Original Research Article

Directly and indirectly determinable rate constants in Michaelian enzyme-catalyzed reactions

ABSTRACT

Backed by kinetic schemes, attempts have been made to derive equations for the calculation of all zero-order first-order rate constants (ZOFORC) for the activation of the enzyme-substrate (ES) complex and its deactivation. The values of ZOFORC, including the kind for the dissociation of the enzyme-product complex (EP) to free enzyme (E) and product (P), are hardly reported. The methods of research were primarily Bernfeld and Lineweaver methods. The goal of the research was to determine ways for the utilization of experimental data for the determination of verifiable and quantifiable rate constants, with the following objectives: 1) To derive equations for the first-order rate constants for the activation of ES and its deactivation, respectively; 2) To quantify by calculation the first-order rate constant for product release; 3) To ultimately quantify the rate constants; and 4) To advise the reactor, process, chemical engineers, *etc.* in different industrial concerns on the usefulness of the rate constants. The value of ZOFORC for the dissociation of EP to free E and P is 3.155 exp. (+5)/min; the values of the rate constant for activation and deactivation are 3.513 exp. (+4) and 2.377 exp. (+8)/min, respectively. Ultimately, it is imperative for all stakeholder groups to devise means of controlling the enzymatic rate of catalysis by manipulating the magnitudes of the rate constant for activation and deactivation in particular. The derived equations can be fitted to the experimentally generated and calculated data. A future research project should entail conducting the assay under optimum conditions so as to verify

possible variations in the ZOFORC values when compared with values generated outside optimum conditions.

Keywords: Alpha-amylase; first-order rate constants; activation; deactivation; enzyme-substrate complex; zero-order kinetic parameters.

1.0 INTRODUCTION

When one examines the literature with a strong interest in the rates of reactions catalyzed by enzymes, one comes up with the finding that there are a lot of kinetic schemes with which to generate kinetic equations, rates, and rate constants; the most popular and simplest of such schemes is: E + S ⇌ ES → E + P, where E, S, ES, and P are the symbols for the enzyme, the substrate, the enzyme-substrate complex, and the product, respectively. The scheme has been described as hypothetical [1]. However, the scheme illustrates the formation of an enzyme-substrate complex, without which progress towards product formation would be impossible. As stated elsewhere [2], the earliest known mechanism of enzyme action (or function) is anchored on the "induced fit" hypothesis or model of Daniel Koshland, Jr. [3] and the "lock and key hypothesis" of Emil Fischer [4]. As a result, it appears that any discussion or concern about the rates of enzyme-catalyzed reactions cannot be complete without mentioning the mechanism of binding. Incidentally, the induced-fit model seemed to have a vague meaning, leading to a more acceptable enzyme's conformational transition (change) model (1). The conformational change of this enzyme during encounter and interaction with substrate is thought to be the one that determines the specificity steps (1). This notwithstanding, an earlier opinion is that the induced fit model seems to be gaining ground with the advent of the "conformation selection" principle [5–10], which postulates that all of the potential conformations of a given protein preexist and that once the ligand selects the most favored conformation, induced fit occurs and conformational change takes place [1].

The generation of rate equations and rate constants requires kinetic schemes far more complex than the generalized simple case given earlier. On the question of the complexity of kinetic schemes that can instill fear in undergraduates, one has the following to advise upon, as discussed elsewhere [11]: Every scheme generated needs to be explained in a stepwise manner. Still, there has been an exceedingly complex derivation of kinetic equations. These are rate and rate constant equations based on the rapid equilibrium assumption or combined assumptions of equilibrium and steady-state, where, respectively, Michaelis and Menten [12] and Briggs and Haden [13] were the original contributors [14].

The paper by Strickland *et al.* [15] *has* as its goal the determination of dissociation constants and specific rate constants of enzyme-substrate complexes, achieved via two different schemes that depict two different mechanisms of enzymatic action; the equations derived based on the schemes, though simple, were not based on a detailed stepwise derivation. Simulations were largely explored for

data generation and fitting the derived equations. In this research, a scheme formulated by Johnson (1) is explored for the derivation of the equations for the first-order rate constant and the quantification of the kinetic constant. The equations are not regularly featured in most enzymology studies. The scheme is thought to be very generalizable to hydrolytic enzymes, such as the amylolytic enzyme.

There is no doubt that the rate constants are very important from the standpoint of biological application, reactor design, drug detoxification and clearance, *etc.* But there is hardly any detailed explanation of how such equations were derived. There are also a few attempts to fit the derived equations to experimental data for quantification by the calculation of multiple kinetic parameters. It is a well-documented fact in the literature [1, 11, 15, 16] that an enzyme-catalyzed reaction has different stages, viz., encounter-complex formation, binding of substrate to the enzyme, conformational transformation to activated complex formation, bond breaking and making (the chemistry), and the release of product and free enzyme. This is possible if the activated complex formation is not aborted. If, for any undefined reason, it is suddenly aborted, the ES that is weakly bound together breaks down into free E and S. These issues have been highlighted in the literature [11].

Besides, the methods of study other than transient-phase kinetic studies using the stopped-flow method [17] for the determination of the first-order rate constants k_3, for the process, EP → E + P (this needed an update for correctional purposes), k_{ES}, for the process, E + S → ES, and k_{-1}, for the process, ES → E + S, are given in the literature [18]. Nonetheless, backed by kinetic schemes, attempts have been made in this research to derive equations for the calculation of all zero-order first-order rate constants (ZOFORC) for the activation of the enzyme-substrate (ES) complex and its deactivation, k_{+2} and k_{-2}, respectively. The values of ZOFORC, including the kind for the dissociation of the enzyme-product complex (EP) to free enzyme (E) and product (P), are hardly reported. Considering the importance of rates in medicine, pharmaceutics, nutrition, engineering, *etc*., and in order to be able to utilize experimental data for the determination of verifiable and quantifiable rate constants as an important goal, it has become very imperative to conduct this research with the following objectives: 1) To derive equations for the first order rate constants, k_{+2} and k_{-2}, for the processes, ES → $E^{\#}S^{\#}$ and $E^{\#}S^{\#}$ → ES, respectively; 2) To quantify by calculation the first order rate constant for product release; 3) To ultimately quantify the rate constants, k_{-2} and k_{+2}; and 4) To advise the reactor, process, chemical engineers, *etc.* in different industrial concerns on the usefulness of k_{-2} and k_{+2}.

