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A new mathematical method for determining the enantiomeric ratio in lipase-catalyzed reactions

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ABSTRACT

We present a new mathematical method for determining the enantiomeric ratio (E) during lipase-catalyzed kinetic resolutions. The method involves the fitting of a model to profiles of adimensionalized concentrations of the two enantiomers of the chiral substrate, plotted against degree of conversion. The model equations are presented for a reversible reaction involving bi-bi ping-pong kinetics in which the chiral substrate enters second and the chiral product leaves second. However, it is also shown that the method is easy to modify for analysis of resolutions involving other chiral substrate-product pairs and of resolutions in which the behavior of the system can be approximated by irreversible uni-uni kinetics. We show that our method retains several advantageous features of existing methods that help to ensure accuracy.

1. Introduction

The production of single enantiomers from racemates of chiral intermediates is becoming of increasing importance in the production of chiral agrochemicals and pharmaceuticals [1]. In the pharmaceutical industry, enantiopure chiral drugs constitute approximately 36% of the overall market [2,3]. At the turn of the millennium, the size of the market for these drugs was increasing by approximately 30% per year, with an estimated value of US $151.9 billion in 2002 [4,5].

One of the most commonly used methods for producing pure enantiomers from racemates is enzymatic kinetic resolution. The preference that an enzyme has for reacting with one enantiomer over the other in this process is expressed by the enantiomeric ratio E, which, for a uni-uni reaction (i.e. $S \rightarrow P$), is defined by the following ratio of specificity constants:

$$E = \frac{k_S^R}{k_S^S} = \frac{k_{cat}^R / K_M^R}{k_{cat}^S / K_M^S} \quad (1)$$

For reactions that involve more than one substrate, the expression for E is similar, but the catalytic and saturation constants used vary, depending on the reaction mechanism and on which of the substrates is chiral.

A kinetic resolution will be most successful, in terms of the enantiomeric purity of the desired enantiomer (i.e. either a high ee_s or a high ee_p), when the value of E is either much greater or much smaller than unity. In order to screen enzymes for the purpose of choosing the best one for the resolution of a particular racemate, it is therefore necessary to have a reliable and easy to use method for determining the value of E that each enzyme has for reaction with the racemate.

Lipases are very commonly used in enzymatic kinetic resolutions, since they are able to catalyze various reactions with a range of different types of substrates and, importantly, they often have high preference for reacting with one of the enantiomers [6,7]. The aim of the current work is to demonstrate a new mathematical method for the determination of E in lipase-catalyzed kinetic resolutions.

2. Materials and methods

2.1. Case studies and reaction schemes

Lipase-catalyzed reactions occur by the ping-pong bi-bi mechanism, which involves a compulsory sequence of binding of substrates and release of products. The second substrate to enter and the second product to leave the active site are the chiral species in both of the reactions used as case studies:

$$\text{first substrate} + \text{second substrate} \rightarrow \text{first product} + \text{second product} \quad (2a)$$

Abbreviations: E, enantiomeric ratio.

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vinyl acetate + (*R*, *S*)-alcohol → acetaldehyde + (*R*, *S*)-alcohol acetate (2b)

2-chloroethyl butyrate + (*R*, *S*)-1-phenoxypropan-2-ol → 2-chloroethanol + (*R*, *S*)-1-phenoxypropan-2-yl butyrate (2c)

For this case, it is possible to show that the enantiomeric ratio is given by an expression analogous to Eq. (1), where k_{cat} is considered for the forward direction of Eqs. (2a)–(2c) and K_M is the saturation constant for the chiral substrate.

Two reaction schemes are considered: the irreversible uni-uni case and the reversible bi-bi ping-pong case where the second substrate to enter and the second product to leave are chiral. In this paper the chiral substrate and the chiral product will always be represented as *S* and *P*, respectively, with the appropriate superscript to identify the enantiomer (either *R* or *S*). Non-chiral substrates and products will be represented by letters without superscripts. Using this notation, the irreversible uni-uni reaction scheme can be written as:

$$S^R \rightarrow P^R \quad (3a)$$

$$S^S \rightarrow P^S \quad (3b)$$

while the scheme for each of the reactions presented in Eq. (2) can be represented as a set of three reactions:

$$A + S^R \leftrightarrow N + P^R \quad (4a)$$

$$A + S^S \leftrightarrow N + P^S \quad (4b)$$

$$P^R + S^S \leftrightarrow S^R + P^S \quad (4c)$$

The reaction described by Eq. (4c) is not desirable, but may occur.

