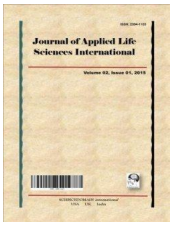

Journal of Applied Life Sciences International

14(3): 1-16, 2017; Article no.JALSI.36862
ISSN: 2394-1103

Unifying Equation and Rate Constant for Amylolysis of Glycosidic Bond

Ikechukwu Iloh Udema[1*]

[1]Owa Alizomor Mixed Sec. Sch. Owa Alizomor, Ika North East/Ude International Concepts Ltd. (862217), B. B. Agbor, Delta State, Nigeria.

Author's contribution

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

Article Information

DOI: 10.9734/JALSI/2017/36862
Editor(s):
(1) Palanisamy Arulselvan, Institute of Bioscience, Universiti Putra Malaysia, Malaysia.
(2) Shahira M. Ezzat, Department of Pharmacognosy, Faculty of Pharmacy, Cairo University, Egypt.
Reviewers:
(1) Michael Ioelovich, Israel.
(2) Marcoaurélio Almenara Rodrigues, Federal University of Rio de Janeiro, Brazil.
Complete Peer review History: http://www.sciencedomain.org/review-history/21644

Original Research Article

Received 20th September 2017
Accepted 24th October 2017
Published 30th October 2017

ABSTRACT

Objectives: The objectives of this research were mainly to determine the rate of amylolysis of glycosidic bond and to formulate a dimensionally consistent quadratic equation for the determination of the changes in the concentration of substrate, molar concentration of product and the parameter exp (*k t*) where *k* and *t* are the pseudo-first order rate constant and duration of assay respectively.
Study Design: Theoretical and Experimental.
Place and Duration of Study: Chemistry & Biochemistry Department, Research Division of Ude International Concepts limited (RC: 862217). The research lasted for about 3 months between May and Sep, 2017.
Methodology: Bernfeld method of enzyme assay was adopted for the generation of data.
Results: The quadratic form of the equation for the quantification of the product of amylolysis gave results that were not significantly different from the use of usual equations (***P*** > 0.05). The rate of hydrolysis of the glycosidic bond was 11851/min and the rate of formation of the product was 23686/min.
Conclusion: The quadratic form of the equation for the quantification of the product of amylolysis has a unifying value. It gives results that are similar to that obtainable from the usual equations. The rate of hydrolysis of the glycosidic bond is ~ 1/2nd the rate of formation of the product. The making and breaking of bond seem to be the rate limiting step.

**Corresponding author: E-mail: udema_ikechukwu99@yahoo.com;*

Keywords: Quadratic equations; rate of hydrolysis of glycosidic bond; rate constant for product formation; pseudo-first order constant.

1. INTRODUCTION

This research focuses mainly on a polymer called potato starch and *Aspergillus oryzea* alpha amylase (EC 3.2.1.1). It is not out of place to state that starch is an important source of energy and a major constituent of the human diet on account of which it is chemically and enzymatically processed into a variety of different products such as starch hydrolysates, glucose syrups, fructose, maltodextrin derivatives or cyclodextrins, used in food industry [1]. In addition to that, the sugars produced can be fermented to produce bioethanol [1-3]. It is known that starch can positively influence the textural properties of many foods and is widely used in food industries and has industrial applications such as a thickening agent, colloidal stabilizer, gelling agent, bulking agent and water retention agent [4]. Successful utilization of starch for most purposes, in food, paper, textile, fuel alcohol, detergent *etc* industries [1] is cognately tied to the efficiency of amylases. Hence hydrolysis of starch from various sources is widely studied [2-8]. Kinetic parameters for most enzymes, hydrolases in particular are regularly studied for different reasons [9-14]. For the purpose of thermodynamic characterization of enzyme catalyzed reaction, amylolytic activity in particular, the rate constant (k_2) often called turn over number [15] is determined at different temperatures by many investigators [16-20]. Every rate constant is quite important to the process or chemical engineers who need to design the reactor in a cost effective way for the optimization of production objectives, *viz*: bioethanol, simple sugars production *etc* [2,3]. Amylolytic activity may also be studied for other purposes such as determination of amylose-amylopectin ratio [5]. Incidentally, a well known formalism, Michaelis-Menten equation and direct expression such as $v_{max}/[E_0]$ are for the determination of k_2. But as originally cited by Schnell and Maini [21] here is another equation [22-24] such as:

$$v = \frac{k_2}{2}\left(\frac{\left(K_m + [E_0] + [\check{S}]\right)}{-\sqrt{\left(K_m + [E_0] + [\check{S}]\right)^2 - 4[\check{S}][E_0]}}\right) \quad (1a)$$

where v, k_2, K_m, and $[E_0]$ are the velocity of hydrolysis of starch, rate constant for the production of reducing sugar maltose, Michaelis-Menten constant, and the concentration of the enzyme respectively; $[\check{S}] = [S] + [C] = [S_0] - [P]$[21] where [*S*], [$S_0$], [*C*], and [*P*] are the concentration of free substrate, total concentration of substrate, concentration of enzyme-substrate complex, and concentration of product respectively. The parameter $[\check{S}]$ expresses the sum of the mass concentration of free substrate and substrate involved in complex formation with the enzyme as being equal to mass concentration of the substrate at zero time less mass concentration of the product and, it was according to Schnell and Maini [21] intended to correct the expression for substrate mass balance given hitherto as $[\check{S}] = [S] + [C]$. This implies that $[S_0] = [S] + [C] + [P]$. The relevant issue is mass conservation and for the purpose of emphasis further explanation may be needed. Now on the issue of conservation law, $\Delta[S_0]$ is also the mass of the product which does not include the water molecule involved in the hydrolytic attack on the glycosidic bond. For every one out of $\phi - 1$ hydrolytic bonds hydrolyzed by the amylolytic action of the alpha amylase, one molecule of maltose is yielded. Therefore, the number of moles of the reducing sugar maltose is equal to the number of moles of water molecule (or even the number of glycosidic bonds equal to the number of maltose molecules yielded). For alpha amylase, maltose is the product within at least a short duration of assay; but the yielding of one molecule of maltose is only equivalent to the loss of two glucose molecules combined in one maltose molecule. But within the short duration of assay (and even as $t \to \infty$) the entire polysaccharide is never hydrolyzed by alpha amylase.

The issues to be raised about Eq. (1a) are: 1) The dimensional consistency considering the fact that, though v may be expressed in g/L.min, it is usually measured in mol/L.min while k_2 in its standard form is measured in dimensionless quantity per unit time and 2) the mass conservation principle, to which, $(K_m + [E_0] + [\check{S}])$ may be amenable, but, by so doing may not be in agreement with the dimension of v and k_2. However, with the understanding that mass concentration is not in any way the same as molar concentration, if the molar mass of the substrate and enzyme are known and by dividing the mass concentrations with corresponding molar mass, the result obtained for k_2 will be

consistent with the usual unit. Any other issue (s) that may arise will be for feature consideration.

