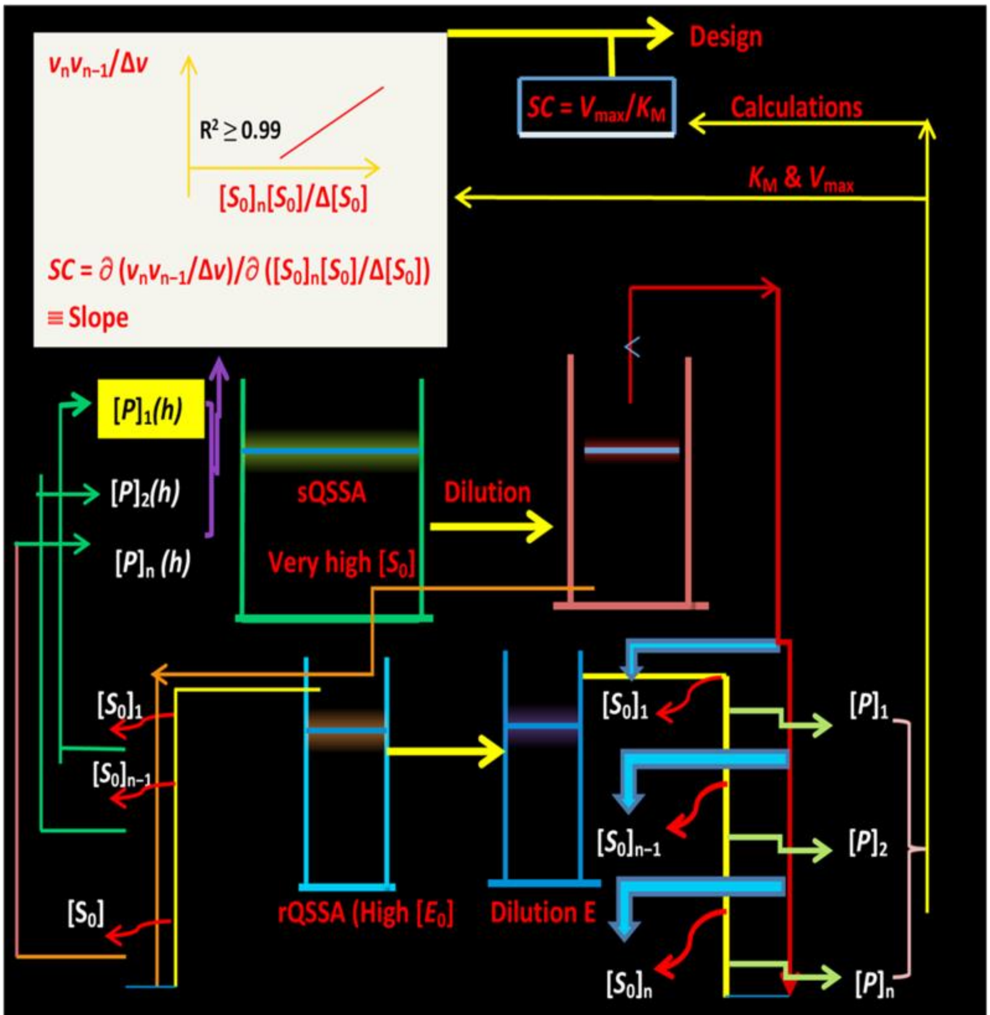
Linking the Pre-steady-state, Steady-state, and Zero-order kinetic parameters together for Industrial applications

Abstract: The debate is not whether or not tQSSA in particular or rQSSA in general is generalizable to a range of substrate concentrations; rather, it is imperative to recognize the fact that sQSSA demands that $[E_T] \ll [S_T]$ while with rQSSA, $[S_T] \ll [E_T]$; each presents kinetic parameters different from the other.

The value of the specificity constant (*SC*) stated earlier, obtained by the traditional V_{max} to K_M ratio (V_{max}/K_M), did not differ (note once again that V_{max}/K_M in M/g min can be converted to 1/g min by dividing the former by $[E_T]$, and if the molar mass of the substrate is known, the latter can also be divided by it). There appeared not to have been a way of linking pre-zero-order kinetics to zero-order kinetics so as to garner key kinetic parameters at very high industrial concentrations of the substrate. The objectives were the derivation of equations that can be explored in relating pre-zero-order (pre-steady-state (prss)) to zero-order kinetic parameters (ZOK), such as the Michaelis-Menten constant (K_M), maximum velocity of catalysis (V_{max}), and specificity constant (*SC*), to be evaluated. The V_{max} for the higher industrial-type concentration of the enzyme (alpha-amylase) was 7812.5 micromoles/l/min, while the K_M was 115.1 g/l. The *SC* obtained by calculation, either by the new equation (Eq. (25b)) or the ratio V_{max}:K_M, was 67.88 micromoles l/g min. Surprisingly, as compared to the literature, the *SC* obtained by the new graphical method was 275.4 micromoles l/g min using sub-K_M values of substrate concentrations. The prss V_{max} and K_M were 2348.62 ± 479.94 micromoles l/g min and 7.41 ± 1.77 g/l, respectively. There is a justification for an equation linking PRSS and the ZOK, which can enhance reactor design. The equation linking PRSS to the ZOK kinetic parameters was derived. With the equation, the K_M for a very high industrial concentration of the substrate and the enzyme that would have been impossible was made possible. Future studies may focus on assays at high concentrations of the enzyme and sub-K_M concentrations of the substrate so as to observe a repeat of higher *SC*. Note that if the concentration of one enzyme is twice (or more) the concentration of another enzyme of the same kind, the V_{max} of the first should be twice the V_{max} of the latter given saturating concentrations of the substrate for each enzyme; the parameter that is constant is the catalytic first-order rate constant (k_{cat}); it may be theoretically assumed that the K_M can follow the same order as with the V_{max}. Apart from a very high concentration of the gelatinized starch that retards the mobility of the enzyme, the spectrophotometer has an upper limit of its power to read color development.

Keywords: Sub-Michaelis-Menten substrate concentrations; specificity constant; Michaelis-Menten constant; maximum velocity of catalysis; high substrate concentrations; alpha-amylase.



$[S_0]$ and $[P]$: initial concentration of substrate and product respectively

rQSSA: reverse quasi-steady-state approximation

sQSSA: standard quasi-steady-state approximation

SC: specificity constant

H: high

v: initial rate

K_M & V_{max}: Michaelis-Menten constant and maximum rate respectively

E & $[E_0]$: enzyme and initial concentration of enzyme

Graphcal abstract

INTRODUCTION

Beyond the concern for a definite nomenclature for kinetic constant that seems to be faced with conflicting interpretation and the methodology that can give a definite value without resorting to the separate determination of maximum velocity, V_{max}, of catalysis and Michaelis-Menten constant, K_M, there is also a need for methods by which such a kinetic constant (specificity constant, SC) can be determined, even under very high concentrations of the substrate. It appears the challenge of methodology has been met as observed in the literature, [1] but the contentious issue of nomenclature, that is, the exact name of the ratio, $V_{max}:K_M$, lingers on. This does not stop the design of the reactor or the batch facility, but doing so without information about the kinetic parameters may not produce a good prototype, let alone the ultimate industrial setting. There is substantial information about assaying at very low concentrations of the enzyme, which supports the condition that validates the Michaelis-Menten equation which following historical antecedence is re-christened the "Henri-Briggs-Haldane-Michaelis-Menten" (HBHMM) equation, [2] and standard quasi-state-state approximation (assumption) (sQSSA); issues regarding this and other assumptions, reverse QSSA (rQSSA), total QSSA (tQSSA), and reactant stationary assumption (RSA) can be found in one way or another in several literature materials. [3-7] It should be made clear that the abbreviation 'HBHMM' is intended to honor the earlier contributors to what later became the Michaelis-Menten equation. High-ranking adherents of MM notation may not be comfortable with this alternative name.

