Original Research Article

Enzymatic Kinetic issues and Controversies surrounding Gibbs free energy of Activation and Arrhenius activation energy.

ABSTRACT

Background: The equation of the difference between reverse and forward Gibbs free energy of activation ($\Delta\Delta G_{\mathrm{ES}^{\#}}$) reflects Michaelis-Menten constant (K_{M}) in both directions; this may not be applicable to all enzymes even if the reverse reaction is speculatively Michaelian. Arrhenius activation energy, E_{a} and $\left(\left(E_{\mathrm{a}} - \Delta G_{\mathrm{ES}^{\#}}\right)/R\,T\right)$ are considered = $\Delta G_{\mathrm{ES}^{\#}}$ and K_{M} respectively. The equations are considered unlikely.

Objectives: The objectives of this research are: 1) To derive what is considered as an appropriate equation for the determination of the difference in $\Delta G_{\mathrm{ES}^{\#}}$ between the reverse and forward directions, 2) calculate the difference between the reverse and total forward $\Delta G_{\mathrm{ES}^{\#}}$, and 3) show reasons why $E_{\mathrm{a}} \neq \Delta G_{\mathrm{ES}^{\#}}$ in all cases.

Methods: A major theoretical research and experimentation using Bernfeld method.

Results and discussion: A dimensionless equilibrium constant K_{ES} is given. Expectedly, the rate constants were higher at higher temperatures and the free energy of activation with salt was < the Arrhenius activation energy, E_{a}; $\Delta\Delta G_{\mathrm{ES}^{\#}}$ ranges between

67 - 68 kJ/mol.

Conclusion: The equations for the calculation of the difference in free energy of activation ($\Delta\Delta G_{\mathrm{ES}^{\#}}$) between the forward and reverse directions and a dimensionless equilibrium constant for the formation of enzyme-substrate (*ES*) were derivable. The large positive value of the $\Delta\Delta G_{\mathrm{ES}^{\#}}$ shows that the forward reaction is not substantially spontaneous; this is due perhaps, to the nature of substrate. The equality of Arrhenius activation energy (E_a) and $\Delta G_{\mathrm{ES}^{\#}}$ may not be ruled out completely but it must not always be the case; the presence of additive like salt can increase the magnitude of E_a well above the values of the $\Delta G_{\mathrm{ES}^{\#}}$. A dimensionless equilibrium constant for the net yield of *ES* seems to be a better alternative than K_M. The E_a unlike $\Delta G_{\mathrm{ES}^{\#}}$ requires at least two different temperatures for its calculation.

Keywords: Aspergillus oryzea alpha-amylase; reverse rate constant; pre- and steady- state rate constant for the formation of enzyme-substrate complex; Gibbs free energy of activation and Arrhenius activation energy; dimensionless equilibrium constant.

1. INTRODUCTION

The word thermodynamic is from Greek words for “heat” and “power” and it is the study of heat, work, energy and the changes they produce in the states of a system [1]. It is also defined as the study of the relation of temperature to the macroscopic properties of matter [1]. It is not just temperature, but other elements of thermodynamics that are of concern. Hence, there has been great interest in the thermodynamics of biological processes

[2]. While thermodynamic activation parameter of interest had been on enthalpy of activation, there had been a shift in recent time towards free energy of activation [2].

According to low *et al*. [3] the Gibbs free energy of activation ($\Delta G_{\mathrm{ES}^{\#}}$) and the enthalpy of activation ($\Delta H^{\#}$) for the formation of the enzyme-substrate complex, *ES* (where # means activation; ΔG and ΔH are the free energy and enthalpy changes respectively) have been used as indexes of catalytic efficiency. This is despite the reliance by biologist on E_a (activation energy) as index of catalytic efficiency. This is as a result of the fact that while E_a could be a good index for catalytic efficiency, in terms of the enzymes' ability to reduce the energy barrier, it is still contingent upon uniformity or similarity in the entropy of activation for all conditions for the same or different enzyme [2]. Whatever be the case, an appropriate interpretation of E_a and $\Delta G_{\mathrm{ES}^{\#}}$ is needed in order to generate relevant data; this is against the backdrop of the claim that E_a and $\Delta G_{\mathrm{ES}^{\#}}$ are equal [4]. Appropriate data (even if based on improvisation) enables proper characterisation, in terms of changes in conformational stability of biomolecules due to temperature changes [5] and the effect of osmolyte [7]. This is for the purpose of application in various industrial establishments [8], the pharmaceutical and food industries in particular; all industries may be important but the most important is food industry whose role is food preservation in raw and processed form. The formulation of infant food and preparation of all kinds of balanced diet without consideration for thermodynamic imperatives for storage leads to wastage. However, there seems to be either a misinterpretation resulting in controversy surrounding the difference between activation energy of the forward and reverse reaction in the scheme

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P \qquad (1a)$$

Where k_1, k_2, k_{-1}, E, S, ES, and P are 2nd order rate constant, rate constant for the formation of product, reverse rate constant, enzyme, substrate, enzyme-substrate complex, and product respectively. The mathematical model presents the Michaelis-Menten constant K_M as one applicable in the forward and reverse direction [4]. There is need to bring into relevance appropriate enzymatic kinetic constants based on appropriate equations. It seems kinetic issues at steady-state in literature may run into conflict if rate constant (or the turnover number) is generally used regardless of reaction systems' conditions, either pre-steady-state or steady-state. Another major controversy lies in the fact that E_a is equated with $\Delta G_{ES^{\#}}$. There should be a way out of the controversial issues. Therefore, the objectives of this research are: 1) To derive what is considered as an appropriate equation for the determination of the difference in $\Delta G_{ES^{\#}}$ between the reverse and forward directions, 2) calculate the difference between the reverse and forward Gibbs free energy of activation, and 3) show reasons why $E_a \neq \Delta G_{ES^{\#}}$ in all cases.

