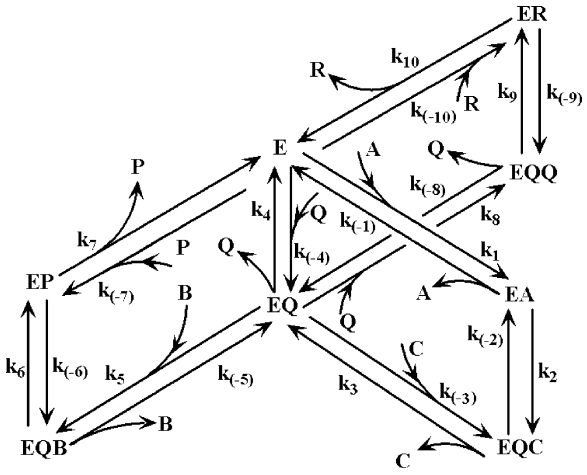

Fig. 3. The proposed three-dimensional schematic diagram of PAC biotransformation and its related by-products for the determination of rate equations by the King and Altman method: pyruvate (*A*), benzaldehyde (*B*), carbon dioxide (*C*), PAC (*P*), acetaldehyde (*Q*), acetoin (*R*) and free PDC (*E*). The definitions of binary and ternary species are listed in the nomenclature.

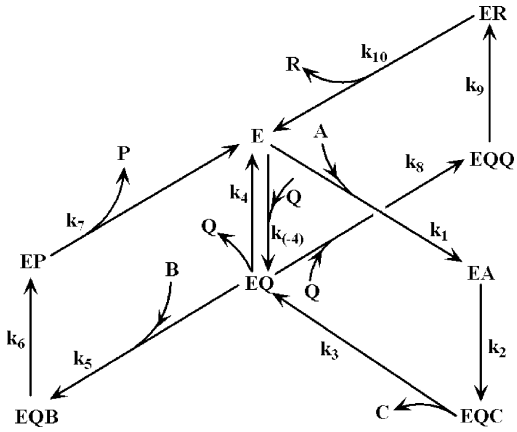

Fig. 4. Simplification of the proposed three-dimensional schematic diagram in Fig. 3 by neglecting backward rate constants except $k_{(-4)}$.

and benzaldehyde results in the new compounds of acetoin and PAC. Alternatively, acetaldehyde can be released from the hydroxyethyl-TPP.

2.1.2. Simplification of the proposed reaction mechanism

The following reverse reactions have been assumed to be negligible, to simplify the proposed reaction mechanism:

(i) The decarboxylation pathway of PDC was simplified by neglecting the reverse reactions, as this direction would involve ligation of CO_2 (EQC to EA) which was considered unfavourable ($k_{(-1)} = 0$, $k_{(-2)} = 0$, $k_{(-3)} = 0$).

(ii) The nucleophilic attacks of 'active acetaldehyde' within the ternary complexes EQB and EQQ, which lead to the formation of binary complexes EP and ER, were assumed to be irreversible ($k_{(-6)} = 0$, $k_{(-9)} = 0$). Also the reverse reactions of the preceding association of benzaldehyde (*B*), or free acetaldehyde (*Q*) with EQ, were neglected ($k_{(-5)} = 0$, $k_{(-8)} = 0$). The assumption of $k_{(-9)} = 0$ might be consistent with the kinetic data for the forward reaction resulting in acetoin production by brewer's yeast PDC [13,14].

(iii) Direct interactions of either the product PAC (*P*) or by-product acetoin (*R*) with the PDC enzyme were considered negligible, therefore, $k_{(-7)} = 0$ and $k_{(-10)} = 0$, respectively. The assumption of $k_{(-10)} = 0$ might be consistent also with the kinetic data which demonstrated acetoin production by brewer's yeast PDC [13,14].

The general simplified reaction mechanism is shown in Fig. 4.