1.1 Significance

The nascent equations enabled the determination of the life span of various individual steps in an enzyme-catalyzed reaction pathway. The results obtained should be a working guide for the design of appropriate kinetic experiments for both medical (pharmaceutical) and nonmedical (industrial) applications. There is always a transition state, which is regarded as activated ES in this research. Its rate of formation and transformation into enzyme product complexes could be very useful in the conceptualization of models relevant to industrial design. The research has shown that the sum of the durations of the individual reaction steps is approximately equal to the total time for each catalytic cycle in the forward direction. This is equally applicable to the backward reaction.

2.0 THEORY

In this section, the kinetic schemes in the literature and selected kinetic equations are to be revisited, and new equations are to be derived and evaluated.

2.1 Examination of simple and practical kinetic schemes

There are a lot of kinetic schemes in the literature [1, 11, 16, 17]. The most reoccurring, as stated earlier, is the scheme given as: E+S⇌ES → E + P. However, the scheme that is more similar to the kind of scheme to be explored in this research is the kind given by Johnson (1). This is scheme 1 below. In that scheme, the author was of the opinion that the rate constant (k_3) for the process EP → E + P is much greater than k_2, which was equated to k_{cat} (the catalytic rate, the turn-over number). The author has the impression that equilibrium exists between ES and EP. However, by indicating that the rate constant for the process EP → ES is equal to zero, there is an admission that such a process does not occur if, in particular, the enzyme in this research is a hydrolase lacking the power to synthesize. These two views of the author are in line with the goal of this research, which entails showing that the overall duration of the enzyme-catalyzed reaction pathway is greater than the specific duration of each phase of the reaction pathway.

Furthermore, enzymes primarily accelerate reactions by lowering the "energy barrier" via the formation of an activated enzyme-substrate complex ($E^{\#}S^{\#}$). The complex may exist for an infinitesimal duration, either proceeding to EP or ES if unfavorable conditions arise for whatever reason. Thus, in a scheme such as E + S ⇌ ES ⇌ $E^{\#}S^{\#}$ → EP→P + E, the rate constant for the process ES →$E^{\#}S^{\#}$ is designated as k_{+2}, while the rate constant for the process $E^{\#}S^{\#}$→ES is designated as k_{-2}. For the

purpose of simplification, k_3 may stand for the rate constant for the process $E^{\#}S^{\#} \rightarrow EP \rightarrow P$ that remains irreversible.

Scheme 1: Formation of enzyme-substrate complex (ES), transformation to enzyme-product complex (EP), and dissociation into free enzyme and product [1].

The enzyme-substrate complex is formed following the occurrence of encounter-complex formation (Figure 1). This takes longer because of the frequency of encounter-complex formation, which depends on the speed of reactants under the prevailing influential thermodynamic temperature. The number of molecules of ES formed per unit time is the first-order rate constant designated as k_{ES} while the second rate constant is given as usual as k_1.

$$E + S \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} ES \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} EP \xrightarrow{k_3} E + P \qquad \text{Scheme} \qquad (1)$$

As explained in the literature [11], there could be an activated complex formation given as $E^{\#}S^{\#}$, which represents a transition intermediate between ES and EP. This is such that the likelihood of EP reversing to ES is impossible as long as the enzyme is neither a synthase nor a synthetase; however, there is a strong likelihood that $E^{\#}S^{\#}$ reverses back to ES for whatever reason. Assuming that $E^{\#}S^{\#}$ and EP have very similar life spans, there is a possibility of the process $ES \rightleftharpoons E^{\#}S^{\#} \rightarrow EP$ such that the real equilibrium lies between ES and $E^{\#}S^{\#}$. The second scheme, which summarizes the issues raised, is stated as follows:

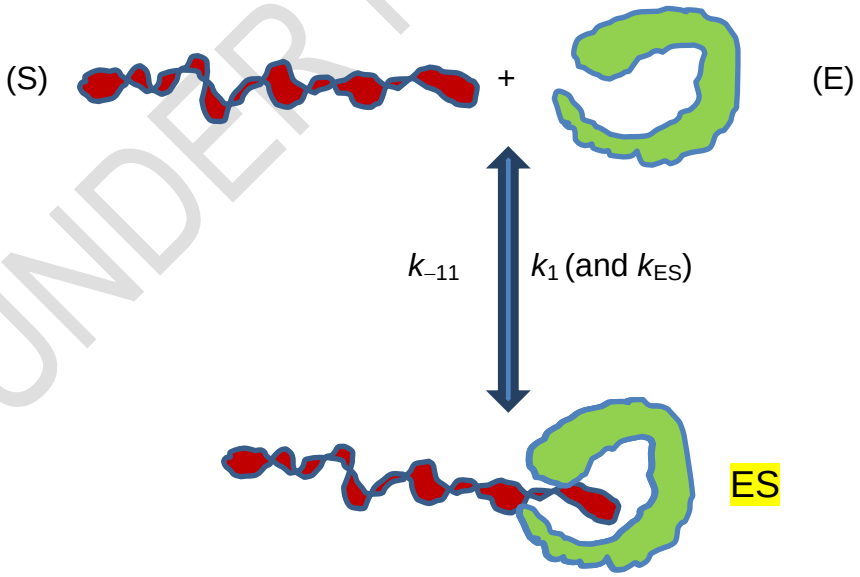

Figure 1: Illustration of substrate (S) combing with the enzyme (E) in a physico-chemical process to form ES; k_{-11} and k_1 are the 1st-order rate constants for the dissociation of ES to free E and free S and the association of E and S to form ES, respectively. k_{ES} is the reciprocal of the duration of ES formation. The point illustrated is that there is duration of the dissociation of ES to free S and free E; the duration is the life span of the ES that may not be activated for product formation or that may have been deactivated.

As soon as stable ES is formed, the first chemistry of the process begins. This is the formation of the transition state (TS), otherwise referred to as the activated ES complex (Figure 2); this is a necessary step before breaking and making bonds. For whatever reason, the TS may become deactivated into ES, with the possibility of the latter dissociating into E and S.

