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Abstract

Kinetic studies of homogeneous enzyme reactions where both the substrate and enzyme are soluble have been well described by the Michaelis Menten (MM) equation for more than a century. However, many reactions are taking place at the interface of a solid substrate and enzyme in solution. Such heterogeneous reactions are abundant both in vivo and in industrial application of enzymes but it is not clear whether traditional enzyme kinetic theory developed for homogeneous catalysis can be applied. Since the molar concentration of surface accessible sites (attack-sites) often is unknown for a solid substrate it is difficult to assess whether the requirement of the MM equation is met. In this paper we study a simple kinetic model, where removal of attack sites expose new ones which preserve the total accessible substrate, and denote this approach the substrate conserving model. The kinetic equations are solved in closed form, both steady states and progress curves, for any admissible values of initial conditions and rate constants. The model is shown to merge with the MM equation and the reverse MM equation when these are valid. The relation between available molar concentration of attack sites and mass load of substrate is analyzed and this introduces an extra parameter to the equations. Various experimental setups to practically and reliably estimate all parameters are discussed.

Keywords: Enzyme kinetics, Michaelis Menten, parameter estimation

1. Introduction

Kinetic studies of enzyme catalysis make up a cornerstone in quantitative biochemistry, which has fueled the development of both fundamental understanding and technical applications of enzymes. The vast majority of work in this area addresses homogenous reactions in the aqueous bulk. This implies that enzyme, substrate(s) and product(s) are diffusible species, and that the rate of their interconversion can be described by conventional rate equations.

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The simplest possible enzyme reaction may be described by the microkinetic scheme

$$e + s \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} es \overset{k_2}{\rightarrow} e + p \,, \tag{1}$$

which has been used for more than a century to rationalize experimental measurements [1, 2]. Typically, this reaction implies a fast formation of complex, es, followed by a longer lasting state where $\frac{d}{dt}es \approx 0$. This is known as the quasi steady state condition and it may prevail until substrate depletion becomes important [3, 4, 5]. This timescale separation puts the study of scheme (1) into the mathematical framework of singular perturbed systems [6, 7] where constructing short term and long term solutions of the system is a typical approach.

During the quasi steady state, the rate of product formation, v_{ss}^{mm} can be computed by the Michaelis Menten (MM) equation

$$v_{ss}^{mm} = \frac{k_2 E_0 S_0}{S_0 + K_m} \,. \tag{2}$$

A sufficient criterion to infer quasi steady state leading to equation (2) is ([8, 9])

$$E_0 << S_0 + K_m \,. \tag{3}$$

Equation (2) allows estimation of K_m and k_2 by performing a series of experiments with different substrate concentrations provided inequality (3) is fulfilled. A voluminous amount of work has been put in mathematical analysis and approximation of reaction (1), based on pseudo first order kinetics, slow-fast dynamics, clever use of a new variable $S + ES$ leading to the total quasi steady state assumption, and discussion of the importance of almost constant substrate concentration during the initial phase [10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22]. We recommend the review [5] for an introduction and overview.

While equation (2) underlies the typical experimental approach where enzyme is saturated with substrate, one can deduce a similar expression as substrate is saturated with enzyme, the reverse Michaelis Menten (rMM) equation

$$v_{ss}^{rmm} = \frac{k_2 E_0 S_0}{E_0 + K_m} \,, \tag{4}$$

with validity criterion [23]

$$S_0 << E_0 + K_m \,. \tag{5}$$

However, the underlying premise of a homogeneous environment is not justified for a large group of enzyme reactions both in vivo [24] and industrial applications [25]. The group includes enzymes in a well mixed, homogeneous liquid interacting with an interface of a solid substrate, and it is not clear whether traditional enzyme kinetic theory developed for homogeneous catalysis can be applied in such cases. Examples of this type of enzyme process include digestive

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reactions in the intestine and numerous technical enzyme applications such as stain removal, dough conditioning and textile bleaching [25].

Another important case is biomass conversion where cellulose is hydrolyzed by selected cellulases. This process is essential for natural carbon cycling and for industrial production of large scale biofuels based on lignocellulosic feedstocks [26, 27]. A review of kinetic models for these processes can be found in [28] and an example of stochastic modelling can be found in [29]. One specific enzyme-substrate system is illustrated in figure 1.