Special attention can be given to $k_{(-4)}$ which represents the rate constant for the formation of EQ from free PDC and free acetaldehyde. It has been suggested by Rosche et al. [15] that this reaction might be a characteristic of bacterial PDC as PAC formation from benzaldehyde and acetaldehyde was observed with *Zymomonas mobilis* [16–18] and *Zymobacter palmae* [15], while 97 species of yeast including *Candida utilis* did not convert acetaldehyde and benzaldehyde to detectable concentrations of PAC (with the methods employed) even though PAC was produced from pyruvate and benzaldehyde [15]. According to the investigation by Chen and Jordan [13], brewer's yeast PDC (EC 4.1.1.1) was able to convert acetaldehyde, in absence of pyruvate, to acetoin but only to a limited extent. At 40 °C and pH 6.0, these authors reported that acetoin was formed 60–100 times faster from pyruvate than from acetaldehyde. As k_8–k_{10} are common to both reactions, it was suggested that EQ is produced 60–100 times faster from pyruvate than from acetaldehyde. Therefore, formation of EQ from free acetaldehyde could be neglected for *C. utilis* PDC ($k_{(-4)} = 0$). Any inhibition effects of substrates, PAC, or by-products have been assumed to be negligible at this stage of model development.

2.1.3. Rate equations for the general simplified reaction

The law of mass action has been applied to the general simplified reaction (Fig. 4), which could apply to both bacteria and yeast PDC, and the following rate equations have been obtained:

Rate of PAC production

$$\left.\frac{\mathrm{d}[P]}{\mathrm{d}t}\right|_i = k_7[\mathrm{EP}_i] \tag{1}$$

Rate of benzaldehyde uptake

$$\left.\frac{\mathrm{d}[B]}{\mathrm{d}t}\right|_i = -k_5[\mathrm{EQ}_i][B_i] \tag{2}$$

Rate of pyruvate uptake

$$\left.\frac{\mathrm{d}[A]}{\mathrm{d}t}\right|_i = -k_1[E_i][A_i] \tag{3}$$

Rate of acetoin production

$$\left.\frac{d[R]}{dt}\right|_i = k_{10}[ER_i] \quad (4)$$

Rate of acetaldehyde production

$$\left.\frac{d[Q]}{dt}\right|_i = k_4[EQ_i] - k_{(-4)}[E_i][Q_i] - k_8[EQ_i][Q_i] \quad (5)$$

Rate of CO_2 production

$$\left.\frac{d[C]}{dt}\right|_i = k_3[EQC_i] \quad (6)$$

An enzyme deactivation rate equation also needs to be included to take account of the deactivating influence of benzaldehyde on PDC. It describes the rate of enzyme deactivation based on 'total enzyme concentration' (E_0) and not 'free enzyme concentration' (E), which is one component of E_0. The distinction of these terms is necessary since in the current model development some of the rate (Eqs. (3) and (5)) include a term for free enzyme.

Rate of enzyme deactivation

$$\left.\frac{d[E_0]}{dt}\right|_i = -(k_{d1} + k_{d2}[B_i])[E_{0i}] \quad (7)$$

The rate equation describing PDC deactivation shown in Eq. (7) is tested and confirmed in earlier studies [3]. It exhibits a first order deactivation of PDC by benzaldehyde (B) up to a concentration of 200 mM. k_{d1} represents the inherent enzyme deactivation constant in 2.5 M MOPS buffer in absence of benzaldehyde and k_{d2} is a benzaldehyde deactivation coefficient in the range of 0–200 mM benzaldehyde.

2.1.4. The King and Altman procedure

The method of King and Altman [1] was selected for the present study. This method has been used by other authors [19,20] as a basis for development of further methods for analysis and derivation of rate equations for complex systems.

From the proposed simplified PAC biotransformation mechanism shown in Fig. 4, there are eight enzyme species: free enzyme (E), four binary enzyme-substrate or enzyme-product complexes (EA, EQ, EP, and ER), and three ternary complexes (EQB, EQQ, and EQC). A reaction is represented by an arrow directed to or away from the specified enzyme complex. The rate constant and accompanying substrate (where applicable) for the reaction are also given on each arrow. Reaction patterns were established according to the King and Altman [1] procedure.

For a pattern to be valid, it must satisfy the following criteria:

(i) arrows must connect all forms of enzyme complexes in the reaction mechanism, a pattern that visits all eight enzyme species therefore consists of seven arrows;
(ii) connected arrows must not form closed loop(s);
(iii) all arrows in the pattern must be pointing in the direction that leads to the formation of the enzyme complex under investigation.

A combinatorial theorem was applied with the above criteria using the subroutines designed in Visual Basic 6.3 of Microsoft® Excel 2002 to select the valid reaction patterns.