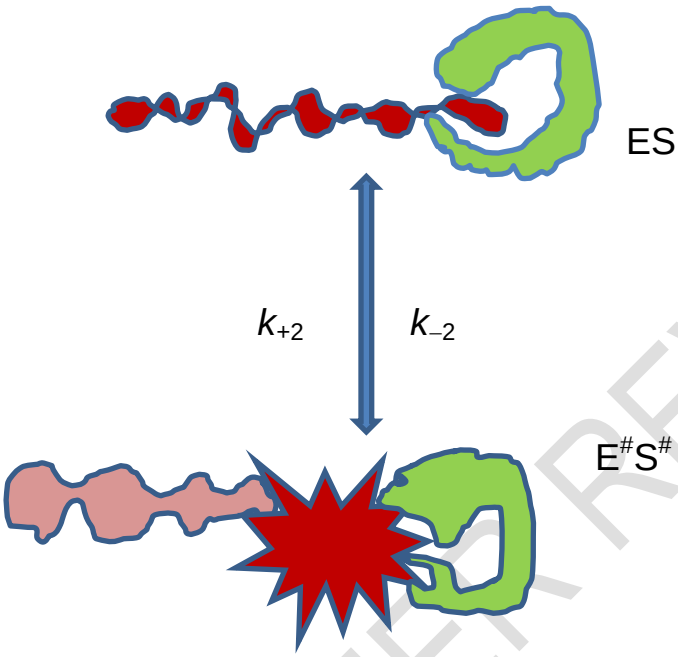

Figure 2: The illustration of the first chemistry of the reaction between the enzyme and the substrate shows that the enzyme decreases the "energy barrier"—the lowering of the activation energy—preceding bond breaking and making The activated enzyme-substrate (ES) complex (known as the transition state, TS) may either proceed to enzyme-product complex formation after the chemistry or, for whatever reason, may be deactivated and reverse to ES, which may dissociate to free enzyme, E, and free substrate, S.

Modified scheme 1, which shows the presence of an activated enzyme-substrate complex

$$E + S \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} ES \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} E^{\#}S^{\#} \rightarrow EP \overset{k_3}{\rightarrow} E + P \qquad \text{Scheme} \qquad (2)$$

The complex $E^{\#}S^{\#}$ has an infinitesimal life span, proceeding either to EP or ES; EP breaks up into E and P as quickly as it is formed. There is every possibility that the transition state can proceed to EP after bond breaking and making (Figure 3); once achieved, there is no question of EP reversing to TS, let alone ES, because the enzyme is neither a synthase nor a synthetase. Therefore, the process depicted above is summarized as "$E + S \rightleftharpoons ES \rightarrow E + P$." If the duration of the process ($EP \rightarrow E + P$) is "t", the first-order rate constant is denoted as k_3 (this is = 1/t).

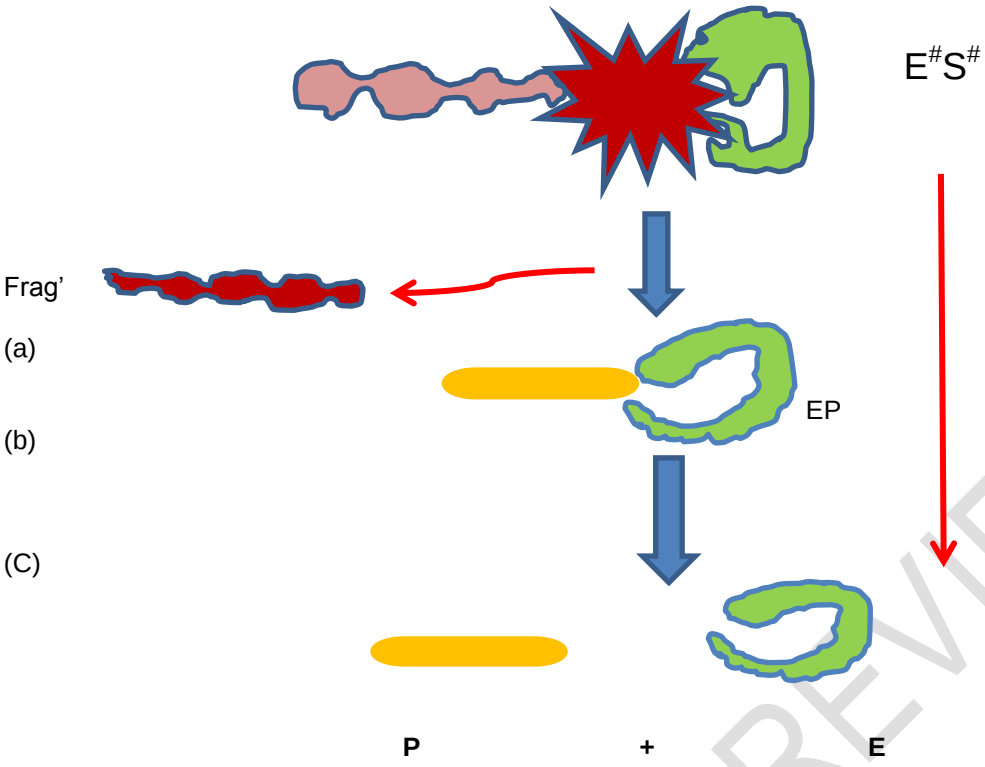

Figure 3: The illustration of the making and breaking of bond within the active-site before product release. If a polymer like starch is a substrate, a substrate fragment may be the first to be released. (a) A substrate fragment is released as applicable to a polymer; (b) the enzyme-product (EP) complex remains for a transient duration after the chemistry; (b) while the enzyme-product (EP) complex remains for a transient duration after the chemistry; (c) the product combines noncovalently with the active site to form an enzyme-product complex, which dissociates into free enzyme and product. Frag' denotes a fragment of the polymer of starch.