At this juncture, it is important to comment that the hydrolysis of a tetrasaccharide, trisaccharide, and disaccharide presents results that can be used to accurately estimate the amount of the substrate transformed into product. This is unlike polysaccharides whose degree of polymerization (ϕ) values are quite large requiring much longer duration of assay on the assumption that the polysaccharide is totally amenable to total amylolysis. For instance if *n* moles of maltose are totally hydrolyzed, 2*n* moles of glucose should be yielded; thus the rate constant for the production of glucose molecules is twice the rate constant for the hydrolysis of the substrate ($[\Delta S]/342[E_0]$ were $[\Delta S]$ is mass concentration of maltose as an example if consideration is given to the fact that mass conservation is not an issue when preparing the standard curve using maltose as standard) for the hydrolysis of maltose (the degree of polymerization = 2). The issue is that unlike polysaccharide it is possible to determine the rate constant for the conversion of substrate like maltose with well known molar mass from the rate constant for product formation because both maltose and glucose are reducing agents. Using spectrophotometer for instance, if *x* mole/L of maltose is totally hydrolyzed, the absorbance may be A_x; but 2*x* mole/L of glucose should be yielded such that the absorbance should be 2 A_x. Thus if different concentrations of the substrate maltose for instance is enzymatically hydrolyzed, the molar concentration of the product must be twice the molar concentration of substrate hydrolyzed within the chosen duration of assay. If the direct linear plot of velocity, *v* of hydrolysis versus concentration of substrate is carried out, the maximum velocity of hydrolysis, v_{max} expressed often as the maximum molar concentration of the product must be twice the maximum molar concentration of the substrate hydrolyzed. Yield comes after hydrolysis. The situation with respect to high molecular weight starch cannot be as straightforward as applicable to maltose. So far, researches have always been on hydrolysis of polysaccharide for diverse reasons without attention on the means of producing the reducing sugars. This appears to be a strange view; but there is time for translational motion leading to effective collision, catalytic orientation, breaking of bond or more technically the amylolysis of glycosidic bonds which require one water molecule per bond, and departure of product [15,25]. Thus the objectives of this research are mainly to determine the rate of amylolysis of glycosidic bond and to formulate a dimensionally consistent quadratic equation for the determination of the changes in the concentration of substrate, molar concentration of product and the parameter exp (*k t*) where *k* and *t* are the pseudo-first order rate constant and duration of assay respectively.

2. THEORY: FORMULATION OF UNIFICATION QUADRATIC EQUATION AND RATE CONSTANT FOR THE AMYLOLYSIS OF GLYCOSIDIC BOND

$$\frac{\Delta[S_0]}{M_s}(\phi - 1)N_A = \text{Number of glycosidic bonds } (b_G) \quad (1b)$$

where ϕ, $[S_0]$, N_A, and M_S are the degree of polymerization per polysaccharide molecule, mass concentration of the substrate, Avogadro's number, and molar mass of substrate which ensures dimensionless parameter, b_G; if the entire chain of a polysaccharide is hydrolyzed, the value of M_S may be the molar mass of the molecule. For the purpose of elucidation, it should be stated that, if the degree of polymerization of starch molecule for instance is designated as ϕ the number of glycosidic bonds per polysaccharide molecule is $\phi - 1$; there is a single covalent bond otherwise called glycosidic bond between every two glucose moieties. If $\Delta[S_0]$ is the mass of the substrate (or perhaps the total mass of the portion of each polysaccharide hydrolyzed) and M_s remains as defined, then, the total number of endo-glycosidic bonds hydrolyzed to yield maltose molecules is as expressed in Eq. (1b) for the following reasons. Each polysaccharide molecule contain a number of glucose molecules = the degree of polymerization (ϕ). One mole of polysaccharide contains $N_A\phi$ molecules of glucose; if $\Delta[S_0]$ is mass concentration of the substrate as polysaccharide hydrolyzed, then ($\Delta[S_0]$/molar mass) $N_A\phi$ molecules of glucose may be yielded if there is total hydrolysis. But with alpha amylase, maltose may be the only reducing agent at least within a short duration of assay such that the number of maltose molecules that may be yielded if $\Delta[S_0]$ is hydrolyzed is ($\Delta[S_0]$/molar mass) $N_A\phi/2$ because the degree of polymerization of maltose is two. It is obvious that $\Delta[S_0]$/ molar mass) N_A » 1. This means therefore, that the number of glycosidic bonds per mol = (ϕ -1) N_A; one polysaccharide molecule contains ϕ - 1; yielding one maltose molecule per polysaccharide molecule involves the hydrolysis

of one out of ϕ - 1 per molecule of polysaccharide. But there cannot be just unscientifically only one polysaccharide in say 0.01 g/L of starch if the same amount is hydrolyzed. Thus if 2.0 exp (-6) mol/L of maltose was yielded hypothetically, the number of maltose molecules is 2.0 exp (-6)N_A; this is equivalent to 2.0 exp (-6)N_A glycosidic bonds.

However, for the purpose of this investigation, a simple expression for the total number (ϕ) of glucose molecules in one polysaccharide otherwise called degree of polymerization is given by rearranging Eq. (1b) to give:

$$\phi = 1 + \frac{M_S b_G}{\Delta[S_o] N_A} \quad (1c)$$

$$\phi = \frac{M_S}{162} \quad (2)$$

Additional explanation is that one mole of any polyglucan contains 1 mole of its degree of polymerization; one molecule should, therefore, contain a number of glucose moieties equal to the degree of polymerization. Since [*P*] is molar concentration of maltose, twice its value should be the molar concentration of glucose, as long as ϕ remains the degree of polymerization. Equation 2 is based on the well known fact that the molar mass of the polysaccharide can be estimated from the product of ϕ and dehydrated glucose (relative molar mass = 162).

$$\Delta[S_0] = [S_0](1 - e^{-kt}) \quad (3a)$$

where *k* and *t* are the pseudo-first order rate constant for the utilization of the substrate and duration of assay respectively and for convenient sake, exp (*k t*) is used in place of e^{kt}. In line with step by step approach and for the purpose of elucidation, Eq. (3a) need to be analyzed (and for this purpose see Eq. (26) [21]) as follows:

$$\text{In}\ \frac{[S_0]}{[S]} = k\ t \quad (3b)$$

$$\frac{[S_0]}{[S]} = \exp(k\ t) \quad (3c)$$

$$[S] = \frac{[S_0]}{\exp\ (k\ t)} \quad (3d)$$

where $\exp\ (k\ t)$ is > 1 and $[S]$ is the concentration of substrate remaining at the end of assay or when the assay is terminated. Therefore,

$$\Delta[S_0] = [S_0] - [S] = [S_0] - \frac{[S_0]}{\exp\ (k\ t)}$$

Simplification gives Eq. (3a). Thus as time $\rightarrow\infty$, 1/exp (*k t*) $\rightarrow$ zero and consequently the concentration of the substrate hydrolyzed $\rightarrow$ [S_0].

Before proceeding further, it is important to state that the equations could be shorter by 4-5 steps but one has to avoid equations which cannot be easily corrected if error is observed and if several steps are avoided. A stepwise presentation is thus adopted. Besides, the issue of number of bonds broken has been of interest in the past [26]. Though not so clear, Marchal et al. [26] seems to have determined pseudo-first order rate constant by fitting an exponential function through the data points (mmol bonds hydrolyzed per kg dry weight and water, as a function of time) for each experiment: *b*h(*t*) = *A*(1 – exp(−*Bt*)), in which *b*h = number of bonds hydrolyzed [mmol/(kg dw + H_2O)], *A* = maximum number of bonds hydrolyzed [mmol/(kg dw + H_2O)], and *B* = pseudo-first-order reaction constant (min^{-1}). Most important issue recognized by the authors [26] is the addition of water to the hydrolytic process. In this paper, however, the number of bonds that can be hydrolyzed given appropriate hydrolase is ϕ -1 where, ϕ is the number of glucose molecules combined in a polysaccharide otherwise called the degree of polymerization.

