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Direct Estimate of the Specificity Constant: A Possibility or a Fluke? Pre-steady-state Substrate Concentrations and Enabling Mathematical Equations

Ikechukwu Iloh Udema
Department of Chemistry and Biochemistry, Research Division, Ude International Concepts LTD (862217), B. B. Agbor, Delta State, Nigeria

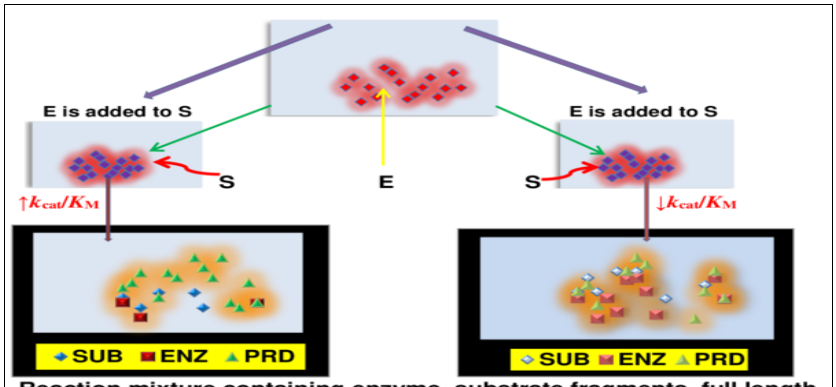
Corresponding Author: **Ikechukwu Iloh Udema**

Abstract

Recently, a high-ranking scientist proposed the need for direct estimation of the specificity constant, rather than calculation based on the determination of maximum velocity and the Michaelis–Menten constant (K_M). The goal of this work is to develop new formulas for the following: the saturating substrate concentration ($[S_T]$); the instantaneous initial rate, also called the "burst phase-like" rate; the zero-arbitrary determination of the pre-steady-state (PSS) substrate concentration, which is suitable for PSS assays; the direct estimation of the specificity constant (SC) in a PSS scenario; and its corresponding $[S_T]_0$ ($\ll K_M$). In order to show the proposed equations' applicability and robustness, the paper also aims to quantitatively evaluate them. The research was theoretical as well as experimental. The Bernfeld method of enzyme assay provides evidence for it. For three distinct enzyme concentrations, the SC values from the two most recent approaches range from 2,197.546 to 11,101.74 L/g. min using one way and from 2,185.649 to 13,860.014 L/g min using the other. For all three concentrations, the SC's sub-K_M values fall between 1,304.368 and 7,943 L/g min. The corresponding $[S_T]_0$ and burst-phase-like initial rate (v_0) range from 0.171 to 3.752 g/L and 14.26 to 55.448 µmol /min respectively. Lower enzyme concentrations result in greater SC values; formulas for directly computing SC under circumstances that validate the reverse and conventional quasi-steady-state approximations could be developed. SC and catalytic efficiency are two quite distinct ideas. Computation cannot be totally avoided.

Keywords: *Aspergillus Oryzae* Alpha-Amylase, Specificity Constant, Catalytic Efficiency, "Burst Phase-Like" Initial Rates and Corresponding Concentration of Substrate, Michaelis-Menten Constant, Second-Order Rate Constant for the Formation of Enzyme-Substrate Complex

Graphical Abstract



Reaction mixture containing enzyme, substrate fragments, full length substrate polymer, and products

Figure 1 **Figure 2**

The symbols SUB (S), ENZ (E), and PRD stand for substrate, enzyme, and product, respectively, for the purposes of this investigation. It is implied that the catalytic efficiency in Figure 1 is higher than that shown in Figure 2 due to the lower number density (red) of the enzyme molecules in Figure 1 and the higher number density of the product (light green) for the same concentration of the substrate (darker blue).

1. Introduction

Recent and past investigators have shown remarkable interest in Michaelian kinetics in light of the need to characterize the putative enzyme being assayed either for the record or for industrial applications [1, 2]. A significant number of such investigations concern the desire to optimize the production of precursors for biofuel based on the hydrolytic action of specified enzymes. Most of the time, pre-steady-state (PSS) and steady-state (SS) assays are carried out [3-7]. Burst phase, pre-steady-state, and steady-state are distinct from zero-order kinetics, and they are expected to kinetically contribute to the characterization of an enzyme whose application can be on the basis of an informed decision. These rate constants are important for proper modeling, either for further experimental or industrial design. The challenge lies in the choice of suitable substrate concentrations and the range of such concentrations. The waste-to-wealth concept is best achieved industrially upon application of a kinetic model; thus, research has focused on how to best convert cellulose to the precursors of biofuels [8-11] and possibly solvents for other applications in the pharmaceutical and food industries. The control of diabetes has prompted recent studies on the digestibility of recalcitrant starch and the inhibition mechanism of alpha-amylase [12-15].

Regardless of the user's goal, whether it be saccharification and liquefaction as steps toward producing biofuels, polar solvents, and biomass conversion before further degradation into simpler biomolecules such as trisaccharides and short oligosaccharides or controlling simple sugar consumption during a meal, it is necessary to control the enzyme's catalytic efficiency. Research has been conducted to better understand the mechanisms that lead to the catalytic efficiency of enzymes, as reported in the literature [16-20]. Catalytic efficiency can be defined as an enzyme's capacity to function at an optimal level with a small amount of substrate. This appears to be a function of the Michaelis–Menten constant (K_M). Conversely, enzyme specificity is defined as the enzyme's ability to selectively identify, exhibit preference for, and consistently binds to the same substrate, even in the presence of other substances or substrate analogues.

The issue of proficiency arises. It is defined in several ways. One not-so-clear definition is that the enzyme's proficiency (k_{cat}/K_M)/k_{uncat} (where k_{cat} and k_{uncat} are the first-order catalytic rate constant and the equivalent rate constant without a catalyst, respectively) is "the equilibrium constant for the conversion of the transition state (TS) of the uncatalyzed reaction in water, and perhaps the enzyme-catalyzed reaction in water, into the TSE complex" [19]. Another definition is that it is "the catalytic efficiency (a second-order rate constant = k_{cat}/K_M) divided by the first-order rate constant for the uncatalyzed reaction in water" [17]. While catalytic proficiency is clearly defined, catalytic efficiency is used interchangeably with the specificity constant (SC) [18]. Some even suggest that k_{cat}/K_M quantifies enzyme specificity, efficiency, and proficiency [20], despite the exclusive definition of catalytic proficiency by other high-caliber scientists [17, 19].

Although acknowledging the potential applications of catalytic efficiency is important, concerns have recently emerged about using the V_{max}/K_M ratio to measure enzyme performance, particularly when the enzyme is used as an industrial catalyst. The parameter V_{max} is the maximum rate of catalysis when the enzyme is saturated with substrate. Using it can result in misinterpreting the performance index, which can be problematic when selecting different variants for industrial applications [21]. Note that V_{max}/K_M should not be used as is, even if the biochemist understands that a variable is missing from the simple expression given for SC. V_{max}/K_M should consistently be stated as k_{cat}/K_M (or V_{max}/K_M [E_T]), where [E_T] is the molar concentration of the enzyme).

