# Substrate Mass Conservation in Enzyme Catalyzed Amylolytic Activity

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*Author's contribution*

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**Short Research Article**

## ABSTRACT

**Aims:** The aims of the research were 1), to derive simple equations that can be used to determine mass concentration of reaction mixture components, and 2), to determine the mass concentration of free substrate, total mass concentration of substrate involved in enzyme-substrate complex formation, and mass concentration of partially digested parent starch, otherwise called fragments at the end of different durations of assay.
**Study Design:** Theoretical and Experimental.
**Place and Duration of Study:** Department of Chemistry and Biochemistry, Research Division, Ude International Concepts LTD (862217), B. B. Agbor, Delta State, Nigeria; Owa Alizomor Secondary School, Owa Alizomor, Ika North East, Delta State, Nigeria. The research, including derivation of equations lasted between 18th April, 2017 and 24th June, 2017.
**Methodology:** Bernfeld method of enzyme assay was used. Assays were carried out on *Aspergillus oryzea* salivary alpha amylase. Various parameters were determined by substituting relevant experimental data to the formulated equations.
**Results:** The mass concentrations of the free undigested starch, the enzyme-substrate complex, and the fragments showed decreasing trend with time. The exception was at highest duration with respect to the concentration of the fragment. The concentrations of the remaining substrate

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calculated using two different approaches, and the new approach (Eq. (31)) in this research were not statistically different ($P > 0.05$). The difference between the change in the concentration of the substrate per unit time, $\Delta[S_0]$/min, and the corresponding product formed per unit time, $[P]$/min, was not statistically different ($P > 0.05$).

**Conclusion:** Different algebraic equations were successfully derived and were used to determine various components of the hydrolyzate at the end of the duration of assay. The hydrolyzate is composed of different components. The sum of the components was very similar to total concentration of substrate, in conformity with substrate mass conservation law.

## 1. INTRODUCTION

The intense studies of alpha amylase (E.C.3 2.1.1.) and polysaccharides go beyond mere academic exercise and interest. Thus naturally occurring polysaccharides and different sources of alpha amylase are investigated for their solubilizability and amylolytic potential respectively. This is *vis-à-vis* the economic advantage, lower cost of exploring raw starch for the intended purposes such as food, paper, textile manufacture [1], and manufacture of single-use biodegradable plastic items *etc* [2]. If gelatinized starch is the substrate, the presence of undigested fibre, fragments, and indeed, various valuable hydrolyzate [3] with different composition *etc* may not be precluded let alone with native starch despite very long duration of assay.

However, most kinetic studies of enzymes involve the assay of the enzymes over a short period of time. This implies that there are likely to be undigested starch, either in raw or gelatinized form, which may exist as fragment, free complete polysaccharide chain, strongly and loosely bound polysaccharide to the enzyme. The fundamental issue is that the total mass of reactant (s) should be equal to the mass of the product (s). In this regard, there is the concept of substrate and enzyme mass conservation, which demands that,

$$[E_0] = [E](t) + [C](t) \tag{1}$$

$$[S_0] = [S](t) + [C](t) + [P](t) \tag{2}$$

where $[E_0]$, $[S_0]$, and $[E_0](t)$ are the total concentrations of the enzyme and substrate respectively, and the concentration of the free enzyme in time, $t$; while $[P_0](t)$ is the concentration of the product. Equations (1) and (2) certify mass conservation law [4,5]. The concentration, $[C](t)$ appears in Eqs (1) and (2) but so long as the different concentrations of the

enzyme, total enzyme, free enzyme, and complex are in mol/L, the same cannot be said of its appearance in Eq (2) where the unit of concentration is g/L. Thus the mass concentration = $M_2 [C](t) \neq M_3 [C](t)$ (where $M_2$ and $M_3$ are the molar masses of the enzyme and parent polysaccharide chain respectively), even if the stoichiometric ratio of substrate to protein in the complex is 1:1, except on the ground that $M_2 = M_3$.

