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Rate constants are determinable outside the original Michaelis–Menten mathematical formalism wherein the substrate concentration range is ≈ 1.6 → 4.8 times enzyme concentration: A pre-steady-state scenario and beyond

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Abstract

For some time now, there has been growing interest in pre-steady-state (PSS) kinetic parameters for whatever reasons, the measurement of which needs high-tech equipment capable of transient time-scale duration of assay. The proposition, however, is that all kinetic parameters, PSS and beyond, can be determined with appropriate PSS derivable equations and the usual Michaelis-Menten (MM) and Briggs-Haldane (BH) equations, respectively. The objectives of the research were: 1) To derive equations, for the determination of reverse rate constant when the substrate concentration, [S] « MM constant, K_M, 2) determine by calculation, the reverse rate constant, forward rate constant, and consequently, show that it is possible to determine rate constant often seen to be masked within original MM cum BH mathematical formalism, and 3) validate corollaries from the derivation that justify procedural issue. Theoretical, experimental (Bernfeld method), and computational methods were explored. Pre-steady-state equations for the determination of kinetic parameters, the reverse rate constant, k_{-1}, for the process ES → E + S, the 2nd order rate constant, k_1, and the rate, v_1, for the formation of enzyme-substrate complex, ES, were derived. The derived originating equations with associated corollaries were validated and have been seen to be capable of reproducing experimental variables and kinetic parameters; rate constants that seemed masked in MM formalism were unmasked. Steady-state (SS) cum zero order kinetic parameters were » their PSS values. "Negative" catalytic efficiency (k_{-1}/K_M) was » "positive" catalytic efficiency, (k_{cat}/K_M), with lower [E_T]. In conclusion, the equations for PSS kinetic parameters were derivable. Previously masked kinetic parameters in the MM/BB mathematical formalism can now be calculated using MM data; thus, all kinetic parameters can be determined regardless of the reaction pathway's state, PSS, and SS. PSS kinetic parameters were « SS/zero order values.

Keywords: *Aspergillus oryzea* alpha-amylase; Pre-steady-state; Steady-state; Kinetic parameters; Catalytic efficiency

1 Introduction

Pre-steady-state enzyme kinetics has been of regular interest in the past and recently. It has either been investigated to achieve scientific insight, thereby creating new knowledge, or applied in the total characterisation of single-substrate-enzyme reactions [1-4]. There have been investigations tailored towards the determination of rate constants associated with pre-steady-kinetics, the so-called burst phase [5, 6]. In all those investigations, hi-tech instrumentation was employed [3, 4], as is often recommended for any pre-steady-state kinetic study. Since assays need to be carried out at an infinitesimal time scale, such advanced instrumentation is inevitable, though the question that remains is; can every institution make such a facility available on demand? This is with some "third world" nations in view. There are,

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however, constraints in the use of some of the hi-tech equipment, one of which is the unavailability of signals for measurement of rates, as may be applicable to rapid-stopped flow methods unlike chemical-quench-flow methods [1].

The chemical-quench-flow method allows direct measurement of the conversion of substrate to product [1]. Nonetheless, there seems to be a progression from one method of kinetic study to another. The steady-state method is the first, followed by the pre-steady-state method, and finally by the single-turnover method. Pre-steady-state analysis, on the other hand, necessitates the use of a high $[S_T]/[E_T]$ ratio [7], a relic of QSSA characterisation ($[S_T] \gg [E_T]$) [8] as well as $[E_T] \ll ([S_T] + K_M])$ [9, 10]. While a high $[E_T]/[S_T]$ ratio is required for single-turnover [7], this is a relic of an earlier definition of quasi-steady-state (rQSSA), which had a $\partial[E_T]/\partial t \approx 0$ introduced previously by Segel and Slemrod [10].

The partial departure from steady-state condition (SS condition) seems attributable to the inability of investigators to define or prove a reaction pathway; the data obtained from SS condition provides only indirect information to define the enzymatic pathway, and since SS cum zero order parameters, k_{cat} (catalytic rate constant, often referred to as turnover number rate [11]), and K_M are complex functions of all the reactions occurring at the enzyme surface, individual reaction steps are masked or overshadowed by those terms, and resolution may be impossible [1, 2]. The limitations are resolved by examination of the reaction pathway by transient-state kinetic methods, whereby the enzyme is seen as a stoichiometric reactant, allowing individual steps in a pathway to be sorted out by direct measurements [1].

Incidentally, the choice of pre-steady-state is known to have its shortcomings in that there are factors that reduce the amplitude in a pre-steady-state burst experiment and there is difficulty in resolving the product and intermediate from excess substrate. Excess enzyme (high $[E_T]/[S_T]$ ratio), which prevents catalytic cycling, limits the bound substrate to a single turnover, allowing the chemical step of the reaction to be isolated and accurately determined as the first-order rate constant [7]. So much about pre-steady-state, notwithstanding, the proposition in this research is that without strict adherence to a very infinitesimal time regime, rate constants in a pre-steady-state scenario, steady-state and post-steady-state (or overall state, from burst phase, first order ... to full length of zero order) can be determined given suitable duration of assay and substrate concentration that excludes substrate depletion.

With focus on ES dissociation and the process EP $\rightarrow$ E + P (where EP is the enzyme product complex), this research addresses how reverse rate constant for the process ES $\rightarrow$ E + S can be determined under conditions such as when $K_M \gg$ substrate concentration, $[S_T]$. Though the range of $[S_T]$ is such that most of the different $[S_T]$ are $< K_M$, nevertheless, there is always a need for a high $[S_T]/[E_T]$: But $[E_T]$ must be higher than what would have been the case, if steady-state is to be investigated. Thus, this research focuses on rate constants outside the domain of original Michaelis–Menten mathematical formalism where, substrate concentration range is $\approx 1.6 \rightarrow 4.8$ times enzyme concentration not just in a pre-steady-state scenario alone but inclusive of much-talked about steady-state and beyond.

To observe the burst phase, pre-steady-state kinetics requires a high $[S_T]/[E_T]$ ratio [7]. Both the length of the assay and the relatively high $[S_T]$ to $[E_T]$ ratio can be used to calculate the reverse rate constant, especially in a pre-steady state scenario. A detailed procedure is based on the usual equation derived on the basis of what Srinivasan [7] referred to as the assumption of rapid equilibrium (as done by Michaelis-Menten [12]) and steady state (as modified by Briggs and Halden [8]). This research has the following objectives. 1) To derive equations for the determination of the reverse rate constant when the $[S_T] \ll K_M$, 2) determine by calculation the reverse rate constant, forward rate constant, and consequently, show that it is possible to determine the rate constant often seen to be masked within the original MM cum BH mathematical formalism, and 3) validate derivation corollaries that justify the procedural issue.

2 Theory

2.1 Review of some kinetic schemes and kinetic equations

The underlying issues to be addressed in this section are the kinetic schemes encountered in the literature, with the view to qualitatively demystify such schemes while restricting the research to no more than three of such schemes for derivational purposes. No equations are intended to arise from all the schemes. The need for demystification is predicated on the comments in the literature. "The fact that the kinetic parameters can assume such complex forms may come as a surprise to many students who have been used to the repeated use of the scheme, E + S $\rightleftharpoons$ ES $\rightarrow$ E + P, to teach the meaning of these parameters" [7]. Incidentally, life is all about learning, and those who must teach must adopt a step-by-step approach showing subtle details regardless of the concerns of publishers, who, of course, would gain more if publications attracted a wider readership because of the presentation of papers in a simple manner as earlier described. The question is who is a student? There are classes of students. A teacher who does not read or learn may

end up as a bad teacher. In spite of the complexity described above, steady-state kinetic parameters help by defining the lower limit for the value of k_{cat} and k_{cat}/K_M [7]. Even if related, post-docs in other fields may find it difficult to understand equations whose origin cannot be traced, if further investigation or application is pursued as an objective. Being on the same page as such people is not out of place.

For a long time, researchers have carried out experiments to determine the reverse rate constant, k_{-1}, for the dissociation of an enzyme-substrate complex, ES, into free enzyme, E, and free substrate, S. Also included among the rate constants are the 2nd rate constant, k_1, for the formation of ES; the rate constant, k_2, for the formation of enzyme-product complex, EP; the reverse rate constant, k_{-2}, for the reformation of ES from EP; the rate constant, k_3, for the release of products, P and E; and the 2nd other rate constant, k_{-3}, for the reformation of EP. The equation, otherwise called a scheme, below gives a picture of the preceding issue.

$$\mathrm{E + S} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{ES} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \mathrm{E'P} \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} \mathrm{E + P} \tag{1a}$$

Other similar issues (schemes (2) and (3)) are available in the literature [7, 13–14]. Schemes (4), as in the literature [6], (2), and (3) below have kinetic equations, but none was given a stepwise derivation. Therefore, procedural issues cannot be learnt. There is a high possibility that intermediates depicted in kinetic schemes exist, but such must be of a very infinitesimal time scale, ≤ μs time-scale. There must be time for each pre-catalytic event and various stages of catalytic events, as described and quantified in the literature [15, 16].