3. Results

3.1. Application of King and Altman procedure

The patterns derived from the general simplified reaction (Fig. 4) corresponding to each enzyme complex are presented in Fig. 5. The procedure treats the reactions as pseudo first order and also imposes an assumption of quasi steady state.

These patterns were then used in further model development. Eqs. (1–6) are not particularly useful because they contain the various enzyme complexes (EQ, ER, EP, and EQC) whose concentrations are not measurable experimentally. For obtaining a usable set of rate equations based on experimental data, the King and Altman procedure uses patterns to create expressions for each enzyme complex related to measurable concentrations of pyruvate, acetaldehyde, and benzaldehyde.

The term 'kappa product' was used by King and Altman [1] to define the multiplication of the product of all rate constants (known as 'rate constant product') with the substrate concentration variables for all arrows of a specific pattern.

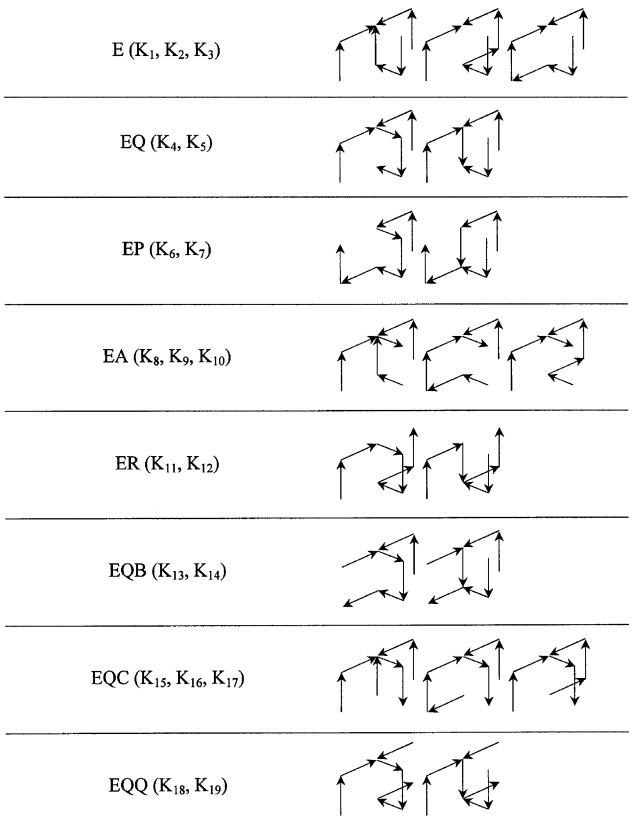

Fig. 5. The patterns for each enzyme species derived with the King and Altman procedure from the general simplified reaction mechanism in Fig. 4.

Table 1
The sum of kappa products corresponding to each enzyme species for the simplified model (Fig. 4) and the simplified model for yeast PDC ($k_{(-4)} = 0$)

Enzyme species	Sum of kappa products	
	General simplified model	Simplified model for yeast[a] ($k_{(-4)} = 0$)
E	$K_1 + K_2[Q_i] + K_3[B_i]$	$K_1 + K_2[Q_i] + K_3[B_i]$
EQ	$K_4[A_i] + K_5[Q_i]$	$K_4[A_i]$
EP	$K_6[A_i][B_i] + K_7[Q_i][B_i]$	$K_6[A_i][B_i]$
EA	$K_8[A_i] + K_9[A_i][B_i] + K_{10}[Q_i][A_i]$	$K_8[A_i] + K_9[A_i][B_i] + K_{10}[Q_i][A_i]$
ER	$K_{11}[Q_i][A_i] + K_{12}[Q_i][Q_i]$	$K_{11}[Q_i][A_i]$
EQB	$K_{13}[A_i][B_i] + K_{14}[Q_i][B_i]$	$K_{13}[A_i][B_i]$
EQC	$K_{15}[A_i] + K_{16}[A_i][B_i] + K_{17}[Q_i][A_i]$	$K_{15}[A_i] + K_{16}[A_i][B_i] + K_{17}[Q_i][A_i]$
EQQ	$K_{18}[Q_i][A_i] + K_{19}[Q_i][Q_i]$	$K_{18}[Q_i][A_i]$
$\sum$	$K_1 + (K_4 + K_8 + K_{15})[A_i] + K_3[B_i] + (K_2 + K_5)[Q_i]$ + $(K_6 + K_9 + K_{13} + K_{16})[A_i][B_i] + (K_{10} + K_{11} + K_{17} + K_{18})[Q_i][A_i]$ + $(K_7 + K_{14})[Q_i][B_i] + (K_{12} + K_{19})[Q_i][Q_i]$	$K_1 + (K_4 + K_8 + K_{15})[A_i] + K_3[B_i] + K_2[Q_i]$ + $(K_6 + K_9 + K_{13} + K_{16})[A_i][B_i] + (K_{10} + K_{11} + K_{17} + K_{18})[Q_i][A_i]$

[a] For the simplified model of yeast PDC, $K_5 = K_7 = K_{12} = K_{14} = K_{19} = 0$, because $k_{(-4)} = 0$.

