# ESTIMATION OF SELECTIVITIES IN REACTIONS INVOLVING THE PING PONG BI BI MECHANISM

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## ABSTRACT

Lipases and β-galactosidases catalyze reactions by the Ping Pong bi bi mechanism. The selectivities of these enzymes are important parameters for kinetic models of processes involving these enzymes, but it is challenging to obtain reliable estimates of these selectivities. To address this challenge, our group recently developed a new parameterization of the Ping Pong bi bi kinetic equation, expressing it in terms of specificity constants. Here, we review our recent work, in which we used this new parameterization as the basis of a method for estimating selectivities in esterification and transesterification reactions catalyzed by lipases and in transgalactosylation reactions catalyzed by β-galactosidases. Importantly, our method gives reliable estimates of selectivities, since phenomena such as denaturation and inhibition of the enzyme do not interfere with the analysis, even if they occur.

**Keywords:** Lipase. β-galactosidase. Specificity constant. Transesterification. Transgalactosylation.

## 1 INTRODUCTION

Several biotechnologically important enzymes catalyze reactions by the Ping Pong bi bi mechanism. Two examples are lipases, which are used to catalyze hydrolysis, esterification and transesterification reactions to produce a wide range of products, and β-galactosidases, which are used to catalyze transgalactosylation reactions to produce prebiotic galactooligosaccharides.[1-6] The design and optimization of industrial processes for the production of these products can be guided by appropriate kinetic models. These processes typically involve several competing reactions, and the selectivities of the enzyme for these reactions determine the maximum yields that can be obtained of the desired product, so these selectivities are important model parameters.[7-9]

It is a challenge to estimate selectivities in systems in which many competing reactions occur. Various authors have estimated the selectivities of their enzymes by fitting kinetic models, based on time as the independent variable, to progress curves, plotted as functions of time.[1,3,4,6] However, in addition to selectivities, such models include several saturation constants and inhibition constants. The large number of parameters in the model means that serious problems of correlation between parameters can occur, such that the estimated selectivities are not reliable.[3] These problems can be overcome through the use of a newly parameterized version of the Ping Pong bi bi equation, expressed in terms of specificity constants.[10] For a single irreversible reaction that follows the Ping Pong mechanism, namely "A + B → P + Q", this newly parameterized equation has the form:

$$v = k_A[A]k_B[B]\frac{[E]_T}{d} = k_{AB}[A][B]\frac{[E]_T}{d} \quad (1)$$

where $k_A$ and $k_B$ are the specificity constants of the enzyme for substrates A and B, respectively. These can be multiplied to give a single "combined specificity constant", $k_{AB}$. The denominator of this equation, $d$, contains several specificity constants, saturation constants and inhibition constants, but, since the denominator cancels out of the final equation set, it is not important.[7-9]

Here, we review our recent work, in which we have shown that this parameterization of the Ping Pong bi bi equation can be used as the basis of a method for estimating enzyme selectivities in systems involving several competing reactions.

## 2 MATERIAL & METHODS

Our method for determining selectivities is based on a set of ordinary differential equations expressing stoichiometric balances on each of the reaction species, but with the percentage degree of reaction rather than time as the independent variable. The definition of percentage degree of reaction varies between systems. In esterification and transesterification reactions, it reflects how many functional groups of the substrate have been esterified or transesterified. In transgalactosylation reactions, it is based on the percentage consumption of the galactosyl donor, lactose. The use of percentage degree of reaction as the independent variable means that the balance equations are expressed as ratios of expressions of the type shown in Eq. (1). The factor $[E]_T/d$ cancels out of these ratios. Further, one reaction is taken as the reference reaction and its combined specificity constant is used to divide all other specificity constants, giving a set of "relative combined specificity constants", which represent selectivities of the enzyme for the reaction of interest relative to the reference reaction. Other selectivities can be calculated from the set of relative combined specificity constants. These mathematical transformations result in a set of equations in which the selectivities are the only parameters and these selectivities are estimated by using them as fitting parameters to adjust the model to reaction profiles plotted against the percentage degree of reaction.

## 3 REVIEW

In previous work, we applied our method of determining selectivities to the lipase-catalyzed production of two molecules that can be used as biolubricants, namely bis(2-ethylhexyl) azelate and triesters of trimethylolpropane (TMP),[7,8] and also to the production of galactooligosaccharides, catalyzed by a β-galactosidase.[9] Below, we summarize the results of our analyses.