Here are derived equations [4].

$$[S](t) = [S_0] \exp(-k_1 [E_0]t) \tag{3}$$

$$[C](t) = [S_0] (1 - \exp(-k_1 [E_0]t)) \tag{4}$$

$$[C](t) = [S_0] (\exp(-k_2 t)) \tag{5}$$

where $k_1$ and $k_2$ are $2^{nd}$ order rate constant and rate of production of products respectively. Of course, $-k_1 [E_0]$ gives pseudo-first order rate constant. However, substitution of Eqs (3) and (4) into substrate mass conservation equation, Eq. (2), gives result which shows that "$[S_0] = [S_0] + [P](t)$"; this, obviously, should not be the case. Meanwhile, most values of $k_2$ are » unity; with $t \ll \infty$, say, 0.5 min and $k_2$ hypothetically = 100/ min, $[C](t) = \sim 1.94 \exp(-22) [S_0]$. It is obvious that $[C](t) \to$ zero when $t \geq 1$min. Therefore, the aims of the research are 1), to derive simple equations that can be used to determine mass concentration of reaction mixture components, and 2), to determine the mass concentration of free substrate, total mass concentration of substrate involved in enzyme-substrate complex formation, and mass concentration of partially digested parent starch, otherwise called fragments at the end of different durations of assay.

### 1.1 Theory

With progress in reaction, both products and fragments in 1:1 ratio increase in quantity; the

free native polysaccharide decreases in mass concentration. With time, also, there is an increase in encounter complex formation. Thus there are weak interactions characterized with higher kinetic energy in addition to actual enzyme-substrate complex with lower kinetic energy. The encounter complex dissociates and re-associates under thermal influence and 'cage effect'. But as products are formed with time, the concentration of enzyme-substrate decreases because the concentration of the substrate decreases with time.

Ultimately, the concentration of the free native substrate is inversely proportional to the concentration of the product, the reducing equivalent, and to all complexes and fragments of polysaccharides, $S_{fr}$, at the end of assay within specified duration, following the termination of assay by the addition of the oxidizing agent. Since there are weak associations in encounter formation, interactions of the enzyme with the substrate via sites other than the active sites [6, 7] giving concentration of a complex that is non convertible to products, interaction via the active site, giving the well known enzyme substrate complex [ES], and with presence of free fragments of the polysaccharide, the concentration of the free native polysaccharide, $[S_f]$ $(t)$ can be given as:

$$[S_f]\ (t) = [S_0] - ([C]\ (t) + [S_{fr}]\ (t) + [P]\ (t)) \quad (6)$$

where $[C]$ $(t)$ is redesignated as the sum of the mass concentrations of the substrate involved in all enzyme-substrate formations. Subtraction of $[P]$ $(t)$ from total initial substrate concentration gives $[S_0]$ exp $(- k\,t)$ where the later is expressed as:

$$[S_0]\ \exp(-k\,t) = [C]\ (t) + [S_{fr}]\ (t) + [S_f]\ (t) \quad (7)$$

Equation (7) can be understood given the expression, $d[S]/dt = k\ [S]$ [8], such that the outcome of basic integration gives, In $([S_0]/[S_R])$ $= k\ t$ where $[S_R]$ $(t)$ is the remainder of the substrate after the termination of catalytic action. Therefore, $[S_R] = [S_0]$ exp $(- k\,t)$.

Thus,

$$[C]\ (t) + [S_{fr}]\ (t) = [S_0]\ \exp (- k\,t) - [S_f]\ (t) \quad (8)$$

Nonetheless, there is the possibility that within the short duration of assay there may be fragments that constitute substrates for further hydrolysis, the limit dextrin or indigestible

fraction. Hence $[S_{fr}]$ $(t)$ and $[S_f]$ $(t)$ can be combined to give $[S_F]$ $(t)$. Then, $[C](t) = ([S_0]/\exp (k\,t)) - [S_F]$ $(t)$. Besides, it should be made clear that, so long as there is substrate, the formation of enzyme substrate complex is a continuous process such that, at the moment the reaction is terminated, some complex may have been formed, but cannot be transformed to product since the process is time dependent and the presence of added oxidizing agent cannot allow further transformation.

## 1.2 Formulation of Mathematical Models

Meanwhile, one of two approaches need to be explored for separate determination of $[S_f]$ $(t)$ via quadratic equation, such that the sum of $[C](t)$ and $[S_{fr}](t)$ designated as $[S_{FRC}]$ $(t)$ can be determined according to Eq. (8), but neither $[S_{fr}]$ $(t)$ nor $[C](t)$ can be determined directly. The other approach entails the direct determination of the sum of $[S_{fr}]$ $(t)$ and $[S_f]$ $(t)$ (designated as $[S_{FRF}]$ $(t)$), and $[C](t)$ via quadratic equation. In order to adapt the approaches or methods, the equations have to be formulated based on simple principle that is testable. The concentration of the free undigested starch is inversely proportional to the concentration of the substrate hydrolyzed $([S_f]$ $(t) = \vartheta$ exp $(k\,t)$ $/[S_0]($ exp $(k\,t) - 1)$ where $\vartheta$ is a constant). Also, with increasing $[S_{FRC}]$ $(t)$ $(=[C](t) + [S_{fr}](t))$, there should be a decreasing $[S_f]$ $(t)$. However,

$$[S_{FRC}]\ (t) = (\exp (k\,t))^{-1}[S_0] - [S_f]. \quad (9)$$

Thus a general equation such as the following can be stated.