It is clear that the most cost-effective means for optimizing industrial yield is under the condition that validates sQSSA. This is so because a much lower concentration of the expensive enzyme than the substrate is required. If coupled with a suitable immobilizer, it can be used several times with a much longer life span. Research articles on immobilization [8-11] are pieces of evidence suggestive of the increasing importance of the immobilization of enzymes and their application in industries in particular. Nonetheless, while the kinetics of immobilized enzymes may differ from those of free enzymes, the starting point that can offer insight is the assay of the free enzyme. Therefore, for the first time, linking the pre-steady-state, steady-state, and zero-order kinetic parameters together for industrial applications may not be out of place with the aim of circumventing the bottleneck associated with the determination of kinetic parameters using industrial-scale high concentrations of the substrate in particular. Information about the kinetic parameters is pivotal to industrial design. With this, the study is carried out with the objectives of deriving equations that can be explored in relating pre-zero-order (otherwise known as pre-steady-state (prss)) to zero-order kinetic parameters, such as the Michaelis-Menten constant (K_M), maximum velocity of catalysis (V_{max}), and specificity constant (SC), and ultimately evaluating the equations with experimental data.

THEORY

In this section, the originating equations are briefly reviewed, and the equation linking the pre-steady-state, steady-state, and zero-order kinetic parameters together for industrial and allied applications is derived. Consequential corollaries are also drawn. In real Michaelian kinetics, the rate of catalysis may be partially linear (the correlation coefficient, R, may be < 1) with respect to the first 3 to 4 different concentrations of the substrate. With higher concentrations, the dependence of initial rate on $[S_0]$ follows a rectangular hyperbola, giving the expression of a zero-order. This plot, however, does not take into account the ratio of maximum velocity to the sum of the Michaelis-Menten constant and any initial concentration of the substrate as applicable to the Michaelis-Menten equation. It is not possible to include the molar mass of the substrate in Michaelis-Menten equation because it will cancel out.

Hence, an equation of ratios that recognizes the nonlinearity factor (ϕ) (which can also be given other related meanings according to the setting) of the original Michaelis-Menten equation and catalytic cycles (M_S/M_2) is adopted, as expressed in Eq. (1) below. It can be shown that such an equation is amenable to double reciprocal transformation and, as such, does not necessarily depart from Michaelian formalism. The derivation of the equations begins with a simple intelligible equation relating two ratios, one of which is greater than the other: This is such that the ratio $a_y > b_y$, and as such, a_y is equal to fb_y ($f > 1$). Such a relationship is not uncommon in several studies, as may be applicable to rQSSA and tQSSA, in which, for instance, the concentration of the enzyme is several folds greater than the concentration of the substrate on a mole-to-mole basis. Also relation such as $[E_0]/(K_M+[S_0]) < 1$is not uncommon in the literature; it simply means that $(K_M+[S_0])/[E_0] = f$ (where $f > 1$). This effectively gives another interpretation to phi (ϕ) which could be seen as the number of times one ratio is greater than other (e.g. $[S_0] + [E_0]M_S/M_2$) may be ϕ–fold greater than $v_i/(v_i + V_{max})$). Therefore, it would be inaccurate to say that the anonymous statement that the word "phi" employed in this study has no physical significance. This appears to be proof of efforts to stifle creative thought.

The majority of underdeveloped and slightly developing nations have been working to strengthen their economies for far too long without realizing that drastic industrialization was necessary. It appears that the mindset of certain developed economies promotes a low degree of industrialization, which results in poverty. Such attitudes include anything that can thwart great ideas along industrial lines of thought when such countries are ever advancing techniques of ongoing industrialization. A net exporter of manufactured goods and services is incomparable to a net exporter of raw materials". Therefore, the issue of how to handle extremely high substrate concentrations on an industrial scale that place significant hydrodynamic limits on the enzymes because of increased viscosity is crucial. However, researchers have expressed concern on this issue. The issue will be discussed latter in this study.

Summarized review of the originating equations

$$\frac{[S_0]}{[S_0]+\frac{M_S}{M_2}[E_0]} = \frac{v_i}{v_i+V_{max}}\phi, \qquad (1)$$

where $[S_0]$, V_{max}, M_2, M_S, and v_i are the mass concentration of the substrate, the maximum velocity of catalysis of the enzyme, the molar mass of the enzyme, the representative molar mass (taken to be molar mass of the parent polymer, the polysaccharide) of all the substrates of different physical forms, the parent polymer, fragments, *etc*., resulting from a number of catalytic cycles, and the initial rate, respectively; $[E_0]$ and ϕ are the mass concentration of the enzyme and nonlinearity factor respectively. As a guide, recall that the Michaelis-Menten equation is given as: $v_i = V_{\max}[S_0]/(K_M + [S_0])$. The elucidation of the meaning of ϕ is given in the next paragraph.

The plot of $([S_0] + [E_0]M_S/M_2)$ versus $[S_0]$ can yield a perfect linearity, similar to the plot of $v_i/(v_i + V_{\max})$ versus v_i but, a plot of $v_i/(v_i + V_{\max})$ versus $[S_0]$ cannot yield a perfect linearity unlike the plot of $([S_0] + [E_0]M_S/M_2)$ versus $[S_0]$. This current view illustrated in Figures 1 and 2 correct earlier view elsewhere [1]. They are respectively based on experimental (Table 1) and hypothetical data.

