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Turnover and Catalytic Cycle Frequency Determination Based on Molar Mass-Dependent Model Equations

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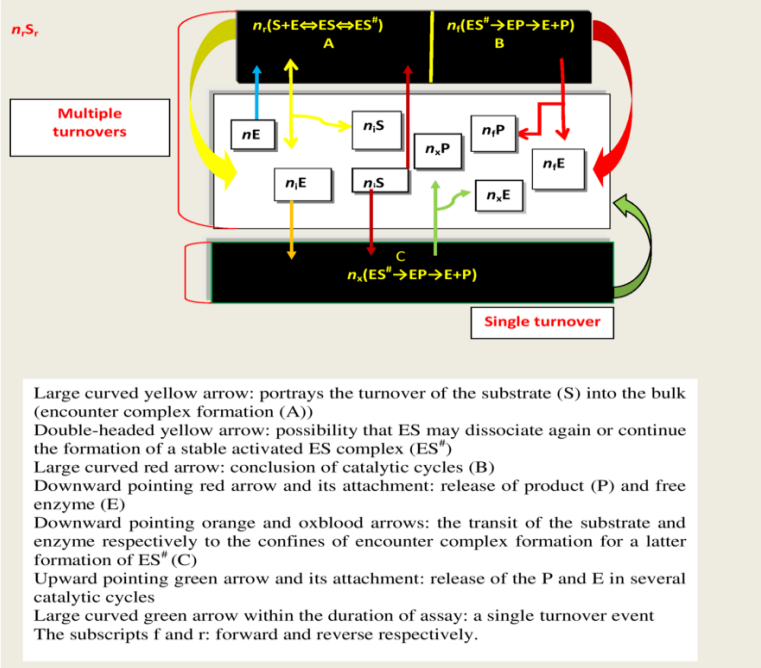
Abstract

There were conflicting definitions and misrepresentations of turnover frequency (TOF), catalytic cycle frequency (CCF), and catalytic first-order rate constant (k_{cat}) in the literature. Based on the Benfield and Lineweaver-Burk methods, data were generated. The results indicated that the CCFs for the forward (~ 0.0025-1.58 *exp*. (+17)/s) and reverse (~ 0.0003-5.4 *exp.* (+18)/s) directions showed an increasing trend with higher concentrations of the enzyme; this was applicable to the TOF. The number of fragments per molecule of an enzyme in the forward direction was 0.19-1.23 *exp.* (+5). In conclusion, TOF and CCF are different parameters, and the former in particular was not the same as k_{cat}, while the latter was a constant; TOF and CCF vary. Strictly speaking, TOF was equal to $f(M_3)$. Besides, CCF per molecule (> 1) of the enzyme is greater than TOF (< 1). This can be verified using sucrase in a future study; a larger data set is needed if starch is a substrate in order to reevaluate the models and address statistical concerns. **PACS:** 87.15.RJ; 87.14.ej

Keywords: *Aspergillus Oryzae* Alpha-Amylase, Catalytic Cycle Frequency, First-Order Catalytic Rate Constant, Gelatinized Insoluble Potato Starch, Turnover Number, Turnover Number Frequency

Graphical Abstract



Large curved yellow arrow: portrays the turnover of the substrate (S) into the bulk (encounter complex formation (A))
Double-headed yellow arrow: possibility that ES may dissociate again or continue the formation of a stable activated ES complex ($ES^{\#}$)
Large curved red arrow: conclusion of catalytic cycles (B)
Downward pointing red arrow and its attachment: release of product (P) and free enzyme (E)
Downward pointing orange and oxblood arrows: the transit of the substrate and enzyme respectively to the confines of encounter complex formation for a latter formation of $ES^{\#}$ (C)
Upward pointing green arrow and its attachment: release of the P and E in several catalytic cycles
Large curved green arrow within the duration of assay: a single turnover event
The subscripts f and r: forward and reverse respectively.

1. Introduction

Concerns about the appropriate turnover number and frequency have been raised numerous times [1-3]. When polymers are the substrate, this characteristic is crucial. Therefore, a quick summary of starch-related concerns is required. The two primary components of starch, a crucial polymer, are straight-chain, smaller molecular weight amylose and branched-chain, greater molecular weight amylopectin. Both types are homoglucans. All forms of glucan consist solely of alpha-D-glucose moieties connected by alpha-1, 4 glycosidic bonds, but amylopectin possesses alpha-1, 6 glycosidic linkages at the branch points.

The endoglycolytic activity of alpha-(1,4)-glucan glucanohydrolases, including alpha-amylase, affects starch, which is made up of alpha-glycosidic linkages. It is clear that the different sources of natural starch satisfy both the short-term energy supply and, eventually, the long-term molecular energy requirements of people. In addition to its uses in electronics, medication delivery, antimicrobials, and structural materials, starch is also used in the pharmaceutical business to produce encapsulating agents, laundry, and biofuel [4]. The degree of starch polymerization, which is a key component in determining the molar mass of starch, is the most significant factor that can satisfy any interest in starch.

The molar mass of the polymer is very relevant because it is the key to the determination of the catalytic cycles, turnover number, frequency of catalytic cycling, and turnover frequency. The hydrolysis of starch produces fragments during investigative research to ascertain the efficiency and other vital attributes of the enzyme for both laboratory (research) and industrial applications. Such fragments boast the time-dependent evolution of crowding effects that can compromise the efficiency of the free enzyme molecules. The frequency of collisions and nonspecific interactions between the enzyme and surrounding fragments, some of which exhibit noncatalytic configurational orientation to the enzyme, can extend the residence time of the enzyme molecules in any non-catalytically oriented complex, in addition to the impact of high viscosity [5]. This can also be facilitated by the fact that the many shorter pieces have a larger surface area than the parent polysaccharide, which is known to slow down the diffusion of water molecules [6].

There has been increasing interest in issues of initial rates (v_i), not necessarily at times approximately equal to zero but for all times referring to different durations of assay. This is with a view to addressing the problem of accurate kinetic parameters that are vital in most industrial, medical, and scientific research. The kinetic parameters must be addressed in the context of definite quasi-steady-state assumptions, as regularly advocated in the literature [7]. Whether or not proponents of single turnover catalytic events are aware of it, the fundamental premise is the reverse quasi-steady-state assumption, which is predicated on the idea that in these assays the molar concentration ($[E_T]$) of the enzyme is greater than the molar concentration ($[S_T]$) of the substrate [8, 9]. Using a single-turnover approach to prevent catalytic cycling is the key to monitoring chemical processes. Single-turnover conditions, which are utilized to isolate events at the enzyme's active site without catalytic cycling, allow all substrates to take part [10, 11].

