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Effect of Different Durations of Assay and Substrate Concentrations on Kinetic Parameters of Amylase with Criteria for Validity in Focus

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Author's contribution

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

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ABSTRACT

Introduction: Researchers adopt different duration of the assay for a given concentration of the enzyme using different substrate concentration ranges. The kinetic parameters (KP) may not be the same for the different duration of the assay. Therefore, it is imperative to determine the KP arising from different durations of the assay and different substrate concentrations. It is also necessary to investigate the validity of the KP given the substrate concentration regime used for assay.
Method: The experiment entails Bernfeld method of enzyme assay for the generation of data. (Modified or alternative) direct linear plots and Lineweaver – Burk plots were carried out using Microsoft Excel. The validity of kinetic parameter was examined using the validating equations such as standard quasi-steady-state approximation (sQSSA), total QSSA (tQSSA), reverse QSSA (rQSSA), and reactant stationary assumption (RSA).
Results and Discussion: Kinetic parameters, KP, Michaelis – Menten constant (K_m) and maximum velocity of hydrolysis (v_{max}) generated from different linear plots generally differed in magnitude.

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Larger magnitude of KP was generally observed at the shorter duration of the assay. The pseudo first-order rate constant, *k* was higher at lower substrate concentration. The KP values satisfy the condition for the validity of rQSSA regardless of the duration of the assay.
Conclusion: Increasing duration of assay of *Aspergillus oryzea* alpha amylase leads to a decrease in the magnitude of kinetic parameters, enzyme-substrate complex dissociation constant (k_s) and maximum velocity of catalysis (v_{max}) and increasing concentration of the substrate leads to decreasing magnitude of the pseudo first-order rate constant, *k* for the utilization of substrate. The duration of the assay does not influence or alter the criterion for the validity of kinetic parameters if the potato starch concentration range is « enzyme concentration.

Keywords: Aspergillus oryzea alpha amylase; kinetic parameters; quasi-steady state approximations; different duration of the assay; different substrate concentration; criteria for the validity of kinetic parameters.

ABBREVIATIONS

MM *: Michaelis–Menten;*
LWB *: Lineweaver Burk Plot;*
DLP *: Direct linear plot;*
ADLP *: Alternative direct linear plot;*
sQSSA *: Standard quasi-steady state approximation;*
rQSSA *: Reverse quasi-steady state approximation;*
tQSSA *: Total quasi-steady state approximation;*
RSA *: Reactant stationary assumption.*

1. INTRODUCTION

The effect of substrates concentration on enzyme-catalysed reactions has been studied for over a century. The observation has always been that the rate or velocity (*v*) of hydrolysis increases with increasing concentration of the substrate, reaching maximum velocity (v_{max}) as the concentration of substrate tends to infinity [1]. There is also, the observation that the velocity of hydrolysis of polysaccharides in particular decreases with higher substrate concentration due to the effect of increasing viscosity [2]. This raises the question as to whether or not saturation phenomenon may be accounted for in part regarding the effect of hydrodynamic forces.

To this end, it has been postulated that the steady-state kinetics of the majority of enzyme-catalysed reactions are described regarding mechanisms that predict a hyperbolic relationship between the steady-state velocity of product formation, *v*, and the substrate concentration, [*S*] [1]. The mathematical expression known as Michaelis – Menten (MM) equation for this relationship is:

$$v = \frac{v_{\max}[S_T]}{K_m + [S_T]} \quad (1)$$

Where $[S_T]$ and K_m are total substrate concentration and MM constant respectively; *v* and v_{max} are the velocities of hydrolysis and maximum velocity of hydrolysis respectively. The objection against this model is that a plot of *v* versus $[S_T]$ gives rectangular hyperbola on account of which it is impossible to accurately measure v_{max} and K_m because the asymptotes cannot be approached closely enough thereby necessitating the need for direct linear plot that may also lack precision if point of intersection of lines cannot be defined precisely [1]. It was found, however, that in the 21st century there is software that can be used to carry out a nonlinear plot of *v* versus $[S_T]$ [3]. It is not certain, however, whether there is software for the execution of direct linear plot (or if it is as readily available as the software for the nonlinear plot).

It has also been reported that substrate unbinding increases the velocity of enzyme-catalysed reaction [4]. There is also the issue of conformational change upon substrate binding to an enzyme [5, 6]. Despite the different opinions regarding the ways kinetic parameters are generated and coupled with the issue of the validity of such data as may be determined by the various condition for the validity of various QSSA, there is no concern about what the effect of different duration of the assay on the kinetic parameters might be. There is also no much concern about what the effect of different concentration of the substrate might be on the pseudo - first order constant for the hydrolysis of the polysaccharide. Thus the need to examine the values of pseudo-first order rate constant and what is often termed turnover number (rate constant for the formation of the product, k_2.) has become very imperative using *Aspergillus oryzea*

alpha-amylase (EC 3.2.1.1) as a case study. Another question is could the turn over number ($v_{max}/[E_T]$) satisfy the condition for the validity of any of the QSSA? However, direct determination of k_2 is precluded for brevity since the validity of v_{max} tantamount to the validity of k_2.

It has been reported that experimental measurements rarely determine rates or rather the velocity of enzymatic action, hydrolytic action for instance, directly [7]. Rather, substrate or product concentrations are determined at various times, and rates are calculated from the change in concentration with time. This process of differentiating the data is according to Schnell and Mendoza [8,9] inexact and if according to Duggleby [10] as previously cited [7], the assay method is not on a continuous basis and the change in concentration is not linear with time, the rate determination may be unreliable. This should be expected if the saturation point of the enzyme is gradually approached yielding as intended in the original Michaelis-Menten formalism hyperbolic curve that presents different slope/gradient at almost every point. In this research, the enzyme is assayed at different durations. While it is a well-known fact that nonlinear regression is now used to determine MM parameters, there is also the evolution of direct linear plot (DLP) [1] and recently modified (or alternative) DLP (ADLP) [11,12]. However, the approach described by Kerminski and Domino [12] is not very clear, but it is similar to ADLP, *i.e*. $[S_i]/V_i$ versus $1/V_i$ not as points but as straight lines passing through $[S_i]/V_i$, 0 and 0, $1/V_i$.

Despite the emergence of better ways of extracting kinetic parameters, there have been calls for the validation of kinetic parameters by applying the condition for the validity of various quasi-steady state assumptions also called approximations (QSSA) beginning from standard QSSA (sQSSA) [13], reverse QSSA (rQSSA) [14,15], total QSSA (tQSSA) [15,16], and recently reactant stationary assumption (RSA) [17]. The objectives of this research are: 1) To examine the effect of different duration of assay on kinetic parameters determined by DLM, ADLM and Lineweaver Burk method, 2) To investigate the effect of different substrate concentration regime on the magnitude of pseudo-first order rate constant for the transformation of substrate, 3) Examine if different duration of assay affects the validity of kinetic parameters as determined from different condition for the validity of various QSSA.

2. MATERIALS AND METHODS

2.1 Materials

Aspergillus oryzea alpha-amylase (EC 3.2.1.1) and potato starch were purchased from Sigma – Aldrich, USA. Hydrochloric acid, sodium hydroxide, and sodium chloride, were purchased from BDH Chemical Ltd, Poole England. Tris, 3, 5-dinitrosalicylic acid, maltose, and sodium potassium tartrate tetrahydrate were purchased from Kem light laboratories Mumbai India, and Distilled water was purchased from local market. The electronic weighing machine was purchased from Wenser Weighing Scale Limited and 721/722 visible spectrophotometer was purchased from Spectrum Instruments China. *p*H meter was purchased from Hanna Instruments, Italy. The water bath was purchased from Hospibrand, USA.