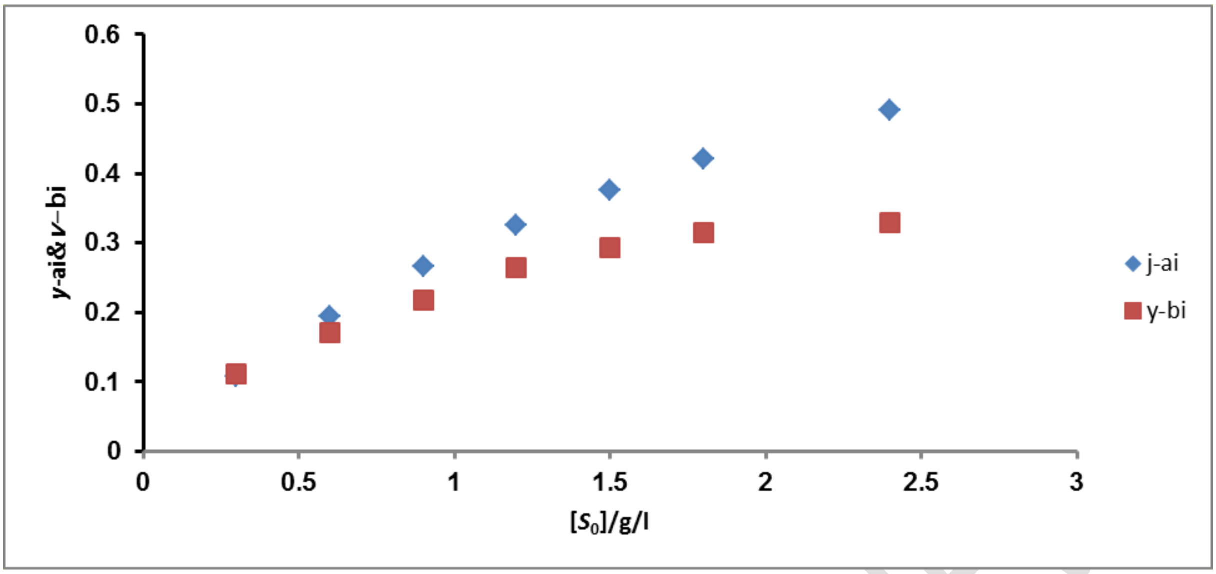


Figure 1: Illustrations of linear relationship between [S_0]/([S_0]+[E_0]M_3/M_2) and [S_0] (legend j-ai) and the nonlinear relationship between v_i (v_i+ V_{max}) and [S_0] (legend j-bi). Such relation is based on rQSSA because the substrate concentration range is sub-K_M value ([E_T] > [S_T]) as observed in Table 1

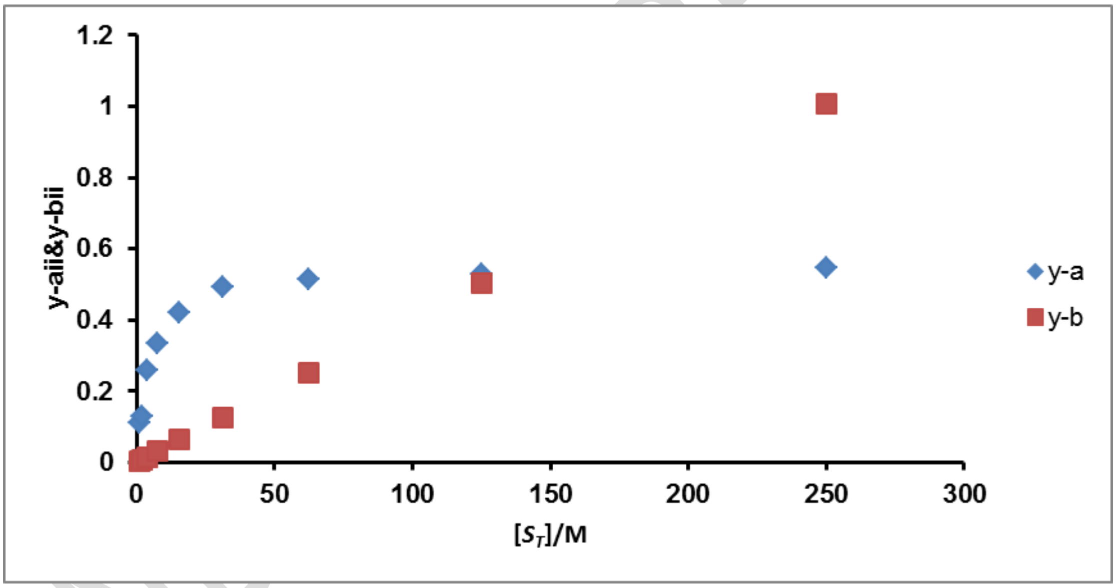


Figure 2: Illustrations of linear relationship between [S_0]/([S_0]+[E_0]M_3/M_2) and [S_0] (legend j-aii) and the nonlinear relationship between v_i (v_i+ V_{max}) and [S_0] (legend j-bii). Such relation is based on sQSSA because the substrate concentration range is mainly » K_M and [E_T] values. The hypothetical data (velocities of product formation and release) were generalized to 0.0002 g/l (~3.846 nM) given substrate concentrations ranging from 1.0 to 300 µM.

Note that as long as ([S_0] + [E_0]M_S/M_2) > v_i/(v_i + $V_{\max}$), division of the fomer by the latter should always yield dimensionless value that > 1. However, the most important issue is that, any assay that can create Michaelian kinetics must give a polynomial with negative coefficient of the leading term where, in particular, the initial substrate concentrations are < the K_M. Therefore, ϕ is the expected reciprocal of the slope of such a plot. One should also be aware that, there are different catalytic cycles for different concentrations of the substrate. Each subsequent catalytic cycle is faced with a mixture of substrates, parent polymers, long fragments, oligosaccharides, *etc*. In other words, catalysis can continue even when the entire parent polymer is exhausted. Therefore, there should always be substrates with different degree of polymerization and consequetly, different molar masses. The molar mass of the parent polysaccharide, M_S, is therefore adopted (this may appear arbitrary in order to simplify the process).

Connection of Eq. (1) with well known Michaelis-Menten equation

The connection of Eq. (1) with Michaelis-Menten equation is shown by first expanding Eq.(1) as follows:

$$\phi v_i[S_0] + \frac{v_i M_S[E_0]}{M_2}\phi = v_i[S_0] + [S_0]V_{max}$$

Since ϕ is equal to two, a simplification should give:

$$v_i\left([S_0] + \frac{2M_S[E_0]}{M_2}\right) = [S_0]V_{max}$$

$$v_i = [S_0]V_{max}/\left([S_0] + \frac{2M_S[E_0]}{M_2}\right)$$

Upon rearrangement of Eq. (1), one gets:

$$\frac{1-\phi}{V_{max}} = \frac{M_S}{M_2[S_0]V_{max}}[E_0]\,\phi - \frac{1}{v_i}, \qquad (2)$$

Further rearrangement gives:

$$\frac{1}{v_i} = \frac{M_S}{M_2[S_0]V_{max}}[E_0]\,\phi + \frac{\phi-1}{V_{max}}, \qquad (3)$$

and as such, ϕ should be equal to two if $(\phi-1)/V_{max}$ is equal to the reciprocal of the maximum velocity of catalysis of the higher concentration of the enzyme. If so, Eq. (3) (a double reciprocal equation), which is derived from Eq. (1), can be rearranged to reproduce the Michaelis-Menten equation: $2M_S[E_0]/M_2$ is equivalent to the Michaelis-Menten constant, K_M. This view is premised on the well-known fact that there are two major assumptions, namely rQSSA and sQSSA; the third, tQSSA, is said to be a generalization to all ranges of substrate concentrations, unlike rQSSA and sQSSA, which, respectively, are strictly for the inequalities $[E_T] \gg [S_T]$ and $[E_T] \ll [S_T]$: these are the possible assumptions that influenced the derivation of Eq. (3) from Eq. (1). Besides both being subject to double reciprocal transformation, the only difference is that one gives K_d (rQSSA) while the other gives K_M (sQSSA). Therefore, a rearrangement of Eq. (3) should give the Michaelis-Menten equation stated above. Rearrangement of Eq. (1) should also reproduce the Michaelis-Menten equation. This may not be the case if a pre-zero-order (pre-steady-state) scenario is applicable. Where the pre-steady-state is the case, the following should be applicable:

Linking the pre-steady-state parameters with zero-order parameters

$$\frac{\phi-1}{V_{max}\,(H)} = \frac{1}{V_{max}^{prss}}, \qquad (4)$$

$V_{max}(H)$ and (V_{max}^{prss}) are the maximum velocity at the higher (H) concentration of the enzyme, calculated based on the maximum velocity of a lower concentration of the same enzyme and the pre-zero-order (or either the pre-steady-state (*prss*), mixed-order state, *etc*.) maximum velocity under the same condition, respectively. Their origins are explained in subsequent paragraphs. But realize that in linking the pre-steady-state parameters with zero-order parameters, the value of ϕ cannot be defined quantitatively without the value of (V_{max}^{prss}).

