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Higher precision in initial rates may be achievable: A test of a pseudo-statistical method

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Abstract

There has been a concerted effort at establishing the best method for the measurement of initial rates for various purposes, including the calculation of kinetic parameters, the maximum velocity (V_{max}), and the Michaelis-Menten constant (K_M). The objectives of this research are: 1) to derive equations without K_M for the determination of the V_{max} in particular and *vice versa*; 2) to determine the K_M and V_{max} with other equations other than the Michaelian equation; and 3) to subject the calculated and extrapolated kinetic parameters to pseudo-statistical remediation where necessary as a test of their viability and usefulness. The study was experimental and theoretical. It is supported by the Bernfeld method of enzyme assay. By graphical means, the V_{max} and K_M values for galactosidase respectively range between 163 and 185 μM/min and between 2.07 and 2.77 mg/L; the range by calculations is 177 and 214 μM/min and 2.45 and 3.311 mg/L, subject to pseudo-statistical remediation. Overall, the ranges of V_{max} and K_M values for alpha-amylase from both the graphical method and calculation are, respectively, 1.095 to 1.018 mM/min and 18.15 to 20.554 g/L. The equations for the determination of the K_M and V_{max}, which are respectively invariant with respect to each other, were rederived. The initial rates must not be characterised by a mixture of conditions that validate different quasi-steady-state assumptions if the true K_M and V_{max} are of interest. The new pseudo-statistical method for the remediation of error in all measurements, if necessary, is viable, useful, and robust.

Keywords: *Aspergillus oryzae* alpha-amylase (EC. 3.2.1.1); Beta-galactosidase (EC.3.2.1.23); Maximum velocity; Michaelis-Menten constant; Correctional mathematical methods; Pseudo-statistical Method

1. Introduction

For more than a century, scientists, the biochemist in the subfield of enzymology, and allied subjects have devoted much attention to the issue of Michaelian kinetic parameter measurement, first executed through the linear transformation of the "Michaelis-Menten [1] equation. However, the latter notwithstanding, Briggs and Haldane [2] played a pivotal role. Also Michaelis-Menten recognised the role of Henri V [3]. To this end, it would have been proper to name the equation the "Henri-Briggs-Haldane-Michaelis-Menten" (HBHMM) equation. A greater motivation for this coinage is reserved for the result and discussion sections. All the while, a hyperbolic curve relating the variation of initial rates with the corresponding concentrations of the substrate has been regularly observed. For the avoidance of doubts and conceptual misrepresentations, the initial rate in this research is defined as an experimental variable generated within a chosen duration of assay without substrate depletion; this is different from the rate generated within an infinitesimal time-scale prior to the pre-steady-state stage. This implies that the HBHMM model is, *ab initio,* a nonlinear equation, and therefore, it is unwarranted to expect a linear transformation to yield a perfect linear curve even if the data were perfectly generated, *i.e.,* the total absence of outliers being insinuated. Sometimes, either

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unknown to the researcher or due to indifference all or two (or more) [4], initial rates may be in a direct proportion to the concentration of the substrate such that the first initial rate (v_i) and its corresponding concentration of the substrate [S_T] are respectively half the next v_i and the corresponding [S_T]; any double reciprocal plot with the two or more v_i must create a small negative intercept [5].

According to Matyska and Ková [6], the concerns expressed by enzymologists and statisticians are that the variance σ^2 of raw experimental data is unknown in most enzymological practice since the experiments are conducted no more than twice, which is not sufficient for the determination of σ^2. It is therefore necessary to accept some assumptions about the value and structure of this variance in most real experiments. However, assumptions must be treated with strong reservation if applied science, medicine, or safety issues are involved. In a trial-and-error mode, a pseudo-weighting method was developed to bring the raw data much closer to perfection, given a set of rules in place. The concern for the elimination of error has expression in the use of equations such as:

$$v_i = \frac{V_{max}[S_I]_i}{K_M+[S_I]_i} + e_i \quad (1)$$

Equation (1) is nothing but the HBHMM equation with an error function, where, as usual, v_i, V_{max}, K_M, [S_T], and e_i are the initial reaction rates obtained from steady-state experiments, the maximum reaction rate, the Michaelis-Menten (MM) constant, and random error components. It was not certain how e_i can be measured.

The best methods of estimating kinetic parameters are, according to Matyska and Kovář [6], the jack-knife Marquardt methods, all of which require a step-by-step approach for adequate comprehension by less gifted scholars in statistics. While V_{max} and K_M can be calculated as intercept and slope from the straight line obtained in a plot of [P]/t vs. ln(1-[P]/[S]t)/t, the procedure cannot give statistically reliable values of the parameters because the errors associated with [P] appear in both the dependent and the independent variable [7]. Thus, most investigations by investigators many years ago [7, 8] were tailored towards the determination of statistical methods for estimating the MM kinetic parameters. The method of least squares gained acceptance with time but gave poor results in the absence of correct weighting, though "bi-weight" regression appeared to be a better option if applied to MM kinetics [9]. This was in response to the failure of almost every linear transformation model to give parameters that are substantially free from errors. This further gave rise to alternative linear transformations: the direct linear transformation model popularised by several researchers [9, 10] and the reciprocal variant [11].

Most, if not all, statistical approaches need statistical packages with which to improve the quality of parameters. If they care, the users of such packages need to be aware of the statistical limitations or validity of the weighting routines incorporated into commercially available packages. Perhaps a good example is the R package by Aledo [4]. With the availability of software packages [4, 12], nonlinear regression took centre stage in all attempts to generate reliable Michaelian parameters. Whichever method, a number of substrate concentrations not less than six is required (eight and above is much better) for enzyme assay. This study is therefore, aimed at ways of achieving a higher precision of initial rates given a pseudo-statistical method. On account of the myriad of reservations expressed against various methods, linear transformation in particular, for the estimation of Michaelian kinetic parameters, the objectives of this research are: 1) to derive equations without K_M for the determination of the V_{max} in particular and *vice versa*; 2) to determine the K_M and V_{max} with other equations other than the HBHMM equation; and 3) to subject the calculated and extrapolated kinetic parameters to the pseudo-statistical remediation where necessary as a test of its viability and usefulness.

1.1. Significance of study

The subjecting of initial rates to a mathematical analysis in order to identify potential sources of errors that could compromise the quality of the result of the study is very useful; the errors such as direct proportionality between initial rates and the corresponding concentration of substrate leading to negative intercepts in double reciprocal plots suggest an incidence of conditions that justify reverse quasi-steady-state approximation (QSSA), though the condition that validates standard QSSA (sQSSA) is the intention. Further progress demands correctional treatment in line with the methods enunciated in addition to the pseudo-statistical remediation method derived and applied in this research. Fewer replications with concomitant savings in time and material could be an added advantage.

1.2. Theory

Partial reviews of the derivation in a posted pre-print [13] and directly from the usual Michaelis-Menten equation are, respectively:

$$v = \frac{v_{\max}\left([S]K_{\mathrm{M}} - [S]^2\right)}{K_{\mathrm{M}}^2 - [S]^2} \tag{2}$$

$$K_{\mathrm{M}} = \frac{[S]_{\mathrm{i}}[S]_{\mathrm{j}}(v_{\mathrm{j}} - v_{\mathrm{i}})}{[S]_{\mathrm{j}}v_1 - [S]_{\mathrm{i}}v_{\mathrm{j}}} \tag{3}$$

Equations (2) and (3) being general equations lead to the following:

$$K_{\mathrm{M}} = \frac{[S]_{\mathrm{i}}[S]_2(v_2 - v_{\mathrm{i}})}{[S]_2 v_{\mathrm{i}} - [S]_{\mathrm{i}}v_2} \tag{4a}$$

In Eq. (4a), *i* stands for the values of the initial rate and the corresponding concentration of the substrate between the first and the $(n-1)^{\text{th}}$ sample. The second equation is:

$$K_{\mathrm{M}} = \frac{[S]_1[S]_{\mathrm{n}}(v_{\mathrm{n}} - v_1)}{[S]_{\mathrm{n}}v_1 - [S]_1 v_{\mathrm{n}}} \tag{4b}$$

where *i* in the former equation, Eq. (4b), is, in this case, always referring to the first (number 1) initial rate and the first concentration of the substrate, *n* (this could be between 2 and ∞) is always the number of the sample.

$$K_{\mathrm{M}}^2 = \frac{v_{\max}[S_{\mathrm{T}}]_1 K_{\mathrm{M}}}{v_1} - \frac{v_{\max}[S_{\mathrm{T}}]_1^2}{v_1} + \frac{v_1[S_{\mathrm{T}}]_1^2}{v_1} \tag{5}$$

$$K_{\mathrm{M}}^2 = \frac{v_{\max}[S_{\mathrm{T}}]_2 K_{\mathrm{M}}}{v_2} - \frac{v_{\max}[S_{\mathrm{T}}]_2^2}{v_2} + \frac{v_2[S_{\mathrm{T}}]_2^2}{v_2} \tag{6}$$

Equations (5) and (6) are the same, so,

$$\frac{v_{\max}[S_{\mathrm{T}}]_2 K_{\mathrm{M}}}{v_2} - \frac{v_{\max}[S_{\mathrm{T}}]_2^2}{v_2} + \frac{v_2[S_{\mathrm{T}}]_2^2}{v_2} = \frac{v_{\max}K_{\mathrm{M}}}{v_1} - \frac{v_{\max}[S_{\mathrm{T}}]_1^2}{v_1} + \frac{v_1[S_{\mathrm{T}}]_1^2}{v_1} \tag{7a}$$

Rearrangement of Eq. (7a) gives:

$$\frac{([S_{\mathrm{T}}]_2^2 v_1 - v_2[S_{\mathrm{T}}]_1^2)v_{\max}}{v_2 v_1} = v_{\max}K_{\mathrm{M}}\frac{[S_{\mathrm{T}}]_2 v_1 - [S_{\mathrm{T}}]_1 v_2}{v_1 v_2} + [S_{\mathrm{T}}]_2^2 - [S_{\mathrm{T}}]_1^2 \tag{7b}$$

