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Mathematical model with phosphate activation effect validates batch (*R*)-phenylacetylcarbinol biotransformation process utilizing *Candida tropicalis* pyruvate decarboxylase in phosphate buffer

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Novelty: The existing (*R*) - phenylacetylcarbinol (PAC) biotransformation model in literatures did not include activation effect of buffering species into the enzyme deactivating rate equation, thereby background deactivation and buffering activation effects could not be discerned. A new mathematical model which incorporated activation effect, using 250 mM phosphate buffer as an example, was able to predict and validate PAC batch biotransformation systems reasonably well and could pave the way for model applications in more complex processes.

ABSTRACT

The new (*R*) - phenylacetylcarbinol (PAC) batch biotransformation kinetics for partially purified *Candida tropicalis* TISTR 5350 pyruvate decarboxylase (PDC) were characterized and validated with a comprehensive mathematical model in 250 mL scale with 250 mM phosphate buffer / pH 7.0. PDC could convert initial 100 / 120 mM benzaldehyde / pyruvate substrates to the statistical significantly highest ($p \leq 0.05$) maximum PAC concentration (95.8 ± 0.1 mM) and production rate (0.639 ± 0.001 mM min⁻¹). A parameter search strategy aimed at minimizing overall residual sum of square (RSS_T) based on a system of six ordinary differential equations was applied to PAC biotransformation profiles with initial benzaldehyde / pyruvate concentration of 100/120 and 30/36 mM. Ten important biotransformation kinetic parameters were then elucidated including the zeroth order activation rate constant due to phosphate buffer species (k_a) of $(9.38 \pm < 0.01) \times 10^{-6}$ % relative PDC activity min⁻¹ mM⁻¹. The validation of this model to independent biotransformation kinetics with initial benzaldehyde / pyruvate concentration of 50 / 60 mM resulted in relatively good fitting with RSS_T , mean sum of square error (MSE), and coefficient of determination (R^2) values of 662, 17.4, and 0.9863, respectively.

Keywords: *Candida tropicalis*; phenylacetylcarbinol; pyruvate decarboxylase; mathematical model; biotransformation; phosphate buffer

Introduction

Microbial biotransformation has been extensively used worldwide in the pharmaceutical industry followed by the food and agriculture sectors^{1,2} for almost 20 years. Revenue generated by microbial biotransformation steadily increased from USD 0.39 trillion in 2001 to USD 1.25 trillion in 2019 within the pharmaceutical industry. The food and agriculture sectors had estimated USD 1.13 trillion (2019) and USD 1.84 billion (2018) in revenue respectively from microbial biotransformation³⁻⁵. Although chemocatalysts can offer the relatively high catalytic activity and selectivity for some reactions^{6,7}, a number of organic compounds transformation processes still rely heavily on biocatalysts to achieve the desired level of enantioselectivity⁸⁻¹⁰. Thus, biocatalysts including enzymes, cells organelles, and whole cells in either native or artificially constructed forms¹¹ have been widely used in the production of both high-volume / low-value compounds such as ethanol¹²⁻¹⁷ and low-volume / high-value chemical species including (*R*)-phenylacetylcarbinol (PAC)^{2,15,18-24}.

The biochemical production of PAC was firstly demonstrated in Germany and later commercialized for ephedrine production²⁵⁻²⁷. PAC could be produced through *in vivo* direct microbial transformation process with some strategies of benzaldehyde feeding using growing cells of yeasts, fungi, and bacteria²⁸⁻³⁰. This biotransformation process can be conducted *in vitro* by using non-viable whole cells^{15,17,24} and partially purified pyruvate decarboxylase (PDC) enzyme^{18,20,28,29,31-34}. The detailed reaction mechanism of PAC biotransformation was clearly elucidated¹⁸. Advantages of using partially purified PDC include prevention of benzyl alcohol or PAC-diol, by-products that are often formed when PAC biotransformation is performed in parallel with microbial cultivation process^{32,35}. Moreover, the isolated enzymes could be recycled and reused during a biotransformation process²⁷. The partially purified

Candida utilis (ICI Australia) could produce 190.4 mM PAC with molar yield as high as 95.3% based on benzaldehyde^{31,32,35}. The PAC concentration could generally be increased through fed-batch processes with either pyruvate (in the form of pyruvic acid) or benzaldehyde dosing protocols using 2.5 M or 20 mM of 3-morpholinopropane-1-sulfonic acid (MOPS) buffer^{18-20,33}. However, the heat labile property and cost-prohibitive nature of MOPS buffer (USD 1.09/g in comparison with only USD 0.02/g for phosphate buffer) were considered major obstacles to the industrial scale application of this buffering compound³⁴.