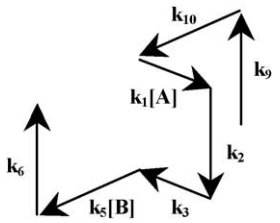

Fig. 6. As an example of kappa product derivation, the procedure is explained for the sixth entry of Fig. 5, a pattern for EP. The corresponding rate constant product and kappa product of this pattern are $k_1k_2k_3k_5k_6k_9k_{10}$ and $k_1k_2k_3k_5k_6k_9k_{10}[A][B]$ or $K_6[A][B]$, respectively. K_6 is the abbreviated term for the rate constant product derived from this Figure.

As an example, the kappa product derivation is illustrated for the enzyme complex EP in Fig. 6.

Palmer [21] illustrated clearly the application of King and Altman procedure in the derivation of the rate equations for single-substrate Michaelis–Menten kinetics and other more complicated reactions. The following relationship was used in the present study as a simplified version of determinant solutions from King and Altman:

$$\frac{[\text{enzyme complex species}]}{[E_0]} = \frac{\text{sum of kappa products for a particular enzyme species}}{\text{sum of all kappa products for all enzyme species}} \quad (8)$$

Table 2
The corresponding rate constant products of K_1–K_{19} symbols

Symbol	Rate constant product	Symbol	Rate constant product
K_1	$k_2k_3k_4k_6k_7k_9k_{10}$	K_{11}	$k_1k_2k_3k_6k_7k_8k_9$
K_2	$k_2k_3k_6k_7k_8k_9k_{10}$	K_{12}	$k_2k_3k_6k_7k_8k_9k_{(-4)}$
K_3	$k_2k_3k_5k_6k_7k_9k_{10}$	K_{13}	$k_1k_2k_3k_5k_7k_9k_{10}$
K_4	$k_1k_2k_3k_6k_7k_9k_{10}$	K_{14}	$k_2k_3k_5k_7k_9k_{10}k_{(-4)}$
K_5	$k_2k_3k_6k_7k_9k_{10}k_{(-4)}$	K_{15}	$k_1k_2k_4k_6k_7k_9k_{10}$
K_6	$k_1k_2k_3k_5k_6k_9k_{10}$	K_{16}	$k_1k_2k_5k_6k_7k_9k_{10}$
K_7	$k_2k_3k_5k_6k_9k_{10}k_{(-4)}$	K_{17}	$k_1k_2k_6k_7k_8k_9k_{10}$
K_8	$k_1k_3k_4k_6k_7k_9k_{10}$	K_{18}	$k_1k_2k_3k_6k_7k_8k_{10}$
K_9	$k_1k_3k_5k_6k_7k_9k_{10}$	K_{19}	$k_2k_3k_6k_7k_8k_{10}k_{(-4)}$
K_{10}	$k_1k_3k_6k_7k_8k_9k_{10}$		

After determination of the kappa products sum for each enzyme species, an expression for the concentration of each enzyme complex related to the concentrations of pyruvate, benzaldehyde, and acetaldehyde can be obtained from Eq. (8). Substitution of these revised enzyme complex expressions derived from the King and Altman procedure in Eqs. (1–6) resulted in a theoretical model representing the biotransformation of PAC and its related by-products based on the simplified mechanism.

The summation of kappa products derived for each complex for both bacterial and yeast PDC are listed in Table 1. The last row of Table 1 is the summation expression of all kappa products from the top rows. The symbols K_1–K_{19} are the abbreviations of rate constant products as listed in Table 2.

Using Eq. (8) and the kappa product expressions given in Table 1 (column 3), the new expressions for the concentrations of the various enzyme complexes expressed as fractions of total enzyme activity were derived for yeast PDC. These now consist only of rate constants and concentrations of measurable species.

