Graphical Abstract

Title: “Turnover and Catalytic cycle frequency Determination based on Molar mass-dependent Model equations”

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The large turning yellow arrow portrays the turnover of the substrate (S) into the bulk for possible interaction with the enzyme for complex formation within the confines of the encounter complex formation (A); the double-headed yellow arrow illustrates the possibility that ES may dissociate again or continue the formation of a stable activated ES complex (ES#); the large curved red arrow signifies the conclusion of catalytic cycles (B); the downward pointing red arrow and its attachment point to the release of product (P) and free enzyme (E); the downward pointing orange and oxblood arrows indicate the transit of the substrate and enzyme respectively to the confines of encounter complex formation for a latter formation of ES# (C); the upward pointing green arrow and its attachment indicate the release of the P and E in several catalytic cycles in a single turnover event signified by the large curved arrow within the duration of assay. Note THAT any number of moles ($n$) multiplied by Avogadro’s number gives the number of participating molecules. The subscripts f and r stand for forward and reverse respectively.
Highlights

Title: Turnover and Catalytic cycle frequency Determination based on Molar mass-dependent Model equations

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- Turnover frequency (TOF) is lowest with high concentration ([E₀]) of the enzyme
- Turnover number (TON) and TOF are respectively highest and lowest for low [E₀]
- Catalytic cycle frequency is highest for the highest concentration of the enzyme
- TON and TOF are functions of the molar mass and [E₀]
- TOF which varies is not equal to 1st order catalytic rate constant
**Title:** Turnover and Catalytic cycle frequency Determination based on Molar mass-dependent model Equations

**Running title:** Turnover frequency depends on substrate molar mass.

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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 ($k_{\text{cat}}$) in the literature. Based on the Benfield and Lineweaver-Burk methods, data were generated. The results showed that the CCF for the forward (3.63 exp. (+7)/s) and reverse (1.196 exp. (+9)/s) and the TOF (8.294 exp. (−4)/s) were respectively the highest and lowest for the highest concentration $[E_0]$ of the enzyme. The number of fragments per molecule of an enzyme was $= f([ET])$, where $[ET]$ was in molar units and it was $\gg [ST]$ (in molar units), and where $[ST] \gg [ET]$, it was $= f([ST])$. In conclusion, TOF and CCF were different, and in particular, the former was not the same as $k_{\text{cat}}$, while the latter was a constant. TOF and CCF vary. Strictly speaking, TOF was $= f(M_3)$. This can be verified with sucrase in a future study.

**Keywords:** Aspergillus oryzae alpha-amylase, catalytic cycle frequency, first-order catalytic rate constant, gelatinized insoluble potato starch, turnover number, turnover number frequency.

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1. **Introduction**

So many concerns have been expressed regarding what should be the turnover number and turnover frequency [1-3]. This is important when polymers are the substrate. Hence, a brief overview of issues about starch cannot be out of place. Starch is a very important polymer composed mainly of branched-chain, larger molecular-weight amylopectin and straight-chain, smaller molecular-weight amylose. The two forms are homoglucans. They are composed of only $\alpha$-D-glucose moieties linked by an $\alpha$-1, 4glycosidic bond in all kinds of glucan, while the amylopectin has an $\alpha$-1, 6-glycosidic bond at the branch points. Starch composed of $\alpha$-glycosidic bonds is subject to the endoglycolytic activity of $\alpha$-(1, 4)-glucan glucanohydrolases such as $\alpha$-amylase. It is obvious that the short-term supply of energy and ultimately the long-term molecular energy needs of humans are met by the various sources of natural starch. Beyond the need for food, starch has applications in the pharmaceutical industry for the production of encapsulating agents, laundry, and biofuel production, in addition to its applications in electronics, drug delivery, antimicrobials, and structural materials [4]. Whatever the interest in starch, the most important factor that can satisfy such an interest is the degree of polymerization of starch; this is a major determinant of the molar mass of 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. Apart from the effect of high viscosity, the frequency of collisions and nonspecific interactions of the enzyme with surrounding fragments, some of which present
noncatalytic configurational orientation to the enzyme, can increase the residence time of the enzyme molecules in any non-catalytically oriented complex [5]. This can further be orchestrated by the increased surface area presented by the numerous shorter fragments than the parent polysaccharide, a scenario 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]. Known or unknown to the advocates of single turnover catalytic events, the underlying assumption is the reverse quasi-steady-state assumption based on the premise that in such assays \([E_0] \gg [S_0]\) [8, 9]. The essence is to pursue a single-turnover approach and thereby prevent catalytic cycling in order to measure chemical steps. To isolate events at the active site of the enzyme without catalytic cycling, single-turnover conditions are utilized so that all of the substrate will participate in the 'single-turnover' [10, 11]. Against these backdrops, unlike in the literature, the goal of this study is to contrast turnover number, turnover frequency, number of catalytic cycles, and frequency of catalytic cycles per unit time with each other. The main objectives are the derivation of equations for the determination of all parameters, the evaluation of the equations with experimental data, and the determination of the number of substrate fragments per molecule of the enzyme. Here, a molar mass-based and a double reciprocal plot model linked to the number of catalytic cycles are essential for the primary determination of the number of catalytic cycles per a given number of moles of the enzyme in a reaction mixture.
1.1 Significance Statement