The equations (Eqs (1) and (2)) [1] to be revisited are given below.

$$k_{\text{cat}} = \frac{k_{+2}k_3}{k_{+2}+k_{-2}+k_3} \tag{1}$$

$$K_{\text{M}} = \frac{k_{+2}k_3+k_{-1}(k_{-2}+k_3)}{k_1(k_{+2}+k_{-2}+k_3)} \tag{2}$$

In order to ascertain the likely validity of the equations to be derived based on Eqs (1) and (2), both equations are examined. The outcome shows that both equations have a common denominator if Eq. (2) is recast as follows: From Eq. (1) is the following:

$$k_{+2} + k_{-2} + k_3 = k_{+2}k_3/k_{\text{cat}}$$

From Eq. (2) is the following:

$$k_{+2} + k_{-2} + k_3 = [k_{+2}k_3 + k_{-1}(k_{-2} + k_3)]/k_1K_{\text{M}}$$

Therefore,

$$k_{+2}k_3/k_{\text{cat}} = [k_{+2}k_3 + k_{-1}(k_{-2} + k_3)]/k_1K_{\text{M}} \tag{3a}$$

Making k_{+2} subject of the formula gives:

$$k_{+2} = \frac{k_{-1}k_{cat}(k_{-2}+k_3)}{k_3 k_1 K_M - k_{cat}k_3} \quad (3b)$$

Making k_{+2} the subject of the formula in Eq. (1) yields:

$$k_{+2} = \frac{k_{cat}(k_{-2}+k_3)}{k_3 - k_{cat}} \quad (3c)$$

Hence, Eq. (3c) is the equivalent of Eq. (3b) such that:

$$\frac{k_{-1}k_{cat}(k_{-2}+k_3)}{k_3 k_1 K_M - k_{cat}k_3} = \frac{k_{cat}(k_{-2}+k_3)}{k_3 - k_{cat}}$$

Making K_M subject of the preceding formula gives after simplification:

$$K_M = \frac{k_3(k_{-1}+k_{cat}) - k_{-1}k_{cat}}{k_3 k_1} \quad (4)$$

Meanwhile, $K_M k_1$ is = k_{-1} + k_{cat} such that the expansion of Eq. (4) gives first:

$$k_{-1}\,k_3 - k_{-1}\,k_{cat} + k_{cat}\,k_3 = k_3\,k_{-1} + k_3\,k_{cat} \quad (5)$$

Elimination of common terms in the immediate preceding equation gives:

$$-k_{-1}k_{cat} = 0 \quad (6)$$

The result indicated by Eq. (6) shows that there may be a problem in either Eq. (1) or Eq. (2). Such a conclusion may be too early at this stage.

However, Eqs (1) and (2) are revisited through a different route. From the two equations, the following is given:

$$\frac{k_{+2}k_3}{K_M k_1} + \frac{k_{-1}(k_{-2}+k_3)}{K_M k_1} = \frac{k_{+2}k_3}{k_{cat}} \quad (7)$$

Factorizing and simplifying gives:

$$k_{+2}(K_M k_1 k_3 + k_{cat}k_3) = k_{cat}\,k_{-1}(k_{-2}+k_3) \quad (8a)$$

$$k_{+2} = \frac{k_{cat}k_{-1}(k_{-2}+k_3)}{K_M k_1 k_3 - k_3 k_{cat}} \quad (8b)$$

Directly from Eq. (1) is Eq. (3c) and bringing Eqs (3c) and Eq. (8b) together gives

$$k_{-1}/(k_1 k_3 K_M - k_3 k_{cat}) = 1/(k_3 - k_{cat}) \quad (9)$$

Realizing that k_1K_M is = k_{-1} + k_{cat} and upon rearrangement of Eq. (9) one obtains:

$$\frac{k_{-1}(k_3 - k_{cat})}{k_3} + k_{cat} = k_1K_M = k_{-1} + k_{cat} \quad (10a)$$

Equation (10a) leads to:

$$k_{-1}(k_3 - k_{cat}) = k_3k_{-1} \quad (10b)$$

Equation (10b) has two possible outcomes:

$$-k_{cat} = 0 \quad (11a)$$

$$-k_{-1}k_{cat} = 0 \quad (11b)$$

Equation (11b), which is a reproduction of Eq. (6) and Eq. (11a), gives enough evidence for the invalidity of either Eq. (1) or Eq. (2).

2.2 The derivation of the equations for the calculation of kinetic parameters that are not discernible in a reaction pathway

There is an argument that the data obtained in the steady-state provide only indirect information to define the pathway [1]. The parameters k_{cat} (catalytic rate) and K_M (Michaelis-Menten constant) are regarded as steady-state parameters, even though the latter is attained at substrate concentration at half the maximum velocity of catalysis, which is far from pre-steady-state, closer to steady-state, and next to the zero-order zone if the substrate concentration ($[S_0]$) at zero time is saturating. To begin with, the following may be true:

$$\frac{1}{k_{cat}} = t_{ES} + \frac{1}{k_{+2}} + \frac{1}{k_3} \quad (12)$$

where t_{ES} is the duration for the formation of ES whose derivation is as described in the literature [18]. Equations (41c–44) of the paper [18] are being rederived to correct a technical error that was unfolded recently in the light of this research. This underpins the danger of abandoning a stepwise approach in derivations, for which regret and apology are expressed to the scientific community. However, the equation [18] from which t_{ES} (=1/k_{ES}) is calculated does not present any technical problem of a derivational nature.

From Eq. (12) is the following:

$$\frac{1}{k_{+2}} = \frac{k_{ES}k_3 - k_{cat}k_3 - k_{cat}k_{ES}}{k_{cat}k_{ES}k_3} \quad (13a)$$

$$k_{+2} = \frac{k_{cat} k_{ES} k_3}{k_{ES} k_3 - k_{cat} k_3 - k_{cat} k_{ES}} \quad (13b)$$

At this juncture, one can point out that, if $[S_0]$ is much greater than $[E_0]$, and the duration of the assay (t) is very short or rather transient, exp. ($-k\,t$) $[S_0]$ (k is the pseudo-first-order rate constant for the utilization of substrate) should be approximately equal to $[S_0]$. The duration of the assay is much shorter than the normal laboratory duration of the assay. The time scale of the assay in question is a micro-minute (or less). This time scale can be calculated for each value of $[E_0]$ for different substrate concentrations, as described in the literature [18]. The implication is that the rate (v_1) of formation of the enzyme-substrate complex (ES) is:

$$v_1 \left(\text{or } \frac{d[ES]}{dt}\right) = k_{ES}[E_0] \quad (14)$$

where a first-order rate constant, k_{ES} is approximately equal to $k_1[S_0]$. Equation (14) notwithstanding, a method for the determination of the maximum value of k_{ES} has been described in the literature [18]. The method for the determination of lower values of k_{ES} has been shown in the literature [11]. In order to derive the equation for k_{-2}, one should recall Eq. (12), and following that, state first the equation of k_{-1} in terms of time as follows:

$$\frac{1}{k_{-1}} = \tau_- + t_{-2} \quad (15)$$

where τ_- (*i.e.*$1/k_{-11}$) and t_{-2} are the durations of the dissociation of ES to free E and free S and the duration for the transformation or deactivation of $E^{\#}S^{\#}$ to ES respectively.