$$[S_f] = \beta\ \exp (k\,t)\ /[S_0]( \exp (k\,t) - 1)\ [S_{FRC}]\ (t) \quad (10)$$

Substitution of Eq. (9) into Eq. (10) gives:

$$[S_f] = \beta\ (\exp (k\,t))^2\ /[S_0]( \exp (k\,t) - 1)\ ([S_0] - [S_f]\ \exp (k\,t)) \quad (11)$$

where $\beta$ is a constant

Rearrangement of Eq. (11) gives:

$$[S_f]\ [S_0] - [S_f]^2\ \exp (k\,t) = \beta\ (\exp (k\,t))^2\ /[S_0]( \exp (k\,t) - 1) \quad (12)$$

Substitution of $\vartheta$ exp $(k\,t)$ $/[S_0]($ exp $(k\,t) - 1)$ into Eq. (12) gives:

$$\vartheta\ \exp (k\,t)\ [S_0]/\ [S_0]( \exp (k\,t) - 1) - \vartheta^2\ (\exp (k\,t))^3\ /\ [S_0]^2( \exp (k\,t) - 1)^2 = \beta\ (\exp (k\,t))^2\ /[S_0]( \exp (k\,t) - 1) \quad (13)$$

Simplification and rearrangement of Eq. (13) gives:

$$(\exp(kt) - 1)/(\exp(kt))^2 = (\vartheta/[S_0]^2) + \beta(\exp(kt) - 1)/(\exp(kt)\,\vartheta\,[S_0]). \quad (14)$$

If $(\exp kt)^{-1}[S_0]$ is without fragments, the value of $[S_f]$ is a theoretical possibility. The usefulness of Eq. (14) is reserved for the method subsection.

However, with time there is a possibility of the presence of fragment. Thus Eq. (10) can be re-stated as:

$$[S_{FRC}] = \beta \exp(kt)/[S_0](\exp(kt) - 1)\,[S_f]\,(t) = (\exp(kt))^{-1}[S_0] - [S_f] \quad (15)$$

Equation (15) can be transformed into a quadratic equation with which to determine two roots. Hence,

$$[S_f]^2 - (\exp(kt))^{-1}[S_f] + \beta \exp(kt)/[S_0](\exp(kt) - 1) = 0 \quad (16)$$

The two roots are:

$$[S_f](low) = [[S_0](\exp(kt))^{-1} - \{([S_0](\exp(kt))^{-1})^2 - 4\beta \exp(kt)/(\exp(kt) - 1)[S_0]\}^{\frac{1}{2}}]/2 \quad (17)$$

$$[S_f](high) = [[S_0](\exp(kt))^{-1} + \{([S_0](\exp(kt))^{-1})^2 - 4\beta \exp(kt)/(\exp(kt) - 1)[S_0]\}^{\frac{1}{2}}]/2 \quad (18)$$

Incidentally,

$$[S_{FRC}](t)\ (i.e.\ [S_0](\exp(kt))^{-1} - [S_f](low)) = [[S_0](\exp(kt))^{-1} + \{([S_0](\exp(kt))^{-1})^2 - 4\beta \exp(kt)/(\exp(kt) - 1)[S_0]\}^{\frac{1}{2}}]/2 \quad (19)$$

The second approach entails the formulation of another quadratic equation for the determination of $[S_{FRF}](t)\ (i.e.\ [S_{fr}](t) + [S_f](t))$ and $[C](t)$.

$$[S_{FRF}](t) = \zeta/[C](t)[P](t) = \zeta/([S_0](\exp(kt))^{-1} - [S_{FRF}](t))[P](t) \quad (20)$$

Once again Eq. (20) is a theoretical possibility if $[C](t)$ and $[P](t)$ only exist in the reaction mixture. If so, $[S_{FRF}](t) = [S_f](t)$; but the simultaneous presence of fragments would always alter the proportionality constant, thereby necessitating the need for the transformation to quadratic equations.