Our previous research showed that, among fifty microbial strains, *C. tropicalis* TISTR 5350 and 5306 were the best yeast strains for PAC production. The two strains yielded 70% increase in volumetric PDC carboligase activity (0.39 ± 0.06 U mL⁻¹) and 58% increased PAC production (19.8 ± 3.4 mM)²¹. However, the strain TISTR 5306 was most suitable for longan extract medium^{16,21-23,34}. Cells disruption strategy with 15 µg mL⁻¹ silver nanoparticles was effective in reducing the treatment time to 3 min prior to purification steps of the released PDC from whole cells of *C. tropicalis* TISTR 5350²³. The subsequent investigation on the best strategy to obtain partially purified PDC revealed that the specific PDC activity obtained from 50% (v/v) acetone precipitation was statistically higher than 40 - 60% (w/v) ammonium sulphate counterparts²³. In order to minimize cost, prevent relatively high overall losses of enzyme activity through purification process, and avoid utilization of cost ineffective MOPS buffer, Khemacheewakul et al.³⁴ used whole cells *C. tropicalis* TISTR 5350 in single phase PAC production and benzaldehyde deactivation studies with various concentrations of phosphate buffer (20 mM - 2.5 M). A thorough examination indicated that 1 M phosphate buffer was optimal for PAC production (28.6 ± 2.3 mM) and provided additional activation effect to PDC stability. The average PDC activation rate constant of 1 M phosphate buffer was higher than 20 mM by more than 9,050 times. Leksawasdi et al.² projected that in a circulated system of recycled phosphate buffer and related co-factors utilizing whole cells of *Saccharomyces cerevisiae* and *C. tropicalis* co-culture as biocatalysts, the costing of phosphate buffer would be USD 0.751 per 1 kg of produced PAC. The similar investigation on the partially purified PDC of *C. tropicalis* TISTR 5350 is thus required to evaluate relevant PAC production characteristics and assess whether the newly developed deactivation rate equation can interact well to the other five rate equations in a PAC biotransformation mathematical model.

The objectives of present study are to investigate the effects of initial substrates concentration pairs (benzaldehyde/pyruvate of 30/36, 50/60, and 100/120 mM) and phosphate buffer concentration levels (20, 250, 500, and 1 M; pH 7.0) on PAC production, PAC formation rate, and PAC molar yields using partially purified *C. tropicalis* TISTR 5350 in the 250-mL single phase batch biotransformation system. In addition, the recently improved mathematical model comprised six ordinary differential equations plus PDC-activation effect by phosphate buffering species is used to determine ten kinetic parameters based on biotransformation profiles of the 30/36 and 100/120 mM initial benzaldehyde / pyruvate concentration pairs. The model is then validated with an independent batch biotransformation system using initial substrates concentration pair of 50/60 mM followed by assessment of relevant statistical parameters.

Materials and methods

Microorganism. *C. tropicalis* TISTR 5350 was purchased from Thailand Institute of Scientific and Technological Research (TISTR, Pathum Thani, Thailand) before subculturing into primary stock²¹. The microbial stock was kept in 60% (v/v) glycerol solution and maintained at -70°C with regular assessments of cells viability by using a haemocytometer¹⁶. The viable cells count was $92.5 \pm 1.8\%$ ²¹. *C. tropicalis* TISTR 5350 was chosen based on optimal PAC production and volumetric PDC carboligase activity in Yeast - Malt (YM) medium^{16,21–23,34}.

Cultivation of microbes and production of partially purified PDC. The cultivation of preseed and seed was done in 0.1 and 1 L yeast media as described by Khemacheewakul et al.³⁴. In order to produce enough wet biomass for the production of partially purified PDC, the cultivation was carried out at 30°C for 36 h in a 16 l stainless steel bioreactor containing 10 l inoculum medium with a composition similar to those published previously by our group²³. The methodologies for preparation of cells pellet, glass bead pretreatment, subsequent precipitation with 50%(v/v) acetone, and production of partially purified PDC have already been stated elsewhere^{22,23}.

PDC production in phosphate buffer with various buffer and initial substrate concentration levels. The pH-controlled, single-phase batch biotransformation system was carried out for 210-min with a sampling interval of 30 min at 4°C in 250 mL phosphate buffer (pH 7.0)³⁴. This buffer is also known as the Gomori buffer because it contains the K_2HPO_4 and KH_2PO_4 conjugated acid-base pair³⁶. A full factorial design experiment with four phosphate buffer concentration levels (20, 250, 500, and 1 M) and three pairs of initial benzaldehyde/pyruvate substrate concentration levels (30/36, 50/60 and 100/120) were conducted. A total of $4 \times 3 = 12$ groups was studied in quintuplicates. The rationale for selecting phosphate buffer at these four concentration levels utilized also by previous studies had already been elaborated in details elsewhere³⁴. Partially purified PDC was added as a biocatalyst while 1 mM thiamine pyrophosphate (TPP) and 1 mM $MgSO_4 \cdot 7H_2O$ were also included as cofactors^{29,35}. Maximum PAC production level (mM), maximum PAC formation rate ($mM \min^{-1}$), and corresponding PAC molar yield (the ratio of PAC being produced over substrate being consumed) for each substrate pair at the maximum PAC production level were measured using high performance liquid chromatography (HPLC). The maximum PAC formation rate was computed by dividing maximum PAC production level at each time point with its corresponding reaction time (30, 60, 90, 120, 150, 180, and 210 min). The initial volumetric PDC carboligase activity in each experiment was maintained between 1.1 – 1.5 U carboligase mL^{-1} .

Analytical Methods. The collected samples were separated into liquid buffering and enzymatic portions through centrifugation process prior to respective sample pretreatment stages as described in literatures^{19,20,29,32,33,35}. PAC, benzaldehyde, benzyl alcohol, and benzoic acid concentration levels were determined by using HPLC as previously described by Rosche et al.²⁹. Acetoin was analyzed by HPLC equipped with the HPX-87H column³⁷. Pyruvate concentration was determined spectrophotometrically by using the enzymatic $NADH + H^+$ coupled assay method with lactate dehydrogenase based on the modified method from Czok and Lamprecht³⁸. Acetaldehyde concentration was determined in the same manner as the

pyruvate assay with the replacement of lactate dehydrogenase with alcohol dehydrogenase based on a modified method from Bernt and Bergmeyer³⁹. One unit PDC carboligase activity was defined as the rate of which 1 μmol PAC was produced from equimolar benzaldehyde and pyruvate per min in carboligase buffer at pH 6.0 and 25°C¹⁸.