The current work pivots around the hypothesis that when a soluble enzyme degrades an insoluble substrate like cellulose, it is likely that a temporary condition occurs in which s is nearly constant. See [30] (supporting information) for an experimental validation. In this study it was found that cellulolytic enzymes, which work at the solid-liquid interface show steady-state kinetics even when the accessible surface of the substrate was covered with enzyme (substrate saturation). This stationarity relies on the balance between the enzymatic removal of substrate attack sites that are initially accessible and the exposure of new (originally inaccessible) attack sites as a result of the enzymatic break-down. This may happen as the enzyme-substrate complex only converts some of the attacked cellulose strand hence leaving a free attack site when the complex dissociates, or it may happen through a complete removal of a layer of substrate, with the subjacent layer having as many free attack sites as the substrate layer just removed. This type of stationarity is unknown for homogeneous reactions and fundamentally different from quasi steady-state in such systems. We will call this approach the substrate conserving model, and in its simplest form it may be captured by the microkinetic scheme

$$e + s \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} es \overset{k_2}{\rightarrow} e + s + p \,, \tag{6}$$
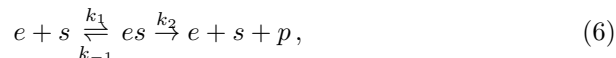
which is presented here for the first time to our knowledge. It is important to note that from a molecular point of view the substrate, s, at the left side of the scheme is not the same as the substrate s on the right side of the scheme. However, from a kinetic point of view they are the same. The substrate reappear on the right side due to the nature of an insoluble substrate and not due to an autocatalytic step. In that sense our scheme is fundamentally different from a cyclic or replicative system [12, 31], where the product of the reaction is resynthesized or reused as substrate for other reactions. Experimental work on cellubiohydrolase supporting reaction scheme (6) is in progress by our group.

One key challenge for any kinetic description of a reaction with insoluble substrate reaction arises from ambiguities regarding the molar concentration of the substrate [28, 32]. Thus, some measure of the number-density of substrate is needed in conventional rate equations, and this problem becomes particularly intricate in attempts to apply a quasi steady-state assumption, which has proven extremely useful in enzyme kinetics. Specific problems with this assumption occurs because a condition for its validity, inequality (3), becomes abstruse for an insoluble particle. Inspired by basic ideas within traditional adsorption theory, we address this by introducing the density of attack sites for the enzyme
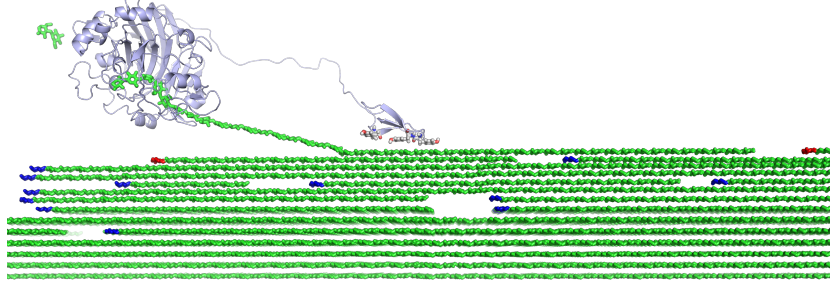
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Figure 1: Illustration of the substrate conserving model for a cellulose degrading enzyme such as the cellobiohydrolase Cel7A (cartoon representation). The enzyme attacks accessible reducing ends (attack-sites) on the surface of the cellulose crystal. These attack sites are shown as red sticks. Following the attack of these sites the enzyme degrades the cellulose strands (green). Degradation of the strands exposes other attack sites (blue sticks) that were initially inaccessible. This in turn opens up for degradation of hidden substrate. The substrate conserving model assumes that this leads to a constant number of accessible attack sites for a limited time. The figure was made in Pymol(R) using the PDB files 4C4C and 2MWK).

on the substrate surface. By attack sites we mean loci on the substrate surface that can combine productively with the enzyme. Although such sites are not evenly distributed in the suspension, we suggest that their overall density makes up a useful surrogate of a molar substrate concentration. Therefore, the molar concentration of substrate, S, is assumed to be related to the mass load (i.e. concentration in units of g/L) of substrate $\hat{S}$ by

$$S = \Gamma \hat{S} \,, \tag{7}$$

where Γ is a conversion factor of available attack sites per mass unit substrate which can be determined from adsorption experiment of the substrate. In the discussion we will return to an alternative way of estimating Γ.