The paper by Reuveni *et al* [17] is seen to have pointed out time (T) distribution for each event; catalytic and non-catalytic events; binding, unbinding, and catalysis ($\langle T_{on} \rangle + \langle \min T_{cat}, T_{off} \rangle$), *etc*, where the subscripts, on, cat, and off, respectively, define the times as binding time, catalytic time, and unbinding time. As in other literature [15, 16], a view has been held to the effect that the mean turnover time, the average time it takes a single enzyme to produce a single molecule of product [17], is always greater than the mean time for binding and catalysis combined. All the schemes depict events, each of which has a life span. The most likely scheme may be the one containing the activated forms, otherwise hereinafter referred to as the catalytic conformational transition state of the enzyme and substrate, leading to a scheme such as:

$$\mathrm{E + S} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{ES} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \mathrm{E'S'} \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} \mathrm{E'P} \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} \mathrm{E + P} \tag{1b}$$

To avoid too many schemes, suffice to state that E′S′ may just proceed to EP in which the bond between P and E is purely physicochemical, subject to thermal and any other plausible physical factors as applicable to ES.

$$\mathrm{E + S} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{ES} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \mathrm{EP} \overset{k_3}{\rightarrow} \mathrm{E + P} \tag{2}$$

$$\mathrm{E + S} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{ES} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \mathrm{E.X} \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} \mathrm{EP} \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} \mathrm{E + P} \tag{3}$$

$$\mathrm{E + S} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{ES} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \mathrm{E'P} \overset{k_3}{\rightarrow} \mathrm{E + P} \tag{4}$$

$$\mathrm{E + S} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{ES} \overset{k_2}{\rightarrow} \mathrm{E + P} \tag{5}$$

Schemes (1a) and (4), every other thing being the same as in other scheme, presuppose that the enzyme underwent a conformational transition to a catalytic state that enabled the formation of product; scheme 1a, however, presents the possibility that the substrate can bind to the free enzyme which may not necessarily be in a conformational transition state unlike what it was in the conformational transition state (the catalytic state)-product complex, E′P. The binding of the product (if applicable) or the substrate to the enzyme in whatever state, may be purely biophysical, whereas the breaking of bond (*e.g*. glycosidic bond) and the making of bond such as H — O — in maltose or product released is purely the biochemistry involved in the process, the catalytic event. Is amylase for instance both a hydrolase and a synthase or synthetase? This question is relevant considering schemes (1a) and (3) where respectively conformational transition state enzyme-product complex and initial state enzyme-product complex are reformed. Schemes (2), (3), and (5) do not present the enzyme in a catalytic conformational state (or better still, a conformational transition state suitable for catalytic function).

Scheme (5) seems to effectively summarise the events within the catalytic domain preceding product release that signal measurable biological activity of the enzyme. For instance … ES ⇌ E.X ⇌ E.P …, ES ⇌ E′S′ ⇌ E′P… *etc* are catalytic events that have duration and consequently catalytic rate. The general objective is product formation in any relevant setting, be it industrial, biological, *etc*. Unbinding of substrate in particular, may therefore, increase the turnover time of any such enzyme molecule, even if the overall effect, as claimed in the literature [17], is an enhanced rate of enzymatic turnover. "Unbinding may censor the tail of the catalysis time distribution and hence prevent a situation in which the substrate is "stuck" in the ES state for an undesirably long period of time. On the other hand, unbinding will inevitably require renewed binding and catalysis, and hence entails a severe time penalty "[17], the delay and associated time wasting. One must not lose sight of the fact that unbinding from the catalytic site may be associated with low or compromised affinity of the enzyme and substrate for each other. However, the release of either enzyme or substrate from a sequestered condition, which otherwise reduces the velocity of hydrolysis [18], may compensate for the time lost without necessarily increasing the rate of catalysis, considering the time lost during the unavailability of either the enzyme or substrate. What is ultimately important is the release of either enzyme or substrate from the ES complex that cannot be transformed into a product.

Beginning from the second order differential equation [1] $\partial[E]/\partial t = -\partial[ES]/\partial t = -k_1[E][S]$ is the equation, $[ES] = [E_T](1 - \exp.(-k_1[S_T])\,t)$, where $k_1[S_T]$ is taken to be pseudo-first order rate constant (k_{obs}) as long as $[S_T] \gg [E_T]$ ($[S_T]$ and $[E_T]$ are the total substrate and enzyme concentrations respectively). The equation can be recast into $(v_{max} - v)/v_{max} = \exp.(k_1[S_T])\,t)$ such that a plot of In. $((v_{max} - v)/v_{max})$ versus t should give a slope equal to $k_1[S_T]$. But the magnitude of this slope should, rather be too low. In the same text can be found, the equation $k_{obs} = k_1[S_T] + k_{-1}$. But $k_1[S_T]$ has already be defined as k_{obs}. However, the former may seem reasonable if various values of k_{obs} are obtained from different time course experiments for different $[S_T]$. In this case lower range of a given substrate concentration range must also be $\gg [E_T]$. A worrying scenario is an equation given as: $[ES]/[E_T] = k_1[S_T](1 - \exp(-k_{obs}\,t))/(k_1[S_T] + 1)$; $k_{obs}\,t$ is a dimensionless quantity and $k_1[S_T]$ in the denominator has a pseudo-first order rate unit given as per unit time. Since one (1) in the denominator has no unit, then the entire equation lacks scientific meaning.

However, the pseudo-first order rate constant defined in other literature [16] is on the basis of rQSSA, in which the $k = [E_T]\,k_1$, appears generalisable. It was also observed that rQSSA and sQSSA take the same mathematical form, such that K_S (dissociation constant) = k_{-1}/k_1 (rQSSA) and $K_M = (k_{-1} + k_{cat})/k_1$ (sQSSA) may be applicable [19]. With this background information, the following equation in the literature [16] was derived:

$$\text{In}\ \frac{[E_T]}{[E_T] - [ES]} = \frac{(k_{-1} + k_{cat})[S_T](1 - \exp(-k\,t))}{K_M k} \tag{6}$$

Looking at Eq. (6), one sees that $[S_T]$ and K_M belong to the same chemical species, and therefore, their units can cancel out. In other words, $[S_T](1 - \exp(-k\,t))$ is the mass of the product produced in time, t ($t \ll 1$; $t \leq$ μs time scale), such that division by K_M, cancels the unit. The important issue is that Eq. (6) is dimensionally valid and consistent.

2.2 Derivation of pre-steady equations for the determination of reverse rate constant associated with unbinding (dissociation) of substrate from enzyme-substrate complex.

Because the burst phase lasts only a few seconds ($t_{(\ll 1)}$), the substrate concentration ($[S_F]$) remaining after quenching (stopping) must be the initial concentration, $[S_T]_{(t=0)}$. With regard to the very short duration ($t \leq$ m s time scale) of the assay, both pre-steady-state and single turn-over events have been described as transient kinetic methods. It may, therefore, appear that pre-steady-state kinetics with $[E_T] \gtrsim [S_T]$ unlike steady-state, where $[E_T] \ll [S_T]$, may present conditions that either satisfy the demands of rQSSA and sQSSA (RSA) or one of them mainly or none at all. The issue is

that the amount of product formed under a millisecond time scale is « amount in 1 minute or more. The duration of the assay in this research was 3 min. There is a need, therefore, to investigate whether or not the determination of rate constants outside the domain of the original Michaelis–Menten cum Briggs and Haldane equation may not be impossible in a pre-steady-state scenario and beyond.

Recall that, at steady-state, the sum of the rates (velocities, v_{-1}, dissociation of ES to E and S and v, dissociation of ES, E'P, EP to E and P) of all dissociation reactions is equal to the rate, v_1 of the formation of ES. This starting point justifies the assertion that steady-state and transient state kinetic studies complement each other, and analysis in the steady-state should be a prelude to the proper design and interpretation of experiments using transient-state kinetic methods [1]. This assertion may stand the test of time as long as the pre-steady-state model cannot be separated from the steady-state model. Thus,

$$v_1 = k_1[E_F][S_T] = \frac{(k_{-1}+k_{cat})}{K_M}[E_F][S_T] \quad (7)$$

Where, k_{-1}, k_{cat}, and k_1 are the reverse rate constant for the process ES → E + S, ES, E'P, EP → E + P, and the formation of ES complex respectively. Also,

$$v_1 = k_{-1}\left(\frac{v_{max}}{k_{cat}} - [E_F]\right) \quad (8)$$

Because $v_1 = v_{-1} + v$, where v_{-1} is = k_{-1} $[ES]$ and $[ES]$ is = $(v_{max}/k_{cat}) - [E_F]$: Thus, Eqs (7) and (8) are the same. Where $[E_F]$ is the free enzyme concentration that may be ≅ $[E_T]$, though, it is prudent to recall that transient kinetics is also under consideration without necessarily exploring transient kinetics equipment; this does not imply that the infinitesimal concentration of the product should be ignored in every step. In terms of the transient or pre-steady-state regime, the infinitesimal time scale and $[S_T]$ « K_M are two sides of the same coin. This is so because, in such a scenario, the reaction path is still behind the steady-state regime that requires more time and much higher $[S_T]$. However, if a scenario with $[S_T]$ « K_M but not an infinitesimal time scale is in mind, the latter can still serve the purpose of this research if Michaelis-Menten formalism is applicable. Part of what is needed is suitable derivable equations. Hence,

$$\frac{k_{-1}+k_{cat}}{K_M}[E_F][S_T] = k_{-1}\left(\frac{v_{max}}{k_2} - [E_F]\right) + v \quad (9)$$

Meanwhile,

$$[E_F] = [E_T] - [ES] = (v_{max} - v)/k_{cat} \quad (10a)$$

Therefore, if $[E_F]$ at the left hand side (LHS) in Eq. (9) is replaced by Eq. (10a), the result is

$$\frac{k_{-1}+k_{cat}}{K_M}\ \frac{v_{max}-v}{k_{cat}}\ [S_T] = k_{-1}\left(\frac{v_{max}}{k_{cat}} - [E_F]\right) + v \quad (10b)$$