In contrast to the literature, this study compares turnover number, turnover frequency, number of catalytic cycles, and frequency of catalytic cycles. The main objectives are to derive and evaluate equations using experimental data (for the computation of all parameters, including the estimation of the number of substrate fragments per molecule of the enzyme). A molar mass-based model and a double reciprocal plot model connected to the number of catalytic cycles are essential for the primary calculation of the number of catalytic cycles for a given number of moles of the enzyme in a reaction mixture. It is equally pertinent to state that this unpublished article was posted at the ChemRxiv preprint server, located in highly advanced scientific communities, and awaited faults to be found by advanced scientists, but to no avail. After more than 2 years of waiting, this study reexamined the models and identified some flaws (notable was dimensional inconsistency in a few equations); corrections were made, and fresh data that influenced new conclusions were determined. Please visit 10.26434/chemrxiv-2023-0sjpl for the purpose of comparison!

1.1 Significance Statement

In light of the different definitions of turnover number (TON) and its frequency and the misrepresentation of catalytic cycle (CC) and its frequency, this study has shown that all parameters are different; while the first-order catalytic rate constant (a zero-order Michaelian value) is the same for different concentrations of the same enzyme under the same conditions, TON and CC frequencies vary. The TON and CC and their corresponding frequencies are valid, distinct kinetic parameters. All the enabling model equations have predictive significance in that one can estimate TON and CC given any concentration of the substrate. These can constitute working guides for bioreactor engineers and technologists for biofuel, food, and pharmaceutical applications.

2. Theory

For the Michaelian type of reaction mixture, the relation between the initial rates and the concentration of the substrate is nonlinear; usually a polynomial—the quadratic kind—is the outcome with a higher coefficient of determination than the linear regression outcome. This is not to imply that with appropriate software (GraphPad, for instance), a hyperbolic curve is no longer relevant. Thus, a factor, φ, is introduced in the following relation to account for nonlinearity. The factor also expresses the possibility that the ratio $[S_0]/([S_0] + M_S[E_0]/M_2)$: $v_i/(v_i + V_{max})$ is not equal to one. This is intended to address a situation in which the substrate concentration, $[S_T]$ (in molar concentration unlike $[S_0]$ in mass concentration), is ≫ the enzyme concentration, $[E_T]$ (in molar concentration unlike $[E_0]$ in mass concentration), such that the following equation may hold.

$$\frac{[S_0]}{[S_0]+\frac{M_S}{M_2}[E_0]} = \frac{v_i}{v_i+V_{max}}\varphi \quad (1)$$

Where v_i is the initial velocity of catalytic action less than the maximum velocity (V_{max}).

Keep in mind that $[E_0]/M_2$ (*i.e.*, $[E_T]$) is, as stated earlier, the molar concentration of the enzyme explored for the assay such that if M_S (*may be a combination of molar masses of polymer fragments and fewer parent polymers*) was to be the real molar mass of the substrate, then $M_S[E_0]/M_2$ should be

the equivalent of the maximum mass concentration of the substrate that combined with the total concentration of the enzyme at the asymptotic level or maximum velocity of catalysis. Therefore, the maximum velocity, V_{max}, is the asymptotic kind because it is ≫ the mixed order maximum velocity (V_{max}^{prss}), where the $[S_0]$ ranges between values that are ≪ the Michaelis-Menten constant, K_M, and values < K_M. It is clear that while M_2 is definitely the molar mass of the enzyme if known *ab initio*, the molar mass of the substrate cannot be definitely represented by M_S for reasons that are germane in light of total Michaelian kinetics. This is to be revisited following derivation for its meaning. Expanding the equation and re-factorizing give, respectively, the following:

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

$$[S_0](v_i - \varphi) = \frac{M_S}{M_2}[E_0]v_i\varphi - V_{max}[S_0] \quad (3)$$

Dividing through first by $[S_0]$ and then, by v_i (V_{max}) gives:

$$\frac{1-\varphi}{V_{max}} = \frac{M_S}{M_2[S_0]V_{max}}[E_0]\varphi - \frac{1}{v_i} \quad (4)$$

Rearrangement gives:

$$\frac{1}{v_i} = \frac{M_S}{M_2[S_0]V_{max}}[E_0]\varphi + \frac{\varphi-1}{V_{max}} \quad (5)$$

Given the slope (S_L) and the intercept (I_{NT}) from the double reciprocal plot, the following should be obtained:

$$I_{NT} = \frac{\varphi-1}{V_{max}} \quad (6)$$

However, the product of V_{max} and I_{NT} is = 1 such that φ is = 2.

$$S_L = \frac{M_S}{M_2V_{max}}[E_0]\varphi \quad (7)$$

Therefore,

$$M_S = \frac{S_L M_2 V_{max}}{2[E_0]} \quad (8)$$

Although literature data is available for the evaluation of Eq. (8), an introspective evaluation is carried out using hypothetical data such as: S_L = 19872; V_{max} = 8 *exp.* (−5) M/min; M_2 = 52 *exp.* (+3) g/mol.; and $[E_0]$ = 0.00025 g/L. With these parameters, the value of M_S is 206668800 g/mol. This is by far higher than the known molar mass of starch; values between 6 and 9 exp. (+ 7) g/L are not unusual. For any catalytic action, a polymer, either the parent polymer or the fragment, constitutes a molecule with its own weight. Therefore, it is the sum of all the polymers that participated in all the catalytic cycles of all the enzyme molecules that is relevant. Hence, M_S is ≫ the true molar mass of the parent substrate, where $[E_T]$ is ≪ $[S_T]$. Meanwhile, recall that $V_{max}/[E_T]$ is the catalytic first-order rate constant, k_{cat}, and the product of the slope, S_L, and V_{max} is equal to the K_M. Thus, Eq. (8) is written as:

$$M_S = \frac{K_M M_2}{2[E_0]} \quad (9)$$

It is therefore not unreasonable to postulate that Eq. (8) cannot be restricted to the substrate concentration at half maximal velocity; it should cover other substrate concentrations that are either < or > the K_M. The magnitude of M_S/M_3 *i.e.* turnovers (№) can either be < or ≫ 1; it may be = 1; it may also be either ≤ or ≥ 1 in accordance with the concentration of the enzyme and the concentration of the substrate. The value of № is therefore, defined mathematically as:

$$№ = \frac{K_M M_2}{2[E_0]M_3} \left(\text{or } № = \frac{K_d M_2}{2[E_0]M_3}\right) \quad (10)$$

Where M_3 is the molar mass of the substrate. The lower the catalytic cycle frequency at low $[E_0]$, the higher the value of №; it expresses the number of times the substrate needs to be turned in for the hydrolytic effect of the enzyme.