The remainder of the paper is organized as follows: The differential equations corresponding to (6) are formulated, and first the steady state equations are considered. This leads to simple and realistic equations that can readily be applied to experimental data. Then, the time dependent solution is derived for any values of initial conditions and rate constants. The results are compared to the MM and rMM equations subject to their specific conditions of validity.

2. Analysis

We will derive the governing equations of reaction (6) using the molar concentration of substrate, S. After derivations we will convert to substrate load by (7) which is the quantity that is normally known and controllable in experiments.

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Using the convention $\dot{x} = \frac{dx(t)}{dt}$ and capital letters for concentrations of species, the reaction scheme (6) leads from the law of mass action to the equations

$$\dot{E} = -k_1 E \cdot S + (k_{-1} + k_2)\, ES \tag{8a}$$

$$\dot{ES} = k_1 E \cdot S - (k_{-1} + k_2)\, ES \tag{8b}$$

$$\dot{S} = -k_1 E \cdot S + (k_{-1} + k_2)\, ES \tag{8c}$$

$$\dot{P} = k_2 ES \tag{8d}$$

with $E(0) = E_0, S(0) = S_0, ES(0) = P(0) = 0$. The two conserved quantities (substrate preservation, enzyme preservation), $\dot{S} + \dot{ES} = 0$, $\dot{E} + \dot{ES} = 0$ means equation (8) is equivalent to

$$\dot{E} = -k_1 E \cdot S + (k_{-1} + k_2)\, ES \tag{9a}$$

$$ES = E_0 - E \tag{9b}$$

$$S = E - E_0 + S_0 \tag{9c}$$

$$\dot{P} = k_2 (E_0 - E)\ . \tag{9d}$$

The equation of $\dot{E}$ is uncoupled, hence the differential equation for P can be solved after E is calculated. Rewriting $\dot{E}$ using the expressions for ES and S gives

$$\dot{E} = -k_1 E^2 - bE + c \tag{10}$$

with

$$b = k_1 (S_0 - E_0) + k_2 + k_{-1}\ , \quad c = (k_{-1} + k_2)\, E_0\ . \tag{11}$$

Using the Michaelis - Menten constant

$$K_m = \frac{k_{-1} + k_2}{k_1} \tag{12}$$

the roots of equation (10) can be formulated for any positive parameter values and initial conditions

$$E_\pm = \frac{1}{2}\left(-(S_0 - E_0 + K_m) \pm \sqrt{(S_0 - E_0 + K_m)^2 + 4 K_m E_0} \right)\ . \tag{13}$$

As $E_- < 0$, $E_+ > 0$, then E_+ is the only physical realizable solution to $\dot{E} = 0$, and it is globally stable since $\dot{E} > 0$ for $0 \le E < E_+$ and $\dot{E} < 0$ for $E > E_+$. The globally stable solution is a consequence of the conservation of enzyme and substrate in scheme 6. Hence, E_+ represents the free enzyme concentration at steady state, which we will denote E_{ss}. The formulation in

equation (13) illustrates that the argument to the square root is always positive, but an equivalent useful formulation is

$$E_{ss} = \frac{1}{2} \left(-(S_0 - E_0 + K_m) + (S_0 + E_0 + K_m) \sqrt{1 - 4 \frac{S_0 E_0}{(S_0 + E_0 + K_m)^2}} \right).$$

(14)

The steady state concentration of the complex ES is then by equation (9b)

$$ES_{ss} = \frac{1}{2} (S_0 + E_0 + K_m) \left(1 - \sqrt{1 - 4 \frac{S_0 E_0}{(S_0 + E_0 + K_m)^2}} \right),$$

(15)

and v_{ss} is k_2 times this expression by equation (8d)

$$v_{ss} = \frac{1}{2} k_2 (S_0 + E_0 + K_m) \left(1 - \sqrt{1 - 4 \frac{S_0 E_0}{(S_0 + E_0 + K_m)^2}} \right),$$