2.2 Method

Twenty grams of potato starch was mixed in 100mL of distilled water and boiled at 100°C for 3 minutes, cooled to room temperature, and a decrease in volume was corrected by topping the volume with distilled water to 100mL to give 20 g/L as stock. Dilution of the stock was made to give different concentrations ranging from 10-20 g/L. A stock solution of the enzyme was prepared by dissolving 0.01 g of the enzyme in a buffer tablet solution (*p*H=7) to which is added 15 mL NaCl (aq) (0.9 g/100mL) and 15 mL $CaCl_2$ (5 mmol/L) to a final volume of 100 mL. The concentrations are stock solution concentration and diluted stock solution giving a final concentration of 0.04 g/L. A capsule of chloramphenicol was added to the solution of the enzyme to prevent any trace of microbial attack. The assay was carried out according to the method described by Bernfeld [18]. Spectrophotometer readings were taken at a wavelength of 540 nm. The extinction coefficient was ~ 181 L/mol.cm. Kinetic parameters were determined by LWB plot [19], direct linear plot (DLP) [11], and alternative DLP (ADLP) [12]. Microsoft Excel was used to draw the lines linking the x any y points (observations) on the x and y-axis respectively. To retain the lines drawn, the highest data point on the side representing the y-axis in the table of the variable (the points or observation) is left while the lower data points are deleted.

The test of the validity of kinetic parameters, K_m, in particular, was according to various QSSA /

RSA mathematical formalism. Concerning sQSSA, the equation below containing only one constant that has to be determined separately is derived from original Schnell equation [17]:

$$\frac{[E_T]}{K_m+[S_T]} \ll \left(1+\frac{k_{-1}}{k_2}\right)\left(1+\frac{[S_{T(mol)}]}{K_m}\right) \quad (2)$$

With regard to what Tzafriri [16] referred to as tQSSA when $[E_T]$ » $[S_T]$, the equation of validation is:

$$\varepsilon = \frac{k_2 K_m}{2[S_{T(mol)}](k_{-1}+k_2)}\left\{\frac{([E_{T(mol)}]+K_m+[S_{T(mol)}])}{\sqrt{([E_{T(mol)}]+K_m+[S_{T(mol)}])^2-4[E_{T(mol)}][S_{T(mol)}]}}-1\right\} \ll 1 \quad (3)$$

Where, k_{-1}, $[E_{T(mol)}]$, and $[S_{T(mol)}]$ is the reverse rate constant for the disintegration of an enzyme-substrate complex, *ES* (or *C*), the molar concentration of the enzyme, *E*, and the molar concentration of the substrate, *S*, respectively. However, Schnell and Maini [15] objection against QSSA when d[*C*]/d*t* ≈ 0 at high enzyme concentration ($[E_T]$ »$[S_T]$) as against d[*S*]/d*t* ≈ 0 prompted another validating equation given below in favour of rQSSA [20].

$$\frac{k_2 K_m}{(k_{-1}+k_2)[E_{T(mol)}]} \ll 1 \quad (4)$$

Next is the condition for the validity of RSA [14, 17,21] and described as a more general condition for the sQSSA to be valid [15]. The condition is:

$$\frac{[E_{T(mol)}]}{K_m+[S_{T(mol)}]} \ll 1 \quad (5)$$

2.3 Statistical Analysis

The median values were adopted and the standard deviation from median was according to Hozo et al. [22].

3. RESULTS AND DISCUSSION

To examine the effect of different substrate concentrations on the magnitude of pseudo-first order rate constant, different concentration of the substrate was prepared and used to assay the enzyme at different durations. This approach has been implicated to be very useful in resolving the basic problem of the inaccuracy of the velocity determination, and being measurement of time course, or progress curve as often known, it has, according to Schnell and Maini [7], the advantage of yielding multiple experimental points from a single reaction experimental assay or perhaps a single reaction mixture rather than multiple reaction mixtures with different substrate concentrations at the same duration of assay, with the same concentration of enzyme. This implies that from a single reaction mixture, an aliquot (0.5 - 1 mL) can be withdrawn/pipetted at specified time interval into 0.5 – 1 mL oxidising reagent to terminate the reaction and for colour development.

Using progress curves usually decreases the number of experimental assays by at least a factor of five [7]. This may be the case if only one substrate concentration is used. If different substrate concentrations are applicable and different duration of assay is intended, it is possible to determine the pseudo-first order rate constant by plotting $\mathrm{In}\,\frac{[S_T]}{[S]_{(t)}}$ versus *t*, and Michaelis – Menten parameters, K_m, if saturation phenomenon is observed; with replicates, the assay can be a very daunting task and tedious as observed in this investigation. To examine the conditions for the validity of various QSSAs multiple assays were carried out on the specific concentration of the enzyme at different ranges of substrate concentration. The effect of duration of the assay on kinetic parameters was also investigated. Both original direct linear plot (DLP) [1] and modified DLP [11] (otherwise called alternative DRP in this research) were used for the determination of kinetic parameters.

To determine the effect of duration of the assay, different length of times was spent in the assay of the enzyme (as stated earlier, enzyme concentration, $[E_T]$ = 0.04 g/L) at water bath temperature of 36 ^{o}C. Kinetic parameters (KP) generated from different plots after different durations of the assay are shown in Table 1. The magnitude of KP (Table 1) generated from different plots DLP (Fig. 1), LWB plot (Fig. 2), and ADLP (Fig. 3) after the one-minute duration of the assay are in the following order: DLP < LWB < ADLP. The DLP (Fig. 4), LWB plot (Fig. 5), and ADLP (Fig. 6) after two minutes duration of assay yielded KP values (Table 1) that are different in magnitude. Although the magnitude of the parameters from LWB plot and ADLP differed, they are nevertheless very close or similar. Lower magnitudes of the parameters were yielded with DLP.

The different magnitude of KP values (Table 2) from DLP (Fig. 7), LWB plot (Fig. 8), and ADLP (Fig. 9) was also observed after three minutes

duration of the assay. ADLP and LWB plot yielded similar v_{max} values but differed in K_m values. Lowest KP values were obtained with DLP. Once again the KP values (Table 1) from DLP (Fig. 10), LWB plot (Fig. 11), and ADLP (Fig. 12) differ. The v_{max} value from LWB plot is > v_{max} from ADLP while the converse is the case with K_m values. DLP yielded lower KP values.

The variation of kinetic parameters with time was also examined by plotting the parameters versus different duration of the assay. With LWB plot, the generated K_m values showed a decreasing trend with time (Fig. 13a). But for the value of K_m at 2 min duration, all K_m values generated from ADLP plot are higher than those generated from LWB plot (Fig. 13a). With DLP, the K_m values showed a decreasing trend with time as applicable to the result obtained from LWB plot (Fig. 13b).

The maximum velocity, v_{max}, attainable and generated by LWB plot and ADLP showed decreasing trend with time; the v_{max} values from LWB plot and ADLP were similar at 3 min duration of assay but different at other durations of assay, with higher values generated by ADLP at other durations of assay (Fig. 14a). With DLP, the v_{max} values were lower than values from LWB plot; both methods produced a result showing decreasing trend with time (Fig. 14b).