The purpose of Eq. (1b) and (3a) and their appearance in subsequent equations is for the formulation of a quadratic equation which can be used to determine exp (*k t*) and the rate constant for the hydrolysis of the glycosidic bond given specified [S_0] and [*P*] without prior information about *k*. It should be made clear that, Eq. (1b) and (1c) are likely if the molar mass of the substrate is known. Nonetheless, the equations show mass concentration of the substrate converted to product. Consequently, the mass of the product is implied in line with mass conservation law. Therefore, division by the molar mass of the product, if clearly defined and certain gives the number of moles per unit volume. If other products that cannot be detected spectrophotometrically and glucose are present in the reaction mixture at the end of assay, then the absorbance read using maltose as standard may not be accurate. Therefore, it is necessary to be certain that only maltose is the only reducing sugar. However, the following relationship may hold:

$$[P]N_A = \frac{[S_0]}{M_S}\left(1 - \frac{1}{\exp(k\,t)}\right)\left(\frac{M_S}{162} - 1\right)N_A \quad (4)$$

Equation (4) is premised on the fact that for every mole of maltose yielded, one mole of the glycosidic bond is hydrolyzed. For further elucidation, recall Eq. (1b) where $\phi = \frac{M_S}{162}$ (Eq. (2)) and $\Delta[S_0]$ defined in Eq. (3a). Substitution of Eq. (3a) into Eq. (1b) gives Eq. (4). The glycosidic bonds are in the polysaccharide. Therefore, the molar concentration of the product, maltose, represents unambiguously, the number of moles of the bonds hydrolyzed.

$$[P] = \frac{[S_0](\exp(k\,t)-1)\left(\frac{M_S}{162}-1\right)}{M_S \exp(k\,t)} \quad (5)$$

For general comprehension it should be seen that exp (*k t*) and exp (- *k t*) are convenient way of presenting $e^{k\,t}$ and $e^{-k\,t}$ respectively. Therefore, $(\exp(k\,t) - 1) = e^{k\,t} - 1$ and $\frac{1}{\exp(k\,t)} = e^{-k\,t}$. Rearrangement of Eq. (5) gives:

$$\frac{[S_0]}{162} - \frac{[S_0]}{M_S} = \frac{\exp(k\,t)[P]}{(\exp(k\,t)-1)} \quad (6)$$

$$\frac{[S_0]}{M_S} = \frac{[S_0]}{162} - \frac{\exp(k\,t)[P]}{(\exp(k\,t)-1)} \quad (7)$$

Let Eq. (8a) below holds temporarily for the purpose of brevity.

$$\zeta = [S_0]\frac{1}{162} - \frac{\exp(k\,t)[P]}{(\exp(k\,t)-1)} \quad (8a)$$

$$M_S = \frac{[S_0]}{\zeta} \quad (8b)$$

If the entire chain of a polysaccharide is hydrolyzed, the value of M_S may be the molar mass of the molecule. This postulation is advanced because only a portion of the chain is hydrolyzed one after the order. The sum of all the portion of the polysaccharide molecules hydrolyzed presents a common molar mass. This implies that the reducing sugar produced within specified duration of assay is not necessarily from one polysaccharide.

Just as k_2 is expressed as $v_{max}/[E_0]$, where k_2, v_{max}, and $[E_0]$ are the rate constant for the production of reducing sugar, maltose for instance, maximum velocity of the production of the reducing sugar, maltose in this case, and the molar concentration of the enzyme, the equivalent rate constant, $k_{2[S]}$ (not a pseudo-rate constant) for the transformation of the substrate *i.e*. the hydrolysis of the glycosidic bond at the active site is:

$$k_{2[S]} = \frac{2\Delta[S_0]\,k_2}{2vtM_3\phi} \quad (9a)$$

Equation (9a) is applicable so long as M_3 is = 324 g/mol. (*i.e*. 2 ×162 g/mol) and ϕ can only be retained if $\Delta[S_0]/M_3$ is the number of moles of the product maltose which is equivalent to $2\Delta[S_0]/M_3$ glucose molecules. Ultimately the integer 2 in the denominator and nominator cancel out. If $\Delta[S_0]/M_3$ is the molar concentration of maltose yielded division by $\phi k_{2[S]}$ is postulated to give perhaps crudely but reasonably the molar concentration of the substrate which formed complex and got transformed to product. The integer 2 is introduced to account for the fact that the degree of polymerization of maltose is two. Equation (9a) can be explained as follows: If twice the number of moles of the product of hydrolysis of starch is divided by its degree of polymerization it gives the number of moles of substrate, $\frac{2\Delta[S_0]}{M_3\phi}$ transformed to product (given that $\Delta[S_0]$ is also equal to the mass concentration of the product in line with mass conservation law). Thus $\frac{\Delta[S_0]}{M_3 k_{2[S]}\phi t} = \frac{v}{k_2}$ where $k_{2[S]}$ has earlier been defined. This implies that 162 ϕ = relative molecular mass of the substrate, but, as stated earlier, it may in this case conjecturally be the relative molar mass of the fragment of the polysaccharide hydrolyzed because within the short duration of assay, it is not likely that the entire chain plus the branch chain is digested. This is very much applicable to alpha amylase. Consequently, Eq. (9a) can be re-written as:

$$k_{2[S]} = \frac{\Delta[S_0]\,k_2}{2vtM_S} \quad (9b)$$

where M_S is redefined as the molar mass of the part of the polysaccharide transformed.

The term transformation is simply the breaking and making of bonds before the departure of the product. In Eq. (9a), $\frac{v}{k_2} : \frac{\Delta[S_0]}{tM_3 k_{2[S]}\phi} = 1$ is simply an expression of the ratio of the molar concentration of the enzyme involved in complex formation (a well known parameter) to the molar concentration of the substrate that was transformed to product or more precisely, the number of hydrolyzed glycosidic bonds, given the value of M_3. In Eq. (9b), $k_{2[S]} \propto 1/M_S$. Thus,

$$k_{2[S]} = \frac{\Theta}{M_S} \quad (10)$$

where, for the purpose of brevity, Θ is $\frac{\Delta[S_0]\,k_2}{2vt}$.

Substituting Eq. (8b) for M_S in Eq. (10) gives:

$$k_{2[S]} = \frac{\Theta\,\zeta}{[S_0]} \equiv \frac{\Theta\left(\frac{[S_0]}{162} - \frac{[P]\exp(k\,t)}{(\exp(k\,t)-1)}\right)}{[S_0]} \quad (11)$$

where ζ is defined in Eq. (8a).

$$[S_0]k_{2[S]} = \Theta\frac{[S_0]}{162} - \frac{\Theta[P]\exp(k\,t)}{(\exp(k\,t)-1)} \quad (12)$$

If $k_{2[S]}$ is replaced by Eq. (9b) the result is:

$$[S_0]\Delta[S_0]\frac{k_2}{2vtM_S\phi} = \Theta\frac{[S_0]}{162} - \frac{\Theta[P]\exp(k\,t)}{(\exp(k\,t)-1)} \quad (13)$$

Rearrangement of Eq. (13) gives:

$$[S_0]\Delta[S_0]\frac{k_2}{vt} = \Theta[S_0]\frac{4M_S}{162} - \frac{\Theta[P]\exp(k\,t)4M_S}{(\exp(k\,t)-1)} \quad (14)$$

Equation (14) can be rearranged to give:

$$\Delta[S_0] = \frac{\left(\Theta\,[S_0]\frac{4M_S}{162} - \frac{\Theta[P]\exp(k\,t)4M_S}{(\exp(k\,t)-1)}\right)v\,t}{[S_0]k_2} \quad (15)$$

Equation (9b) could be rearranged to give:

$$v\,t = \frac{k_2\Delta[S_0]}{2M_S K_{2[S]}} \quad (16)$$

As simple as Eq. (16) may be, it nevertheless needs further elucidation otherwise one may feel that $k_2 = k_{2[S]}$. This calls for the invocation of the conservation law. If one mole of the substrate is hydrolyzed, the number of times hydrolytic actions occur is ϕ-1 which represents the number of glycosidic bonds per molecule of the polysaccharide or the number of moles of water molecules needed. If $\Delta[S_0]$ is hydrolyzed, then $N_A(\Delta[S_0]/M_S)(\phi\text{-}1)$ is the number of glycosidic bonds if M_S is the molar mass of the substrate. If not, the number of glycosidic bonds is $N_A\,\Delta[S_0]/M_3$ where N_A is Avogadro's number and M_3 =324 g/mol. To be clearer, the molar concentration of substrate hydrolyzed = 2[*P*]/ ϕ. Additional explanation is that one mole of any polyglucan contains 1 mole of its degree of polymerization; one molecule should, therefore, contain a number of glucose moieties equal to the degree of polymerization. Since [*P*] is molar concentration of maltose, twice its value should be the molar concentration of glucose, as long as ϕ remains the degree of polymerization.