Expansion of Eq. (10b) at the LHS gives:

$$\frac{k_{-1}}{k_{cat}K_M}(v_{max} - v)[S_T] + \frac{(v_{max} - v)[S_T]}{K_M} = k_{-1}\left(\frac{v_{max}}{k_{cat}} - [E_F]\right) + v \quad (10c)$$

Division through Eq. (10c) by k_{-1} gives,

$$\frac{(v_{max} - v)[S_T]}{k_{cat}K_M} + \frac{(v_{max} - v)[S_T]}{k_{-1}K_M} = \frac{v_{max}}{k_{cat}} - [E_F] + \frac{v}{k_{-1}} \quad (10d)$$

Making $[E_F]$ subject of the formula gives:

$$[E_F] = \frac{v_{max}}{k_{cat}} + \frac{v}{k_{-1}} - \frac{(v_{max} - v)[S_T]}{K_M k_{cat}} - \frac{(v_{max} - v)[S_T]}{K_M k_{-1}} \quad (11)$$

Meanwhile, Eq. (9) can be re-expressed as:

$$\frac{k_{-1}+ k_{cat}}{K_M}[E_F][S_T] = k_{-1}\frac{v}{k_{cat}} + v \quad (12)$$

Because $\frac{v_{max}}{k_{cat}} - [E_F] = [ES]$(or v/k_{cat}). Therefore, if Eq. (11) is substituted into Eq. (12), the result is:

$$k_{-1}\frac{v}{k_{cat}} + v = \frac{k_{-1}+ k_{cat}}{K_M}[S_T]\left(\frac{v_{max}}{k_{cat}} + \frac{v}{k_{-1}} - \frac{(v_{max} - v)[S_T]}{K_M k_{cat}} - \frac{(v_{max} - v)[S_T]}{K_M k_{-1}}\right) \quad (13a)$$

Putting $k_{-1}\frac{v}{k_{cat}} + v$ in the form $\frac{k_{-1}+k_{cat}}{k_2}\ v$ and substitute back into Eq. (13a) to enable elimination of common factor and following the cross multiplication by k_{cat} gives:

$$v = \frac{k_2}{K_M}[S_T]\left(\frac{v_{max}}{k_{cat}} + \frac{v}{k_{-1}} - \frac{(v_{max} - v)[S_T]}{K_M k_{-1}}\right) \quad (13b)$$

Note that $\frac{(v_{max} - v)[S_T]}{K_M}$ in Eqs. (13b) and (11) is equal to v and, as such, $v\ /k_{-1}$ can be eliminated thereby defeating the objective of defining and quantifying k_{-1}. Therefore, Eq. (13b) needs to be opened, v redefined as $-\partial[S_T]/\partial t$, divided by $[S_T]$, and cross multiplied by ∂t to give:

$$-\frac{\partial[S_T]}{M_{alt}[S_T]} = \frac{v_{max}\,\partial t}{K_M} - \frac{\partial[S_T]\,k_{cat}}{M_{alt}\,K_M k_{-1}} - \frac{v_{max}\,[S_T]\,\partial t}{K_M^2} - \frac{\partial[S_T]\,[S_T]}{M_{alt}\,K_M^2} - \frac{v_{max}\,[S_T]k_{cat}\,\partial t}{K_M^2\,k_{-1}} - \frac{\partial[S_T]\,[S_T]\,k_{cat}}{M_{alt}\,K_M^2\,k_{-1}} \quad (13c)$$

Where, M_{alt} is the molar mass of the product, maltose, to account for the concentration of it when there is substrate partial depletion, even if infinitesimal. Integration gives:

$$\frac{\ln([S_T]_{(t=0)}/[S_T]t)}{M_{alt}} = \frac{v_{max}\,\Delta t}{K_M} - \frac{\Delta[S_T]k_{cat}}{M_{alt}\,K_M k_{-1}} - \frac{v_{max}\,[S_T]\,\Delta t}{K_M^2} - \frac{\Delta[S_T]\,([S_T]_{(t=0)} + [S_T]t)}{2\,M_{alt}\,K_M^2} - \frac{v_{max}\,[S_T]k_{cat}\,\Delta\,t}{K_M^2\,k_{-1}} - \frac{\Delta[S_T]\,([S_T]_{(t=0)} + [S_T]t)\,k_{cat}}{2\,M_{alt}\,K_M^2\,k_{-1}} \quad (14)$$

Where, $[S_T]_{(t=0)}$ and $[S_T]_t$ are the substrate concentration at time, t = 0 and after chosen duration of assay, respectively. Recall that Michaelis-Menten cum Briggs and Haldane equation reduces to $v = v_{max}[S_T]_{(t=0)}/K_M$ if $[S_T]_{(t=0)} \ll K_M$ such that at almost any chosen $t \ll 1$ hour (even 1 – 5 minutes let alone, « 1 minute, the millisecond time scale for instance), the magnitude of $\Delta[S_T] \cong 0$, and consequently, $[S_T]_{(t=0)}/[S_T]_t \cong 1$. With the two conditions, $[S_T] \ll K_M$ and time ≤ millisecond time scale, in mind, Eq. (14) becomes

$$\frac{v_{max}\,\Delta t}{K_M} - \frac{v_{max}\,[S_T]\,\Delta t}{K_M^2} - \frac{v_{max}\,[S_T] k_{cat}\,\Delta t}{K_M^2\,k_{-1}} = 0 \quad (15a)$$

Divide through Eq. (15a) by $\Delta t\, v_{max}/K_M$ to give:

$$1 - \frac{[S_T]}{K_M} - \frac{[S_T]k_{cat}}{K_M\,k_{-1}} = 0 \quad (15b)$$

Rearrangement and making k_{-1} subject of the formula in Eq. (15b) gives:

$$k_{-1} = \frac{[S_T]k_{cat}}{K_M - [S_T]} \quad (16)$$

Equation (16) confirms the earlier stance that $[S_T] \ll K_M$; the implication implied in the equation is that there should be various values of k_{-1}. The latter should be increasing with increasing values of $[S_T]$ similar to what is expected from Michaelis-Menten cum Briggs and Haldane equation expressed as $v/[E_T] = k_{cat}\,[S_T]/(K_M + [S_T]) < k_{cat}$. Such values will always be $< k_{cat}$ as long as $K_M - [S_T] > [S_T]$.

Other derivations arise if the equation below is simplified. First, k_{-1} (= $k_{cat}v_{-1}/v$) is substituted into Eq. (16) to give:

$$\frac{k_2 v_{-1}}{v} = \frac{[S_T]k_{cat}}{K_M - [S_T]} \quad (17a)$$

Rearrangement gives:

$$v_{-1} = \frac{[S_T]\,v}{K_M - [S_T]} \quad (17b)$$

Once again, v_{-1} may be increasing with every value of $[S_T] \ll K_M$, but it will always be $< v$ as long as $K_M - [S_T] > [S_T]$. Also, given that $v_1 = v_{-1} + v$ then,

$$v_1 = \frac{[S_T]\,v}{K_M - [S_T]} + v \quad (18a)$$

Simplification gives:

$$v_1 = \frac{K_M v}{K_M - [S_T]} \quad (18b)$$

Looking at Eq. (18b), one may mistakenly, if not naively, think that v_1 could be higher with larger K_M values, which in most texts may suggest low affinity of the substrate for the enzyme. However, it is self-evidence that $(K_M - [S_T]) < K_M$ so that $K_M/(K_M - [S_T])$ will always be >1. This reflects the fact that v_1 is a sum of two positive parts. If K_M is large, v may be low, so that v_1 should also be low. The variable v_1 can also be derived in terms of v_{max}. Given that, $v/v_{max} = [S_T]/K_M$, one can substitute the reciprocal of it into Eq. (17b) in its other form given as:

$$v_{-1} = \frac{v}{\frac{K_M}{[S_T]} - 1} \quad (19a)$$

This gives:

$$v_{-1} = \frac{v}{\frac{v_{max}}{v} - 1} \quad (19b)$$

Rearrangement gives:

$$v_{-1} = \frac{v^2}{v_{max} - v} \quad (19c)$$

Substitution of Eq. (19c) into $v_1 = v_{-1} + v$ gives:

$$v_1 = \frac{v^2}{v_{max} - v} + v \quad (20a)$$

Simplification leads to:

$$v_1 = \frac{v_{max}\,v}{v_{max} - v} \quad (20b)$$

2.3 Validating derived equations

Validity should be the case if $v = v_{max}\,[S_T]/K_M$ can be reproduced as follows. Equations (18b) and (20b) are the same. Thus,

$$\frac{K_M v}{K_M - [S_T]} = \frac{v_{max}\,v}{v_{max} - v} \quad (21a)$$

Expansion gives:

$$v_{max}\,K_M - v_{max}\,[S_T] = v_{max}\,K_M - K_M\,v \quad (21b)$$

Elimination of common variables and rearrangement gives, $v = v_{max}\,[S_T]/K_M$ as expected. Given, $v_1 = v_{-1} + v$ also,

$$\frac{v_{max}v}{v_{max} - v} = \frac{[S_T]\,v}{K_M - [S_T]} + v \quad (22a)$$

Elimination of v and cross multiplication by $(v_{max} - v)$ yields

$$v_{max} = \frac{[S_T]\,(v_{max} - v)}{K_M - [S_T]} + v_{max} - v \tag{22b}$$

Elimination of v_{max} and rearrangement gives first,

$$v = \frac{[S_T]\,v_{max} - [S_T]\,v}{K_M - [S_T]} \tag{22c}$$

Expansion, elimination of v $[S_T]$, and rearrangement give $v = v_{max}\,[S_T]/K_M$ as expected. However, given $v = v_{max}[S_T]/K_M$ once again, $\frac{K_M v}{K_M - [S_T]} = \frac{[S_T]\,v}{K_M - [S_T]} + v$. Simplification and rearrangement give $\frac{K_M}{K_M - [S_T]} = \frac{K_M}{K_M - [S_T]}$. This can be described as an indirect validation of the originating equations.