Rearrangement of Eq. (7b) gives:

$$v_{\max}K_{\mathrm{M}} = \frac{([S_{\mathrm{T}}]_2^2 v_1 - v_2[S_{\mathrm{T}}]_1^2)\, v_{\max}}{[S_{\mathrm{T}}]_2 v_1 - [S_{\mathrm{T}}]_1 v_2} - \frac{v_2 v_1([S_{\mathrm{T}}]_2^2 - [S_{\mathrm{T}}]_1^2)}{[S_{\mathrm{T}}]_2 v_1 - [S_{\mathrm{T}}]_1 v_2} \tag{7c}$$

Equation (4) can now be substituted into Eq. (7c), to give after rearrangement the following:

$$V_{\max}\frac{[S]_1[S]_2(v_2 - v_1)}{[S]_2 v_1 - [S]_1 v_2} = \frac{([S_{\mathrm{T}}]_2^2 v_1 - v_2[S_{\mathrm{T}}]_1^2)\, v_{\max}}{[S_{\mathrm{T}}]_2 v_1 - [S_{\mathrm{T}}]_1 v_2} - \frac{v_2 v_1([S_{\mathrm{T}}]_2^2 - [S_{\mathrm{T}}]_1^2)}{[S_{\mathrm{T}}]_2 v_1 - [S_{\mathrm{T}}]_1 v_2} \tag{8}$$

Cancellation of common term or factor and rearrangement gives:

$$V_{\max} = \frac{v_2 v_1([S_{\mathrm{T}}]_1^2 - [S_{\mathrm{T}}]_2^2)}{[S_{\mathrm{T}}]_1[S]_2(v_2 - v_1) - [S_{\mathrm{T}}]_2^2 v_1 + v_2[S_{\mathrm{T}}]_1^2} \tag{9a}$$

Equation (9a) clearly shows how the $V_{\max}$ depends on a two-substrate concentration product in both the denominator and the nominator for its calculation. A general equation that should be applied after adjustment in the kinetic variables, following the appropriate equation (s) given in this research, is:

$$V_{\max} = \frac{v_{\mathrm{n}}v_{((\mathrm{n}-1))}\left([S_{\mathrm{T}}]_{((\mathrm{n}-1))}^2 - [S_{\mathrm{T}}]_{\mathrm{n}}^2\right)}{[S_{\mathrm{T}}]_{((\mathrm{n}-1))}[S]_{\mathrm{n}}\left(v_{\mathrm{n}} - v_{((\mathrm{n}-1))}\right) - [S_{\mathrm{T}}]_{\mathrm{n}}^2 v_{((\mathrm{n}-1))} + v_{\mathrm{n}}[S_{\mathrm{T}}]_{((\mathrm{n}-1))}^2} \tag{9b}$$

The calculation should cover the variables, v_1, v_2 …, and $v_{\mathrm{n}-1}$ and the corresponding substrate concentrations, $[S]_1$, $[S]_2$ …, and $[S]_{\mathrm{n}-1}$. The n^{th} variable should be used consistently till the $\mathrm{n}^{\text{th}}-1$ variable is reached. When the kinetic variables are almost perfectly generated or measured, using high precision equipment, any of the equations for $V_{\max}$ can be used

for its calculation. However preliminary investigation in this research has shown that it is better to adopt Eq. (9a) or equivalent equation in the literature [13] but stated herein shortly because, such enables the earlier disclosure of un-Michaelian trend whereby $[S_T]_n v_{n-1} - [S_T]_{n-1} v_n$ is either negative or zero. Since, in this research, details and a step-by-step approach are matters of policy rather than haste and convenience, another general equation is hereby given as follows:

$$V_{max} = \frac{v_i v_n ([S]_n - [S]_i)}{[S]_n v_i - [S]_i v_n} \tag{9c}$$

In Eq. (9c) *i* stands for the values of the initial rate and the corresponding concentration of the substrate between the first and the $(n-1)^{th}$ sample. Equation (9c) needs to be used to check the first three data. The second equation can take the form:

$$V_{max} = \frac{v_1 v_n ([S]_n - [S]_1)}{[S]_n v_1 - [S]_1 v_n} \tag{9d}$$

where *i* in the former equation, Eq. (9c) is, in this case, always refers to the first initial rate and the first concentration of the substrate; *n* (this could be between 2 and ∞) is always the number of sample as stated earlier.

The corresponding equation of K_M is derived as follows. Given the equation in the literature [13], written as below, one can derive the corresponding equation of K_M as follows:

$$v_{max} = \frac{v_1 v_2 ([S]_2 - [S]_1)}{[S]_2 v_1 - [S]_1 v_2} \tag{10}$$

Equation (10) can be substituted into Eq. (7c) to give:

$$\frac{v_1 v_2 ([S]_2 - [S]_1)}{[S]_2 v_1 - [S]_1 v_2} K_M = \frac{([S_T]_2^2 v_1 - v_2 [S_T]_1^2) \frac{v_1 v_2 ([S]_2 - [S]_1)}{[S]_2 v_1 - [S]_1 v_2}}{[S_T]_2 v_1 - [S_T]_1 v_2} - \frac{v_2 v_1 ([S_T]_2^2 - [S_T]_1^2)}{[S_T]_2 v_1 - [S_T]_1 v_2} \tag{11a}$$

Cancellation of common factors and rearrangement gives:

$$K_M = \frac{([S_T]_1 [S_T]_2^2 - [S_T]_2 [S_T]_1^2)(v_2 - v_1)}{([S_T]_2 - [S_T]_1)([S_T]_2 v_1 - [S_T]_1 v_2)} \tag{11b}$$

Equation (11b) gives exactly the same results when fitted to kinetic variables and substrate concentrations as it is in earlier derivation in the literature [13]. Here the approach partially evaded the direct use of Michaelis-Menten equation, but reaffirmed the procedural validity now and in the past [13]. Again, the general form of Eq. (11b) is given as:

$$K_M = \frac{\left([S_T]_{(1\to(n-1))} [S_T]_n^2 - [S_T]_n [S_T]_{(1\to(n-1))}^2\right)(v_n - v_{(1\to(n-1))})}{([S_T]_n - [S_T]_{(1\to(n-1))1})([S_T]_n v_{(1\to(n-1))} - [S_T]_{(1\to(n-1))} v_n)} \tag{11c}$$

The second possibility is that, if Eq. (9b) is substituted into original Michaelis-Menten equation one gets the equation for K_M without V_{max} given, after simple steps, as:

$$K_M = \frac{v_2 [S_T]_1 ([S_T]_2 - [S_T]_1) - [S_T]_1 ([S_T]_2 v_1 - [S_T]_1 v_2)}{[S_T]_2 v_1 - [S_T]_1 v_2} \tag{12}$$

What must not be ignored is that, be it linear regression or nonlinear regression, the curve follows the line of best-fit in order to generate kinetic parameters in which the effect of outliers is minimised on the basis of compromise rather than rectification. Therefore, the parameter generated cannot be seen as being entirely dependent on the original experimental data laden with errors. If there are reasons for the use of the experimental variables obtained from the experiment, then the parameters should be substituted into the original equation, the Michaelis-Menten equation, for instance, in order to calculate those variables, such as velocities corresponding to the measured substrate concentrations, assuming that the measurement was error-free. Alternatively, the substrate concentrations need to be calculated because, peradventure, there may have been inaccurate pipetting of the solution or a mixture of insoluble substrate and solvent. In all these cases, the pipetting of the enzyme solution may be considered error-free. At this juncture, there is a need to point out that something similar, but with a minor difference, in the overall "structure" of the equation is available in the literature, perhaps for the inhibition case [14]. There has always been criticism against

any form of regression; surprisingly, nonlinear least squares fitting technique is included [12] despite the application of software. Indeed, software seems unable to correct errors.

2. Materials and methods

2.1. Materials

Aspergillus oryzae alpha-amylase (EC 3.2.1.1) and insoluble 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. An electronic weighing machine was purchased from Wenser Weighing Scale Limited and 721/722 visible spectrophotometer was purchased from Spectrum Instruments, China; pH meter was purchased from Hanna Instruments, Italy.

2.2. Methods

The enzyme was assayed according to the Bernfeld method [15] using gelatinised potato starch. Reducing sugar produced upon hydrolysis of the substrate using maltose as a standard was determined at 540 nm with an extinction coefficient equal to 181 L/mol.cm. A concentration equal to 1 g/100 mL of potato starch was gelatinised at 100 °C for 3 min and subjected to serial dilution after making up for the loss of moisture due to evaporation to give concentrations ranging between 4 and 10 g/L for the assay in which $[S_T] \gg [E_T]$. A concentration of 0.01 g/100 mL of *Aspergillus oryzae* alpha-amylase was prepared by dissolving 0.01 g of the enzyme (as the stock) in 100 mL of Tris-HCl buffer at pH = 6.9. The assay of the enzyme was carried out with an enzyme concentration of 1 mg/L. The duration of the assay was 3 minutes at 20 °C.

The determination of K_M is according to Eqs (4, 13). The V_{max} was obtained by fitting the Eq. (10) to the unweighted velocity data in this experiment and in the literature [4]. Equations (11b), (12), and (9) were left out because of a time constraint; otherwise, the same result is expected using either Eq. (4) or Eq. (10), as the case may be. The equations ($v_1 \rightarrow v_3$) [13] and those derived in this research ($v_4 \rightarrow v_9$) used to correct the variables, the velocities (v_1, v_2, v_3, v_4, v_5, v_6, v_7, v_8, and v_9) of enzymatic action, are:

$$v_1 = \frac{v_2 v_3 [S_T]_1^2([S_T]_3 - [S_T]_2)}{[S_T]_1[S_T]_2[S_T]_3(v_3 - v_2) + [S_T]_1^2(v_2[S_T]_3 - v_3[S_T]_2)} \quad (13)$$

$$v_2 = \frac{([S_T]_1[S_T]_2[S_T]_3 - [S_T]_1^2[S_T]_2)\, v_1 v_3}{[S_T]_1^2 v_3([S_T]_3 - [S_T]_2) + v_1([S_T]_1[S_T]_2[S_T]_3 - [S_T]_1^2[S_T]_3)} \quad (14)$$

$$v_3 = \frac{([S_T]_1[S_T]_2[S_T]_3 - [S_T]_1^2[S_T]_3)v_1 v_2}{[v_1([S_T]_1[S_T]_2[S_T]_3 - [S_T]_1^2[S_T]_2) - [S_T]_1^2 v_2([S_T]_3 - [S_T]_2)} \quad (15)$$