2.2. Mathematical method

The model is based on Eqs. (5)–(14), which are presented in Table 1. Eq. (5) defines the adimensionalized concentrations used in the model. The data obtained experimentally are the fractions of the two enantiomers of the chiral substrate (denoted as F_S^S and F_S^R) and the relative fractions of the two enantiomers of the chiral product (denoted as F_P^S and F_P^R), which are defined by Eq. (6). The adimensionalized concentrations of the substrate and product enantiomers are related, according to the stoichiometry of the reaction, by Eq. (7). It is assumed that the original substrate mixture is racemic and there is no product at time zero. In this case, the zero-time adimensionalized concentrations of the two enantiomers (S_o^S and S_o^R) are given by Eq. (8). Eqs. (6)–(8) can be used to derive Eq. (9), which expresses S^S and S^R in terms of the originally measured relative fractions of the enantiomers.

The mathematical analysis is based on a method that can be applied to any system of sequential and parallel reactions catalyzed by the same enzyme. The deduction of the equations has been described in detail previously [8–10]. The equations use the degree of conversion (ξ) as the independent variable; if the starting reaction mixture contains a racemate of the chiral substrate and no chiral product, the degree of conversion is given by Eq. (10). When the analysis is applied to the irreversible uni-uni scheme shown in Eqs. (3a) and (3b), the final equation set is that given by Eq. (11). On the other hand, when the analysis is applied to the reversible bi-bi ping-pong scheme shown in Eqs. (4a)–(4c), the final equation set is given by Eq. (12), where the expressions for the individual rates are those given by Eq. (13). It is possible to express all other concentrations that appear in Eq. (13) as functions of S^R and S^S, as given by Eq. (14). After substitution of Eqs. (13) and (14) into Eq. (12), the final differential equation set for the reversible bi-bi ping-pong case contains two dependent variables, S^R and S^S, and three parameters, the enantiomeric ratio (*E*), the equilibrium constant for the reaction (K_{eq}) and the selectivity factor α^R.

For given values of parameters (either *E* alone or *E*, K_{eq} and α^R), the FORTRAN subroutine DRKGS [11], which uses a 4th order Runge-Kutta algorithm with automatic step size adjustment, was used to integrate the equation set (either Eq. (11) or Eq. (12)) to obtain values of S^R and S^S as functions of ξ. When parameter estimation was undertaken, the sum of squares of residuals (SSR), given by Eq. (15) in Table 1, was minimized.

Table 1
Model equations.

Description and equation	Eq. no.
Adimensionalized concentrations[a]	
$S^R = \frac{[S^R]}{[S^R]_o+[S^S]_o}$; $S^S = \frac{[S^S]}{[S^R]_o+[S^S]_o}$; $A = \frac{[A]}{[S^R]_o+[S^S]_o}$; $N = \frac{[N]}{[S^R]_o+[S^S]_o}$	(5)
Relative fractions of enantiomers	
$F_S^S = \frac{S^S}{S^S+S^R}$; $F_S^R = \frac{S^R}{S^S+S^R}$; $F_P^S = \frac{P^S}{P^S+P^R}$; $F_P^R = \frac{P^R}{P^S+P^R}$	(6)
Stoichiometric relationships between substrate and product enantiomers	
$S^R + P^R = S_o^R$; $S^S + P^S = S_o^S$	(7)
Zero-time adimensionalized concentrations of the two enantiomers	
$S_o^S = S_o^R = 0.5$	(8)
Adimensionalized concentrations as a function of measured relative fractions	
$S^S = 0.5\left(1 - \left(1 - \frac{F_S^S}{F_S^R}\right) / \left(1 - \frac{F_P^R}{F_P^S} \cdot \frac{F_S^S}{F_S^R}\right)\right)$; $S^R = S^S\left(\frac{F_S^R}{F_S^S}\right)$	(9)
Degree of conversion	
$\xi = 1 - (S^R + S^S)$	(10)
Differential equations for irreversible uni-uni scheme	
$\frac{dS^R}{d\xi} = -\frac{E.S^R}{E.S^R+S^S}$; $\frac{dS^S}{d\xi} = -\frac{S^S}{E.S^R+S^S}$	(11)
General form of differential equations for reversible bi-bi ping-pong scheme	
$\frac{dS^R}{d\xi} = -\frac{r^R}{r^R+r^S}$; $\frac{dS^S}{d\xi} = -\frac{r^S}{r^R+r^S}$	(12)
Identities of reaction rates in Eq. (12) for the reaction shown in Eqs. (4a)–(4c)	
$r^R = S^R\left(E.A + \frac{P^S}{\alpha^R}\right) - P^R\left(\frac{E.N}{K_{EQ}} + \frac{S^S}{\alpha^R}\right)$; $r^S = S^S\left(A + \frac{P^R}{\alpha^R}\right) - P^S\left(\frac{N}{K_{EQ}} + \frac{S^R}{\alpha^R}\right)$	(13)
Expressions, in terms of S^R and S^S, for other variables that appear in Eq. (13)	
$A = A_O - (1 - S^R - S^S)$; $N = 1 - S^R - S^S$; $P^R = 0.5 - S^R$; $P^S = 0.5 - S^S$	(14)
Objective function for curve fitting[b]	
$SSR = \sum_{i=1}^{n}(S_{ei}^R - S_{pi}^R)^2 + \sum_{i=1}^{n}(S_{ei}^S - S_{pi}^S)^2$	(15)

