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Characterization of α-amylases isolated from *Cyperus esculentus* seeds (tigernut): Biochemical features, kinetics and thermal inactivation thermodynamics

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ABSTRACT

In this study, α-amylases from fresh and dried *Cyperus esculentus* seeds were isolated and characterized for biochemical and thermodynamic properties. The α-amylases (FTNE-α-amylase and DTNE-α-amylase) were optimally active at 60–70 °C, pH 7.0 (FTNE-α-amylase) and pH 6.0 (DTNE-α-amylase) with activation energies (Ea) of 3.56 and 6.81 kJ mol^{-1}, respectively. Both enzymes retained above 70% of their hydrolytic activities after 1 h at pH 6.0–8.0. The enzyme activities were more enhanced in the presence of divalent metal ions (Ca^{2+}, Mg^{2+}, Zn^{2+}, Cu^{2+}, Ba^{2+}, Co^{2+}, Pb^{2+}, and Hg^{2+}) than monovalent ions. SDS, cyclohexanol, ether and diethylamine inhibited them except EDTA. Using soluble starch as substrate, DTNE-α-amylase had higher V$_{max}$, K$_{cat}$ and lower K$_m$ than FTNE-α-amylase. Both enzymes expressed high substrate preferences for yam flour amidst other substrates with catalytic efficiencies (K$_{cat}$/K$_m$) of 2.71 and 4.44 mL$^.$ mg^{-1}sec^{-1}, respectively. The higher catalytic efficiency of DTNE-α-amylase was due to its transition state stabilization (ΔG_{E-S} = -0.90 kJ mol^{-1} and ΔG_{E-T} = -2.22 kJ mol^{-1}). Fourier Transformed-Infrared (FTIR) spectroscopy revealed changes in C–O stretching and -N-H bending in the substrate-bound enzymes. Thermal inactivation of the amylases followed first-order rate kinetics and was non-spontaneous ($\Delta G^{\#}$>0). FTNE-α-amylase half-lives and D-values at 70 and 80 °C, and Z-value (256.41 °C), were however higher than in DTNE-α-amylase (Z-value = 63.29 °C; t$_{1/2}$ = 22.43 min at 70 °C) indicating that FTNE-α-amylase is more thermally stable than DTNE-α-amylase and would be more industrially applicable as a thermostable starch-hydrolyzing enzyme.

1. Introduction

Alpha amylases (E.C. 3.2.1.1) are enzymes that catalyze the hydrolysis of α-(1,4)-glycosidic linkages in starch components and related polysaccharides to release maltose (Metin et al., 2010; Avwioroko et al., 2018). Amylases are important enzymes of great significance for biotechnology, constituting a class of industrial enzymes having approximately 25% of the world enzyme market (Rajagopalan and Krishnan, 2008). It is a key enzyme in the production of starch derivatives and has also found reputable application in fabrics designing industries, baking industry, pharmaceuticals and detergents making industries (Gupta et al., 2008; Sindhu et al., 2017). They can be obtained

from several sources including plants, animals and microorganisms. Today the use of industrial enzymes such as amylases in starch processing industry has almost completely replaced chemical hydrolysis of starch (Gupta et al., 2003; Tanyildizi et al., 2005; Avwioroko and Tonukari, 2014; Tonukari et al., 2016).

Although over the years, attention of researchers has been drawn to isolation and characterisation of alpha amylases, invertases and other vital industrial enzymes from fungi and bacteria, in the last few decades till recently research interest is now being shifted or diversified to less considered aspects of enzyme sources such as plants and grains. Amylase from germinating rice grain (Liu et al., 2018), wheat seed (Singh and Kayastha, 2014), and barley grains (Sheng et al., 2018) have been

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reported in literature. Though efforts made by researchers in recent past have increased global knowledge on some enzymes from plant or vegetable sources, not much progress has been made relative to the bulk of information on enzymes from microbial sources in the National Center for Biotechnology Information (NCBI) and other protein data bases.