For the purpose of clarity, as stated earlier, $V_{\max}(H)$ is the maximum velocity of catalysis at a higher concentration of the enzyme. But this can only be achieved with a much higher concentration of the substrate ($[S_0] \gg [E_0]$). The inequality is useful and relevant if the molar masses of the enzyme and substrate are equal. Therefore, it may be more appropriate to adopt the mole concept such that $[S_T] \gg [E_T]$, where $[S_T]$ and $[E_T]$ are, respectively, the molar concentrations of the substrate and the enzyme (the subscript T has no physical meaning other than to distinguish the molar quantity from mass concentration). It means that one can determine, for adoption, the molar concentration of the enzyme by dividing its mass concentration by its molar mass. This can be observed in the subsequent equations to be derived. When $[S_0] \gg [E_0]$ (or rather $[S_T] \gg [E_T]$), standard quasi-steady-state approximation and Michaelian formalism are relevant. The determination of the K_M for high concentrations of the enzyme and even for high saturating concentrations of the substrate in an industrial setting is the problem.

Regarding the meaning of M_S $[E_0]/M_2$ and determination of $V_{max}(H)$

In an assay in which [E_0] $\gg$ the concentration of the substrate, which is also < the K_M, the appropriate condition is a reverse quasi-steady-state approximation or assumption; in such a scenario, v_i is directly proportional to [S_0]. In this regard, it has been shown that K_d = M_S [E_0]/M_2; [2] therefore, as long as K_d < K_M, then K_M should be equal to ϕM_S [E_0]/M_2. It does not mean that, in the circumstances, all molecules of the enzyme have been saturated, hence the factor 'ϕ'. It is neither about the ratio M_S:M_2 nor any fictitious interpretation. But at saturation, [E_T] combines fully with [S_T] on a mole-to-mole basis for a single-active-site enzyme. The molar concentration of the enzyme that combined with the substrate is equal to [ES], as is the molar concentration of the substrate that combined. Since the molar masses of the enzyme and the substrate are different, the respective mass concentrations are also different, such that M_S [ES] $\neq$ M_2 [ES]; so it stands to reason that M_S [ES] may not be equal to neither K_d nor K_M. The K_M, which is the main object of this investigation, is not an exception. Hence, as long as [E_0] is the mass concentration, division by M_2 and multiplication by M_S and ϕ are not out of the question. The physical interpretation of ϕ already implied in the preceding text is that the K_M may just be ϕ-fold larger than M_S [E_0]/M_2 irrespective of the name given to. ϕ. Although, without unintended doubt, ϕ M_S [E_0]/M_2 has a clear physical meaning, information about ϕ cannot easily be garnered, the simplifying assumption made earlier notwithstanding. But there is a way out of it for the purpose of this research.

Every biochemist, and any one in related field, knows that the maximum velocity (V_{max}) of catalysis is directly proportional to the concentration of the enzyme [E_T]. Given the same conditions, once the V_{max} is known for the same enzyme, the first-order rate constant, that is the catalytic rate constant (k_{cat}) is the same for any concentration of the enzyme as long as the assay was conducted using the saturating substrate concentration regime. This is undergraduate-level information that cannot be ignored in any high-level dissertation.

The Unknown Michaelis-Menten constant (K_M) as reflected in Eq. (7) is, as stated earlier, ϕ-fold greater than M_S [E_0]/M_2 (the unit of [E_0] is g/l); if one is conversant with assumptions or approximation in the literature, he or she would have been aware of standard quasi-steady-state (sQSSA), total QSSA (tQSSA), reverse QSSA (rQSSA) *etc*. In order to specify any of such assumptions, either the substrate concentrations [S_T] or [E_T] are compared with each other. Hence, [E_T] $\gg K_M$, [E_T] $\gg$ [S_T] *etc*., and *vice versa*, corresponding to tQSSA or rQSSA and sQSSA respectively. [4-6] Thus, if [S_T] in mol. /l $\gg$ [E_T] also, in mol. /l, it stands to reason, why the K_M could be ϕ-fold greater than M_S [E_0]/M_2 (Eq. (7)). The debate is not whether or not tQSSA in particular or rQSSA in general is generalizable to a range of substrate concentrations; rather, it is imperative to recognize the fact that sQSSA demands that [E_T] $\ll$ [S_T] while with rQSSA, [S_T] $\ll$ [E_T]; each presents kinetic parameters different from the other.

The color expected following the reaction with the reducing agent could be too high for the spectrophotometer (spec.) if [E_T] $\gg$ [S_T]; dilution of such a solution to achieve reduced color intensity is not advised. Different concentrations of the enzyme under the same conditions of assay possess different K_M and maximum velocities. The only parameter that is constant is the catalytic constant, k_{cat}. On the other hand, the maximum velocity of catalysis at higher concentrations of the same enzyme where [E_T]$\gg$[S_T] (on mole-mole basis) is designated as V_{max}^{prss} ; the spec can measure such velocities and lower initial velocities ($V_{max}^{prss} \ll V_{max}(H)$) if the duration of assay is short.

$$\phi = \frac{V_{max}(H) + V_{max}^{prss}}{V_{max}(H)}, \tag{5}$$

$$K_M = \frac{\phi M_S [E_0]}{M_2}, \tag{6}$$

As stated earlier, [E_0]/M_2 where M_2 is the molar mass of the enzyme, is the same as [E_T] (the molar concentration) even if a ratio of the molar mass (in g/mol.) of the substrate to the molar mass of the enzyme appears in any equation, including Eq. (6).

$$\frac{K_M}{\phi} = \frac{M_S [E_0]}{M_2}, \tag{7}$$

Substituting Eq. (5) into Eq. (7) gives:

$$\frac{M_S [E_0]}{M_2} = \frac{K_M V_{max}^{prss}}{V_{max}(H) + V_{max}^{prss}}, \tag{8}$$

Solving for K_M gives:

$$K_M = \frac{M_S [E_0]}{M_2 V_{max}^{prss}} \left(V_{max}(H) + V_{max}^{prss}\right), \tag{9}$$

One can wonder as to the workability of Eq. (9) considering the fact that there are two different maximum velocities, more so where $V_{max}^{prss} \ll V_{max}(H)$; indeed, the latter is the zero-order maximum velocity of the much higher concentration of the enzyme, which may be between 40- and 60-fold lower in concentration than some of the concentration of the substrate at the lower end of the substrate concentration range chosen by the experimenter. An interested person may wish to know how to determine V_{max}^{prss} and $V_{max}\,(H)$, which are maximum velocities at sub-K_M and above-K_M concentrations, respectively. The catalytic rate constant, $k_{cat,}$ can be generated at very low concentrations of the enzyme given low but saturating concentrations of the substrate. Moving forward, the maximum velocity of the high-concentration enzyme can be computed based on the relation $V_{max}\,(H) = k_{cat}\,[E_T]$. The low concentration range of the substrate explored in the assay of the low concentration enzyme can then be used to assay the high concentration enzyme to give $V_{max}\,(L)$; $V_{max}\,(H) \gg V_{max}\,(L)$. Given these data, the molar mass of the substrate (M_S) and high mass concentration (E_0) of the enzyme and its molar mass (M_2) and substituting all into Eq. (9), and solving gives the K_M of the high concentration enzyme.