Hypothesis testing. Averages and standard errors were calculated from experimental data in quintuplicates. The results obtained were statistically analyzed using SPSS for Windows 22.0 (SPSS Inc., Chicago, IL, USA) and the analysis of variance using Duncan's Multiple Range Test (DMRT) at $p \leq 0.05$ ^{15,24,40}.

Construction of PAC biotransformation model. The PAC biotransformation model for *C. tropicalis* TISTR 5350 in this study consisted of six rate equations that described the main product (PAC) formation (1), substrates (pyruvate and benzaldehyde) consumption (2, 3), by-products (acetaldehyde and acetoin) formation (4,5) as well as deactivation of pyruvate decarboxylase with inorganic phosphate activation effect (6). Eq. (1) to (5) were derived and determined based on the combined King and Altman schematic strategy as well as the initial rate studies by Leksawasdi et al.^{18–20}. Eq. (6) was modified and developed by Khemacheewakul et al.³⁴ for whole cells *C. tropicalis* TISTR 5350. This equation combined both the PDC deactivation effect by background species excluding buffer concentration and benzaldehyde (k_{d1} , k_{d2}) as well as the PDC activation effect by inorganic phosphate species ($\text{K}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$) within the 20 mM to 1 M concentration range (k_a). The complete simulation profile for each species of PAC biotransformation kinetics was constructed based on Euler–Cauchy numerical integration with a time increment of 0.01 h¹⁸. The description for each parameter / variable is given in the nomenclature section.

$$\left. \frac{dP}{dt} \right|_i = V_p \left(\frac{K_b B_i^h}{1 + K_b B_i^h} \right) \left(\frac{A_i}{K_{ma} + A_i} \right) E_i \quad (1)$$

$$\left. \frac{dA}{dt} \right|_i = -\left. \frac{dP}{dt} \right|_i - \left. \frac{dQ}{dt} \right|_i - 2 \left. \frac{dR}{dt} \right|_i \quad (2)$$

$$\left. \frac{dB}{dt} \right|_i = -\left. \frac{dP}{dt} \right|_i \quad (3)$$

$$\left. \frac{dQ}{dt} \right|_i = V_q A_i E_i - V_r A_i Q_i E_i \quad (4)$$

$$\left. \frac{dR}{dt} \right|_i = V_r A_i Q_i E_i \quad (5)$$

$$\left. \frac{dE}{dt} \right|_i = \begin{cases} 0, & t < t_{lag} \\ -(k_{d1} + k_{d2} \cdot B) E_i + k_a \cdot P, & t \geq t_{lag} \end{cases} \quad (6)$$

Strategy for parameters estimation and model validation. The initial values of each parameter for Eq. (1) – (5) were averaged from values reported in previous initial rate and simulation studies of a high-buffering-capacity biotransformation system^{18,19} using partially purified PDC from *C. utilis* in 2.5 M MOPS buffer (Table 2). The initial values for the zeroth order activation rate constant due to phosphate buffer species (k_a) as well as the other three parameters (k_{d1} , k_{d2} and t_{lag}) in Eq. (6) were obtained from a recent PDC deactivation study using whole cells PDC from *C. tropicalis* TISTR 5350³⁴ in 20 mM – 1 M phosphate buffer. Parameter estimation and model validation were performed using the customized and well - established subroutines written in Microsoft Visual Basic for Applications (VBA) 6.3 for Microsoft EXCEL^{®12,14,18,19,34}. Specifically, the minimization of total residual sum of square (RSS_T) between experimental data and simulated values from the model, and the convergence search criterion (CSC) of lesser than 1% of RSS_T were set as objective functions while the mean square error (MSE) and the coefficient of determination (R^2) were calculated simultaneously. RSS_T for each set of biotransformation profiles is the summation of individual RSS values for product and by-products formation, substrates consumption, and enzyme deactivation profiles as shown in Eq. (7). MSE is the ratio of RSS_T and available degree of freedom (DOF) for each system. DOF is the number of experimental data points being regressed minus the total number of parameters in the mathematical model. Biotransformation profile data from the 30 / 36 and the 100 / 120 mM initial benzaldehyde / pyruvate concentration pairs were used for parameter estimation in this biotransformation model. The error estimation of each parameter was evaluated using the standard error of each time point as described by Khemacheewakul et al.³⁴.

$$RSS_T = RSS_A + RSS_B + RSS_Q + RSS_R + RSS_E \quad (7)$$

Where A refers to pyruvate concentration, B refers to benzaldehyde concentration, Q refers to acetaldehyde concentration, R refers to acetoin concentration, and E refers to relative enzyme activity.

The estimated parameters were then validated by being implemented in Eq. (1) – (6) and numerically integrated to simulate the biotransformation profiles of an independent data set obtained from the 50/60 mM substrate pair. The assessment of relevant statistical parameters (RSS_T , MSE, R^2) was then made to evaluate the quality of fit.