The pseudo-first-order rate constant (k), values showed an irregular pattern with time; with substrate concentration ranging from 10 – 14 g/L, there was a decreasing trend in the magnitude of k values but remained constant with substrate concentrations = 14 and 15 g/L. A further decrease occurred with substrate concentration ranging from 15–17 g/L and then remained constant with substrate concentration ranging from 17–20 g/L (Fig. 15). No one should speculate that these results should be generalizable given the same assay condition. But it is likely that similar trend may emerge with other assay conditions, where, in particular, $[E_T] \gg [S_T]$.

As shown in Table 2, the kinetic parameters, MM constant in particular (as it is supposed to be, if certain condition is fulfilled) from various kinds of plots, DLP, LWB, and ADLP at different duration of assay satisfied the condition for the validity of rQSSA [17] in a definite manner. This is to imply that a definite statement can be made about the ratio of $[S_{T(mol)}]$ to $[E_{T(mol)}]$ on mole to mole basis, *i.e*. $[S_{T(mol)}] \ll [E_{T(mol)}]$. This is unlike the condition for the validity of tQSSA which seems to be satisfied regardless of what the ratio of $[S_{T(mol)}]$ to $[E_{T(mol)}]$ may be. This is to say that there is no way one can know if the condition under which the kinetic parameters were generated by whatever plot satisfies the condition for the validity of Michaelian formalism, the hyperbolic curve relating v to $[S_{T(mol)}]$ or $[S_T]$ [1]. A Michaelian formalism, for emphasis, is one in which steady-state kinetics of a majority of enzyme-catalysed reactions are adequately described regarding mechanism (s) that predicts a hyperbolic relationship between the steady hyperbolic velocity, v, and the concentration, $[S_{T(mol)}]$ [1]. There is also the proposition that Michaelian behaviour is still assured if the relative steady-state populations of free enzyme states are independent of substrate and product concentration [23]. This seems to suggest that enzyme may possess little or no strong interactive binding with the substrate as to be seen to be freely available. But this is possible if $[S_{T(mol)}]$ is overwhelming « $[E_{T(mol)}]$ perhaps, to some extent in this investigation.

Observed in this investigation is the validity of kinetic parameters from the different duration of the assay when the substrate concentration is much higher, ($[S_T]$ = 20 g/L) but on the basis of the condition for the validity of sQSSA. This brings ambiguity on the condition for the validity implied in Eq. (2) considering the fact that at a lower concentration of the substrate, the kinetic parameters were not valid because $[E_T]/([S_T] + K_m) \gg 1$if it is assumed that K_m is exactly what it stands for. For a variable, y, to be » another variable, x, y should not be < 1.5 – 2 – fold > than x: Thus, it seems to be generally valid, the right hand side (RHS) of the inequality (Eq. 2) should be » 1 as to imply that the assay was conducted under condition in which $[S_T] \gg K_m$ for the most part that can guarantee a valid prediction of a hyperbolic relationship between steady state v and $[S_T]$.

What seems to be important in the light of this research finding is that it is either $[E_{T(mol)}]$ is « $[S_{T(mol)}]$ or » $[S_{T(mol)}]$. The relevance of this submission is that the RHS of Eq. (2) is always > 1 and therefore, one might conclude, albeit wrongly, that the left-hand side (LHS) might be < RHS, but that is possible if $[E_{T(mol)}] \ll [S_{T(mol)}]$, thereby satisfying the condition for the validity of sQSSA. But on the contrary, if $[S_{T(mol)}] \ll [E_{T(mol)}]$, the condition for the validity of rQSSA should apply. Since K_m or equilibrium dissociation constant (K_s) is generated using a range of

substrate concentrations, then, K_m and K_s should be seen to be valid under the condition for the validity of validating equations such as intended with Eq. (2) and Eq. (5) [17] regardless of the concentration of the substrate, *S*, within the concentration range used.

Schnell [17] posits that "$[S_{T(mol)}]$ » $[E_{T(mol)}]$ is one of the conditions for the steady – state assumption to be valid, but more importantly, it is a condition for the validity of the RSA". On the basis of this, therefore, if the kinetic parameters do not satisfy the condition for the validity of RSA (Eq. (5)), they cannot satisfy the condition for the validity of sQSSA (Eq. (2)) because both equations have a common factor, $\frac{[E_T]}{K_m+[S_T]}$. While admitting that the condition for the validity should not be restricted to $[S_{T(mol)}]$ » $[E_{T(mol)}]$, but includes $[S_{T(mol)}] \approx [E_{T(mol)}]$ [17], because regardless of the value of K_m on mole – mole basis, $\frac{[E_T]}{K_m+[S_T]}$ where $[E_{T(mol)}] \approx [S_{T(mol)}]$, must always be < 1. It may also be « 1if K_m is very large; in such situation, k_{-1} may be $\geq k_2$ with the implication that the RHS of Eq. (2) should always be > 1 regardless of the value of $[S_{T(mol)}]$. But the LHS of Eq. (2) being < 1 even if $[E_{T(mol)}] \approx [S_{T(mol)}]$, does not guarantee that the expectation for the hyperbolic relationship between *v* and $[S_T]$ is fulfilled without which the Michaelian condition may be violated, and consequently, the condition for the validity of sQSSA should also be violated.

It must be made abundantly clear that every different concentration of the enzyme has a different saturating concentration of the substrate, and, consequently, different K_m and v_{max}. The fact that the kinetic parameters seem to be valid at higher $[S_T]$, means that substrate concentration range does not address the need for the saturating concentration of substrate, *S* for the concentration of the enzyme, *E* used in this research.

The purpose of presenting the hand-drawn lines between *v* and $[S_T]$, and between $1/v$ and $[S_T]/v$ is to show evidence of imperfections associated with DLP and ADLP respectively (Fig. 1, Fig. 3, Fig. 4, Fig. 6, Fig 7, Fig. 9, Fig. 10, and Fig. 12). It is essential to emphasise that velocity data for [*S*] values below K_m value in a direct linear plot of *v* versus [*S*] may not yield straight lines intersecting at a common point if in particular [*E*] > [*S*]. This aspect is the essence of this research. Any strong case of intersection is likely to be a result of very avoidable manipulation or theoretical abstraction. The data generated from the plots are more important as they are intended to show the effect of different duration of the assay on kinetic parameters and implication for their validity. Looking at the DLPs, the number of non-intersecting lines or parallel lines appeared to decrease with longer duration of the assay. ADLPs always give intersection perhaps due to the inverse relationship between $1/v$ and $[S_T]/v$. Further to this, is the approach given below by which $K_{m(ij)}/V_{(ij)}$, and $1/V_{(ij)}$ is calculated [12].

$$\frac{K_{m(ij)}}{V_{(ij)}} = \frac{\left[(V_j - V_i)\left(\frac{S_j}{V_j} - \frac{S_i}{V_i}\right)\right]}{\left[(S_j - S_i)\left(\frac{V_i}{S_i} - \frac{V_j}{S_j}\right)\right]} \quad (6)$$

Equation (6) may serve the interest of researchers who comprehend the issue connected to the approach, though, one may realise that sometimes $\frac{S_j}{V_j} = \frac{S_i}{V_i}$ or $\frac{V_i}{S_i} = \frac{V_j}{S_j}$, such that, $\frac{K_{m(ij)}}{V_{(ij)}} = 0$, if clearly understood. This may be a possibility where there is a common intersection. What seems to be important is that the equation or the approach as in this research may be applied to non-enzymatic systems such as drug-receptor interactions [12] similar to the suggestion that there is a need for new tools including mathematical models in particular applicable to system biology and computer software intended to achieve more accurate results [24] as may be applicable to pharmaceutical research. This is again where the issue of the validity of kinetic parameters on the basis of the condition for the validity of various QSAA/RSA becomes imperative.