Cyperus esculentus L. var. *sativus* is an edible perennial sedge that produces subterranean tuberous rhizomes, commonly known as ''tiger-nut'' (Oladele et al., 2010; Vega-Morales et al., 2019). It belongs to the family of *Cyperacheae* reported to be highly beneficial to health (Oladele et al., 2010). The underutilized tiger-nut is becoming increasingly relevant in industry as it is gradually gaining popularity in the production of yogurt, especially in developing countries. It is perceived to be rich in various enzymes which could find potential application in industrial processes such as fermentation, starch hydrolysis etc (Adama et al., 2014; Tonukari et al., 2015). However, most of these enzymes in *C. esculentus* seeds (tigernut) with such potential industrial relevance are yet to be fully studied and reported in literature.

The present research was aimed to determine the biochemical features, kinetics and thermal inactivation thermodynamic properties of alpha amylases isolated from fresh and dried *Cyperus esculentus* seeds (tigernut).

2. Materials and methods

2.1. Plant materials and chemicals used

Fresh *Cyperus esculentus* seeds (tigernut) (Fig. 1) were obtained from Ede market in Osun State, Nigeria. The fresh tigernut seeds were spread under sunlight on an inert surface to dry-up to a constant weight sample referred to as dried tigernut seed. All the chemicals and buffers used were of analytical grade.

2.2. Preparation of enzyme extract

50 g of fresh and dried *C. esculentus* seeds were separately homogenized in 150 mL ice-cold extraction buffer (50 mM sodium acetate buffer, pH 5.5) using a laboratory blender (Singh and Kayastha, 2014). The homogenate was then passed through a two layered pre-washed muslin cloth to obtain a filtrate. The filtrate was further purified by centrifugation at 3000*g* for 10 min. The supernatants arising from both the fresh and dried *C. esculentus* seed extracts were stored at 4 °C and later used for analysis as crude enzymes (FTNE-α-amylase and DTNE--α-amylase, respectively).

Fig. 1. Images of (a) fresh and (b) dried *Cyperus esculentus* seeds (tigernut). The fresh tigernuts are oval in shape with milk colour appearance mixed with brown patches, whereas the dried tigernuts are wrinkled, with dark-brown appearance. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

2.3. Alpha amylase activity assay

Activities of FTNE-α-amylase and DTNE-α-amylase were determined following the starch-iodide method described by Singh and Kayastha (2014) with little modifications. The hydrolytic activity of α-amylase was routinely assayed using soluble starch as substrate. 0.5 mL of 1% w/v soluble starch solution, 0.3 mL phosphate buffer (0.1 M, pH 6.0) and 0.1 mL distilled water were incubated at 70 °C in a water bath for 10 min. The enzymic reaction was started by addition of 0.1 mL of suitably diluted (1:1) enzyme extract. The reaction was stopped after 5 min by adding 0.5 mL 1 N HCl followed by rapid cooling. To 0.2 mL of the reaction mixture, 0.1 mL potassium iodide solution (2% KI and 0.2% iodine) was added. The final volume was made to 5 mL with distilled water and absorbance was read at 610 nm. One unit of α-amylase activity was defined as the amount of enzyme that caused a decrease in absorbance by 0.005 in starch-iodine colour, under the assay conditions.

2.4. Biochemical characterisation of FTNE-α-amylase and DTNE-α-amylase

2.4.1. Determination of optimum temperature and activation energy

The effect of temperature on the activity of alpha amylases from fresh and dried *C. esculentus* seeds was investigated by carrying out the amylase assay at different temperature conditions (30–80 °C). The optimum temperature range was obtained from a plot of relative activity versus temperature (°C). Using values obtained from the assay of the effect of temperature on enzyme activity, a graph of natural logarithm of activity (ln V) against reciprocal of temperature (T^{-1}) (in Kelvin) was plotted and the energy of activation (E_a) determined from the slope as follow (Pal and Khanum, 2011):

$$Slope = \frac{-E_a}{R} \tag{1}$$

where R is the universal gas constant (8.314 J mol^{-1}K^{-1}).