Expansion of Eq. (20) gives:

$$[S_0](\exp(kt))^{-1}[S_{FRF}](t) - [S_{FRF}]^2(t) = \zeta/[P](t) \quad (21)$$

Meanwhile,

$$[S_{FRF}](t) = [S_0](\exp(kt))^{-1} - [C](t) \quad (22)$$

Substitution of Eq. (22) into Eq. (21) gives:

$$[S_0](\exp(kt))^{-1}([S_0](\exp(kt))^{-1} - [C](t)) - ([S_0](\exp(kt))^{-1} - [C](t))^2 = \zeta/[P](t) \quad (23)$$

Meanwhile, $[C](t)$ decreases as $[P](t)$ increases. Then if $[C](t) = \varphi/[P](t)$, where $\varphi$ is a proportionality constant, then the following may hold after substitution into Eq. (23) and its expansion.

$$([S_0](\exp(kt))^{-1})^2 - [S_0](\exp(kt))^{-1}\varphi/[P](t) - ([S_0](\exp(kt))^{-1})^2 + 2[S_0](\exp(kt))^{-1}\varphi/[P](t) - (\varphi/[P](t))^2 = \zeta/[P](t). \quad (24)$$

Simplification and rearrangement of Eq. (24) gives:

$$(\exp(kt))^{-1}[P](t) = (\varphi/[S_0]) + \zeta[P](t)/\varphi[S_0] \quad (25)$$

Once again, the application of Eq. (25) comes under method subsection. Meanwhile, Eq. (21) can be transformed into a quadratic equation with which to determine $[S_{FRF}](t)$ (low) and $[S_{FRF}](t)$ (high).

$$[S_{FRF}]^2(t) - [S_0](\exp(kt))^{-1}[S_{FRF}] + \zeta/[P](t) = 0 \quad (26)$$

$$[C](t) = [S_{FRF}](t)\ (low) = \{[S_0](\exp(kt))^{-1} - [([S_0](\exp(kt))^{-1})^2 - 4\zeta/[P](t)]^{\frac{1}{2}}\}/2 \quad (27)$$

$$[S_0](\exp(kt))^{-1} - [C](t) = [S_{FRF}](t)\ (high) = \{[S_0](\exp(kt))^{-1} + [([S_0](\exp(kt))^{-1})^2 - 4\zeta/[P](t)]^{\frac{1}{2}}\}/2. \quad (28)$$

The most important deduction is that:

$$[S_{FRF}](t)\ (high) - [[S_0](\exp(kt))^{-1} - \{([S_0](\exp(kt))^{-1})^2 - 4\beta \exp(kt)/(\exp(kt) - 1)[S_0]\}^{\frac{1}{2}}]/2 = [S_{fr}](t) \quad (29)$$

$$[S_{FRC}](t)(high) - \{[S_0](\exp(kt))^{-1} - [([S_0](\exp(kt))^{-1})^2 - 4\zeta/[P](t)]^{\frac{1}{2}}\}/2 = [S_{fr}](t) \quad (30)$$

Thus,

$$[S_0](\exp(kt))^{-1} = [C](t) + [S_f](t) + [S_{fr}](t). \quad (31)$$

## 2. MATERIALS AND METHODS

### 2.1 Materials

#### 2.1.1 Chemicals

*Aspergillus oryzea* alpha amylase (EC 3.2.1.1) and potato starch were purchased from Sigma – Aldrich, USA. Tris 3, 5 – dinitrosalicylic acid, maltose, and sodium potassium tartrate tetrahydrate were purchased from Kem light laboratories Mumbai, India. Hydrochloric acid, sodium hydroxide, and sodium chloride were purchased from BDH Chemical Ltd, Poole England. Distilled water was purchased from local market. The molar mass of the enzyme is ~ 52 k Da [9,10].

#### 2.1.2 Equipment

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

### 2.2 Methods

The enzyme was assayed according to Bernfeld method [11] using gelatinized potato starch whose concentration was 20 g/L. The duration of assay ranges from 1-5 min. Reducing sugar produced upon hydrolysis of the substrate using maltose as standard was determined at 540 nm with extinction coefficient equal to ~ 181 L/mol.cm. A suspension of raw potato starch in distilled water (2 g + 100 mL of distilled water), was gelatinized at 100°C for 3 min, and cooled to room temperature after making up for the loss of moisture due to evaporation. A mass concentration = 2 mg/L of *Aspergillus oryzea* alpha amylase was prepared in Tris HCl buffer at *p*H = 6.

#### 2.2.1 Determination of pseudo-first order rate constant for the hydrolysis of starch

The reducing sugar produced at the end of different duration of assay, following the addition of 3, 5 – di-nitro-salicylic acid (DNS) was converted to mass concentration while taking into cognizance the fact that for every mole of maltose produced, 1 mole of water molecule must be utilized or added to the glycosidic bond resulting to an increase in the mass of the product, fragment and reducing sugars.