The real mass of the product which includes a water molecule is 342 [*P*] > $\Delta[S_0]$ = 324[*P*]. This is the case because the mass of the substrate starch, a polysaccharide, takes into account of the fact that it is formed by condensation reaction that entails loss of water molecules: To be clearer, for every glycosidic bond formed, one water molecule is lost or released. Meanwhile 162 is usually a well known relative molar mass of "dehydrated" glucose (180-18). It applies to any monosaccharide moiety in polysaccharide including maltose as a substrate or product. If starch, a polysaccharide (formed by condensation reaction that always results in loss of water for every glycosidic bond formed) is a substrate as in this research, the production of maltose by the amylolytic action of alpha amylase requires the addition of water molecule to that part of the polysaccharide, the glycosidic bond, where one water molecule was lost during the formation of the polysaccharide. Thus maltose (342 g/mol) the product in this case has the mass of one water molecule less than the combined mass (360 g/mol) of two glucose molecules. Strictly for the purpose of illustration, if the degree of polymerization of an oligosaccharide is 10, then the number of maltose moieties should be 5: But the molar mass of the oligosaccharide $\neq$ 342 $\times$ 5 g/mol, otherwise the molar mass of maltose should also be 360 g/mol. Therefore, the only way to obey the mass conservation law is to take into account the gain or loss of water as the case may be.

Therefore, the addition of water molecule to the polysaccharide for each molecule of maltose yielded should ultimately give total mass of product, maltose whose "hydrated" mass should be > than the mass of the substrate hydrolyzed. Hence, $\Delta[S_0]$=324[*P*]. This takes into account mass conservation law [26-29] and accounts for the fact that hydrolysis involves the uptake of water molecule which adds to the total weight of the product and substrate [26, 29], otherwise the actual mass of the product is 342 [*P*].

2.1 Determination of the 1st Slope

The purpose of Eq. (16) is the determination of a slope ($S_{L\ (1)}$) which could be used to find an expression for M_S. The slope from the plot of *v t* (or [*P*]) versus $\Delta[S_0]$ ($[S_0]$ exp ((*k t*) –1)/exp (*k t*)) can be expressed as:

$$S_{\mathrm{L}(1)} = \frac{k_2}{2M_{\mathbf{S}}k_{2[\mathrm{S}]}} \quad (17a)$$

Equation (17a) leads to Eq. (17b).

$$k_{2[S]} = \frac{k_2}{2M_{\mathbf{S}}S_{\mathrm{L}(1)}} \quad (17b)$$

From Eq. (17a)

$$M_{\mathbf{S}} = \frac{k_2}{2S_{\mathrm{L}\,(1)}k_{2[\mathrm{S}]}} \quad (18)$$

Substitution of Eq. (18) into Eq. (15) gives:

$$\Delta[S_0] = \begin{pmatrix} \Theta[S_0]\frac{k_2}{162S_{\mathrm{L}(1)}k_{2[\mathrm{S}]}} \\ -\frac{\Theta[P]\exp(k\,t)k_2}{(\exp(k\,t)-1)S_{\mathrm{L}(1)}k_{2[\mathrm{S}]}} \end{pmatrix} v\frac{t}{[S_0]k_2} \quad (19)$$

Meanwhile, [*P*] = *v t* and Eq. (18) is substituted into Eq. (10) to give:

$$k_{2[\mathrm{S}]} = \frac{2\Theta}{k_2}\,S_{\mathrm{L}(1)}k_{2[\mathrm{S}]} \quad (20)$$

$$\Theta = \frac{k_2}{2S_{\mathrm{L}(1)}} \quad (21)$$

Rearrangement of Eq. (19) and substitution of Eq. (21) into it gives the following after simplification,

$$\Delta[S_0] = \Theta\begin{pmatrix} \frac{[S_0]k_2}{162S_{\mathrm{L}(1)}k_{2[\mathrm{S}]}} \\ -\frac{[P]\exp(k\,t)\,k_2}{(\exp(k\,t)\,-\,1)S_{\mathrm{L}(1)}k_{2[\mathrm{S}]}} \end{pmatrix} v\frac{t}{[S_0]k_2}$$

$$= \frac{k_2}{2k_{2[\mathrm{S}]}S^2_{\mathrm{L}(1)}}\left(\frac{[S_0]}{162} - \frac{[P]\exp(k\,t)}{(\exp(k\,t)-1)}\right)v\frac{t}{[S_0]} \quad (22)$$

2.2 Determination of 2nd Proportionality Constant

A plot of $\Delta[S_0]$ versus $\left(\frac{[S_0]}{162} - \frac{[P]\exp(k\,t)}{(\exp(k\,t)-1)}\right)v\frac{t}{[S_0]}$ gives a 2nd slope, $S_{\mathrm{L}\,(2)}$ expressed as:

$$S_{\mathrm{L}(2)} = \frac{k_2}{2k_{2[\mathrm{S}]}\,S^2_{\mathrm{L}(1)}} \quad (23)$$

Thus,

$$k_{2[\mathrm{S}]} = \frac{k_2}{2S^2_{\mathrm{L}(1)}\,S_{\mathrm{L}(2)}} \quad (24)$$

Equations (17b) and 24 give similar results if $S_{\mathrm{L}(1)}$ is accurately known.

In order to determine a unification, a quadratic equation without complex number ($\sqrt{-x}$) there is need to introduce what may be defined as correction factor, β into Eq. (4) as explained in appendix section. Several steps ultimately lead to $\Delta[S_0]^2\,S^2_{\mathrm{L}(1)}\frac{k_{2[S]}}{k_2} - \frac{\Delta[S_0][P]}{162} + \frac{[P]^2}{\beta} =$. Several steps are in the manuscript in preparation where another homologue is addressed. However, Eq. (16) in this section is replaced by $v\,t = \frac{k_2\Delta[S_0]}{M_3K_{2[\mathrm{S}]}}$ where M_3 is the mass of the product given that mass conservation is obeyed. The detail of the derivation is in the appendix section which begins from Eq. (A1).

3. MATERIALS AND METHODS

3.1 Materials

Aspergillus oryzea alpha amylase and soluble 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, while potassium iodide was purchased from Merck Germany. Distilled water was purchased from local market.

3.2 Equipment

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. Water bath was purchased from Hospibrand, USA.

3.3 Methods

Stock solution of soluble potato starch was prepared by mixing 1 g in 100 ml of distilled water and subjected to heat treatment at 100°C for 3 minutes, cooled to room temperature, and decrease in volume was corrected by topping the volume with distilled water to 100 mL to give 1.0 g%. Stock solution of the enzyme was prepared by dissolving 0.01g in 100 mL trisHCl buffer (*p*H=6) at room temperature. The determination of rate of hydrolysis of the glycosidic bond is given in its simplest form as Eq. (24). Assay was according to Bernfeld method [30] for the quantification of the molar concentration of reducing sugar, maltose and kinetic parameter, maximum velocity of hydrolysis in particular was by Lineweaver-Burk [31] and direct linear [32] plots. The value obtained from direct linear plot was adopted.