2.4.2. Effect of pH on enzyme activity and determination pH optimum

The effect of pH on the activities of α-amylases from fresh and dried *C. esculentus* seeds was determined at pH values ranging from 2-10 at 70 °C following the above described assay protocol (Singh and Kayastha, 2014). The buffers used were glycine HCl 0.05 M (pH 2–3), sodium acetate 0.05 M (pH 4–5), phosphate 0.05 M (pH 6–8) and glycine–NaOH 0.05 M (pH 9–10) (Avwioroko et al., 2015). The optimum pH value was obtained from a plot of relative activity against pH values.

2.4.3. Enzyme pH stability

The pH stability of the amylases was investigated at acidic, neutral and alkaline conditions (pH 6, 7 and 8). FTNE-α-amylase and DTNE-α-amylase (0.1 mL) were mixed with 0.3 mL 50 mM buffer (pH 6, 7 and 8) and then refrigerated at 4 °C for 1 h, followed by determination of enzyme activity at the assay temperature (70 °C).

2.4.4. Effect of metal ions and chelating agent

To ascertain the effect of metal ions (NaCl, KCl, CaCl$_2$, MgCl$_2$, NH$_4$Cl, ZnCl$_2$, CuSO$_4$, AlCl$_3$, AgNO$_3$, FeCl$_3$, BaCl$_2$, CoCl$_2$, PbCl$_2$, HgCl$_2$) and chelating agent (ethylenediamine tetraacetic acid, EDTA) on activities of FTNE-α-amylase and DTNE-α-amylase, the enzymes' activities were determined in the presence of each of the effector molecules at concentrations of 1 and 10 mM following the described assay protocol. The enzyme activity in the absence of the effectors was taken as 100% and relative activity was calculated (Avwioroko et al., 2015).

2.4.5. Effect of organic solvents

The effects of organic solvents (cyclohexanol, ethanol, methanol, diisopropylether, ether and diethylamine) on FTNE-α-amylase and DTNE-α-amylase activities were also determined by incubating the assay

reagents with each solvent at 10 and 20% v/v concentration, separately, for 10 min at 70 °C before initiating enzyme catalysis. The enzyme activity in the absence of the organic solvent(s) was taken as control (100%) and relative activity was calculated.

2.4.6. Effect of inhibitors and denaturing agents

The effects of inhibitors and denaturing agents such as 1,3-dinitrobenzene, DTNB [5,5-dithiobis (2-nitrobenzoic acid)], sodium dodecyl sulphate (SDS), urea, and thiourea, on FTNE-α-amylase and DTNE-α-amylase were also ascertained by determining the enzymes' activities in the presence of 1 and 10 mM concentrations of the aforementioned effector molecules using the standard assay procedure.

2.4.7. Determination of V_{max}, K_m, K_{cat} and enzyme's substrate preference

The crude α-amylases (FTNE-α-amylase and DTNE-α-amylase) were subjected to hydrolysis of different starch-containing substrates including cassava (*Manihot esculenta*) starch, cassava flour, wheat grain flour, white yam (*Dioscorea rotundata*) flour, corn (*Zea mays*) flour and soluble starch (as control). The hydrolytic activities were measured at different substrate concentrations (1–10 mg/mL) following the amylase assay protocol described above (Singh and Kayastha, 2014). Kinetic constants (V_{max} and K_m) of FTNE-α-amylase and DTNE-α-amylase were obtained from Lineweaver-Burk plots (Supplementary data S1 and S2; Eq. (2)), while turnover number (K_{cat}) (Eq. (3)) and enzyme's substrate preference (Eq. (4)) were calculated as described by (Ferreira et al., 2018).