In the 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 (graph pad, for instance), a hyperbolic curve is no longer relevant. Thus, a factor, φ is introduced in the following relation to account for nonlinearity. This is intended to address a situation in which the substrate concentration, \([S_0]\), is \(\gg\) the enzyme concentration, \([E_0]\), such that the following equation may hold.

\[
\frac{[S_0]}{[S_0] + \frac{M_S}{M_2}[E_0]} = \frac{v_i}{v_i + V_{\text{max}}\varphi},
\]

(1)

Therefore, the maximum velocity, \(V_{\text{max}}\), is the asymptotic kind because it is \(\gg\) the mixed order maximum velocity \((V_{\text{max}}^{\text{press}})\), where the \([S_0]\) ranges between values that are \(\ll\) the Michaelis-Menten constant, \(K_M\), and values \(<K_M\). Let it be made clear that while \(M_2\) is definitely the molar mass of the enzyme if known \(\textit{ab initio}\), the molar mass of the substrate cannot be definitely represented by \(M_S\) for reasons that are germane in the 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:

\[
[S_0](v_i + V_{\text{max}}) = \frac{M_S}{M_2} [E_0] v_i \varphi + v_i \varphi [S_0],
\]

(2)

\[
[S_0](v_i - \varphi) = \frac{M_S}{M_2} [E_0] v_i \varphi - V_{\text{max}} [S_0].
\]

(3)

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

\[
\frac{1 - \varphi}{V_{\text{max}}} = \frac{M_S}{M_2[S_0]V_{\text{max}}} [E_0] \varphi - \frac{1}{v_i},
\]

(4)

Rearrangement gives:

\[
\frac{1}{v_i} = \frac{M_S}{M_2[S_0]V_{\text{max}}} [E_0] \varphi + \frac{\varphi - 1}{V_{\text{max}}},
\]

(5)

Given the slope (\(S_L\)) and the intercept (\(I_{NT}\)) from the double reciprocal plot (Eq. (1)), the
following should be obtained:

\[
I_{NT} = \frac{\varphi - 1}{V_{\text{max}}},
\]

(6)

However, the product of \(V_{\text{max}}\) and \(I_{NT}\) is = 1 such that \(\varphi\) is = 2.

\[
S_L = \frac{M_S}{M_2V_{\text{max}}} [E_0] \varphi,
\]

(7)

Therefore,

\[
M_S = \frac{S_L M_2 V_{\text{max}}}{2[E_0]},
\]

(8)