2. Theory

There are issues with the equations for the determination of Arrhenius activation energy and free energy of activation when such equations are transformed into linear form for graphical purpose that is often ignored in literature. The free energy of activation enunciated in literature [4] has minor issue. The equation of the free energy of activation is seen to be the same as the Arrhenius equation of activation energy. The mathematical form or equation seems useful but their claims need to be examined. A well known equation found in literature [4] is

$$v = -\frac{\mathrm{d}[S]}{\mathrm{d}t} = \frac{k_B T}{h}[S]\exp\left(\frac{-E_a}{RT}\right) \quad (1b)$$

Where k_B, T, R, E_a, and h are Boltzmann constant, thermodynamic temperature, universal gas constant, activation energy, and Planck constant respectively; v, $[S]$, and t are the velocity of amylolysis, concentration of substrate, and duration of assay. As stated in another manuscript addressing different issue, what seems to be ignored in literature is that v has to be the mass concentration of substrate converted to product per mL of enzyme solution per min, and, in line with mass conservation law, it is also the mass concentration of maltose (if it is assumed that maltose is the only reducing sugar); thus division of Eq. (1b) by molar mass of maltose becomes very imperative. Thus, $[S]/M_p$ (where M_p is molar mass of maltose) is equivalent to the molar concentration of bonded maltose molecules in a given mass concentration of the substrate. It is very likely however, that a plot of v versus $[S]$, all in molar units should have a value different from the plot of In ($[S_0]/[S]_{(t)}$) versus t ($[S_0]$ and $[S]_{(t)}$ are respectively, substrate concentration in time t = 0 and in time, t). The most important issue is that, the pseudo-first order rate constant (k) is $= \frac{k_B T}{h} \exp\left(\frac{-E_a}{R\,T}\right)$. From the latter, the value of E_a can be calculated. Yet the rearrangement of the equation gives

$$E_a = R\,T\,\mathrm{In}\frac{k_B T}{h\,k} \qquad (1c)$$

The issue regarding Eq. (1c) is that it is the same as the direct equation for the calculation of free energy of activation. This, if it is not a mistake, seems to suggest that, $\Delta G_{ES^{\#}} = E_a$. This is a key controversial issue of this section that will be addressed shortly. At this juncture one may need to state that $\Delta^{\#}G^{0}$, the standard format is avoided for the sake of simplicity. The free energy change associated with $ES^{\#}$, under experimental condition of subsaturating substrate concentration [4] (*i.e*. substrate concentration at which the maximum velocity cannot be achieved even if such concentration is > the Michaelis – Menten constant, K_M) [4, 9] is:

$$\Delta G_{\mathrm{ES}^{\#}} = -R\,T\,\mathrm{I\,n}\frac{k_{\mathrm{cat}}}{K_{\mathrm{S}}} + R\,T\,\mathrm{I\,n}\frac{k_{\mathrm{B}}T}{h} = 0 \quad (2)$$

Where, k_{cat} and K_{s} are turnover number (or rate of formation of product) and enzyme-substrate complex dissociation constant and the parameter $\Delta G_{\mathrm{ES}^{\#}}$ is the free energy of activation. In order to reveal important issue in Eq. (2), it needs to be rearranged to give

$$\Delta G_{\mathrm{ES}^{\#}} = R\,T\left(\mathrm{I\,n}K_{\mathrm{S}} + \mathrm{I\,n}\frac{k_{\mathrm{B}}T}{h\,k_{\mathrm{cat}}}\right) \quad (3)$$

The issue in Eq. (3) is that despite the fact that K_{S} is given as k_{-1}/k_1 (where k_{-1} and k_1 are reverse rate constant for the process $ES \rightarrow E + S$, and 2nd order rate constant for the formation of *ES*) in which its unit is L/mol/min (though it can be in L/g/min), the impression seem to be that it is dimensionless. If not, Eq. (3) cannot be valid because $\exp\left(\left(\Delta G_{\mathrm{ES}^{\#}}/RT\right) - \mathrm{I\,n}k\mathrm{B}T\hbar\, k\mathrm{cat} \neq K_{\mathrm{S}}\right.$ whose unit is either L/mol/min or L/g/min.

If two different substrates or enzymes are compared by applying Haldane relationship [4] from the perspective of equilibrium constant given as $K_{\mathrm{eq}} = \frac{(k_{\mathrm{cat}}/K_{\mathrm{S}})_1}{(k_{\mathrm{cat}}/K_{\mathrm{S}})_2}$, there may be no issue of dimensional inconsistency. However, the original form of Haldane relationship for subsaturating [*S*] is given as:

$$K_{\mathrm{eq}} = \frac{(k_{\mathrm{cat}}/K_{\mathrm{S}})_{\mathrm{f}}}{(k_{\mathrm{cat}}/K_{\mathrm{S}})_{\mathrm{r}}} \quad (4a)$$

Where, the subscripts, r and f, denote reverse and forward directions respectively. The equation for the saturating [*S*] is:

$$K_{\mathrm{eq}} = \frac{(k_{\mathrm{cat}}/K_{\mathrm{M}})_{\mathrm{f}}}{(k_{\mathrm{cat}}/K_{\mathrm{M}})_{\mathrm{r}}} \quad (4b)$$

Copeland [4] sees Eq. (4a) and Eq. (4b), as the Haldane relationship, which provides a useful measure of the directionality of an enzymatic reaction under a specific set of solution conditions. Both equations may be rewritten as

$$K_{\mathrm{eq}} = \frac{[P]_{\mathrm{eq}}}{[S]_{\mathrm{eq}}} \tag{4c}$$

However, the author [4] refers to enzymes that can catalyse the backward reaction. One may add that there is one ultimate direction, either product-free enzyme direction ($k_2 > k_{-1}$) or substrate-free enzyme direction ($k_{-1} > k_2$). The important concern is that while $v = k_2 [E] [S] /K_S$ and $v_1 = k_1 [E][S]$, the velocity of hydrolysis and the velocity of formation of *ES* respectively, it is not certain if $v_r = k_r[E][P]$ (where k_r is also a 2nd order rate constant for the reverse (r) process, $EP \leftarrow E + P$) is practicable for the same reaction system. In either direction, the ratio k_{cat} /K_M can be related to the free energy difference between the free reactants (*E* and *S*, in the forward direction) and the transition state complex ($ES^{\ddagger}$). If the free energy of the reactant state is normalised to zero, the free energy difference is defined by [4, 9]:

$$\Delta G_{\mathrm{f}}^{\#} = -R\,T\,\mathrm{In}\left(\frac{k_{\mathrm{cat}}}{K_{\mathrm{M}}}\right)_{\mathrm{f}} + R\,T\,\mathrm{In}\frac{k_{\mathrm{B}}T}{h} \tag{5}$$

Once again, there is need to recall that k_{cat} refers to maximum molar concentration of maltose, the product, divide by $[E_0]$ in molar unit. Reasonably, K_M the mass concentration of substrate at half maximum velocity of amylolysis should be equivalent to the molar concentration of maltose yet to be released from the glycosidic bond of the polysaccharide.