$$\frac{1}{V} = \frac{1}{V_{max}} + \frac{K_m}{V_{max}} * \frac{1}{[S]} \tag{2}$$

$$K_{cat} = \frac{V_{max}}{[E_t]} \tag{3}$$

Where Et is enzyme total.

$$\text{Enzyme's substrate preference} = \frac{K_{cat}}{K_m} \tag{4}$$

The free energy of substrate binding (ΔG_{E-S}) and free energy of transition state formation (ΔG_{E-T}) at the assay temperature (70 °C) were calculated as follow (Saleem et al., 2005; Ferreira et al., 2018):

$$\Delta G_{E-S} = -RTIn\left(\frac{1}{K_m}\right) \tag{5}$$

$$\Delta G_{E-T} = -RTIn\left(\frac{K_{cat}}{K_m}\right) \tag{6}$$

2.4.8. Fourier transformed infrared (FTIR) spectroscopy

The characteristic functional groups and bonding pattern of the FTNE-α-amylase and DTNE-α-amylase were studied using Fourier Transformed Infrared (FTIR) spectroscopy both in the absence and presence of their substrate (soluble starch). FTIR spectra were recorded between 4000 and 400 cm^{-1} using FTIR-8400S spectrophotometer (Shimadzu Corporation, Kyoto, Japan) at a resolution of 4.0 cm^{-1} with KBr. All the spectral measurements were performed at 25 ± 2 °C.

2.5. Enzyme thermal stability and thermal deactivation thermodynamics

To ascertain the enzymes' thermal stabilities, α-amylases from fresh and dried *C. esculentus* were incubated at 60, 70 and 80 °C for various time intervals (10–60 min). Thereafter, aliquots were withdrawn for determination of enzyme activity (V) following the assay procedure previously described. At each temperature investigated (60, 70 and 80 °C), the thermal deactivation constant (K_d) was obtained as the gradient of the plot of In V against time (min). Decay constants or half-lives ($t_{1/2}$) and D-values of each enzyme (FTNE-α-amylase and DTNE-α-amylase) at each temperature were calculated as a function of K_d as

follow (Pal and Khanum, 2011; Ferreira et al., 2018):

$$Halflife, t \, {}^1\!/{}_2 = \frac{In2}{K_d} \tag{7}$$

$$D - value = \frac{In10}{K_d} \tag{8}$$

The D-value denotes the time taken (min) for an enzyme or bioactive molecule to lose 90% of its activity. The Z-value (in °C) of the enzymes, which refers to temperature increase in degree Celsus required to reduce an enzyme's D-value by 10%, was determined from the slope of the plot of Log D-value against temperature (°C) as follow:

$$Slope = \frac{-1}{Z} \tag{9}$$

The activation energy for the thermal denaturation (E_d) of alpha amylases from fresh and dried *C. esculentus* seeds was determined from the slope of the Arrhenius plot of In K_d versus 1/T (in Kelvin) (Pal and Khanum, 2011; Ferreira et al., 2018):

$$Slope = \frac{-E_d}{R} \tag{10}$$

The changes in enthalpy of activation of denaturation ($\Delta H^{\#}$), Gibbs free energy of activation of denaturation ($\Delta G^{\#}$) and entropy of activation of denaturation ($\Delta S^{\#}$) were determined using the following equations (Pal and Khanum, 2011; Ferreira et al., 2018):

$$\Delta H^{\#} = E_d - RT \tag{11}$$

$$\Delta G^{\#} = -RT.In\left(\frac{K_d.h}{K_b.T}\right) \tag{12}$$

$$\Delta S^{\#} = \frac{\Delta H^{\#} - \Delta G^{\#}}{T} \tag{13}$$

Where h is Planck's constant (6.626×10^{-34} J min^{-1}), K_b is Boltzmann constant (1.38×10^{-23} J K^{-1}), R is universal gas constant (8.314 J $mol^{-1}K^{-1}$), K_d is the thermal deactivation constant (min^{-1}) and T is temperature (in Kelvin).