3.2. Rate equations from the simplified reaction mechanism for yeast PDC

3.2.1. PAC production

Algebraic rearrangement of Eq. (9) obtained from Eq. (1) and the expression of EP from column three of Table 1, results in Eq. (10).

$$\left.\frac{\mathrm{d}[P]}{\mathrm{d}t}\right|_i = \frac{k_7K_6[A_i][B_i][E_{0i}]}{\sum} \quad (9)$$

$$\left.\frac{\mathrm{d}[P]}{\mathrm{d}t}\right|_i = \frac{[A_i][B_i][E_{0i}]}{\left(\frac{1}{k_1} + \left(\frac{1}{k_2} + \frac{1}{k_3}\right)[A_i]\right)\left(\frac{k_4}{k_5} + [B_i] + \frac{k_8}{k_5}[Q_i]\right) + [A_i] \times \left(\frac{1}{k_5} + \left(\frac{1}{k_6} + \frac{1}{k_7}\right)[B_i] + \frac{k_8}{k_5}\left(\frac{1}{k_9} + \frac{1}{k_{10}}\right)[Q_i]\right)} \quad (10)$$

The following assumptions have been made to simplify Eq. (10):

(i) From the biotransformation profiles obtained experimentally [22], the concentration of acetaldehyde $[Q_i]$ was significantly lower than those of benzaldehyde and pyruvate. Thus $[Q_i]$ was neglected in the above PAC rate equation.
(ii) Assuming the rate constants leading to PAC (k_5, k_6, k_7) to be large, allows further simplification by neglecting the third term of the denominator in Eq. (10).

After simplification and rearrangement, Eq. (11) is obtained. This has the form of an equation with two-substrate Michaelis–Menten type kinetics for each substrate as illustrated in Eq. (12).

$$\left.\frac{d[P]}{dt}\right|_i = \frac{\left(\frac{k_2k_3}{k_2+k_3}\right)[A_i][B_i][E_{0i}]}{\left(\frac{1}{k_1}\frac{k_2k_3}{k_2+k_3}+[A_i]\right)\left(\frac{k_4}{k_5}+[B_i]\right)} \quad (11)$$

$$v = \frac{V_p[A_i][B_i][E_{0i}]}{(K_{ma}+[A_i])(K_{mb}+[B_i])} \quad (12)$$

A double substrate kinetic model had been applied also by other authors [17,18] to predict a profile of continuous PAC production in an enzyme membrane reactor using a potent mutant of *Zymomonas mobilis* PDC.

3.2.2. Benzaldehyde consumption

Eq. (2) and the expression for EQ in Table 1 are used in solving for rate equation of benzaldehyde consumption. The resulting equation is shown as Eq. (13):

$$\left.\frac{d[B]}{dt}\right|_i = -\frac{k_5K_4[A_i][B_i][E_{0i}]}{\sum} \quad (13)$$

Because $k_5K_4/\sum$ is in fact $k_7K_6/\sum$ (from information in Tables 1 and 2), Eq. (13) can then be rearranged as shown in Eq. (14). The equation between benzaldehyde consumption and PAC formation can also be obtained by inspection of Fig. 4 under a quasi steady state assumption:

$$\left.\frac{d[B]}{dt}\right|_i = -\frac{k_7K_6[A_i][B_i][E_{0i}]}{\sum} = -\left.\frac{d[P]}{dt}\right|_i \quad (14)$$

3.2.3. Acetoin production

Substitution of Eq. (4) with kappa product expression for ER given in Table 1 results in Eq. (15). The term V_r is introduced in Eq. (16) to replace $k_{10}K_{11}/\sum$:

$$\left.\frac{d[R]}{dt}\right|_i = \frac{k_{10}K_{11}[Q_i][A_i][E_{0i}]}{\sum} \quad (15)$$

$$\left.\frac{d[R]}{dt}\right|_i = V_r[Q_i][A_i][E_{0i}] \quad (16)$$

3.2.4. Acetaldehyde production

The rate equation describing the formation of acetaldehyde (Eq. (17)) can be obtained from Eq. (5) and from Table 1 for $[E]$ and [EQ]. The term V_q for acetaldehyde formation replaces $k_4K_4/\sum$:

$$\left.\frac{d[Q]}{dt}\right|_i = \frac{k_4K_4[A_i][E_{0i}] - k_8K_4[Q_i][A_i][E_{0i}]}{\sum} \quad (17)$$