$$v_4 = \frac{([S_T]_1[S_T]_4[S_T]_5 - [S_T]_1^2[S_T]_4)\, v_1 v_5}{[S_T]_1^2[S_T]_5(v_5 - v_1) + [S_T]_1[S_T]_4([S_T]_5 v_1 - [S_T]_1 v_5)} \quad (16)$$

$$v_5 = \frac{([S_T]_1^2[S_T]_5 - [S_T]_1[S_T]_4[S_T]_5)\, v_1 v_4}{[S_T]_1^2([S_T]_5 - [S_T]_4)v_4 - ([S_T]_1[S_T]_4[S_T]_5 - [S_T]_1^2[S_T]_4)v_1} \quad (17)$$

$$v_6 = \frac{([S_T]_1^2[S_T]_6 - [S_T]_1[S_T]_5[S_T]_6)\, v_1 v_5}{[S_T]_1^2([S_T]_6 - [S_T]_5)v_5 - ([S_T]_1[S_T]_5[S_T]_6 - [S_T]_1^2[S_T]_5)v_1} \quad (18)$$

$$v_7 = \frac{([S_T]_1^2[S_T]_7 - [S_T]_1[S_T]_6[S_T]_7)\, v_1 v_6}{[S_T]_1^2([S_T]_7 - [S_T]_6)\, v_6 - ([S_T]_1[S_T]_6[S_T]_7 - [S_T]_1^2[S_T]_6)\, v_1} \quad (19)$$

$$v_8 = \frac{([S_T]_1^2[S_T]_8 - [S_T]_1[S_T]_7[S_T]_8)\, v_1 v_7}{[S_T]_1^2([S_T]_8 - [S_T]_5)\, v_7 - ([S_T]_1[S_T]_7[S_T]_8 - [S_T]_1^2[S_T]_7)\, v_1} \quad (20)$$

$$v_9 = \frac{([S_T]_1^2[S_T]_9 - [S_T]_1[S_T]_8[S_T]_9)\, v_1 v_8}{[S_T]_1^2([S_T]_9 - [S_T]_5)\, v_8 - ([S_T]_1[S_T]_8[S_T]_9 - [S_T]_1^2[S_T]_8)\, v_1} \quad (21)$$

The graphing approaches were a double reciprocal plot and a plot based on Eq. (4) for K_M and on Eq. (10) for V_{max}, where respectively, the *x*-axis is taken as $f([S], v)$ and the *y*-axis is taken as $f([S]^2, v)$, and $f([S], v)$ and $f(v^2, [S])$.

2.3. Statistics

Duplicate assays for each substrate were deliberately adopted in this research, not just to reduce time and cost but also to serve as a preliminary test of the mathematical equations derived so as to verify robustness and consistency. As in the previous publication [13], the pseudo-weighting factors for the products and substrates are given in a summarised version as follows:

$$\beta_p \equiv \frac{v_1}{v_2} + \frac{v_2}{v_3} + \cdots + \frac{v_{(n-1)}}{v_n} \tag{22}$$

The pseudo-weighting factor for the substrate is given as:

$$\beta_s \equiv \frac{[S_T]_1}{[S_T]_2} + \frac{[S_T]_2}{[S_T]_3} + \cdots + \frac{[S_T]_{(n-1)}}{[S_T]_n} \tag{23}$$

The coefficients, β_s **and** β_p, are taken to be a weighting factor for the fractional contribution of each substrate and each product to the excess (or, generally speaking, the error) observed in the summation results. The summation equations for the V_{max} and K_M are:

$$\sum_2^n V^*_{max} = \frac{v_2 v_1([S_T]_2 - [S_T]_1)}{[S_T]_2[S_T]_1 v_1 - [S_T]_1^2 v_2} + \frac{v_3 v_1([S_T]_3 - [S_T]_1)}{[S_T]_3[S_T]_1 v_1 - [S_T]_1^2 v_3} \ldots + \frac{v_{n-1} v_1([S_T]_{n-1} - [S_T]_1)}{[S_T]_{n-1}[S_T]_1 v_1 - [S_T]_1^2 v_{n-1}} \tag{24}$$

where, v_i is the original velocity of enzymatic action without weighting or any treatment, and, n is the total number of different concentrations of the substrate; i = 1, 2, 3, …

$$\sum_2^n K^*_M = \frac{v_2[S_T]_1([S_T]_2 - [S_T]_1) - [S_T]_1([S_T]_2 v_1 - [S_T]_1 v_2)}{[S_T]_2 v_1 - [S_T]_1 v_2} + \frac{v_3[S_T]_1([S_T]_3 - [S_T]_1) - [S_T]_1([S_T]_3 v_1 - [S_T]_1 v_3)}{[S_T]_3 v_1 - [S_T]_1 v_3} + \cdots + \tag{25}$$

where, $[S_T]_i$ is the original concentration of the substrate and, n is the total number of different concentrations of the substrate; i = 1, 2, 3, …

The mathematically and pseudo-statistically determined V_{max}, $V_{max(p\text{-}stat)}$, is [13]:

$$V_{max_{(p-stat)}} = \sum_2^n V^*_{max} \left(1 - \frac{1}{\beta_p\left[(n-1)\beta_p\right]^{1/(n-1)}}\right) \Big/ (n-1) \tag{26}$$

The corresponding K_M is:

$$K_{M_{(p-stat)}} = \sum_2^n K^*_M \left(1 - \frac{1}{\beta_s\left[(n-1)\beta_s\right]^{1/(n-1)}}\right) \Big/ (n-1) \tag{27}$$

The arithmetic means (AV) are:

$$V_{max}(AV) = \sum_2^n V^*_{max}/(n-1) \tag{28}$$

$$K_M(AV) = \sum_2^n K^*_M/(n-1) \tag{29}$$

$$V_{max_{(p-stat)}} = \sum_1^n V^*_{max} \left(1 - \frac{1}{\beta_p\left[n\,\beta_p\right]^{1/n}}\right) \Big/ n \tag{30}$$

$$K_{M_{(p-stat)}} = \sum_1^n K^*_M \left(1 - \frac{1}{\beta_s\left[n\,\beta_s\right]^{1/n}}\right) \Big/ n \tag{31}$$

$$V_{max}(AV) = \sum_1^n V^*_{max}/n \tag{32}$$

$$K_{\mathrm{M}}(AV) = \sum_1^n K_{\mathrm{M}}^*)/n \quad (33)$$

Equations (30), (31), (32), and (33) apply where double reciprocal plots are explored for the determination of the kinetic parameters. Standard deviations (SD) were calculated using Microsoft Excel with different sample numbers (n) for each parameter for different enzymes; values are reported as mean ± SD.

3. Results and discussion

This section is best introduced with an overview of the equations derived in this research. Separate different equations for the calculation of K_{M} and V_{max} that give the same results are an expression of robustness and consistency, and most importantly, the validity of a procedural issue. To accomplish the goal of validity, the equations had to be evaluated by graphical means, beginning with the double reciprocal plot and then, ten plots based on some of the derived equations (Figures 1 → 11). The double reciprocal plots, otherwise called Lineweaver-Burk plots (LWB) [16], using the un-weighted (UNW) and recalculated (RC) initial rates (Table 1), showed that the result in the literature [4], if mistakes are excluded, is far higher than the results shown in Figure 1 and Table 2.

Table 1 Unweighted and recalculated initial rates or velocities

SN.	Beta-galactosidase (EC.3.2.1.23)		*Aspergillus oryzae* alpha amylase (EC. 3.2.1.1)	
1	UNW velocities data/ μM/min [4]	RCV data/μM/min	Data given as arithmetic mean of each UNW velocity /μM/min	RCV data/μM/min
2	3	3.122449127	171.15	177.503376
3	6	6.181818182	219.05	215.108142
5	17	15	259.55	250.4857109
6	48	27.54545455	285.25	281.430301
7	101	90.3919266	311.85	311.862502
8	121	126.457077	329.45	355.606156
9	139	148.706315	-	-
10	152	180.4739919	-	-
11	181	189.4642622	-	-

UNW stands for un-weighted velocity. For the benefit of convenience, the substrate, o-nitrophenyl-β-D-galactopyranoside (ONPG) concentrations (mM) [4] are: 0.05, 0.1, 0.25, 0.5, 2.5, 5, 8, 20, and 30. Gelatinised insoluble potato starch concentrations (g/L) used for the assay are: 4, 5, 6, 7, 8, and 10. Mean of two determinations was the case for this research as applicable to *A. oryzae* alpha-amylase.

The Figures are deliberately included for immediate visual examination of issues observed or raised; hence, the tables remain complimentary rather than of procedural importance. With the LWB plot, the results (Table 2) were compared as follows: The literature on UNW initial rates [4] with partly RC initial rates plus UNW initial rates (this research) gave kinetic parameters that were greater than those given by fully RC initial rates (this research), with correlation coefficients, R, ranging between 0.998 and 1 (Figure 1). The reported results [4], based on software-assisted nonlinear regression, the V_{max} and K_{M}, were less than what was observed in this research, where values were generated

graphically by LWB plots and other plots based on derived equations. The LWB plot for *A. oryzae* was not shown, but the results are shown in Table (2).

Table 2 Michaelian parameters determined according to Lineweaver-Burk method with data in the literature as applicable to EC.3.2.1.23 [4] and in this research (EC 3.2.1.1).

SN.	**Beta-galactosidase (EC.3.2.1.23)**		***Aspergillus oryzae* alpha amylase (EC. 3.2.1.1)**	
1	V_{max} using UNW/μM/min	833.333	*Av* V_{max} using UNW /mM min	1.166 (1.018)
2	K_M using UNW/mM	13.75	av K_M using UNW/g/L	22.323 (18.346)
3	V_{max} using RCV($v_1 \rightarrow v_4$) & UNW($v_5 \rightarrow v_9$)/μM/min	243.902 (214.097)	V_{max} using RCV($v_1 \rightarrow v_6$)/mm/min	1.164 (1.016)
4	K_M using RCV($v_1 \rightarrow v_4$) & UNW($v_5 \rightarrow v_9$)/mM	4 (3.311)	K_M using RCV($v_1 \rightarrow v_6$)/g/L	22.090 (18.154)
5	V_{max} using RCV ($v_1 \rightarrow v_9$)/μM/min	217.391 (190.674)	-	-
6	K_M using RCV ($v_1 \rightarrow v_9$)/mM	3.435 (2.844)	-	-

UNW and RCV stand for unweighted and recalculated velocities of enzymatic action, respectively. The average of duplicate values of V_{max} and the average of duplicate values of K_M were taken from two plots. All values in brackets are outcomes of the pseudo-statistical treatment of the kinetic parameters generated from both raw and recalculated or corrected initial rates. The pseudo-statistical factors defined by Eqs (26) and (27) are 0.877791 and 0.8278, respectively, for beta-galactosidase, and 0.872989 and 0.821833, respectively, for alpha-amylase.