The reciprocal of V_{max}/K_M is obtained from the double reciprocal plot and yet may not address the concern of eminent scientist [20], who prefers direct information about k_{cat}/K_M without any form of calculation. Thus, this study aims to determine whether a direct estimate of the specificity constant is possible or merely a fluke. Ultimately, this study presents pre-steady-state substrate concentrations and the corresponding mathematical equations with which to examine such scenarios. The main objective of this study is to develop a new formula for directly calculating SC without making any judgments. The other objectives are to: 1) determine the equations for SC in a PSS scenario with an instantaneous initial rate (also known as the "burst phase-like" rate) and the corresponding $[S_T]_0$; 2) find a zero-arbitrary PSS substrate concentration suitable for PSS tests; and 3) evaluate the robustness and usefulness of the generated equations.

1.1 Significance

The study revealed that estimating the specificity constant requires calculations. However, separately estimating the maximum velocity (V_{max}) of catalytic action and the Michaelis–Menten constant (K_M) to find the k_{cat}/K_M ratio may not be necessary. Nevertheless, a single-step calculation is required to determine SC using the derived equations. The original Lineweaver-Burk equation and the modified forms derived in this study, particularly the equation for the pre-steady-state case, require only a single-step calculation. When the starting rate is plotted versus substrate concentrations (which are below the K_M, or when [E_T] is much greater), the pre-steady-state equation replaces an incorrect determination of SC, which, otherwise, is an aspect of rQSSA ($d[S_T]/dt \approx 0$). A straightforward formula for calculating what appears to be an instantaneous (or rather, a "burst phase-like") starting rate and its matching substrate concentration has also been provided by the study. By examining the equation derived in this study, pre-steady-state studies can avoid an arbitrary selection of substrate concentrations < K_M.

2. Theory

In this section, two equations are to be derived; one is based on the approach in the literature [22]. The other equation is based on a new principle. This notwithstanding, the recent direct linear method for estimating kinetic parameters is quite fortunate. In this case, no calculation is done; just the median of various points of intersection gives the direct estimate of V_{max} and K_M. The direct linear plot [23] and the alternative variants [24] are graphical means of determining the kinetic parameters. The former requires separate determination of the parameters followed by calculation, whereas the alternative variant gives a direct value

for the SC after taking the reciprocal of the K_M-to-V_{max} ratio if initial rates were accurately generated. Thus, the reciprocal variant of the direct linear plot seems to be the first graphical approach for the determination of SC. In this study, a mathematical approach and a new graphical method are to be investigated.

2.1 Derivation based on approach in the literature [22]

Most of the initial rate (v) is plotted versus the initial concentration [S_T] of the substrate, but a plot of v versus [E_T] is not out of the question. Similarly, a plot of 1/ v versus 1/[E_T] is not out of the question. Indeed, it is more appropriate to plot v versus [E_T] as a prelude to any Michaelian investigation of any suitable enzyme. Based on the Michaelian principle, v is not directly proportional to [S_T] because there is the presence of the latter in the denominator in addition to the K_M. In this regard, Matyska and Kovář [25] strongly acknowledged that the Michaelis-Menten equation is a nonlinear equation. Hence,

$$v \propto [E_T] \tag{1a}$$

Therefore,

$$v = k_{int}[E_T] \tag{1b}$$

Where k_{int} is a first order rate constant for each concentration of the substrate (S).

This suggests that every enzyme concentration, ranging from 4 to 6, is tested for every substrate concentration, ranging from 6 to 10 or higher. Different [E_T] values result in different k_{int} values.

$$[S_T]_1 < [S_T]_2 < [S_T]_3 < [S_T]_4 < [S_T]_5 < [S_T]_6 < [S_T]_7 \ldots [S_T]_8 < [S_T]_9 < [S_T]_{10} < [S_T]_n \tag{2}$$

The different k_{int} values for different values of [S_T] are:

$$k_{int}^{\rightarrow 1} < k_{int}^{\rightarrow 2} < k_{int}^{\rightarrow 3} < k_{int}^{\rightarrow 4} < k_{int}^{\rightarrow 5} < k_{int}^{\rightarrow 6} < k_{int}^{\rightarrow 7} \ldots k_{int}^{\rightarrow 8} < k_{int}^{\rightarrow 9} < k_{int}^{\rightarrow 10} < k_{int}^{\rightarrow n} \tag{3}$$

The values in Eqs (2) and (3) can be subjected to the alternative variant of the direct linear plot such that the median can give exactly k_{cat}/K_M. This could be very tedious and less accurate if more than six different [S_T] values are used. Again, assaying for different concentrations of E (up to 4 or more) can also be tedious and take a lot of time. If the values of $1/k_{int}$ are plotted versus different values of 1/[S_T], the 1/slope gives the SC value. The equation is given as:

$$1/k_{int} = \frac{d(1/k_{int})}{[S_T]d(1/[S_T])} + 1/k_{cat} \tag{4}$$

Where $(^{d(1/k_{int})}/_{d(1/[S_T])}$ is the slope, the equivalent of SC. The condition that satisfies standard quasi-steady-state (sQSSA) must be guaranteed for each value of [E_T] in order to be accurate. A short duration (30–40 seconds) of assay is advisable in order to avoid significant substrate depletion; the concentration of the substrate at the lower part of the range may be several folds (not < 4-fold) > the highest value of [E_T]. The following is derived from Eq. (4):

$$K_M = \frac{\partial(1/k_{int})}{\partial(1/[S_T])} k_{cat} \tag{5a}$$

$$SC = 1/\left(\frac{\partial(1/k_{int})}{\partial(1/[S_T])}\right) \tag{5b}$$

Thus, the concern for direct estimation of SC has been addressed by Eq. (5b), but the process leading to it can be tedious. Furthermore, high precision is demanded, which makes the use of automated devices inevitable. It is also very necessary to ensure that the substrate concentration regime is such that the lowest concentration of the substrate is ≫ the highest concentration of the enzyme, strictly on a mole-mole basis. A very short duration of the assay is desirable.

2.2 Derivation based on an alternative principle, the variation of the reciprocal of initial rates with the product of the ratios of substrate concentrations

To begin this section, the view in the literature [20] needs to be examined. Taking the equation in the literature given as:

$$v = \frac{k_{sp}[S_T]}{\left(1 + k_{sp}\frac{[S_T]}{k_{cat}}\right)} \tag{6a}$$

Where k_{sp} is specificity constant. The issue with Eq. (6) is that the experimental variable v is not clearly defined in light of the meaning of k_{cat}. Based on the meaning of the latter, " v " should be a pseudo-first-order constant for product formation at the initial stage. The author's comment that certain algebraic equations may be considered trivial is unnecessary because

neglecting fundamentals can lead to flawed theses, even if they contain advanced mathematics that few in the same field can understand, including junior-level professionals.