Initially, Serrano et al.[7] applied the method to the lipase-catalyzed transesterification of diethyl azelate with 2-ethylhexan-1-ol, using data of Gómez et al.[1] Since azelaic acid is a dicarboxylic acid, this system involves two competing reactions:

*Reaction 1: diethyl azelate + 2-ethylhexan-1-ol → ethanol + ethyl 2-ethylhexyl azelate* (2)

*Reaction 2: ethyl 2-ethylhexyl azelate + 2-ethylhexan-1-ol → ethanol + bis(2-ethylhexyl) azelate* (3)

These reactions were irreversible in the experiments of Gómez et al.,[1] due to the removal of ethanol by evaporation during the reaction[1]. Serrano et al.[7] showed that the selectivity of Novozym 435 for reaction 2 over reaction 1 was 0.37. One way of interpreting this selectivity is to consider a situation in which the concentrations of diethyl azelate and ethyl 2-ethylhexyl azelate are equal at a particular instant during the reaction; in this case, reaction 2 will proceed at only 37% of the rate of reaction 1.

Later, Mitchell et al.[8] applied the method to the esterification of trimethylolpropane (TMP). TMP has three alcohol groups, such that three esterification reactions can occur:

*Reaction 1: Fatty acid + TMP → water + TMP monoester* (4)

*Reaction 2: Fatty acid + TMP monoester → water + TMP diester* (5)

*Reaction 3: Fatty acid + TMP triester → water + TMP triester* (6)

Two selectivities can be defined for this system: (i) $S_M$ is the selectivity of the enzyme for catalyzing reaction 2 in relation to reaction 1; (ii) $S_D$ is the selectivity of the enzyme for catalyzing reaction 3, again, in relation to reaction 1. Mitchell et al.[8] determined these selectivities for various datasets from the literature, for which water was removed during the process, making the reactions irreversible (Table 1). Since all selectivities are less than 1 and $S_M > S_D$, it can be concluded that the specificity of the lipases for esterifying TMP is greatest for unesterified TMP, lower for the monoester and lower still for the diester.

**Table 1** Selectivities estimated for the lipase-catalzyzed esterification of trimethylolpropane.

| Data source | Enzyme | Fatty acid | $S_M$ | $S_D$ |
|---|---|---|---|---|
| Åkerman et al.[2] | Novozym 435 | oleic acid | 0.371 | 0.029 |
| Bornadel et al.[3] | Novozym 435 | oleic acid | 0.886 | 0.091 |
| Tao et al.[4] | lipase of Candida sp. 99-125 | caprylic acid | 0.748 | 0.314 |

In relation to the production of galactooligosaccharides, Mitchell and Krieger[9] analyzed data of Guerrero et al.[5] and Vera et al.[6] for a system involving the production of GOS3 (Gal-Gal-Glc), GOS4 (Gal-Gal-Gal-Glc) and GOS5 (Gal-Gal-Gal-Gal-Glc) from lactose, catalyzed by the β-galactosidase of *A. oryzae*. This system involves various competing transgalactosylation and hydrolysis reactions,:

*Reaction 1 – production of GOS3: Lactose + Lactose → glucose + GOS3* (7)

*Reaction 2 – production of GOS4: Lactose + GOS3 → glucose + GOS4 ()* (8)

*Reaction 3: production of GOS5: Lactose + GOS4 → glucose + GOS5* (9)

*Reaction 4 – primary hydrolysis of lactose: Lactose + water → glucose + galactose* (10)

*Reaction 5 – secondary hydrolysis of GOS3: GOS3 + water → lactose + galactose* (11)

*Reaction 6 – secondary hydrolysis of GOS4: GOS4 + water → GOS3 + galactose* (12)

*Reaction 7 – secondary hydrolysis of GOS5: GOS5 + water → GOS4 + galactose* (13)

The production of galactooligosaccharides is kinetically controlled, with the maximum yields of the galactooligosaccharides depending on the selectivity of the enzyme for the transgalactosylation reactions in relation to the primary and secondary hydrolysis reactions. Table 2 shows the selectivities obtained in the analysis.