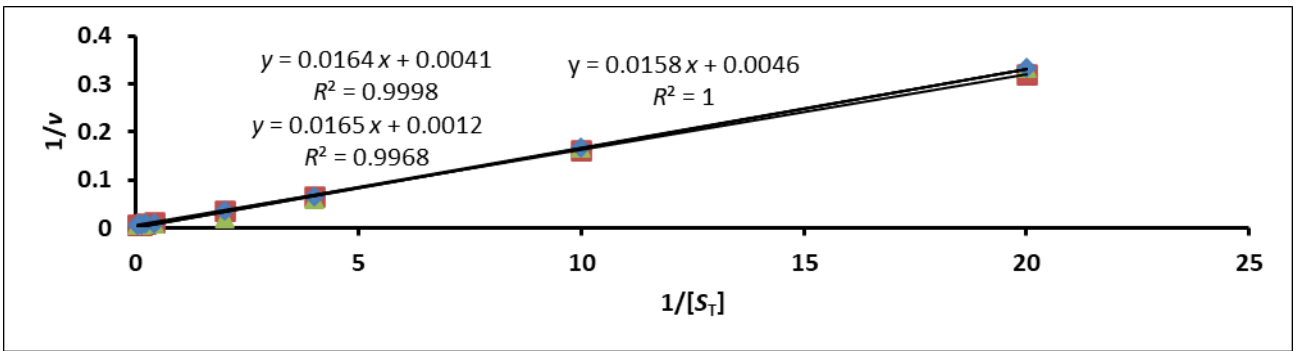

Figure 1 Double reciprocal plot, Lineweaver-Burk plot, using directly original (▲) data (unweighted) in the literature [4] for comparative and confirmation/validation purposes. Other legends are partially recalculated variables (◆), $v_1 \rightarrow v_4$, and totally recalculated variables (■), $v_1 \rightarrow v_9$. Relevant equations in this research were fitted to the unweighted data for the purpose of recalculating each velocity, the initial rate, v_i of enzymatic action as may be applicable. The V_{max} and K_M for the unweighted v_i are respectively 833.333 μM/min and 13.03 g/L; for the partly corrected vi the values are respectively 243.9 μM/min and 4 g/L; for the totally corrected v_i the values are respectively 217.391 μM/min and 3.435 g/L.

Calculated kinetic parameters based on the derived equations are shown in Table 3. It needs to be made clear that those Michaelian parameters (to be emphatic), K_M and V_{max}, are functions of total substrate concentration [S_T] and velocity, v_i of enzymatic action. Hence, the much-discussed transient assays must not only be in terms of time scale; they must also take into account the substrate concentration regime if Michaelian kinetics is in view. If [S_T] range $\ll K_M$ and [S_T] $\ll$ [E_T] (total enzyme concentration), the Michaelian formalism (sQSSA) ceases to be relevant, becoming more of a case of rQSSA. In this case, the v_i becomes directly proportional to [S_T]. Under such circumstances, Eqs (4a), (9a), (10), (11b), (12), *etc.* become invalid if intended for the calculation of K_M or V_{max}, as the case may be. In a perfect direct proportionality, $[S_T]_n v_{n-1} - [S_T]_{n-1} v_n = 0$, as observed with unweighted data in the literature (see footnote under Table 3); one can even insinuate that $[S_T]_n v_{n-1} - [S_T]_{n-1} v_n < 0$ is better than a zero outcome because at least a negative value

of the kinetic parameter would have been achieved, as observed in this research and recorded under Table 3 as a footnote. Both are emphatically invalid. However, such a possibility cannot be ruled out if a single bond substrate is the case, as is applicable to disaccharides and perhaps o-nitrophenyl-β-D-galacto-pyranoside [4]. In general, this may be the case where $[S_T]$ « $[E_T]$ and $[S_T]$ « K_M such that v_i remains directly proportional to $[S_T]$ for at least up to five different $[S_T]$. In such situation, $v/[S_T]$ or $[S_T]/v$ for up to five different $[S_T]$ must be constant. But this situation may not be in line with Michaelian kinetics.

Table 3 Michaelian parameters, determined by fitting relevant equations in this research to data in the literature (with respect to EC 3.2.1.23 [4]), and in this research (with respect to EC 3.2.1.1).

SN.	**Beta-galactosidase (EC 3.2.1.23)**		***Aspergillus oryzae* alpha amylase (EC 3.2.1.1)**	
1	$V_{max\text{-}p\text{-}s}$ (RCV)/μM/min	177.133±30.565	$V_{max\text{-}p\text{-}s}$ (RCV)μM/min	1095.832±39.032
2	Average (n = 8)/μM/min	201.794±34.821	Average(n=5) /μM/min	1255.270±44.711
3	$K_{M\text{-}p\text{-}s}$ (RCV) /mM	2.446±0.438	$K_{M\text{-}p\text{-}s}$ (RCV)/g/L	20.554±2.318
4	Average (n = 8)/mM	2.955±0.529	Average (n=5) */g/L*	25.081±2.821

The sample size, otherwise referred to as the number of different $[S_T]$ and consequently the number of different velocities (*vs.*), is 9 for b-galactosidase and 6 for *A. oryzae* alpha amylase; the effective sample size is, however, 8 and 5, respectively, based on the number of times V_{max} and K_M were calculated as described. The total V_{max} and K_M were subjected to pseudo-statistical treatment and adjustment according to Eqs (17) and (18), respectively, which are intended to eliminate excess contributions to the kinetic parameters due to error(s), if there was/were any. With literature [4] values of 3 and 6 μM/min per $[S_T]_1$ and $[S_T]_2$, respectively, $[S_T]_2\, v_1 - [S_T]_1\, v_2 = 0$, while in this research, it is = 0.02045 mMg/min.L The subscript p-s is the abbreviated form of p-stat, the pseudo-statistically adjusted parameter.

There is a very strong point in emphasising the need to examine the accuracy of the measured and the experimentally generated variables, v_1, v_2, and v_3, in particular. To achieve this, more specific equations such as Eq. (4a), Eq. (9c), Eq. (12), and Eq. (10), can be used. In all, $[S_T]_2 v_1 - [S_T]_1\, v_2$ must not yield a negative or zero value. A better value must be greater than 1. The results as quantitative values were obtained first by a double reciprocal plot (Figure 1) using data from the literature [4]. Both the raw initial rate data and the corrected version in the literature [4] and in this research are shown in Table 1. All results show that the raw (unweighted) data overestimated kinetic parameters due to the doubling of v_i with $[S_T]_2$, which is also twice the first substrate concentration, $[S_T]_1$. As noted elsewhere [5], the other initial rates that did not follow the same pattern were annulled by attenuation rather than total elimination, the effect of the negative intercept. But with partial correction of the initial rates ($v_1 \rightarrow v_4$), the kinetic parameters, the K_M and V_{max}, are reduced in magnitude but the values are higher than those for the total corrections, covering the 9 initial rates. The values obtained after the pseudo-statistical treatment (this means multiplying the initial results by a decimal integer defined by Eqs (26) and (27) if necessary) were expectedly lower than the untreated (Table 2). The results, such as 190.7 micro M/min and 2.84 mg/L from the correction of all v_i values and 214.1 micro M/min and 3.3 mg/L for the partly corrected v_i values, are not widely different from the literature values of 180 micro M/min and 2.5 mg/L [4].

The values that were overestimated due to the first two initial rates for galactosidase are also due to conditions that invalidate the Michaelis-Menten equation (re-christened in this research as the HBHMM equation) and the associated quasi-steady-state assumption such that $[S_T]_n v_{n-1} - [S_T]_{n-1} v_n$ should be equal to zero. Subjecting such overestimated kinetic parameters as 833.33 μM/min and 13.75 g/L (Table 2) to a pseudo-statistical treatment is ruled out because it is of no value. However, such overestimation cannot be ruled out, assuming accurate values of initial rates, if Michaelian kinetics is out of the question in preference for single turnover kinetics [17]. In general, this may be the case where $[S_T]$ is ≪ $[E_T]$ and $[S_T]$ ≪ K_M such that v (note that v is also initial rate) remains directly proportional to $[S_T]$ for at least up to five different $[S_T]$. In such a situation, $v/[S_T]$ or $[S_T]/v$ for up to five different $[S_T]$ must be constant. But this situation may not be in line with Michaelian kinetics.

As a result, it was critical to evaluate the equations, by plotting f (v^2, $[S_T]$) versus f (v, $[S_T]$) where the y-axis is equivalent to $(v_n v_{n-1}([S_T]_n - [S_T]_{n-1}))$ and x-axis is equivalent to $([S_T]_n v_{n-1} - v_n[S_T]_{n-1})$ and f ($[S_T]^2$, v) versus f (v, $[S_T]$) where the y-axis is equivalent to $([S_T]_n[S_T]_{n-1}(v_n - v_{n-1}))$ and x-axis is equivalent to $([S_T]_n v_{n-1} - v_n[S_T]_{n-1})$, to yield respectively the V_{max} and K_M. All results (equation of linear regression) observed were displayed as an inset and written as a footnote under each corresponding Figure, namely, Figures 2 and 3 for *A. oryzae* alpha amylase and Figures (4 → 7) for beta-galactosidase. The results (1.034 mM/min and 19.296 g/L) from Figures 2 and 3, based on Eqs (9a) and (11b), respectively, were similar to the values (1.016 mM/min and 18.154 g/L) yielded after subjecting the initial results (Table 2) from the LWB plot to a pseudo-statistical treatment. The magnitude of kinetic parameters obtained was < than that obtained by the LWB method.