Therefore, if x mol/L of maltose was produced 18x g/L must be subtracted from say, 342x g/L of product. This ensures mass conservation. Thus after time *t*, the remaining undigested substrate is given as: dry $[S_0]$ – $324vt$ (synthesis of polysaccharides require the loss of water molecules while depolymerization, otherwise called saccharification involves hydration, the addition of water molecules to the glycosidic bond). Then, ln $([S_0]/([S_0] - 324\,v\,t))$ is plotted versus *t* in order to obtain the pseudo-first order rate constant, the slope.

#### 2.2.2 Determination of proportionality constant (β) for the determination of the mass concentration of free un-hydrolyzed starch molecules

The proportionality constant is determined by combining the slope and intercept of the plot of $(\exp(k\,t) - 1)/(\exp(k\,t))^2$ versus $(\exp(k\,t) - 1)/(\exp(k\,t))$ (Eq. (14)). Thus, $\beta$ = Intercept. $[S_0]^3$. Slope. Substitution of $\beta$ into the quadratic equation, Eq. (16), gives after evaluation the two roots, lower root as the mass concentration of the free undigested starch and the sum of $[C](t)$ and $[S_{fr}](t)$ as the higher root.

#### 2.2.3 Determination of proportionality constant (ζ) for the determination of the mass concentration of the substrate involved in complex formation with the enzyme

The proportionality constant is obtained by combining the intercept and slope of the plot of $(\exp(k\,t))^{-1}[P](t)$ versus $[P](t)$ (Eq. (25)). Thus $\zeta$ = Intercept. $[S_0]^2$. Slope. Substitution of $\zeta$ into the quadratic equation, Eq. (26), gives after evaluation the two roots, lower root as the mass concentration of the substrate involved in complex formation with the enzyme, $([C](t))$, and $([S_{fr}](t) + [S_f](t))$ or Eq. (22) as the higher root.

### 2.3 Statistical Analysis

The values obtained are expressed as mean±SD. Each parameter is an average of the values from eight determinations. Standard deviation (SD) was determined using Microsoft Excel. Graph Pad software (www.graphpad.com/quick calcs /*t*-test) was used to carry out unpaired *t*-test for significant difference between the means obtained from two different methods viz: Direct application of $[S_0]$ / $\exp(k\,t)$ and the sum, $[C](t)) + [S_{fr}](t) + [S_f](t)$ for the calculation of the remaining substrate and, between $\Delta[S_0]$/min and $[P]$/min.

## 3. RESULTS AND DISCUSSION

Apart from the recorded velocity of hydrolysis of starch, $v$ (Table 1), other data generated depended completely on the application or fitting of the derived equations to different data that are dependent on $v$. The presence of partially digested starch polymers requires the derivation of quadratic equations, Eq. (16) and Eq. (26). Fitting of Eq. (16) and Eq. (26) to appropriate proportionality constant, $\beta$ and $\zeta$ respectively, and other data including $324vt$ and $\exp(k\,t)$, as the case may be, yields after evaluation the mass concentrations of un-hydrolyzed free starch, $[S_f](t)$ and starch involved in complex formation with the enzyme, $[C](t)$ respectively. Substitution of $[S_f](t)$ and $[C](t)$ into Eq. (29) and Eq. (30) respectively, gives, after calculation, the same result for the mass concentration, $[S_{fr}](t)$ of partially digested substrate for each duration of assay. It should be pointed out that the existence of fragment and $[C](t)$ in particular, may not be in doubt because greater part of the time is spent by the enzyme in the transformation of the substrate in the active site, while given the usual Einstein's relationship, $\tau = l^2/D$ (where $l$, $\tau$, and $D$ are the duration of root mean square distance otherwise called average interparticle distance, average transit, and diffusion coefficient), it could be seen that $\tau$ is very small compared to the time spent in the active site during catalysis [12-14].

In order to determine $\beta$ and $\zeta$, plots of $(\exp(k\,t) - 1)/(\exp(k\,t))^2$ versus $(\exp(k\,t) - 1)/(\exp(k\,t))$ (Fig. 1) and $(\exp(k\,t))^{-1}[P](t)$ versus $[P](t)$ (Fig. 2) were carried out. The coefficients of determination as shown (inset) in Fig. 1 and Fig. 2 were expectedly high (~1).