Table 1. Calculated parameters viz $\Delta[S_0]$, exp ($k\ t$), $k_{2[S]}$ and k_2

Calculated parameters	Time/min						
	1	**1.5**	**2**	**2.5**	**3**	**3.5**	**5**
$\Delta[S_0]$/g/L	0.167	0.234	0.348	0.479	0.518	0.592	0.897
$\chi[S_0]$/g/L	0.185±0.006	0.277±0.008	0.369±0.011	0.460±0.014	0.551±0.016	0.641±0.019	0.910±0.026
Exp ($k\ t$)	1.0094±0.0003	1.0141±0.0046	1.0188±0.0061	1.0235±0.0008	1.0283±0.0009	1.0331±0.0011	1.0477±0.0016
Exp ($k_c\ t$)	1.0084±0.0009	1.0118±0.0001	1.0177±0.0006	1.0245±0.0014	1.0266±0.0001	1.0305±0.0010	1.0470±0.0011
χ_{Qua}	1.00842	1.01183	1.01773	1.02448	1.02659	1.03053	1.04694
$\boldsymbol{k_{2[S]}}$(1/min)	(11851.94)±93)/min						
k_2(1/min)	(23686.0±34)/min(from direct linear plot)						
	(24803.34±51.94)/min (from Lineweaver-Burk plot)						

$\chi = \frac{exp(k\ t)-1}{exp\ (k\ t)}$, *$\Delta[S_0]$ is the amount of substrate hydrolyzed (= 324 × average [P]); $[S_0]$, $k_{2[S]}$ and k_2 are the mass concentration of the substrate, rate of hydrolysis/amylolysis of glycosidic bond, and the rate of production of the reducing sugar, maltose; k ((9.311 ± 0.299)exp (-3) ≡ mean ± SD)/min is the slope of the plot of ln ($[S_0]$/($[S_0]$-324[P])) versus t; k_c (calculated pseudo-first order rate constant)= ln ($[S_0]$/($[S_0]$-324[P]))/t; χ_{Qua} is from quadratic equation (Eq.(A.25)); χ_{Qua} is obtained by substituting average values of [P] per duration of assay into the quadratic equation*

The determination of rate constant for the hydrolysis of endo-glycosidic bond requires the determination of the first and second slopes: This is where assaying of the enzyme to generate data-velocity of hydrolysis with substrate concentration ranging from 6-16 g/L, maximum velocity of hydrolysis obtained from direct linear plot and via linear Lineweaver-Burk plot, and ultimately rate constant for product formation- is indispensible. The pseudo-first order rate constant k, was obtained from the plot of In $[S_0]$ – In $([S_0]\text{-}324[P])$ versus t. In order to verify the validity of the quadratic equation and any other equation the first slope from the plot of $[P]$ versus $\Delta[S_0]$ (Fig. 1) was substituted into Eq. (22) and Eq. (25) for the determination of $\Delta[S_0]$ and exp (kt) respectively. The determination of rate constant for the hydrolysis of the glycosidic bond requires a plot of $\Delta[S_0]$ versus $\left(\frac{[S_0]}{162} - \frac{[P]\exp(k\,t)}{(\exp(k\,t)-1)}\right) v \frac{t}{[S_0]}$ or the simplified form, $\left(\frac{1}{162} - \frac{[P]}{\Delta[S_0]}\right)[P]$ as shown under Fig. (2). The first and second slopes are then substituted into Eq. (24) to give after calculation the rate constant for the hydrolysis of the glycosidic bond.

3.4 Statistical Analysis

Significant difference is tested using internet based graph pad (www.graphpad. com/quick calcs /*t*-test). Micro-soft Excel was used to determine standard deviation (n=8).

4. RESULTS AND DISCUSSION

To begin with, it was not too clear what may be the significance of the quadratic equations as alternative to direct equations for the calculation of $\Delta[S_0]$, $[P]$, and exp $(k\,t)$ until Eq. (1a) was located in literature. All are unification equations. While the use of calculated pseudo-first order rate constant (designated as k_c as shown under Table 1) in the expression $[S_0]$ (exp $(k_c\,t)$ -1)/exp $(k_c\,t)$ gives the same results as 324 $[P]$, it is not so with the use of graphically determined pseudo-first order rate constant k, the slope of the plot of In $[S_0]$ – In $([S_0]\text{-}324[P])$ versus t (Table 1). There was no significant difference (***P*** > 0.05). There was also no significant difference between the result obtained from the substitution of data into the quadratic equation and 324 $[P]$ (***P*** > 0.05). There was also no significant difference between exp $(k\,t)$, exp $(k_c\,t)$ and χ_{Qua} (χ_{Qua} = exp $(k\,t)$ obtained from quadratic equation) (***P*** > 0.05). The question then is what is the 'big deal' using any of determined parameters against the backdrop of the observed 'no significant differences'. The most important objective is the determination of the rate of amylolysis of the glycosidic bond which may be confused with the rate constant for the formation of product. This required two plots, one for the determination the first slope and for the second slope as in shown in Fig. 1 and Fig. 2 respectively. This constant, k_2 is an over-all constant covering catalytic orientation, binding, breaking of covalent bond and making of covalent bond, departure of product fragment, and the enzyme; there cannot be binding if there was no effective collision between the enzyme and the substrate in the first place [15,25]. The important issue is that each aspect has its duration. The slowest is the breaking and making of bond. The rate constant for amylolytic action is (11851.94)±93)/min (mean ± SD where df = 7)

An important deduction in the light of Eq. (A.26) is that it is possible to predict the value of $[P]$ if the values of $k_{2\,[S]}$, $[S_0]$, k, and k_2 are known at a specified condition and duration of assay. The issue is that while $[P]$ (or $v\,t$) = $k\,[S_0]t$, the value of $[P]$ in g/L will appear to be indefinite with different duration of assay or any enzyme activity. For the purpose of illustration a hypothetical case such as assay over a duration of 5 hours (300 min) given experimental value of k = 9.331875 exp(−3)/min in this research need to be considered. If $[S_0]$ is 20 g/L (for the determination of k) as in this research, $[P]$ should be = 55.99 g/L ≡ 0.17 mol/L if maltose is the product and, in this case $v \cong 0.187$ g/L/min. Thus the value of $[P]$ is indefinite with time for the same concentration of the substrate. However, if the operation, $-\int_0^t \frac{\mathrm{d}[S]}{[S]} = \int_0^t k\,\mathrm{d}t$, is carried out, the result is first, $[S_t] = [S_0] \exp(-k\,t)$ and then, $[P] = [S_0](1 - \exp(-k\,t))$. With the latter, $[P]$ = 18.783 g/L which observes mass conservation law because $[P] < [S_0]$. This is applicable to Eq. (A.26) which appears to be more complex without very visible advantage until closer examination.

As it is often the case, the rate of product formation, the rate constant is very often reported in most kinetic studies much more than the rate constant for the transformation of the substrate in the active site or rather the rate of breaking of glycosidic bond and making of any other covalent bond as observed in this research. A simple illustration of this issue is the hydrolysis of any disaccharide. As earlier stated for any one mole of substrate (maltose which can be detected by

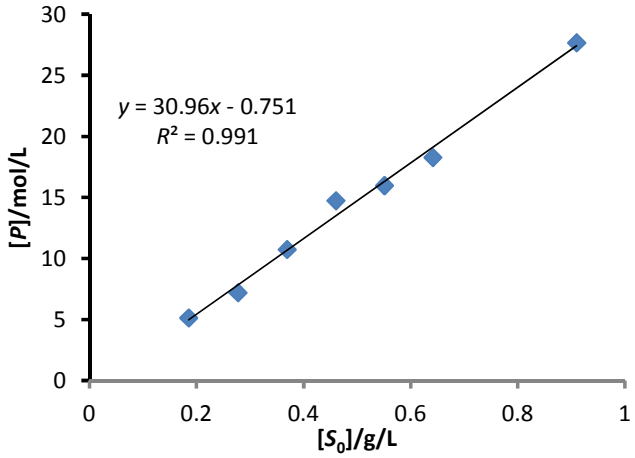

Fig. 1. Plot of *v t i.e.* [*P*] versus Δ[S_0] for separate determination of the first ($S_{L(1)}$) of two slopes