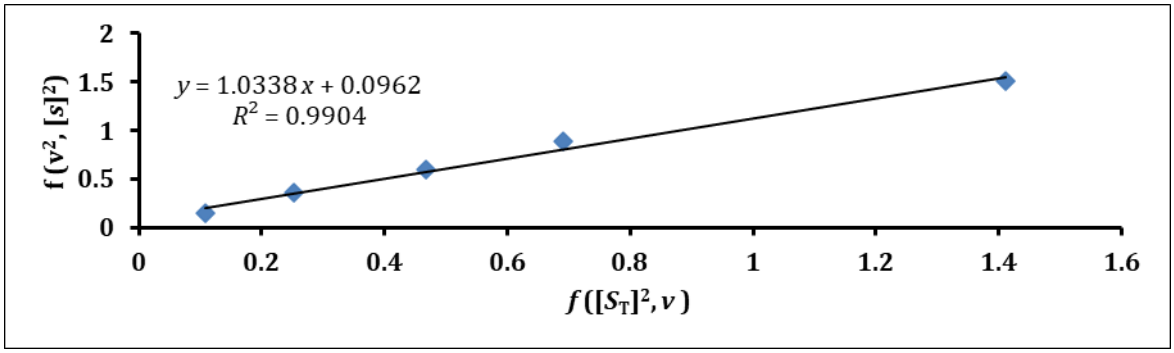

Figure 2 Determination of maximum velocity of enzymatic action, V_{max} by graphical method based on Eq. (9a)**.** The ordinate, $y = f(v^2,\ [S]^2) \equiv v_n v_1([S_T]_1^2 - [S_T]_2^2)$ and the abscissa, $x = f(v,\ [S]^2) \equiv [S_T]_1[S]_2(v_2 - v_1) - [S_T]_2^2 v_1 + v_2[S_T]_1^2$: The inset shows that V_{max} = 1.034 exp. (– 3) M/mL/min; R is ≈ *0.99.* Data is from this research covering the assay on alpha-amylase.

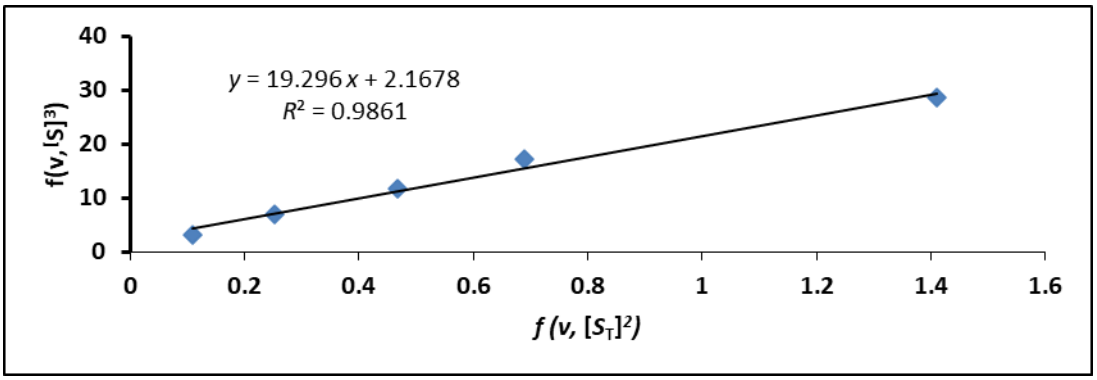

Figure 3 Determination of MM constant, K_M by graphical method based on Eq. (4/11b)
The ordinate, $y = f(v,\ [S_T]^3) \equiv [S]_1^2\ v_n([S_T]_n - [S_T]_1) - [S_T]_1^2\ ([S_T]_n v_1 - [S_T]_1 v_n)$ and the abscissa, $x = f(v, [S_T]^2) \equiv [S_T]_n [S_T]_1 v_1 - [S_T]_1^2\ v_n$: The inset shows that K_M = 19.296 g/L; $R \approx 0.99$. Data is from this research.

The second set of plots (Figures (8) → (11)) were plots of $v_n v_i$ $([S_T]_n - [S_T]_i)$ versus $[S_T]_n\ v_i - [S_T]_i v_n$. Fitting the equations to the recalculated variables (the velocities) and then plotting gave magnitudes of values that were < those observed in LWB plots, with R values being perfectly = 1 in one instance. However, such values were not widely different from those obtained from weighted linear and nonlinear regression in the literature [4]. The results garnered from using partly corrected v_i values ($v_1 \rightarrow v_4$) and fully corrected v_i values are quite lower than the values garnered from a plot (LWB plot) using the raw data.

The values of the parameters V_{max} (Figure 4 and Eq. (9c)) and K_M (Figure 5 and Eq. (4a) as percentages of inaccurate parameters are respectively 23.08 and 22.03 %; the pseudo-statistically remediated values are 168.83 mM/min and 2.508 g/L, which correspond to the initial measurements of 192.33 μM/min and 3.03 g/L, respectively. Here, one sees that the initial measurements were not overestimates, even if they were > than those in the literature report. The literature report [4], however, reveals a burden of error in the initial rates, which may have been attenuated by the mechanism and assumptions of the nonlinear regression software package. One must, however, admit that only one of the eight replicates of the initial rates was made available in the literature. It was sufficiently useful for the illustration of the facts and principles advanced in this research.

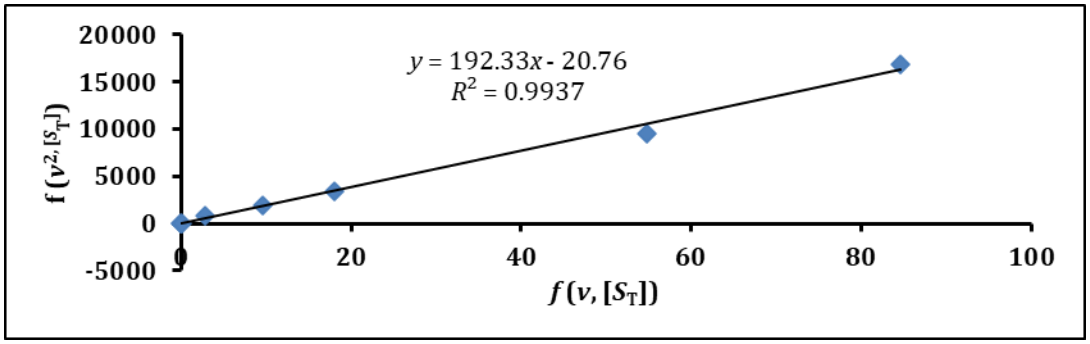

Figure 4 Determination of maximum velocity of enzymatic action, V_{max} by graphical method based on Eq. (9c). The ordinate, $y = f(v^2, [S]) \equiv v_n v_{n-1}([S]_n - [S]_{n-1})$ and the abscissa, $x = f(v, [S]) \equiv [S]_n v_{n-1} - [S]_{n-1} v_n$: The inset shows that $V_{max} \approx 192.33$ μM/min (23.08 % of the inaccurate value); R *is* ≈ 0.99. The pseudo-statistically remediated value is 168.826 μM/min. The original velocities, v_1, v_2, v_3, and v_4 were recalculated according to corresponding equations, Eq. (13) → Eq. (16). The original data explored is in the literature [4].

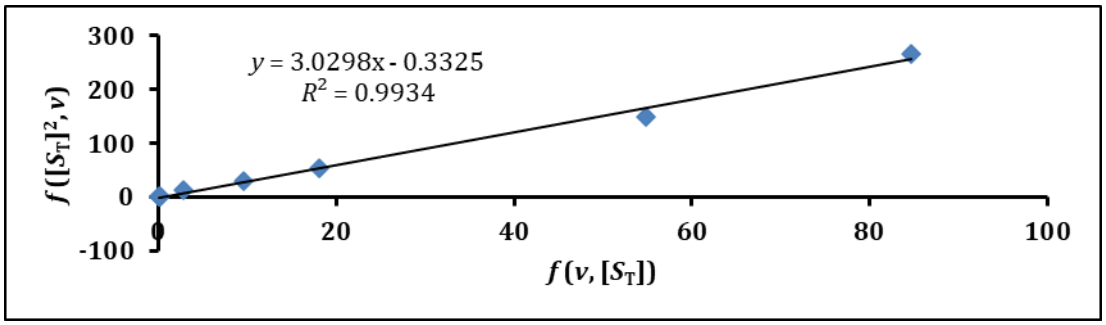

Figure 5 Determination of MM constant, K_M by graphical method based on Eq. (4a).
The ordinate, $y = f(v, [S]^2) \equiv [S_T]_n[S_T]_{n-1}(v_n - v_{n-1})$ and the abscissa, $x = f(v, [S_T]) \equiv [S_T]_n v_{n-1} - [S]_1 v_{n-1}$ the inset shows that $K_M \approx 3.03$ mM (22.04 % of inaccurate value); $R \approx 0.99$. The pseudo-statistically remediated value is 2.508g/L. The data explored is in the literature. The original velocities, v_1, v_2, v_3, and v_4 [4] were calculated according to corresponding equations, Eq. (13) → Eq. (16).

Using all corrected v_i values, the values of the parameters garnered, K_M (Figure 6 and Eq. (4a)) and V_{max} (Figure 7 and Eq. (9c) as percentages of inaccurate parameters, are respectively 24.35 and 22.35 %; the pseudo-statistically remediated values are 163.52 micro M/min and 2.508 g/L, which correspond to the initial measurement of 186.285 micro M/min and 3.348 g/L, respectively. Here, one sees that the initial measurements were not overestimates, even if they were greater than those in the literature report. Using Figure 8 and Eq. (9d) for V_{max} and Figure 9 and Eq. (4b) for K_M, coupled with the use of all corrected initial rates, the values of the parameters as percentages of inaccurate parameters are, respectively, 25.28 and 24.20 %; the pseudo-statistically remediated values are 184.687 micro M/min and 2.755 g/L, which correspond to the initial measurements of 3.348 g/L and 186.285 micro M/min.