Results

PDC production in phosphate buffer with various buffer and initial substrate concentration levels. The current study investigated a single-phase batch biotransformation process using partially purified *C. tropicalis* TISTR 5350 PDC as a biocatalyst. The maximum PAC production levels ($P_{\max}$), maximum PAC formation rates ($r_{P_{\max}}$), and PAC molar yields with respect to each substrate ($Y_{P/B}$ and $Y_{P/A}$) under different initial substrate and phosphate buffer concentration levels are tabulated in Table 1. The 100 / 120 mM initial benzaldehyde / pyruvate substrate pair in 250 mM phosphate buffer showed the highest statistical significantly highest ($p \leq 0.05$) $P_{\max}$ (95.8 ± 0.1 mM) and $r_{P_{\max}}$ (0.639 ± 0.001 mM min⁻¹) with corresponding $Y_{P/B}$ and $Y_{P/A}$ of 0.99 ± 0.01 and 0.88 ± 0.01 based on benzaldehyde and pyruvate, respectively.

Table 1: Concentration level effects of phosphate buffer (Ph_b) and initial substrates (benzaldehyde (B) as well as pyruvate (A)) on maximum PAC production levels ($P_{\max}$), maximum PAC formation rates ($r_{P_{\max}}$), as well as corresponding PAC - substrate molar yields based on benzaldehyde ($Y_{P/B}$) and pyruvate ($Y_{P/A}$) being consumed at maximum PAC production level in single - phase biotransformation systems with initial volumetric enzyme activity between 1.1 - 1.5 U carboglycase mL⁻¹.

(B/A) (mM)	Ph_b (mM)	$P_{\max}$ (mM)		$r_{P_{\max}}$ (mM min ⁻¹)		$Y_{P_{\max}}$ for each substrate							
						$Y_{P_{\max}/B}$				$Y_{P_{\max}/A}$			
(30/ 36)	20	15.3 ± 0.26	f	0.128 ± 0.001	f	0.87 ± 0.01	c	0.62 ± 0.01	g				
	250	25.8 ± 0.14	e	0.143 ± 0.001	f	<u>0.97 ± 0.01</u>	<u>ab</u>	<u>0.97 ± 0.04</u>	<u>ab</u>				
	500	25.4 ± 0.28	e	0.169 ± 0.001	ef	0.95 ± 0.01	b	0.66 ± 0.01	f				
	1,000	15.5 ± 0.09	f	0.103 ± 0.001	g	<u>0.97 ± 0.01</u>	<u>ab</u>	0.81 ± 0.01	e				
(50/ 60)	20	40.6 ± 0.21	c	0.193 ± 0.001	e	<u>0.99 ± 0.01</u>	<u>a</u>	<u>0.99 ± 0.01</u>	<u>a</u>				
	250	39.2 ± 0.62	c	0.261 ± 0.001	d	<u>0.99 ± 0.04</u>	<u>a</u>	0.93 ± 0.01	c				
	500	39.5 ± 1.14	c	0.188 ± 0.005	e	<u>0.96 ± 0.03</u>	<u>ab</u>	<u>0.99 ± 0.03</u>	<u>a</u>				
	1,000	35.2 ± 0.13	cd	0.168 ± 0.001	ef	<u>0.98 ± 0.00</u>	<u>ab</u>	0.90 ± 0.02	d				
(100/ 120)	20	83.7 ± 0.46	b	0.465 ± 0.001	c	<u>0.98 ± 0.01</u>	<u>ab</u>	0.95 ± 0.01	bc				
	250	<u>95.8 ± 0.11</u>	<u>a</u>	<u>0.639 ± 0.001</u>	<u>a</u>	<u>0.99 ± 0.01</u>	<u>a</u>	0.88 ± 0.01	d				
	500	85.5 ± 0.65	b	0.407 ± 0.001	c	<u>0.99 ± 0.01</u>	<u>a</u>	<u>0.99 ± 0.01</u>	<u>a</u>				
	1,000	89.7 ± 0.62	b	0.598 ± 0.001	b	<u>0.99 ± 0.01</u>	<u>a</u>	<u>0.98 ± 0.01</u>	<u>ab</u>				

The numbers with the same alphabet (a - g) indicated no statistical significantly difference ($p > 0.05$) for comparison between different rows of the same column within each group of initial substrates concentration pair. Bold and underline numbers and alphabets showed the statistical highest values within each column.

The reaction time resulting in the $P_{\max}$ for each run could be determined by dividing $P_{\max}$ with $r_{P_{\max}}$ whose value was the multiple of 30 min (30, 60, 90, 120, 150, 180, and 210 min). Rounding-off errors of the tabulated values might result in non-integer reaction times which should be rounded to the nearest multiple of 30 min, for instance, reaction time for PAC biotransformation with initial substrates (B , A) of (30, 36) mM in 250 mM phosphate buffer producing $P_{\max}$ of 25.8 mM was $25.8 \text{ mM} / 0.143 \text{ mM min}^{-1} = 180.42 \approx 180 \text{ min}$.

PAC biotransformation model and model validation. Simultaneous numerical integration of rate equations Eq. (1) - (6) was implemented to simulate a combined biotransformation kinetic profile of two benzaldehyde/pyruvate concentration pairs: 30/36 and 100/120 mM. The simulation profiles of the optimized parameters are shown in Fig. 1(a) and 1(b). The initial searching values were extracted from Leksawasdi et al.^{18,19} and Khemacheewakul et al.³⁴ (Table 2). Optimization of these kinetic values by a grid-search parameter estimation strategy improved fitting of the model as evident by lowering of RSS_T from 67,465 to 3,462 and MSE from 1,775 to 91.1 (19.5 times improvement). Model fitting statistics RSS_T , MSE and R^2 for individual biotransformation kinetic profiles are presented in Table 3. These were 936, 24.6 and 0.9717 for the 30/36 mM initial benzaldehyde / pyruvate concentration levels and 2,526, 66.5 and 0.9787 for the 100/120 mM levels, respectively.