$$v_{ps} = \frac{k_{sp}[S_T]}{1+k_{sp}\frac{[S_T]}{k_{cat}}} \quad (6b)$$

Where v_{ps} is given as initial rate divided by the molar concentration of the enzyme.
Equation (6b) sets the standard for the subsequent derivations. But before then, a better impression regarding SC is as follows: The impression about SC is that it provides a lower limit for the second-order rate constant for substrate binding, while k_{cat} provides a lower limit for each first-order rate constant following substrate binding. The notion of lower limit is best interpreted in terms of the reciprocal of k_{cat}, which gives the duration of the catalytic cycle; this, as observed in the literature [26, 27], should be equal to the sum of the reciprocals of the first-order rate constants of the individual reaction steps. This means that the individual first-order rate constant is greater than the overall catalytic rate constant, referred to as the lower limit [20].
There is a substrate concentration that may be lower than the lowest concentration in the range chosen for the enzyme assay. This concentration is as follows:

$$[S_T]_0 = b_0 K_M \quad (7)$$

Where b_o (this is < 1) is a dimensionless constant for a given substrate concentration range for a given concentration of E under a defined condition of assay, temperature, pH, *etc.* The value of $[S_T]_0$ is a specific substrate concentration that may be less than K_M. With other concentrations that are also less than $[S_T]_0$, the initial rates are directly proportional to the substrate concentrations. The following relationships are reasonable:

$$[S_T]_1 = \beta_0[S_T]_0 \quad (8)$$

Where β_o is the number of times $[S_T]_1$ is > $[S_T]_0$
Substituting Eq. (7) into the equation gives:

$$[S_T]_1 = \beta_0\, b_0\, K_M \quad (9)$$

By the same line of argument, the relationship between other higher concentration of the substrate and Eq. (7) is given as follows:

$$[S_T]_2 = \beta_1\,[S_T]_1 = \beta_1\beta_0\, b_0 K_M \quad (10)$$

$$[S_T]_3 = \beta_2\,[S_T]_2 = \beta_2\,\beta_1\beta_0\, b_0 K_M \quad (11)$$

$$[S_T]_4 = \beta_3\,[S_T]_3 = \beta_3\,\beta_2\,\beta_1\beta_0\, b_0 K_M \quad (12)$$

A general equation is given as:

$$[S_T]_n = \beta_{n-1}\,[S_T]_{n-1} = \beta_{n-1}\,\beta_{n-2}\,\beta_{n-3}\ldots\,\beta_0\, b_0 K_M \quad (13)$$

One can then write the Michaelian equation based on the above equations as follows.

$$v_{ps} = \frac{k_{cat}[S_T]_n}{K_M+[S_T]_n} \quad (14a)$$

$$v_{ps} = \frac{k_{cat}\,\beta_{n-1}\beta_{n-2\ldots}\,\beta_2\beta_1\beta_0 b_0 K_M}{(K_M+\beta_{n-1}\beta_{n-2\ldots}\,\beta_2\beta_1 b_0 K_M)} \quad (14b)$$

Linear transformation of Eq. (14b) gives:

$$\frac{1}{v_{ps}} = \frac{1}{k_{cat}\beta_{n-1}\beta_{n-2\ldots}\,\beta_2\beta_1\beta_0 b_0} + \frac{1}{k_{cat}} \quad (15)$$

A plot of the reciprocal of v_{ps} versus the reciprocal of $\beta_{n-1}\,\beta_{n-2}\,\beta_{n-3}\ldots$ gives intercept whose reciprocal gives the catalytic rate constant. Meanwhile, the slope (S_L) from such a plot gives:

$$S_L = \frac{1}{k_{cat}\,\beta_0 b_0} \tag{16}$$

Recall that $\beta_0\, b_0$ is given as $[S_T]_1/K_M$, then Eq. (16) can be restated as:

$$S_L = \frac{K_M}{k_{cat}[S_T]_1} \tag{17}$$

Then SC is given as:

$$SC = \frac{1}{S_L[S_T]_1} \tag{18}$$

Equation (18) suggests that SC is dependent on the first and lowest substrate concentration in the range of substrate concentrations chosen.

To set the record straight, recall the premise for the formulation of Eq. (15): despite the well-known double reciprocal transformation of the Michaelian equation by Lineweaver and Burk (LB), there does not seem to be an equation for the direct determination of the specificity constant [20]. The challenge in using LB is generating accurate initial rates. The plot of the reciprocal of the initial rates versus the reciprocal of the substrate concentration implied by equation [20], $1/v = (1/k_{sp}[S_T]) + 1/k_{cat}$, needs to give way to the following equation.

$$\frac{1}{k_{int}} = \frac{K_M}{k_{cat}[S_T]} + \frac{1}{k_{cat}} \tag{19}$$

Where k_{int} is already defined in Eq. (1b). Note that, in principle, Eq. (4) is similar to Eq. (19). The difference is that Eq. (4) is derived from multiple enzyme concentration assays, whereas Eq. (19) applies to a single enzyme concentration. Both are double reciprocal equations.

Selecting an appropriate substrate concentration range and assay time is crucial for PSS kinetic modeling. When selecting distinct $[S_T]$, it is important to avoid being arbitrary. The problem is that, unlike amylose or amylopectin, there is no definitive value for the molar mass of a complete starch molecule, even after choosing an appropriate $[S_T]$:$[E_T]$ ratio that satisfies the requirements of a standard quasi-steady-state approximation (sQSSA) at the most basic level (which requires knowledge of the molar mass of the substrate, starch, and large-molecular-weight proteins). If the molar mass of the substrate is not known with certainty, a nanoscale concentration of the enzyme with coefficients less than 5 is preferable (*e.g.*, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, or 5nmol/L). Avoid the mass-mass ratio; otherwise, a misleading result is inevitable. In deriving the simple equations, the importance of the general equation, Eq. (13), becomes apparent in a different context. One can plot v_{n-1}/v_n versus $[S_T]_{n-1}/[S_T]_n$ to generate a straight-line equation under the premise that v_{n-1}/v_n is directly proportional to $[S_T]_{n-1}/[S_T]_n$ and is partly constant, yielding the following:

$$\frac{v_{n-1}}{v_n} = \frac{[S_T]_{n-1}}{[S_T]_n} S_\chi + \chi \tag{20}$$

Where S_χ and χ are the slope and intercept respectively. Equation (20) leads to the following:

$$\frac{v_0}{v_1} = \frac{[S_T]_0}{[S_T]_1} S_\chi + \chi \tag{21}$$

Equation (21) can be rearranged to give:

$$\frac{1}{v_1} = \frac{[S_T]_0}{v_0[S_T]_1} S_\chi + \frac{\chi}{v_0} \tag{22}$$

The first plot establishes the values of S_χ and χ. Since v_0 and $[S_T]_0$ are constants for a given substrate concentration range and enzyme concentration for the assay, Eq. (2) allows us to determine the initial rate before the end of the assay duration, which may be much less than three minutes. This timescale may be transient, like the initial rate. One can rewrite a general equation for Eq. (22) as follows:

$$\frac{1}{v_1} = \frac{[S_T]_0}{v_0[S_T]_1} S_\chi + \frac{\chi}{v_0} \tag{22}$$

Equation (22) is a modified version of the Lineweaver-Burk equation [28] and can determine the initial rate that is not measurable when the enzyme is added to the substrate, regardless of the effect of magnetic stirring. The unmeasurable initial rate, v_0 can be considered a "burst-like initial rate." The equation for v_0 is derived from Eq. (22) as follows:

$$\frac{v_0}{\chi} = V_{\max} \tag{23}$$

$$v_0 = \chi V_{\max} \tag{24}$$

The equation for the corresponding concentration of the substrate ($[S_T]_0$) is derived as follows:

$$\frac{K_M}{V_{\max}} = \frac{[S_T]_0}{v_0} S_\chi \tag{25}$$

$$[S_T]_0 = \frac{K_M}{S_\chi V_{\max}} v_0 \tag{26a}$$

Substituting Eq. (24) into Eq. (26a) gives:

$$[S_T]_0 = \frac{K_M}{S_\chi} \chi \tag{26b}$$

It is clear that v_0 and $[S_T]_0$ are directly proportional. This direct proportionality should also be demonstrated for all values of $[S_T]_0$ that are less than $[S_T]_0$ and K_M. However, this cannot be extended infinitely because the ideal values are defined by values smaller than $[S_T]_0$. It is plausible that $v_0/[S_T]$ is approximately equal to SC (slightly less than V_{max}/K_M).