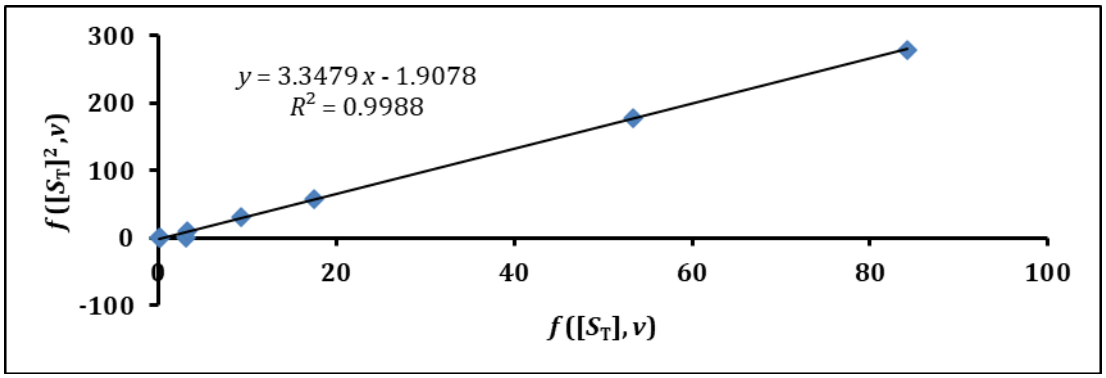

Figure 6 Determination of MM constant, K_M by graphical method based on Eq. (4a).
The ordinate, $y = f(v, [S_T]^2 \equiv [S_T]_n[S_T]_1(v_n - v_1)$ and the abscissa, $x = f(v, [S_T]) \equiv [S_T]_n v_1 - [S_T]_1 v_n$: The inset shows that $K_M \approx 3.348$ mM (22.04 % of the inaccurate value); $R \approx 0.999$. The data explored is in the literature. The pseudo-statistically remediated value is 2.772 g/L. The original velocities, v_1, v_2, v_3, v_4, v_5, v_6, v_7, v_8, and v_9 [4] were recalculated according to corresponding equations, Eq. (13) → Eq. (21).

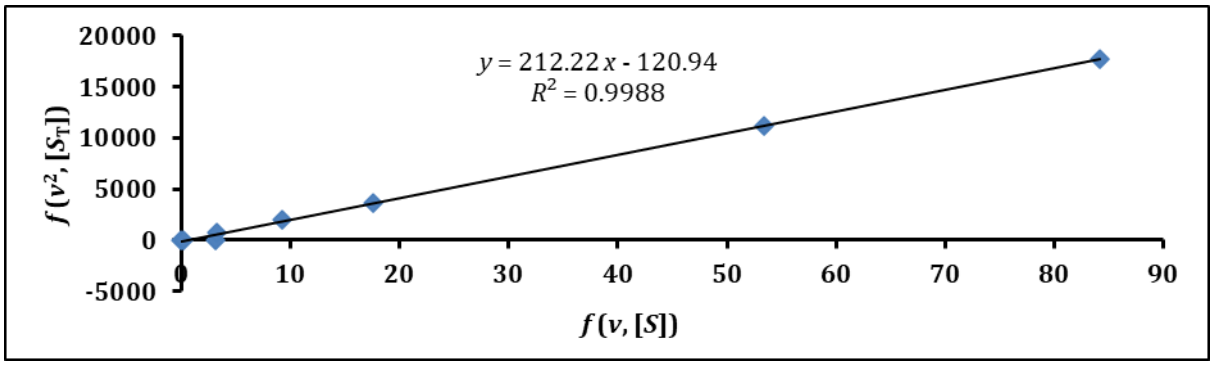

Figure 7: Determination of maximum velocity of enzymatic action, V_{max} by graphical method based on Eq. (9c). The ordinate, $y = f(v^2, [S_T]) \equiv v_n v_1([S_T]_n - [S_T]_1)$ and the abscissa, $x = f(v, [S_T]) \equiv [S_T]_n v_1 - [S_T]_1 v_n$: The inset shows that $V_{max} \approx 212.22$ μM/min (22.354 % of the inaccurate value); $R \approx 0.999$. The pseudo-statistically remediated value is 163.519 μM/min. The original velocities, v_1, v_2, v_3, v_4, v_5, v_6, v_7, v_8, and v_9 were recalculated according to corresponding equations, Eq. (13) → Eq. (21). The original data explored is in the literature [4].

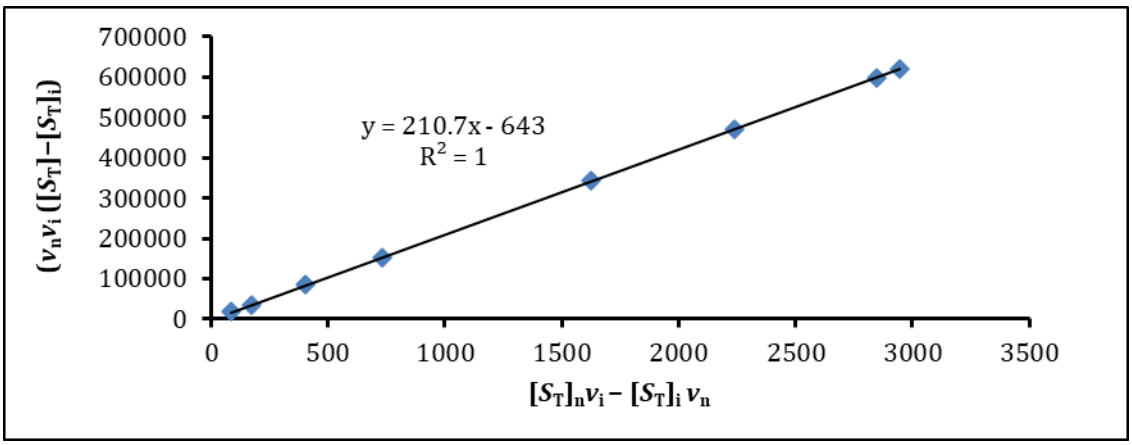

Figure 8 Determination of maximum velocity of enzymatic action, V_{max} by graphical method based on Eq. (9d). The ordinate, $y = f(v^2, [S_T]) \equiv v_n v_i([S_T]_n - [S_T]_i)$ and the abscissa, $x = f(v, [S_T]) \equiv [S_T]_n v_i - [S_T]_i v_n$; i is always = 1. The inset shows that $V_{max} \approx 210.7$ μM/min (25.284 % of the inaccurate value); $R = 1$. The pseudo-statistically remediated value is 184.687 μM/min. The original velocities, v_1, v_2, v_3, v_4, v_5, v_6, v_7, v_8, and v_9 were recalculated according to corresponding equations, Eq. (13) → Eq. (21). The original data explored is in the literature [4].

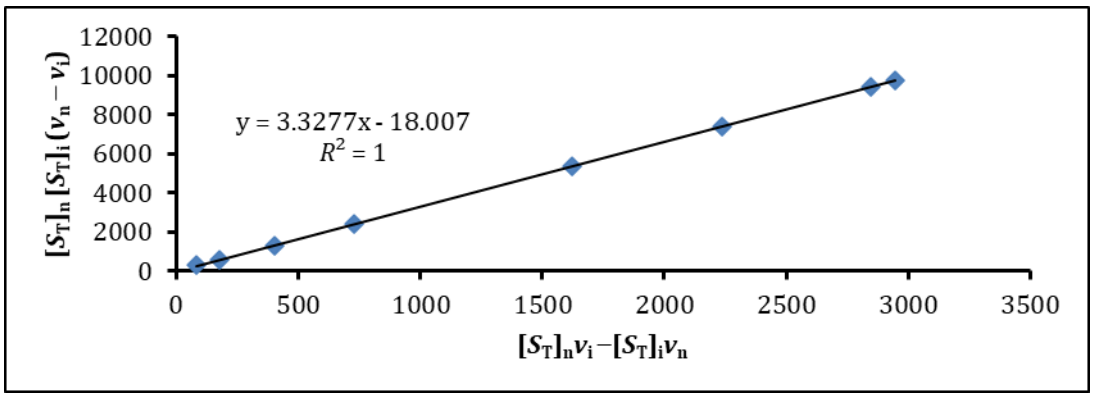

Figure 9 Determination of MM constant, K_M by graphical method based on Eq. (4b). The ordinate, $y = f(v, [S]^2) \equiv [S_T]_n[S_T]_i(v_n - v_i)$ and the abscissa, $x = f(v, [S_T]) \equiv [S_T]_n v_i - [S_T]_i v_n$: The inset shows $K_M \approx 3.3277$ mM (23.528 % of inaccurate value); $R = 1$. The pseudo-statistically remediated value is 2.755 g/L. The data explored is in the literature. The original velocities [4], v_1, v_2, v_3, v_4, v_5, v_6, v_7, v_8, and v_9 were recalculated according to corresponding equations, Eq. (13) → Eq. (21).

Timing errors do not just arise because of a failure to terminate reactions consistently. It also arises if the duration of the assay is such that it totally depletes the substrate before the expiry of the time regime where the lower end of the concentration is the case. But if the upper range of the concentration is the case, the reaction continues until termination by the experimenter. This amounts to a timing error. It does not matter if the duration is on the millisecond time scale. The equations given in this research serve to correct such errors in kinetic variables for the first three assays at three different concentrations of the substrate, as noted in the literature [13]. It is not certain whether computer software can make such adjustments or corrections. Besides, the question, though in a different context, "is there anything left to say on enzyme kinetic constants and quasi-steady state approximation?" [18] seems, to be given a partial answer in this research. There may be more to say yet.

Surprisingly, fitting the equations to the unweighted data and plotting, yielded values (2.498 mM and 196.07 μM/min) that were greater than those obtained, using the recalculated velocity data, but with abysmally low correlation coefficient, R (0.474) with respect to K_M. The K_M was therefore similar to the 2.5 mM obtained by weighted linear and nonlinear regression in the literature. This is a pointer to the efficacy of the equations. It must be emphasised again that the values do not represent the ultimate high precision value but rather a substantial improvement. Thus, using Figure 10 and Eq. (9d) for V_{max} and Figure 11 and Eq. (4b) for K_M, coupled with the use of all unweighted initial rates, the values of the parameters as percentages of inaccurate parameters are, respectively, 23.528 and 18.16 %; the pseudo-statistically remediated values are 172.108 μM/min and 2.067 g/L, which correspond to the initial measurements of 196.07 μM/min and 2.498 g/L, respectively.