However, DLP, ADLP, and LWB plot were used to generate kinetic parameters at the different duration of assay: Results from DLP and ADLP were compared with results from LWB plot in a graphic manner (Fig. 13a, Fig. 13b, Fig. 14a, and Fig. 14b). The observed higher kinetic parameters (K_m (or K_s) and $v_{max} = [E_{T(mol)}]k_2$) at the shorter duration of the assay, in general, may be for reasons that are not farfetched. Before proceeding further, one should recall MM 1913 research in which sucrose was the substrate subject to one hydrolytic action of invertase (EC 3.2.1.1) [24]. The values of K_s and K_m, in particular, depend on whether or not the substrate concentration approached saturation as was the case with the research by the authors. This is to say that for any given $[E_T]$, there is a definite K_m that may be valid as long as the condition for the validity of sQSSA is satisfied. This is unlike polysaccharide, potato

starch in this research, subject to several amylolytic action because when the enzyme involved in complex formation is released as implied in the scheme, $E + S \rightleftharpoons ES \rightarrow E + P + S_F$ (where S_F is polysaccharide fragment or shorter polysaccharide after every hydrolytic action), S_F is also part of the remaining substrate [25] which like the parent polysaccharide can form separately another complex with free enzyme. Thus at the initial transient, $t \leq 1/k_2$ for instance, the substrates are mainly parent polysaccharide with a complete degree of polymerisation (DP). When x mol of reducing sugar, maltose, is yielded by the action of alpha-amylase, 2x mol of glucose is lost from the parent polysaccharide yielding shorter polysaccharide which becomes a different substrate for the free enzyme as t becomes > $1/k_2$. Thus as $t \rightarrow > 1/k_2$, there is a mixture of parent polysaccharide and shorter polysaccharide otherwise called fragments [25].

This different polysaccharide, shorter and longer polysaccharide/oligosaccharide (or dextrin, maltotriose, maltopentaose *etc* [2,26] may present different K_m or K_s and v_{max} values for the free enzyme. A C-4 glucan cannot exhibit the same K_m/K_s values as a C-7 glucan! The cumulative effect of the presence of shorter polysaccharide with longer duration of the assay is the different K_m or K_s and v_{max} which can be calculated in different ways as follows:

$$\Delta K_{\mathrm{m}}\ (\mathrm{or}\ \Delta K_{\mathrm{s}}) = -\int_{t \leq \frac{1}{k_2}}^{t \gg \frac{1}{k_2}} \mathrm{d}K_{\mathrm{m}}(\mathrm{or}\ \mathrm{d}K_{\mathrm{s}}) \qquad (7)$$

The simple mean of all the K_m (or K_s) generated at the different duration of assay, as the case may be, can also be taken as follows:

$$K_{\mathrm{m}}(\mathrm{av})\ (or\ K_s(\mathrm{av})) = \frac{\sum_1^{\Theta} K_{\mathrm{m}}(or K_{\mathrm{s}})}{\Theta} \qquad (8)$$

Where Θ is the number of different duration of assay. Perhaps, a time-weighted average may be better and it is given as follows:

$$K_{\mathrm{m}}(\mathrm{wt})\big(or\ K_s(\mathrm{wt})\big) = \frac{k_{\mathrm{m}(t_1)}(\mathrm{or}K_{\mathrm{s}(t_1)})t_1 + k_{\mathrm{m}(t_2)}(\mathrm{or}K_{\mathrm{s}(t_2)})t_2 + k_{\mathrm{m}(t_3)}(\mathrm{or}\ K_{\mathrm{s}(t_3)})t_3 \ldots}{t_1 + t_2 + t_3 + \cdots} \qquad (9)$$

Equations (7), (8), and (9) are not intended to impress the mathematically minded scholars but to emphasise the fact that the kinetic parameters can never be the same with polysaccharides at different duration of assay; each duration has its own probable kinetic parameter, and the shortest possible duration of assay generates kinetic parameters that are more reflective of what may be expected from the parent polysaccharides. It seems at the duration of assay « 1 min, the K_m (or K_s) may be higher than any of the values obtained in 1 min assay from all the different plots, 28.74 g/L from ADLP, for instance. At this juncture, it must be made clear that if the kinetic parameters generated are validated on the basis of the condition for the validity of rQSSA, then instead of K_m, K_s (k_{-1}/k_1 where k_1 is the second order rate constant for the formation of ES.) should be the parameter.

Examination of the effect of different substrate concentration reveals that the pseudo-first order rate constant (k) for the transformation of the substrate into products may generally not be the same for all substrate concentrations (Fig. 15). It seems the substrate at different concentration may constitute itself as a substrate and sterically hinder direct contact of the vulnerable functional group with the active site for every collision of the enzyme molecule with the substrate molecule as to suggest an element of randomness (or stochasticity) in the interaction of enzyme and substrate. Thus, while MM equation may have been described as a deterministic rate equation [27], the data to be generated from enzyme assay is in part, a product of the stochastic process. Therefore, though the concentration of the substrate may be very high, not all collision is effective; collision may be between the substrate and site other than the active site. This is despite the observation that although the driving force for ligand binding is often ascribed to the hydrophobic effect, electrostatic interactions executed via electrostatic steering also influence the binding process of both charged and nonpolar ligands [25,28]. However, it seems the most important source of constraint is increasing viscosity associated with gelatinized starch at higher concentration that impedes the diffusional mobility of the enzyme [2,29], a hydrodynamic interaction which explains why protein self-diffusion at biological volume fractions is found to have slowed down to 20 % of the dilution limit. There is also the observation that when "an enzyme is incubated with its substrate, the rate of catalysis will decline with time due to the combined effects of substrate utilisation and product accumulation" [10]. As stated earlier, concerning kinetic constant generated at a different duration of the assay, the occurrence of a mixture of different polysaccharides with progress in time may also affect the values of k at different substrate concentration.

Table 1. Kinetic parameters generated from different Plots after different durations of assay

1 minute duration of assay			
Type of plot	DLP	LWB	ADLP
K_m (g/L)	8.0	23.79 ± 0.01	28.74 ± 11.30
v_{max} (mM/mL.min)	1.0	1.87 ± 0.00	2.13 ± 0.55
2 minutes duration of assay			
Type of plot	DLP	LWB	ADLP
K_m (g/L)	4.5	14.88 ± 0.01	14.17 ± 8.17
v_{max} (mM/mL.min)	0.78	1.21 ± 0.01	1.18 ± 0.34
3 minutes duration of assay			
Type of plot	DLP	LWB	ADLP
K_m (g/L)	4.0	8.64 ± 0.01	13.89 ± 4.19
v_{max} (mM/mL.min)	0.7	0.90 ± 0.00	0.90 ± 0.02
5 minutes duration of assay			
Type of plot	DLP	LWB	ADLP
K_m (g/L)	3.50	5.73 ± 0.01	10.40 ± 7.16
v_{max} (mM/mL.min)	0.55	6.50 ± 0.00	0.80 ± 0.02

DLP, ADLP, and LWB are direct linear plot, alternative direct linear plot and Lineweaver Burk plot respectively; K_m and v_{max} are Michaelis – Menten constant and maximum velocity of hydrolysis of starch.