The model validation was done on an independent biotransformation kinetic profile with initial benzaldehyde / pyruvate concentration levels of 50 / 60 mM. The relatively good quality of fitting could be observed visually as shown in Fig 1(c). RSS_T , MSE, and R^2 of the fitted model were 662, 17.4, and 0.9863, respectively (Table 3).

Table 2: Initial searching values and optimized values of kinetic parameters with corresponding RSS_T , MSE, and R^2 of PAC biotransformation model for partially purified PDC from *C. tropicalis* TISTR 5350 using initial substrates (pyruvate and benzaldehyde) concentration levels of (30/36) and (100/ 120) mM as well as initial volumetric enzyme activity between 1.1 – 1.5 U mL⁻¹.

Kinetic parameters	Units	References	Initial searching values	Optimized values
V_P	mM min ⁻¹ % ⁻¹	(I)	1.55×10^{-2}	$(6.71 \pm 0.20) \times 10^{-2}$
K_B	mM ^{1-h}	(I)	9.00×10^{-5}	$(1.01 \pm 0.02) \times 10^{-4}$
H	no unit	(I)	1.98	1.94 ± 0.01
K_{ma}	mM	(I)	4.84	0.70 ± 0.01
V_q	min ⁻¹ % ⁻¹	(I)	6.38×10^{-6}	$(1.68 \pm 0.11) \times 10^{-5}$
V_r	min ⁻¹ mM ⁻¹ % ⁻¹	(I)	9.88×10^{-7}	$(6.06 \pm 0.37) \times 10^{-6}$
k_{d1}	min ⁻¹	(II)	8.89×10^{-3}	$(5.99 \pm 0.23) \times 10^{-3}$
k_{d2}	min ⁻¹ mM ⁻¹	(II)	3.30×10^{-5}	$(1.33 \pm < 0.01) \times 10^{-5}$
k_a	% min ⁻¹ mM ⁻¹	(II)	1.34×10^{-5}	$(9.38 \pm < 0.01) \times 10^{-6}$
t_{lag}	min	(II)	1.00	0.42 ± 0.37
RSS_T			67,465	3,462
MSE			1,775	91.1
R^2			0.7176	0.9772

(I): Initial rate and batch biotransformation kinetics studies by Leksawasdi et al.^{18,19} whose values were averaged and the relevant enzyme activity unit was normalized to relative percentage; (II): Benzaldehyde deactivation kinetics with inorganic phosphate buffer activation effect by Khemacheewakul et al.³⁴. Full name of each parameter was given in nomenclature section.

Table 3: Apparent initial concentration values of chemical species (enzyme activity (*E*), pyruvate (*A*), benzaldehyde (*B*), PAC (*P*)) in the batch biotransformation processes utilized by PAC biotransformation model with corresponding individual RSS, MSE, and R^2 for partially purified PDC from *C. tropicalis* TISTR 5350. The initial volumetric enzyme activity for each experiment was between 1.1 – 1.5 U mL⁻¹ with experimental benzaldehyde and pyruvate concentration levels of (30/36), (50/60), and (100/120) mM.

Species	Units	Initial concentration of chemical species in PAC biotransformation model for each experimental B/A pair		
		30/36 [†]	50/60 [‡] (validation)	100/120 [†]
<i>E</i>	%	89.4	96.4	114
<i>A</i>	mM	35.2	59.1	116
<i>B</i>	mM	30.9	50.4	100
<i>P</i>	mM	0.01	0.01	0.01
RSS _T		936	662	2,526
MSE		24.6	17.4	66.5
R ²		0.9717	0.9863	0.9787

[†]These experimental pairs were used in parameters determination process

[‡]The validity of parameters in PAC biotransformation model was confirmed by this experimental pair

Table 4: Comparison of normalized kinetic parameters from literatures and current study.