Although literature data is available for the evaluation of Eq. (8), an introspective
evaluation is carried out using hypothetical data such as: \(S_L = 19872\); \(V_{\text{max}} = 8 \exp. (-5)\)
\(M/\text{min}; M_2 = 52 \exp. (+3) \text{ g/mol.}; \text{and } [E_0] = 0.00025 \text{ g/L.} \) 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 \(\gg\) the true molar mass of the parent
substrate, where $[E_0]$ is $\ll [S_0]$. Meanwhile, recall that $V_{\text{max}}/[E_0]$ is the catalytic first-order rate constant, $k_{\text{cat}}$, and the product of the slope, $S_L$, and $V_{\text{max}}$ is equal to the $K_M$. Thus, Eq. (8) is written as:

$$M_S = \frac{K_M M_2}{2[E_0]}$$  \hspace{1cm} (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 $< \text{ or } >$ the $K_M$. The value of $M_S/M_3$ ($№$) can either be $< \text{ or } \gg 1$; it may be $= 1$. The number of turnovers ($№$) is therefore defined mathematically as:

$$№ = \frac{K_M M_2}{2[E_0]M_3}$$  \hspace{1cm} (10)

where $M_3$ is the molar mass of the substrate. The magnitude of $№$ can either be $\leq$ or $\geq 1$ in accordance with the concentration of the enzyme and the concentration 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.

Most importantly, recall that a polymer has a degree of polymerization, and a single turnover is not about one polymer participating in any transformation to a product. With disaccharide as substrate, for instance, and a known large molar mass of sucrase and a relatively lower molar mass of sucrose, $№$ could be very large (as large as a value $\geq \exp{(+6)}$ if $[E_0] \ll 1$). Note for the purpose of derivation that, given the saturating concentration of the substrate for different concentrations of the same enzyme under the same conditions of assay, $k_{\text{cat}}$ is constant in principle. But with higher $[S_0]$, if gelatinized, the viscosity may be very high, thereby hindering translational diffusion of the molecules of the enzyme; this may affect the $K_M$ and $V_{\text{max}}$. On the other hand, given the same substrate concentration regime, two different concentrations of the enzyme may give different $V_{\text{max}}$ and different $K_M$ values. One of them may not be a true $K_M$ (and $V_{\text{max}}$) because one of the enzyme
molecules may not have been saturated. Hence, $K_M/[E_0]$ ([E_0] is in mass concentration units) and $V_{\text{max}}/[E_T]$ ([E_T] is in molar units) can differ. As such, a turnover frequency (regarded as $k_{\text{cat}}$, albeit not exactly so) may not be the same as the number of catalytic cycles per unit time.

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 $\gg$ [$E_T$] (both concentrations must be in molar units). Here, one argues that the number of catalytic cycles may not be the same as the turnover number. Besides, the questions arising from Eq. (10) are as follows:

What is the number of cycles of the enzymatic actions needed for the production of a maximum number of the product, maltose, for instance? What frequency or number of cycles of catalytic action (hydrolysis) per unit time is adequate for the production of the maximum number of molecules of the product within the duration of the assay? Note that Eq. (10) expresses the turnover number due to a given mass concentration of the enzyme. Then the question is: what is the number of cycles per mole per unit time ($f_r^\oplus$), in seconds?

$$f_r^\oplus = \frac{2 \times 1000 K_M M_2 k_{\text{cat}}}{2[E_0] M_3 V_{\text{max}} V_{\text{rn}} \tau_+},$$

(11a)

where the figures 1000, $V_{\text{rn}}$, and 2 are the conversion factors from the liter to the milliliter, the reaction mixture volume ($2 \text{ mL in this study}$), and an integer following the half maximum velocity at substrate concentration equal to the $K_M$. The life span of the catalytic cycles leading to the formation of the product within the duration of assay is designated as: $\tau_+$. Therefore, Eq. (11a) becomes:

$$f_r^\oplus = \frac{1000 K_M M_2 k_{\text{cat}}}{[E_0] M_3 V_{\text{max}} V_{\text{rn}} \tau_+},$$

(11b)
In Eq. (12), \([S_0]\) represents any concentration of the substrate that is either < or > the \(K_M\).