Above all, keep in mind that not all processes produce products with a single turnover and that a polymer has a degree of polymerization greater than one. For instance, due to the large and low molar masses of sucrase and disaccharides like sucrose, respectively, the number of turnovers (№s) could be rather high (up to a value of ≥*exp.* +6). This would happen if the concentration of the substrate was more than that of the enzyme, which may be far lower than one international unit. For the purpose of derivation, it should be noted that, for different enzyme concentrations under the same assay conditions, k_{cat} is theoretically constant at a saturating substrate concentration. However, if gelatinized, a larger $[S_0]$ might lead to very high viscosity, which would impede the enzyme molecules' translational diffusion and perhaps have an impact on the K_M and V_{max}. On the other hand, two different concentrations of the enzyme may produce different V_{max} and K_M values when using the same substrate concentration regime. One of these values may be inaccurate because some of the enzyme molecules may have been unsaturated. Therefore, $K_M/[E_0]$ and $V_{max}/[E_T]$ (where $[E_T]$ is in molar units) can differ for different enzyme concentrations. As a result, the turnover frequency (or just "the turnover number") and the number of catalytic cycles per unit time (or just "the number of catalytic cycles"), represented by k_{cat} (t k_{cat}), are not necessarily the same.

As stated earlier, the literature seems to be replete with arguments about what a turnover number and turnover frequency should be. Issues regarding this are reserved for the discussion section. Equation (10) is strictly applicable to conditions that validate the Michaelian equation for short, foremost being that $[S_T]$ is ≫ $[E_T]$ (recall that both concentrations must be in molar units). Additionally, the following queries result from Eq. (10): For example, how many cycles of enzymatic activity are required to produce the maximal amount of maltose? How many cycles of catalytic action (hydrolysis) per unit of time are sufficient for the assay to produce the greatest number of molecules of the product? Observe that the turnover number resulting from a specific mass concentration of the enzyme is expressed in Eq. (10). This raises the question: how many catalytic cycles are there per mole of the enzyme per second

($\overset{+}{\text{fr}}$©)? The response, given that $V_{rn}.V_{max}/k_{cat}$ is equal to the number of moles of the enzyme in a reaction volume, is as follows:

$$\overset{+}{\text{fr}}© = \frac{2\times1000\,K_M M_2 k_{cat}}{2[E_0]M_3 V_{max} V_{rn}\tau_+} \quad (11)$$

Where the figures 1000, V_{rn}, and 2 are the conversion factors from the liter to the milliliter, the reaction mixture volume (2 mL in this study), and an integer following the use of half maximum velocity at substrate concentration equal to the K_M (see Eq. (10)); τ_+ is the life span of the catalytic cycles leading to the formation of the product within the duration of the assay. If the velocity of catalysis is at a substrate concentration less than the K_M, Eq. (11) is restated as:

$$\overset{+}{\text{fr}}© = \frac{1000\,[S_0]M_2 k_{cat}}{[E_0]M_3 v_i V_{rn}\tau_+} \quad (12)$$

In Eq. (12), $[S_0]$ may also be greater than K_M. Another version of Eq. (11), given the definition or equation of the k_{cat}, is given as follows:

$$\overset{+}{\text{fr}}© = \frac{1000\,K_M M_2^2}{[E_0]^2 M_3 V_{rn}\tau_+} \quad (13)$$

Under well-defined quasi-steady-state assumptions, such as rQSSA, sQSSA, or tQSSA [12-15], and with very accurate initial rates that follow a polynomial, one can determine the kinetic parameters, including K_M, as explained in multiple preprint presentations [16-18]. Such an equation in its simplest form for the determination of the K_M is given as follows:

$$K_M = \frac{[S_0]_n[S_0]_{n-1}(v_n - v_{n-1})}{([S_0]_n v_{n-1} - [S_0]_{n-1} v_n)} \quad (14)$$

Therefore, following the substitution of Eq. (14) into Eq. (13), the latter takes the form given as follows.

$$\overset{+}{\text{fr}}© = \frac{1000\,M_2^2}{[E_0]^2 M_3 V_{rn}\tau_+}\frac{[S_0]_n[S_0]_{n-1}(v_n - v_{n-1})}{([S_0]_n v_{n-1} - [S_0]_{n-1} v_n)} \quad (15)$$

Equation (12) can also take the form:

$$\overset{+}{\text{fr}}© = \frac{1000\,[S_0]M_2^2 V_{max}}{[E_0]^2 M_3 v_i V_{rn}\tau_+} \quad (16)$$

Similarly, Eq. (16) can take on a different form using the same reasoning that leads to Eq. (15), which results in Eq. (18). But first, Eq. (17) is given as follows:

$$V_{max} = \frac{v_n v_{n-1}([S_0]_n - [S_0]_{n-1})}{([S_0]_n v_{n-1} - [S_0]_{n-1} v_n)} \quad (17)$$

$$\overset{+}{\text{fr}}© = \frac{1000[S_0]M_2^2}{[E_0]^2 M_3 v_i V_{rn}\tau_+}\frac{v_n v_{n-1}([S_0]_n - [S_0]_{n-1})}{([S_0]_n v_{n-1} - [S_0]_{n-1} v_n)} \quad (18)$$

In a laboratory, the volume of a reaction mixture is very small. A small amount of the reaction mixture is used to estimate all parameters and products when measuring a catalytic cycle (CC) in an industrial setting. The total CC and the number of catalytic cycles per mole per unit time ($\overset{+}{\text{fr-Ind}}$©) applicable to a larger industrial vessel (whose volume is labeled $V_{(ind)}$) over the assay's duration are given as follows [19]:

$$\overset{+}{\text{fr-Ind}}© = \frac{exp.(+6)\,K_M M_2 k_{cat} V_{(ind)}}{[E_0]M_3 V_{max} V_{rn}^2 \tau_+} \quad (19)$$

Equation (19) is a modification of Eq. (11). This is with the understanding that, $V_{(ind)}$ is in liters while, as usual V_{rn} is in cubic centimeters, thereby necessitating the introduction of another conversion factor.

$$\overset{+}{\text{fr-Ind}}© = \frac{\exp.(+6)[S_0]M_2 k_{cat} V_{(ind)}}{2[E_0]M_3 v_i V_{rn}^2 \tau_+} \quad (20)$$