Kinetic para- meters	Units	*Leksawasdi et al. ¹⁸		†Leksawasdi et al. ¹⁹		‡Current study	
V_P	mM min ⁻¹ % ⁻¹	$(1.30 \pm 0.02) \times 10^{-2}$	c	$(1.56 \pm 0.03) \times 10^{-2}$	b	$(\underline{6.71 \pm 0.20}) \times 10^{-2}$	<u>a</u>
K_B	mM ^{1-h}	$(\underline{1.00 \pm < 0.01}) \times 10^{-4}$	<u>a</u>	$(\underline{0.80 \pm < 0.01}) \times 10^{-4}$	<u>a</u>	$(\underline{1.01 \pm 0.02}) \times 10^{-4}$	<u>a</u>
h	no unit	$\underline{2.18 \pm 0.58}$	<u>a</u>	$\underline{1.77 \pm 0.47}$	<u>a</u>	$\underline{1.94 \pm 0.01}$	<u>a</u>
K_{ma}	mM	$\underline{7.91 \pm 8.21}^{\S}$	a,b	$\underline{6.33 \pm 0.10}$	<u>a</u>	0.70 ± 0.01	b
V_q	min ⁻¹ % ⁻¹	$(0.61 \pm < 0.01) \times 10^{-5}$	b	$(0.03 \pm < 0.01) \times 10^{-5}$	c	$(\underline{1.68 \pm 0.11}) \times 10^{-5}$	<u>a</u>
V_r	min ⁻¹ mM ⁻¹ % ⁻¹	$(0.98 \pm < 0.01) \times 10^{-6}$	b	$(0.01 \pm < 0.01) \times 10^{-6}$	c	$(\underline{6.06 \pm 0.37}) \times 10^{-6}$	<u>a</u>
k_{d1}	min ⁻¹	$(0.04 \pm < 0.01) \times 10^{-3}$	c	$(0.05 \pm < 0.01) \times 10^{-3}$	b	$(\underline{5.99 \pm 0.23}) \times 10^{-3}$	<u>a</u>
k_{d2}	min ⁻¹ mM ⁻¹	$(0.33 \pm < 0.01) \times 10^{-5}$	c	$(0.40 \pm < 0.01) \times 10^{-5}$	b	$(\underline{1.33 \pm < 0.01}) \times 10^{-5}$	<u>a</u>
1k_a	% min ⁻¹ mM ⁻¹	N/a ¹		N/a ¹		¹ (9.38 ± < 0.01) × 10 ⁻⁶	
t_{lag}	min	$\underline{314 \pm < 1}$	<u>a</u>	252 ± < 1	b	0.42 ± 0.37	c
Prediction capability		Interpolation		Extrapolation		Interpolation	
Validated initial (<i>B</i> , <i>A</i>) in mM		(100, 120)		(400, 600)		(50, 60)	
$^{\P}\text{RSS}_T$		1,610		11,000		<u>662</u>	
$^{\P}\text{MSE}$		28.2		282		<u>17.4</u>	
$^{\P}\text{R}^2$		~ 0.99		~ 0.97		~ 0.99	

Values with different capital alphabets (a - c) in the same row indicated statistically significant difference ($p \leq 0.05$).

Statistical parameters with the highest statistically significant ($p \leq 0.05$) or best values were bolded and underlined.

*2.5 M MOPS with (B/A) of (50/60), (100/120), (150/180) mM using initial volumetric PDC activity of 1.1 – 3.4 U mL⁻¹

†2.5 M MOPS with (B/A) of (400/600) mM using initial volumetric PDC activity of 8.4 U mL⁻¹

‡1 M phosphate with (B/A) of (30/36), (50/60), (100/120) mM using initial volumetric PDC activity of 1.1 – 1.5 U mL⁻¹

§Large error of K_{ma} was reported by this literature.

¹ k_a was only included in current study, Khemacheewakul et al.³⁴ reported k_a value of $(13.4 \pm 8.6) \times 10^{-6}$ % min⁻¹ mM⁻¹ which was not statistical significantly different ($p > 0.05$) from current study

[¶]These statistical parameters were normalized across previous studies of Leksawasdi et al.^{18,19} and current study so that RSS_T, MSE, and R² were comparable.

Figure 1: Simulated profiles of PAC (P , ●) biotransformation systems with experimental initial substrates (pyruvate – (A , ■) and benzaldehyde (B , ▲)) concentration levels (mM) of (a): (30/36) and (b): (100/120) after RSS_T minimization as well as predicted profile for validation purpose with initial substrates concentration level of (c): (50/60) mM based on optimized parameter values as indicated in Table 2. The corresponding values of RSS_T , MSE, and R^2 for each profile are shown in Table 3. The error bars had already been incorporated to all experimental data sets but were found to be relatively small for most of data sets. The initial volumetric enzyme activity (E , □) was $1.1 - 1.5 \text{ U mL}^{-1}$. The formation of by-products such as acetaldehyde (Q , ×) and acetoin (R , +) was not detected.

Discussion

The results from the effects of phosphate buffer and initial substrates concentration levels could be compared to those reported by Khemacheewakul et al.³⁴ when whole cells of *C. tropicalis* TISTR 5350 was used as biocatalyst instead. Whole cells biotransformation in 1 M phosphate buffer using initial 30 / 40 mM benzaldehyde / pyruvate substrates concentration levels could produce $P_{\max}$, equivalent $r_{P\max}$, $Y_{P/B}$, and $Y_{P/A}$ of $28.6 \pm 2.3 \text{ mM}$, $0.566 \pm 0.014 \text{ mM min}^{-1}$, 0.95 ± 0.08 and 0.71 ± 0.06 , respectively. The values of $P_{\max}$ from both studies could not be directly compared due to different initial substrate concentration levels. Nevertheless, the results of $r_{P\max}$ and $Y_{P/A}$ were still comparable and statistically higher ($p \leq 0.05$) by $12.9 \pm 0.3\%$ and $23.9 \pm 2.0\%$, respectively for the current study which might suggest the beneficial effect of using a lower phosphate buffer concentration level (250 mM) for PAC biotransformation system with partially purified PDC. In fact, $P_{\max}$ ($15.5 \pm 0.1 \text{ mM}$) and $r_{P\max}$ ($0.103 \pm 0.001 \text{ mM min}^{-1}$) of partially purified *C. tropicalis* TISTR 5350 were significantly affected ($p \leq 0.05$) by 1 M