Another version of Eq. (11b), given the definition or equation of the \(k_{cat}\) is given as:

\[
\frac{+\left(\phi\right)}{[E_0]} \frac{500 [S_0]M_2 k_{cat}}{[E_0]M_3 v_i V_{rn} \tau^+},
\]  

(12)

where one should recall that \([E_0]\) is the mass concentration of the enzyme. Besides, as explained in several preprint presentations [12-14], given very accurate initial rates under well-defined quasi-steady-state assumptions, which may be either of the following: rQSSA, sQSSA, or tQSSA, [7, 15-17], initial rates that follow a polynomial [13, 14] can enable the determination of the kinetic parameters, including the \(K_M\), as desired in this study instead of going through several plots. Such an equation in its simplest form is given as:

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

(14)

\[
\frac{+\left(\phi\right)}{[E_0]} = \frac{1000 K_M M_2^2}{[E_0]^2 M_3 V_{rn} \tau^+},
\]  

(15)

Equation (12) can also take the form:

\[
\frac{+\left(\phi\right)}{[E_0]} = \frac{500 [S_0]M_2 V_{max}}{[E_0]^2 M_3 v_i V_{rn} \tau^+},
\]  

(16)

Similarly, Eq. (12) can assume another version following the same argument leading to Eq. (16).

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

(17)

\[
\frac{+\left(\phi\right)}{[E_0]} = \frac{500 [S_0]M_2^2}{2[E_0]^2 M_3 v_i V_{rn} \tau^+ ([S_0]_n v_{n-1} - [S_0]_{n-1} v_n)},
\]  

(18)

Given that the volume of a reaction mixture in a laboratory setting is small and that, in the quantification of catalytic cycle (CC) in an industrial setting, a small volume of the reaction mixture is used for the estimation of all parameters and products, the total CC applicable in a larger industrial vessel (whose volume is designated as \(V_{(ind)}\)) is summarily given as follows: [19, 20] the number of catalytic cycles within the duration of the assay is:
\[ V_{\text{max}} = \frac{\exp(+6) K_M M_2 k_{\text{cat}} V_{\text{ind}}}{[E_0] M_3 V_{\text{max}} V_{\text{rn}}^2 \tau_+}, \]  

This is with the understanding that, \( V_{\text{ind}} \) is in liters while, as usual \( V_{\text{rn}} \) is in cubic centimeters, thereby necessitating the introduction of another conversion factor.

\[ \frac{V_{\text{ind}}}{V_{\text{rn}}} \]

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

\[ \frac{1000 M_2^2 k_{\text{cat}}}{[E_0]^2 M_3 SC V_{\text{rn}} \tau_+}, \]  

\[ \frac{1000 M_2^2 k_{\text{cat}} V_{\text{ind}}}{[E_0]^2 M_3 SC v_{\text{rn}}^2 \tau_+}, \]

Meanwhile, what may tentatively be regarded as the duration (\( \tau_+ \)) of catalytic cycles in the forward direction—the formation and the release of product—is given by the following equation:

\[ \frac{1000 K_M M_2^2}{[E_0]^2 M_3 V_{\text{rn}} \tau_+} = \frac{\bar{N}_P}{t}, \]

where \( \bar{N}_P \) and \( t \) are the number of molecules of the product, maltose and the duration of assay respectively.

\[ \tau_+ = \frac{1000 K_M M_2^2 t}{[E_0]^2 M_3 V_{\text{rn}} \bar{N}_P}, \]

The reciprocal of \( \tau_+ \) gives the frequency of the catalytic cycle in the product-destined direction.

\[ \frac{1}{\tau_+} = \frac{1000 K_M M_2^2 V_{\text{rn}} \bar{N}_P}{1000 K_M M_2^2 t}, \]

Equation (25) satisfies the process, \((E+S\rightleftharpoons \text{ES}^{\#} \rightarrow \text{EP} \rightarrow E+P) (\frac{\text{fr}}{\text{fr}} \text{©})\); the 2\textsuperscript{nd} process is given the following equation:

\[ \frac{1}{\tau_-} = \frac{1000 K_M M_2^2 V_{\text{rn}} \bar{N}_S}{1000 K_d M_2^2 t}, \]
In Eq. (26), $K_d$ is the equilibrium dissociation constant of the ES into free substrate and free enzyme molecules, while $\hat{N}_S$ is the number of substrate molecules released. 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:

\[
\frac{1}{\tau^+} = \frac{f_r \circ \text{pres}}{1000K^\text{pres}_M M^2_2 t}, \tag{27}
\]

\[
\frac{1}{\tau^-} = \frac{f_r \circ \text{pres}}{1000K^\text{pres}_d M^2_2 t}, \tag{28}
\]