(16)

which is a main result of the present work. Examples of v_{ss} for varying initial conditions are shown in figure 2. The steady state solutions (14), (15), (16) are globally valid and attracting for any positive $E_0, S_0, k_1, k_{-1}, k_2$. These expressions may be particularly practical for insoluble substrates because the free enzyme concentration can readily be measured after substrate and bound enzyme have been removed by centrifugation. Then, from measurements of v_{ss} and E_{ss}, one may estimate k_2 as

$$k_2 = \frac{v_{ss}}{E_0 - E_{ss}}.$$

(17)

2.0.1. Approximation of steady state solution

A simpler expression of v_{ss} may be practical for parameter estimation based on experimental data. Such an approximation will now be considered. The argument to the square root in the steady state equations is strictly positive by equation (13) which means the power series of the square root is always valid in the variable

$$y = \frac{S_0 E_0}{(S_0 + E_0 + K_m)^2}$$

(18)

with explicit expression

$$1 - \sqrt{1 - 4y} = \sum_{n=1}^{\infty} \frac{(2n)!}{(2n - 1)(n!)^2} y^n$$

(19)

All terms of the series in (19) are positive, hence a truncation at any order will underestimate the true value. The linear order expansion is

$$\sqrt{1 - 4y} = 1 - 2y + \epsilon$$

(20)

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where the remainder or error, ϵ, by Taylor's formula with Lagrange's remainder is

$$\epsilon = 2c^2 \quad \text{for some } 0 < c < y \tag{21}$$

confining ϵ by

$$\epsilon < 2y^2 . \tag{22}$$

Disregarding the ϵ term, we use the power expansion to linear order around zero

$$\sqrt{1 - 4y} \approx 1 - 2y . \tag{23}$$

With the linear order approximation (denoted by superscript 1) to equations (14), (15), (16) we get

$$E_{ss}^1 = E_0 \frac{E_0 + K_m}{S_0 + E_0 + K_m} \tag{24a}$$

$$ES_{ss}^1 = \frac{E_0 S_0}{S_0 + E_0 + K_m} \tag{24b}$$

$$v_{ss}^1 = k_2 \frac{E_0 S_0}{S_0 + E_0 + K_m} . \tag{24c}$$

While easily obtained, these formulas are a useful result of the present work. The formulas are symmetric in E_0 and S_0 which is to be expected from scheme (6), meaning k_2 and K_m can be inferred from saturation experiments of either substrate or enzyme. Formula (24c) which may be seen as an extended MM equation, has been derived in [31, 23] subject to a quasi steady state assumption of scheme (1). In the substrate conserving model, equation (24) is simply a linear approximation to a true steady state.

2.1. Time dependent solution

As the polynomial in equation (10) can always be factorized, we can integrate the equation using separation of variables and partial fraction decomposition

$$E(t) = \frac{E_+ - E_-}{1 + c_1 e^{-\frac{t}{\tau}}} + E_- \tag{25}$$

with

$$c_1 = \frac{E_+ - E_0}{E_0 - E_-} \tag{26}$$

and

$$\tau = \frac{1}{k_1 (E_+ - E_-)} \tag{27}$$

The enzyme-substrate complex is then

$$ES(t) = E_0 - E_- - \frac{E_+ - E_-}{1 + c_1 e^{-\frac{t}{\tau}}} . \tag{28}$$

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Integrating $\dot{P} = k_2 ES$ and using $P(0) = 0$ then gives

$$P(t) = k_2 \left((E_0 - E_-) t - \tau (E_+ - E_-) \ln \left(\frac{e^{\frac{t}{\tau}} + c_1}{1 + c_1} \right) \right) . \tag{29}$$

The formulas (25), (28), (29) provide closed form expressions of the time evolution of scheme (6) for any values of the initial concentrations and rate constants. The time scale τ describes when the system is close to steady state. Inserting the values of $E_\pm$ from equation (13) in equation (27)

$$\tau = \frac{1}{k_1 \sqrt{(S_0 - E_0 + K_m)^2 + 4 K_m E_0}} \tag{30}$$

which is identical to the formula in [33] for initial transient when analyzing the MM equation. The lowest order approximation to the square root using equation (23) is

$$\tau \approx \frac{1}{k_1 (E_0 + S_0 + K_m)} \tag{31}$$

which provides an a priori estimate of the time scale for approach to the steady state with prior knowledge on order of magnitude of k_1 and K_m.