phosphate buffer in comparison with the whole cells counterpart ($45.8 \pm 4.4\%$ and $81.8 \pm 0.5\%$ lowered, respectively) when subjected to similar initial substrates concentration level as shown in Table 1. As PDC is an intracellular enzyme, the application of this enzyme in the form of whole cells may protect the enzyme against high phosphate buffer concentrations through the mechanism of mass transfer limitation due to diffusion hindrance. However, catalytic rates may be compromised⁴¹. This was also in agreement with Rosche et al.³² who revealed that 600 mM phosphate buffer had greater level of inhibitory effect to partially purified PDC from *Rhizopus javanicus* NRRL 13161 than MOPS buffer at a similar concentration level resulting in lower PAC concentration being produced. MOPS buffer was then chosen as buffer of choice for subsequent studies^{18–20,28,33}. In fact, partially purified *C. utilis* PDC in 2.5 M MOPS buffer also expressed the highest r_{Pmax} when initial benzaldehyde / pyruvate concentration level was 100/120 mM¹⁸. Evidently, $Y_{P/B}$ was not affected by phosphate buffer concentration at 250 mM and 1 M as both molar yields were in the vicinity of unity and not statistically significant different ($p > 0.05$) from one another.

Several authors had discussed advantages and disadvantages of using phosphate species as reaction buffers for PDC^{2,25,32,34,42}. Juni et al.⁴² pointed out that high phosphate concentration might help prevent proteolytic enzymes from deactivating PDC, thereby enhancing the PDC stability while strengthening association of important prosthetic cofactors such as TPP with the enzyme. Such protective effect from a high phosphate buffer concentration observed in this study may also be applicable to PDC from *R. javanicus* NRRL 13161, because it possesses a relatively high PAC formation rate³². *R. javanicus* was formerly ranked as a potential candidate for the best PAC producer. However, the possibility of large-scale enzyme production from this filamentous fungal strain was eventually abandoned due to unsolvable proteolytic enzyme problems (unpublished result). Van Urk et al.²⁵ discovered allosteric inhibition of phosphate species on *S. cerevisiae* PDC in a greater extent than that of *C. utilis*. Using phosphate buffer for PAC biotransformation with PDC from *C. tropicalis* TISTR 5350 may be advantageous when conducted in an optimal concentration for each enzyme form (i.e., 1 M for whole cells PDC³⁴ and 250 mM for partially purified PDC (this study)). Phosphate buffer also offers cost-saving advantage. Leksawasdi et al.¹⁷ proposed that utilization of phosphate buffer in a 1 kg PAC production system would maintain the cost ratio of reaction buffer to total production at 25.9%, which is much lower than a cost ratio of 94.9% for MOPS buffer³⁴.

Evidently, the developed PAC biotransformation model provided good fitting within the investigated range of 30/36 to 100/120 mM initial benzaldehyde/pyruvate concentration levels for partially purified PDC from *C. tropicalis* TISTR 5350. Comparison of all ten kinetic parameters determined in the current study to those with comparable normalized units in the literature is given in Table 4. k_a was included to PAC biotransformation for the first time in current study. Khemacheewakul et al.³⁴ reported k_a value of $(13.4 \pm 8.6) \times 10^{-6} \% \text{ min}^{-1} \text{ mM}^{-1}$ which was not statistical significantly different ($p > 0.05$) from current study. The inclusion of $k_a.P$ term in Eq. (6) allowed the effect of buffering species to be separated out from the original enzyme deactivation model which contained only k_{d1} , k_{d2} , and t_{lag} . The absence of $k_a.P$ term in previous studies would result in averaging out of buffer species activation effect to aforementioned three parameters with relatively less quality of fit as shown in Table 4. The developed mathematical model thus had potential for both interpolating and extrapolating predictive capability for relatively high buffering systems (2.5 M MOPS and 250 mM

phosphate) across two yeast strains (*C. utilis* and *C. tropicalis*) covering initial volumetric PDC carboligase activity between 1.1 - 8.4 U mL⁻¹ and initial benzaldehyde and pyruvate concentration levels between (30, 36) to (400, 600) mM^{18-20,34}.

The statistical significantly highest ($p \leq 0.05$) overall rate constant for the formation of PAC (V_p) by using partially purified PDC from *C. tropicalis* TISTR 5350 indicated that PDC from this strain produces PAC faster than that from *C. utilis* by 4-5 times^{18,19}. The improvement in value of V_p was thus in agreement with finding in previous section where 250 mM phosphate buffer was utilized. Further comparison was made between intrinsic binding constant (K_b) and Hill coefficient (h) for benzaldehyde whose values for both partially purified PDC from *C. utilis* and *C. tropicalis* was not statistical significantly different ($p > 0.05$) with validated range of mean between $(0.80 - 1.01) \times 10^{-4}$ mM^{1-h} and 1.77 - 2.18, respectively^{18,19}. It was possible that partially purified PDC from both strains exhibited similar allosteric and sigmoidal behaviors toward benzaldehyde. In addition, h value of ~2 suggests that PDC might operate in a dimeric subunit form during PAC biotransformation¹⁸⁻²⁰. The lower affinity constant value for pyruvate (K_{ma}) in the current study implies a greater level of binding between partially purified PDC from *C. tropicalis* TISTR 5350 and pyruvate than the *C. utilis* counterpart by 9-11 times. In fact, the observably higher overall rate constants for the formation of both acetaldehyde (V_q) and acetoin (V_r) in the current study may be the result of increased affinity toward pyruvate of this decarboxylating enzyme^{18-20,25,31}. Partially purified PDC from *C. tropicalis* TISTR 5350 was more prone to deactivation effect by benzaldehyde than the *C. utilis* counterpart as indicated by a much higher first-order benzaldehyde deactivation coefficient (k_{d2}) and shorter lag time for *C. tropicalis* PDC. However, the use of phosphate buffer could negate a portion this deactivating effect by providing additional activation effect. The multiplication between zeroth-order activation rate constant due to phosphate buffer (k_a) with PDC stabilizing effect of 250 mM phosphate buffer resulted in an activation rate of $(2.34 \pm < 0.02) \times 10^{-3}$ % min⁻¹. This rate was lower than the activation rates when whole cell PDC in 1 M (1.34×10^{-2} % min⁻¹) and 20 mM (1.48×10^{-6} % min⁻¹) phosphate buffer were used³⁴.