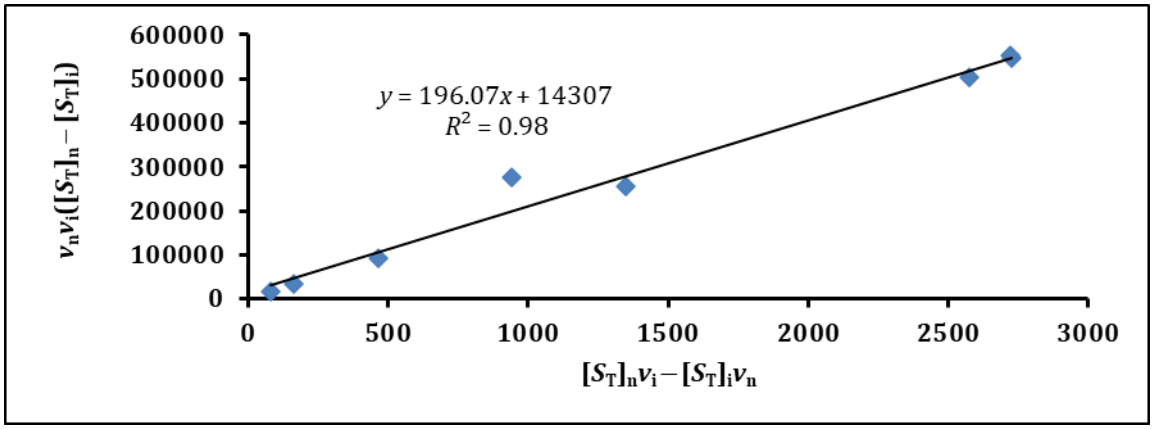

Figure 10 Determination of maximum velocity of enzymatic action, V_{max} by graphical method based on Eq. (9d). The ordinate, $y = f(v^2, [S_T]) \equiv v_n v_i([S_T]_n - [S_T]_i)$ and the abscissa, $x = f(v, [S_T]) \equiv [S_T]_n v_i - [S_T]_i v_n$; i is always = 1. The inset shows that V_{max} is = 196.07 μM/min (23.528 % of the inaccurate value); R = 1. The pseudo-statistically remediated value is 172.108 μM/min. The original velocities (unweighted), v_1, v_2, v_3, v_4, v_5, v_6, v_7, v_8, and v_9 were used. The original data explored is in the literature [4].

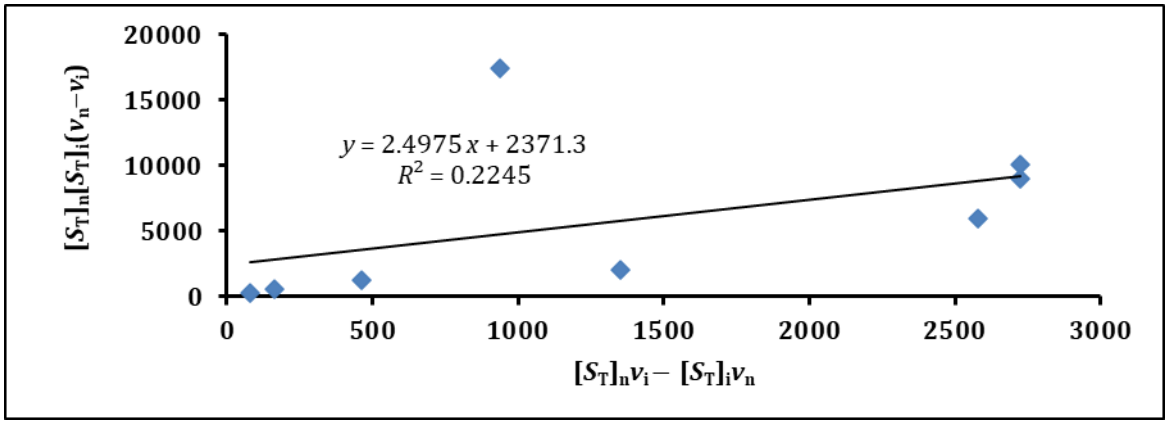

Figure 11 Determination of MM constant, K_M by graphical method based on Eq. (4b). The ordinate, $y = f(v, [S_T]^2) \equiv [S_T]_n[S_T]_i(v_n - v_i)$ and the abscissa, $x = f(v, [S_T]) \equiv [S_T]_n v_i - [S_T]_i v_n$: The inset shows that $K_M \approx 2.498$ mM (18.16% of inaccurate value); R is ≈ 0.474. The pseudo-statistically remediated value is 2.067 g/L. The data explored is in the literature. The original velocities (un-weighted), v_1, v_2, v_3, v_4, v_5, v_6, v_7, v_8, and v_9 [4] were used.

The outcome of this study notwithstanding, one must bear in mind that if there is no error in all measurements (be it 8 or more replicates for each substrate) under conditions that justify the Michaelian equation and underlying assumptions, there cannot be any need for statistical remediation for generating kinetic parameters; thus, the requirement for statistical soundness and absence of any calculation is out of the question [9]. As opined in a recent preprint report [19], there may be calculations depending on the approach to the solution to any problem of interest. For instance, what has been regarded as the best form of the kinetic parameter, the specificity constant (*SC*), must be calculated given a single intersection in a reciprocal variant of the direct linear plot by taking the reciprocal of the ratio of K_M to V_{max}. But if errors are inevitable even with the use of high-tech devices, then the initial rates must be subjected to correctional treatment, which should ultimately reduce the number of intersections to a minimum.

3.1. Graphic summary of issues addressed in this study

Here, virtually all issues raised in this study are given a graphic summary. This begins with a scenario in which $[E_T] \gg [S_T]$ (Figure 12). The reciprocal of the intercept gives a very high value (overestimation of the maximum velocity, V_{max} (16667 mM/min) and consequently an overestimated Michaelis-Menten constant, K_M (K_M value = 106.668 g/L)) in a double reciprocal plot. The K_M and V_{max} values are not true representatives of Michaelian parameters.

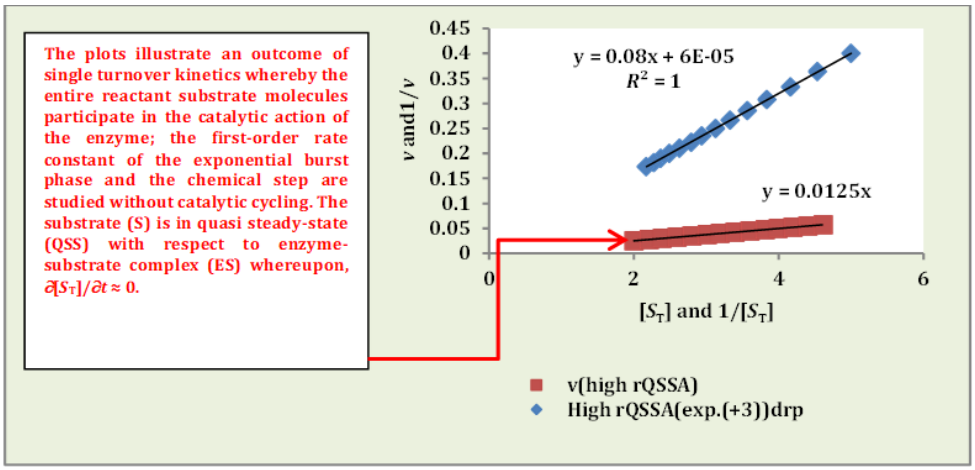

Figure 12 Plots where conditions that validate a very high incidence of rQSSA are the case: $[E_T] \gg [S_T]$. Plot of v_1 to v_5 versus $[S_T]_1$ to $[S_T]_5$ gave equation of linear regression (double reciprocal plot (drp)) such as: $y = (0.08x - 0.0002)$ exp. (+3). A drp plot of all values of v versus all values of $[S_T]$ gave a linear regression equation such as: $y = (0.08\ x + 6$ exp. $(-05))$ exp. (+3). (■) stands for a linear regression of v versus $[S_T]$ ($y = 0.0125x$); (◆) stands for a linear regression of $1/v$ versus $1/[S_T]$. $[S_T]_n v_{n-1} - [S_T]_{n-1} v_n$ = zero in all data points.

Figure 13 neither validates exclusively the rQSSA (or tQSSA) nor the sQSSA; neither rQSSA nor sQSSA is completely satisfied. The K_M and V_{max} values are not exactly what are expected of conditions that validate sQSSA, though they seem high, and a polynomial with a negative coefficient of the leading term as observed notwithstanding. A polynomial with a higher coefficient of determination (R^2) than a linear plot has a tendency towards Michaelian kinetics.

The fact, however, is that instances abound in which biological functions of the enzyme are effected where the concentration of the enzyme is of the same order of magnitude as its concentration of the substrate; there may also be instances where the concentrations at the upper end of the substrate concentration range are about the same order of magnitude as the actual Michaelis-Menten constant of the enzyme while the enzyme's concentration, though lower, cannot be totally saturated. These scenarios have often led researchers to insinuate formally that the concentration of the enzyme need not be much less than the concentration of the substrate in order for the sQSSA, or Michaelian kinetics, or equation to be valid [21]. Ultimately, what is important is the realisation that the Michaelian-Menten constant is a mixed-order constant per a given concentration of the enzyme. The Michaelis-Menten constant must not be the same for different concentrations of the same enzyme with the same substrate under the same condition.