$$\frac{1}{k_{-1}} = \frac{1}{k_{-11}} + \frac{1}{k_{-2}} \quad (16)$$

where k_{-11} is the first-order rate constant for the dissociation of ES to free E and free S. One can make k_{-2} subject of the formula in Eq. (16) to give:

$$k_{-2} = \frac{k_{-1} k_{-11}}{k_{-11} - k_{-1}} \quad (17)$$

Given that $k_{-1} = K_M k_1 - k_{cat}$, the K_M can be expressed in terms of all the rate constants except k_{+2} and k_{-1} as follows: First $K_M k_1 - k_{cat}$ is substituted into Eq. (17) to give:

$$k_{-2} = \frac{(K_M k_1 - k_{cat}) k_{-11}}{k_{-11} - (K_M k_1 - k_{cat})} \quad (18a)$$

After rearrangement, K_M can be expressed as:

$$K_M = \left(\frac{k_{-11}(k_{-2}+k_{cat})+k_{-2}k_{cat}}{k_{-2}+k_{-11}}\right)/k_1 \quad (18b)$$

Following the example of Eq. (18b), and given that $k_{cat} = K_M k_1\text{-}k_{-1}$, the K_M can also be expressed in terms of all the rate constants except k_{+2} and k_{cat} as follows: First, $K_M k_1\text{-}k_{-1}$ is substituted into Eq. (13b) to give:

$$k_{+2} = \frac{(K_M k_1 - k_{-1})k_{ES}k_3}{k_{ES}k_3 - (K_M k_1 - k_{-1})k_3 - (K_M k_1 - k_{-1})k_{ES}} \quad (19a)$$

After rearrangement, K_M can be expressed as:

$$K_M = \left(\frac{k_{ES}k_3 k_{+2}}{k_{ES}k_3 + (k_3 + k_{ES})k_{+2}} + k_{-1}\right)/k_1 \quad (19b)$$

There is a need at this juncture to state that the process "$E^{\#}S^{\#} \rightarrow$ EP" is the actual catalytic event, which can be given its exclusive duration without necessarily specifying the duration of $E^{\#}S^{\#}$ formation separately from the duration of the transition to EP.

It is not the characteristic of scientists to ignore what they consider trivial based on their conception of the simplicity of the mathematical content of an article, but to do so may lead to misinformation and ambiguity. Thus, while admitting that Eqs (18b) and (19b) can give similar results, any difference should be the result of approximations of the original raw data. What should not be ignored are the equations as corollaries, which express the mutual dependence of k_{-1} and k_{cat}. Thus, bringing Eqs (18b) and (19b) together, simplifying, and making k_{-1} and k_{cat} subjects of the formula gives:

$$k_{cat} = \left[\left(\frac{k_{ES}k_3 k_{+2}}{k_{ES}k_3 + (k_3 + k_{ES})k_{+2}} + k_{-1}\right)(k_{-11} + k_{-2}) - k_{-11}k_{-2}\right]\frac{1}{k_{-11}+k_{-2}} \quad (20)$$

$$k_{-1} = \frac{k_{cat}(k_{-11}+k_{-2}) + k_{-11}k_{-2}}{k_{-11}+k_{-2}} - \frac{k_{ES}k_3 k_{+2}}{k_{ES}k_3 + (k_3 + k_{ES})k_{+2}} \quad (21)$$

3.1. MATERIALS AND METHODS

3.1.1 Materials

3.1.1.1 Chemicals

The enzyme that was assayed is *Aspergillus oryzae* alpha-amylase (EC 3.2.1.1), and insoluble potato starch was the substrate; both were purchased from Sigma-Aldrich, USA. Tris 3, 5-di-nitro-salicylic 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 approximately 52 kDa [19, 20]. Distilled water was purchased from the local market. As a word of caution, readers of this paper should be aware that the use of the same enzyme in articles by the same author(s) is strictly due to budgetary constraints; however, this is not a serious concern because each paper addresses different issues, such as the evaluation of new models.

3.1.1.2 Equipment

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

3.2 Methods

3.2.1 Preparation of reagents and assay

The method of assaying the enzyme is Bernfeld's method [21], which uses gelatinized potato starch, whose concentration range is 5–10 g/L. The reducing sugar produced upon hydrolysis of the substrate at 20 ^{o}C using maltose as a standard was determined at 540 nm with an extinction coefficient approximately equal to 181 L/mol.cm. The assay took 3 minutes to complete. A mass concentration equal to 1.667 mg/L of *Aspergillus oryzae* alpha-amylase was prepared in a Tris-HCl buffer at *p*H 6.9; there were special considerations in the choice of *p*H and temperature. The evaluation of new equations was the only overriding interest.

3.2.2. The determination of rate constants

The pseudo-first-order rate constant for the utilization of gelatinized starch is determined as described in the literature [22]. The second-order rate constant for the formation of the enzyme-substrate (ES) complex and the duration of its formation are determined as described elsewhere [18]. While $V_{max}(k_{cat}[E_0])$ is a well-known parameter, $V_{-max}(k_{-1}[E_0])$ for the release of free E and S from ES is not well-known. The first-order rate constant, k_{-1}, for the release of free E and free S is also determined as described in the literature [18]. The Lineweaver-Burk [23] plot was used for the determination of the K_M and V_{max}. Equations (13b) and (17) were for the calculation of k_{+2} and k_2, respectively. The determination of k_3 is as described in the literature [18], but a corrected version is stated herein as follows:

$$\mathrm{In}\left(\frac{K_M k}{k_{cat}[S_0]}\mathrm{In}\frac{[E_0]-[ES](t_{cat}-\tau)}{[E_0]}+1\right)=k\,\tau \quad (22a)$$

$$\mathrm{In}\left(\frac{K_d k_-}{k_{-1}[S_0]}\mathrm{In}\frac{[E_0]-[ES](t_{cat}-\tau)}{[E_0]}+1\right)=k_-\,\tau_- \quad (22b)$$

To refresh your memory, the rate constants k_{cat} and k_{-1} represent the catalytic first-order rates for the processes ES → $E^{\#}S^{\#}$→EP→E+P and $E^{\#}S^{\#}$ → ES→E + S, respectively. The durations τ and τ- are the durations for the processes leading to the release of product and free enzyme and free enzyme and substrate, respectively.