Furthermore, Eq. (11) for the routine laboratory setting and Eq. (19) for the industrial setting are rewritten to reflect the presence of the specificity constant (*SC*).

$$\overset{+}{\text{fr}}© = \frac{1000\,M_2^2 k_{cat}}{[E_0]^2 M_3 SC\, V_{rn}\tau_+} \quad (21)$$

$$\overset{+}{\text{fr-Ind}}© = \frac{exp.(+6)\,M_2^2 k_{cat} V_{(ind)}}{[E_0]^2 M_3\, SC\, v_{rn}^2 \tau_+} \quad (22)$$

Meanwhile, what may tentatively be regarded as the duration (τ_+) of catalytic cycles in the forward direction—the formation and the release of product—can be derived from the equation based on Eq. (13):

$$\overset{+}{\text{fr}}© = \frac{1000 K_M M_2^2}{[E_0]^2 M_3 V_{rn}\tau_+} = \frac{\tilde{N}_P}{t} \quad (23)$$

Where $\tilde{N}_P$ and t are the number of molecules of the product (maltose) per mol of the enzyme and the duration of the assay respectively. Thus,

$$\tau_+ = \frac{1000 K_M M_2^2 t}{[E_0]^2 M_3 V_{rn}\tilde{N}_P} \quad (24)$$

The reciprocal of τ_+ gives the frequency of the catalytic cycle (fr_{cc}^+) in the product-destined direction.

$$\frac{1}{\tau_+} = fr_{cc}^+ = \frac{[E_0]^2 M_3 V_{rn}\tilde{N}_P}{1000 K_M M_2^2 t} \quad (25)$$

Equation (25) satisfies the process, (E+S⇔ES$^{\#}$⋯→EP⋯→E+P) (fr_{cc}^+); the 2nd, *i.e.,* the reverse process (ES$^{\#}$ ⇔ E+S) is given the equation as follows:

$$\frac{1}{\tau_-} = fr_{cc}^- = \frac{[E_0]^2 M_3 V_{rn}\tilde{N}_S}{1000 K_d M_2^2 t} \quad (26)$$

In Eq. (26), K_d is the equilibrium dissociation constant for the conversion of the weakly activated, unstable enzyme-substrate complex (ES$^{\#}$) into free substrate and free enzyme molecules. Meanwhile, '$\tilde{N}_S$' is the number of substrate molecules released per mole of enzyme. Note that Eqs (25) and (26) serve the criteria that validate sQSSA or Michaelian zero-order kinetics. For the rQSSA case, the same structure of the equations applies, but the equations need to be restated to reflect prevailing reality as follows:

$$\frac{1}{\tau_+^{\text{pres}}} = fr_{cc}^{+\text{pres}} = \frac{[E_0]^2 M_3 V_{rn} \tilde{N}_P}{1000 K_M^{\text{pres}} M_2^2 t} \quad (27)$$

$$\frac{1}{\tau_-^{\text{pres}}} = fr_{cc}^{-\text{pres}} = \frac{[E_0]^2 M_3 V_{rn} \tilde{N}_S}{1000 K_d^{\text{pres}} M_2^2 t} \quad (28)$$

With respect to Eq. (26), K_d must be calculated for the rQSSA case given the equation:
$k_{-1} + k_{cat} = K_M k_1$; $k_d = K_M - k_{cat}/k_1$ where k_{cat}/k_1 is the well-known Van Slyke and Cullen constant [20] and k_1 is the 2nd-order rate constant for the formation of the ES. In order to reflect the clear and subtle differences between Eq. (26) and Eq. (28), the former is rewritten as:

$$\frac{1}{\tau_-} = fr_{cc}^{-} = \frac{[E_0]^2 M_3 V_{rn} \tilde{N}_S}{1000(K_M - k_{cat}/k_1) M_2^2 t} \quad (29)$$

Again, Eq. (28) assumes the same structure as Eq. (29), but different methods and equations are needed for the determination of k_1 [21]. Equation (28) is permissible on the condition that a plot of initial rates (v_is) versus $[S_0]$ yields a polynomial curve containing a negative coefficient of the leading term in addition to the fact that, $[E_T]$ is either only > $[S_T]$ or it is ≈ $[S_T]$ (it may also be about the same concentration as the K_M); if the $[E_0]$ is ≫$[S_0]$, a polynomial plot giving the same coefficient of determination as the linear plot (R^2 = 1) will not be a valid input to any computation. The linear plot gives directly a slope equal to $V_{max}^{\text{pres}}/K_d$ (where, as in anywhere in the text, pres. stands generally for pre-steady-state and steady state before zero-order state); however, following the same argument leading to the former, K_d is given as $[E_0]M_3/M_2$. Where either $[E_T]$ is > $[S_T]$ or it is ≈ $[S_T]$ (≈K_M) the equation takes the form:

$$\frac{1}{\tau_-} = fr_{cc}^{-\text{pres}} = \frac{[E_0]^2 M_3 V_{rn} \tilde{N}_S}{1000(K_M^{\text{pres}} - k_{cat}^{\text{pres}}/k_1^{\text{pres}}) M_2^2 t} \quad (30)$$

A preprint [17] has revealed what the situation is like where the initial rate, v_i, is consistently directly proportional to the substrate concentration range explored for the assay. Under such a scenario (if $\tilde{N}_S$ is the maximum number of substrate molecules per mole), the equation should be:

$$\frac{1}{\tau_-} = fr_{cc}^{-} = \frac{[E_0] V_{rn} \tilde{N}_S}{1000 M_2 t} \quad (31)$$

It would seem that there is only one constant of proportionality given that Eq. (31) goes for an enzyme concentration greater than the concentration of the substrate, leaving no room for the estimation of k_1. This problem can be solved using the method outlined in the literature [21]. Therefore, using k_1 in the formula $K_d = k_{-1}/k_1$, where k_{-1}, given as $K_d k_1$ and multiplied by $[E_0]/M_2$, yields the molar concentration of any substrate molecules that may have dissociated but must rapidly rebind because of the enzyme's oversaturating concentration. The number of such substrate molecules per mole of the enzyme is needed. Thus, $\tilde{N}_S$ = *exp.* (−3) $K_d k_1$ $[E_0] v_{rn} N_A t$ *exp.* (+3)$M_2/[E_0]$ v_{rn} $M_2 = K_d k_1 t N_A$. Therefore, in Eq. (31), $\tilde{N}_S$ is *for a scenario where v is strictly directly proportional to [S0].* If not $\tilde{N}_S$ is = ($K_d k_1 - k_{cat}$) tN_A. For the product case, $\tilde{N}_P$ = *exp.* (−3)V_{max} $v_{rn} N_A t$ *exp.* (+3)$M_2/[E_0] v_{rn} = V_{max}$ $N_A t$ $M_2/[E_0]$. To give the equations for visualization at once, substitutions of $\tilde{N}_P$ and $\tilde{N}_S$ into Eq. (25) and Eq. (26) respectively give respectively the following equations:

