Kinetic constraints and features imposed by the immobilization of enzymes onto solid matrices: A key to advanced biotransformation

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The kinetics of immobilized enzymes can not be analyzed by means of the simple Michaelis-Menten concept, which generally fails to describe the immobilized state due to both its probable barriers, and because the active concentration of the enzyme approaches, or even exceeds this of its substrate(s). In such cases, the various experimental data are usually treated by complex rate equations comprising too many parameters acquiring different natures and meanings, depending on both the properties of the immobilization state and the experimental conditions; thus, more likely, only apparent values of the Michaelis-Menten kinetic parameters can be estimated experimentally. Likewise, immobilization is often a key method in optimizing the operational performance of enzymes, in both laboratory and industrial scale, and affects considerably the kinetics in non-aqueous and non-conventional media due to several issues as the structural changes of the enzyme molecule, the heterogeneity of the system, and the partial or total absence of water. In this work a theoretical approach is described on the formulation of simplified rate equations, reflecting also the actual mass balances of the reactants, in the case where esterification synthetic reactions are catalyzed by immobilized lipases, in either a non-aqueous organic solvent or in a non-solvent system.

Keywords: Enzyme kinetics, Immobilized enzymes, Esterification, Non-solvent systems

Immobilized enzymes have found extensive applications during the last decade in the industrial processes due to either high priced enzymes and/or augmented demands for higher yields of free-enzyme-products1. Immobilization on various solid matrices seems to be advantageous by providing multiple uses of enzyme in continuous reactors, by enhancing abilities as fast handling of the reaction courses through immersing/withdrawing of biocatalyst in the reaction medium or via additional effects caused by the other reactants2,3. The immobilization of enzymes, which is generally carried out on matrices either non-porous or into porous, may affect both their catalytic properties (activity, stability, etc) as well as their physical and chemical properties (structure, hydrophobicity, geometry, etc), and it can be achieved by either attachment onto solid carriers, or by cross-linking, encapsulation in membranes and in microcapsules, and/or by precipitation in organic solvents, etc4,5. Immobilized enzymes are physically separated from the bulk solvent, are permeable to all reactant molecular species, and often exhibit increased stability due to a more rigid tertiary protein structure, which is not regarded as contributing always in efficient catalysis6; however, this latter rigidity is not necessary a disadvantage (e.g. auto hydrolysis of proteases is eliminated) as immobilized enzymes can be re-used in continuous reactors and moreover are protected, within reasonable limits, from thermal and/or pH inactivation7. As example, let it be the esterification of short-chain fatty acids catalyzed by immobilized lipases, where although the pH-value in the neighborhood of the enzyme molecule is lower considerably, however, immobilization prevents the acid inactivation of lipase molecules, under certain circumstances; further complexities are introduced in the systems of esterification processes, as the formed water surrounds the lipase molecules, affects its conformation, and causes inconvenience to the

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mathematical modeling of the reaction system due to other sources (e.g. profound diffusional effects, agitation speed, and others)\(^8\).

When the aggregates of immobilized enzyme are sunk in aqueous solvent, then it is usually assumed that a boundary double layer (Helmotz-Stern) is formed, and that all species of the system should be diffused from the bulk solvent through the double layer in order to reach the enzyme surface and react, and vise versa; however, this is not the case in non-aqueous organic solvents whose polarity is a decisive factor in the occurrence and importance of diffusional effects\(^9,10\). An important issue of the complications due to phase heterogeneity, is the concept of \(pH\), and its measured value, whose the known meaning in dilute aqueous solutions is not operative in the case of reaction involving immobilized enzyme, depending also on the composition of the bulk solvent (presence of organic solvents, polarity of organic solvents, etc)\(^7,11,12\).