[a] Zero-time concentrations are indicated by the subscript "*o*".
[b] The subscript "*ei*" represents the *i*th experimental data point and the subscript "*pi*" represents the corresponding model prediction.

2.3. Cultivation to produce lipase

Burkholderia cepacia LTEB11 was cultivated in a 125 mL Erlenmeyer flask containing 50 mL Luria Bertani (LB) Miller broth (NaCl 10 g/L, bacteriological tryptone 10 g/L, yeast extract 5 g/L) at 37 °C and 150 rpm for 8 h. 1 mL of the culture broth was then inoculated into a 500 mL Erlenmeyer flask containing 150 mL of a medium containing (in g/L): KNO_3 3.54, K_2HPO_4 1.0, $MgSO_4{\cdot}7H_2O$ 0.5, NaCl 0.38, $FeSO_4{\cdot}7H_2O$ 0.01, yeast extract 5.0 and 1% (v/v) commercial olive oil (Gallo brand). After incubation for 72 h at 37°C and 150 rpm, the medium was centrifuged at 12,500 × *g* for 20 min at 4 °C.

2.4. Free and immobilized lipase preparations

To produce 'free lipase', the culture supernatant was frozen and subsequently lyophilized. 'Immobilized lipase' was produced according to [12]. Accurel MP 1000® powder was wetted with ethanol for 30 min and then washed twice with ethanol–water (50:50, v/v) and once with water. It was then added to the culture supernatant in the proportion of 1 g of Accurel for every 25 mg of protein, determined according to [13], in order to obtain maximal adsorption [14]. The mixture was left overnight at 25 °C at 200 rpm. The liquid phase was removed by filtration through Qualy® filter paper, and the solid support was then delipidated: 20 mL of a solution of chloroform:butanol (9:1) was added per gram of solid material, the mixture was stirred at 200 rpm for 10 min at 25 °C and the solids were recovered by filtration. This procedure was repeated until the organic phase separated by filtration did not contain free fatty acids or triglycerides, as determined by thin-layer chromatography, using hexane:diethyl-ether:acetic acid (7:3:0.1) as the mobile phase. The immobilized enzyme was then dried in a desiccator for 16 h at 4 °C and stored at 4 °C.

2.5. Determination of lipase activity

The *p*NPP (*p*-nitrophenyl palmitate, Sigma) method in aqueous solution was used [15]. 1 mL of solution A (3 mg of *p*NPP in 1 mL of 2-propanol) was added to 9.0 mL of solution B (50 mM pH 7 phosphate buffer, Triton X-100, 0.44%, gum arabic 0.11%, w/v), dropwise, with intense stirring. For free lipase, 0.9 mL of this mixture was then transferred to a cuvette and a 0.1 mL sample containing the enzyme was mixed in. For immobilized lipase, the reaction was initiated by adding 1 mg of immobilized enzyme to the 10 mL of reaction medium. The mixture was stirred at 200 rpm, with samples being removed at intervals. The molar absorptivity coefficient of *p*-nitrophenol (*p*NP) at pH 7.0 was determined as 9.8×10^3 L/(mol cm) at 410 nm. A unit of activity was defined as the liberation of 1 μmol/min of *p*NP (*p*-nitrophenol).