$$\frac{1}{\tau_+} = fr_{cc}^{+} = \frac{[E_0] M_3 V_{max} V_{rn} N_A}{1000 M_2 K_M} \quad (32a)$$

$$\frac{1}{\tau_-} = fr_{cc}^{-} = \frac{[E_0]^2 M_3 K_d k_1 V_{rn} N_A t}{1000 M_2^2 K_d t}$$

$$= \frac{[E_0]^2 M_3 k_1 V_{rn} N_A}{1000 M_2^2} \quad (32b)$$

$$= \frac{[E_0]^2 M_3 k_{-1} V_{rn} N_A}{1000 M_2^2 K_d} \quad (32c)$$

Equation (32c) should be suitable for saturation kinetics *i.e.*, Michaelian kinetics. (sQSSA) whereby $k_{-1} = K_M k_1 - k_{cat}$. Where conditions leading to the adoption of Eq. (31) prevail (the symbol V_{max} has to be changed to V_{max}^{Pres} if $[E_T] \gg [S_T]$), fr_{cc}^{+} can take the form as follows:

$$fr_{cc}^{+} = \frac{[E_0] V_{rn} \tilde{N}_P}{1000 M_2 t} = \frac{V_{max}^{Pres} v_{rn} N_A}{1000} \quad (33a)$$

$$fr_{cc}^{-} = \frac{[E_0] V_{rn} \tilde{N}_S}{1000 M_2 t} = \frac{[E_0] V_{rn} K_d k_1 N_A t}{1000 M_2 t}$$

$$= \frac{[E_0] V_{rn} K_d k_1 N_A}{1000 M_2} \quad (33b)$$

A little insight into the origin of Eq. (33b) for the record is given as follows:

$$V = v_{-1} + v_{+1} = k_1 [S_0][E_T] \quad (33c)$$

Where v_{-1} and v_{+1} are the velocity of the breakdown and formation of *ES* respectively; Eq. (3c) originates from a of part Michaelian derivation for short. It can be restated as follows: As stated earlier, $V = k_{-1}[ES] + [ES]k_{cat}$, and given that $[ES] = [E_T][S_0]/K_d$ where $[E_T] \gg [S_T]$ then, $\frac{(k_{-1} + k_{cat})[E_T][S_0]}{K_d} = k_1 [S_0][E_T]$. If a single (or less than one) turnover is a possibility, then $k_{-1} \to 0$; thus, $k_{cat}/K_d = k_1$ and there should not be any saturation kinetics such that the latter equation should be restated as follows: $k_1 = k_{cat}^{Pres}/K_d$ (or $M_2 V_{max}^{Pres}/[E_0]K_d$): Given the latter, the parameter, $\tilde{N}_P$ can be determined. Equation (33b) is suitable for $[E_0] \gtrsim 0.02$ g/L assayed with substrate concentrations ranging between 0.3 and 3 g/L (or less) given that the ratio of the molar mass of the latter to the former is ≈ 1241:1.

2.1 Linking the equations of catalytic cycle frequency to turnover number

Solve for $[E_0]$ in Eq. (10) and substitute into Eq. (33a) to give the equation as follows:

$$fr_{cc}^{+} = \frac{V_{max} V_{rn} N_A}{2000№} \quad (34a)$$

Solve for $[E_0]$ in Eq. (10) and substitute into Eq. (32b) to give the following equation:

$$fr_{cc}^{-} = \frac{M_2^2 K_d^2 M_3 k_1 V_{rn} N_A}{4 \times 1000 M_3^2 M_2^2 №^2}$$

$$= \frac{K_d^2 k_1 V_{rn} N_A}{4000 M_3 №^2} \quad (34b)$$

Restate Eq. (33a) as follows:

$$fr_{cc}^{+} = \frac{(K_d k_1 - k_{-1}^{Pres})[E_0] v_{rn} N_A}{1000 M_2} \quad (35a)$$

Where $k_{cat}^{Pres} = K_d k_1 - k_{-1}^{Pres}$. Solving for ($K_d$) in Eq. (10) and substitute into Eq. (35a) gives the equation as follows:

$$fr_{cc}^{+} = \frac{(2[E_0] M_3 № k_1 - M_2 k_{-1}^{Pres})[E_0] v_{rn} N_A}{1000\, M_2^2} \quad (35b)$$

Solve for (K_d) in Eq. (10) and substitute into Eq. (33b) to give the equation as follows:

$$fr_{cc}^{-} = \frac{[E_0]^2 M_3 № V_{rn} k_1 N_A}{500\, M_2^2} \quad (36a)$$

In addition to the spec-detectable products, an enzyme molecule that hydrolyzes a polymer substrate will also create fragments ($N_{frag.}$) of the parent polysaccharide, which is starch. The spectrophotometer-detectable product and polymer fragment are produced concurrently, despite the fact that each enzyme molecule at the end of the duration of the assay creates more product molecules than fragment molecules. Nonetheless, it is postulated that the number of fragments is inversely proportional to the turnover number and directly proportional to the number of products, assuming, however, single-digit proportionality constant.
Thus,

$$N_{\text{frag.}} = \frac{V_{max} V_{rm} N_A t\ exp.(-3)}{№} \quad (37a)$$

$$= \frac{2[E_0] M_3 V_{max} V_{rm} N_A t\ exp.(-3)}{K_M M_2} \quad (37b)$$

Equation (37b) is derived by substituting Eq. (10) into Eq. (37a). If half maximum velocity is considered, the equation is given as follows:

$$N_{[\text{frag}/2].} = \frac{[E_0] M_3 V_{max} V_{rm} N_A t\ exp.(-3)}{K_M M_2} \quad (38)$$

If the number of fragments are of interest at velocities of hydrolysis less than V_{max}, then, the equation is as follows:

$$N_{\text{frag.}} = \frac{2[E_0] M_3 v\, V_{rm} N_A t\ exp.(-3)}{K_M M_2} \quad (39)$$

The number of fragments per molecule of the enzyme is expressed as follows:

$$\frac{N_{\text{frag.}}}{N_{[E_T]}} = \phi_{\text{frag}} = \frac{2 M_3 V_{max} t}{K_M} \quad (40)$$