With respect to Eq. (26), $K_d$ must be calculated for the sQSSA case given the equation:

\[
k^{-1} + k_{\text{cat}} = K_M k_1; \quad k_d = K_M - k_{\text{cat}}/k_1 \text{ where } k_{\text{cat}}/k_1 \text{ is the well-known von Slyke and Cullen constant} \text{[21]} \text{ and } k_1 \text{ is the 2nd-order rate constant for the formation of the ES. In order to reflect the clear and subtle differences between Eq. (26) and Eq. (28), the former is rewritten as:}
\]

\[
\frac{1}{\tau^-} = \frac{f_r \circ \text{pres}}{1000\left(K_M - k_{\text{cat}}/k_1\right) M^2_2 t}. \tag{29}
\]

Again, Eq. (28) assumes the same structure as Eq. (29), but different methods and equations are needed for the determination of $k_1$ [22, 23]. Equation (28) is permissible on the condition that a plot of initial rates ($v_i$) versus $[S_0]$ yields a polynomial curve containing a negative coefficient of the leading term in addition to the fact that $[E_T]$ (in molar concentrations) is either only $> [S_T]$ (in molar concentrations) or it is $\approx [S_T]$ (it may also be about the same concentration as the $K_M$); if the $[E_0]$ is $\gg [S_0]$, 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 slope of the linear plot gives directly a slope equal to $V_{\text{max}}^{\text{pres}}/K_d$ (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 $[E_0]$ is $> [S_T]$ or it is $\approx [S_T]$ ($\approx K_M$) the
The equation takes the form:

$$\frac{1}{\tau_+} = \frac{[E_0]V_{rn}\bar{\hat{S}}}{1000(K_{M}^{\text{pres}} - k_{\text{cat}}^{\text{pres}}/k_1^{\text{pres}})M_2^2t},$$  \quad (30)

A preprint [13] has revealed what the situation is like where the initial rate, \(v_i\), is consistently directly proportional to the substrate concentration ([S_0]) range explored for the assay. Under such a scenario, the equation should be:

$$\frac{1}{\tau_-} = \frac{[E_0]V_{rn}\bar{\hat{S}}}{1000M_2t},$$  \quad (31)

Given that Eq. (31) goes for an enzyme concentration \(\gg\) the concentration of the substrate, it would appear that there is one constant of proportionality given by leaving no room for the determination of \(k_1\); this challenge can be overcome by the method described in the literature. [22]. Thus, with \(k_1\) in the equation, \(K_d = k_{-1}/k_1, k_{-1}\) given as \(K_d k_1\) and multiplied by \([E_T]\) gives the molar concentration of the substrate molecules that may have dissociated, if any, but must quickly rebind due to the oversaturating concentration of the enzyme. Therefore, in Eq. (31), \(\bar{\hat{S}}\) is = \(\exp(-3)\). \(K_d k_1[E_T]V_{rn}N_A t\). For the product case, \(\bar{\hat{P}}\) is = \(\exp(-3)\). \(V_{max} V_{rn} N_A t\). To give the equations for visualization at once, substitutions give the following:

$$\frac{1}{\tau_+} = \frac{[E_0]V_{rn}\bar{\hat{P}}}{1000M_2t} = \frac{[E_0]\exp(-6)\max V_{rn}^{2}N_A}{M_2},$$  \quad (32)

$$\frac{1}{\tau_-} = \frac{[E_0]V_{rn}\bar{\hat{S}}}{1000M_2t} = \frac{\exp(-6)K_d k_1[E_0]^2V_{rn}^{2}N_A}{M_2^2},$$  \quad (33)