An important lesson from Eq. (26a) is that, when $[S_T]$ is assumed to be much smaller than K_M, the original Michaelian equation should not be rewritten as $v = V_{max}\,[S_T]/K_M$. Such an expression is reserved for a PSS and possibly a burst phase scenario in which K_M cannot validly be defined as the substrate concentration at half the maximal velocity or rate of catalysis. Instead, the correct equation is $v = [S_T]_{(PSS)}/K_d$, where $[S_T]_{(PSS)}$, K_d, and V_{max}^{PSS} are the PSS substrate concentration, the enzyme-substrate (ES) dissociation constant, and the PSS maximum velocity, which is much smaller than the zero-order maximum velocity. The literature has previously explained similar problems [22, 26]. Keep in mind that K_M is equivalent to $K + K_d$, where K stands for the Van Slyke and Cullen constants [29]. Consequently, there is no provision for $K + K_d$ in either $V_{max}\,[S_T]/K_M$ or $[S_T]_{(PSS)}/K_d$. The issue of K_M misuse, which is frequently organized by the living giants of biochemistry, does not need to be addressed by the giants of enzymology like Van Slyke-Cullen, Michaelis, and Menten. These giants appear to have an attitude that "knowledge begins and ends with me" because of the enormous amount of knowledge they have amassed over the years.

Here we are again with issue arising from Eq. (26a); this is just about making v_0 subject of the formula as follows:

$$v_0 = S_\chi V_{\max}[S_T]_0/K_M \tag{27a}$$

Equations 26 and 27a apply to all values of $[S_T]$ that are greater than or equal to $[S_T]_0$ and less than or equal to K_M; they do not apply only to $[S_T]_0$ and v_0. However, $dv_n/d[S_T]_n$ must always equal $v_0/[S_T]_0$. One of the main research questions is how to independently determine the value of $v_0/[S_T]_0$. Plotting v_n values against $[S_T]_n$ using equation 27b results in a linear curve whose slope ($S_{PSS\chi}$) should equal $= S_\chi V_{max}/K_M$.. In other words, $v_0/[S_T]_0$ equals $S_{PSS\chi}$. The equation is:

$$v_n = S_\chi V_{\max}[S_T]_n/K_M \tag{27b}$$

The presence of a pre-determined slope (S_χ) allows the accurate estimation of V_{max}/K_M from:

$$V_{\max}/K_M = S_{PSS\chi}/S_\chi \tag{28}$$

Thus, SC can be given as:

$$\mathrm{SC} = S_{PSS\chi}/S_\chi\,[E_T] \tag{29}$$

If it is equal to $v_0/[S_T]_0$, then equation (29) can be used to determine zero-order SC. Several approaches have been derived in addition to the LW approach. None of them are without calculation. Assays must be conducted for different concentrations of E before deriving Eq. (19). A single assay must be conducted if the LW approach is used. However, it is not without calculation because the slope must be divided by $[E_T]$. The most direct and minimal calculation is the alternative direct linear approach, which immediately yields $1/V_{max}/K_M$ (or $1/k_{cat})/K_M$ if $[E_T]/v$ is plotted versus $[E_T][S_T]/V$. However, this approach still requires calculation because one must take the reciprocal of $1/k_{cat}/K_M$ to quantify SC. This is in addition to the initial calculation of v_{PS} (*i.e.*, the initial rate divided by the molar concentration of the enzyme). Equation (29) requires information about substrate concentrations that are much less than the K_M. Firstly, it should be noted that initial rates obtained from substrate concentrations much greater than $[E_T]$ will produce a polynomial curve of the quadratic kind when plotted versus $[S_T]$. Thus, a general equation for the estimation of PSS can be derived from a polynomial of the following kind:

$$y = -\alpha x^2 + \beta x + \gamma \tag{30}$$

Differentiation with respect to *x* gives:

$$\mathrm{d}y/\mathrm{d}x = -2\alpha x + \beta \tag{31}$$

If one recalls that *v* (which is *y*) = k_{cat} [*ES*], then,

$$\mathrm{d}v = k_{cat}\mathrm{d}[ES]/dx = -2\alpha x + \beta \tag{32}$$

Integrating yields:

$$k_{cat}\Delta[ES] = -\alpha x^2 + \beta\Delta x \tag{33}$$

Bearing in mind that under SS condition, ($^{\Delta[ES]/\Delta t}$) may be ≈ zero. Therefore,

$$-\alpha x^2 + \beta\Delta x = 0 \tag{33}$$

Here, x refers to [S_T] and Δ x refers to Δ[S_T] (= molar mass (M_{alt}) of product × v × duration (t) of assay). The equation for PSS concentration of substrate is:

$$[S_T]_{PSS} = \sqrt[2]{\beta M_{alt} vt/\alpha} \tag{34}$$

2.3 Another direct approach besides the alternative variant of direct linear plot

Another direct approach with generalizable attributes is based on an alternative method for determining kinetic parameters, as described in the literature [30]. The equation is derived as follows: The equations for maximum velocity in the pre-steady state, the steady state, and the post-steady state (the zero-order scenario) and either the enzyme-substrate (ES) dissociation constant (K_d) or the Michaelis–Menten constant (K_M), depending on the case, are as follows:

$$V_{max} = \frac{v_n v_{n-1}([S_T]_n - [S_T]_{n-1})}{[S_T]_n v_{n-1} - [S_T]_{n-1} v_n} \tag{35}$$

$$K_M(or\ K_d) = \frac{[S_T]_n[S_T]_{n-1}(v_n - v_{n-1})}{[S_T]_n v_{n-1} - [S_T]_{n-1} v_n} \tag{36}$$

$$\frac{V_{max}}{K_M(K_d)} = \frac{v_n v_{n-1}([S_T]_n - [S_T]_{n-1})}{[S_T]_n[S_T]_{n-1}(v_n - v_{n-1})} \tag{37}$$

Rearrangement of Eq. (37) gives:

$$\frac{v_n v_{n-1}}{v_n - v_{n-1}} = \frac{[S_T]_n[S_T]_{n-1}}{[S_T]_n - [S_T]_{n-1}} \frac{V_{max}}{K_M(or\ K_d)} \tag{38}$$

A plot of $\frac{v_n v_{n-1}}{v_n - v_{n-1}}$ versus $\frac{[S_T]_n[S_T]_{n-1}}{[S_T]_n - [S_T]_{n-1}}$ gives directly a slope equal to the specificity constant, SC.

Preliminary calculations must be made, as shown by Eq. (38). Plotting the data yields the SC without additional calculations, except for dividing by the molar concentration of the enzyme and, if necessary, multiplying by the molar mass of the substrate. But keep in mind that K_M must be replaced by K_d if the beginning rates are directly proportional to the substrate concentrations and if these concentrations are both less than K_M and much less than the enzyme concentration, as previously said.

For a pre-steady-state scenario, one alternative approach is to consider that v_i is directly proportional to the sub-Michaelian substrate concentration and that the enzyme concentration is much greater than [S_T]. Note that, in such a scenario, the regression coefficient must be ≥ 0.999, and the maximum velocity should correspond to the pre-steady state with a K_d. Similarly, v_n could be 2 v_{n-1}, just as $[S_T]_n$ could be $2[S_T]_{n-1}$.