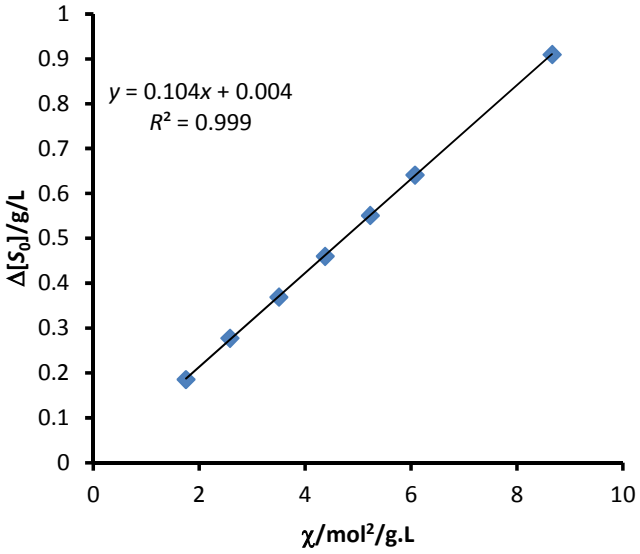

Fig. 2. Plot of Δ[S_0] versus χ *i.e.* $\left(\frac{1}{162}-\frac{[P]}{\Delta[S_0]}\right)[P]$ to give a 2nd slope, $S_{L(2)}$

the spectrophotometer) hydrolyzed, two moles of glucose (or glucose and fructose) are yielded; thus rate constant for product formation is twice the rate constant for the transformation of the substrate via the hydrolysis of the glycosidic bond because the v_{max} for the formation of product is twice the v_{max} for the transformation of the substrate. If a given mass of a polysaccharide is the substrate for an appropriate enzyme, complete hydrolysis of *n* moles of the polysaccharide should give *n*Φ moles of the monosaccharide. Therefore, the expression, $v = k\,[S_0]$, is purely equation of velocity of reaction which, if it remains constant (which is very unlikely, given different [S_0]), enables one to determine the magnitude of *P* after a given time that seem to be indefinite.

Sometimes a new model, mathematical model in particular may not possess immediate applicability except in the feature. Nonetheless the quadratic equations derived so far have unifying character such that various kinetic parameters are brought together in the same equation like Eq. (1a). However, any where [*P*] (*i.e. v t*) or *v* appears in the equations is a target for replacement with Michaelis – Menten equation $v = \frac{v_{max}[S_0]}{K_m+[S_0]}$. This can enable the

determination of the relationship between virtually all kinetic parameters including k_2; it could also be used to predict the duration of assay given predetermined concentration of product desired with available concentration of enzyme and substrate. Every research has predetermined scope such that one need to reserve for further investigation this issue of the application of the quadratic equation derived in this research.

5. CONCLUSION

Despite the uncertainty as to what the application of the transformation into quadratic form for the quantification of either the product or the substrate might be it was however, successfully derived and compares with Eq. (1a). The results obtained from such quadratic transformations are not significantly different from the result obtainable from the use of the usual equations. The most important objective of this research is the determination of the rate of hydrolysis of glycosidic bond; the result showed that it is ~ $1/2^{nd}$ the usual rate constant for the formation of product leaving one to conclude that the rate determining step is the making and breaking of bond. The fast steps may be the frequency of collision which may be higher at higher concentration of reactants and also, velocity of product departure.

ACKNOWLEDGEMENTS

I hereby express my profound gratitude to Madam R. U. Udema who kindly paid for the article processing fee despite her challenging commitment.

COMPETING INTERESTS

Author has declared that no competing interests exist.

REFERENCES

1. Paula Monteiro de Souza, Pérola de Oliveira e Magalhães. Application of microbial alpha amylase in industry – a review. Brazilian Journal of Microbiology. 2010;41:850-861.
2. Ruiz MI, Sanchez CI, Torrres RG, Molina DR. Enzymatic hydrolysis of cassava starch for production of bioethanol with a Colombian wild yeast strain. J. Braz. Chem. Soc. 2011;22(12):2337-2343.
3. Uthumporn U, Zaidul ISM, Karim AA. Hydrolysis of granular starch at sub-gelatinization temperature using a mixture of amylolytic enzymes. Food Bioprod. Process. 2010;88:47-54.
4. Jaspreet Singha J, Kaurb L, McCarthy OJ. Factors influencing the physico-chemical, morphological, thermal and rheological properties of some chemically modified starches for food applications — A review. Food Hydrocolloids. 2007;21:1–22.
5. Herrero-Martínez JM, Schoenmakers PJ, Kok WTh. Determination of the amylose-amylopectin ratio of starches by iodine-affinity capillary electrophoresis. J. Chromatogr. A. 2004;1053:227-234.
6. Absar N, Zaidul ISM, Takigawa S, Hashimoto N, Matsuura-Endo C, Yamauchi H, et al. Enzymatic hydrolysis of potato starches containing different amounts of phosphorus. Food Chem. 2008;1-6.
7. Kasprzak MM, Laerk HN, Larsen FH, Knudsen KEB, Pedersen S, Jørgensen AS. Effect of enzymatic treatment of different starch sources on the *in vitro* rate and extent of starch digestion. Int. J. Mol. Sci. 2012;13:929-942.
8. Slaughter SL, Ellis PR, Butterworth PJ. An investigation of the action of porcine pancreatic alpha amylase on native and gelatinized starches. Biochimica *et* Biophysica Acta. 2001;1525:29-36.
9. Presečki AV, Blažević ZF, Vasić-Rački D. Complete starch hydrolysis by the synergistic action of amylase and glucoamylase: Impact of calcium ions. Bioprocess Biosyt Eng. 2013;1555-1562.
10. Lim LH, Macdonald DG, Hill GA. Hydrolysis of starch particles using immobilized barley alpha amylase. Biochem Eng J. 2003;13:53–62.
11. Stoisser T, Brunsteiner M, Wilson DK, Nidetzky B. Conformational flexibility related to enzyme activity: Evidence for a dynamic active-site gatekeeper function of Tyr215 in *Aerococcus viridans* lactate oxidase. Sci. Rep. 2016;6:1-13.
12. Brooks HB, Davidson VL. A method for extracting rate constants from initial rates of stopped-flow kinetic data: Application to a physiological electron-transfer reaction. Biochem. J. 1993;294:211-213.
13. Christensen T, Stoffer BB, Svensson B, Christensen U. Some details of the reaction mechanism of glucoamylase from *Aspergillus niger* kinetic and structural

studies on Trp52dPhe and Trp317hPhe mutants. Eur. J. Biochem. 1997;250:638-645.