But both Eq. (4a) and Eq. (4b) are dimensionless. Nonetheless the controversial issue in contention is that, according to Copeland [4],

$$E_{\mathrm{a}} = \Delta G_{\mathrm{ES}^{\#}} = -R\,T\,\mathrm{In}\left(\frac{k_{\mathrm{cat}}}{K_{\mathrm{M}}}\right)_{\mathrm{f}} + R\,T\,\mathrm{In}\left(\frac{k_{\mathrm{B}}T}{h}\right) \tag{6a}$$

It is controversial because both apparent thermodynamic (ΔG^0) and free energy of activation ($\Delta G_{ES^\#}$) have the same unit. Free energy = RT In X where X must be dimensionless. Now, $\Delta G^0 = -RT$ In K_M (or $-RT$ In K_S) where R, T, K_M and K_S are gas constant, thermodynamic temperature, Michaelis-Menten constant and enzyme-substrate complex dissociation constant respectively; $\Delta G_{ES^\#} = RT$ In ($k_B T/hk_x$) where k_x is any kind of 1st order rate constant. One can see that $k_B T/hk_x$ unlike K_M (or K_S) is dimensionless and if so how can both ΔG^0 and $\Delta G_{ES^\#}$ possess the same unit? This is clearly another controversial issue.

Before proceeding further, there is need to justify Eq. (6a). It is known that $\Delta G_{ES^\#} = \Delta H_f^\# - T\Delta S_f^\#$ and since $\Delta H_f^\# = E_a - RT$, it means that $-RT - T\Delta S_f^\#$ should be equal to zero $\left(RT = -T\Delta S_f^\#\right)$. The implication is that $\Delta S_f^\#$ must always possess a negative value. Rearrangement of Eq. (6a) gives:

$$E_a = \Delta G_{ES^\#} = R\,T\,\mathrm{In}(K_M)_f + R\,T\,\mathrm{In}\frac{k_B T}{h\,k_{cat}} \tag{6b}$$

Recall that the Gibbs free energy of activation for the formation of product, P is given as: $\Delta G_P^\# = RT\,\mathrm{In}\frac{k_B T}{h\,k_{cat}}$ but $\mathrm{In}\left(\left(E_a - R\,T\,\mathrm{In}\frac{k_B T}{h\,k_{cat}}\right)/R\,T\right)$ derived from Eq. (6b) $\neq (K_M)_f$. This is another controversial issue that needs to be re-examined but before then,

$$\Delta G_{ES^\#} = E_a - RT - T\Delta S_f^\# \tag{7a}$$

The magnitude of Gibbs free energy of activation as against E_a is seen to be the true energy barrier, and consequently, Gibbs free energy of activation unlike E_a can be used as a quantitative index of catalytic efficiency [3]. This is despite the view by Copeland [4] that the over-all activation energy E_a (this can be called Arrhenius activation energy) is composed of ΔG_{ES} and $\Delta G_{k_{cat}}$; the term $\Delta G_{k_{cat}}$ (this can be called Gibbs activation energy) is the amount of energy that must be expended to reach the transition state while ΔG_{ES} is the net energy gain

that results from the realisation of *ES* binding energy gain [4]. The questions are: Does $\Delta G_{\mathrm{ES}^{\#}}$ possess exclusive rate constant (or turn-over number) given as part of Eyring equation and is $E_a = \Delta H^{\#} + R\,T$ no longer relevant?

Meanwhile, the free energy of reaction is given as $\Delta G = -RT \ln([P]/[S])$ [4] $\equiv -RT \ln(v_2\, t\, M_p/[S])$ where v and t are the velocity of catalysis and duration of assay respectively. This goes to show that $\exp(-\Delta G/RT) = K_x$ must be a dimensionless equilibrium constant. There is need therefore, to restate Eq. 6b after some derivations. Meanwhile a proposed equation is $k_1 = (k_{-1} + k_2)\, M_p/K_M$ (in a submitted manuscript) with the understanding that any [*S*] including K_M (or K_S) is equivalent to a number of moles of maltose = $[S]/M_p$ and K_M/M_p (or K_S/M_p) in a bonded state. Meanwhile, the velocity (v_1) of formation of *ES* is given as $v_1 = v_{-1} + v_2$ (or $(k_{-1} + k_2)[ES]$) where v_{-1} is the velocity of dissociation of *ES* into *E* and *S*. However, the equation is applicable to steady state condition such that the rate of breakdown of *ES* ($-\partial[ES]/\partial t = (k_{-1} + k_2)[ES]$) is equal to the rate of its formation ($\partial [ES]/\partial t = k_1 [E][S] = (k_{-1} + k2_{[ES]})$ [4]. This is strangely unlike the report that at steady state $v = \partial$ [P]/∂t (https://en.wikipedia.org). The implication is that both the formation and breakdown of *ES* exercise 1st order rate constant ($v_1/[ES] = k_{-1} + k_2$). But it is better if the 1st order rate constant is determined for each substrate during pre-steady-state. Meanwhile according to [10], the net rate of formation of *ES* is given as:

$$\Delta v_1 = \frac{\partial [ES]}{\partial t} = k_1([E_0] - [ES])[S] - (k_{-1} + k_2)[ES] \tag{7b}$$

The equation $v_1 = k_1\,[E]_{(t)}\,[S]_{(t)}$ can be transformed into

$$v_1/[ES] = \frac{k_{-1}}{K_S}\left(\frac{[E_0]}{[ES]} - 1\right)([S_T] - 342\,tv) = k_{es} \tag{7c}$$

A double reciprocal plot of k_{es} versus [*S*] gives an intercept whose reciprocal gives the maximum k_{ES} as the pre-steady-state 1st order rate constant for the formation of *ES* in a way similar to the steady-state situation described above. However, a mathematical argument may challenge Eq. (7b) and Eq. (7c); ∂ [*ES*]/∂ *t* ought to be directly proportional to [E_0] and [S_0]. The [*ES*] increases as the free enzyme concentration, [E_f] decreases with increasing [S_0] within the same duration of assay. Therefore, [*ES*] α 1/[E_f] and α [S_0]. Nonetheless, ∂[*ES*]/∂ *t* can be jointly and directly proportional to [E_f] and [S_0] (α [E_f] [S_0]) as long as [E_f][S_0] as well as [E_f][S_f] is increasing with increasing [S_0]. It must be made clear that $k_1([E_0]-[ES])[S_f]$ may be < $(k_{-1}+k_2)[ES]$. However, $k_1([E_0]-[ES])[S_0]$ may be >$(k_{-1}+k_2)[ES]$. What needs to be considered is that during a pre-steady-state condition maximum velocity is not attained (absence of zero order); this implies that the rate constant is < v_{max} /[E_0]. This is obvious given that k_{2red} = *v*/[E_0] = k_2[S_0]/ ([S_0] + K_M) (k_{2red} is the reduced rate constant); a similar equation may be applicable to k_{-1}. Unlike the former, the later holds whether or not [S_0] > K_M (or K_S) as long as k_1 = (k_{-1} + k_2) /K_M (or k_{-1}/K_S). The equations, Eq. (7b) and Eq. (7c) are respectively relevant to the schemes, *E* + *S* ⇌ *ES* ⇌ $ES^{\#}$ and *E* + *S* ⇌ *ES* whenever each occurs separately in any part of the reaction mixture. Thus before steady-state, rate constants may be $k_2\frac{[S_0]}{[S_0]+K_M}$ (or *v*/[E_0]) and v_{-1}/[E_0]. Thus, Eq. (7b) is restated as

$$\Delta v_1 = \frac{\partial [ES]}{\partial t} = k_1([E_0]-[ES])[S] - (v_{-1}+v)\frac{1}{[E_0]}[ES] \qquad (7d)$$