Thus,

$$v_{n-1} = [S_T]_{n-1}\frac{V_{max}^{pr-ss}}{K_d}, \tag{39}$$

$$v_n = [S_T]_n\frac{V_{max}^{pr-ss}}{K_d}, \tag{40}$$

3. Materials and Methods

3.1 Materials

3.1.1 Chemicals

As in a previous study [31], *Aspergillus oryzae* alpha-amylase (EC 3.2.1.1) and potato starch were purchased from Sigma-Aldrich, USA. Tris, 3, 5-dinitrosalicylic acid, maltose, and sodium potassium tartrate tetrahydrate were purchased from Kem Light Laboratories in Mumbai, India. Hydrochloric acid, sodium hydroxide, and sodium chloride were purchased from BDH Chemical Ltd., Poole, England. Distilled water was purchased from the local market. The molar mass of the enzyme is ~ 52 kDa [32, 33].

3.1.2 Equipment

An electronic weighing machine was purchased from Wensar Weighing Scale Limited, and a 721/722 visible spectrophotometer was purchased from Spectrum Instruments, China; a *p*H metre was purchased from Hanna Instruments, Italy.

3.2 Methods

The enzyme was assayed according to the Bernfeld method [34] using gelatinized potato starch; three different concentrations of the enzyme were assayed. In this study, a mass concentration of 0.002 g/L was explored, given a mass concentration range of substrate equal to 0.3–3 g/L. The other concentrations of the enzyme that were assayed were 0.0005 and 0.0002 g/L, given a mass concentration range of substrate equal to 5–10 g/L. Reducing sugar produced upon hydrolysis of the substrate at room temperature using maltose as a standard was determined at 540 nm with an extinction coefficient equal to 181 L/mol·cm. The duration of the assay was 3 minutes. A mass concentration of 2 mg/L of *Aspergillus oryzae* alpha-amylase was prepared in Tris-HCl buffer at *p*H = 7. The assay was conducted at room temperature (21-23°C).

As explained in the literature [31], the equation below can be used to determine the second-order rate constant provided that $[E_T]$ is greater than most, if not all, of the substrate concentrations within the chosen range.

The equation is however, not suitable for the case in which $[E_T] \ll [S_T]$.

$$\ln\{[E_T]/([E_T] - [ES])\} = (k_{-1} + k_{cat})[S_T](1 - exp.(-kt)/K_M k, \quad (41)$$

The pseudo-first-order rate constant, k for the hydrolysis of starch is generated as described in the literature [31, 35]. The equation for the case in which $[E_T] \ll [S_T]$ is being reviewed to simplify the derivation procedure for the manuscript under preparation. The equation is given as follows:

$$k_1 = \frac{M_2^2 V_{max}}{[E_T]_{(g)} K_M M_2 - M_3 [E_T]_{(g)}^2} \quad (42)$$

Where $[E_T]_{(g)}$ and M_3 are the mass concentration of the enzyme and molar mass of the substrate, the insoluble potato starch, respectively.

4. Results and Discussion

Although the issue of inconsistency in the applicable Michaelis–Menten constant has been acknowledged and supported in a recent preprint publication, the starting point of this section is to reiterate the claim that the velocity (initial rates, v_i) equations of the catalytic reaction have been used to determine kinetic parameters outside of the conditions for which they are valid. To highlight this issue, an equation was derived for an arbitrary choice of substrate concentrations, as explained in Eq. (34). The calculated values of $[S_T]$, based on Eq. (34), were substituted into a polynomial equation to determine the corresponding sub-K_M. For clarity, sub-K_M substrate concentrations are those that are much smaller than the initial rates. It should be noted that, as stated in the literature [31], the polynomial must possess a negative coefficient of its leading term. However, this is strictly for illustration purposes. Otherwise, after calculating the sub-K_M concentrations of the substrate, an assay of the same or higher enzyme concentration needs to be conducted. A typical reverse quasi-steady-state approximation (rQSSA) is demonstrated by plotting the initial rates versus the corresponding sub-K_M substrate concentrations. Figures 1 and 2 illustrate this for enzyme concentrations of 0.0002, 0.0005, and 0.002 g/L. The rQSSA relation is a linear curve with a coefficient of determination typically ≥ 0.999. This holds true as long as the concentration range is less than the putative K_M value of the enzyme and, better yet, less than $[E_T]$ [23, 37]. In such a scenario, the zero-order SC cannot be inferred from data points that validate only rQSSA, partially validate sQSSA, or validate both rQSSA and sQSSA [38, 39].

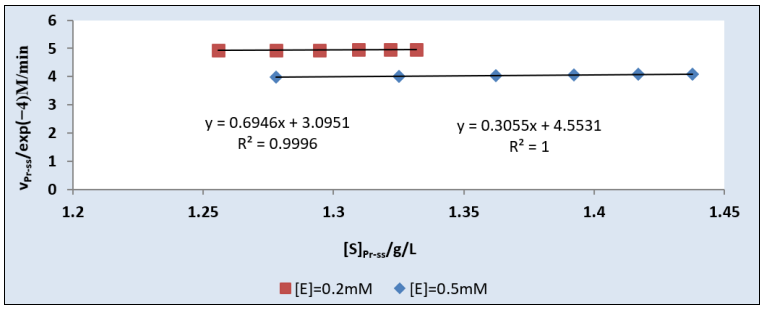


Fig 1: Plot illustrating the non-Michaelian characteristics of initial rates which is directly proportional to the sub-Michaelis-Menten constant concentration of the substrate where the concentrations of the enzyme are 0.0002 g/L (■) and 0.0005 g/L (◆). The SC for 0.0005 g/L is = 7223.84 L/g. min; SC for 0.0002 g/L is = 7943 L/g min

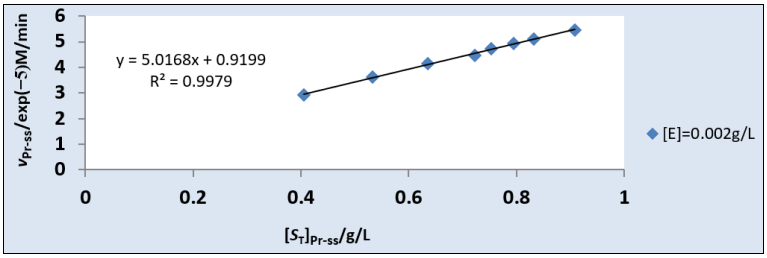


Fig 2: Plot illustrating the non-Michaelian characteristics of initial rates which is directly proportional to the sub-Michaelis-Menten constant concentration of the substrate where the concentration of the enzyme is 0.002 g/L. The SC value is 1304.368 L/g min