Meanwhile, if one recalls Eq. (11) where $[E_F]$ can be redefined as $v\,K_M\,[S_T]/k_2$ such that:

$$\frac{vK_M[S_T]}{k_{cat}} = \frac{v_{max}}{k_{cat}} + \frac{v}{k_{-1}} - \frac{(v_{max} - v)[S_T]}{K_M k_{cat}} - \frac{(v_{max} - v)[S_T]}{K_M k_{-1}} \tag{23a}$$

Then Eq. (23a) is rearranged to give:

$$\frac{1}{k_{-1}}\left(v - \frac{(v_{max} - v)[S_T]}{K_M}\right) = \frac{vK_M}{[S_T]k_2} + \frac{(v_{max} - v)[S_T]}{K_M k_{cat}} - \frac{v_{max}}{k_{cat}} \tag{23b}$$

Realising that $v = \frac{(v_{max} - v)[S_T]}{K_M}$ and eliminating k_{-1} leads to two quadratic equations. Beginning from

$$\frac{vK_M}{[S_T]\,k_{cat}} + \frac{(v_{max} - v)[S_T]}{K_M k_{cat}} - \frac{v_{max}}{k_{cat}} = 0 \tag{23c}$$

They are:

$$v\,K_M - \frac{v\,[S_T]^2}{K_M} - v_{max}[S_T] + \frac{v_{max}\,[S_T]^2}{K_M} = 0 \tag{23d}$$

Rearrangement in two different ways gives:

$$\left(\frac{v_{max}}{K_M} - \frac{v}{K_M}\right)[S_T]^2 - v_{max}[S_T] + vK_M = 0 \tag{24}$$

$$vK_M^2 + (v_{max} - v)[S_T]^2 - v_{max}[S_T]K_M = 0 \tag{25}$$

From Eq. (24), $[S_T]$ is given as:

$$[S_T] = \frac{\left(v_{max} \pm \sqrt[2]{v_{max}^2 - 4\,v\,(v_{max} - v)}\right)K_M}{2\,(v_{max} - v)} \tag{26}$$

$$K_M = \frac{v_{max} \pm \sqrt[2]{v_{max}^2 - 4\,v\,[S_T]^2\,(v_{max} - v)}}{2\,v} \tag{27}$$

Also, Eq. (25) is rearranged to give an expression to v.

$$v = \frac{v_{max}\,([S_T]K_M - [S_T]^2)}{K_M^2 - [S_T]^2} \tag{28}$$

Equations (26), (27), and (28) may not be used to evaluate kinetic data and independent variables obtained indirectly through linear transformation of the Michaelis-Menten cum Briggs and Haldane equations, direct linear, or nonlinear methods, because a simpler version of each can be derived from the Michaelis-Menten cum Briggs and Haldane equations given respectively as: $[S_T] = v\,K_M\,/(v_{max} - v\,)$, $K_M = [S_T]\,(v_{max} - v\,)/v$ and the well-known Michaelian transformation. The higher and lower positive roots give respectively the Michaelis-Menten constant and $[S_T]$ considering Eqs (26) and (27). The main message intended is that the originating equations and derived equations are valid for all time. Note that all equations for the same parameter can always give the same result if variables are very accurate, as expected if stopped and quench-flow measurements are explored. Another way to evaluate the equations is to recalculate the variables, *i.e.*, the velocities of enzyme action for all the concentrations of the S after substituting the v_{mas} and K_M into the canonical Michaelian equation for confirmation.

2.4 Derivation of a general equation for the determination of reverse rate constant

From Eq. (16), is the following:

$$\frac{K_M - [S_T]}{k_2} = \frac{[S_T]}{k_{-1}} \tag{29a}$$

Dividing through by K_M gives

$$\frac{1}{k_{cat}}\left(1 - \frac{[S_T]}{K_M}\right) = \frac{[S_T]}{k_{-1}\,K_M} \tag{29b}$$

From the equation $v \cong k_2\,[E_F]\,[S_T]\,/K_M$ ($[E_F] \cong [E_T]$, but the magnitude of $[E_F]$ cannot be ignored for the purpose of this derivation) is the equation:

$$\frac{[S_T]}{K_M} = \frac{v}{k_{cat}[E_T]} \tag{30}$$

Substitution of Eq. (30) into Eq. (29b) gives:

$$\frac{1}{k_{cat}}\left(1 - \frac{v}{k_{cat}\,[E_F]}\right) = \frac{v}{k_{-1}k_{cat}\,[E_F]} \tag{31}$$

Meanwhile,

$$[E_F] = [E_T] - [ES] = (\,v_{max} - v\,)\,/k_{cat} \tag{32}$$

And,

$$[E_F] = K_M\, v \,/\, k_{cat}\,[S_T] \tag{33}$$

Substitution of Eq. (32) and Eq. (33) into Eq. (31) on the left hand side and right hand side respectively gives after simplification:

$$\frac{1}{k_{cat}}\left(1-\frac{v}{(v_{max}-v)}\right) = \frac{[S_T]}{k_{-1}\,K_M} \tag{34a}$$

Equation (34 a) is rearranged to give Eadie – Hofstee – like equation ($y = -\,m\,x + c$ where y, m, x, and c are the dependent variable, slope, independent variable and a constant respectively)

$$v = -\frac{(v_{max}-2\,v)}{[S_T]}\frac{k_{-1}\,K_M}{k_{cat}} + v_{max} \tag{34b}$$

Equations (34 a) and (34 b) do not address the concern in the literature [20] that v appears on both side of original Eadie –Hofstee equation and that $v/[S_T]$ is not an independent variable. Note however, that log $v/(v_{max} - v)$ is plotted versus log $[S_T]$ for co-operative enzymes [21]. However, the equation contains twice, the v_{max} such that the intercept may not accurately reproduce the original v_{max} if it were to be known *a priori*. Equation (34b) can be written as:

$$v_{max} - v = \frac{(v_{max}-2\,v)}{[S_T]}\frac{k_{-1}\,K_M}{k_{cat}} \tag{34c}$$

Then, another approach may be needed. Unlike Eq. (34C), where, k_{-1} is = $f([S_T])$, another equation where k_{-1} is = $f([E_T])$ is to be determined. Since $[E_T]$ is not much less than $[S_T]$ (yet, $[S_T] > [E_T]$), one can open-up Eq. (34b), and substitute (K_M + $[S_T]$) v/ $[S_T]$ into it to give:

$$v = \left(-\frac{K_M+[S_T]}{[S_T]^2}v + \frac{2v}{[S_T]}\right)\frac{k_{-1}\,K_M}{k_{cat}} + \frac{K_M+[S_T]}{[S_T]}v \tag{34d}$$

Cancellation of common terms and rearrangement of Eq. (34c) gives:

$$\left(-\frac{K_M+[S_T]}{[S_T]} + 2\right)\frac{k_{-1}\,K_M}{k_{cat}} + K_M = 0 \tag{35a}$$

Further cancellation of common terms and rearrangement of Eq. (35a) in which (K_M + $[S_T]$) / $[S_T]$ = v_{max}/v gives:

$$2 - \frac{v_{max}}{v} = -\frac{k_{cat}}{k_{-1}} \tag{35b}$$

Since $v_{max} = k_{cat}\,[E_T]$ rewriting Eq. (35b) gives:

$$-\frac{k_{cat}}{k_{-1}}v = 2v - [E_T]k_{cat} \tag{35c}$$

Making v subject of the formula in Eq. (35c) yields:

$$v = \frac{[E_T]k_{cat}k_{-1}}{k_{cat}+2\,k_{-1}} \tag{36}$$

With very close observation, one sees Eq. (36) as one similar to Michaelian equation as to imply that:

$$\frac{[S_T]}{[S_T]+K_M} = \frac{k_{-1}}{k_{cat}+2\,k_{-1}} \tag{37}$$

Making k_{-1} subject of the formula reproduces Eq. (16). However, the slope of the plot of v values versus different $[E_T]$ should give a slope (S_{lope}) given as:

$$S_{lope} = \frac{k_{cat}k_{-1}}{k_{cat}+2\,k_{-1}} \tag{38}$$

Then, k_{-1} is then given as:

$$k_{-1} = \frac{S_{lope}}{k_{cat}-2\,S_{lope}} \tag{39}$$

The reciprocal variant can be given as:

$$\frac{1}{v} = \frac{1}{[E_T]}\frac{k_{cat}+2\,k_{-1}}{k_{cat}k_{-1}} \tag{40}$$

Equation (40) can then be expanded and rearranged to give:

$$\frac{1}{v} = \frac{1}{[E_T]k_{-1}} + \frac{2}{[E_T]k_{cat}} \tag{41}$$

The maximum velocity, v_{max}, may not be attained if different saturating concentrations of $[S_T]$ are required for different $[E_T]$. As a result, Eq. (40) cannot be considered final because it is based on a series of $[S_T]$ for the assay of different $[E_T]$; each $[E_T]$, $1 \rightarrow 4$ (or more), is assayed for each substrate concentration. Different magnitudes of the slope are expected for different $[S_T]$ [16]. The slope is plotted versus the reciprocal of the different $[S_T]$. The direct linear approach, the reciprocal variant [22], may not be impossible. A positive intercept is expected if the original experimental variables are free from error.