The molarity balancing of PAC formation was examined by observing PAC molar yield based on each substrate. The ranges for $Y_{P/B}$ were 0.85 - 0.97, 0.89 - 0.99, and 0.93 - 0.99 for the initial benzaldehyde and pyruvate concentration pairs of 30/36, 50/60, and 100/120 mM, respectively, indicating the closing molarity balance. On the contrary, corresponding ranges of $Y_{P/A}$ of 0.80 - 0.97, 0.81 - 0.93, and 0.78 - 0.89 were not uniform among the tested concentration pairs. Similar phenomenon was also observed in other PAC biotransformation systems^{18-20,29,31,32,34,35}. The formation of relatively volatile by-products such as acetaldehyde from pyruvate generally resulted in lower ranges of $Y_{P/A}$ in comparison with $Y_{P/B}$. The absence of acetaldehyde concentration in the reaction buffer and some losses in pyruvate molarity balance confirmed the volatility nature of this compound¹⁸⁻²⁰. Benzyl alcohol, benzoic acid and acetoin were also not detected in the current study. Benzyl alcohol and its derivatives are often formed from side reactions of either alcohol dehydrogenase or other oxidoreductases when growing yeast cells were used in a PAC biotransformation process, resulting in a loss of up to 30 - 40% benzaldehyde^{26,43}.

Conclusions

The partially purified PDC from *C. tropicalis* TISTR 5350 with initial volumetric enzyme activity between $1.1 - 1.5 \text{ U mL}^{-1}$ produced PAC at the optimized levels ($95.8 \pm 0.1 \text{ mM}$ and $0.639 \pm 0.001 \text{ mM min}^{-1}$) in 250 mM phosphate buffer. The improved set of PAC biotransformation mathematical model fitted well to the kinetic biotransformation profiles of two initial concentration levels of benzaldehyde and pyruvate of 30/36 and 100/120 mM. The independent prediction of 50/60 mM benzaldehyde/pyruvate profile validated the interpolation ability of the developed model with corresponding RSS_T , MSE, and R^2 of 662, 17.4, and 0.9863, respectively. Such mathematical model will be useful for further optimization of a more complex biotransformation process, for instance, development of feeding strategies in fed batch or continuous systems.

Nomenclature

A	pyruvate concentration (mM)
B	benzaldehyde concentration (mM)
CSC	convergence search criterion (no unit)
E	relative enzyme activity (%)
h	Hill coefficient for benzaldehyde (no unit)
i	iteration loop identifier for numerical integration
k_a	zeroth order activation rate constant due to phosphate buffer (% relative enzyme activity $\text{min}^{-1} \text{ mM}^{-1}$ of phosphate buffer)
k_{d1}	first order reaction time deactivation constant (min^{-1})
k_{d2}	first order benzaldehyde deactivation coefficient ($\text{mM}^{-1} \text{ min}^{-1}$)
K_b	intrinsic binding constant for benzaldehyde (mM^{1-h})
K_{ma}	affinity constant for pyruvate (mM)
MSE	mean square error (no unit)
N/a	not available
P	<i>R</i> -phenylacetylcarbinol concentration (mM)
PAC	<i>R</i> -phenylacetylcarbinol
Ph_b	phosphate buffer concentration (mM)
PDC	pyruvate decarboxylase enzyme
Q	acetaldehyde concentration (mM)
r	formation rate (mM min^{-1})
R	acetoin concentration (mM)
R^2	coefficient of determination (no unit)
RSS	residual sum of squares (no unit)
t	time (min)
T	total substrates and products species within the reaction buffer
t_{lag}	lag time (min)
U	carboligase activity of PDC (unit)
V_p	overall rate constant for the formation of PAC ($\text{mM min}^{-1} \%^{-1}$)
V_q	overall rate constant for the formation of acetaldehyde ($\text{min}^{-1} \%^{-1}$)
V_r	overall rate constant for the formation of acetoin ($\text{min}^{-1} \text{ mM}^{-1} \%^{-1}$)
Y	molar yield of <i>R</i> -phenylacetylcarbinol on each substrate (no unit)

Data Availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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Competing interests

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

Author contributions

J.K. and N.L. designed the study. J.K. performed the experiments, collected, and analyzed the data. N.L. and Y.P. checked the validity of model. J.K., N.L., K.P., S.S., and C.M. performed statistical analyses. J.K. and N.L. wrote the initial draft of the manuscript. S.T., R.N., C.T., Y.P., P.R., K.J., K.P., S.S., C.M., T.C., P.S., A.R., N.T.N.T., S.W., S.R.S. revised the manuscript. J.K. and N.L. wrote the final version of the manuscript. All authors reviewed the final version of the manuscript.

Figures

Figure 1

please see the manuscript file for the full caption