Table 2. Validation of kinetic parameters from different duration of assay on the basis of the condition for the validity of various QSSA and RSA

With substrate concentration =10 g/L in 1 min duration of assay			
QSSA/RSA	LWB	ADLP	DLP
sQSSA(2)	L=2.00; R = φ1.42	L = 1.75; R = φ1.35	L = 3.76; R = φ 2.25
tQSSA(3)	ε = μ0.20« 1	ε = μ0.21« 1	ε = μ0.10 « 1
rQSSA(4)	μ 0.35 «1	μ 0.42 «1	μ 0.12 « 1
RSA(5)	2.00 > 1	1.75 »1	3.76 »1
K_m/g/L	23.79	28.74	8.00
With substrate concentration = 20 g/L in 1 min duration of assay			
QSSA/RSA	LWB	ADLP	DLP
sQSSA(2)	L =1.55; R = φ 1.84	L = 1.39; R = φ1.70	L = 2.42; R = φ3.50
tQSSA(3)	ε = μ0.20 « 1	ε = μ0.21 « 1	ε = μ0.11 « 1
rQSSA(4)	μ 0.35 «1	μ 0.42 « 1	μ 0.12 « 1
RSA(5)	1.55 > 1	1.39 > 1	2.42 »1
K_m/g/L	23.79	28.74	8.00
With substrate concentration = 10 g/L in 2 min duration of assay			
QSSA/RSA	LWB	ADLP	DLP
sQSSA(2)	L = 2.72; R = φ 1.67	L = 2.80;R = φ1.71	L = 4.67; R = φ3.22
tQSSA(3)	ε = μ0.15« 1	ε = μ0.15« 1	ε ≈ μ0.07« 1
rQSSA(4)	μ0.22« 1	μ0.21 «1	≈ μ0.07 «1
RSA(5)	2.72»1	2.80 »1	4.67 »1
K_m/g/L	14.88	14.17	4.50
With substrate concentration = 20 g/L in 2 min duration of assay			
QSSA/RSA	LWB	ADLP	DLP
sQSSA(2)	L = 1.94; R = φ 2.34	L = 1.98;R = φ2.41	L = 2.76; R = φ5.44
tQSSA(3)	ε = μ0.16« 1	ε = μ0.16« 1	ε ≈ μ0.07« 1
rQSSA(4)	μ0.22« 1	μ0.21 «1	≈ μ0.07 «1
RSA(5)	1.94»1	1.98 »1	2.76 »1
K_m/g/L	14.88	14.17	4.50

With substrate concentration = 10 g/L in 3 min duration of assay			
QSSA/RSA	LWB	ADLP	DLP
sQSSA(2)	L = 3.63.; R = φ 2.16	L = 2.83;R = φ1.73	L = 4.84; R = φ3.5
tQSSA(3)	ε = μ0.11« 1	ε = μ0.15« 1	ε ≈ μ0.06« 1
rQSSA(4)	μ0.13« 1	μ0.21 «1	≈ μ0.06 «1
RSA(5)	3.63»1	2.83 »1	4.84 »1
K_m/g/L	8.64	13.89	4.00
With substrate concentration = 20 g/L in 3 min duration of assay			
QSSA/RSA	LWB	ADLP	DLP
sQSSA(2)	L = 2.36; R = φ 3.31	L = 2.00;R = φ2.44	L = 2.82; R = φ6.00
tQSSA(3)	ε = μ0.12« 1	ε = μ0.16« 1	ε ≈ μ0.07« 1
rQSSA(4)	μ0.13« 1	μ0.21 «1	≈ μ0.06 «1
RSA(5)	2.36»1	2.00 »1	2.82 »1
K_m/g/L	8.64	13.89	4.00
With substrate concentration = 10 g/L in 5 min duration of assay			
QSSA/RSA	LWB	ADLP	DLP
sQSSA(2)	L = 4.10; R = φ 2.75	L = 3.32;R = φ1.96	L = 5.01; R = φ3.86
tQSSA(3)	ε = μ0.08« 1	ε = μ0.12« 1	ε = μ0.05« 1
rQSSA(4)	μ0.08« 1	μ0.15 «1	≈ μ0.05 «1
RSA(5)	4.10»1. 1	3.32 »1	5.01 »1
K_m/g/L	5.73	10.40	3.50
With substrate concentration = 20 g/L in 5 min duration of assay			
QSSA/RSA	LWB	ADLP	DLP
sQSSA(2)	L = 2.63; R = φ 4.49	L = 2.23R = φ2.41	L = 2.88; R = φ6.71
tQSSA(3)	ε = μ0.09« 1	ε = μ0.13« 1	ε ≈ μ0.06« 1
rQSSA(4)	μ0.08« 1	μ0.15 «1	≈ μ0.05 «1
RSA(5)	2.63»1	2.23 »1	2.88 »1
K_m/g/L	5.73	10.40	3.50

$\varphi = \left(1 + \frac{k_{-1}}{k_2}\right)$; $\mu = \frac{k_2}{k_{-1}+k_2}$; *L and R are left hand side and right hand side of the inequality. The numbers in parenthesis are equation numbers in the text, theoretical section to be specific*

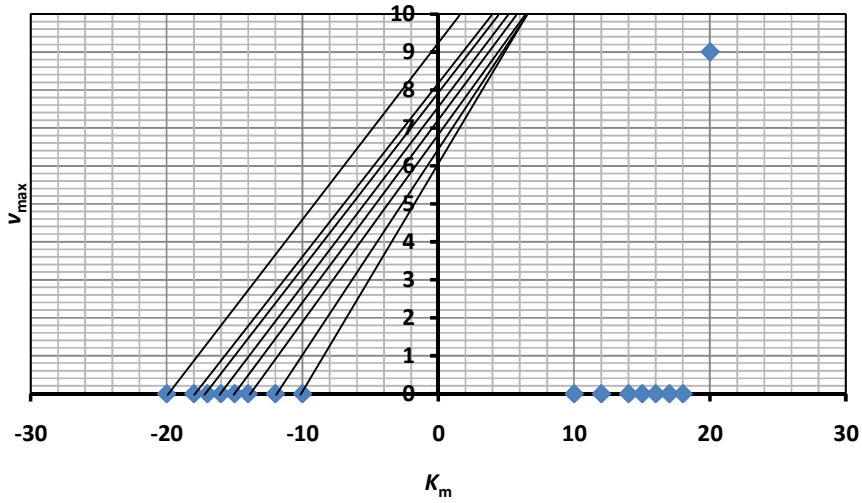

Fig. 1. Direct linear plot where the substrate concentration ranges from 10 – 20 g/L and duration of assay is 1 min. It seems there is only one intersection at upper margin of the graphical area; v_{max} ≈1.00 mM/mL.min; K_m ≈ 8.00 g/L (all data are median values)