14. Ewers D, Becher T, Machtens JP, Weyand I, Fahlke C. Induced fit substrate binding to an archeal glutamate transporter homologue. Proc Natl Acad Sci U.S.A. 2013;110(30):12486–12491.
15. Reuveni S, Urbakhc M, Klafterc J. Role of substrate unbinding in Michaelis-Menten enzymatic reactions. Proc Natl Acad Sci U.S.A. 2014;4391-4396.
16. D' Amico S, Marx JC, Gerday C, Feller G. Activity – stability relationships in extremophilic enzymes. J. Biol. Chem. 2003;276(10):7891–7896.
17. Gheibi N, Saboury AA, Haghbeen K, Movahedi – Moosavi AA. The effect of some osmolytes on the activity and stability of mushroom tyrosinase. J. Biosci. 2006;32(3):355–362.
18. D' Amico S, Gerday C, Feller G. Structural determinants of cold adaptation and stability in a psychrophilic a – amylase. Biologia, Bratislava. 2002;11(57/Suppl): 213–219.
19. Tanaka A, Hoshino E. Thermodynamic and activation parameters for the hydrolysis of amylose with Bacillus alpha amylase in a diluted anionic surfactant solution. J. Biosci. Bioeng. 2002;93(5):485–490.
20. Udema II. The effect of additives and temperature on the velocity of hydrolysis of raw starch with human salivary alpha - amylase. Int. J. Biochem. Res. Rev. 2016;10(2):1–17.
21. Schnell S, Maini PK. Enzyme kinetics at high enzyme concentration. Bull Math. Biol. 2000;62:483-499.
22. Goldstein A. The mechanism of enzyme-inhibitor-substrate reactions. J. Gen. Physiol. 1944;27:529–580.
23. Cha S, Cha CJM. Kinetics of cyclic enzyme systems. Mol. Pharmacol. 1965;1: 178–189.
24. Reiner JM. Behavior of enzyme systems. New York: Van Nostrand Reinhold Company; 1969.
25. Udema II. Space-time and entropic characterization of *Aspergillus oryzea* alpha amylase catalyzed reaction. Int. J. Biochem. Res. Rev. 2016;15(2):1-12.
26. Marchal LM, Jonkers J, Franke JTH, de Gooijer CD, Tramper J. The effect of process conditions on the alpha amylolytic hydrolysis of amylopectin potato starch: An experimental design approach. Biotechnol. Bioeng. 1999;60(3):348-357.
27. Bérangère F, Laurent P, Agnes P, Claude-Gilles D. Methodology for bioprocess analysis: Mass balances, yields and stoichiometries, stoichiometry and research- The importance of quantity in biomedicine. Innocenti A, Editor. InTech, Croatia, China; 2012.
    Available:Https://www.intechopen.com
28. Beard DA, Liang SD, Quian H. Energy balance for the analysis of complex metabolic net works. Biophys. J. 2002;83: 79-81.
29. Udema II. Substrate mass conservation in enzyme catalyzed amylolytic activity. Int. J. Biochem Res. 2017;8(1):1-10.
30. Bernfeld P. Amylases, alpha and beta. Methods. Enzymol. 1955;1:49–152.
31. Lineweaver H, Burk D. The determination of enzyme dissociation constants. J Am Chem Soc. 1934;56(3):658–666.
32. Eisenthal R, Cornish-Bowen A. The direct linear plot: A new graphical procedure for estimating enzyme kinetic parameters. Biochem. J. 1974;139:715-720.

APPENDIX A

A closer look at this section, the appendix section, will reveal that it is intended for a different purpose, the derivation of unification equation which called for the introduction of a factor designated as, β. This does not appear in the corresponding Eq. (4) in theoretical section. Another difference is the presence of M_3 (the molar mass of the product-maltose) in this section which should culminate to the equations that may not be found in the theoretical section. Also, the degree of polymerization (ϕ) does not appear in any of the derivation in this section. Both theoretical and appendix section contains derivations but the main objective is the determination of rate constant for the hydrolysis of endo-glycosidic bonds distinct from overall rate constant for product formation that entail stages as enunciated in the main text. The rate constants, $k_{2[S]}$ and $\boldsymbol{k_{2[S]}}$ were conveniently left with same alphabets otherwise they represent different parameters namely $\frac{\Delta[S_0]\,k_2}{vtM_3}$ (Eq.A.7) and rate constant for the hydrolysis of endo-glycosidic bond respectively.

$$[P]N_A = \frac{\boldsymbol{\beta}[S_0]}{M_3}\left(1 - \frac{1}{\exp(k\,t)}\right)\left(\frac{M_3}{162} - 1\right)N_A \tag{A.1}$$

where **β** is the correction factor (this may be referred to as a proportionality constant) if M_3 is taken as the molar mass of the product, maltose. Equation (A.1) is premised on the fact that for every mole of maltose yielded, one mole of the glycosidic bond is hydrolyzed.

$$[P] = \frac{\frac{\boldsymbol{\beta}[S_0](\exp(kt)-1)}{\exp(kt)}\left(\frac{M_3}{162}-1\right)}{M_3} \tag{A.2}$$

Rearrangement of Eq. (A.2) gives:

$$\frac{[S_0]}{162} - \frac{[S_0]}{M_3} = \frac{\exp(k\,t)[P]}{\boldsymbol{\beta}(\exp(k\,t)-1)} \tag{A.3}$$

$$\frac{[S_0]}{M_3} = \frac{[S_0]}{162} - \frac{\exp(k\,t)[P]}{\boldsymbol{\beta}(\exp(k\,t)-1)} \tag{A.4}$$

Let Eq. (A.5) below holds temporarily for the purpose of brevity.

$$\zeta = [S_0]\frac{1}{162} - \frac{\exp(k\,t)[P]}{\boldsymbol{\beta}(\exp(k\,t)-1)} \tag{A.5}$$

$$M_3 = \frac{[S_0]}{\zeta} \tag{A.6}$$

It is necessary to bear in mind that the reducing sugar produced within specified duration of assay is not necessarily from one polysaccharide.

Just as k_2 is expressed as $v_{max}/[E_0]$, where k_2, $v_{max,}$ and $[E_0]$ are the rate constant for the production of reducing sugar, maltose for instance, maximum velocity of the production of the reducing sugar, maltose in this case, and the molar concentration of the enzyme, the equivalent rate constant, $k_{2[S]}$ (not a pseudo-rate constant) for the transformation of a given amount of the substrate, being equal to the mass of the product in line with conservation law is:

$$\boldsymbol{k_{2[S]}} = \frac{\Delta[S_0]\,k_2}{vtM_3} \tag{A.7}$$

The term transformation is simply the breaking and making of bonds before the departure of the product. In Eq. (A.7), $\frac{v}{k_2}:\frac{\Delta[S_0]}{tM_3\boldsymbol{k_{2[S]}}}$ is simply an expression of the ratio of the molar concentration of the enzyme involved in complex formation to the molar concentration of the substrate that was transformed to product or more precisely, the number of hydrolyzed glycosidic bonds, given that the value of M_3 is known. However, if $\Delta[S_0]$ is taken as mass of product, in line with mass conservation

principle, then M_3 becomes the molar mass of the product - maltose for instance - and $\boldsymbol{k_{2[S]}}$ should be $\cong k_2$. The approximation is indicated because of imperfection in every assay.

$$= \frac{\Delta[S_0]\, k_2 \zeta}{vt[S_0]} \tag{A.8}$$

Equation (A.8) is obtained by replacing M_3 with Eq. (A.6).

In Eq. (A.7), $\boldsymbol{k_{2[S]}} \ \alpha\ 1/M_3$. Thus,

$$\boldsymbol{k}_{2[S]} = \frac{\Theta}{M_3} \tag{A.9}$$

where, for the purpose of brevity, Θ is $\frac{\Delta[S_0]\, k_2}{vt}$.