As Table 1 shows, the values of $v$ are similar. However, the trends in the values of other parameters are of greater concern since they were obtained from assays at different durations. The values of $[C](t)$ (the mass concentration of the substrate involved in complex formation) formed or existing at the moment the assay was terminated at the end of each duration of assay, decreased with time because there may have been increasing time available for the enzyme to catalyze the hydrolysis of the substrate against the backdrop of the fact that it takes much shorter time for effective collision to be made by the enzyme with substrate [12]. In the same vein, there is decreasing trend in the values of $[S_f](t)$. The values of $[S_{fr}](t)$ showed increasing trend with time, but the magnitude of the differences is not high. It is important to realize that the fragments are also substrate for subsequent hydrolysis with time. As such, the concentration ($[S_{fr}](t)$) may decrease with time as it seems to be within 5 min, the highest duration of assay. The duration of most enzyme catalyzed hydrolysis of starch as carried out by investigators [15-18] ranges from 10 min - 24 hr. Within such long duration it is very likely that the magnitude of $[S_f](t)$ would have decreased to very low concentration. The possibility of the existence of fragments cannot be in doubt because of different degree of digestibility of starch from different sources. Sub-fractions, according to the rate of digestion, rapidly digestible starch (RDS), slowly digestible starch (SDS), and resistant starch (RS) have been reported [17].
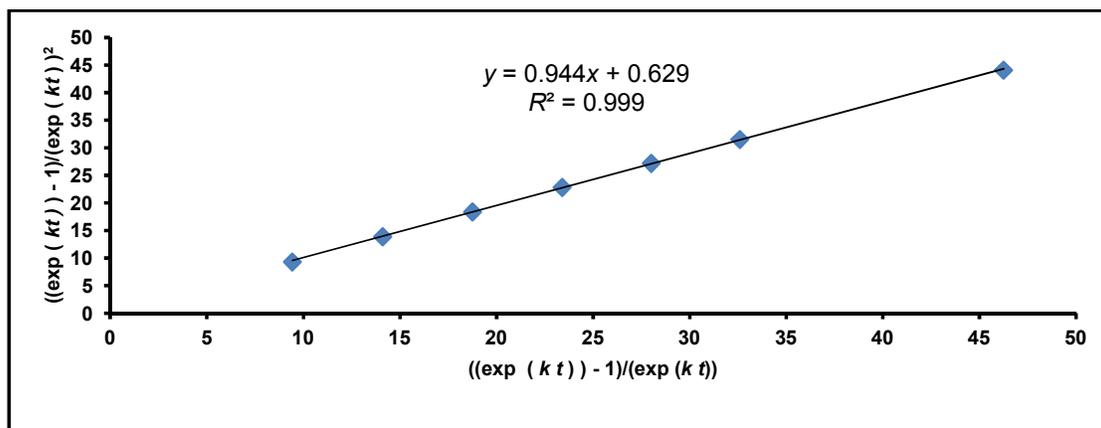


**Fig. 1. A plot for the determination of proportionality constant (β) with which to calculate the mass concentration of undigested substrate**

*The parameters, k and t, are the apparent rate constant and duration of assay respectively. Eight plots were carried out. The mean±SD of β is reported (4.460869±0.651606). This value is used as reported*

**Table 1. Mass concentration of some different components of the reaction mixture at the end of different duration of assay**

| $T$ (min) | 1.0 | 1.5 | 2.0 | 2.5 | 3.0 | 3.5 | 5 |
|---|---|---|---|---|---|---|---|
| $v$ (U/ml) | 515.55±61.02 | 480.33±3.75 | 518.90±18.10 | 588.96±33.69 | 532.87±1.27 | 522.48±15.85 | 553.63±12.53 |
| $[C](t)$ (g/L) | 1.27±0.04 | 0.89±0.00 | 0.61±0.00 | 0.43±0.00 | 0.40±0.40 | 0.36±0.00 | 0.23±0.00 |
| $[S_f](t)$ (g/L) | 1.28±0.04 | 0.84±0.03 | 0.63±0.02 | 0.50±0.01 | 0.42±0.01 | 0.36±0.01 | 0.25±0.00 |
| $[S_{fr}](t)$(g/L) | 17.27±0.04 | 17.99±0.02 | 18.38±0.01 | 18.61±0.00 | 18.63±0.00 | 18.64±0.01 | 18.60±0.02 |
| $[S_0](\text{Exp}kt)^{-1}$(g/L)(i) | 19.81±0.01 | 19.72±0.01 | 19.63±0.01 | 19.54±0.01 | 19.45±0.02 | 19.35±0.02 | 19.09±0.03 |
| $[S_0](\text{Exp } k\, t)^{-1}$ (g/L) (d) | 19.81±0.01 | 19.67±0.01 | 19.63±0.01 | 19.54±0.01 | 19.45±0.02 | 19.36±0.02 | 19.09±0.03 |
| Exp $(k\,t)$/exp(-3) | 1009.37±0.28 | 1017.02±0.43 | 1018.84±0.57 | 1023.60±0.72 | 1028.39±0.87 | 1033.27±1.02 | 1047.76±1.48 |
| $\Delta[S_0]$(mg/L.min) | 185.73±5.59 | 185.33±5.51 | 184.92±5.54 | 184.47±5.52 | 184.03±10.91 | 184.01±5.47 | 182.33±9.03 |
| $[P]$ (mg/L.min) | 176.32±20.87 | 164.57±0.57 | 183.90±6.19 | 202.94±12.86 | 182.25±0.46 | 178.69±5.42 | 189.34±12.17 |