It was hypothesized that, for any given enzyme concentration assayed within an arbitrarily chosen substrate concentration range, there is always a "burst-like" initial rate corresponding to the substrate concentration. Figures 3 and 4 illustrate this concept for $[E_T]$ = 0.002 g/L and $[E_T]$ = 0.0002 and 0.0005 g/L, respectively. The "burst-like" initial rate and its corresponding $[S_T]_0$ are defined, respectively, by Equations (24) and (26b). The latter appears to be higher or lower than the calculated sub-K_M concentration of the substrate. With 0.0002 and 0.0005 g/L of enzyme, the sub-K_M concentration ranges are 1.2558–1.3332 g/L and 1.278–1.438 g/L, respectively. At the relevant $[E_T]$ values (0.0002 and 0.0005 g/L), it is surprising that the corresponding substrate concentrations ($[S_T]_0$ = 3.752 and 2.457 g/L) for burst-like initial rates (v_0, Table 1) exceed the calculated sub-K_M values. Though these values are less than the substrate concentration range (5-10 g/L) chosen for the assay, they are nevertheless greater than the corresponding K_M values (Table 1). The situation is totally different when the enzyme concentration is 0.002 g/L. The $[S_T]_0$ value is less than the Michaelis-Menten-like K_M. This implies that K_M is better described as an enzyme-substrate dissociation constant applicable when $[E_T] > [S_T]$ or $[S_T] \neq [E_T]$. It is also possible that $[S_T]$ may be approximately equal to $[E_T]$. In any case, the burst-like initial rates of 55.448 and 43.78 mM/min at 0.0002 and 0.0005 g/L, respectively, were greater than the calculated initial rates corresponding to the sub-KM concentration ranges of 49.367–49.599 and 39.856–40.794 mM/min (Table 1). Values reported for 0.002 g/L of the enzyme were calculated differently. The burst-like initial rate of 14.26 mM/min is less than the initial rates of 29.447–54.572 mM/min corresponding to the sub-K_M $[S_T]$ value. The situation might be different if the assay were carried out using sub-K_M concentrations to generate rates rather than calculations. An important finding is that a practical and theoretical guide exists for determining a lower substrate concentration regime, which may be greater than or less than K_M, depending on the enzyme concentration. This may imply the need to define the condition(s) that should characterize the kinetic parameters. This characterization should be relevant to either rQSSA or sQSSA.

Table 1: Kinetic parameters with emphasis on specificity constant

$[E_T]$/g/L	0.0002	0.0005	0.002
RVDLP			
SC/L/g min.	13000	3687.943	2280.702
V_{max}/ **µM/min**	78.125	104.167	166.667
K_M (K_d)/g/L	1.5625	2.938	1.9
LWBP			
SC/L/g min.	13800.425	3044.586	2196.688
V_{max}/ **µM/min**	75.512	105.954	201.410
K_M (K_d)/g/L	1.423	3.693	2.384
Equations (15 & 18)			
SC/L/g min.	13860.014	3045.531	2185.649
Equation (38)			
SC/L/g min.	11101.74	3045.12	2197.546
v_0/**µM/min**	55.448	43.78	14.26
$[S_T]_0$/g/L	3.752	2.457	0.171

RVDLP and LWBP stand for reciprocal variant of direct linear plot and Lineweaver-Burk plot respectively; v_0 stands for "burst initial rate". The corresponding substrate concentration is $[S_T]_0$.

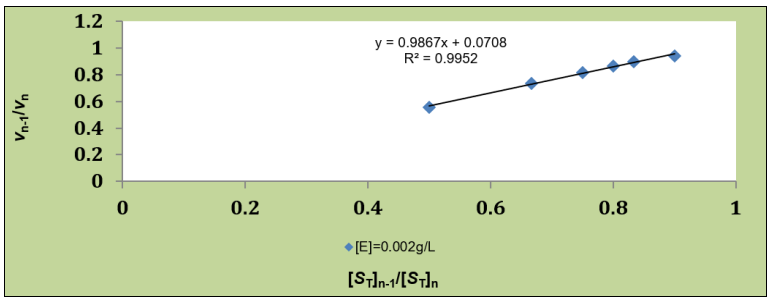


Fig 3: The ratio of initial rates as a function of the ratio of the corresponding concentration of the substrate, plotted versus the latter where $[E_0]$ = 0.002 g/L. $[S_T]_n > [S_T]_{n-1}$ and $v_{n-1} < v_n$ where n is the number of assays (or "population size") for the initial rates, v, and concentrations of the substrate

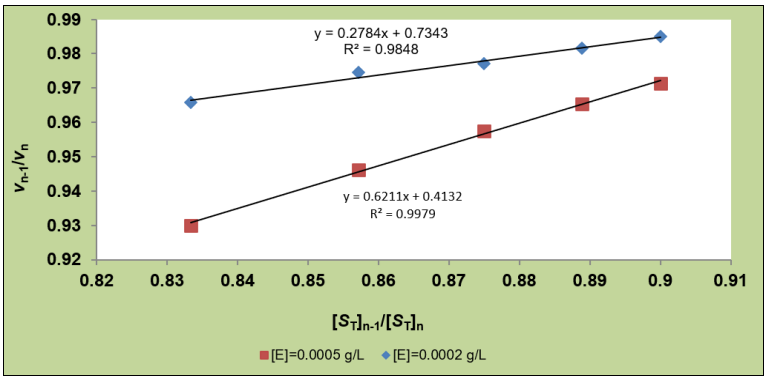


Fig 4: The ratio of initial rates as a function of the ratio of the corresponding concentration of the substrate, plotted versus the latter where $[E_0]$ = 0.0002 g/L (◆) and 0.0005 g/L (■)

Despite the surprising outcome of this study, it is important to note that the observed differences in $[S_T]_0$ and K_M (and K_d, if applicable) values are due to conditions such as $[E_T]$ being greater than most $[S_T]$ values, approximately equal to $[S_T]$, and less than $[S_T]$. The much lower observed Michaelis-Menten-like, K_M value with an $[E_T]$ value of 0.002 g/L results from the condition that validates rQSSA, in which a single turnover event characterizes the catalytic cycle. This implies that most substrate molecules undergo enzymatic action with a higher k_1 value. This also explains the literature [37] view that the inequality cannot be sustained. It was observed that, with very high enzyme concentrations, K_M is small. This study observed K_M values of 2.384 and 1.9 g/L for $[E_T]$ = 0.0002 g/L, resulting from LWBP and RVDLP, respectively (Table 1). The reported values for $[E_T]$ = 0.0002 g/L are the lowest (Table 1).

The main objective of this study is to derive equations for directly estimating SC. For this purpose, Eq. (15) for the plot and Eq. (18) for direct SC estimation with a single calculation become relevant. Figures 5 and 6 show the necessary slopes for $[E_T]$ = 0.0002, 0.0005, and 0.002 g/L. Dividing the slope by the first $[S_T]$ in the chosen concentration range gives the SC, as shown in Table 1. The SC values compare as follows: 0.0002 > 0.0005 > 0.002 g/L. This is applicable to the V_{max} values. Note, however, that four versions were provided. The oldest approach is the Lineweaver-Burk plot (LWBP), which produced values similar to those of the direct linear plot's reciprocal variant (RVDLP) and one of the newest approaches, Eq. (18) in this research. The second newest approach is represented by Eq. (38). Figures 7 and 8 were created to illustrate the latter approach; in this case, only preliminary calculations need to be carried out. Figure 7 illustrates $[E_T]$ = 0.0002 g/L, and Figure 8 illustrates $[E_T]$ = 0.0002 and 0.0005 g/L. The slope has a direct value of SC. The SC values for 0.0005 and 0.002 g/L are similar for all versions, but not for 0.0002 g/L, perhaps due to measurement error. In all cases, however, the SC values with sub-K_M $[S_T]$, as displayed in Figures 1 and 2, are in the following order: 0.0002 > 0.0005 > 0.002 g/L.