3 Material and methods

3.1 Chemicals

Aspergillus oryzea alpha amylase (EC 3.2.1.1), and insoluble potato starch, were purchased from Sigma – Aldrich, USA. Hydrochloric acid, sodium hydroxide, Tris 3, 5 – di-nitro-salicylic acid, maltose, and sodium potassium tartrate tetrahydrate were purchased from Kem light laboratories Mumbai India. Distilled water was purchased from local market.

3.2 Equipment

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

3.3 Methods

A stock solution of soluble potato starch was prepared by mixing 1 g in 100 mL of distilled water and subjected to heat treatment at 100 °C for 3–6 minutes, cooled to room temperature, and the decrease in volume due to evaporation was corrected by topping the volume with distilled water to 100 mL to give 1.0 g%. Different concentrations of the substrate used in all assays were prepared by adding different volumes of distilled water: 5 mL, 4 mL, 3 mL, 2 mL, 1 mL, and 0.0 mL to 5 mL, 6 mL, 7 mL, 8 mL, 9 mL, and 10 mL of heat treated starch, respectively. The *p*H of tris–HCl buffer changes with temperature, and this may affect the solubility of the substrate. This informed the need to use a neutral solvent to prepare different solutions of the gelatinised substrate. One stock solution of the enzyme was prepared by dissolving 0.01 g of the enzyme in 100 mL of tris–HCl buffer, whose *p*H is 7. Different concentrations of the enzyme were prepared by serial dilution of its stock solution by 60-, 40-, 30, and 20-fold dilutions of the stock solution. These correspond to mass concentrations of 1.6667 mg/litre, 2.5 mg/litre, 3.333 mg/litre, and 5 mg/litre, respectively. Assay of the enzyme was carried out according to the Bernfeld method [23]. The enzyme's hydrolytic activity was stopped after three minutes by adding 1 mL of 3, 5-di-nitro salicylic acid solution to a 2 mL reaction mixture containing 1 mL of substrate and 1 mL of enzyme. Spectrophotometric readings were taken at 540 nm and the molar absorption coefficient was 181.1 liters mol^{-1}. cm^{-1} based on the usual Beer–Lambert equation, $A_{540nm} = \varepsilon\, C\, l$ where, for the purpose of emphasis and clarity, C, and l are the molar absorption coefficient, molar concentration of product, and path length respectively, while A_{540nm} is the absorbance. Kinetic parameters such as Michaelis–Menten constant (K_M) and maximum velocity (v_{max}) of hydrolysis were determined according to the Lineweaver–Burk method [24]. The molar mass of *Aspergillus oryzea* alpha-amylase assayed is 52.10 kDa [25].

3.4 Statistical Analysis

All values from two determinations were expressed as mean.

4 Results and discussion

Equations applicable to near pre-steady-state conditions were derived which portray substrate concentrations, $[S_T]$, that are < $[E_T]$ such that $\partial[E_T]/\partial t \rightarrow 0$. This is to imply saturation of the enzyme is not applicable to the earlier transient. However, in this research, it is "a go-between scenario" in that neither the enzyme nor the substrate is overwhelmingly exposed to very high concentrations of each other, though $[S_T] \gtrsim [E_T]$ at the lower end of the substrate concentration range. The experimental findings seem to show that without very important transient assay equipment, the stop-flow and quench flow technique equipment, one can estimate rate constants, the reverse rate constant for ES → E + S. Thus, even if the assay was done, on a time scale much higher than millisecond time or even much less than the latter scale, values of kinetic parameters obtained can be seen to be different from what is expected of a steady-state and post-steady state condition. This is illustrated first in Table 1 and Table 2 where, respectively, the values of k_{-1} range from 1.9 → 3.4 k/min corresponding to the substrate concentration range, 5 →10 g/L, as obtained using Eq. (41) and the values of the rate of formation of ES, v_1, which showed an increasing trend with higher $[S_T]$ for each $[E_T]$. This result, with respect to k_{-1} is, however, different from the result (Table 3) obtained using Eq. (34c)/(34b), even though Eq. (41) was derived from the former. The reason may constitute a research question for the feature.

Meanwhile, in order to adequately illustrate almost all issues and advance answers for comprehensive understanding at all levels, including undergraduate level, double reciprocal plots (Fig. 1a and 1b) and reciprocal variants of direct linear plots (Fig. not shown) as well as a plot of $1/v$ versus $1/[E_T]$ (Fig. 2) were done. Figures (1a) and (1b) are respectively for the determination of v_{max} and K_M for each $[E_T]$ and for the determination of catalytic

efficiency/specificity constant and the corresponding K_M and k_{cat}. The graphical determination of k_{-1} for each $[S_T]$ is according to Fig. (2). Perhaps as the reaction pathway approaches v_{max}, the higher concentration of the enzyme becomes less exposed to available substrate as it is unable to rebind as soon as there is either product release or substrate release from the ES complex. It can be seen as enzyme concentration dependent, as it was observed for each concentration of the substrate with an increasing concentration of the enzyme in the plot of $1/v$ vs. $1/[E_T]$ (Fig. 2)) based on Eq. (41), whose inverse slope gave the value of k_{-1} as shown in Table (1). Further plots of the slope from the plot of $1/v$ versus $1/[E_T]$ for each $[S_T]$ (Eq. (41)) versus $1/[S_T]$ (though unusual, it is not different from plotting $[E_T]/v$ versus $1/[S_T]$ (double reciprocal plot, drp) that gives the intercept as the reciprocal of k_{cat}) gave much higher results (Table 3) than the reciprocal variant of direct linear plot (rvd). No figure is shown for this.

Table 1 Primary kinetic parameter and variables

$[S_T]$/g/L	$(1/\partial v/1/\partial[E_T])(k_{-1})$/exp.(+3) /min	$\partial v/\partial[E_T]$/exp.(+3)/min	Velocities of enzymatic action/μM/min			
			$[E_T]_1$	$[E_T]_2$	$[E_T]_3$	$[E_T]_4$
5	1.896	8.844	83.41	166.20	274.25	638.65
6	2.128	9.540	90.15	193.30	320.20	693.50
7	2.513	10.304	104.50	228.00	336.80	762.00
8	2.987	11.129	123.35	247.35	393.85	828.20
9	3.261	11.341	134.25	259.00	425.15	850.30
10	3.414	12.401	140.40	266.70	431.20	923.00
Upper limit of valid pre-steady-state $[S_T]$/g/L			2.11	1.62	2.02	2.44

Velocities of amylolysis for different enzyme concentrations $[E_T]$, $[E_T]_1 \rightarrow [E_T]_4$ (≈ 3.205, 4.808, 6.410, and 9.615) exp. (– 8) M. v being α $E_T]$ gives slope, $\partial v/\partial[E_T]$, equivalent to first order rate constant, the primary kinetic parameter, which is < k_{cat} reached when the enzyme is saturated. A drp plot of $1/v$ versus $1/[E_T]$ according to Eq. (41) gave slopes $(1/\partial v/1/\partial[E_T])$ whose reciprocal gave, k_{-1} for each $[S_T]$ according to Eq. (41). Mean of velocity values from two determinations for each $[S_T]$ for purely illustrative purpose at this early stage of this research were given.

Table 2 Velocities of the formation of enzyme-substrate complex ES

v_1/μM/min			
$[E_T]1$	$[E_T]2$	$[E_T]3$	$[E_T]4$
115.889	242.594	392.720	1128.915
129.322	305.010	494.296	1312.403
161.047	401.438	535.002	1581.432
210.659	465.522	694.897	1895.950
244.571	508.730	798.636	2015.903
265.780	539.141	820.255	2478.784

The velocities, v_1, of the formation of ES were calculated for each concentration of the enzyme ($[E_T]_1 \rightarrow [E_T]_4$) by fitting, Eq. (20b) to experimental variables, v and maximum velocities of amylolysis, v_{max}.

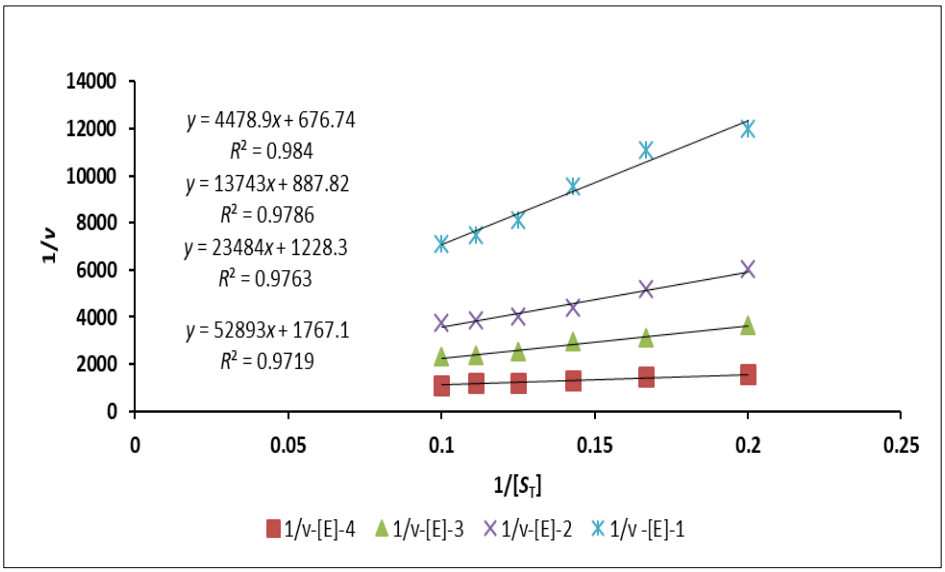


Figure 1a Determination of Michaelian parameters. The legends, (■), (▲), (×), and (ж), denote, double reciprocal plots for $[E_T]4$, $[E_T]3$, $[E_T]2$, and $[E_T]1$ for the determination of respective maximum velocity, v_{max}, and Michaelis-Menten constant, K_M