Meanwhile v_{-1} = k_{-1}[*ES*] and *v* = k_2 [*ES*] and substitution into Eq. (7d) gives

$$\Delta v_1 = k_1([E_0]-[ES])[S] - (k_{-1}+k_2)\frac{[ES]^2}{[E_0]} \qquad (7e)$$

Similar to Eq. (7c), division of Eq. (7e) by [*ES*] gives a 1st order rate constant such as

$$k_{\mathrm{ES}} = k_1\left(\frac{[E_0]}{[ES]} - 1\right)[S] - (k_{-1} + k_2)\frac{[ES]}{[E_0]} \tag{7f}$$

Thus a dimensionless equilibrium constant for the net yield of *ES* is: $K_{\mathrm{ES}} = [ES]k_{-1}/k_{\mathrm{ES}}[E_0]$.

In the light of what Copeman [4] called Haldane relationships, Eq. (4a) and Eq. (4b) there is need to introduce Eq. (7c) to account for the sum of two free energies of activation in the two forward directions leading to activated complex formation $E + S \rightleftharpoons ES \rightleftharpoons ES^{\#}$ and for the formation of products, $ES^{\#} \rightarrow E + P$.

$$\Delta G^{\#} = R\,T\,\mathrm{In}\left(\frac{k_{\mathrm{B}}T}{h\ k_{\mathrm{ES}}}\right)_{\mathrm{f}} + R\,T\,\mathrm{In}\frac{k_{\mathrm{B}}T}{h\,k_{\mathrm{cat}}} \tag{8}$$

Equation (8) presents no dimensional issue. One should not lose sight of the fact that deactivation can lead ultimately to dissociation into *E* and *S*. Thus this can result to the difference in free energy of activation between the reverse direction ($ES \rightarrow E + S$) and the forward direction ($E + S \rightarrow ES$ (or $ES^{\#}$)) *i.e.* $\Delta\Delta G_{\mathrm{ES}^{\#}} = RT\,\mathrm{In}\frac{[ES]k_{-1}}{[E_0]k_{\mathrm{ES}}}$. Expansion gives:

$$RT\,\mathrm{In}\frac{[ES]k_{-1}}{[E_0]k_{\mathrm{ES}}} = R\,T\,\mathrm{In}\,K_{\mathrm{ES}} = \Delta G^0_{\mathrm{ES}} \tag{9}$$

Where $K_{\mathrm{ES}}\left(i.e.\frac{[ES]k_{-1}}{[E_0]k_{\mathrm{ES}}}\right)$ may be seen as a dimensionless equilibrium constant; ΔG^0_{ES} and K_{ES} (a dimensionless parameter) are the Gibbs free energy of *ES* formation and equilibrium constant respectively.

However, from the relationship between pseudo-first order rate constant and activation energy stated above, the equation of activation energy is given as shown in Eq. (1b). What seem to be ignored is exposited as follows. Combining Eq. (6a) and Eq. (1b) gives

$$R\,T\,\mathrm{In}\left(\frac{k_{\mathrm{B}}T}{h\,k}\right) = -R\,T\,\mathrm{In}\left(\frac{k_{\mathrm{cat}}}{K_{\mathrm{M}}}\right)_{\mathrm{f}} + R\,T\,\mathrm{In}\frac{k_{\mathrm{B}}T}{h} \tag{10a}$$

Then after expansion of Eq. (10a) and elimination of common factors one obtains

$$\mathrm{I\,n}\,\frac{1}{k} + \mathrm{I\,n}\,\frac{1}{K_\mathrm{M}} = -\mathrm{I\,n}\,k_\mathrm{cat} \tag{10b}$$

Simplification gives

$$kK_\mathrm{M} = k_\mathrm{cat} \tag{11a}$$

$$K_\mathrm{M} = \frac{k_\mathrm{cat}}{k} \tag{11b}$$

A serious examination of Eq. (11a) should reveal that it is for a dimensionless parameter, although *ab initio*, K_S or K_M is in mol/L or generally, g/L. However, the unit of MM constant is well known. Therefore, the concept of ideal state may be introduced as was the case in literature [11]. Thus Eq. (11b) can be restated as $K_\mathrm{M} = \frac{k_2}{k} K_\mathrm{M}^0$, where K_M^0 is the hypothetical (the reference state) MM constant equal to 1 g/L. Another issue with the modified form of Eq. (11b) restated as Eq. (11c) below is that any calculation should yield very high magnitude of MM constant because *k* is often « 1 but > 0.

$$K_\mathrm{M} = \frac{k_\mathrm{cat}}{k} K_\mathrm{M}^0 \tag{11c}$$

It would, therefore, appear that Eq. (5) and Eq. (6a/6b) are dimensionally inconsistent. The task is to adopt an alternative equation to Eq. (4b) which seems unusual. An equation given below could be most appropriate considering the definitions of k_{cat} and k_{-1}.

$$\Delta\Delta G_{\mathrm{ES}^\#} = -R\,T\,\mathrm{I\,n}\,\frac{(k_\mathrm{cat})_\mathrm{f}}{(k_{-1})_\mathrm{r}} \tag{12a}$$

The determination of k_{-1} is briefly described in method's subsection. However, Eq. (12a) excludes the scheme or process, $E + S \rightleftharpoons ES \rightleftharpoons ES^\#$ where k_{ES} is applicable; its inclusion means that Eq. (12a) can be written as

$$\Delta\Delta G_{\mathrm{ES}^\#} = R\,T\,\mathrm{I\,n}\,\frac{k_\mathrm{B}T[ES](k_{-1})_\mathrm{r}}{[E_0]h(k_\mathrm{ES})_\mathrm{f}(k_\mathrm{cat})_\mathrm{f}} \tag{12b}$$

Equation (12b) represents the difference in free energy between the forward and backward reactions for the enzymes which cannot catalyse the process, *EP* ← *E* + *P*.