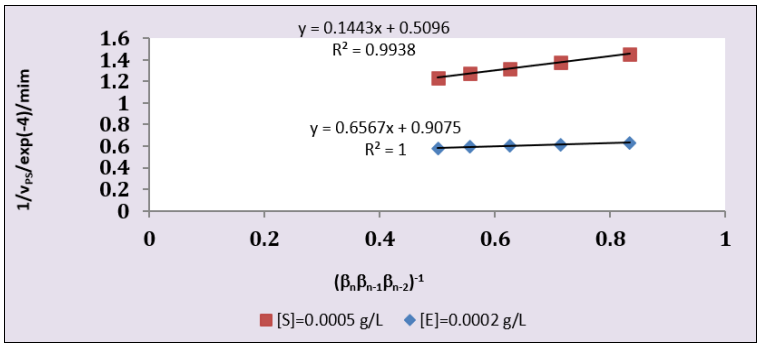


Fig 5: The reciprocal of initial rates as a function of the product of the ratio of substrate concentrations where the concentrations of the enzyme are 0.0005 g/L (■) and 0.0002 g/L (◆). $\beta_{n-1} = [S_T]_n/[S_{n-1}]$

The possibility of error in Figures 5 and 6 can be minimized or eliminated if a careful, stepwise approach is adopted for every calculation to determine the product of substrate concentration ratios. It is important that the initial rates be free of substantial error. This method is highly generalizable because it can be applied to conditions that validate either rQSSA or sQSSA. The initial substrate concentration within the chosen concentration range is important because it is part of the equation for calculating SC. Therefore, it must be accurately measured.

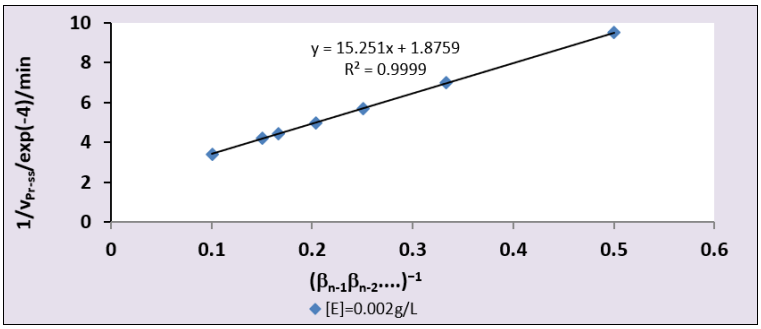


Fig 6: The reciprocal of initial rates as a function of the product of the ratio of substrate concentrations where the concentrations of the enzyme is 0.002 g/L. Note that taking the reciprocal of the slope (which is k_{cat}) divide by $[S_T]_1$ (the first substrate concentration in the range) and multiplying by the molar concentration of the enzyme gives ≈ 8.41 exp. (−5) mol./g min

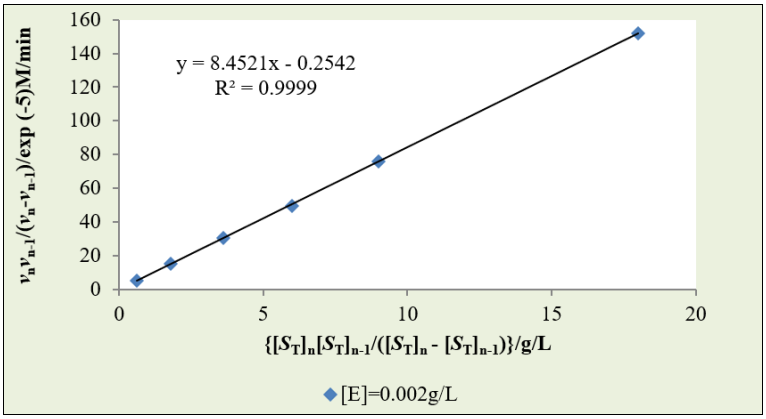


Fig 7: Direct determination of SC by alternative graphical approach: The ratio of the product of different initial rates divided by their difference to the corresponding product of different concentrations of substrate divided by their difference where the concentration of the enzyme is 0.002 g/L

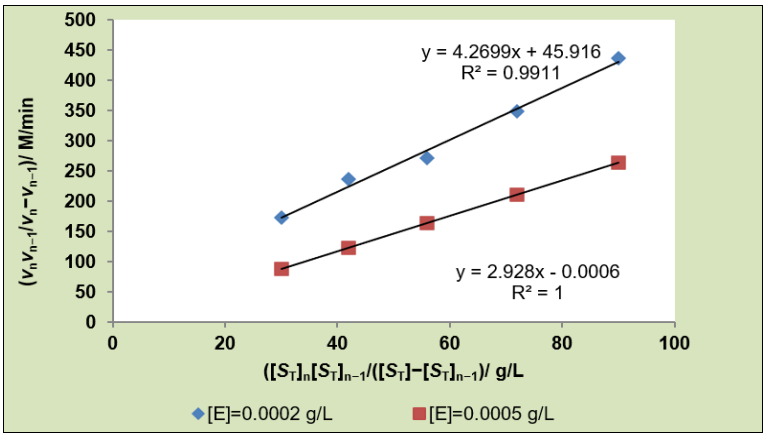


Fig 8: Direct determination of SC by alternative graphical approach: The ratio of the product of different initial rates divided by their difference to the corresponding product of different concentrations of substrate divided by their difference where the concentrations of the enzyme are 0.0002 g/L (◆) and 0.0005 g/L (■)

The purpose of most industrial enterprises is to optimize production, which requires effective control of the production process. These products may be processed foods or drugs, including prescription and over-the-counter medications. Enzymes are extremely important here because their ability to reduce the energy barrier (Arrhenius and Gibbs free energy of activation) can drastically affect the efficiency of industrial processes. This may explain why renowned scholars and researchers emphasize direct estimates of catalytic specificity. Even under normal assay conditions, some reactions may be enthalpically controlled, while others may be entropically controlled. The possibility that an enzyme-catalyzed reaction is driven by both enthalpy and entropy cannot be ruled out. There are many illustrations of energy curves and diagrams in the literature, including Wikipedia. Nevertheless, Figures 9 and 10 are valuable for clarifying the issues in contention. When an enzyme identifies its substrate and binds to it catalytically, both the substrate and the enzyme are in the correct configurational and conformational orientation following effective binding. This reduces the energy barrier, which is the specificity question.

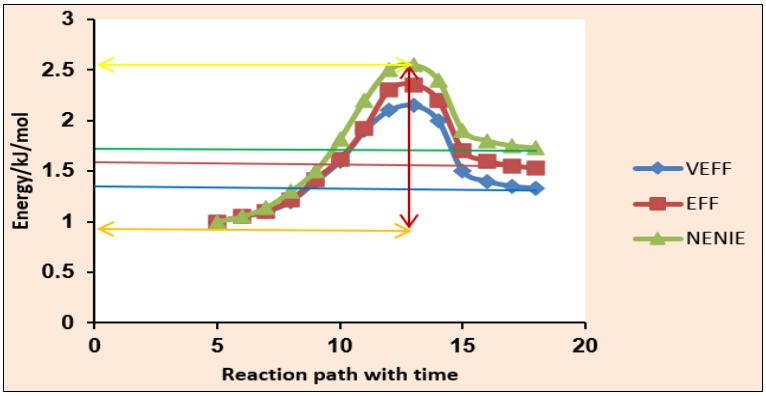


Fig 9: The endothermic case (hypothetical)

The endothermic reactions are very peculiar with most enzyme-catalyzed reaction, notable of which is alpha-amylase amylolysis of glucans, if in particular the reaction conditions falls outside the usual. The blue curve, expresses the fact that the enzyme substantially reduced the activation, thereby becoming very efficient (VEFF), with the highest proficiency and specificity; the red curve follows next as one that is efficient (EFF), while the green curve may illustrate a situation whereby the enzyme is neither efficient nor inefficient (NENIE). The red doubled-headed arrow illustrates the energy barrier height; the orange doubled-headed arrow illustrates the upper energy barrier height-either Arrhenius activation energy or the Gibbs free energy of activation that must be added to the reactants for the reaction to proceed; the lower double-headed yellow arrow illustrates the ground-state energy level of the reactants; while the blue, green, and oxblood horizontal lines mark off the higher ground-state energy level of the products, for the very efficient, efficient and neither efficient nor inefficient enzyme respectively. In these cases the product is less stable than the reactant.