*The parameters, v, [C](t), [S_f](t), [S_{fr}](t), and [S_0] are the velocity of enzyme catalyzed hydrolysis of the substrate, starch, mass concentration of substrate involved in complex formation with the enzyme, mass concentration of free un-hydrolyzed substrate, mass concentration of the fragments of the polysaccharide, and total mass concentration of the substrate; (i) and (d) represent determination of the remaining substrate by taking the sum of [C](t), [S_f](t), and [S_{fr}](t), and directly using [S_0]/exp (k t) respectively. The values of (i) and (d) were expected to be equal. The values of exp (k t) and other parameters are approximated to 2 decimal places. The total amount of ∆[S_0] per duration of assay ranges from 185.73 – 911.65 mg/L which corresponds to duration of assay ranging from 1 – 5 min.*
*The corresponding values of [P] per duration of assay range from 176.32 – 946.7 mg/L*
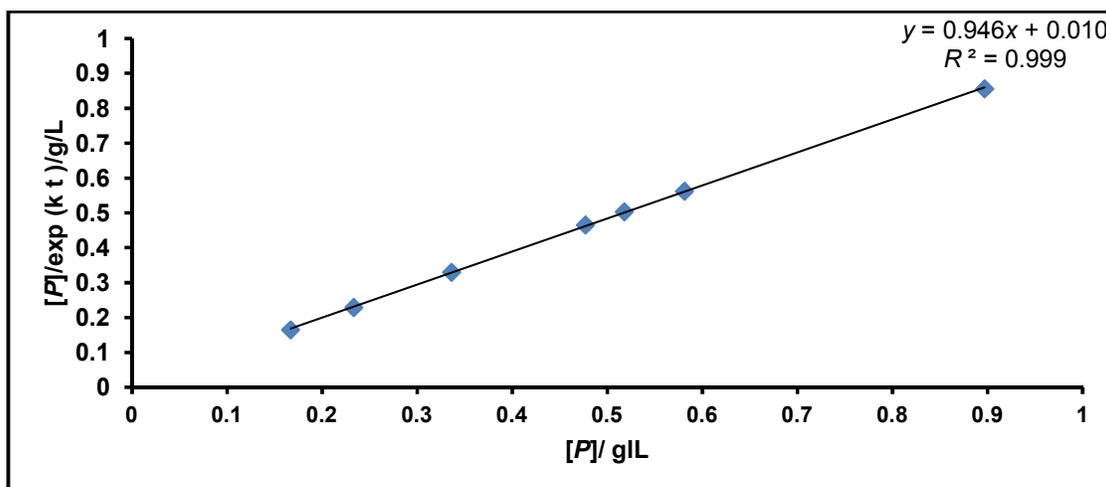
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**Fig. 2. A plot for the determination of proportionality constant with which to calculate the mass concentration of substrate involved in complex formation with the enzyme**

*The parameters, k, t, and [P] are the apparent rate constant, duration of assay and mass concentration of the product respectively. Eight plots were carried out. The mean±SD of $\zeta$ is reported (3.9269±0.1959) g/L. This value was used as reported*