The approach by Buurma *et al* [11], the concept of ideal reference state is adopted but not with any presumption of validity because $K_{\mathrm{M}}/K_{\mathrm{M}}^{0}$ does not amount to an experimental dimensionless equilibrium constant. Hence, Eq. (5) can be restated as:

$$\Delta G_{\mathrm{f}}^{\#} = -R\,T\,\mathrm{In}\left(\frac{k_{\mathrm{cat}}}{K_{\mathrm{M}}/K_{\mathrm{M}}^{0}}\right)_{\mathrm{f}} + R\,T\mathrm{In}\frac{k_{\mathrm{B}}T}{h} \qquad (13a)$$

Equation (13a) remains speculative. Otherwise Eq. (8) may be more appropriate. The general equation of the free energy of activation is:

$$\Delta G_{\mathrm{f}}^{\#} = R\,T\mathrm{In}\frac{k_{\mathrm{B}}T}{hk_{\mathrm{x}}} \qquad (13b)$$

Where, k_{x} may be either k_{cat} or k. The linearised form of Eq. (13b) is given as

$$\mathrm{In}\frac{k_{\mathrm{x}}}{T} = \mathrm{In}\frac{k_{\mathrm{B}}}{h} - \frac{\Delta G_{\mathrm{f}}^{\#}}{R\,T} \qquad (13c)$$

Equation (13c) can also be found in literature [IUPAC]. Equation (13c) is simply a rearrangement of Eyring equation given as $G_{\mathrm{ES}^{\#}} = RT\left(\mathrm{In}\left(\frac{k_{\mathrm{B}}T}{h}\right) - \mathrm{In}\,(k_{\mathrm{cat}})\right)$ [4]. On the other hand Arrhenius equation of activation energy as in most standard text books [1] is given as

$$k_{\mathrm{x}} = Ae^{-E_a/RT} \qquad (14a)$$

Where, *A* is a well known pre-exponential (frequency) factor. The author [13] proposes an apparent activation energy given as

$$E_{\mathrm{app}} \equiv R\left(\frac{\partial \mathrm{In}\,k_{\mathrm{x}}}{\partial {}^{1}/_{T}}\right)_{\mathrm{P}} \qquad (14b)$$

Equation (14b) is clearly a slope from the plot of In k_{x} versus 1/*T*.

There is an insinuation that such original equation is suitable for a less precise rate-temperature data and in particular, those covering a narrow temperature range. For the

analysis of more precise rate-temperature data, particularly those covering a wide temperature range, *A* is seen to be proportional to *T* raised to a power *m* (though there is no evidence that *m* is a positive integer), so that the equation is restated as [13]:

$$k_{\mathrm{x}} = A^{!} T^{m} e^{-E_a/RT} \tag{14c}$$

There is no need for an alternative to (Eq. (14a)) because a lot of works on the effect of temperature on enzyme catalysed reactions (14, 15) have been carried out. Meanwhile,

$$\mathrm{I\,n}\frac{k_{(2\mathrm{x})}}{k_{(1\mathrm{x})}} = \frac{E_a}{R}\left(\frac{T_2 - T_1}{T_1 T_2}\right) \tag{15}$$

The results may not always be the same for any pairs of different temperatures. Unlike Eq. (13c) that may not be appropriate, Arrhenius equation has its linear form usually given as

$$\mathrm{I\,n}\, k_x = \mathrm{I\,n}\, A - \frac{E_a}{R\,T} \tag{16a}$$

Equation (16a) can be restated as

$$RT\ \mathrm{I\,n}\ \frac{A}{k_x} = E_a \tag{16b}$$

Unlike Eq. (13b) which can be used directly to calculate $\Delta G_{\mathrm{f}}^{\#}$, Eq. (16b) cannot be used directly to calculate E_{a} because *A* is not known *ab initio* until graphically determined.

In the light of the reservation expressed against original Arrhenius, there is need to relate original equation of free energy of activation to its arithmetic form as follows.

$$\Delta G_{\mathrm{ES}^{\#}} = E_{\mathrm{a}} - R\,T - T\Delta S_{\mathrm{f}}^{\#} = R\,T\ \mathrm{In}\frac{k_{\mathrm{B}} T}{h\,k_x} \tag{17}$$

Rearrangement leads to a similar result found on the website (https://www.en.Wikipedia.org/wiki/Activation_energy). The results are:

$$\mathrm{I\,n}\frac{k_x}{T} = \mathrm{I\,n}\frac{k_{\mathrm{B}}}{h} + 1 + \frac{\Delta S_{\mathrm{f}}^{\#}}{R} - \frac{E_{\mathrm{a}}}{RT} \tag{18}$$

The controversy given Eq. (14a) lies in the second equation of exponential factor given as ((https://www.en.Wikipedia.org/wiki/Activation_energy):

$$A = \frac{k_B T}{h} \exp\left(1 + \frac{\Delta S_f^{\#}}{R}\right) \quad (19)$$

For clarity, it needs to be stated that Eq. (18) can be rearranged to give $\mathrm{In}\, k = \mathrm{In}(k_B T/h) + 1 + \Delta S^{\#}/R - E_a/RT$ which enables the formulation of Eq. (19) because In $A = \mathrm{In}(k_B T/h) + 1 + \Delta S^{\#}/R$ which is part of the former. Clearly, Eq. (19) contains for an intercept, an independent and dependent variable, the temperature and entropy of activation respectively as it ought to be. But this is not in agreement with the original Arrhenius equation (Eq. 14a). It is rather unclear why $\mathrm{In}\frac{k}{T}$ should not be plotted versus 1/*T*. Doing so may yield result different from that expected from the plot of In*k* versus 1/*T*. Furthermore, it should give an intercept, the exponential factor in which $\Delta S^{\#}$ should be constant against the usual for a given temperature range. The former approach Eq. (13a) may speculatively serve for the purpose of comparison as alternative to Eq. (13c) for biological systems whose physiological temperature range differs in line with classifications such as psychrophiles, mesophiles, and thermophiles. This is against the backdrop of what Arcus [16] referred to as assumption often made with respect to the equation $k = \kappa k_B T \exp(-\Delta G^{\#}/RT)/h$ to the effect that $\Delta H^{\#}$ and $\Delta S^{\#}$ are independent of temperature and hence that $\Delta G^{\#}$ varies with temperature according to the Gibbs equation: However, the same authors [16] hold the valid view that a number of investigators have noted deviations from the equation when plotting temperature versus enzyme-catalyzed rates, as to imply a more complex temperature dependence for these systems.