2.6. Resolution of secondary allylic alcohols

The reaction medium contained 450 U of *p*NPP-hydrolyzing activity, 0.5 mmol of a racemate of the secondary allylic alcohol, 22 mmol vinyl-acetate (Acros Organics, Belgium) and 7 mL of hexane (Vetec, Brazil). The reaction was carried out at 37 °C in an orbital shaker at 180 rpm. The racemates of the secondary allylic alcohols were obtained by chemical synthesis according to [16].

2.7. Determination of relative fractions of enantiomers

Substrates and products were separated on a gas chromatograph (Varian model 3800) with a β-cyclodextrin chiral column. Analysis conditions were: 1 μL sample, flame ionization detector at 280 °C, carrier gas He at 5.5 mL/min, temperature gradient from 40 to 170 °C at 2 °C/min. The relative fractions of the enantiomers (F_S^S and F_S^R, F_P^S and F_P^R) were calculated from the relative peak areas.

3. Results

3.1. Fit of the model to experimental data for the resolution of secondary alcohols

The irreversible uni-uni model represented by Eq. (11) was used to fit data obtained during the resolution of racemates of several secondary alcohols, as described by Eq. (2b). Although the reaction follows the reversible bi-bi ping-pong scheme described by Eqs. (4a)–(4c), good fits were obtained in all cases (Fig. 1). The good fit is not surprising, since the initial concentration of the non-chiral substrate (vinyl-acetate) was 44-fold greater than the initial concentration of the racemate of the secondary allylic alcohol. With such a large excess of the non-chiral substrate, the reversible bi-bi equation system described by Eq. (12) is very closely approximated by the irreversible uni-uni equation system described by Eq. (11) [17].

3.2. Fit of the model to literature data

The model, represented by Eq. (12), was applied to literature data [18] for the resolution of (*R*,*S*)-1-phenoxypropan-2-ol, as described by Eq. (2c). This reaction also follows the scheme given in Eqs. (4a)–(4c). The authors undertook reactions with four different initial adimensionalized concentrations of the non-chiral acyl donor (A_o = 1.5, 3, 5 and 10). With a single set of parameters, obtained by fitting all four data sets simultaneously, good fits were obtained for all profiles (Fig. 2). A notable feature of these graphs is that S^R, after initially decreasing, begins to increase as the reaction nears equilibrium. This occurs because the high values of P^R and S^S that occur during the kinetic resolution favor the reaction described by Eq. (4c). The tendency of this side reaction to occur depends on the value of α^R.

4. Discussion

This paper presents a new mathematical method for determining the enantiomeric ratio (*E*) from data obtained during a kinetic resolution. The proposed method has several of the advantages of the methods reviewed by Straathof and Jongejan [19]. It is also easier to apply than the method described by Anthonsen et al. [18] for determining *E* in reversible bi-bi reactions. In addition, it is readily adaptable to situations other than those shown in the case studies. These points are discussed separately below.

4.1. Advantages of the proposed method for determination of E

The proposed method shares the advantages of several of the methods for determining *E* that were reviewed by Straathof and Jongejan [19]. Although the methods reviewed were for the determination of *E* in uni-uni reactions, the advantages discussed below apply to both uni-uni and bi-bi reactions.

The first advantage is that, experimentally, it is simply necessary to determine the relative fractions of the two enantiomers of the chiral substrate (i.e. F_S^S and F_S^R) and the relative fractions of the two enantiomers of the chiral product (i.e. F_P^S and F_P^R). This avoids the need for quantitative handling of samples and calibration, thus eliminating a potential source of error [19].

The second advantage is that the proposed method is based on the removal of multiple samples, taken at various degrees of conversion. This leads to estimates of *E* that are statistically more reliable than those obtained when simple equations, such as the Chen equation [20], are used with data obtained at a single degree of conversion. Further, if the multiple data points are collected over a wide range of ξ values, then it is possible to detect systematic deviations between the data profile and the best-fitting curve. Possible causes of such systematic deviations will be discussed later.

The third advantage is that, since the proposed method is based on the degree of conversion, it is not affected by interfering phenomena that are common in kinetic resolutions, such as enzyme deactivation and substrate or product inhibition. Although these phenomena do slow the reaction, when the degree of conversion is used as the independent variable, the variables and parameters that describe their effects cancel out of the equation system [8–10,19].