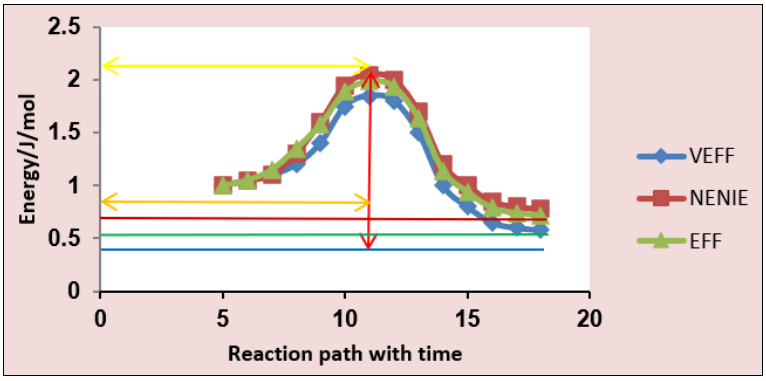


Fig 10: The exothermic case (Hypothetical)

Similar to the endothermic case, exothermic reactions are also peculiar to alpha-amylase amylolysis of glucans. The blue curve shows that the enzyme substantially reduces activation, becoming highly efficient (VEFF) with the highest proficiency and specificity. The green curve shows an efficient (EFF) enzyme, and the red curve shows an enzyme that is neither efficient nor inefficient (NENIE). The red double-headed arrow illustrates the height of the energy barrier; the orange double-headed arrow illustrates the upper energy barrier height, either the Arrhenius activation energy or the Gibbs free energy of activation that must be added to the reactants for the reaction to proceed. The lower orange double-headed arrow illustrates the ground-state energy level of the reactants, while the blue, green, and oxblood horizontal lines mark the much lower ground-state energy level of the products for the very efficient, efficient, and neither efficient nor inefficient enzymes, respectively. In these cases, the product is more stable than the reactant.

To the best of our knowledge, apart from the proposal by the esteemed scholar Johnson [20] in the field of biochemistry that a direct estimate of SC is desirable, no one has attempted to derive new equations or quantify the values of SC in the past, as this study has done. However, one could speculate that there is always motivation for such a desire, even if there is no evidence of it. Chemical engineers, physical chemists, etc., may suggest benefits or the desirability of such equations. Nevertheless, the two newest approaches are undeniably the most effective. This is because, when there are many data points, a direct linear plot or its reciprocal variant can be unwieldy and prone to error if software is not applicable. Outliers are always likely with LWBP given its imprecise initial rates.

The principle of efficiency is a fundamental concept in classical mechanics, both at the elementary and postdoctoral levels. Thus, an enzyme's suitability for industrial applications can be evaluated based on its catalytic efficiency. Without information about the reverse rate constant, the efficiency of a chosen enzyme can be quickly determined by multiplying the ratio of SC to k_1 by 100. As shown in Table 2, lower enzyme concentrations under conditions that validate the Michaelian equation and sQSSA exhibit the highest SC values, regardless of the direct approach. In this study, the enzyme concentrations are compared as follows: 0.0002 > 0.0005 > 0.002 g/L. Besides other engineering and technical issues, a reactor's efficiency depends on the enzyme's capacity to reduce the energy barrier. Thus, a higher catalytic rate and a lower reverse rate constant can result in a very high SC and, consequently, a very high catalytic efficiency of 100 SC/k_1. One can undoubtedly conclude that SC is different from catalytic efficiency. Unlike SC, information about the second-order rate constant (k_1) and SC is needed to determine catalytic efficiency. The latter and SC should not be interchanged for any reason.

Table 2: Catalytic efficiency expressed as SC as a percentage of second order rate constant, k_1 for the formation of enzyme-substrate complex

$[E_T]$/g/L	0.0002	0.0005	0.002
RVDLP	84.113	78.874	4.674
LWBP	89.292	65.115	4.501
Equations (15 & 18)	89.678	65.135	4.479
Equation (38)	71.831	65.126	4.503

The table of values is an out of the application of the equation: $k_1 = (k_{cat} + k_{-1})/K_M$; k_1 is a sum of two parts. Hence a fraction of it contributed by SC (k_{cat}/K_M) multiplied by 100 gives the percentage contribution which is equivalent to catalytic efficiency, SC.

5. Summary

Two new equations were derived for the direct estimation of the specificity constant (SC). These equations are in addition to those for a non-arbitrary choice of pre-steady-state (PSS) or sub-K_M concentrations of substrate, "burst phase-like" rates, and their corresponding substrate concentrations $[S_T]_0$. SC values from the two newest methods, using three different enzyme concentrations, range from 2,197.546 to 11,101.74 L/g min using one method and from 2,185.649 to 13,860.014 L/g min using the other method. The sub-K_M SC values for the three different enzyme concentrations range between 1,304.368 and 7,943 L/g. min. The burst-phase-like initial rate (v_0) and the corresponding $[S_T]_0$ range from 14.26 to 55.448 mM/min and from 0.171 to 3.752 g/L, respectively. In all cases, the enzyme with the lowest concentration possesses the highest parameter values. The concept of SC differs greatly from that of catalytic efficiency.

6. Conclusion

The pre-steady-state (PSS) substrate concentrations ([S_T]s) and the corresponding mathematical equations for examining such scenarios were presented. Equations were presented for computing the specificity constant (SC) for the PSS scenario with an instantaneous initial rate (also known as the "burst phase-like" rate), as well as the corresponding [S_T]$_0$ («K_M) for a non-arbitrary choice of PSS or sub-K_M substrate concentrations suitable for the PSS test and for the saturating [S_T] scenario. In the end, assessing the equations for usefulness and robustness demonstrated that directly predicting the SC is feasible and not a coincidence. Future research will examine whether an original v_0/[S_T]$_0$ ratio can be generated by plotting initial rates versus substrate concentrations below [S_T]$_0$ while keeping the enzyme concentration constant. This approach can enhance the determination of zero-order SC at substrate concentrations below the K_M.

7. Author Contribution

The sole author designed, analyzed, interpreted and prepared the manuscript.

8. Dedication

This study is dedicated to Delta State Governor Sheriff Oborevwori and the government officials who support citizens' rights. It reflects the commitment of the former military governor of the defunct Mid-West State, Retired Brigadier General Samuel Osaigbovo Ogbemudia, and his commissioner, Chief Edwine Kiagbodo Clark. They were recognized for their non-derisive and non-divisive policies.

9. Disclaimer (Artificial Intelligence)

Author(s) hereby declare that no generative AI technologies such as Large Language Models (ChatGPT, COPILOT, *etc*.) and text-to-image generators have been used during the writing or editing of this manuscript. s of the article.

10. Disclaimer

The preprint document is available in these links:
https://doi.org/10.1101/2023.04.09.536186
https://www.researchgate.net/publication/369952471

11. Competing Interests

There are no known competing financial interests, non-financial interests, or personal ties that could have influenced the work presented in this publication, according to the sole author's declaration. The only issue is the monthly pension, which is much less than two USD per day and may not have been recommended by the World Bank or the IMF.

12. Acknowledgement

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13. References

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