As stated earlier, the Gibbs free energy of activation is always calculated (see far right of Eq. (15)). Otherwise, a plot of $\mathrm{In}(k/T)$ versus 1/*T* (which is unusual) would mean that the slope is equal to $-\Delta G_{ES^{\#}}/R$ (see Eq. (13c)). The same plot can also give the slope as $-\frac{E_a}{RT}$

(Eq. (16)). This represents another controversial outcome. Then the question is where does one go from here? Nonetheless, accepting $\Delta G_{\mathrm{ES}^{\#}}/R$ as slope only leads to a conclusion that sometimes, $\Delta G_{\mathrm{ES}^{\#}} = E_{\mathrm{a}}$ on the condition that $\Delta S^{\#}$ is equal to *R* but opposite in sign but of questionable validity. This is highly controversial considering the fact that, Eq. (16) *ab initio*, clearly specified E_a/R as a slope if In (k_x/T) is plotted versus 1/*T*. Nonetheless the slope remains the same regardless of the form of the equation including $\mathrm{In}(k/T) = (\Delta S^{\#}/R) + \mathrm{In}(k_{\mathrm{B}}/h) - \Delta H^{\#}/RT$. It would appear therefore, that neither the linearisation of Erying equation nor the impression that $\Delta G_{\mathrm{ES}^{\#}}$ is consistently = E_{a} is valid. It is very likely that E_{a} is either > or < $\Delta G_{\mathrm{ES}^{\#}}$. The equality of $\Delta G_{\mathrm{ES}^{\#}}$ and E_{a} may be conditional rather than mathematical in nature.

3. MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals

Porcine pancreatic alpha amylase (PPAA) (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. Calcium chloride was purchased from Lab Tech Chemicals, India. The molar mass of the enzyme is 55 k Da [17].

3.1.2 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.

3.2 Methods

The method reported here is as previously adopted but restated here for quick reference [18]. The enzyme was assayed according to Bernfeld method [19] using raw potato starch whose concentration range was 5-10 g/L. Reducing sugar produced upon hydrolysis of the substrate at room temperature using maltose as standard was determined at 540 nm with extinction coefficient equal to ~ 181 L/mol.cm. The duration of assay was 5 min. 500 μg/mL of porcine pancreatic alpha-amylase was prepared in Tris HCl buffer at *p*H = 7.4 as described elsewhere [18]. An assay of the enzyme was done with and without calcium chloride in a total reaction mixture of 3 mL composed of 1 mL of substrate (raw potato starch), 1 mL of enzyme, 0.5 mL of calcium chloride and 0.5 mL of distilled (or 1 mL of distilled water where calcium chloride is not included in the reaction mixture). Assay was conducted at 310.15 K in an improvised water-bath. The primary kinetic parameters, K_M and v_{max} were extrapolated from double reciprocal plot of Lineweaver-Burk [20].The duration of the formation of *ES* is given as [submitted manuscript]

$$k\,t = \mathrm{I\,n}\frac{1}{1-\frac{[E_T]M_{alt}}{[S_0]}\mathrm{I\,n}\frac{[E_T]}{[E_T]-[ES]}} \tag{20}$$

Where, *k* and *t* (this « 1 s) are the pseudo-first order rate constant and the duration of *ES* formation respectively. A plot of the left hand side (LHS) versus *k* gives a slope = *t*; *k* is determined according to the equation [submitted manuscript]:

$$k = M_{\text{alt}}\left(\frac{v_{\max} \pm \sqrt[2]{v_{\max}^2 - 4\,S_{\text{lope}-1}[\text{S}_0]}}{2[S_0]}\right) \quad (21)$$

The sum, $k_{-1} + k_{\text{cat}}$, is determined according to the equation [submitted manuscript]:

$$\text{I n}\frac{[E_0]}{[E_0]-[ES]} = \frac{(k_{-1}+k_{\text{cat}})[S_0]}{K_{\text{M}}k}(1 - e^{-k\,t}) \quad (22)$$

The product of slope and K_{M} gives $k_{-1} + k_{\text{cat}}$. The 2nd order rate constant, k_1 for the formation of *ES* is given as [submitted manuscript]:

$$\text{I n}\frac{[E_0]}{[E_0]-[ES]} = \frac{k_1[S_0]}{k\,M_{\text{alt}}}(1 - e^{-k\,t}) \quad (23)$$

3.3 Statistical analysis

The values of the velocities of hydrolysis of starch are expressed as mean ± SD; sample size, *n*, is equal to 4. A method described by Hozo et al. [21] was used to determine the SD. The mean values of velocities from different duration of assay were used for the determination of relevant parameters.

4. RESULTS AND DISCUSSION

In the light of the kinetic issues presented in theory section, there is need to justify it with experimental results. The enzyme PPAA was assayed to generate the velocities of amylolysis with different concentration of the substrate at different temperatures ranging between 298.15 and 333.15 K. The results were used to determine calculationally and graphically all kinetic and thermodynamic activation parameters (Table 1) as may be applicable.

It is obvious that for any time regime much greater than $1/k_2$, a substantial amount of the substrate may have been converted to a product. Hence, Eq. (7b) may be relevant. However, the appearance of k_2 seems to suggest that an approach of $d[_{\text{ES}}]/dt$ to zero (zero-

order kinetics) has been achieved. If d[*ES*]/d*t* is greater than zero, then k_1 ([E_T] – [*ES*]) should be greater than (k_{-1}+k_2) [*ES*]. The results (Table 1) obtained by substituting k_2 obtained from the plot of 1/v_{exp} versus 1/([S_0] – [*P*]), ([*S*] – [*P*]), [*ES*] given as v_{exp}/k_2 and other parameters do not show that k_1 ([E_T– [*ES*]) is always > (k_{-1} + k_2) [ES] for every value of ([*S*] – [*P*]) unlike for every value of [*S*]. However, when ([*S*] – [*P*]), [*ES*] given as v_{cal}/k_2, and other parameters are substituted into the equation (Eq. (7b)), the result showed that d[*ES*]/d*t* = 0; this implies a steady-state condition whereby k_1 ([E_T – [*ES*]) = (k_{-1}+k_2) [*ES*]. This was exactly the case where [*S*], [*ES*] as v_{cal}/k_2 and other parameters were substituted into Eq. (7b) (Table 2). This was not the case at-all where k_2 obtained from the plot of 1/v_{exp} versus 1/[*S*], [*ES*] (*i.e*. v_{exp}/k_2) and other parameters were substituted into the same equation (k_1 ([E_T – [*ES*]) was not > (k_{-1}+k_2) [*ES*] for all values of [*S*]). The results from substitution of [*S*] – [*P*] into the equation showed that k_1 ([E_T] – [*ES*]) < (k_{-1}+k_2) [*ES*] (Table 2). The observed k_1 ([E_T] – [*ES*]) = (k_{-1}+k_2) [*ES*] where calculated values of velocities of amylolysis were used to calculate *ES* using v_{cal}/k_2 could be as a result of the elimination of what could have been outliers and the establishment of perfect proportionality between *v* and [*S*].