As $k_8K_4/\sum$ is in fact $k_{10}K_{11}/\sum$ (Tables 1 and 2) or V_r, Eq. (17) can be rewritten as

$$\left.\frac{d[Q]}{dt}\right|_i = V_q[A_i][E_{0i}] - V_r[Q_i][A_i][E_{0i}] \quad (18)$$

3.2.5. Pyruvate consumption

By inserting the expression of E from Table 1 to Eq. (3), Eq. (19) is obtained:

$$\left.\frac{d[A]}{dt}\right|_i = -\frac{\begin{array}{c}k_1K_1[A_i][E_{0i}] + k_1K_2[Q_i][A_i][E_{0i}] \\ +k_1K_3[A_i][B_i][E_{0i}]\end{array}}{\sum} \quad (19)$$

Writing $k_1K_1/\sum$ as V_q, and $k_1K_2/\sum$ as V_r, and expressing $k_1K_3/\sum$ as $k_7K_6/\sum$ (Tables 1 and 2), Eq. (19) can be rewritten then as Eq. (20):

$$\left.\frac{d[A]}{dt}\right|_i = -V_q[A_i][E_{0i}] - V_r[Q_i][A_i][E_{0i}] - \left.\frac{d[P]}{dt}\right|_i \quad (20)$$

Further rearrangements of Eq. (20) using Eq. (16) and Eq. (18) results in Eq. (21):

$$\left.\frac{d[A]}{dt}\right|_i = -\left.\frac{d[P]}{dt}\right|_i - \left.\frac{d[Q]}{dt}\right|_i - 2\left.\frac{d[R]}{dt}\right|_i \quad (21)$$

The coefficient of 2 in the rate equation for acetoin reflects the fact that it requires two moles of pyruvate for one mole of its production.

3.2.6. CO_2 production

Eq. (22) is obtained after combination of Eq. (6) with the expression for EQC given in Table 1:

$$\left.\frac{d[C]}{dt}\right|_i = \frac{\begin{array}{c}k_3K_{15}[A_i][E_{0i}] + k_3K_{17}[Q_i][A_i][E_{0i}] \\ +k_3K_{16}[A_i][B_i][E_{0i}]\end{array}}{\sum} \quad (22)$$

As $k_3K_{15}/\sum$ can be written as V_q, $k_3K_{17}/\sum$ as V_r, and $k_3K_{16}/\sum$ as $k_7K_6/\sum$ (Tables 1 and 2), Eq. (22) can be rewritten as Eq. (23):

$$\left.\frac{d[C]}{dt}\right|_i = V_q[A_i][E_{0i}] + V_r[Q_i][A_i][E_{0i}] + \left.\frac{d[P]}{dt}\right|_i \quad (23)$$

The modification of Eq. (23) is performed then in similar way to that for Eq. (20) to obtain Eq. (24):

$$\left.\frac{d[C]}{dt}\right|_i = \left.\frac{d[P]}{dt}\right|_i + \left.\frac{d[Q]}{dt}\right|_i + 2\left.\frac{d[R]}{dt}\right|_i \quad (24)$$

Table 3
Kinetic parameters used in the construction of simulation profiles in Fig. 7a

Kinetic parameters	Unit	Initial searching values (source of values where applicable)	Values
V_p	$\mu mol\ h^{-1}\ U^{-1}$	5.50	4.40
K_{ma}	mM	2.20 [40]	2.64
K_{mb}	mM	42.0 [40]	50.4
V_q	$ml\ h^{-1}\ U^{-1}$	7.70×10^{-4}	7.00×10^{-4}
V_r	$l^2\ h^{-1}\ U^{-1}\ mol^{-1}$	2.60×10^{-5}	2.60×10^{-5}
k_{d1}	h^{-1}	2.64×10^{-3} [3]	3.17×10^{-3}
k_{d2}	$mM^{-1}\ h^{-1}$	1.98×10^{-4} [3]	2.38×10^{-4}
t_{lag}	h	5.23 [3]	4.19

Each parameter was searched within ±20% of initial values.