The number of fragments per molecule of the enzyme for the half maximum velocity case is given as:

$$\frac{N_{[\text{frag}/2].}}{N_{[E_T]}} \left(\text{or } \phi_{0.5\text{frag}}\right) = \frac{M_3 V_{max} t}{K_M} \quad (41)$$

Note that Eq. (37) to Eq. (39) represents the total number of fragments for each concentration of the substrate. In these derivations, a situation where [$\boldsymbol{S}_T$] is not ≫ [$\boldsymbol{E}_T$] and the possibility of [$\boldsymbol{E}_T$] being ≈ [$\boldsymbol{S}_T$] are relevant in line with the notion that the reactant stationary assumption (RSA) [7], standard quasi-steady-state assumption (sQSSA), and reverse QSSA (rQSSA) [15] may be valid in such a scenario. As noted elsewhere [22], the literature review gave the impression, probably in error or due to the absence of self-evaluation, that the total mass of the product may be larger than the total mass of the reactant, in violation of the mass conservation law. This observation motivated this study to derive alternate equations whose application should not violate the mass conservation law.
The result, both mathematical and quantitative, is very relevant to reactants and the polymer substrate in particular. Since the researcher would want to know how much of a starchy diet is digested and how much is undigested, it might help determine what a study scientist in a clinical setting specifically anticipated in the control of diabetes. Industrialists involved in biomass conversion for biofuel production might examine the model for evaluating the efficiency of enzymes and the degree of resistance to digestion of polysaccharide sources. Other applications of enzymes in industries and some processes are food and beverage industry; textile and leather industry; detergent industry; paper and pulp industry; pharmaceutical and fine industry; and waste management and bioremediation [23].
Some other researchers came up with their own definitions of concepts and their possible applications. The turnover rate (or turnover frequency) is, according to Davis (2018) [24], defined as the rate at which a catalytic cycle turns over, and it is regarded as the most suitable basis for comparing the rate of reaction on different catalysts. “The yield of the catalytic reactions is often used as a primary metric of catalytic performance in organic chemistry and homogeneous catalysis communities” [25]. In this regard catalytic rate constant becomes more relevant because it defines the rate of product formation and in particular, specificity constant. “A common criterion for assessing the operational stability of a catalyst is the dimensionless total turnover number (TTN), which is the average number of turnovers per active site over the catalyst's lifetime: If the catalyst's price is known, the cost contribution per catalytic cycle can be calculated” [26].

3. Experimental

3.1 Materials

3.1.1 Chemicals

As in the recent literature [27], *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 kDa [28].

3.1.2 Equipment

An electronic weighing machine was purchased from Wenser Weighing Scale Limited, and a 721/722 visible spectrophotometer was purchased from Spectrum

Instruments, China. A pH meter was purchased from Hanna Instruments, Italy.

3.2 Methods

To generate initial rates and determine kinetic parameters, the reagents and test procedures are according to Bernfeld (1955) [29], as adopted from previously conducted assays in which a mass concentration of 0.02 g/L of *Aspergillus oryzae* alpha amylase was explored, given a mass concentration range of substrate equal to 0.3–3 g/L. The other concentrations of the enzyme that were assayed were 0.002 and 0.0002 g/L, given a mass concentration range of substrate equal to 5–10 g/L [30].

3.3 Statistics

Only duplicate assays were done on the enzyme. Therefore, the mean of each duplicate was used for each calculation. Determination of standard deviation was ruled out because two determinations are neither suitable for parametric nor nonparametric statistics, which otherwise could draw negative remarks from biostatisticians. The research is mainly theoretical, which carries its exclusive score, but it was tested with small data in order to evaluate the models.

4. Result and Discussion

There is ample evidence of interest in calculating the turnover number (TON) and its frequency [31, 32]. While the Lumry–Eyring model [32] is used to estimate the total turnover number (TTN) and the rate constant for spent catalyst replacement [31], the model is somewhat ambiguous. This study presents an alternative method (Equation (9)). This formula shows that the turnover number (symbolized as №) for polysaccharides ranges from 0 to 4 at a given enzyme concentration (see Table 1). Consequently, if the substrate is a polysaccharide with a notably high degree of polymerization, the parameter, №, occasionally has a negligible value for any mass concentration of the enzyme. The turnover number and the corresponding frequency showed decreasing trend in both forward and reverse directions (Table 1). However, there is an inverse relationship between its magnitude and $[E_0]$. Conversely, calculating the number per given time yields values of less than 1/s. TOF is clearly distinct from the catalytic rate constant, which is typically defined as the ratio of the maximum molar concentration of one of the produced products per unit time to the molar concentration of the enzyme. All kinetic parameters except the unusually higher k_{cat} for 0.02 g/L than for 0.002 g/L of the enzyme showed, as expected, an increasing trend in their magnitudes. The parameters were crucial for the determination of other parameters such as CCF, CCF per molecule of the enzyme, and the number of fragments of the substrate molecule (emphatically, a polymer) per molecule of the enzyme (Table 1).

Table 1: Investigated parameters

Parameters	Mass concentration of the enzyme/g/L		
	0.0002	0.002	0.02
k_{cat}/min	20,305.519	5,379.359	6,105.026
k_{-1}/min	3,833.846	35,727.821	201,368.198
K_M or K_M^{Pres}/g/L	1.562364	2.475357	7.412171
K_d/g/L	0.248138837	2.151427535	7.194063057
k_1/L/g. min	15450.53841	16,606.56624	27990.88472
+ CCF/s	2.491 *exp.* (+14)	4.165 *exp.* (+15)	1.579 *exp.* (+17)
− CCF/s	2.961 *exp.* (+14)	2.766 *exp.* (+16)	5.364 *exp.* (+18)
+ CCF/molecule of E	53.76976754	899.0773285	340.7764125
− CCF/molecule of E	63.92117804	687.0430569	11580.3327
+№	3.147	4.986 *exp.* (−1)	1.493 *exp.* (−1)
–№	4.998 *exp.* (−1)	4.334 *exp.* (−1)	1.450 *exp.* (−1)
+TOF/s	1.748 *exp.* (−2)	2.77 *exp.* (−3)	8.294 *exp.* (−4)
–TOF/s	8.33 *exp.* (−3)	2.408 *exp.* (−3)	8.056 *exp.* (−4)
$N_{frag.}/N_{[E_T]}$ or ϕ_{frag}	1.936 *exp.* (+4)	3.237 *exp.* (+4)	1.227 *exp.* (+5)
$N_{frag.}/N_{[E_T/2]}$ or $0.5\phi_{frag}$	0.968 *exp.* (+4)	1.618 *exp.* (+4)	6.135 *exp.* (+4)