In a batch experiment, or reactor, where in particular the enzyme may be immobilized, the concentration of the enzyme, as in an *in vivo* environment, could be several fold higher than the concentration of the substrate, or, as opined elsewhere, [3] it may be of the same order of magnitude as the substrate concentration; meanwhile, the concentration could also be very high in an *in vivo* scenario, let alone in an industrial setting. The concentration of the product is expected to be extremely high, well above the capacity of the spectrophotometer to measure it. It should be noted that the substrate concentration regime that produced the maximum velocity ($V_{max}(L)$) of the lower concentration of the enzyme based on the enabling initial rates can, with a much higher concentration of the enzyme, produce an almost immeasurable concentration of the product. The designer must bear in mind one aspect of interest, either rQSSA (tQSSA) or sQSSA; there are two ways to resolve the problem.

Need for two assays on two different concentrations of the same enzyme

Given a very high concentration of the enzyme for large-scale production for industrial or scientific research, the concentration of the substrate must also be very high, much higher than [E_0] in order to achieve a Michaelian kinetics, otherwise called the zero-order kinetics. However, at high substrate concentrations, the viscosity could also be too high, leading to a hindrance on translational diffusion that can also reduce encounter-complex formation. Macromolecular crowding indicates the presence of nonspecific steric repulsion between the molecules and generates the excluded volume effect [12] where any part of two macromolecules cannot exist in the same place at the same instant of time.

Any situations that impede motion can be described as potential ‘biochemical crowders"— a very high concentration of a homogeneous milieu (highly concentrated gelatinized starch, for instance). It must be understood that with polysaccharides or any polymer as substrate for that matter, at high concentration, though it constitutes crowding in that there may be impediment to free diffusion, the immediate availability of alternative substrate for, first, encounter complex formation, and with the realization that all collisions are not effective, binding may still occur; effective collision must lead to stable enzyme-substrate formation. [13]

Crowding due to high concentration may be in the form of homogeneity, but the high viscosity can slow down the translational diffusion of the smaller enzyme and promote nonspecific interactions (interactions that cannot lead to effective ES formation), which can also increase the residence time of the enzyme, as was attributed to a smaller substrate in the literature. [14] For example, eukaryotic cellular environments are highly crowded, where the estimated total concentration of soluble and insoluble macromolecules, including proteins, nucleic acids, ribosomes, and carbohydrates, in the cytoplasm ranges from 50 to 400 mg/ml and may occupy 30 to 40% of the total cell volume. [3] Such "macromolecular crowding" conditions can affect the conformational dynamics, molecular diffusion, stability, and functional properties of proteins. [15, 16] This may not be different from an industrial setting.

Furthermore, with time, the number of substrate fragments composed of longer parent polysaccharides, fragments that are intermediate in length, and shorter polysaccharides, besides the usual product, maltose, increases. [17] This can contribute to an aggravated crowding effect, as it has been observed that the increased surface areas due to shorter polymers than parent polymers offer a higher potential for crowding. [14] Despite the liquefaction by the amylolytic action of the enzyme, the different fragments and the free product can increase the incidence of nonspecific interactions. The phenomenon of sequestration as it affects the velocity of the catalytic action cannot be ignored at excessively high substrate concentrations. [18] In one sentence, very high substrate concentrations can lead to substrate inhibition (ScienceDirect.com), despite the undisputed saturation phenomenon. Besides, it has been observed for years that high substrate concentrations inhibit microbial growth. Since some enzymes are cytosolic in location while a few others may be membrane-bound, inhibition may be occasioned by osmotic shock or stress.

However, the concentration of the enzyme must indeed be based on the substrate concentration used and the extent of amylolysis envisaged and desired, as excessive use of enzymes will cost a lot for the profit-oriented industry; however, the era of immobilization [8] has taken center stage in this regard. This cannot foreclose the need for pieces of information regarding the enzyme, either in a free-state or immobilized state. After making an appropriate choice of the underlying QSSA, for instance, in industrial applications, the ratio of the substrate concentration to the concentration of the enzyme ($[S_T]/[E_T]$) on mole-mole bases should be 40–80, [3] covering the substrate concentrations from the lowest to the highest.

From the discussion above, there is a compelling reason for any relevant engineer to consider the following steps: Reduce the concentration of substrate and enzyme by several folds, equally applied to the enzyme and substrate. Hypothetically, a substrate can be reduced from a concentration of 800 g/l to 80 g/l; the enzyme may be reduced from 8.045 exp. (−3) g/l down to 8.045 exp. (−4) g/l to give a ratio of approximately 40.035 to 99.54. With this ratio, the Michaelian principle can be observed. The determination of the V_{max} (L) for this much lower concentration ($[E_T](L)$) in molar units leads to the determination of the catalytic first-order rate constant, which ought to be the same for the same enzyme under the same defined conditions. With that, the maximum velocity ($V_{max}(H)$) of the much higher concentration ($[E_T](H)$) of the enzyme is then given as (Since different concentrations of the same enzyme under the same conditions possess different V_{max} (the only parameter constant is k_{cat}), given a higher concentration of one enzyme, its maximum velocity is symbolized by upper case H to distinguish it from the maximum velocity of the lower concentration of the same enzyme, symbolized by upper case L!): k_{cat} $[E_T](H)$; this is where the suggestion that the specificity constant (*SC*) should be regarded as a single kinetic parameter [19] has to be disregarded, but not jettisoned, to achieve a solution. Be it a batch reaction mixture or a reactor scenario that may go on for hours, the duration of the assay for this preliminary or preparatory stage needs to be relatively transient (0.5→1 min) compared to hours in an industrial setting. This can preclude substrate depletion when an assay is conducted.