Considering the effect of crowding with time, the number of fragments left after the duration of the assay has the cognate equation derived as follows:

$$2k_{\text{cat}} = \frac{N_{\text{frag}} + N_AV_{max}V_{rn}M_{alt}/M_3}{N[E_T]t},$$  \quad (34)

$$N_{\text{frag}} = 2N[E_T]t k_{\text{cat}} - \exp(-3)N_AV_{max}V_{rn}M_{alt}/M_3,$$  \quad (35)

For a molecule of the enzyme that acts hydrolytically on the substrate, there are a number of fragments as long as the substrate is a polymer. Thus,
One can also write an equation for \( N_{\text{frag}} \) where half the maximum velocity of catalysis is the case as follows:

\[
N_{\text{[frag/2]}} = N_{[ET]} \tau_s k_c - 5 \exp \left( -4 \right) N_A V_{\text{max}} V_{rn} M_{\text{alt}} t / M_3 N_{[ET]} \tag{37}
\]

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

\[
0.5 \frac{N_{\text{frag}}}{N_{[ET]}} = \phi_{0.5\text{frag}} = t k_c - 5 \exp \left( -4 \right) N_A V_{\text{max}} V_{rn} M_{\text{alt}} t / M_3 N_{[ET]} \tag{38}
\]

Note that Eqs (35) and (36) represent the total number of fragments for each concentration of the substrate.

In these derivations, a situation where \([S_T]\) is not \(\gg [ET]\), the possibility of \([ET]\) being \(\approx [S_T]\) as well as \([S_T]\) being not \(\gg [ET]\) are relevant in line with the notion that the reactant stationary assumption (RSA) [7], standard quasi-steady-state assumption (sQSSA), and reverse QSSA (rQSSA) [18-18] may be valid in such a scenario. As noted elsewhere, the [24] 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 mass conservation law. This 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. It could aid in the determination of what a research scientist in a clinical setting in particular expects in the control of diabetics, for instance, given that the investigator would want to know what amount of a starchy food is digested and what amount is undigested. The industrialist involved in biomass conversion for biofuel production can explore the model for comparing the efficiency of enzymes and the degree of resistance to digestion of polysaccharide sources.
3. Materials and methods

3.1 Materials

3.1.1 Chemicals

As in the recent literature [23], *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 [25].

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.

3.1.2 Methods

The preparation of reagents and methods (Bernfeld) of assay for the generation of initial rates and the determination of kinetic parameters were as previously described [23].

4. Result and discussion

Interest in the estimation of turnover number (TON) and its frequency is known [26, 27]. A not-too-clear application of the quotient of the catalyst turnover number and the rate constant of spent catalyst replacement [26] and the Lumry-Eyring model [27] for the estimation of TTN are known. In this study, an alternative approach has been derived (Eq. 9). The equation shows that, for a given concentration of the enzyme, the turnover number, $N_e$, is comparatively quantified either by the relation $N_e \leq 1$ or $N_e \geq 1$ (Table 1) for polysaccharide. It follows that $N_e$ is not necessarily a large figure where polysaccharide with a characteristically large degree of polymerization is the case for any mass
concentration of the enzyme, but its magnitude is inversely proportional to \([E_0]\). However, 

\[ \text{rate per second gives values that are } \ll 1/s. \]  

It is crystal clear that TOF is not the same as the catalytic rate constant, which is often reported as the ratio of the maximum molar concentration of one of the products to the molar concentration of the enzyme.

If the choice of the research scientist or reactor designer is the condition in line with, rQSSA, where \([E_T] \gg [S_T]\), then it is very probable that all the substrate molecules should be transformed into products within the chosen duration of assay as expected in a single turnover catalytic event without the need for extra recycling of the enzyme molecules for another round of catalytic hydrolysis of the substrate. On the other hand, the choice may be one in which \([S_T] \gg [E_T]\) in which a fraction of the substrate is transformed (turnover event) into the product, leaving behind a substantial amount of free substrate and fragments [24]. The enzyme has opportunity to be recycled meeting any of the free substrate or fragments. Catalytic cycling needs to be classified into positive recycling (product destined) and negative recycling (deactivation of activated ES and ultimately dissociation into free S and E). The focus is always on the positive aspect.