As a consequence, the kinetic behavior of immobilized enzymes has found an extensive interest comprising a variety of published theoretical approaches and experimental works. These attempts were focused on describing and treating observed effects due to the immobilization which influence a number of properties of the enzyme molecule. Such properties were found to be either conformational changes affecting enzyme activity, or enzyme-substrate(s) interactions due to the nature of the solvent (aqueous, organic polar or non polar, occurrence of micelles, etc) and/or the electrostatic charges of the used enzyme and the immobilization matrix; more factors affecting the reactivity of immobilized enzymes are the water content, the kinetic mode of the free enzyme, the rate-controlling step of the observed reaction i.e. whether it is, diffusional or kinetically controlled, etc\(^13\). Nevertheless, it is important to point out that the active concentrations of the immobilized lipases is roughly equal to that of their substrates, and thus the Michaelis-Menten concept should be ruled out\(^14\). This latter condition, which holds always true in the case of synthetic reactions catalyzed by immobilized hydrolases (mainly lipases) in non-conventional media, is apparently not taken into account even in recent published works where their rate equations were generated by ignoring the mass balance of the used substrate(s)\(^5,17\). This work is an attempt to approach theoretically and formulate simplified rate equations, which reflect also the actual mass balances of the reactants, in the case of immobilized lipases onto porous matrices, which catalyzed esterification reaction in an either non-solvent system or in non-aqueous organic solvent.

**Methods**

Suitable arithmetic approaches have been applied by using binomial expansion in converging series to specific enzymatic rate equations. Further arithmetic approximations have been also used in order to simplify awkward equations and avoid imaginary and/or complex solutions. All calculations including binomial expansions and simplifications were performed by the use of both the program “Theorist” for Apple Macintosh computers\(^18\), and the Wolfram Alpha LLC 2010\(^19\).

**Development of the rate equations**- It has been reported that a lipase-esterification process which is carried out in a conventional reaction medium comprising water, among other solvent components, it follows the ping-pong bi-bi mechanism (Cleland’s nomenclature) including substrate inhibition by the used alcohol\(^7,8,15\), according to scheme 1. Therein, the enzyme \(E\) is firstly reacts with a fatty-acid \(Q\), forming the enzyme-substrate complex \(EQ\), which decomposes to water (first product) and acyl-enzyme \(F_1\); this latter it reacts immediately with the present, in relative excess, alcohol \(P_1\) and forms a second enzyme-substrate complex \(F_1P_1\), which is decomposed to free enzyme and the ester \(A_1\). Another reaction has been generally acknowledged, which describes the formation of a non-productive enzyme-substrate complex \(EP_1\) introducing a competitive inhibition by the alcohol; the competitive inhibition by the product \(H_2O\), has been also commented\(^8,15,20,22\).

**Scheme 1**

\[
\begin{align*}
E + Q_1 & \xrightleftharpoons[k_1]{k_4} EQ_1 & \xrightarrow{k_2} & F_1 + H_2O \\
K_{mQ_1} & & \\
F_1 + P_1 & \xrightleftharpoons[k_3]{k_5} F_1P_1 & \xrightarrow{k_4} & E + A_1 \\
K_{mP_1} & & \\
E + P_1 & \xrightleftharpoons[k_5]{k_6} EP_1 \\
K_{iP_1} & & 
\end{align*}
\]
Systems of chemical equations similar to the above one are generally treated by assuming the establishment of an equilibrium or a steady-state condition\textsuperscript{20,23}; then, by taking into account that the relations \( \frac{d[EQ_1]}{dt} = 0 \), \( \frac{d[F_1P_1]}{dt} = 0 \), hold true, the rate equation can be developed by considering also the mass balance equation (1), where \([E]_t\) is the total active concentration of the immobilized lipase.

\[ [E]_t = [E] + [EQ_1] + [F_1] + [F_1P_1] + [EP_1] \quad \cdots (1) \]

Although different esterification processes have been reported, which were catalyzed by immobilized lipases in various reaction media and analyzed in terms of scheme 1, however it seems more reasonable that the experimenter should choose either a reaction medium based on specific organic solvents, or alternatively, a non-solvent system, where esterification would be performed within the reactive alcohol. In the former case the relations \([E]_t \approx [Q_1]_t\), \([P]_t\) hold true, while in the latter case the relations \([E]_t \approx [Q_1]_t\), \([E]_t \ll [P]_t\) and \([Q]_t \ll [P]_t\), hold true\textsuperscript{1,7,15,20}; thus, either two more mass balance equations (2) and (3) should be taken into account (former case), or one more mass balance equation (2) should be taken into account (latter case). In equations (2) and (3) \([Q]_t\) and \([P]_t\) denote the total concentrations of the used acid and alcohol substrates, respectively.

\[ [Q_1]_t = [Q_1] + [EQ_1] \quad \cdots (2) \]

\[ [P]_t = [P_1] + [F_1P_1] \quad \cdots (3) \]