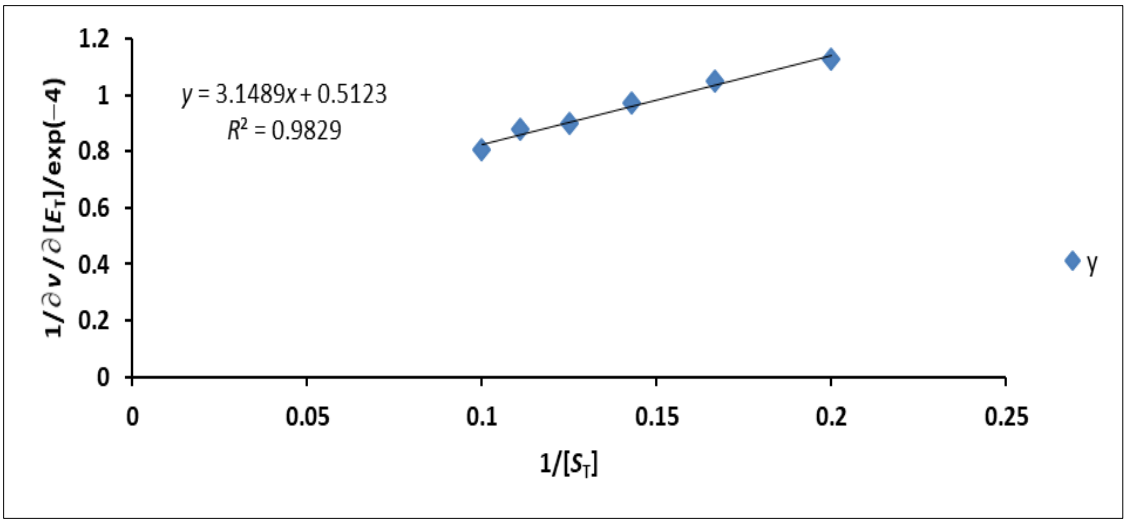


Figure 1b A plot of the reciprocal of the slope of v vs. $[E_T]$ (Table 1) vs. $1/[S_T]$ for the determination of catalytic efficiency and the corresponding k_{cat} and K_M. The results show that catalytic efficiency otherwise called specificity constant [26], is the reciprocal of the slope being ≅ 3175.712 L /g.min; k_{cat} ≅ 19519.813 /min (Table 1) and K_M ≅ 6.147 g/L(Table 1). The values from reciprocal variant of direct (rvd) linear plot are shown in Table 1. Here, the k_{cat}/K_M from rvd is 2848.978 L /g.min

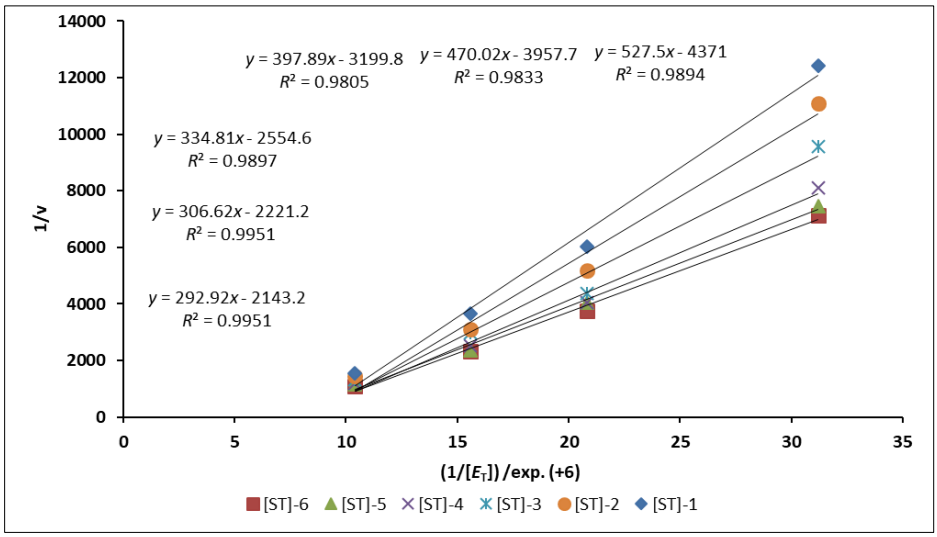


Figure 2 A plot for the determination of the reverse rate constant, k_{-1} for the process, ES → E + S, as $f(1/v, 1/[E_T])$, *i.e.*, 1/slope, $1/(1/\partial v)/(1/\partial[E_T])$, of the plot of $1/v$ vs. $1/[E_T]$ for each S, concentration, $[S_T] = 5 \rightarrow 10$ g/L. The legends (■), (▲), (×), (ж), (●), and (◆) denote, plots where $[S_T]$s are: 5, 6, 7, 8, 9, and 10 g/L respectively.

Table 3 Kinetic parameters arising primarily from Equations (34c) and (41)

Parameters	$[E_T]_1$	$[E_T]_2$	$[E_T]_3$	$[E_T]_4$	**Ñ**
K_M/g/L (drp)	29.932	19.119	15.479	7.243	6.147
„ (rvd)	18.690	16.429	11.364	7.500	6.509
v_{max} /μM/min (drp)	565.899	814.133	1126.354	1551.422	
„ (rvd)	297.619	714.286	909.091	1562.500	
k_{cat}/exp.(+3)min (drp)	17.148	16.934	17.545	16.135	19.520
„ (rvd)	9.286	14.857	14.182	16.250	18.544
k_1/exp. (+3) L/g. min = $(\partial v_1/\partial[S_T])$	1.020	1.282	1.431	2.294	-
k_{-1}/exp.(+3)min = $(K_M k_1 - k_{cat})$	9.788	4.252	2.080	0.958	Ξ
k_{-1}/exp.(+3)min (drp)	-	-	-	-	21.299
„ (rvd)	-	-	-	-	4.196
K_M/g/L (drp)	-	-	-	-	50.217
„ (rvd)	-	-	-	-	10.573
*k_{-1}/exp.(+3)min	1.184	1.625	2.961	7.843	-
SS cum PSS maximum k/ L/g. min.	8.060	-	-	6.170	-
SS cum PSS maximum k_{-1}/min	141.355	-	-	30.025	-

The plot of $(v_{max} - v)$ vs. $(v_{max} - 2v)/[ST]$ (Fig. not shown) gave, *k_{-1} for each $[E_T]$ according to Eq. (34c); Ξ represents results from further plots of the slope from the plot of $1/v$ vs. $1/[E_T]$ for each $[S_T]$ (Eq. (41)) vs. $1/[S_T]$ (akin to double reciprocal plot, drp) and reciprocal variant of direct (rvd) linear plot which gave widely different values. **Ñ** represents results from drp and rvp of $v/[E_T]$ vs. $[S_T]$. A drp plot using data generated according to method in the literature [27] and rvd gave respectively v_{max} (37683.235 μM/min) ≡ k_{cat} (19.595 k/min) and K_M (6.062 g/L) and 41958.04 μM/min ≡ k_{cat} (21.818 k/min) and K_M (8.224 g/L.). These are for the stock solution of the enzyme, whose concentration is 0.1g/L (≡ ≈ 1.923 μM). $[E_T]1 < [E_T]2 < [E_T]3 < [E_T]4$. k_1 values for each enzyme concentration was generated from the slope of the plot of v_1 (Table 2) vs. $[S_T]$. SS and PSS stand for steady-state and post steady-state respectively.

One may recall Eqs. (16), (17b), and (20b) for k_{-1}, v_{-1} and v_1, where, respectively, the values should be increasing as higher concentration of substrate is deployed or reached and where higher v for different concentration of the enzyme is the case. As may be applicable, the $[S_T]$ must be < K_M and $v < v_{max}$. The experiment is not designed for a situation whereby the highest $[E_T]$ should be < the lowest $[S_T]$ though, the former is < 7 →10 g/L of substrate, unlike other values of $[E_T]$. It needs to be stated that writing equation such as $v_1 = v_1 + v = k_1 [E_T] [S_T]$, does not necessarily imply total steady state, rather it is an approximation otherwise called assumption, because v may be infinitesimal, at the initial transient and cannot be ignored sometimes. Thus, this approximation may be applicable: $v_{-1} + v \approx k_1 [E_T] [S_T]$.

Given the values of v_1 in Table 2, the values of the 2nd order rate constants (k_1) for $[E_T]$ were graphically determined by plotting v_1 versus $[S_T]$ (Fig. 3). Expectedly, the values of k_1 showed an increasing trend with higher $[E_T]$. However, as shown in Table 3, the values of k_{-1} exhibited the opposite trend as a result of higher $[E_T]$, implying a trend towards a single turn-over event, which is characteristic of the high $[E_T]/[S_T]$ ratio frequently mentioned in the literature [19].