The input material is the substrate, and the output material is a fraction of the substrate that has been transformed or that has undergone a turnover process during the catalytic cycle to yield the product. Now the question is: how many catalytic cycles were able to transform a given fraction of the substrate polymers? What is the number of molecules of the product yielded from such transformations? Another question that arises is: does it mean that the number of substrate molecules that underwent the turnover processes is the same as the number of catalytic cycles? The answer to the third question is no. 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: \( E + S \leftrightarrow ES \leftrightarrow ES^\# \rightarrow EP \rightarrow E + P \). The forward and backward reactions beginning with \( ES^\# \) are two-step processes. Either of them can be faster than the other. When the \( ES^\# \) dissociates into free \( E \) and \( S \), 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.
**Table 1.** Catalytic cycles per unit time, turnover frequency and the number of unhydrolyzed fragments of the substrate per molecule of the enzyme.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Mass concentration of enzyme /g/L</th>
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<tbody>
<tr>
<td></td>
<td>0.0002</td>
</tr>
<tr>
<td></td>
<td>+ CC/s</td>
</tr>
<tr>
<td></td>
<td>− CC/s</td>
</tr>
<tr>
<td></td>
<td>№</td>
</tr>
<tr>
<td></td>
<td>TOF/s</td>
</tr>
<tr>
<td></td>
<td>NF/E at $V_{\text{max}}$</td>
</tr>
<tr>
<td></td>
<td>NF/E at 0.5$V_{\text{max}}$</td>
</tr>
</tbody>
</table>

$+$ CC/s ($f_r^{+}$) and $-$ CC/s ($f_r^{-}$) are the catalytic cycles in the forward and reverse directions respectively. They are respectively, according to Eqs (32) and (33); № and NF/E are the turnover number and the number of fragments of the substrate polymer per molecule of the enzyme, E. They ($\phi_{\text{frag}}$) and (0.5$\phi_{\text{frag}}$) are respectively according to Eqs (36) and (38) while № is according to Eq. (9).

The analysis and discussion rest on the following premise: "Since catalysis is a kinetic phenomenon, the rate at which catalytic cycles turnover is the essential goal of catalytic research, insofar as it gives detailed information on how the cycle turns over" [2]. But the question is: 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; one of them, the catalytic cycle, gives effect to the other, the turnover. In a catalytic cycle, there are steps: Abortion of any of the chemical steps terminates the turnover of the substrate to the product.
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," and it is taken as a key industrial performance criterion [1, 3, 28]. 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 [29]. 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. [30], who based such work on the equation [31] below.

\[ k_{\text{turn}} = \frac{k_{\text{cat}}[S_0]}{[S_0]+K_M} \]  

Eq. (39)

The equation may not be an outcome of "a supportive and acceptable evolutionary pressure" but a thought process; here, "the turnover rate \(k_{\text{turn}}\) 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" [30]. This definition notwithstanding, \(k_{\text{cat}}\) is given as \(V_{\text{max}}/[E_T]\), and substitution into Eq. (39) with rearrangement gives:

\[ [E_T]k_{\text{turn}} = \frac{V_{\text{max}}[S_0]}{[S_0]+K_M} \]  

(40)

This study comes up with alternative definitions, explanations, and methods of calculation. While the conceptual definition given by Kozuch et al. [1] 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 [1]. Besides, the TOF is, according to Boudart [2], simply defined as the number of revolutions of the catalytic cycle per unit time, generally the second. Rather, the TOF is a function of the catalytic cycle per unit time in seconds. If \(k_{\text{cat}}\) is equivalent to TOF, there cannot be any single turnover event with any concentration of the enzyme considering the
fact that \( k_{\text{cat}} \) times time is \( \gg 1 \). This is applicable to \( v_i/[E_T] \) times time (\( v_i \) can be the lowest initial rate for the lowest concentration of the substrate).