3.3. Simulation profiles: comparison with experimental data

The system of differential equations developed here forms the basis of a time profile for a batch biotransformation process of PAC production. Simultaneous numerical integration of Eqs. (7), (12), (14), (16), (18), and Eq. (21) with the Euler–Cauchy method [24] results in concentration-time profiles for benzaldehyde and pyruvate consumption, PAC, acetaldehyde and acetoin formation and in a profile for PDC deactivation. Eq. (24) for the CO_2 production profile may be relevant for the future design of a feedback control strategy for substrate feeding based on the rate of CO_2 emission.

To illustrate the general characteristics of the model, a simulation profile was generated using kinetic parameters derived from previously published experimental data for production of PAC using PDC from *C. utilis* [22]. Initial benzaldehyde and pyruvate concentrations were 400 and 600 mM in 2.5 M MOPS with initial enzyme activity of 8.4 U carboligase ml^{-1}, pH 6.5 at 6 °C. The starting values of the kinetic parameters are given in Table 3. In the generation of the simulation curves and their optimal fitting to the data, a parameter searching program was applied with each parameter value allowed to 'float' by ±20% from initial values. The results of this searching program designed to minimize the residual sum of squares (RSS) are shown in the simulation curves (Fig. 7a) and the optimal parameter values (Table 3). However, it was evident from these results that PAC production was underestimated initially and overestimated at the end with an overall RSS value of 3.96×10^4.

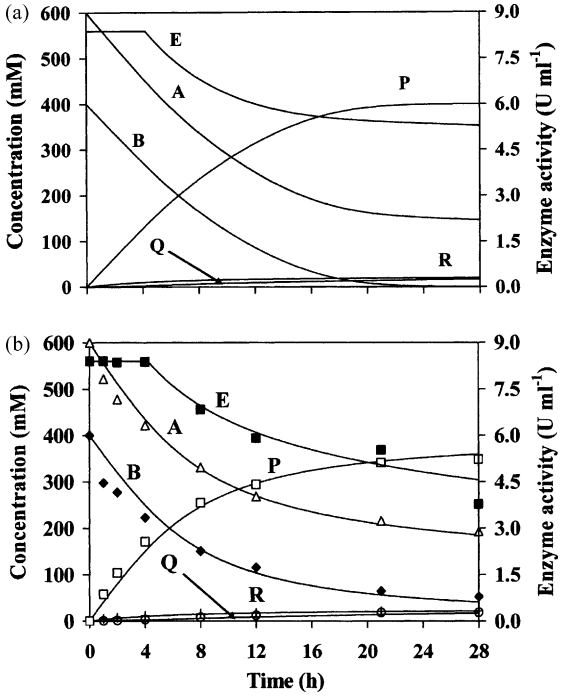

Fig. 7. Simulation profiles from the developed rate equations for PAC production with initial substrates concentration of 600 mM sodium pyruvate and 400 mM benzaldehyde. The experimental data for partially purified *C. utilis* PDC in 2.5 M MOPS, 0.5 mM $MgSO_4$, 1 mM TPP, initial pH 6.5 at 6 °C were published by Rosche et al. [22]. The simulation was based on the PAC rate equation from (a) two-substrate Michaelis–Menten type kinetics for benzaldehyde and pyruvate (Eq. (12)) and (b) modified Eq. (12) with sigmoidal relationship of benzaldehyde [23]. Each capital letter represents substrate, product, or by-product; pyruvate (A), benzaldehyde (B), PAC (P), acetaldehyde (Q), acetoin (R), and relative carboligase activity (E).

Table 4
Kinetic parameters used in the construction of simulation profiles in Fig. 7b

Kinetic parameters	Unit	Initial searching values (source of values where applicable)	Values
V_p	$\mu mol\ h^{-1}\ U^{-1}$	5.50	6.60
K_b	$mM^{-0.77}$	1.00×10^{-4} [23]	8.00×10^{-5}
h	No unit	2.18 [23]	1.77
K_{ma}	mM	2.20 [40]	1.76
V_q	$ml\ h^{-1}\ U^{-1}$	7.70×10^{-4}	7.02×10^{-4}
V_r	$l^2\ h^{-1}\ U^{-1}\ mol^{-1}$	2.60×10^{-5}	2.40×10^{-5}
k_{d1}	h^{-1}	2.64×10^{-3} [3]	3.17×10^{-3}
k_{d2}	$mM^{-1}\ h^{-1}$	1.98×10^{-4} [3]	2.38×10^{-4}
t_{lag}	h	5.23 [3]	4.20

Each parameter was searched within ±20% of initial values.