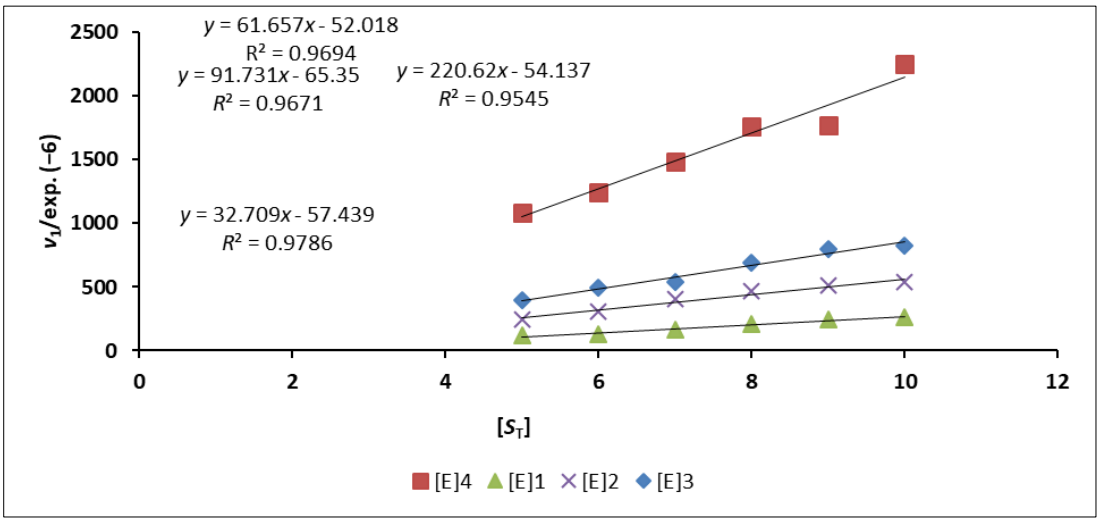


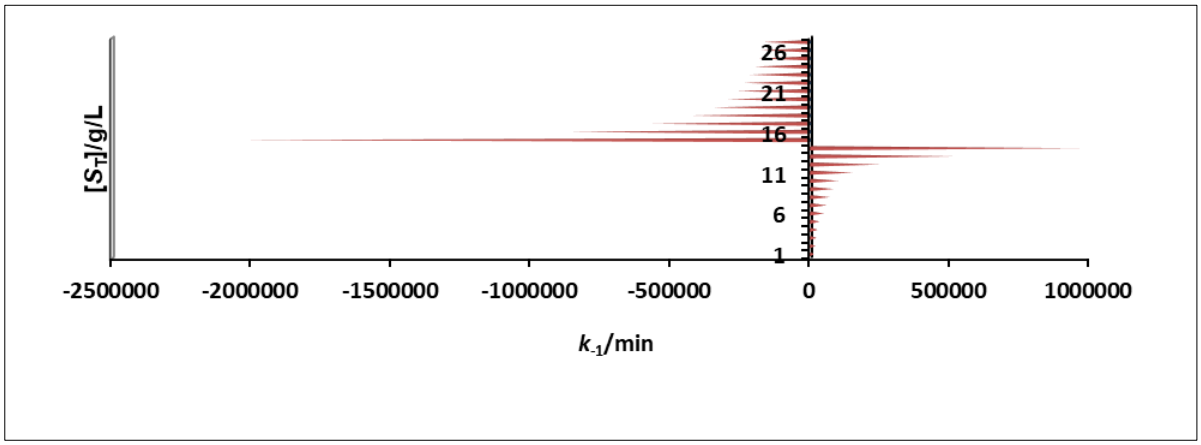
Figure 3 Plots for the determination of 2nd order rate constants from the slope ($\partial v_1 / \partial [S_T]$), being $\equiv k_1 [E_T]$ in the equation, $v_1 = k_1 [E_T] [S_T]$. The plot is for each concentration of the enzyme, $[E_T]_4$ (■), $[E_T]_3$ (▲), $[E_T]_2$ (×), and $[E_T]_1$ (◆)

Concern for k_{-1} has increased in the literature recently, but for different reasons, and other enzymes besides amylase are of interest [4-6]. It is necessary to always determine k_{-1} which can indirectly showcase the forward catalytic potential of any Michaelian enzyme given a clear-cut assay condition. In this regard, Eq. (6) [16] can facilitate the determination of k_1 and consequently enable the calculation of k_{-1} regardless of underlining assumptions. A situation where k_{-1} is either ≥ k_{cat}, does not suggest an encouraging catalytic efficiency, even if both parameters may be high.

In Table 3, can be found the results of a method not usually explored in the determination of catalytic rate. Here, the v values were plotted versus $[E_T]$ the slope of which represents the 1st order rate constant for each $[S_T]$. The slopes, $v/[E_T]$ versus $[S_T]$, were then plotted using the double reciprocal plot, drp, a reciprocal variant of the direct linear plot, rvd, yielding results comparable to those obtained by another method reported in the literature [27] for the enzyme stock solution. As usual, drp gave higher values of k_{cat} but surprisingly lower values of K_M compared with rvd. If the substrate concentration range is (or) $[E_T]$, this could be an effective and efficient method of directly measuring the enzymes' catalytic first order rate constant. This is against the backdrop of the view that the steady-state assumption can be valid for situations where $[E_T]/[S_T]$ 1[11] as long as $K_M > 1$, $K_s k_1/k_{cat} \gg 1$, though the concern is about the question of whether or not the enzyme can reach maximum velocity or saturation at slightly lower $[S_T]$ [26] in a "hyperbolic relationship between the rate of reaction and the substrate concentration". The approach and its results seem to support current interest and emphasis on catalytic efficiency and proficiency [7], made possible by k_{cat} and K_M both of which were determined as described (see Table 3); both (especially k_{cat}) are strictly the property of the enzyme for a given substrate; the v_{max} may vary in accordance with the concentration of the enzyme, but k_{cat} is always a constant. Specificity is a function of k_{cat}/K_M while the net turnover rate is a function of k_{cat} in any enzyme catalysed reaction pathway [26]. Besides being seen as the main steady kinetic parameters, k_{cat}/K_M is regarded as the most important because it quantifies enzyme specificity, efficiency, and proficiency [28].

As stated earlier, the decreasing values of k_{-1} calculated on the basis of the steady-state cum zero order condition or zonation equation are as a result of the higher concentration of the enzyme that needed a much higher concentration of

the substrate. It is well known that at much higher substrate concentrations, the rate of change of velocity as a function of substrate concentration variation becomes less rapid as it approaches asymptomatically a constant called the catalytic rate k_{cat}. At lower substrate concentrations, the velocity could be linearly proportional to the substrate and is regarded as a first order zone as against the zero orders that characterize the asymptotic trend towards v_{max} with higher substrate concentrations [7]. It is strongly believed that k_{cat}/K_M can be measured accurately in this first order zone, but little is mentioned about the reverse rate constant with regard to its implications for net catalytic efficiency. This research has generated k_{-1} values on the basis of Eq. (34c), regarded as one of the pre-steady-state equations. Thus, there should be an upper limit to the substrate concentration within the zone of the pre-steady-state beyond which any calculated result becomes invalid, as first illustrated in Fig. (4); to the right are "spikes" indicating a trend in the direction of increasing valid values of k_{-1} while to the left are the invalid increasing negative magnitude of k_{-1} as a function of $[S_T]$.



The valid values of k_{-1} are to the right of the ordinate while invalid values are to the left at much higher concentration of the substrate. Note of caution: The fig. does not present anything more than illustration with artistic attraction.

Figure 4 A plot showing the domain of valid values of reverse rate constants, k_{-1}, with increasing substrate concentrations which are < K_M

Rearranging Eq. (16) to give the equation below enables the calculation of the upper limit of the substrate concentration within the pre-steady-state domain. Thus,

$$[S_T] = \frac{K_M k_{-1}}{k_{cat} + k_{-1}} \tag{42}$$

The results obtainable from Eq. (42) are given in Table (1). It is doubtful if linearity can be extricated from transient state kinetics, which in most assays is relevant in the lower part of the substrate concentration range adopted for assay. While six (6) different $[S_T]$ were used for assay in this research, an additional 8 →10 $[S_T]$, including compulsorily the four lower concentrations at the lower range shown in Table 1, with an additional 4 → 6 much higher $[S_T]$, say 12 → 30 g/L is recommended; in this case, using the same concentration of enzyme in this research, substrate depletion must be avoided, and, so, the duration of the assay, which need not be transient, may just be 30 s. The fact that kinetic parameters, v_{max} or its equivalent k_{cat} and K_M appear in almost all the equations shows that Michaelian formalism cannot be ignored, and thus, regardless of any other emerging assumptions, reactant stationary assumption (RSA) [11], total QSSA (tQSSA)[29] *etc*, most of $[S_T]$ must be » any $[E_T]$ and the duration of the assay need not be more than 60 s. The assumption that all rate constants can be determined if Michaelis-Menten formalism is applicable is not far from the truth since as stated earlier, it is either Michaelian or kinetic parameters are known *ab initio* given well defined experimental conditions, or they need to be generated with suitable substrate concentration regime; additional evidence to this view is the result (Table 3) for $[E_T]$ = 3.20513 exp. (- 8), $[E_T]_1$, and 9.6154 exp. (- 8) M, $[E_T]_4$, concentrations used for illustration only. The results were obtained on the basis of Eq. (6) with other preliminary supporting calculations, all as described in the literature [16]. The values of k_{-1} and k for $[E_T]_1$ are those for $[E_T]_4$, all of which are » pre-steady-state values," all based on the same time-scale, the 3 minute duration of the assay.