3.3 Statistical analysis

Assays were conducted in triplicate. Micro-Soft Excel was used for the determination of the standard deviation (SD) for the arithmetic mean values.

4. RESULTS

To begin with, it is necessary to point out that the result of this research with respect to usual Michaelian parameters may differ from those reported for the same enzyme in different concentrations and assay conditions in the literature [11, 18]. This is due to different reasons in accordance with the aims and objectives; comparison is taken to be unnecessary because the concerns covering the aims and objectives are quite different, with hardly any data for the first-order rate constants of the kind investigated in the literature. This research has, however, been able to produce equations for the determination of the values of hitherto unmeasurable first-order rate constants for the transition from ES to $E^{\#}S^{\#}$ and from the latter to ES. Similar to the view elsewhere (1), the process $E^{\#}S^{\#}$ → ES is possible, but the process EP → $E^{\#}S^{\#}$, let alone E + P → ES, is thermodynamically unfeasible. Upon algebraic appraisal and analysis of the equations reported in the literature (1), it was observed that the kinetic constants, k_{cat} and K_M, cannot produce the values of the first-order rate constants, k_{-2} and k_{+2}, and if the latter values are known *ab initio* by whatever means, substituting them into the literature equations (1) cannot, beyond unnecessary subjectivity or sentiment, reproduce the elements of the Michaelis-Menten constant, as evidenced in the outcome of algebraic appraisal and analysis (Eqs 6 and 11b), The equations (13b), (17b), (18b), and (19b) derived in this research can positively be used to quantify the corresponding parameter; quantification is, however, restricted to k_{-2} and k_{+2}, though this requires information about the K_M and V_{max}.

Proceeding further requires that certain points be made known. Since the velocity is linearly proportional to the substrate concentration if the latter is low, attributing k_{cat}/K_M to a first-order zone is a little perplexing given that the constant, k_{cat}, is asymptotically approached at much higher substrate concentrations [24]. The admissibility of K_M as one best estimated in a mixed-order or pseudo-first-order zone calls into question the belief (or, mildly speaking, the suggestion) that the ratio k_{cat}/K_M is best measured in a first-order zone [24]. If, as stated categorically, K_M is merely a ratio of the parameters (perhaps the sum of ratios given as $(k_{-1}/k_1) + k_{cat}/k_1$) estimated at the zero-order zone and the first-order zone, then it is an overt contradiction to describe k_{cat}/K_M as a steady-state parameter [24]. This issue was proposed and discussed elsewhere [25]. So, in this study, zero-order kinetic parameters (specifically, the Michaelis-Menten parameters K_M and V_{max}) are used rather than steady-state kinetic parameters, which is significant progress in this section.

As vividly shown in Table 1, the main Michaelian parameters are the zero-order parameters: 10.058 exp. (-4) M/min for the V_{max} and 60.527 g/L for the K_M. The second-order rate constant for the formation of ES is well known in most general text books, but in the rate equation for the formation of ES, the first-order rate constant (preferably a pseudo-first-order rate constant) can be k_1 $[E_0]$ or k_1 $[S_T]$, depending on whether the assay was performed with a fixed concentration of the S and a varying concentration of the E or with a fixed concentration of the E and a varying concentration of the S. The maximum velocity V_{max} for product formation and release differs by 99.4% from the maximum velocity V_{-max} for ES dissociation into free E and free S; this is how k_{-1} compares to k_{cat}. The zero-order dissociation constant is similar to the K_M, the difference being 0.605 % of the K_M.

Table 1. The values of kinetic parameters-the zero-order parameters

Parameters	Values (A-mean ± SD)
V_{max}/ exp.(– 4) M/min	10.058±0.211
V_{-max}/ exp.(– 4) M/min $V_{-max} - V_{max}$	1677.564±28 99.4 % of V_{-max}
K_M/g/L	60.527±3.002
K_d/g/L $K_M - K_d$	60.161±0.989 0.605 % of K_M
K_{ES}/exp.(+6)/min $K_{ES} - k_{-11}$	5.901±0.225 8.914 % of k_{ES}

k_{cat} /exp.(+ 4)/min $k_{+2} - k_{cat}$	3.138±0.007 10.65 % k_{+2}
K_1 /exp.(+4)/L/g.min	8.7±0.14
k_{-1}/exp.(+6)/min $k_{-1} - k_{cat}$	5.234±0.086 99.4 % of k_{-1}
(k_{cat} + k_{-1})/exp.(+6)/min	5.266±0.087
k_3/exp.(+5)/min $k_3 - k_{cat}$	3.155±0.061 90.054 % of k_3
k_{-11}/exp.(+6)/min	5.375±0.013
Eq.(13b):k_{+2}/exp.(+4)/min	3.512±786
Eq.(17): k_{-2}/exp.(+8)/min $k_{-2} - k_{+2}$	2.377±0.546 99.985 % of k_{-2}

A stands for arithmetic and SD means standard deviation.

The first-order rate constants (k_{cat} and k_{+2}) are similar in magnitude but differ, the difference being approximately 10.65 % of the k_{+2}-value. The k_{cat} value differs from the k_3 value by 90.054 % of the latter; the opposing processes, E + S $\rightarrow$ ES and ES $\rightarrow$ E + S, occurred at first-order rates, k_{ES} and k_{-11}, respectively, which differ by approximately 8.914 % of the k_{ES}. The K_M and K_d served the purpose of determining by calculation the values of k_3 and k_{-11}, respectively, as explained in the method section. As one should expect, k_{-1} is calculated by subtracting k_{cat} from the sum of k_{-1} and k_{cat} (Table 1), the equivalent of $K_M k_1$.