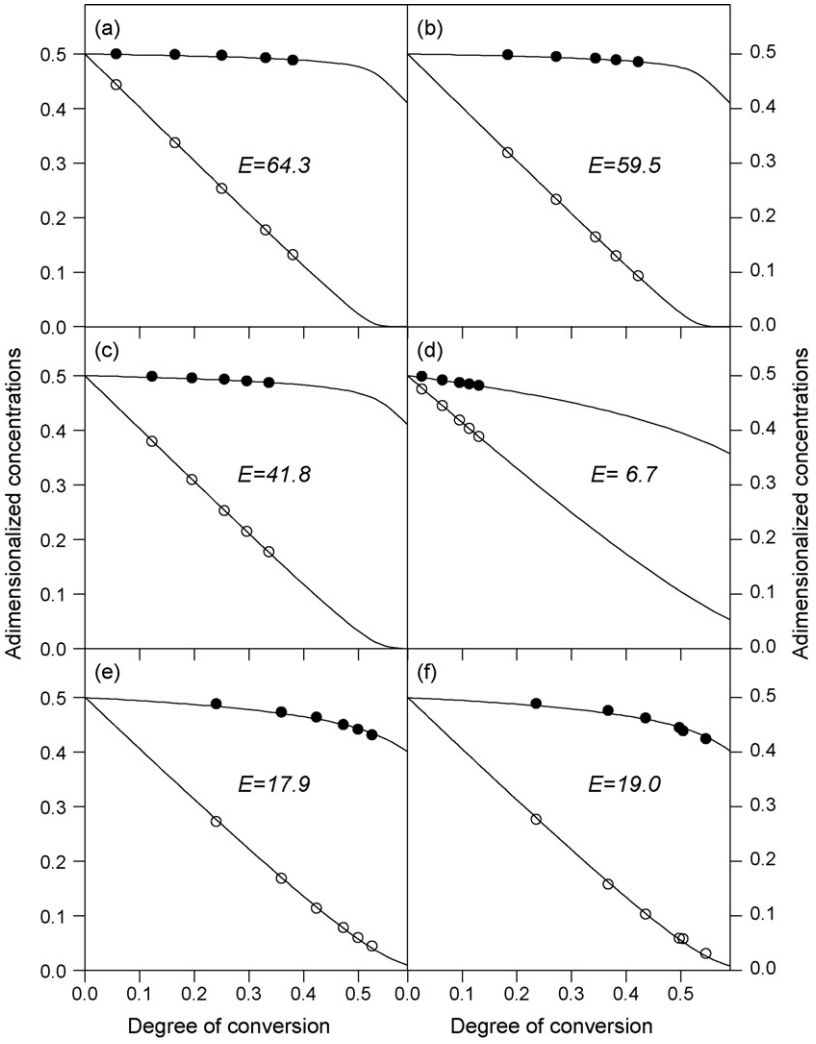

Fig. 1. Experimental results for kinetic resolutions of secondary allylic alcohols. Also shown are the best-fitting model curves and the values of *E* obtained by the fitting procedure. The different plots are for the resolution of: (a) (*R*,*S*)-1-phenylprop-2-en-1-ol; (b) (*R*,*S*)-1-(4-chlorophenyl)prop-2-en-1-ol; (c) (*R*,*S*)-1-(3-methoxyphenyl)prop-2-en-1-ol; and (d) (*R*,*S*)-5-methylhex-1-en-3-ol with *Burkholderia cepacia* lipase immobilized on Accurel and (e) (*R*,*S*)-1-phenylprop-2-en-1-ol; and (f) (*R*,*S*)-*p*-chlorophenylprop-2-en-1-ol with lyophilized free *B. cepacia* lipase. Key: (●) S^S; (○) S^R; (—) best-fitting curve.

The fourth advantage of the proposed method is that it is possible to obtain good estimates from a single experiment involving a racemate. One method for determining *E* involves independent determination of V_{max} and K_M for experiments undertaken with pure preparations of each enantiomer, but this method is highly susceptible to error if the supposedly pure preparations of each enantiomer are in fact contaminated with traces of the other enantiomer [19].

4.2. Flexibility of the proposed method for determining E

We have demonstrated the proposed method for the reversible bi-bi ping-pong mechanism in which the chiral substrate enters second and the chiral product leaves last and also for a situation in which the simplification of irreversible uni-uni kinetics applies. It is easy to adapt the equation set for lipase-catalyzed resolutions involving different chiral substrate-product pairs. In such cases, Eq. (12) will be used, but the expressions for r^R and r^S will be different.