**Table 2** Selectivities of the β-galactosidase of *Aspergillus oryzae* for transgalactosylation and hydrolysis reactions during the prodution of galactooligosaccharides.

| Selectivity | Value for data of Guerrero et al.[5] | Value for data of Vera et al.[6] |
|---|---|---|
| Production of GOS4 over production of GOS3 | 1.50 | 1.68 |
| Production of GOS5 over production of GOS3 | 1.50 | 2.41 |
| Production of GOS3 over primary hydrolysis of lactose | 919 | $3.72 \times 10^8$ |
| Production of GOS3 over secondary hydrolysis of GOS3 | 16 | 6 |
| Production of GOS4 over secondary hydrolysis of GOS4 | 21 | 8 |
| Production of GOS5 over secondary hydrolysis of GOS5 | 47 | 15 |

These selectivities show that, amongst the transglycosylation reactions, the β-galactosidase of *A. oryzae* is more selective for producing GOS4 and GOS5 over GOS3. Despite this, the production of GOS3 dominates, due to the high initial concentrations of lactose used, 50% w/w in the experiments of Guerrero et al.[5] and 1.8 mol/kg in the experiments of Vera et al.[6] Also, the β-galactosidase of *A. oryzae* has a very high selectivity for converting lactose to GOS3 over primary hydrolysis of lactose, so primary hydrolysis is negligible. However, once GO3, GOS4 and GOS5 are produced, significant secondary hydrolysis occurs due to the relatively low values of the selectivities for their production in relation to their secondary hydrolysis (with values ranging from 6 to 47) and the high water content of the system, the initial molar ratio of water to lactose being 26:1 in the experiments of Guerrero et al.[5] and 12:1 in the experiments of Vera et al.[6]

## 4 DISCUSSION

Our method for estimating selectivities has several advantages over methods that have previously been used in enzymatic processes involving the Ping Pong bi bi mechanism. The key feature of our method is the use of percentage degree of reaction as the independent variable, which leads to a set of equations in which the selectivities of the enzyme are the only parameters. As mentioned in the introduction, this reduces problems of correlation between parameters during curve fitting. There are two further advantages. First, the enzyme concentration does not appear in the final equation set. Consequently, even if denaturation occurs during the reaction, it does not affect the analysis. Second, terms describing inhibition cancel out of the equation set during the conversion of the independent variable from time to percentage degree of reaction. Consequently, even if inhibition occurs during the reaction, it does not affect the analysis.

Our method can be applied to any process catalyzed by an enzyme that follows the Ping Pong bi bi mechanism. To do this, one needs to (i) determine the various reactions that occur in the system; (ii) build a model, which will contain rate terms similar to Eq. (1) for each reaction that occurs, and convert the independent variable of the equations from time to percentage degree of reaction; (iii) obtain experimental profiles for the key species, especially the species that will be used to calculate the percentage degree of reaction; (iv) use the selectivities as fitting parameters to fit the model to the experimental profiles for the species, plotted against percentage degree of reaction. once the selectivities have been determined using our method, a simple kinetic model can be fitted to the profiles plotted as a function of time.[9] This fitting will allow estimation of parameters related to saturation, inhibition and denaturation of the enzyme. The resulting time-based model can be used as a tool to guide process scale-up and optimization.

## 5 CONCLUSION

We have reviewed our recent work regarding the estimation of selectivities in biotechnologically important reactions that are catalyzed by enzymes that follow the Ping Pong bi bi mechanism, showing its application to two lipase-catalyzed reactions, namely the esterification of a triol and the transesterification of a dicarboxylic acid, and also to transgalactosylation reactions catalyzed by a β-galactosidase. Our method uses balance equations based on the Ping Pong bi bi kinetic equation, but with the independent variable changed from time to percentage degree of reaction. This eliminates constants related to inhibition, saturation and denaturation of the enzyme, such that the selectivities are the only parameters of the model. This not only reduces problems of parameter correlation but also means that the method is unaffected by inhibition and denaturation of the enzyme, even if these phenomena occur. The selectivities estimated by our method are important parameters in time-based models, which can be used to guide bioreactor design and optimization.

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## ACKNOWLEDGEMENTS


The authors thank CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico) for research productivity scholarships.