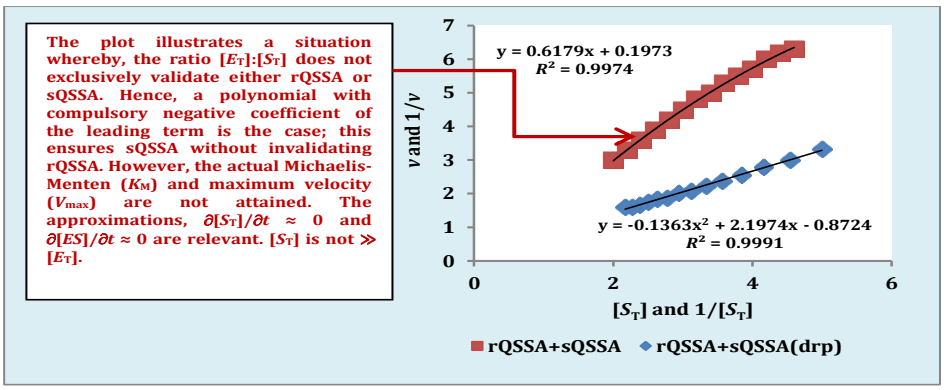

Figure 13 Plots where conditions that neither totally validates an incidence of rQSSA nor sQSSA: Some v values are $\propto$ [S_T] while some are not. Plot of all v values versus all [S_T] values gave equation of linear regression (double reciprocal plot (drp)) such as: y = (0.6179 x + 0.1973) exp. (+3); the resulting V_{max} = 5.068mM/min and the K_M = 3.132g/L. The linear regression of 1/v versus 1/[S_T] gave: y = (0.6682x – 0.0164) exp. (+3) for the plot covering 1/v_1 to 1/v_5. (■) stands for a "polynomial regression" of v versus [S_T]; (◆) stands for a linear regression of 1/v versus 1/ [S_T]. $[S_T]_n v_{n-1} - [S_T]_{n-1} v_n$ is ≠ zero where the v values covers v_7 to v_{14}; $[S_T]_n v_{n-1} - [S_T]_{n-1} v_n$ = zero where the v values covers v_1 to v_6.

Figure 14 is clear evidence that where [E_T] is approximately equal to [S_T], both the polynomial plot without a negative coefficient of the leading term and the linear plot exhibit the same coefficient of determination. Although the symbols for maximum velocity and Michaelis-Menten constant are retained for convenience sake, they are nevertheless not true quantitative representations of Michaelian parameters. The fact is that [E_T] ≈ [S_T] has been noted in the literature, though views such as intra-cellular concentrations of enzymes are usually higher or at least of the same magnitude as their substrates [20] is an exaggeration.

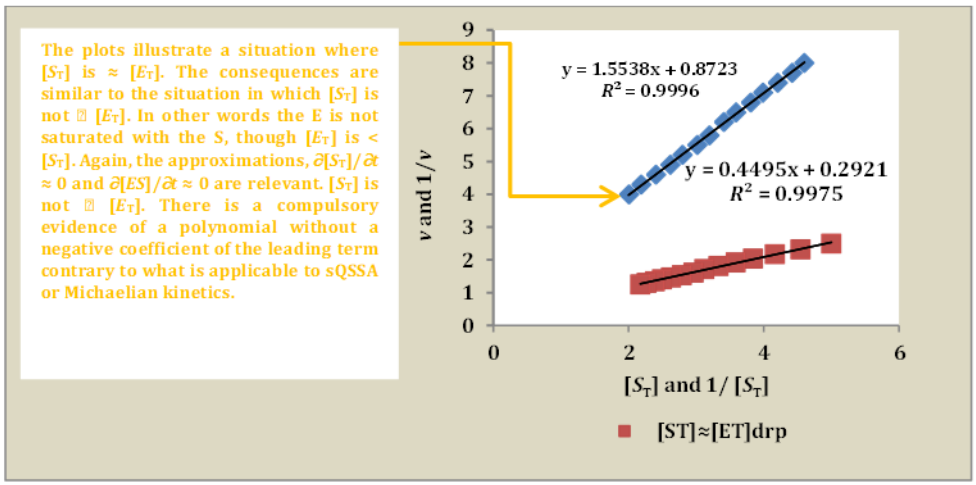

Figure 14 Plots where conditions that validate an incidence of either rQSSA or sQSSA may be the case: Such conditions are [S_T] ≈ [E_T]; [E_T] < K_M. The V_{max} value and K_M value expected from the regression equation (y = 0.4495 x + 0.2921) exp. (+3) from the plot of 1/v versus 1/[S_T] are respectively 3.423 mM/min and 1.54 g/L. (◆) stands for either linear or "polynomial" regression of v versus [S_T]: Both plot show R^2 that is = 0.9996; (■) stands for a linear regression (drp) of 1/v versus 1/[S_T].

Figure 15 epitomises what it takes to comply with the conditions that validate Michaelian kinetics: [S_T] ≫ [E_T], and the values of K_M and V_{max} are truly Michaelian parameters. This implies that they are characteristically Michaelian; this does not imply that a hyperbolic curve could have been observed with the substrate concentration regime explored.

Higher [S_T] cannot be ruled out. The implication is that higher values of the Michaelian-Menten and maximum velocities should be expected, subject to true experimentation for confirmation.

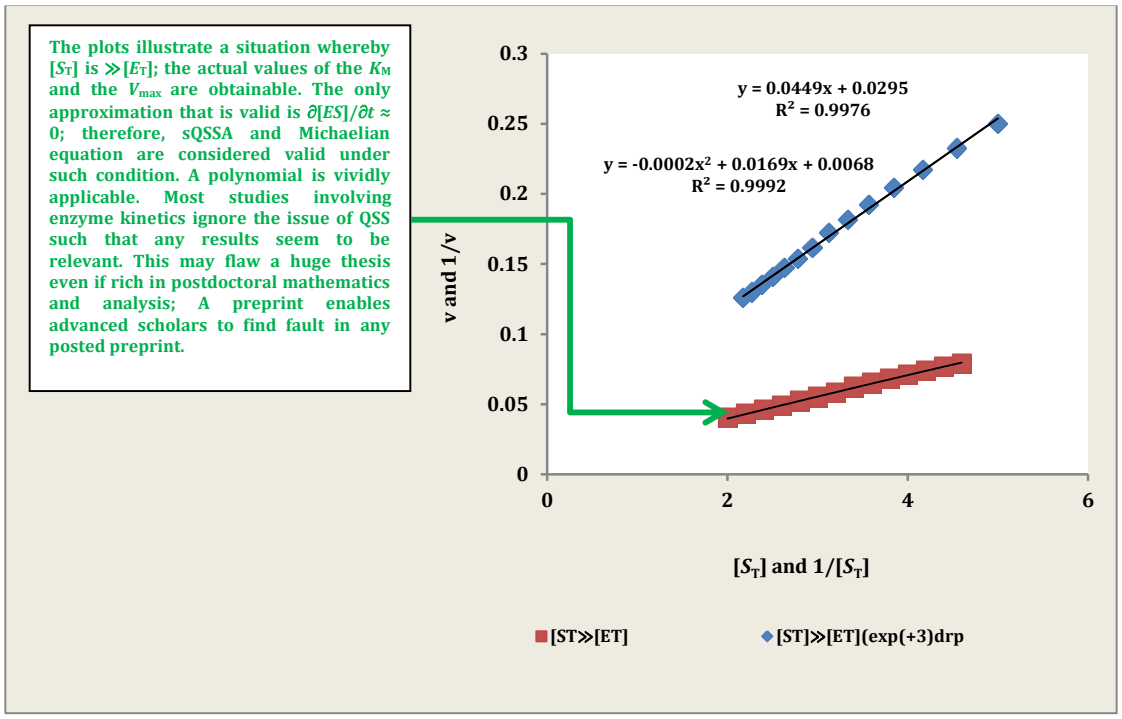

Figure 15 Plots where the condition that validate an incidence of sQSSA (or "Henri-Briggs-Haldane-Michaelis-Menten" (HBHMM) equation) may be the case: Such condition is that [S_T] ≫ [E_T]. The V_{max} value and K_M value expected from the regression equation (y = 0.0449x + 0.0295) exp. (+6) from the plot of 1/v versus 1/[S_T] are respectively 33.898 μM/min and 1.522 g/L. (◆) stands for a linear regression of 1/v versus 1/[S_T]; (■) stands for a polynomial plot of v versus [S_T] with R^2 (0.9992) very similar to a linear regression with R^2 = 0.9991 (not shown).

The graphic summary is intended to remind all and sundry, students and high-ranking scholars in the field, that the issue of QSSA must be reflected in the study of enzyme kinetics because the result of such a study has profound implications for scientific, engineering, and, in particular, medical applications. "To be as imposing as a titanic, does not mean that a titanic-like body is unsinkable". This implies that minor issues that are ignored can ultimately flaw a post-doctoral thesis by high-ranking researchers. Needless to give an example, but what needs to be taken home is that if an enzyme is very active with a given drug (and even food) to be activated, care should be taken to ensure that a low concentration of drug needs to be administered. In the management of diabetics, starchy foods containing resistant starches are recommended for the same reason.

4. Conclusion

The equations for the determination of the K_M and V_{max}, which are respectively invariant with respect to each other, were rederived. These were in addition to other equations for the same purpose and for the correction of initial rates. The recalculated (or corrected) initial rates gave results for kinetic parameters by graphical means, the LWB method, linear regression based on derived equations, and calculations based on derived equations, which represent a remarkable improvement on the LWB-generated results using unweighted results. The V_{max} and K_M values for galactosidase by graphical means respectively range between 163 and 185 mM/min and between 2.07 and 2.77 g/L; the ranges by calculations are 177 and 214 mM/min and 2.45 and 3.311 g/L, subject to pseudo-statistical remediation. Overall, the ranges of V_{max} and K_M values for alpha-amylase from both the graphical method and calculation are, respectively, 1.095 to 1.018 mM/min and 18.15 to 20.554 g/L. Nonetheless, the underlying issue remains the conditions that validate Michaelian or non-Michaelian kinetics for the generation of kinetic parameters. The initial rates must not be a mixture of both if the true K_M and V_{max} are of interest. The new pseudo-statistical method for the remediation of error in all measurements, if necessary, is viable, useful, and robust. A future study should examine the

effect of high-precision instrumentation for assay in conditions that validate specified QSSA so as to verify the desirability of any statistical approach for the remediation of initial rates and kinetic parameters in particular. Such future results based on high-precision instrumentation can then be further compared with results from the current method.

Compliance with ethical standards

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Disclosure of conflict of interest

There is no competing interest; no financial interest with any government or corporate body or any individual.

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Author contribution

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

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Dr. Udema, Ikechukwu Iloh, holds BSc., MSc., and Ph.D. degrees in biochemistry. The first and second degrees were earned from the University of Benin, Benin City, Edo State, Nigeria. The third degree was earned from Ambrose Alli University, Ekpoma, Edo State, Nigeria. He also holds a Post Graduate Diploma (PGD, edu) earned from Delta State University, Abraka, Delta State. He retired as a teacher and a vice principal (adm). His research interests cover life (biochemistry, respiratory physiology, *etc.*) and physical (a few subfields of physics) sciences. His motto is: No one is the beginning or end of knowledge.