When the chiral substrate enters first and the chiral product leaves first, then the reaction scheme is as follows:

$$S^R + B \leftrightarrow P^R + Q \tag{16a}$$

$$S^S + B \leftrightarrow P^S + Q \tag{16b}$$

$$S^R + P^S \leftrightarrow P^R + S^S \tag{16c}$$

The reaction described by Eq. (16c) is undesirable, but may occur.

In this case, the equations for r^R and r^S will be [17]:

$$r^R = S^R\left(E.B + \frac{\alpha^R.P^S}{K_{eq}}\right) - \frac{P^R(E.Q + \alpha^R.S^S)}{K_{eq}} \tag{17a}$$

$$r^S = S^S\left(B + \frac{\alpha^R.P^R}{K_{eq}}\right) - \frac{P^S(Q + \alpha^R.S^R)}{K_{eq}} \tag{17b}$$

When a chiral substrate enters first and the chiral product leaves second, then the reaction scheme is as follows:

$$S^R + B \leftrightarrow N + P^R \tag{18a}$$

$$S^S + B \leftrightarrow N + P^S \tag{18b}$$

In this case, the equations for r^R and r^S will be [17]:

$$r^R = E\left(S^R.B - \frac{N.P^R}{K_{eq}}\right)\left(\frac{\alpha^S N}{K_{eq}} + B\right) \tag{19a}$$

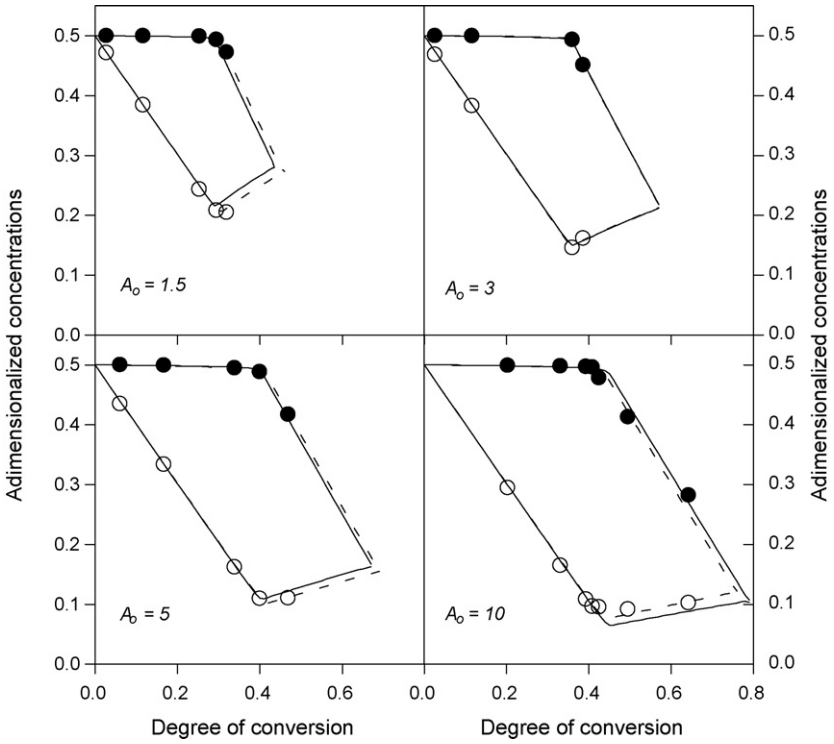

Fig. 2. Fit of the reversible bi-bi ping-pong model to the experimental results of Anthonsen et al. [18] for the transesterification of (*R*,*S*)-1-phenoxypropan-2-ol using 2-chloroethyl butyrate as the acyl donor. Four different initial adimensionalized concentrations of chloroethyl butyrate (A_o) were used. They are indicated on the individual graphs. Key: (●) S^S; (○) S^R; (—) best-fitting curves, corresponding to $E = 174$, $K_{eq} = 0.319$ and $\alpha^R = 98.1$, obtained by simultaneously fitting all four data sets using the method proposed in the current work; (- - -) curves corresponding to the parameter sets obtained by Anthonsen et al. [18], who fitted each data set individually. Their parameter values were: for $A_o = 1.5$, $E = 151$, $K_{eq} = 0.366$ and $\alpha^R = 480$; for $A_o = 3$, $E = 259$, $K_{eq} = 0.317$ and $\alpha^R = 940$; for $A_o = 5$, $E = 130$, $K_{eq} = 0.358$ and $\alpha^R = 0.6$; for $A_o = 10$, $E = 133$, $K_{eq} = 0.259$ and $\alpha^R = 740$.

$$r^S = \left(S^S.B - \frac{N.P^S}{K_{eq}}\right)\left(\frac{\alpha^R N}{K_{eq}} + B\right) \tag{19b}$$

Note that in this case there are two selectivity factors, α^R and α^S.