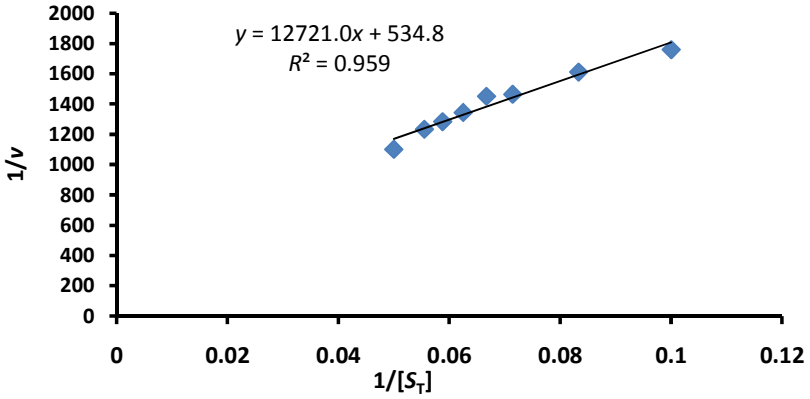

Fig. 2. Lineweaver Burk plot with substrate concentration ranging from 10 – 20 g/L and duration of assay is 1 min: v_{max} = 1.87±0.00 mM/mL.min; K_m = 23.79 ± 0.01 g/L (all data are median values, n = 6)

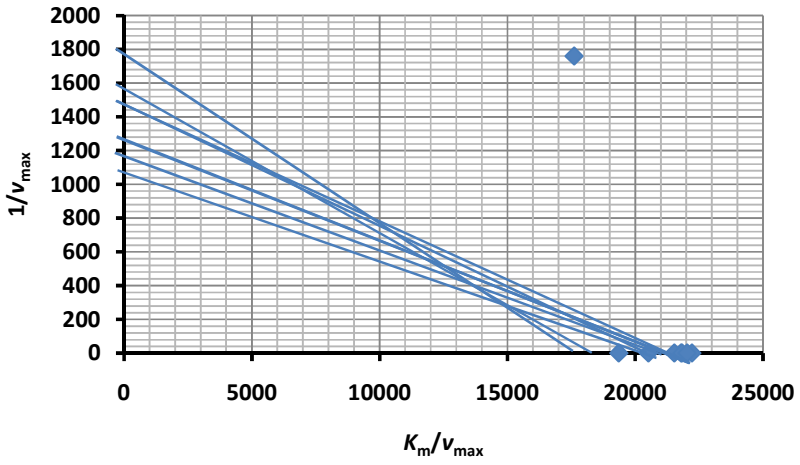

Fig. 3. The alternative direct linear plot where substrate concentration ranges from 10 – 20 g/L and duration of assay is 1 min: v_{max} ≈ 2.13±0.55 mM/mL.min; K_m ≈ 28.74±11.30 g/L (all data are median values, n = 12)

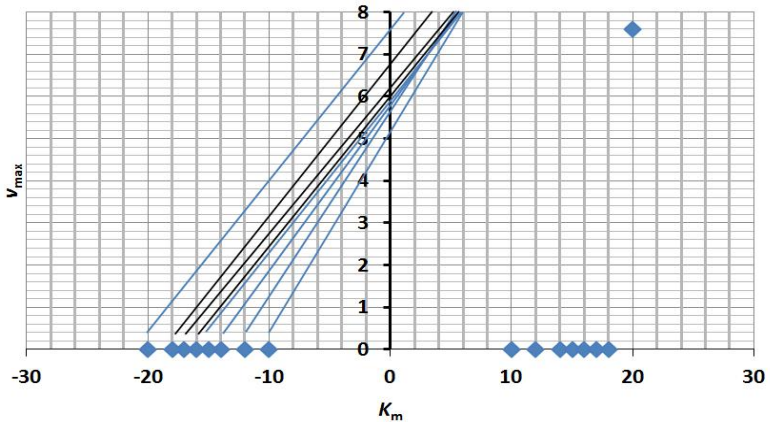

Fig. 4. Direct linear plot where the substrate concentration ranges from 10 – 20 g/L and duration of assay is 2 min: v_{max} ≈ 0.78 mM/mL.min; K_m ≈ 4.50 g/L (all data are median values)

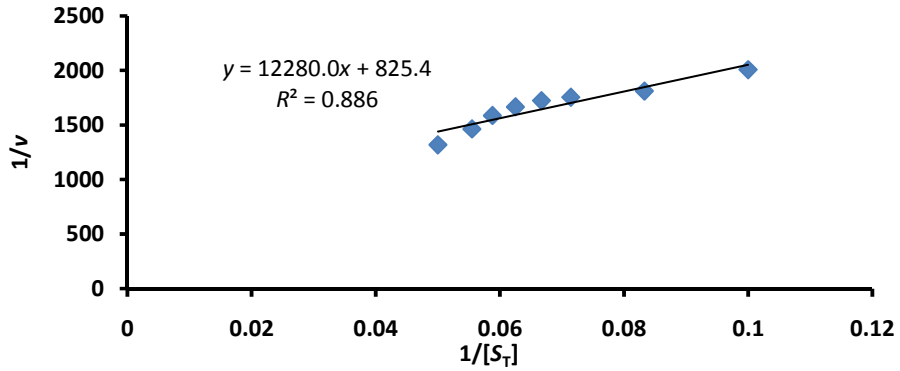

Fig. 5. Lineweaver Burk plot where the substrate concentration ranges from 10 – 20 g/L and duration of assay is 2 min: v_{max} = 1.21± 0.01 mM/mL.min; K_m = 14.88±0.01 g/L (all data are median values, n = 6)

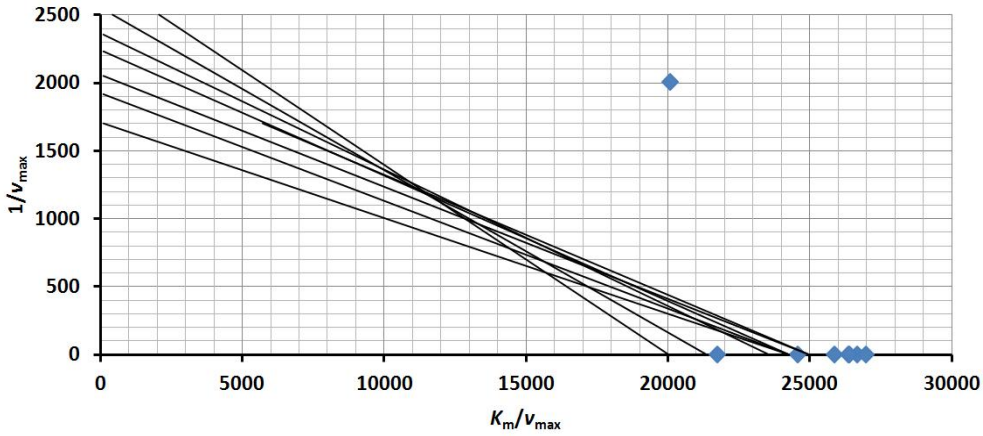

Fig. 6. Alternative direct linear plot where substrate concentration ranges from 10 – 20 g/L and duration of assay is 2 min: v_{max} = 1.18 ± 0.34 mM/mL.min; K_m = 14.17±8.17 g/L (all data are median values, n = 12)