5. DISCUSSION

Next, one may consider matters of general interest connected to the issue of measurements, which this research addressed as part of its goal. It is already known that in any study on enzyme kinetics, there are measureable and unmeasurable quantities [24]. In this research, categorization is expanded to cover 1) primary measurables, 2) secondary measurables, and 3) tertiary measurables. Examples of the primary measurables are the mass concentrations of the substrate, enzymes, coenzymes, and cofactors, if applicable, and environmental conditions such as pH, ionic strength, temperature, and, if necessary, pressure. Examples of the secondary measurables are the initial rates or the velocity of the catalytic action, changes in absorbance as applicable to conformational changes

of the protein as enzyme or substrate, RNA, DNA, *etc*. Examples of tertiary measurables are those parameters that are either graphically or calculationally determined; such include Michaelis-Menten parameters, all first- and second-order rate constants, and the dissociation constant. Therefore, in this research, attention is paid to directly and indirectly determinable (measurable) rate constants in Michaelian enzyme-catalyzed reactions. The measurement in this research falls into the tertiary category. As shown in Table 1, they are first-order rate constants that the equations in the literature cannot adequately address; the rate constants are k_{-2}, k_{+2}, and k_3.

Most importantly, the observed first-order rate constants are greater than the usual rate constants, k_{cat} and k_{-1}. The implication, as noted in the literature [1, 16, 25], is that the overall duration of all the catalytic events, both physico-chemical and biochemical, is greater than the duration of any of the catalytic events. Each of the durations (not shown) is equal to the reciprocal of the zero-order, first-order rate constant. They are in the order of magnitude: $k_{-2}>k_3>k_{+2}$ (Table 1). The corresponding order of magnitude on a time scale is: $1/k_{-2}\ll 1/k_3<k_{+2}$.

The need for stability of enzyme-substrate complexes has been observed in the literature (1, 24, 26). Following an encounter-complex formation, a kinetically driven process, with what has been described as the substrate's right geometry in terms of structural and electrostatic orientations (1), the enzyme-substrate complex is formed: But this is purely governed by physico-chemical factors. The (bio) chemical factor is the function of the enzyme, which, through its internal mechanism, assumes an activated state that can also activate the substrate. This facilitates the breaking and making of bonds so that either a product or a substrate fragment, or both, can be released, depending on the nature of the substrate. Here the view of Van Slyke and Cullen [26] becomes very relevant, though they refer to "ordinary" rather than activated ES ($E^{\#}S^{\#}$). They admit that the process ES $\rightarrow$ E + S can occur because the interaction between S and E is noncovalent and dominated by hydrogen bonding and electrostatic effects, which are strongly subject to environmental perturbation; but upon assumption of the chemistry by the enzyme, a stronger binding interaction is promoted to enable the breaking and making of new bonds for product formation. If, on the other hand, a stronger binding interaction leads to rigid covalent bond formation without any form of structural flexibility, then the enzyme is said to be inhibited. All these point to the importance of a catalytically functional activated complex. As shown in Table 1, the rate constants, k_{-2} and k_{+2}, for the deactivation of $E^{\#}S^{\#}$ to ES and the converse, respectively, are widely different, the difference being approximately 99.985 % of the k_{-2}. This may account for the large value of k_{-1}, which is exceedingly greater than k_{cat}. This may imply that there is a

very high probability (0.99985), $k_{-2}/(k_{-2}+k_{+2})$, of the occurrence of occasional deactivation of the activated ES complex.

It behoves the process, chemical engineers, *etc.* to continue to consider the following fundamental facts: the role of thermodynamic temperature as a driver of kinetic aspects of enzyme-catalyzed reactions beyond kinetic stability; this implies that the speed at which an encounter-complex is formed depends on the translational diffusion coefficient and the rate of effective collision [27, 28]. Therefore, any factor—inorganic or organic osmolyte, genetic modification, or perhaps immobilization—that can sustain the stability of the enzyme while increasing the temperature even above the optimum temperature should be encouraged. In this era of the need for environmentally friendly fuel consumption, the optimization of the production of biofuels requires that the k_{+2} values be boosted while diminishing the k_{-2} values. The concern of clinical dieticians, the pharmaceutical industry, and medics is how best to compulsorily control diabetics; in this regard, ways of boosting the k_{-2} values should be encouraged.

On a matter of general interest and importance arising from this study in light of the concern for validity, stability of ES, *etc.*, one begins by stating that the vast array of literature materials covering enzyme kinetics is evidence of the importance of kinetic parameters; besides, there are no kinetic studies without rates and cognate rate constants. So, a lot of research papers on the kinetics of enzyme-catalyzed reactions with associated models abound in the literature [29-34]. Also, researchers [35, 36] have published kinetic equations of enzyme-catalyzed reactions using kinetic schemes. This is in light of the importance of kinetics and associated rate constants in the control of enzymes in various applications in various industries, viz., paper, food, detergent, fuel ethanol, *etc.* [37].

Kostylev and Wilson [32] recognize the question of steps in enzyme-catalyzed reactions; in agreement with Kopemann [29], the authors believe that some enzyme-catalyzed reactions are time-dependent while others are independent. It would appear that reactions may be surface diffusion-dependent, on account of which the reaction rate is time-dependent purely as a fractal system. This statement implies that if the reaction is not surface-diffusion-dependent, the rate of reaction may not be time-dependent, regardless of whether the system is fractal-like. However, if there are steps in the catalytic pathway, then each step must have a life span and consequently have a rate constant, as evidenced in this investigation with artistic illustrations (Figures 1–3).

The point illustrated by Figure 1 is that there is a duration of the dissociation of ES to free S and free E; the duration is the life span of the ES that may not be activated for product formation or

that may have been deactivated. The most important illustration is that steps are recognized such that the reverse first order rate constant, k_{-1}, is the net rate constant for the process, $E^{\#}S^{\#} \rightarrow ES \rightarrow E + S$; The first order rate constant for $E^{\#}S^{\#} \rightarrow ES$ is k_{-2}. As stated in the text, the overall rate constant for the forward reaction leading to product release is the catalytic rate constant (k_{cat}). Figure 2 illustrates the fact that the activated ES complex (known as the transition state, TS) may either proceed to enzyme-product complex formation after the chemistry or, for whatever reason, may be deactivated and reverses to ES, which may dissociate to free enzyme E and free substrate S. The steps, including the chemistry of the process, the lowering of the "energy barrier" and the breaking and making of bonds, are illustrated in Figure 3. After the chemistry, a substrate fragment is released in a nonprocessive fashion shortly before the release of the free enzyme and product.