Table 1. Experimental velocity of amylolysis, kinetic constants including rate constant from the plot of 1/ v_{exp} versus 1/*([S]+[P])* and rate of formation of *ES.*

v_{exp} (μM/mL.min)	KC - a	KC- val	$k_1([E_0]–[ES]^{(cv)})[S]$ & $(k_2+k_{-1})[ES]^{(cv)}$ (M/min) exp(- 3)	$k_1([E_0]–[ES]^{(ev)})[S]$ (M/min) exp(- 3)	$(k_2+k_{-1})[ES]^{(ev)}$ (M/min) exp(- 3)
86.05±0.44	k_1 1/M/min)	44118	~ 4.849	5.000 $^{(\Phi)}$ 4.853$^{(\ominus)}$	4.825
10.49±0.22	(k_2+k_{-1}) (1/min)	3748.8	~ 5.856	6.024$^{(\Phi)}$ 5.851$^{(\ominus)}$	5.877
11.33±0.17	k_2 (1/min)	66.9	~ 6.428	6.633$^{(\Phi)}$ 6.447$^{(\ominus)}$	6.351
12.58±0.86	k_{-1}	3681.9	~ 6.798	6.989$^{(\Phi)}$	6.805

	(1/min)			$6.796^{(\Theta)}$	
15.12±0.11	K_M (g/L)	29.1	~ 8.507	$8.744^{(\Phi)}$ $8.518^{(\Theta)}$	8.475

v_{exp}, KC – a, (cv), and (ev) are experimental velocity of hydrolysis, kinetic constants which includes rate constant, k_2 ($v_{max}/[E_0]$) from the plot of 1/ v_{exp} versus 1/([S] – [P]), values of [ES] determined using calculated velocities, and values of [ES] determined using experimental velocities respectively; (Θ) and (Φ) mean values obtained by using respectively [S] – [P] and [S] for calculation.

Table 2. Experimental velocity of amylolysis, kinetic constants including rate constant from the plot of 1/ v_{exp} versus 1/[S] and the rate of formation of *ES*.

v_{exp} (μM/mL.min)	KC - a	KC- val	$k_1([E_0]–[ES]^{(cv)})[S]$ & $(k_2+k_{-1})[ES]^{(cv)}$ (M/min) exp(- 3)	$k_1([E_0]–[ES]^{(ev)})[S]$ (M/min) exp(- 3)	$(k_2+k_{-1})[ES]^{(ev)}$ (M/min) exp(- 3)
86.05±0.44	k_1 1/M/min)	31450	~ 3.588	$3.590^{(\Phi)}$ $3.484^{(\Theta)}$	3.575
10.49±0.22	(k_2+k_{-1}) (1/min)	2903.7	~ 4.337	$4.333^{(\Phi)}$ $4.209^{(\Theta)}$	4.357
11.33±0.17	k_2 (1/min)	69.9	~ 4.763	$4.761^{(\Phi)}$ $4.628^{(\Theta)}$	4.770
12.58±0.86	k_{-1} (1/min)	2833.8	~ 5.038	$5.036^{(\Phi)}$ $4.897^{(\Theta)}$	5.223
15.12±0.11	K_M (g/L)	31.6	~ 6.313	$6.323^{(\Phi)}$ $6.159^{(\Theta)}$	6.283

v_{exp}, KC – a, (cv), and (ev) are experimental velocity of hydrolysis, kinetic constants which includes rate constant, k_2 ($v_{max}/[E_0]$) from the plot of 1/ v_{exp} versus 1/[S], values of [ES] determined using calculated velocities, and values of [ES] determined using experimental velocities respectively; (Θ) and (Φ) mean values obtained by using respectively [S] – [P] and [S] for calculation.

In the light of the issues raised in the theoretical section, this paragraph begins with a clear-cut explanation of what Gibbs free energy of activation and Arrhenius activation energy stands for. The minimum amount of energy-kinetic energy- which reactants need to become reactive and proceed to product, is called activation energy. The minimum energy must be equal to what Blamire [22] calls potential chemical energy (PCE) “locked” up in the chemical bonds of the reactants. Gibbs free energy is a part of the PCE that may be available for useful work. Substantial part of the rest may be lost as heat, the entropic expansion outcome.

Table 3. Rate constants and Gibbs free energy of activation with and without salt, $CaCl_{2\,(aq)}$

T (K)	k_2(– salt) (1/s)	$\Delta G^{\#}$(– salt) (kJ/mol)	k_2(+ salt) (1/s)	$\Delta G^{\#}$(+ salt) (kJ/mol)	[*S*] (g/L)	$\left(\Delta\Delta G_{ES^{\#}}\right)^{f-r}$ (kJ/mol)
298.15	0.094±0.057	79.135	0.107±0.033	78.681	5	66.482
310.15	0.618±0.090	77.305	0.876±2.268	76.405	6.25	67.109
318.15	0.749±.0.048	78.859	1.353±0.382	77.293	7	67.290
323.15	0.992±0.316	79.383	2.488±13.166	76.912	7.5	67.557
333.15	3.564±1.863	78.384	3.564±1.863	78.043	10	68.252

T is the thermodynamic temperature. The Arrhenius activation energies with and without calcium chloride are 85.937 and 79.027 kJ/mol respectively; the corresponding pre-exponential factors are 1.64 exp (14) and 7.838 exp (12) respectively. The superscript $f-r$ means total forward Gibbs free energy minus reverse Gibbs free energy of activation according to Eq. (12b); the result is only for assay without salt.

The PCE is an intrinsic property of the reactant molecules; molecular motion which increases the frequency of collision is extrinsic in nature, and it increases the possibility of encounter complex formation. Therefore, Arrhenius activation, E_a covers both intrinsic and extrinsic energies. The absorption of heat from the system and supply of heat (endothermicity) enables the breaking of bonds; this implies overcoming the “energy barrier” and consequently the PCE. A catalyst, abiotic and biotic lowers the amount of heat that needs to be supplied because of its effect of weakening the bond, reducing in process the potential chemical energy, leading to increase in entropy in the transition state complex; the increase in positive entropy means that the free energy of activation would be lowered. In this regard, Arcus [16] opined that the tight binding of the transition state significantly lowers $\Delta G_{ES^{\#}}$ for the reaction, leading to the extraordinary rate enhancements. Free energy of activation is defined as the free energy difference between reactive reactants and the total reactants [1]. In thermodynamics, the Gibbs free energy is a thermodynamic potential that can be used to calculate the maximum of reversible work that may be performed by a

thermodynamic system at a constant temperature and pressure (https://en.wikipedia.org/wiki/Gibbs_free_energy). As this research shows (Table 3 and foot note under Table 3), the Arrhenius activation energy with salt is higher than without salt. Whereas with salt, the Gibbs free energy of activation is lower than without salt. The nature of the substrate, raw starch in this research, may be a contributory factor in this regard. The presence of additive in the reaction mixture can influence the magnitude of the Arrhenius activation energy and its corresponding pre-exponential factor as shown as footnote under Table 3; both parameters with the salt were larger than without the salt.