3. Discussion

3.1. Comparison to existing results

Pseudo first order approximations to nonlinear dynamics can be useful for study of enzyme kinetics though care must be taken when considering validity conditions [34]. In the case of effectively constant substrate, $S_0 \gg E_0$, the reaction schemes (1) and (6) may be described by pseudo first order approximations

$$e \underset{k_{-1}}{\overset{k_1 S_0}{\rightleftharpoons}} es \overset{k_2}{\rightarrow} e + p \tag{32a}$$

$$e \underset{k_{-1}}{\overset{k_1 S_0}{\rightleftharpoons}} es \overset{k_2}{\rightarrow} e + p + s \tag{32b}$$

where s in (32b) is redundant as subtrate is in excess. Hence these linear differential equations with constant coefficients lead to identical expressions of $\dot{P}(t)$

$$\dot{P}(t) = \frac{k_2 E_0 S_0}{S_0 + K_m} \left(1 - e^{-k_1 (S_0 + K_m) t} \right) \tag{33}$$

which for $t \gg \tau_1 = \frac{1}{k_1 (S_0 + K_m)}$ is close to v_{ss}^{mm}, equation (2).

This match between the substrate preserving equation and the MM equation is evident from figure (2) when comparing the thin black curve and the grey curve. The MM equation (2) scales linearly with E_0 causing collapsing curves

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of $\frac{V_{ss}}{E_0}$ for varying E_0. This is generally not true in the substrate preserving model except when $E_0 << S_0 + K_m$. As an indicator of self consistency notice that τ approaches τ_1 with the given assumption of substrate excess.

A realization of v_{ss}^1, equation (24c), is compared to the MM equation and the rMM equation. The validity of v_{ss}^1 hinges on y being small, (equation (18)). The validity criterion of the MM equation, $E_0 << S_0 + K_m$ ensures y is small, hence guaranteeing that v_{ss}^1 is a good approximation to v_{ss} (equation (16)). Applying $E_0 << S_0 + K_m$ directly to v_{ss}^1, the result is v_{ss}^{mm} by a simple limit consideration. Hence, the substrate conserving model and the MM equation merge within the range of validity of the latter. By completely analogue argument the substrate conserving model and the rMM equation merge within the range of validity of the latter. To illustrate this, an arbitrary choice of 'small' has been chosen to be 0.1 meaning that the MM equation is considered valid when $E_0 \leq 0.1(S_0 + K_m)$, the rMM equation is considered valid when $S_0 \leq 0.1(S_0 + K_m)$ and v_{ss}^1 is valid when $y \leq 0.1$. This splits the $(S_0/K_m, E_0/K_m)$ - space in regions of validity as seen in figure (3), with v_{ss}^1 having the largest validity domain. We stress that equation (16) is valid in the entire $(S_0/K_m, E_0/K_m)$ - plane.

3.2. Estimation of parameters

We will now discuss the typical experimental approach of measuring v_{ss} as a function of E_0 and $\hat{S}_0$, which are readily controllable. Specifically we focus on how to derive the three parameter k_2, K_m and Γ from such experiment. Equation (16) can be expressed using the mass load of the substrate (i.e. the concentration in g/L) from equation (7) instead of molar quantity

$$v_{ss} = \frac{1}{2}k_2 \left(\Gamma\hat{S}_0 + E_0 + K_m\right) \left(1 - \sqrt{1 - 4\frac{\Gamma\hat{S}_0 E_0}{\left(\Gamma\hat{S}_0 + E_0 + K_m\right)^2}}\right), \qquad (34)$$

and its approximation equation (24c) may similarly be described using mass load

$$v_{ss}^1 = k_2 \Gamma \frac{E_0 \hat{S}_0}{\Gamma\hat{S}_0 + E_0 + K_m} . \qquad (35)$$

The formula (34) (or equation (35) if y from equation (18) is small) is a useful expression for experimentalists wishing to estimate Γ, k_2 and K_m.