Under these conditions, and by taking also into account that the produced water molecules are either continuously removed and/or destroyed chemically\textsuperscript{7,24}, the esterification process in scheme 1 can be comprised by the two first simple successive reactions only, where two unireactant enzymatic species i.e. E and F\textsubscript{1}, react with two separate substrates i.e. Q\textsubscript{1} and P\textsubscript{1}, respectively. The third chemical equation of scheme 1, above, should not further be taken into account, and probably it has been introduced in order to both facilitate the fitting of consequent experimental points and reduce the errors, which in turn it seems more likely that were due to the adoption of incorrect kinetic model\textsuperscript{7,8,15,25}, as it has been commented by Gandhi et al and references therein\textsuperscript{26}; then, only the produced water molecules could cause inhibitory effects due to the fact that catalysis by the most of lipases has as prerequisite the binding of substrates in the single oxyanion hole of these enzymes. Finally, and for the sake of clarity we should recall the assumption that herein we will deal with lipases immobilized onto porous matrices, catalyzing an acid/alcohol esterification reaction in non-aqueous non-conventional media. Therefore, diffusional effects should not be taken into account due to the lack of a boundary double-layer, as well as to the contradiction with the assumption that either a steady-state condition or an equilibrium is restored, respectively, and also due to the relatively increased stirring rate\textsuperscript{9,10}.

**Results and Discussion**

In all cases, the establishment of steady-state and/or equilibrium conditions should be also assumed where the relative reaction rates are given in terms of the concentrations of the enzyme-substrate complexes and thus, forms of quadratic equations are used whose only the negative root solutions are valid; additionally, it is required that \([EQ_1]_t = 0\) and \([F_1P_1] = 0\) when either \([Q_1]_t = 0\) or \([E]_t = 0\), and/or \([P]_t = 0\) or \([F_1]_t = 0\), respectively.

**Case A1—** In that case the relations \([E]_t \approx [Q_1]_t\), \([P]_t\) as well as equations (2) and (3) hold true, and thus, for the first two successive enzymatic reactions (scheme 1), the equations (4) and (5) should be taken into account, respectively\textsuperscript{14}; in equations (4) and (5) \(V_{max 1}\), \(V_{max 2}\) and \(V_{k22}\), \(V_{k44}\) are the Michaelis-Menten maximum velocities and the reaction rates of the first and second enzymatic reactions, respectively, and \(K_{mQ1}\) and \(K_{mp1}\) are their corresponding Michaelis-Menten constants.

\[ \frac{V_{k22}}{V_{max 1}} = \frac{[E]_t + [Q_1]_t + K_{mQ1}}{2} \quad \sqrt{\left( \frac{[E]_t + [Q_1]_t + K_{mQ1}}{2} \right)^2 - 4[E]_t [Q_1]_t} \quad \cdots (4) \]

\[ \frac{V_{k44}}{V_{max 2}} = \frac{[F_1]_t + [P]_t + K_{mp1}}{2} \quad \sqrt{\left( \frac{[F_1]_t + [P]_t + K_{mp1}}{2} \right)^2 - 4[F_1]_t [P]_t} \quad \cdots (5) \]

Equations similar to (4) and (5) have been elaborated previously by binomial expansion of the
terms of the square roots in converging series and found that its simpler expression operates as a fair estimate of the enzymatic reaction rate in cases as the examined one\textsuperscript{14}; thus, in a straightforward analogy and treatment of equations (4) and (5), the possible simplest forms which can be attributed to the above equations are given by equations (4a) and (5a), respectively, i.e.:

\[
\frac{v_{k_2}}{V_{max_1}} = \frac{[Q_i]}{[E]_1 + [Q_i]_1 + K_{mQ_i}} \Rightarrow
\]

\[
v_{k_2} = \frac{V_{max_1} [Q_i]}{[E]_1 + [Q_i]_1 + K_{mQ_i}} \quad \ldots (4a)
\]

\[
\frac{v_{k_4}}{V_{max_2}} = \frac{[P_i]}{[F_i]_1 + [P_i]_1 + K_{mP_i}} \Rightarrow
\]

\[
v_{k_4} = \frac{V_{max_2} [P_i]}{[F_i]_1 + [P_i]_1 + K_{mP_i}} \quad \ldots (5a)
\]