In the light of Eq. (7f) and the motivational fact that k_1 ([*E*][*S*]/[*ES*]) = k_{-1} + k_2 (under steady-state condition), the total forward Gibbs energy of activation minus the reverse Gibbs free energy of activation were calculated; the results (Table 3) show that there was much greater reverse reaction as to imply a low affinity of the enzyme for the substrate. Hence $k_{-1} > k_{ES}$ and it is » k_2 (Table 2).Thus, contrary to suggestion elsewhere, $\Delta G_{\mathrm{ES}^{\#}} = E_a$ [4] may not always be the case and, recall that, $E_a = \Delta H^{\#} + RT$ and $RT \neq +T\Delta S^{\#}$ ($R \neq \Delta S^{\#}$). The Gibbs free energy of activation $\Delta G_{\mathrm{ES}^{\#}}$ is the standard Gibbs energy difference between the transition state of a reaction (either an elementary reaction or a stepwise reaction) and the ground state of the reactants [12].

5. CONCLUSION

The equations for the calculation of the difference in free energy of activation ($\Delta\Delta G_{\mathrm{ES}^{\#}}$) between the forward and reverse directions and a dimensionless equilibrium constant for the formation of enzyme-substrate (*ES*) were derivable. The large positive value of the $\Delta\Delta G_{\mathrm{ES}^{\#}}$ shows that the forward reaction is not substantially spontaneous; this is due perhaps, to the

nature of the substrate. The equality of Arrhenius activation energy (E_a) and $\Delta G_{ES^{\#}}$ may not be ruled out completely but it must not always be the case; the presence of additive like salt can increase the magnitude of E_a well above the values of the $\Delta G_{ES^{\#}}$. A dimensionless equilibrium constant for the net yield of *ES* seems to be a better alternative than K_M. The E_a unlike $\Delta G_{ES^{\#}}$ requires at least two different temperatures for its calculation.

COMPETING INTERESTS

Author has declared that no competing interests exist.

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REFERENCES

1. Levine RD. Free energy of activation. Definition, properties, and dependent variables with special reference to "linear" free energy relations. J. Phys. Chem. 1979; 83 (1): 159-170.
2. Johnston IA, Walesby NJ. Molecular mechanisms of temperature adaptation in fish Myofibrillar adenosine triphosphatases. J**.** Comp. Physiol. B. 1977; 119:195–206.
3. Low PS, Bada JL, Somero GN. Temperature adaptation of enzymes: Roles of the free energy, the enthalpy, and entropy of activation. Proc. Natl. Acad. Sci. U.S.A. 1973; 70(2): 430–432.
4. Copeland RA. A practical introduction to structure, mechanism, and data analysis. 2nd ed. New York/Chichester / Weinheim /Brisbane / Singapore / Toronto. Wiley VCH, Inc.; 2002.
5. D' Amico S, Marx J – C, Gerday C, Feller G. Activity – stability relationships in extremophilic enzymes J. Biol. Chem. 2003; 276(10): 7891–7896.

6. Cipolla A, Delbrassine F, Da Lage JL, Feller G. Temperature adaptations in psychrophilic, mesophilic and thermophilic chloride – dependent alpha-amylase Biochemie. 2012; 30: 1–8.

7. Miyawaki O, Dozen M, Nomura K. Thermodynamic analysis of osmolyte effect on thermal stability of ribonuclease A in terms of water activity. Biophy. Chem. 2014; 185: 19-24.

8. Kumar S, Grewal J, Sadaf A, Hemamalini R, Khare SK. Halophiles as a source of polyextremophilic α-amylase for industrial applications. AIMS Microbiol. 2016; 2(1): 1-26.

9. Siddiqui KS, Bokhari SA, Afzal AJ, Gong SG. A novel thermodynamic relationship based on Kramers theory for studying enzyme kinetics under high viscosity. IUBMB Life. 2004; 56(7): 403-407.

10. Schnell S, Maini, PK. Enzyme kinetics at high enzyme concentration, Bull. Math. Biol. 2000; 483-499.

11. Buurma NJ, Pastorello L, Blandermer MJ, Engberts JBFN, Kinetic evidence for hydrophobically stabilized encounter complexes formed by hydrophobic esters in aqueous solutions containing monohydric alcohols. J. Am. Chem. Soc. 2001; 123: 11848–11853.

12. IUPAC. Compendium of Chemical Terminology, 2nd ed. (the "Gold Book"). Compiled by A. D. McNaught and A. Wilkinson. Blackwell Scientific Publications, Oxford (1997).

13. Laidler KJ. The development of the Arrhenius equation. J. Chem. Edu. 1984; 61(6): 494-498.

14. Riaz M, Perveen R, Javed MR, Nadeem H, Rashid MH. Kinetic and thermodynamic properties of novel glucoamylase from *Humicola* sp. Enzyme Microb. Technol. 2007; 41: 558-564.

15. Udema II. The Effect of Additives and Temperature on the Velocity of Hydrolysis of Raw Starch with Human Salivary a – amylase. Int. J. Biochem. Res. Rev. 2016; 10 (2): 1-17.

16. Arcus VL, Prentice EJ, Hobbs JK, Mulholland AJ, Van der Kamp MW, Pudney CR, *et al*. On the temperature dependence of enzyme-catalyzed rates. Biochemistry. 2016; 55: 1681−1688.

17. Gopal BA, Muralikrishna G. Porcine pancreatic α-amylase and its isoforms: Purification and kinetic studies. Int. J. Food Prop. 2009; 12: 571–586.

18. Udema II. In vitro investigation into the effects of ethanol, aspirin, and stabilisers on mesophilic alpha amylases. Thesis, AAU Ekpoma 2013.

19. Bernfeld P. Amylases, alpha and beta. Methods Enzymol. 1955; 1:149–152.

20. Lineweaver H, Burk D. The determination of enzyme dissociation constants. J. Am. Chem. Soc. 1934; 56(3): 658–666.

21. Hozo SP, Djulbegovic B, Hozo I. Estimating the means and variance from the median range and the size of a sample. BMC Med. Res. Methodol. 2005; 5(13):1-10.

22. Blamire J. Energy, molecules and chemical reactions. Science at a distance. 2001; http://www.brooklyn.cuny.edu.