+ CCF (or fr_{cc}^{+}) and – CCF (or fr_{cc}^{-}) are the catalytic cycle frequencies in the forward and reverse directions respectively at maximum velocity of catalysis. They are determined respectively, according to Eqs (32) and (33). +№, –№ +TOF, –TOF, ($N_{frag}/N_{[E_T]}$ or ϕ_{frag}) and ($N_{frag}/N_{[E_T/2]}$ or $0.5\phi_{frag}$) are the turnover number in the forward direction, turnover number in the reverse direction, turnover frequency in the forward direction, turnover frequency in the reverse direction, the number of fragments of the substrate polymer per molecule of the enzyme, E, at maximum velocity, V_{max} and the number of fragments of the substrate polymer per molecule of the enzyme at half maximum velocity respectively; (ϕ_{frag}) and ($0.5\phi_{frag}$) are respectively according to Eqs (36) and (38) while № is according to Eq. (9). The mean of the replicate values of V_{max} were explored. Note too, that, (K_M^{Pres}) signifies the fact that the K_M is not attained with respect to concentrations of E equal to 0.002 and 0.02 g/L ($K_M^{Pres} < K_M$).

If the research scientist or reactor designer chooses a condition that aligns with rQSSA, where $[E_T]$ is much greater than $[S_T]$, then it is highly probable that all substrate molecules will transform into products, as expected in a single-turnover catalytic event within the chosen duration of assay. This eliminates the need for extra recycling of enzyme molecules for another round of catalytic hydrolysis of the substrate. Conversely, the choice may be one in which $[S_T] \gg [E_T]$. In this case, a fraction of the substrate is transformed into the product (turnover event), leaving behind a substantial amount of free substrate and fragments [22]. The enzyme has the opportunity to be recycled and meet any free substrate or fragment. Catalytic cycling can be classified as positive (product-destined) or negative (deactivation of activated ES and dissociation into free S and E). The focus is always on the positive aspect.

The substrate is the input material. A portion of the substrate that has gone through a turnover process to produce the

product during the catalytic cycle is known as the output material. How many catalytic cycles were required to change a specific percentage of the substrate polymers is now the question. How many product molecules are produced as a result of these transformations? Another concern that comes up is whether the number of catalytic cycles and the number of substrate molecules that experienced turnover processes are equal. "No" is the response. The reason is that unbinding of the enzyme substrate complex, ES, may be preceded by either deactivation of the activated ES ($ES^{\#}$) or the latter proceeds to the enzyme product complex, EP, and finally dissociates into free E and P; the two scenarios are depicted as follows: $E + S \leftarrow\cdots ES \Leftrightarrow ES^{\#} \cdots\rightarrow EP \cdots\rightarrow E+P$. The forward and backward reactions beginning with $ES^{\#}$ are two-step processes. Any of them can be faster than the other. When the ES dissociates into free E and S, the purpose of a turnover process does not occur, even if it is a part of the catalytic cycle, with the potential that the free enzyme might locate either a substrate fragment or a full-length substrate polymer.

The analysis and discussion are based on the premise that, since catalysis is a kinetic phenomenon, the essential goal of catalytic research is to understand the rate at which catalytic cycles turn over because this provides detailed information on the process [1]. However, the question remains: What is "turned over," or what "turns over"? Is it the substrate or the enzyme? There are two phenomenal concepts in this statement. The catalytic cycle and turnover are different concepts, with the catalytic cycle giving functional effect to turnover. In a catalytic cycle, there are steps. Aborting any of these steps (initial steps in particular) terminates the conversion of the substrate to the product (*i.e.,* the purpose of the turnover is aborted).

Different definitions of turnover frequency (TOF), catalytic cycle frequency, and catalytic rate constant abound in the literature. The number of catalytic cycles that each enzyme molecule carries out in its lifetime is often called the "total turnover number (TTN)," and it is taken as a key industrial performance criterion [2, 3, 33]. The *TTN* is a dimensionless number, defined as the ratio of moles of product generated divided by the moles of biocatalyst used in a reaction [3]. But this is the definition of a first-order rate constant achieved when the enzyme is saturated with substrate. Another definition is based on the work of Rueveni *et al*. (2014) [35], who based such work on the equation [36] below.

$$k_{\text{turn}} = \frac{k_{\text{cat}}[S_0]}{[S_0]+K_{\text{M}}} \tag{42}$$

The equation may not be an outcome of "a supportive and acceptable evolutionary pressure" but a thought process; here, "the turnover rate k_{turn} is the reciprocal of the mean turnover time $\langle T_{\text{turn}} \rangle$—the average time it takes a single enzyme to produce a single molecule of product" [35]. This definition notwithstanding, k_{cat} is given as $V_{\text{max}}/[E_{\text{T}}]$, and substitution into Eq. (39) with rearrangement gives:

$$[E_{\text{T}}]k_{\text{turn}} = \frac{V_{\text{max}}[S_0]}{[S_0]+K_{\text{M}}} \tag{43}$$

This study comes up with alternative definitions, explanations, and methods of calculation. While the conceptual definition given by Kozuch *&* Martin (2012) [3] supports Eq. (10) if division by time in seconds is done, the mathematical aspect does not give support. The conceptual definition is: From a mathematical perspective, the TOF, like any other rate-based quantity, must be defined as the derivative of the number of turnovers with respect to time [3]. According to Boudart (1995) [1], the TOF is simply defined as the number of revolutions of the catalytic cycles per unit time, typically one second. However, the TOF is actually the number of catalytic cycles per unit time in seconds. If k_{cat} is equivalent to TOF, then there cannot be a single turnover event at any enzyme concentration, considering that k_{cat} times time (either a unit of time or the duration of the assay) is greater than one. The same applies to $v_i/[E_T]$ times time (v_i can represent the minimum initial rate for the minimum substrate concentration).

In this study, the catalytic cycle (CC) refers to the totality of sequential steps, each with a lifespan, that lead to the release of products following the *turnover of the substrate to the catalytic platform of the enzyme.* Therefore, it is the number of CC per second, either in the forward or in the reverse direction that defines the catalytic cycle frequency of an enzyme. It is indisputable that the catalytic rate constant is not equivalent to the TOF or the fr_{cc}, which is also not the same as the reverse first-order rate constant (k_{-1}). A straightforward analogy is sufficient for our undergraduates on the borderline: Two cars are assigned to convey items over the same distance to a location while going at the same speed. Ten items of the same kind can be transported in one car and five in another. Assuming each trip takes 30 minutes and there are 120 goods to deliver, divided by two for each vehicle, it should take 180 and 360 minutes, respectively, for the vehicles to complete the task. The delivery rates are 0.333 and 0.167 per minute, respectively. However, they both have the same number of trips per unit time (0.0333/min).