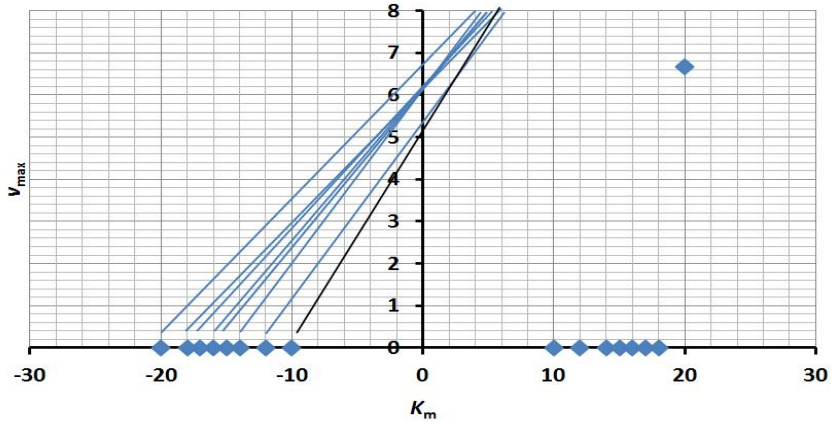

Fig. 7. Direct linear plot where the substrate concentration ranges from 10 – 20 g/L and duration of assay is 3 min (all data are median values): v_{max} ≈ 0.70 mM/mL.min; K_m ≈ 4.00 g/L (all data are median values); it appears that some lines could intersect outside the graphical area

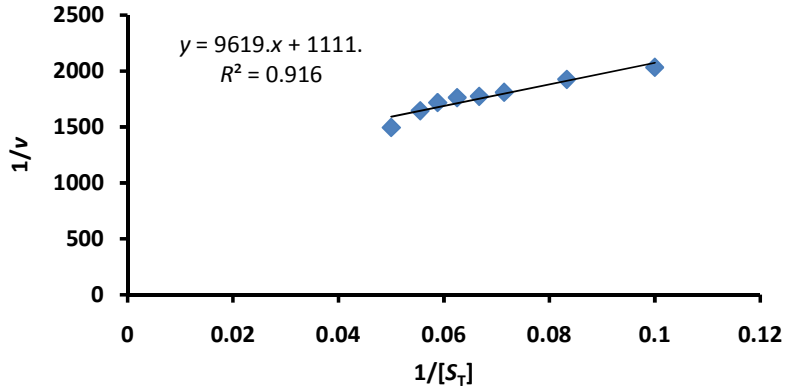

Fig. 8. Lineweaver Burk plot where substrate concentration ranges from 10 – 20 g/L and duration of assay is 3 min: $v_{max} \approx 0.90 \pm 0.00$ mM/mL.min; $K_m = 8.64 \pm 0.01$ g/L (all data are median values)

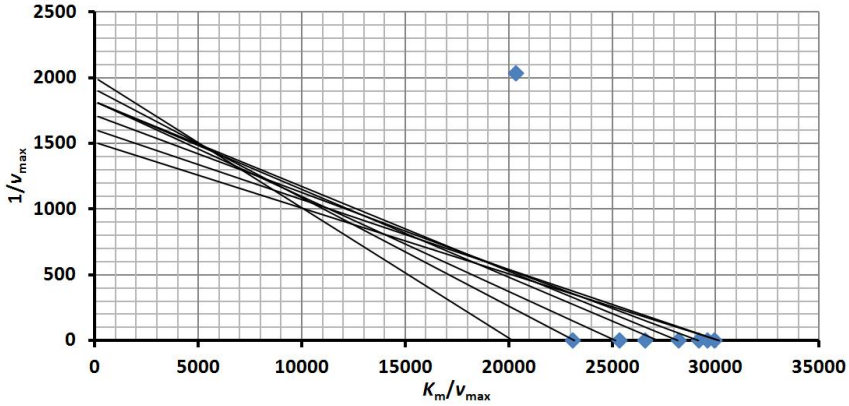

Fig. 9. Alternative direct linear plot where substrate concentration ranges from 10 – 20 g/L and duration of assay is 3 min: $v_{max} = 0.90 \pm 0.02$ mM/mL.min; $K_m = 13.89 \pm 4.19$ g/L (all data are median values, n =11)

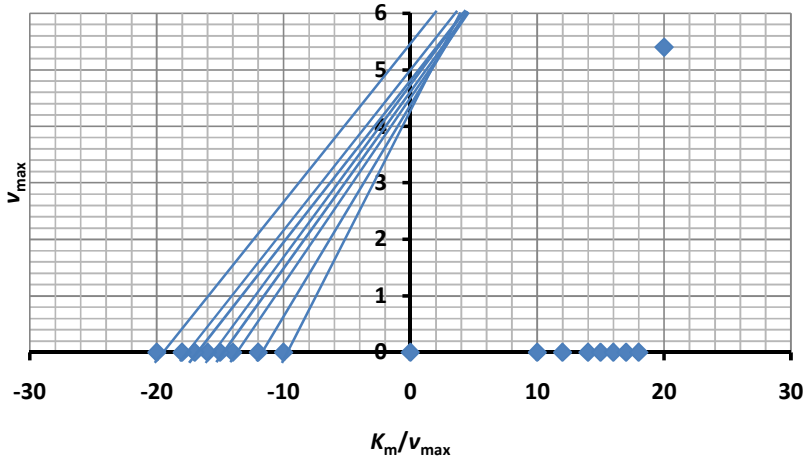

Fig. 10. Direct linear plot where the substrate concentration ranges from 10 – 20 g/L and duration of assay is 5 min $v_{max} = 0.55$ mM/mL.min; $K_m = 3.50$ g/L (all data are median values)

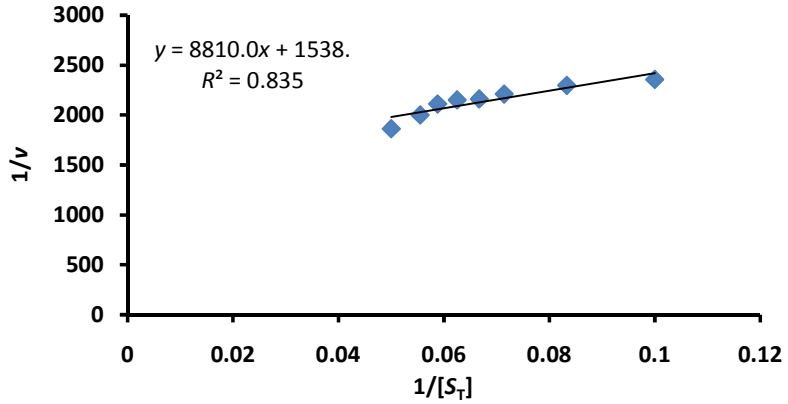

Fig. 11. Lineweaver Burk plot where substrate concentration ranges from 10 – 20 g/L and duration of assay is 5 min (all data are median values): v_{max} = 6.50± 0.00 mM/mL.min; K_m = 5.73±0.01 g/L

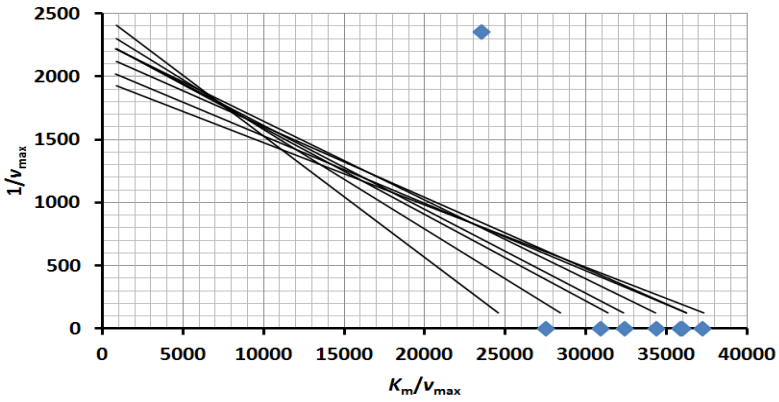

Fig. 12. Alternative direct linear plot where substrate concentration ranges from 10 – 20 g/L and duration of assay is 5 min v_{max} = 0.80± 0.02 mM/mL.min; K_m = 10.40±7.16 g/L (all data are median values, n =12)