The important issue is that there should be mass conservation at the end of enzyme catalyzed reaction. Thus $[S_0]$ /ext $k$ $t$ + [P] should be = $[S_0]$. The remainder, $[S_0]$ /ext $k$ $t$ could be expressed as [C] ($t$) + $[S_{fr}]$ ($t$) + $[S_f]$ ($t$); this, therefore, represents one of two ways of calculating the remainder. According to Schnell and Maini [4], Lim showed that "the expression $[\hat{S}]$ = [S] + [C] is not the substrate mass balance, it is only a definition for the sum of *C* and *S*. The correct conservation law is: $[\hat{S}]$ = [S] + [C] = $[S_0]$ - [P]". The parameter, $[\hat{S}]$ stands for $[S_{FRC}]$ ($t$) (=[C]($t$) + $[S_{fr}]$($t$)) in this research. While [S] represents the free substrate in Lim's formalism, cognizance is taken of free or undigested full-length starch molecules as well as partially digested starch or fragments in this research. As shown in Table 1, the concentrations of the remaining substrate calculated using two different approaches, $[S_0]$ /exp $k$ $t$ (or the equivalent, $[S_0]$ – [P]) and the new approach (Eq. (31)) in this research were not statistically different ($P$ > 0.05). The difference between the change in the concentration of the substrate per unit time, $\Delta[S_0]$/min, and the corresponding product formed per unit time, [P]/min, was not statistically different ($P$ > 0.05). There can be no deviation from the issue of mass conservation principle and as a consequence there is no way, arithmetic operation such as summation or rather, addition and subtraction can be excluded from the determination of reaction mixture component after the termination of the catalyzed reaction contrary to the view by Lim.

According to Kasprzak et al. [17], it has been found that the consumption of easily digestible food causes a rapid rise in blood glucose and substantial fluctuation of hormones in healthy individuals let alone in some diabetic patients. It is in the light of this that it this research has become useful because one can predict the time it would take for the digestion of starch-rich food materials and different composition of a given reaction mixture and guide any informed decision on the amount of feed/food to be presented to animals/human beings, the athletes and diabetics in particular. This implies a form of regulation of the amount of carbohydrate rich diet to individuals with different degree of tolerance to blood glucose. Hence moderate to high resistant starch may present higher concentration of undigested starch and fragments in particular and much less concentration of reducing sugar than much less resistant starches. This is in line with the result in this research where the equation of mass conservation clearly presents very high concentration of fragments compared to other components of the digestion medium.

It should be realized that mass conservation concept has been applied in different ways for solving problem of product and by-product distribution or flux using different models, three-pole model for instant [19]. Mass conservation study coupled with three-pole model, has application in predicting the peritoneal absorption of icodextrin [19]. The products of digestion and different fragments of varying molar mass which

make-up various fraction influence subsequent enzymatic activity, and in particular, the rate of absorption. This shows the usefulness of mass conservation concept ($\Delta m_1 + \Delta m_2 + \Delta m_3 + \Delta m_4 + \Delta m_5 = 0$, where $m$ is the mass of the reactant [19]). This is in line with the age-long universal principle otherwise known as Lavoisier principle, that matter is neither created nor destroyed in chemical reaction and by extension biochemical reaction except there is nuclear reaction.

According to Bérangère et al. [20] the mass balancing theory principles were developed in the late 70s and it has "practical applications mostly in the field of numerical procedures to solve a system for unknown flows or to calculate maximum likelihood estimators in case of over-determined systems" [20]. Pioneering works in this field were carried out by many researchers including Minkevich [21], Roels [22] etc. Thus, it is recognized that biological reaction stoichiometry – defined as a chemical reaction which provides basic information about the nature and the quantities of chemical species consumed and produced – is, as in this research, mandatory for analyzing biological process [20].

While the paper by Beard et al. [23] is not fully understood, being a highly specialized field or subject matter, the mention of stoichiometry which involves mole ratio, makes substrate mass conservation relevant, though a departure from flux balance analysis – changes in the quantities of matter in a reaction mixture for instance – to energy balance analysis has been the case recently. Therefore, mass-balance investigated in this research and energy balance is an important biochemical concept. The concept of mass conservation has application in the reduction of ordinary differential equations into two simpler equations for kinetic studies [24].

## 4. CONCLUSION

In conclusion, various algebraic equations were successfully derived for the determination of various components of the hydrolyzate at the end of the duration of assay. The results showed that there were different components of the reaction mixtures viz: free un-hydrolyzed substrate, partially digested substrate (substrate fragments), enzyme–substrate complex apart from the expected reducing sugar at the end of assay. The substrate mass conservation law was observed because the sum of the components was very similar to the total concentration of the substrate before the commencement of assay.

Further research may entail establishing a link between rate constant for the formation of reducing sugar and pseudo-rate constant for the disappearance of the substrate from the bulk medium.

## COMPETING INTERESTS

Author has declared that no competing interests exist.

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