Although the phenomenon of viscosity becomes attenuated with hydrolysis of the gelatinized polysaccharide, the increasing number of fragments seems to lessen the effect of hydrolytic activity; this may contribute in part to the hyperbolic curve tendency as the rate of increase in product (P) formation ($\Delta\partial[P]/\Delta\partial t$) decreases. Most workers consider this to be a crowding effect [5, 37]. All metrics, including the number of fragments per enzyme molecule, exhibited a rising trend with increased $[E_0]$ (Table 1). The catalytic cycle frequency in the forward (+CCF) and reverse (-CCF) directions ranged between 0.005 and 1.58 *exp.* (+17)/s and between 0.0033 and 5.4 *exp*. (+18)/s, respectively (Table 1). A single turnover event or even a fraction of it is encouraged by a very high catalytic cycle frequency, as most, if not all, of the substrate molecules are converted into products. Consequently, for the highest $[E_0]$, the turnover number (0.149) and the associated frequency (8.294 *exp*. (−4)/s) are the lowest. For the lowest $[E_0]$, the highest values are 3.147 and 1.748 *exp*. (−2)/s, corresponding to TON and TOF, respectively (Table 1).

Different substrate concentrations and ranges appear to affect the number of fragments per molecule with different values of $[E_0]$. When 0.0002 g/L of the enzyme was exposed to greater substrate concentrations (5 to 10 g/L), the lowest value (~ 1.9 *exp*. (+4)) was obtained. At substantially lower substrate concentrations (the same starch, ranging from 0.3 to 3 g/L), the greatest number of fragments per molecule of the enzyme (~ 1.23 *exp.* (+5)) was produced for the highest enzyme concentration (Table 1). The number of fragments per molecule of the enzyme at half maximum velocity, V_{max}

of catalysis is obtained by dividing values at V_{max} by two (Table).Therefore, it seems that two scenarios—$[S_0] \gg [E_0]$ and $[E_0] \gg [S_0]$—produce different extra number of fragments. Fewer oligosaccharides, tetrasaccharides, trisaccharides, *etc*., are expected to make up these quantities. The accuracy of the model equations is tested by the discovery that the total number of maltose units per molar mass of 64.54 *exp.* (+6) g/mol [38] of insoluble potato starch is roughly 1.89 *exp.* (+5), which is greater than the highest number of fragments per molecule of the enzyme (Table 1).

"What methods are used in TOF determination? What is essential in the more accurate calculation of TOF?" [39]. These questions seem to justify the need for this study in light of the fact that industrial application of enzymes deserves suitable models for the design of reactors for industries and laboratory instruments for research. Thus, the questions give a summary of the significance of the study with potential to invoke further research to generate large data set. It is pertinent to state that the higher the enzyme concentration, a trend towards TON ≤ 1 is inevitable if the substrate concentration range is several folds less than the K_M of the enzyme. The parameter that should clearly redefine TOF is the catalytic cycle frequency per molecule of the enzyme which was highest with 0.002 g/L; the order for the forward and reverse direction of catalysis is as follows: 0.002>0.02>0.0002 g/L; the basic reason is that in rQSSA scenario, the substrate concentration range (5-10/g/L) available to an enzyme concentration equal to 0.002 g/L is more or less sufficient for recycling of the enzyme unlike a concentration equal to 0.0002 g/L that does not present sufficient free enzymes which in turn is unlike 0.02 g/L of the enzyme exposed to even much lower concentration (0.3-3 g/L) of the substrate. A question such as the following needs a circumspective consideration. "What is the purpose of the immobilization of the enzyme?" Does it enhance or inhibit recycling of the enzyme whose life span is either certain or uncertain? With this pieces of information generated in this research, it is not out of place to suggest that there is no basis for comparing turnover frequency with CCF per molecule of the enzyme.

5. Conclusions

The various formulas for calculating the catalytic cycle (CC) frequency, turnover number (TON), and number of fragments per enzyme molecule (N_F/E) were developed. The characteristics are different from one another: the speed (or rate) is the TON frequency, and the TON is the number of times it takes a mass concentration of the enzyme to convert the substrate to product. For polysaccharides or any hydrolyzable polymer, TON might be less than, larger than, or equal to 1. The number of CC per unit time is known as the CC frequency; the TON per unit time is not equal to the first-order rate constant (k_{cat}); the CC entails all the events leading to the product's formation and release. The TON frequency may vary, but k_{cat} is expected to be constant for any concentration of the enzyme. A higher concentration of the enzyme ($[E_T] \gg [S_T]$) has the potential to promote a single turnover event because of the very high CC frequency. Larger numbers of fragments of the substrate polymer are possible where rQSSA ($[E_T] \gg [S_T]$) conditions are present. A single turnover event does not imply that only one catalytic cycle should occur. Rather, it's the magnitude of the frequency of CC that guarantees the possibility of a single turnover within the duration of the assay. Regardless of the size of the turnover frequency, the number of catalytic cycle frequency remains higher in magnitude. Future research will examine the proposition that small molar mass substrates and possibly large molar mass enzymes give large TON and TON frequency. Besides this, a larger data set needs to be generated in order to reevaluate the models and address statistical concerns if starch is a substrate.

Even in the absence of dissociation following the release of a product, an enzyme that subjects a polysaccharide to processive hydrolysis does not prevent multiple catalytic cycles. Polysaccharides (or even hexasaccharides) require dissociation of the product and the polysaccharide fragment given an endohydrolase, let alone an exohydrolase. If the polysaccharide is "turned over," the resulting fragments are subject to another round of the catalytic cycle, regardless of excessive enzyme concentration. Polysaccharides, which are subject to multiple turnovers due to fragments even with excess $[E_T]$, are not disaccharides, which are subject to a single turnover with excess $[E_T]$!

6. Author Contribution

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

7. Dedication

I dedicate this study to all my primary school teachers (1965-1972) at the former Pilgrim Baptist Primary School in Ubulu-uku, Aniocha South LGA, Delta State, Nigeria.

8. Disclaimer (Artificial intelligent)

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

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11. Competing Interest

The sole author has declared that he has no known competing interest(s). The only challenge is the significantly less than two USD per day in monthly pension.

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