Rate constants are very essential for both experimental and industrial design; this research produced algebraic models that can be explored for the calculation of the rate constant (Table 1) for the processes $ES \rightarrow E + S$ and $E^{\#}S^{\#} \rightarrow ES$ that are part of the steps in the dissociation pathway ($E^{\#}S^{\#} \rightarrow ES \rightarrow E + S$) in support of the notion of steps or stages in the catalytic cycle (16). The rate constants for the forward direction covering the processes $E + S \rightarrow ES$; $ES \rightarrow E^{\#}S^{\#}$; and $(E^{\#}S^{\#} \rightarrow EP) \rightarrow E + P$ are also given (Table 1). The rate constant in parenthesis is not given. It's not that it isn't a possibility, but the lack of a conceptual framework appears to rule it out for the time being.

The importance of activating an enzyme before assuming catalytic action is well noted in the literature [38]. Enzyme-catalyzed reactions can be evaluated for possible observance of the Arrhenius principle, not just for kinetic stability but also for thermodynamic stability (3). This is very important for the process, production, and chemical engineers. The process $ES \rightarrow E^{\#}S^{\#}$ illustrates activation, while the process $E^{\#}S^{\#} \rightarrow ES$ illustrates deactivation. They provide distinct stages for precisely quantifying the kinetic and thermodynamic stability of reaction mixture species. The pieces of information about on- and off-rate constants presented in this research can enhance both the mechanistic understanding and rational design of enzymes through genetic modification (site-directed mutation) for industrial application and also with the possibility of medical application. The paucity of such information has been a challenge in the application of some enzymes, notably cellulase [39]. On grounds of general interest, it is necessary to state that cellulase is a complex composed of three different enzymes, namely endoglucanase (E.C.3.2.1.4), exoglucanase (E.C.3.2.1.91), and beta glucosidase (E.C.3.2.1.21).

Researchers are also concerned about the validity, based on certain criteria, of various assumptions underlying the derivation of kinetic equations, particularly the Michaelis-Menten equation [40-47]. The implication is that certain kinetic (or Michaelian) constants may be rendered invalid if the criteria, under which the acclaimed assumption is stated, if stated at all, are incorrect. In recognition of the fact that there could be assays in which $[E_0]$ is not much less than $[S_0]$, the possibility of enzyme kinetics far from the sQSSA [39] cannot be ruled out; nonetheless, this research explored enzyme concentrations approximately 2.4–fold lower in concentration than the lowest concentration of the substrate. Despite all of the mathematical exposition and analysis, the main takeaway is that, in addition to the condition of validity, such as the relationship $[E_0]/[S_0] \ll 1$, there is another condition given as $[E_0]/([S_0]+ K_M) \ll 1$ that validates any results arising from an assay in which *ab initio* $[S_0] \gg [E_0]$ [48]. This is the case in this study. Thus, validity is established.

For the purpose of clinical and industrial applications, it is also important that the calculated and graphically determined kinetic constants are accurate. This has prompted concern for alternative means of generating the primary Michaelian parameters by means of direct linear or reciprocal variants [49, 50], in addition to nonlinear regression [51]. In this research, a double reciprocal plot was explored for convenience in the evaluation of nascent equations, which can still be reevaluated with much higher precision data derived from high-tech instrumentation in addition to the use of either nonlinear regression or a direct linear plot.

There has also been concern for the difference between steady-state kinetic parameters, the outcomes of linearity in the relationship between rate and substrate concentration, and zero-order kinetic parameters, the outcomes of nonlinearity—the asymptote [25]. Similar views seemed to have been held years ago, as cited by Bersani and Dell'Acqua [46] with reference to the work of Heineken *et al*. [36]. The authors opined that the assumption that the ES (or C) is approximately constant during the initial transient can be misapplied, leading to a misinterpretation of the quasi-steady assumption. To them, the true situation is that, if an asymptotic expansion of the solution of the ordinary differential equations (ODEs) governing the process is obtained with respect to an appropriate parameter, the sQSSA is the zero-order approximation of the solution. According to Heineken *et al*. [41], the true Michaelian parameters are mixed-order with respect to the K_M and zero-order with respect to the V_{max}, rather than steady-state parameters.

It seems, before now, that no attempt has been made in the literature to quantify the life span (*i.e*., duration) of individual reaction steps frequently referred to in the literature [16, 18, 52] based on

first-order kinetics. Earlier researchers focused on the importance of the specificity constant k_{cat}/K_M and its direct determination [1, 24], with less attention paid to the reverse first-order rate constant for the dissociation of ES to free E and free S until recently [25]. Apart from being one of the very few instances where the life span of various individual steps in an enzyme-catalyzed reaction pathway is quantified, the results so obtained should be a working guide for the design of appropriate kinetic experiments for both medical (pharmaceutical) and non-medical (textile, biofuel, *etc.*, industries) applications. The result has helped to re-emphasize that there must always be a transition state, which is regarded as activated ES in this research, whose rate of formation and transformation to enzyme-product complexes could be very useful in the conceptualization of models relevant to industrial design. The research has shown that, truly, the sum of the durations of the individual reaction steps is less than the total time for each catalytic cycle in the forward direction. This is equally applicable to the backward reaction.

6.0 CONCLUSION

All the equations for the calculation of zero-order first-order rate constants (ZOFORC) for the activation of the enzyme-substrate (ES) complex and its deactivation, k_{+2} and k_{-2}, respectively, were derived. The value of ZOFORC for the dissociation of the enzyme-product (EP) complex to free E and P is 3.155 exp. (+5)/min; the values of k_{+2} and k_{-2} are 3.513 exp. (+4) and 2.377 exp. (+8)/min, respectively. The study has successfully revealed that the dissociation of ES into E and S may not be at a single first-order rate constant; rather, there is first a first-order rate constant for the process $E^{\#}S^{\#} \rightarrow ES$, and then $ES \rightarrow E + S$. Ultimately, it is imperative for all stakeholder groups, including dieticians, medics, paramedics, technologists, engineers, *etc.*, to devise means of controlling the enzymatic rate of catalysis by manipulating the magnitudes of k_{+2} and k_{-2} in particular. The derived equations can be fitted to the experimentally generated and calculated data. Since this research was carried out without regard to the enzyme's optimal conditions, future research should entail conducting assays under optimum conditions so as to verify possible variations in the ZOFORC values when compared with values generated outside optimum conditions.

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