Of course, it is necessary to deduce an appropriate set of relationships amongst the adimensionalized concentrations, equivalent to those shown in Eq. (14), such that the final equations are expressed only in terms of S^R and S^S.

Other adaptations are possible. For example, when the substrate is a racemic ester, it can decompose during storage or handling, meaning that chiral product is present at time zero [19]. This can be taken into account by measuring how much chiral product is present in the zero-time sample and formulating Eqs. (10) and (14) appropriately. Also, if the enantiomers of the chiral substrate are initially present in different amounts, which can happen if the starting material has been previously processed [19], then this is taken into account by using the real zero-time values of S_o^R and S_o^S, instead of simply substituting them with 0.5.

4.3. Comparison with the model of Anthonsen et al.

The method proposed in the current work has similarities with that proposed by Anthonsen et al. [18] but is somewhat easier to use. For the reaction scheme described by Eqs. (4a)–(4c), they used numerical integration to integrate the following equation directly:

$$\frac{d[P^R]}{d[P^S]} = \frac{[S^R](E[A] + [P^S]/\alpha_R) - [P^R](E[N]/K_{EQ} + [S^S]/\alpha_R)}{[S^S]([A] + [P^R]/\alpha_R) - [P^S]([N]/K_{EQ} + [S^R]/\alpha_R)} \tag{20}$$

When E is large, then initially $[P^R]$ will change rapidly for very small changes in $[P^S]$; once P^R is almost completely consumed, $[P^R]$ will change very slowly for large changes in $[P^S]$. This creates difficulty for the numerical integration of Eq. (20), it being necessary to use techniques for stiff equation sets. Although such integration techniques are available, our proposed method simplifies the task by avoiding the need to use them. This is possible because changes in S^R and S^S are described by separate differential equations and the degree of conversion is used as the independent variable.

Our approach has another advantage over that used by Anthonsen et al. [18]. They generated a table of output at evenly spaced degrees of conversion (ξ) and then used cubic spline interpolation to estimate the substrate and product concentrations at other degrees of conversion. Our approach is simpler: we avoid the need for interpolation by obtaining output from the numerical integration for each degree of conversion obtained experimentally.

Finally, Anthonsen et al. [18] carried out the same reaction with several different initial concentrations of the non-chiral substrate. In this case, our strategy of estimating a single set of parameter values from the combined data is statistically sounder than is their strategy of determining a separate parameter set for each different reaction and then estimating each parameter by averaging the values obtained for that parameter from the various different fits.

4.4. Causes of systematic deviations

It is possible for systematic deviations to occur between the experimental profiles for S_R and S_S and the best-fitting curves obtained by the analysis. There are two possible reasons for such deviations.

Firstly, deviations will occur if the model equations are not appropriate for the mechanism being analyzed. For example, the irreversible uni-uni equation can be applied to determine E for bi-bi reactions only under specific conditions [17,19]. It is important to note that when these conditions are not met, if one were to apply the Chen equation [20] to data obtained from a single sample, the

value obtained for E would be erroneous, but it would be impossible to detect this error. Deviations will only be visible if multiple samples are obtained, as in the current method.

Secondly, the value of E might change during the reaction. In some cases the medium properties can change to such a degree that they affect the enantioselectivity of the enzyme [21]. However, such effects are highly complex and at the moment we simply do not have sufficient knowledge to incorporate them into model equations.

5. Conclusions

Our method can be used to analyze reaction profiles to determine the enantiomeric ratio (E) during lipase-catalyzed kinetic resolutions. It applies to reversible reactions involving bi-bi ping-pong kinetics, with different rate expressions being used, depending on the order in which the chiral substrate and chiral product enter and leave the active site. It can be modified for resolutions in which the behavior of the system can be approximated by irreversible uni-uni kinetics. Our method combines several advantages of previously proposed methods and is easy to use.

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