Next is the determination of (V_{max}^{prss}) under conditions that are applicable to mainly rQSSA; this means that the assay of the higher concentration of the enzyme should be conducted with substrate concentrations that are 60- to 100-fold lower than high substrate concentrations, such as a range given hypothetically as follows: 50, 60, 70, 80, 90, 100, 200, 250, 300, 400, 500, 600, 700, and 800 g/L. The initial rates and substrate concentrations are then subjected to a double reciprocal treatment to give (V_{max}^{prss}) and (K_M^{prss}), where the latter is most likely to be $\ll$ the mixed order K_M shortly before the asymptotic state. It is not unlikely that (V_{max}^{prss}) > than the maximum velocity of the lower concentration of the enzyme. The fact that the maximum velocity is always directly proportional to the molar concentration of the enzyme and the notion that different concentrations of the same enzyme have different K_M, which follows from the definition of the latter, do not imply that the K_M must also be proportional to the concentration of the enzyme. However, this cannot be ruled out entirely. If assumed to be so, then Eq. (9) can be used to estimate (V_{max}^{prss}), and given the equation immediately below, [20] one can calculate the putative enzyme-substrate dissociation constant (K_d) that can be substituted into "a far right" rQSSA equation as that is diametrically opposed to the sQSSA equation, the so-called Michaelis-Menten equation, in order to solve for the initial rates (v_i^{prss}). First,

$$K_d = [E_0]\, M_5 / M_2 \, , \tag{10}$$

$$V_{max}^{prss} = \frac{M_5\, [E_0]\, V_{max}(H)}{K_M M_2 - M_5 [E_0]} \, , \tag{11}$$

$$v_i^{prss} = \frac{M_5\, [E_0]\, V_{max}(H)}{K_M M_2 - M_5 [E_0]} \; \frac{[S_0](prss)}{\frac{[E_0] M_5}{M_2}} \, , \tag{12}$$

where $[S_0]$(prss) is the sub-K_M concentration of the substrate. The importance of Eq. (12) goes beyond the estimation of pre-mixed zero-order initial rates; it shows that against the popular standard text book information extracted from journals while writing such books, when, in particular, $[E_0]$ is either $\gg$ $[S_0]$ or ≈ $[S_0]$, Michaelis-Menten equation (re-christened “Henri-Briggs-Haldane-Michaelis-Menten" (HBHMM) equation) [2] should not be transformed into the form: $v_i = V_{max} [S_0]/K_M$ where V_{max} is wrongly intended to denote the maximum velocity of any concentration of the enzyme.

If a plot of all experimentally determined (v_i^{prss}) values are plotted versus all [S$_0$](prss) values (note that all [S$_0$](prss) values must be ≪ K_M) a straight line is expected; this is as it should be in a single turnover catalytic event. The slope (S_L) of such plot, based on Eq. (12) is given as:

$$S_L = \frac{M_S\,[E_0]\,V_{max}\,(H)}{K_M\,M_2 - M_S\,[E_0]}\,/K_d \tag{13}$$

where K_d (M_S [E_0] /M_2) is the dissociation constant of the enzyme-substrate complex.

Revalidation of the equations in two ways

Equations (9) and (13) feature the K_M. Therefore, the rearrangement of Eq. (13) to give the K_M is followed by equating the result with Eq. (9) to give the following:

$$\frac{M_S\,[E_0]\,V_{max}\,(H)}{M_2\,V_{max}^{prss}} + \frac{1}{M_2} = \frac{V_{max}\,(H)}{S_L K_d M_2} + \frac{1}{M_2} \tag{14}$$

A simplification of Eq. (14) reproduces the equation of (V_{max}^{prss}) in terms of the slope from the plot of initial rates versus sub-K_M concentrations of the substrate. The reproduced equation is:

$$S_L = V_{max}^{prss}\,/K_d \tag{15}$$

Equation (15) emanates from the notion that v_i is directly proportional to [S_0] ([S$_0$] being ≪ K_M) as an obvious and inappropriate modification of the "HBHMM" equation.

In a real, unequivocally rQSSA-model, the K_d as defined in a preprint [20] is given as:

$$K_d = \frac{[E_0]\,M_S}{M_2} \tag{16}$$

$$V_{max}^{prss} = S_L\,\frac{[E_0]M_S}{M_2} \tag{17}$$

Equation (17) is again as in the literature. [20] This is the first evidence of its validity. The second piece of evidence is one in which the result should be equal to zero on both sides of the equation. From Eq. (13) is given:

$$V_{max}\,(H) = \frac{K_M\,M_2 - M_S\,[E_0]}{M_S\,[E_0]}\,S_L K_d \tag{18}$$

From Eq. (9) is given:

$$\frac{M_2\,V_{max}^{prss}\,K_M}{M_S\,[E_0]} = V_{max}\,(H) + V_{max}^{prss} \tag{19}$$

Substitution of Eq. (18) into Eq. (19) gives, after rearrangement, the following:

$$\frac{M_2 V_{\max}^{\mathrm{prss}} K_{\mathrm{M}}}{M_S\,[E_0]} - \frac{S_L K_M M_2 K_d}{M_S\,[E_0]} = -\frac{S_L K_M M_S K_d}{M_S\,[E_0]} + V_{\max}^{\mathrm{prss}} \qquad (20)$$

Realizing that $K_d S_L$ is equal to ($V_{\max}^{\mathrm{prss}}$) and canceling common terms reduces Eq. (20) to:

$$\mathrm{LHS} = \mathrm{RHS} = 0 \qquad (21)$$

This exercise is predicated on the observation in the literature that neither of the key kinetic parameters found a valid equation. [21] It is a lesson that should always compel high-ranking scholars to examine what they consider mere algebra, "the mother of all mathematics", which is exact in nature.

Bringing the specificity constant (SC) into relevance.

From Eq. (22) below is derived based in part, on the fact that $V_{\max}(H)$ is equal to SC K_{M}, in Eq. (18):

$$S_{\mathrm{L}} K_{\mathrm{M}} M_2 K_{\mathrm{d}} - S_{\mathrm{L}} [E_0] M_S K_{\mathrm{d}} = M_S K_{\mathrm{M}} [E_0]\, SC \qquad (22)$$

$$K_{\mathrm{M}} = \frac{S_{\mathrm{L}} [E_0] M_S K_{\mathrm{d}}}{S_{\mathrm{L}} M_2 K_{\mathrm{d}} - M_S [E_0]\, SC} \qquad (23)$$

$$SC = \frac{S_{\mathrm{L}} K_{\mathrm{M}} M_2 K_{\mathrm{d}} - S_{\mathrm{L}} [E_0] M_S K_{\mathrm{d}}}{M_S [E_0] K_{\mathrm{M}}} \qquad (24a)$$

$$= \frac{V_{\max}^{\mathrm{prss}} K_{\mathrm{M}} M_2 - V_{\max}^{\mathrm{prss}} [E_0] M_S}{M_S [E_0] K_{\mathrm{M}}} \qquad (24b)$$

N.B.: ($V_{\max}^{\mathrm{prss}}$) is equal to $K_{\mathrm{d}}.S_{\mathrm{L}}$. It is easier to determine the real maximum velocity for low and very high concentrations of the enzyme, as described in this research. Therefore, Eq. (24b) can further be written after substituting Eq. (9) into it as follows:

$$SC = \frac{V_{\max}^{\mathrm{prss}} M_2}{M_S [E_0]} - \frac{V_{\max}^{\mathrm{prss}} M_S}{\frac{M_S^2 [E_0]}{M_2 V_{\max}^{\mathrm{prss}}}\left(V_{\max}(H) + V_{\max}^{\mathrm{prss}}\right)} \qquad (25a)$$

$$= \frac{V_{\max}^{\mathrm{prss}} M_2}{M_S [E_0]} - \frac{M_2 \left(V_{\max}^{\mathrm{prss}}\right)^2}{M_S [E_0]\left(V_{\max}(H) + V_{\max}^{\mathrm{prss}}\right)} \qquad (25b)$$

Equation (25b) seems to challenge a proposal that *SC* stand alone as a kinetic parameter with a positive response to it in a very recent preprint report that shows several ways by which a direct determination of *SC* can be done. The best [1] of all the methods entailed a plot of $v_n v_{n-1}/\Delta v$ versus $[S_0]_n[S_0]_{n-1}/\Delta[S_0]$, giving the slope as *SC* without further calculation except to convert to Ml/gs to l/gs if the v_i values were not divided by $[E_0]$ before the plot.