In this study, the catalytic cycle (CC) is the totality of sequential steps, each with a life span, leading to the release of products, which constitutes the turnover of the substrate. Therefore, the number of CC per second (\( \frac{v}{[E_T]} \)), that is either in the forward or reverse direction is the catalytic cycle frequency. There cannot be any argument about the fact that the catalytic rate constant is not the same as TOF and the catalytic cycle frequency (\( \frac{v}{[E_T]} \)), which are not equivalent to either the reverse first-order rate constant (\( k_{-1} \)) or the \( k_{\text{cat}} \). For our undergraduates at the border line, a simple analogy suffices: "Two vehicles at the same speed with different capacities, one that can transport 10 goods and the other 5 goods of the same kind in every aspect, are assigned to transport the goods over the same distance to a location. If each trip takes 30 minutes and there are a total of 120 goods divided by 2 for each vehicle, then it should take, respectively, 180 and 360 minutes for the vehicles to execute the task. The rate constant for one is 0.333/min and the other is 0.167/min; yet both possess the same number of trips per unit time (0.0333/min)". Thus, the (\( \frac{v}{[E_T]} \)) is not the same as TOF, let alone the \( k_{\text{cat}} \) or \( k_{-1} \), the reverse rate constant, as the case may be.

Although the phenomenon of viscosity becomes attenuated with hydrolysis of the gelatinized polysaccharide, the increasing number of fragments seems to lessen the effect of high viscosity; this may contribute in part to the hyperbolic curve tendency as the rate of increase in product (P) formation (\( \Delta \hat{c}[P]/\Delta \hat{c}t \)) decreases. This is generally regarded as a crowding effect [5, 32]. As shown in Table 1, all parameters except the number of fragments per molecule of the enzyme showed an increasing trend with higher \( [E_0] \). The highest values of catalytic cycle frequency, 3.626 exp. (+7)/s and 1.196 exp. (+9) for the forward and reverse reactions, respectively, were recorded for the highest concentration of the enzyme. A very high catalytic cycle frequency promotes either a singer turnover event...
or even a fraction of it since most, if not all, of the substrate molecules are transformed (turned over) to product. Therefore, the turnover number (0.149) and the corresponding frequency (8.294 exp. (−4)/s) are lowest for the highest $[E_0]$ and highest, 3.147 and 1.748 exp. (−2)/s, for the lowest $[E_0]$ (Table 1).

It appears that different substrate concentrations and the range give different numbers of fragments with different values of $[E_0]$. Higher concentrations of the substrate (5 to 10 g/L), to which 0.0002 g/L was exposed, gave the highest value (1.22 exp. (+5)) of the number of fragments, while much lower concentrations of the substrate (the same starch), ranging between 0.3 and 3 g/L, gave a higher value (3.66 exp. (+4)) of the number of fragments with a higher concentration of the enzyme; it seems therefore that two scenarios, $[S_0] \gg [E_0]$ and $[E_0] \gg [S_0]$, produce a higher number of fragments. These numbers of fragments are expected to be composed of fewer oligosaccharides, tetrasaccharides, trisaccharides, etc. A test of the validity of the model equations is expressed by the observation that the total number of maltose units per molar mass (64.54 exp. (+6)) [33] of insoluble potato starch is ≈ 1.89 exp. (+5), which is > any of the number of fragments.

5. Conclusions

The different equations for the determination of turnover number (TON), catalytic cycle (CC) frequency, and number of fragments per molecule of the enzyme (NF/E) were derived. The parameters differ from each other: the TON is the number of times it takes a mass concentration of the enzyme to convert the substrate to product; the speed (or rate) is the TOF. TON can be <, >, and = 1 for polysaccharides or any hydrolyzable polymer. The CC is the totality of all events leading to the formation of the product and its release; the number of CC per unit time is called the CC frequency; the TON per unit time is not equal to the first-order rate constant ($k_{\text{cat}}$). TOF may vary, but $k_{\text{cat}}$ is expected to be constant for
any concentration of the enzyme. A higher concentration of the enzyme ([E₀]≫[S₀]) 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 ([E₀]≫[S₀]) or sQSSA ([E₀]≪[S₀]) 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. Future research will examine the proposition that small molar mass substrates and possibly large molar mass enzymes give large TON and TOF.

Author contribution

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

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Devotion

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