Therefore, and for the examined case, the total rate of the esterification reaction should depend on the relation between the rate constants \(k_2\) and \(k_4\) (scheme 1). If \(k_2 \ll k_4\) then the total enzymatic reaction rate \(v_{enz}\) would be given by equation (4a), while if \(k_2 \gg k_4\) then the \(v_{enz}\) would be given by equation (5a). Nevertheless, a kineticist would consider that both equations (4a) and (5a) have the general form of equation (6), where the subscript “(any)” is attributed to either any substrate and/or any \(K_m\) and the superscript “app” is assigned to an apparent \(K_m\)-value, which verifies the relations \(K_{mQ_i} < K_{mQ_i}^{app}\) and \(K_{mP_i} < K_{mP_i}^{app}\); thus, equations (4a) and (5a) incorporate the concept of a competitive inhibition one.

\[
v_{enz} = \frac{V_{max} [S_{(any)}]}{[S_{(any)}]_1 + K_{mP_i}^{app}} \quad \ldots (6)
\]

**Case A2**—Herein, the relations \([E]_1 \approx [Q_i]_1, [E]_1 \ll [P_i], [Q_i]_1 \ll [P_i]_1\) and \([F_i][P_i]_1 \ll [P_i]_1\), as well as equation (2), hold true, while equation (3) decays to the form \([P_i]_1 = [P_i]\); thus, for the first two successive enzymatic reactions (scheme 1), the equations (4) and (7) should be taken into account, respectively, where the same notations are valid for their parameters.

\[
\frac{dE}{dt} = \frac{k_1}{k_1} [E]_Q Q + k_2 [F_i]_1 [P_i]_1 - k_3 [E][Q_i] = 0 \quad \ldots (8)
\]

\[
\frac{d[F_i]}{dt} = k_3 [F_i]_1 [P_i]_1 - (k_3 + k_4) [F_i][P_i]_1 = 0 \quad \ldots (10)
\]

The above equations (8) and (9) are solved for \([E]\) (equations 11a and 11b) and are equated to form equation (12); then by solving equation (10) for \([F_i]\) and substituting the \([F_i][P_i]_1\) from equation (12), equation (13) is obtained. Next, substitution to
equation (1) of equations (11a) to (12) is carried out, where the term \([EP]_t\) has been omitted, by taking into account, also, equations (2) and (3), according to the examined case, i.e., (a) when the relations \([E]_t \approx [Q]_1\), \([E]_t \approx [P]_1\), \([E]_t \ll [P]_t\), and \([Q]_t \ll [P]_t\) hold true.

\[
[E] = \frac{k_1[EQ]_1}{[Q]_t} + k_2[F]_1[P]_1
\]

... (11a),

\[
[E] = \frac{(k_1 + k_2)[EQ]_1}{[Q]_t}
\]

... (11b),

\[
[F]_1 = \frac{k_2[EQ]_1}{k_4[Q]_t}
\]

... (12),

\[
[F]_1 = \frac{(k_1 + k_2)[EQ]_1}{k_4[Q]_t}
\]

... (13).

Then, in the (a) situation equation (1) is transformed to equations (14) and (14a), while in the (b) situation equation (1) is transformed to equation (15) and (15a), respectively:

\[
[E]_t = [EQ]_1 \{1 + \frac{k_1 + k_2}{k_4[Q]_t} \} + \frac{k_2}{k_4} \left\{1 + \frac{k_2}{[P]_t} \right\}
\]

... (14),

or

\[
[E]_t = [EQ]_1 \{1 + \frac{k_1}{[Q]_t} + \frac{k_2}{[Q]_t} + \frac{k_4([Q]_t - [EQ]_1)}{[P]_t} \} + \frac{k_2}{k_4} \left\{1 + \frac{k_2}{[P]_t} \right\}
\]

... (14a).