On the issue of the usefulness or significance of this research, one can think of biochemical engineering, where rates are of paramount importance for the purpose of instrumental design, for instance. Sometimes there is a need to control the volume of product such that the forward catalytic efficiency defined as k_{cat}/K_M has to be compromised, giving way to an enhanced "backward catalytic efficiency" defined as k_{-1}/K_M as long as the relationship, $k_1 = k_{-1}/K_M + k_{cat}/K_M$ remains relevant. Holistic characterisation of an enzyme can guide medical and scientific decisions; cyclic nucleotide

phosphodiesterases (PDEs), for example, regulate cellular cyclic nucleotide levels. Pre-steady-state kinetic studies are an important method for fully characterising those enzymes.

Despites what seem to be conflicting opinions, "pre–steady-state kinetics aspiring to observe the burst phase would still use a high substrate/enzyme ratio" [7]; "in a pre-steady-state approach, high concentrations of enzyme are used so that a significant amount of product is formed during the first turnover [30]; and the use of low enzyme concentrations to make steady-state measurements" [30], this study demonstrated that regardless of the enzyme or substrate concentration regime, the $[E_T]/[S_T]$ ratio can be determined on any timescale with relevant assumption (s), though a 30-60 second assay duration may be appropriate to avoid substrate depletion. Once again, despite the contrasting views by the same author, *vis-à-vis* another variant of a view about the same parameter, K_M (seen as a steady-state constant [2]), yet, upon sQSSA treatment, an equilibrium dissociation constant which results, refers to K_M while at the same time, it is also taken to be a ratio of the parameters estimated at the zero-order zone and the 1st order zone [7], effort should be made to determine k_{-1}, having known, k.

Considering the events to the right and left of ES in Schemes 1 → 4, one easily sees ES as a species standing between two equilibrium events, such that to the left is k_{-1}/k_1 and to the right is k_{cat}/k_1, thereby giving the impression that K_M is a "mixed equilibrium constant," rather than a single equilibrium constant. A look at the following (schemes 1→4 notwithstanding) can better throw light on the issue: $E + S \rightleftharpoons ES \rightleftharpoons E^{\#}S^{\#} \rightarrow E + P$ where $E^{\#}S^{\#}$ stands for transient transition state. If the rate constant, k_3 for the process, $E^{\#}S^{\#} \rightarrow E + P$ is » the rate constant, k_{-2} for the process, $E^{\#}S^{\#} \rightarrow ES$, then, $E^{\#}S^{\#}$ must have a very transient existence with implication that, k_{cat} should be at least > k_{-1}. On the contrary, k_{-1} can even be » k_{cat} (Table 3) if, k_{-2} »k_3 as applicable to the lowest $[E_T]$. Again, the process $E^{\#}S^{\#} \rightarrow EP \rightarrow E + P$ may be a possibility if it is understood that there is no covalent bond formation between the enzyme and product, similar to what may be seen to be applicable to ES; in both, a physicochemical interaction and binding could be the case, before transition to $E^{\#}S^{\#}$ if the substrate tightly binds to the active site, overcoming perturbation or product inhibition if the product remains bound to the active site longer than necessary. Overall, emphasis should always be placed on k_{-1}/K_M and k_{cat}/K_M for an effective determination of the net catalytic efficiency of the enzyme. Indeed, k_{cat}/K_M taken as a single parameter is seen as one that quantifies enzyme specificity, efficiency, and proficiency [31]. If this be the case, steady-state, seen to be incapable of revealing mechanistic aspects of substrate conversion to product [32], remains an integral part of the reaction pathway, next to the zero order state; but if K_M is the $[S_T]$ at half maximal velocity, then, instead of K_M and v_{max} (or its preferred equivalent k_{cat}) being referred to as steady-state parameters, they should rather be seen as steady-state cum post steady-state (or zero order zonation) parameters shortly before the end of the zero order state/zone.

Hence, the argument that the low $[S_T]$ defines k_{cat}/K_M [26] seems objectionable if the assumption that when $[S_T] \ll K_M$, MM equation collapses to $v \approx v_{max}[S_T]/K_M$ (though K_M may no longer stand for its original definition, rather it becomes K_S, the equilibrium dissociation constant, k_{-1}/k_1 which stands); what may be shown in such a scenario is a burst phase cum first order state that does not satisfy the requirement for the attainment of maximum catalytic rate Higher substrate concentrations are only needed to extrapolate to get k_{cat} [26]. This, however, can be obtained by nonlinear regression, direct linear plot, *etc*. It takes substrate concentration nearly equal to K_M to signal the transition from steady-state to zero-order state.

Another highly prominent scholar [7] is of the opinion that the first-order zone enables the most accurate determination of the first fundamental steady-state kinetic rate constant k_{cat}/K_M. The concern regarding this "exposition" is that it seems the concept of steady-state, a relatively transient occurrence, has been used to define all kinetic parameters regardless of the zonation of the enzymatic reaction pathway; recall that in any plot of v versus $[S_T]$, what seems to have played out is that the place of K_M is ruled out if the plot is intended to be Michaelian, considering the denominator, ($K_M + [S_T]$), if the former remains unknown until determined by any means. The situation could have been described as one that serves the condition appropriate for rQSSA, if $[E_T] \gg [S_T]$ at the lower concentration range of the substrate. With absolute linearity, with $R^2 = 1$ consistently, for up to 3 → 5 different $[S_T]$, the expectation of first order would have been met. Therefore, the partial descriptive nomenclature for steady-state parameters, such as k_{cat}/K_M [7], is rather a misnomer. Rather, in such a situation, most of the vast population of enzyme molecules is free, such that $[E_T] \approx [E_F]$, where the latter is the concentration of free enzyme, any fraction of time within the duration of the assay. After the burst phase and the immediate 1st order zone, with a higher concentration of the substrate, a larger population of the enzyme forms ES, whose destruction is compensated for by reformation because there is always the availability of free enzyme and substrate within a short duration of the assay. This led to the assumption that $\partial[ES]/\partial t \approx 0$, the steady-state assumption; this led to what should be referred to as the Michaelis-Menten cum Brigs-Haldane equation, such that the parameters obtainable from the use of such an equation can be described as steady-state kinetic parameters. Next are the zero order zones in which virtually all the enzyme molecules have formed ES; dissociation into either E and S or P is the only means of creating free E; otherwise no free enzyme exists with an excess of the substrate molecules before dissociation, unlike in the pre-steady-state and steady-state zones.

Despite sQSSA treatment, an equilibrium dissociation constant which results, though, refers to K_M, while at the same time, it is also taken to be a ratio of the parameters estimated at the zero-order zone and the 1st order zone [7], effort should be made to determine k_{-1}, having known k_1. Indeed, the only valid steady-state parameter is the K_M which is in good agreement with Canela *et al.* preprint report [2]. Being $[S_T]$ at half maximum velocity, v_{max}, goes to show that the latter is only attained at concentrations > K_M. The ratio v $(K_M + [S_T])/[S_T]$ shows that v_{max} is greater than v. It may be necessary to establish the exact $[S_T]$ where v_{max} is attained. The linear transformation of the MM cum BH equation separated the v_{max} and K_M/v_{max}, though error-laden experimental variables (the value*s*) led to overestimates (Table 3) in most cases, being the outcome of the line of best fit. Only $[E_T]_4$ showed a departure from this overestimation tendency. The direct or the reciprocal variant of linear plots and nonlinear regression, though seen to generate more accurate parameters, may or may not give values that are slightly < the real value of v_{max} in particular. The concern that direct determination of catalytic efficiency, also known as the specificity constant [26], should always be the case was addressed in this study by plotting v values versus $[E_T]$ for each $[S_T]$ and then plotting the reciprocal of the slopes versus $1/[S_T]$. The reciprocal of the resulting slope gave k_{cat}/K_M; the reciprocal of the intercept (Fig 1b) gave the k_{cat}. The corresponding K_M was, therefore, calculated. As usual, the values of v_{max} and K_M from drp were slightly > values from rvd (Table 3), while the values of the specificity constant from drp were also slightly > values from rvd, as shown in Fig. 1b. In partial conclusion, in the light of the objectionable issue discussed earlier, Michaelian kinetic parameters, including specificity constant, should be named as steady-state cum zero-order kinetic constants.

5 Conclusion

Equations, not just for the determination of reverse rate constant only, where $[S_T] \ll K_M$, but also where $[S_T] > K_M$ were derived. In other words, pre-steady-state rate constants, 1st (reverse 1st order in particular and forward 2nd order rate constants) which cannot easily be determined where $[S_T] \gg [E_T]$ were possibly determined and consequently unmasked within the original MM cum BH mathematical formalism. Equations that emerged by derivation as corollaries possess a verifiable and reproducible capacity for reproducing both experimental variables and kinetic parameters; procedural issues were thus justified. Derived pre-steady-state equations give kinetic parameters that are < steady-state and beyond values. The determination of rate constants based on the original Michaelis–Menten cum Briggs and Haldane equation may not be impossible under a pre-steady-state setting with or without transient kinetics high-tech measurement equipment. All rate constants can be calculated outside of the Michaelis-Menten formalism. To avoid substrate depletion, a substrate concentration range of 3 or more concentrations $\ll K_M$ and those $\gg K_M$ with a short assay duration of 30 → 60 seconds is recommended. Reports on both negative and positive catalytic efficiency are preferred. Still, Michaelis-Menten kinetic parameters are indispensable for almost all purposes. Pre-steady-state, steady-state, and beyond implies that there are stages in enzyme catalysed pathway; therefore, specific case of steady-state before zero-order zone/state should be the subject of feature investigation.

Compliance with ethical standards

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There is no competing interest.

Author's Contributions

The author, UII conceived, designed, conducted the experiment, analysed, discussed, and wrote the entire paper.

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