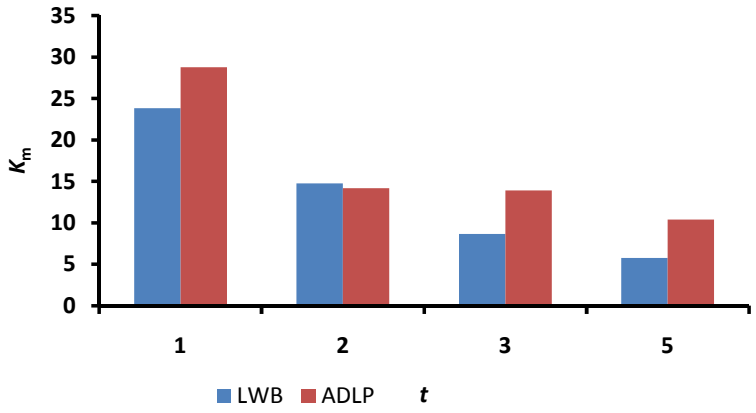

Fig. 13a. Variation of Michaelis-Menten constant, K_m (g/L) with time, t (min). LWB is Lineweaver Burk plot generated data points and ADLP is alternative direct linear plot generated data points

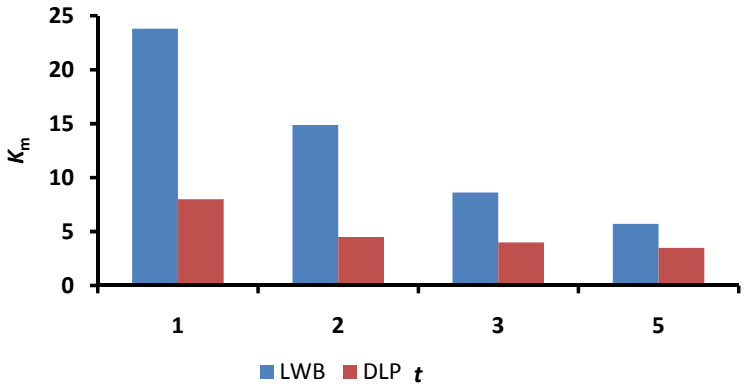

Fig. 13b. Variation of Michaelis-Menten constant, K_m (g/L) with time, t (min). LWB is Lineweaver Burk plot generated data points and DLP is direct linear plot generated data points

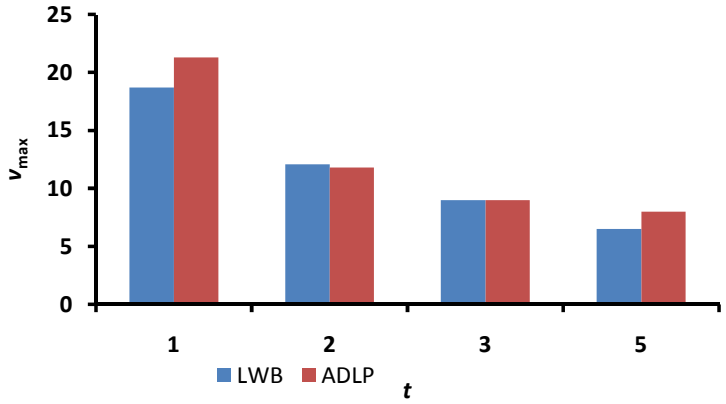

Fig. 14a. Variation of maximum velocity of hydrolysis, v_{max} (exp (-4) M/ml.min) with time, t (min). LWB is Lineweaver Burk plot generated data points and ADLP is alternative direct linear plot generated data points

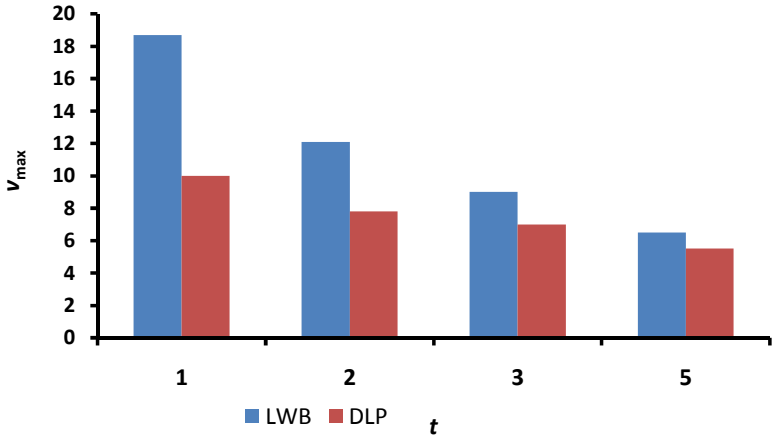

Fig. 14b. Variation of maximum velocity of hydrolysis, v_{max} (exp (-4)M/ml.min) with time, t (min). LWB is Lineweaver Burk plot generated data points and DLP is direct linear plot generated data points

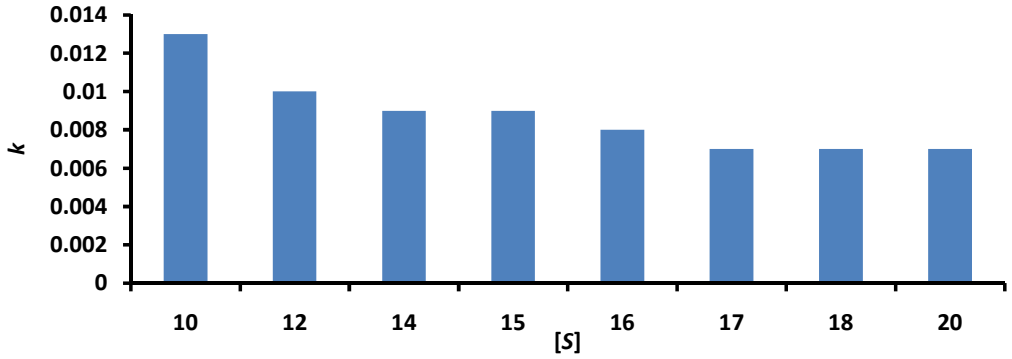

Fig. 15. Variation of pseudo-first rate constant for the hydrolysis (or disappearance of substrate) with substrate concentration: *k* (1/min) is the pseudo-first order rate constant and [*S*], the substrate concentration (g/L)

4. CONCLUSION

Increasing duration of the assay of *Aspergillus oryzea* alpha amylase leads to decrease in the magnitude of kinetic parameters, the enzyme-substrate complex dissociation constant (K_s) and maximum velocity of catalysis (v_{max}) and increasing concentration of the substrate leads to decreasing magnitude of the pseudo-first-order rate constant, *k* for the utilisation of substrate. Thus the duration of the assay does not influence or alter the criterion for the validity of kinetic parameters if the potato starch concentration range is « enzyme concentration. Regardless of the duration of the assay, the kinetic parameters were valid on the basis of the condition for the validity of rQSSA.

COMPETING INTERESTS

Author has declared that no competing interests exist.

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