EXPERIMENTAL

Materials and methods

As in the literature, *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. The molar mass of the enzyme is = 52 k Da. [22] An electronic weighing machine was purchased from Wenser Weighing Scale Limited, and a 721/722 visible spectrophotometer was purchased from Spectrum Instruments, China. A pH meter was purchased from Hanna Instruments, Italy.

Preparation of solutions of reactants and assay.

The enzyme was assayed according to the Bernfeld method [23] using gelatinized potato starches. The average molecular weight of the insoluble potato starch is 6.454 exp. (+7) g/mol. [24] The 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 gelatinized at 100 ^{o}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 $[S_0]$ » $[E_0]$ (0.0002 g/l) as in a previous investigation; 0.3 and 3 g/L for the assay in which $[E_0]$ (0.002 g/L) > $[S_0]$ except where $[S_0]$ is = 3 g/l as in the previous investigation; [20] 0.3 and 3 g/l (with the exception that instead of 2 g/l, 2.4 g/l was explored as one of the values of $[S_0]$ in this research for the assay in which $[E_0]$ (0.02 g/l) is, again, $\gg$ $[S_0]$. A concentration equal to 0.02 g/l of *Aspergillus oryzae* alpha-amylase was prepared by dissolving 0.002 g of the enzyme (as the stock) in 100 ml of Tris HCl buffer at a pH of 7.0. The assay of the enzyme was carried out in 3 minutes at 20 ^{o}C.

Determinations of kinetic parameters

The kinetic parameters of interest are the K_M, $V_{\max}$, ($V_{\max}^{\text{prss}}$), and *SC*; they were determined as described by Eq. (9) with respect to the determination of K_M where $[E_0]$ is = 0.02 g/l and in very recent preprints [1, 25] as well as Eq. (25b). The remediation of initial rates v_2, v_5, v_6, and v_7 corresponding to [S_0] values equal to 0.6, 1.5, 1.8, and 2.4 g/l was according to Eqs (14), (17), (18), and (19) as shown in the literature. [2]

Statistical analysis

Micro-Soft Excel was explored for the determination of standard deviation (SD) where necessary; the degree of freedom is equal to 6.

RESULTS AND DISCUSSION

In order to implement the postulated approaches to the solution to the anticipated problem of the effect of a very high concentration of the substrate, which, though much higher than the concentration of the enzyme, still requires that the enzyme be explored in a relatively higher amount for industrial application, two assays were conducted for two different concentrations of the same enzyme. However, the initial rates from the assay of the enzyme, 0.0002 g/l and 0.002 g/l have already been explored in the literature but represented here for descriptive analysis and comparison. The initial rate values and the corresponding [S_0] values are given in Table 1. The goal remains to identify means of obviating or eliminating entirely the effect of very high substrate concentrations that present effects (reduction in the initial rates) similar to those discussed in the literature. [12, 15, 26-28]

Apart from the concern expressed regarding the high concentration of the substrate in an industrial setting, the observation that biocatalysts and biocatalysis are limited in application due to a poor transition from the laboratory to the process plant, [29] attention has also been drawn to the inadequacy of using the ratio V_{max}/K_M. [30-32] The poor transition from the laboratory to the process plant or reactor, probably orchestrated by an inappropriate choice, or no choice at all, of a suitable QSSA, and the negative hydrodynamic effects associated with high substrate concentration may have accounted for it in a manner that has also impaired the correct application of *SC*. As will be observed shortly, the highest *SC* is attributed to a lower concentration of the enzyme in conditions that validate sQSSA (Table 1); conditions that validate rQSSA give a very high V_{max}, but the *SC* is much lower because of the high concentration of the enzyme used for the assay. The right choice for the process engineer is to adopt a kinetic model that goes with validated rQSSA if the substrate is very expensive compared with the enzyme, which is generally known to be expensive. On the other hand, if the substrate is cheaply available, given the expensive enzyme, the sQSSA-oriented model should be advised.

TABLE 1. Kinetic parameters determined by different methods with different concentrations of the same enzyme

$[S_0]$ (g/l)	**Re. *v_i (Lit.) (µM/min)**	****v_i (This res.) (µM/min)**
0.3	**22.51**	**84.90^a**
0.6	**38.86**	**164.80 (160.75)**
0.9	**55.20**	**240.15^a**
1.2	**67.44**	**303.65^a**
1.5	**77.79**	**366.63 (429.40)**
1.8	**86.65**	**425.46 (468.16)**
2.0 **(2.4 in this study)**	**91.89**	**532.22 (541.50)**
3	**113.19**	**636.10^a**
K_M (Eq. (9))/ kg/l		**115.10**
K_M^{prss} (raw)/kg/l		**8.105±1.94**
V_M^{prss} (raw)/(µM/min)		**2348.26±479.94**
$V_{M(p-stat)}^{prss}$/(µM/min)		**2148.17± 439.05**
SC (Eq. (25b))/ µML/g min		**67.88**
$\partial (v_n v_{n-1}/\Delta v)/ \partial ([S_0]_n[S_0]_{n-1}/\Delta[S_0])$/ µML/g min		**275.43 /($R^2 = 0.9944$)**

$[S_0]$, v_i, Re, res, and Lit stand for the concentration of the substrate, initial rate, remediation, research, and literature respectively; * and ** stand for values in the literature and this research respectively; the values in brackets are the raw data while the superscript, a denotes any value that was not remediated while values without any legend are remediated values.; p-stat stand for the pseudo-statistically remediated mean and standard deviation (SD) values; [25[weighting factor for the fractional contribution of each substrate to the excess concentration observed in the summation result is 5.1; the corresponding value for the product is 5.31837; the p-stat remediation factor for the product (V_{max}) and substrate (K_M) are respectively, ≈ 0.91479 and 0.914278. Assay on the enzyme ($[E_0]$ = 0.02 g/l) gave SC (as the ratio: V_{max}/K_M) = 67.88 µMl/g min. With 0.002 g/l (Lit) [1] the value for the ratio is ~ 87.72 µML/g min while with $\partial (v_n v_{n-1}/\Delta v)/ \partial ([S_0]_n[S_0]_{n-1}/\Delta[S_0])$ it is = 84.92 µMl/g min ($R^2 = 0.996$).