In case of $E_0 << S_0 + K_m$, equation (35) reduces to

$$v_{ss}^1 \approx k_2 \frac{E_0 \hat{S}_0}{\hat{S}_0 + \frac{K_m}{\Gamma}} . \qquad (36)$$

As K_m and Γ are only appearing together as a fraction they are not structurally identifiable [35], meaning that no matter the quality of the experiment, they cannot be estimated individually. However, introducing the MM constant in mass units, $\hat{K}_m = \frac{K_m}{\Gamma}$, equation (36) is the Michaelis Menten equation with
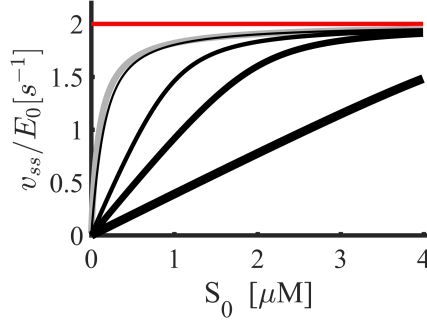
Figure 2: Example of model behaviour for $K_m = 0.1\mu M$, $k_2 = 2s^{-1}$. Black curves are v_{ss} computed from equation (16) with E_0 being 0.1,1,2,5 μM with increasing line width corresponding to increasing E_0. The grey curve is the MM equation (2), and the red curve is the common saturation level, k_2. For decreasing E_0 equation (16) approach the MM equation. When the MM equation is valid, $\frac{v_{ss}}{E_0}$ is constant for varying E_0. As E_0 is increased, the validity criterion of the MM equation, (3), is not fulfilled. In this case, $\frac{v_{ss}}{E_0}$ is not invariant to varying E_0 in the substrate conserving model.
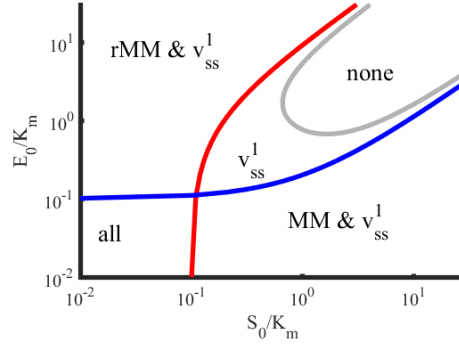

Figure 3: Validity regions of the MM equation (blue curve: $E_0 = 0.1(S_0 + K_m)$) , rMM equation (red curve: $S_0 = 0.1(S_0 + K_m)$), and v_{ss}^1 (grey curve: $y = 0.1$) , with the criteria of v_{ss}^1 being the least restrictive. The validity of v_{ss} from equation (34) is the entire domain.

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substrate mass load instead of molar substrate concentration, and $\hat{K}_m$ instead of K_m. Hence, k_2 and $\hat{K}_m$ are structurally and practically identifiable from saturation experiments with substrate excess when E_0 and $\hat{S}_0$ are known.

Imposing the condition $S_0 << E_0 + K_m$ on equation (35), it reduces to

$$v_{ss}^1 \approx k_2 \Gamma \frac{E_0 \hat{S}_0}{E_0 + K_m} \, , \tag{37}$$

where only K_m and $k_2\Gamma$ can be estimated. However, estimation of $K_m, k_2\Gamma, k_2, \frac{K_m}{\Gamma}$ can be obtained by performing separate experiments with either enzyme excess or substrate excess. This clearly implies individual estimation of all the parameters K_m, k_2, Γ and even provides redundant information which can be used to estimate Γ in two different ways.

Alternatively, one may use experimental conditions with neither substrate or enzyme in significant excess, and estimate k_2, K_m, Γ through non linear regression of equation (34) or equation (35) to a series of experiments with varying E_0 and S_0.

4. Conclusion

In conclusion, the biochemically motivated substrate conserving model leads to a set of simple rate equations which may be used for enzymatic breakdown of solid substrates. The substrate conserving model is symmetric in E and S meaning that substrate excess and enzyme excess yields similar steady state curves. The substrate conserving model provides exact, closed form expressions of steady state values and time progress curves, which are valid for any admissible values of initial conditions and rate constants. Therefore, use of quasi steady state approximations are unnecessary. The model overlaps with the MM equation and rMM equation within their validity domains of respectively substrate or enzyme excess. Considering a linear relation between mass load of substrate and molar substrate provides an extra parameter, Γ, to the steady state equations. To estimate all parameters by v_{ss} measurements one cannot restrict to cases with either substrate excess or enzyme excess. Both have to be considered, or a range where none of them are dominant must be investigated.

5. Acknowledgements

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