Furthermore, by considering equation (14a) as well as the abovementioned conditions for the (a) situation, a quadratic equation is developed whose the normalized form is appeared in equation (16); again, the negative root solution of equation (16) is taken into account. In a straightforward analogy to case A1 we could write equation (16a) which coincides to the general form of equation (6) as we could suggest that \(K_m = \frac{K_m^Q}{[Q]_t} \). Likewise, for the (b) situation a cubic relation is produced as appeared in equation (17); however, due to the fact that \([EQ]_1 \neq 0\), as well as \([Q]_1 \gg [Q]_t\) hold true, then equation (17) can be transformed to its equivalent quadratic equation (18), suggesting that \(K_m^Q = \frac{K_m^Q}{[Q]_t} \). In that latter case. In both equations (16a) and (18a) it has been considered that the relation \(k_2 \ll k_4\) holds true.

\[
[E]_t = [EQ]_1 \{1 + \frac{K_m^Q}{[Q]_t} \} + \frac{k_2}{k_4} \left\{1 + \frac{K_m^Q}{[P]_t} \right\}
\]

... (15).

\[
\frac{[E]_t}{[Q]_t} = \frac{[E]_t}{[Q]_t} \{1 + \frac{K_m^Q}{[Q]_t} \} + \frac{k_2}{k_4} \left\{1 + \frac{K_m^Q}{[P]_t} \right\}
\]

... (15a).

\[
K_m^Q = \frac{V_{max}[Q]_t}{[E]_t} \frac{[E]_t}{[Q]_t} \{1 + \frac{K_m^Q}{[Q]_t} \} + \frac{k_2}{k_4} \left\{1 + \frac{K_m^Q}{[P]_t} \right\}
\]

... (16a).
\[
\left[\text{EQ}_1\right]^2 \left[\text{EQ}_2\right]^2 \left\{ \left[\text{E}\right]_t + \left[\text{P}\right]_t + K_{mQ_1} + K_{mP_1} + [Q_1]_t \right\}
\]
\[
+ \left[\text{EQ}_1\right] \left( k_4 \left[\text{E}\right]_t \left[\text{P}\right]_t + K_{mQ_1} + K_{mP_1} + [Q_1]_t \right)
\]
\[
+ \left[\text{EQ}_2\right] \left( k_2 \left(1 + \frac{k_2}{k_4}\right) k_{34} \right)
\]
\[
= 0 \quad \ldots (17)
\]
\[
\left[\text{EQ}_2\right]^2 \left[\text{EQ}_1\right] \left\{ \left[\text{E}\right]_t + \left[\text{P}\right]_t + K_{mQ_1} + K_{mP_1} + [Q_1]_t \right\}
\]
\[
+ \left[\text{EQ}_2\right] \left( k_4 \left[\text{E}\right]_t \left[\text{P}\right]_t + K_{mQ_1} + K_{mP_1} + [Q_1]_t \right)
\]
\[
+ \left[\text{EQ}_1\right] \left( k_4 \left(1 + \frac{k_4}{k_2}\right) \right)
\]
\[
= 0 \quad \ldots (18)
\]
\[
v_{\text{enz}} = \frac{V_{\text{max}} [Q_1]_t}{ \left[\text{E}\right]_t + [\text{P}]_t + K_{mQ_1} + K_{mP_1} + [Q_1]_t}
\]
\[
+ \left[\text{EQ}_2\right] \left( k_4 \left(1 + \frac{k_4}{k_2}\right) \right)
\]

By simple inspection and perception of equations (4a), (5a), (7), (16a) and (18a) an inherent similarity among them is deduced, although equations (16a) and (18a) are more complicated and over-parameterized ones, and thus should not be recommended.

The choice of the proper rate equation is a matter of fitting the corresponding experimental data and deciding by based on concrete convergence criteria. Nevertheless, the use of non parametric curve fitting methods it should be recommended. In addition, experimenters could take into account previous theoretical approaches, where either the use of a systematic insertion of higher powers of the employed substrate in a virial-like expansion of equation under consideration or the use of the conception of a fractal approach of the same equation is a key to a possible diagnosis of the proper equation.

**Conclusions**

It has been shown that by taking into account the mass balances of the reacting immobilized lipase, as well as that of the utilized acid and/or alcohol, theoretical equations were formulated, in order to describe with accuracy the kinetics of esterification by immobilized lipase onto a porous matrix, in either a non-aqueous organic solvent or in a non-solvent system. By systematic use of the proposed equations, herein, the error due to the ignorance of the fact that the active concentration of the used lipase approaches that of its substrates (acid and/or alcohol) is restored, under the aforementioned reaction conditions; the use of an unadulterated ping-pong-bi-bi mechanism, which is generally accepted and it is followed in bulk aqueous media, it cannot and it should not be applied under the examined, herein, esterification conditions.

**References**