Substituting Eq. (A.6) for M_3 in Eq. (A.9) gives:

$$\boldsymbol{k}_{2[S]} = \frac{\Theta\,\zeta}{[S_0]} \equiv \frac{\Theta\left(\frac{[S_0]}{162} - \frac{[P]\exp(k\,t)}{\boldsymbol{\beta}(\exp(k\,t)-1)}\right)}{[S_0]} \tag{A.10}$$

$$[S_0]\boldsymbol{k}_{2[S]} = \Theta\frac{[S_0]}{162} - \frac{\Theta[P]\exp(k\,t)}{\boldsymbol{\beta}(\exp(k\,t)-1)} \tag{A.11}$$

If $\boldsymbol{k_{2[S]}}$ is replaced by Eq. (A.7) the result is:

$$[S_0]\Delta[S_0]\frac{k_2}{vtM_3} = \Theta\frac{[S_0]}{162} - \frac{\Theta[P]\exp(k\,t)}{\boldsymbol{\beta}((\exp(k\,t)-1)} \tag{A.12}$$

Rearrangement of Eq. (A.12) gives:

$$[S_0]\Delta[S_0]\frac{k_2}{vt} = \Theta[S_0]\frac{M_3}{162} - \frac{\Theta[P]\exp(k\,t)M_3}{\boldsymbol{\beta}(\exp(k\,t)-1)} \tag{A.13}$$

$$\Delta[S_0] = \frac{\left(\Theta\,[S_0]\frac{M_3}{162} - \frac{\Theta[P]\exp(k\,t)M_3}{\boldsymbol{\beta}(\exp(k\,t)-1)}\right)v\,t}{[S_0]k_2} \tag{A.14}$$

Equation (A.7) could be rearranged to give:

$$v\,t = \frac{k_2\Delta[S_0]}{M_3\boldsymbol{K}_{2[S]}} \tag{A.15}$$

The purpose of Eq. (A.15) is the determination of a slope ($\boldsymbol{S}_{\mathbf{L\ (1)}}$) which could be used to find an expression for M_3. The slope from the plot of $v\,t$ (or $[P]$) versus $\Delta[S_0]$ ($[S_0]$ exp (($k\,t$) −1)/exp ($k\,t$)) can be expressed as:

$$\boldsymbol{S}_{\mathbf{L(1)}} = \frac{k_2}{M_3\boldsymbol{k}_{2[S]}} \tag{A.16}$$

Equation (A.16) leads to Eq. (A.17).

$$k_{2[S]} = \frac{k_2}{M_3 S_{\mathrm{L}(1)}} \tag{A.17}$$

From Eq. (A.16)

$$M_3 = \frac{k_2}{S_{\mathrm{L}\,(1)}\boldsymbol{k}_{2[S]}} \tag{A.18}$$

Substitution of Eq. (A.18) into Eq. (A.14) gives:

$$\Delta[S_0] = \left(\Theta[S_0]\frac{k_2}{162S_{\mathrm{L}(1)}\boldsymbol{k_{2[S]}}} - \frac{\Theta[P]\exp(k\,t)k_2}{\boldsymbol{\beta}(\exp(k\,t)-1)S_{\mathrm{L}(1)}\boldsymbol{k_{2[S]}}}\right) v\frac{t}{[S_0]k_2} \tag{A.19}$$

Meanwhile, [*P*] = *v t* and Eq. (A.18) is substituted into Eq. (A.9) to give:

$$\boldsymbol{k_{2[S]}} = \frac{\Theta}{k_2}\,S_{\mathrm{L}(1)}\boldsymbol{k_{2[S]}} \tag{A.20}$$

$$\Theta = \frac{k_2}{S_{\mathrm{L}(1)}} \tag{A.21}$$

Rearrangement of Eq. (A.19) and substitution of Eq. (A.21) into it gives the following after simplification,

$$\begin{aligned}\Delta[S_0] &= \Theta\left(\frac{[S_0]k_2}{162S_{\mathrm{L}(1)}\boldsymbol{k_{2[S]}}} - \frac{[P]\exp(k\,t)\,k_2}{\boldsymbol{\beta}(\exp(k\,t)-\,1)S_{\mathrm{L}(1)}\boldsymbol{k_{2[S]}}}\right) v\frac{t}{[S_0]k_2}\\ &= \frac{k_2}{\boldsymbol{k_{2[S]}}S^2_{\mathrm{L}(1)}}\left(\frac{[S_0]}{162} - \frac{[P]\exp(k\,t)}{\boldsymbol{\beta}(\exp(k\,t)-1)}\right) v\frac{t}{[S_0]}\end{aligned} \tag{A.22}$$

Knowing that *v t* = [*P*], Eq. (A.22) can then be transformed into a quadratic equation as follows. Expansion of Eq. (A.22) gives:

$$\frac{\exp(kt)-1}{\exp(kt)}[S_0]^2\;\frac{\boldsymbol{k_{2[S]}}}{k_2}\;S^2_{\mathrm{L}(1)} = \left(\frac{[S_0]}{162} - \frac{[P]\exp(kt)}{\boldsymbol{\beta}(\exp(kt)-1)}\right)[P] \tag{A.23}$$

Equation (A.23) is as it is after cross multiplication, because $\Delta[S_0] = [S_0]\,(\exp(k\,t)\text{-}1)/\exp(k\,t)$. Further rearrangement gives:

$$\frac{(\exp(k\,t)-1)^2}{(\exp k\,t)^2}[S_0]^2S^2_{\mathrm{L}(1)}\frac{k_{2[S]}}{k_2} = \frac{\frac{[S_0]}{162}(\exp(kt)-1)[\mathrm{P}]}{\exp(k\,t)} - \frac{[P]^2}{\boldsymbol{\beta}} \tag{A.24}$$

Let, for the purpose of simplicity, $\chi = (\exp(k\,t)\text{-}1)/\exp(k\,t)$. Thus,

$$\chi = \frac{\left(\frac{[S_0]}{162}[P] \pm \sqrt{\left(\frac{[S_0][P]}{162}\right)^2 - 4\frac{[P]^2}{\boldsymbol{\beta}}[S_0]^2S_{(1)}{}^2\frac{\boldsymbol{k_{2[S]}}}{k_2}}\right)}{2[S_0]^2S^2_{\mathrm{L}(1)}\frac{\boldsymbol{k_{2[S]}}}{k_2}} \tag{A.25}$$

Equation (A.24) can be rearranged so as to make [*P*] or $\Delta[S_0]$ subject of the formula in a quadratic equation. Thus,

$$[P] = \left(\frac{\frac{\Delta[S_0]}{162} \pm \sqrt[2]{\left(\frac{\Delta[S_0]}{162}\right)^2 - 4\times S^2_{\mathrm{L}(1)}\frac{\boldsymbol{k_{2[S]}}}{\boldsymbol{\beta}k_2}\Delta[S_0]^2}}{2/\boldsymbol{\beta}}\right) \tag{A.26}$$

$$\Delta[S_0] = k_2\left(\frac{\frac{[P]}{162} \pm \sqrt[2]{\left(\frac{[P]}{162}\right)^2 - 4S^2_{\mathrm{L}(1)}\frac{\boldsymbol{k_{2[S]}}}{k_2}[P^2]\frac{1}{\boldsymbol{\beta}}}}{2S^2_{\mathrm{L}(1)}\boldsymbol{k_{2[S]}}}\right) \tag{A.27}$$

As already stated above, $\Delta[S_0] = [S_0]\,(\exp(k\,t)\text{-}1)/\exp(k\,t)$ such that Eq. (A.24) can be rearranged to give:

$$\Delta[S_0]^2\ S_{L(1)}^2 \frac{\boldsymbol{k_{2[S]}}}{k_2} - \frac{\Delta[S_0][P]}{162} + \frac{[P]^2}{\boldsymbol{\beta}} = 0 \quad \text{(A.28)}$$

Determination of **β** requires that $\Delta[S_0]$ be converted to [*P*] (*i.e*. 324×[*P*]) in line with conservation law.

$$\therefore \quad 324^2[P]^2 S_{L(1)}^2 \frac{\boldsymbol{k_{2[S]}}}{k_2} - \frac{324[P]^2}{162} + \frac{[P]^2}{\boldsymbol{\beta}} = 0 \quad \text{(A.29)}$$

Ultimately, 1/**β =** 0.995924 and $\boldsymbol{k_{2[S]}}/k_2$ = 1/324 $S_{L(1)}$ (Eq. (A.16) where M_3 and $S_{L(1)}$ are 324 and 30.99 exp (-4)/mol/g) respectively.

Peer-review history:
The peer review history for this paper can be accessed here:
http://sciencedomain.org/review-history/21644