The above simulation was based on the King–Altman approach, which in its simplified form, decreases to two-substrate Michaelis–Menten type kinetics for benzaldehyde and pyruvate transformation.

However, in previous studies it was found that the effect of benzaldehyde could be best described by a sigmoidal relationship [23]. For this reason, Eq. (12) was modified and K_{mb} replaced by K_b and h, with the latter in the sigmoidal equation (see Table 4). A searching program was again employed with the parameter values allowed to 'float' by ±20%. The optimal simulation curves are shown in Fig. 7b and compared with the experimental data and the related kinetic parameter values are given in Table 4. Under these conditions, the RSS value was decreased to 1.10×10^4 and an improved data fit was evident.

4. Discussion and conclusions

The method of King and Altman used in the current study has been applied by various other authors for the derivation of rate equations from complex reaction systems. These include the reaction of acetylcholine esterase to breakdown the neurotransmitter acetylcholine to acetate and choline [25], the hydrolysis of ATP by (Na,K)-ATPase [26], the reduction of cytochrome-c with reductase [27], the inhibition of xanthine oxidase by uric acid in the reduction of xanthine [28], and endocytosis of sucrose lipoprotein by Hep-G2 cells [29]. The alternative procedure for evaluating King and Altman diagrams was also suggested by Myers and Palmer [30].

The computer algorithm for King and Altman method was given by Olavarria [31] to calculate all patterns, including those containing cycles. Further extension of the method has been made by Zhao [32] to the analysis of relaxation times of enzyme-catalysed reactions and by Mogi [33] to "a graphic transformation method" in obtaining the steady-state distribution of a coupled system. Mazur and Kuchinski [34] related the similarity of King and Altman procedure to probabilistic enzyme kinetics in avoiding the use of the mass action law for deriving rate equations. Topham and Brocklehurst [35] used a King and Altman schematic diagram in an evaluation of the general validity of the Cha [36] method, an alternative method for deriving rate equations.

The advantage of constructing a model from its overall reaction mechanisms is that all neglected variables and rate constants can be recovered if evidence negating the validity of one or more of the assumptions emerges.

In the present study, a simplified mathematical model has been developed which describes the enzymatic conversion of pyruvate and benzaldehyde by PDC to PAC and associated by-products acetaldehyde, acetoin and carbon dioxide. The model incorporates the accepted mechanism of PAC production involving pyruvate decarboxylation by PDC to form an 'active acetaldehyde' enzyme complex, followed by its subsequent reaction with benzaldehyde (or acetaldehyde to form by-product acetoin). It also accounts for the experimental observation that PDC from some bacteria (e.g. *Zymomonas* sp.) can efficiently convert acetaldehyde and benzaldehyde to PAC [37], while PDC from various yeasts and fungi require pyruvate rather than acetaldehyde for the biotransformation [15].

Detailed experimental determination of kinetic parameters and model validation studies over a low value range of initial benzaldehyde and pyruvate concentrations were reported in an earlier publication [23]. In this study, kinetic values were determined from data of three batch biotransformation profiles by *C. utilis* PDC over a range of initial concentrations (viz. 50–150 mM benzaldehyde, 60–180 mM pyruvate, 1.1–3.4 U ml^{-1} enzyme activity). The model with these parameter values was then used to predict a batch biotransformation profile at 120/100 mM initial pyruvate/benzaldehyde (initial enzyme activity 3.0 U ml^{-1}). An acceptable fitting to the profiles of substrates pyruvate and benzaldehyde, product PAC, by-products acetaldehyde and acetoin, as well as enzyme activity level was obtained.

In the present study, it was found that an improved fit of an experimental data set could be achieved if the King–Altman model was modified by replacing the Michaelis–Menten relationship for benzaldehyde with an empirically derived sigmoidal relationship. However, it needs to be recognized that this is an essentially pragmatic approach that does not provide any real insights into underlying mechanisms. Furthermore, additional effects such as substrate and product inhibition may need to be included in a more comprehensive model applicable for higher initial benzaldehyde and pyruvate concentrations and subsequent increased PAC, acetaldehyde and acetoin levels.

The value of the present study is that it develops a model for a complex enzymatic reaction based on the King–Altman procedure which may be used ultimately for further kinetic analysis and optimization of PAC production. It demonstrates also that the model can be extended to higher concentrations of benzaldehyde and pyruvate than previously investigated.

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