The K_M and V_{max} values obtained at sub-K_M concentrations of the substrate are, respectively, 7.4098±1.774 g/l and 2148.6615 mM/min; the zero-order K_M and specificity constant, *SC*, obtained according to Eqs. (9) and (25a or its simplified form, 25b), respectively, are 115.1002 g/l and 67.8757 mM.l/g min. The value of the specificity constant (*SC*) stated earlier, obtained by the traditional V_{max} to K_M ratio (V_{max}/K_M), did not differ (note once again that V_{max}/K_M in M/g min can be converted to 1/g min by dividing the former by $[E_T]$, to give $V_{max}/K_M\,/[E_T]$ and if the molar mass of the substrate is known, the latter can also be divided by it).

The value of *SC* obtained by graphical method in a plot of $v_n v_{n-1}/\Delta v$ versus $[S_0]_n[S_0]_{n-1}/\Delta[S_0]$ whose slope is $\partial\,(v_n v_{n-1}/\Delta v)/\partial\,([S_0]_n[S_0]_{n-1}/\Delta[S_0])$, the equivalent of *SC*, gave an unusually higher value equal to 275.43 mM.l./g min with sub-K_M values of the substrate concentrations than the calculated *SC* based on the relationship V_{max}/K_M and Eq. (25b) in the zero-order category. The underlying raison d'être is that where a higher concentration of the same enzyme is assayed at the sub-K_M concentrations, the velocities of product formation could be much higher than the velocities of the much lower concentration of the same enzyme exposed to saturating concentrations of the same substrate (which are sub-K_M values for the higher concentration of the same enzyme) under the same conditions. Yet, two kinetic values for high-concentration enzymes are sub-zero-order maximum velocity (similar to pre-steady-state maximum velocity) and the K_d rather than the K_M (usually > than K_d) because saturating concentrations of the substrate were not the case. Thus, while the ratio (*SC*) in molar concentration per mass concentration per unit time obtained either by computation based on relevant equations or by graphical means is much higher than for the low-concentration enzyme, nevertheless, *SC* obtained by dividing the value in mM.l./g min by the molar concentration of the high-concentration enzyme could be much lower than for the low-concentration enzyme; this provides an answer to the question as to why a higher value equal to 275.43 mM.l./g min was observed.

A similar trend was observed for the value of $[E_0]$ explored earlier in the literature, [1] which is equal to 0.002 g/l; in this case, the value of *SC* is 84.919 mM.l./g min. Alas, noting is indeed unusual because there is a strong need to be first-order rate conscious; by this is meant that if all values of *SC* in mM.l/g min are divided by the corresponding molar concentration of the enzyme assayed, the trend observed in the literature, [1] *viz*., the *SC* values with sub-K_M [S_T] as displayed (in the literature [1]), which compare in the following order: 0.0002 > 0.0005 > 0.002 g/l can be replicated; indeed, this is the case in this study given that the division by [E_T] in molar concentration presents the following: 2207.894 l/g min for 0.002 g/l of the enzyme in the literature and 716.118 l/g min for 0.02 g/l of the enzyme in this research. This is where the class of QSSA must be clarified. With 0.002 g/L (38.462 nM/L), the [S_0] values were generally not much less than [E_0], unlike 0.02 g/l (384.62 nM/l), which was $\gg$ 8-to 80-fold higher than [S_0], a "far-right case of rQSSA".

As stated earlier, such pieces of information about kinetic parameters are prerequisites for the highly predictable outcome of a data-guided reactor design. One can add that stabilizers that are larger than the enzyme and substrate and other thermostatically controlled measures, among other things, can be sought for and made part of the reactor design. With reference to the works of Rubin-Pitel and Zhao, [33] Carrillo *et al.* [31] posited that non-natural environments include high substrate and/or product concentrations, which, as stated earlier, are very similar to the biological system.

Therefore, the bone of contention should be how to obtain kinetic data relevant to very high substrate concentrations and possibly the concentration of the product, which are regarded as important factors operating in actual bioprocess situations, such as continuous and batch-type reactors, as well as reversible and irreversible processes. [31] On this issue, it is equally advisable to explore the potential of the salting-in and salting-out effects; are there substances that solubilize highly concentrated substrate and that can be filtered out after every reactor function? This remains a question to be considered by the chemical engineer. Some of those salting-in and salting-out agents are either enzyme stabilizers or destabilizers. Information regarding the kinetic parameters, V_{max}, K_M, and *SC*, is only relevant in the desire to establish the substrate concentration that should enable maximum formation of the product by the chosen concentration of the enzyme; it must be noted that a low concentration of the enzyme presents a lower K_M (see [1]) than a higher concentration of the same enzyme (Table 1) under the same conditions for the same substrate as long as a condition that validates the sQSSA or HBHMM equation is in place. The K_M observed for the chosen concentration of the enzyme should guide the preparation of suitable zero-order kinetics. Assessment of variants of the same enzyme for a given specific substrate must be based on well-established optimum conditions for each variant, so that the question of reliability of the outcome of comparison may be out of the question.

Summary

The summary covers all that have been discussed on how the procedure developed and enabling equations can aid in cost-effective design of reactors for production, research *etc*; the significance is also given.

Use of procedure and enabling equation

Thus, Taking note of the reference high concentration of the substrate and the corresponding enzyme, as stock on an industrial scale, that can give zero-order V_{max} and mixed-order *KM, which* for practical purposes is unrealizable, the derived equations offer means of converting the zero-order kinetic parameters of high-concentration enzymes to pre-zero-order values (*e.g.,* 2148.17 ± 439.05 mM/min). Since zero-order values are of interest, the pre-zero-order V_{max} can be matched with appropriate concentrations of the enzyme that can reproduce the catalytic zero-order, first-order rate constant via the equation, $V_{max}^{prss} = k_{cat}[E_T]_{(x)}$ where $[E_T]_{(x)}$ designates yet-to-be-determined practically useful concentrations of the enzyme (though no zero-order k_{cat} value is given, nevertheless it is easily determined by experiment with low concentrations of the enzyme); this can enhance process engineering design that relies on rate constants stated earlier for the optimization of production goals.

Significance of this work

Be it research-oriented institutions, industries such as pharmaceuticals, research, forensic, and diagnostic laboratories, cost-effectiveness without compromising the primary objective must be the watchword. All these endeavors, particularly research, are very expensive in time and materials (especially the enzymes). Therefore, rational use of resources is very necessary. These methods and the relevant equation can aid the miniaturization of facilities for the conduct of research or for production processes. The bottom line is cost-saving and efficiency.

CONCLUSION

The need to derive an equation linking prss and the zero-order kinetic parameter is justified by the result obtained. The derived equations and results as values of kinetic parameters become prerequisites for industrial (batch or reactor) design. Specifically, the equation linking prss to the ZOK kinetic parameters was derived. With the equation, the K_M for a very high industrial concentration of the substrate and the enzyme that would have been impossible was made possible. In order to establish consistency, future studies may focus on assays at high concentrations of the enzyme and sub-K_M concentrations of the substrate so as to observe a repeat of higher *SC*.

Conflict-of-Interest Statement . The author declares no conflict of interest

Informed Consent Statement**.** Not applicable

Human and Animal Rights Statement. Not applicable

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The sole author has declared that he has no known competing financial interests OR non-financial interests OR personal relationships that could have appeared to influence the work reported in this paper.

Disclaimer (Artificial intelligence)

The sole author hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc.) and text-to-image generators have been used during the writing or editing of this manuscript.

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