2.6. Total protein assay

Total protein contents of the crude enzymes (FTNE-α-amylase and DTNE-α-amylase) were determined in triplicates using Biuret method (Gornall et al., 1949) using albumin as protein standard.

2.7. Statistical analysis

Values of enzyme activities obtained were expressed as mean ± standard error of mean (SEM) for triplicate experiments (n = 3). The mean values were compared using One-Way Analysis of Variance (ANOVA) followed by Dunnett's post hoc test. Significant differences were considered at $p < 0.05$.

3. Results and discussion

3.1. Effect of temperature

The activity of alpha amylases from both fresh and dried *C. esculentus* seeds (FTNE-α-amylase and DTNE-α-amylase) was optimum at a temperature range of 60–70 °C and was significantly low (below 100%) at mild temperatures between 30 – 40 °C (Fig. 2). This indicates the tendency of the α-amylases being thermophilic in nature. It is desirable in most biotechnology industries for α-amylases to be able to exhibit their catalytic ability optimally even at moderately high temperatures (Hmidet et al., 2008; Mehta and Satyanarayana, 2013). Previous studies on α-amylase from many plant sources have reported an optimum
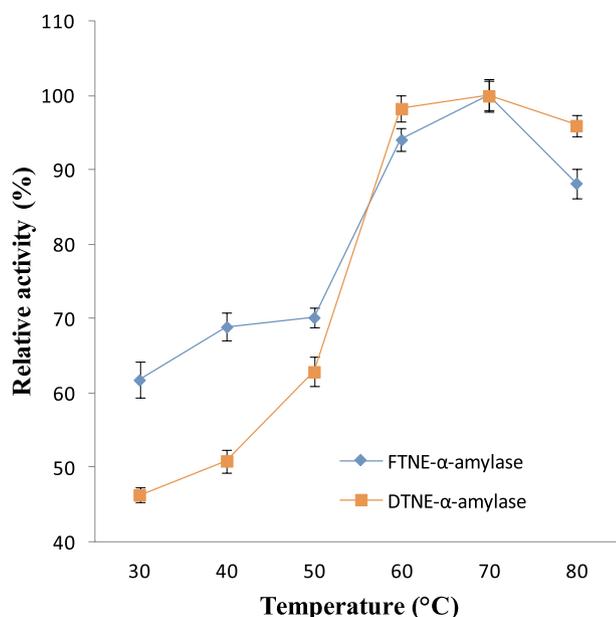
Fig. 2. Effect of temperature on activity of α-amylase from *Cyperus esculentus* seeds. Alpha amylase activities were measured at various temperatures (30–80 °C) at pH 6. The relative activity was calculated by dividing enzyme specific activity obtained at each investigated temperature by that obtained at the normal assay condition (70 °C, pH 6) and the value multiplied by 100. The plots shown are mean ± standard error of mean of relative amylase activities (n = 3) at different temperatures.

Fig. 3. Effect of different pH values on activity of *C. esculentus* seed α-amylases. Alpha amylase activities were measured at various pH values at a constant temperature of 70 °C. The relative activity was calculated by dividing enzyme specific activity obtained at each investigated temperature by that obtained at the normal assay condition (70 °C, pH 6) and the value multiplied by 100. The plots shown are mean ± standard error of mean of relative amylase activities (n = 3) at different pH values.

temperature of 70 °C for α-amylase from germinating soybean seeds and two isoforms from Azuki beans (Mar et al., 2003; Kumari et al., 2010) and 60 °C for α-amylase from mango seed (Afiukwa et al., 2009), which are consistent with our report. In contrast, however, Sottirattanapan et al. (2017) reported an optimum temperature of 50 °C for amylase from root of *Paederia foetida* Linn, which is lower than that obtained in this study.

3.2. Effect of pH and pH stability

The activity of alpha amylase from dried *C. esculentus* seeds was optimum at an acidic pH of 6 and thereafter declined (Fig. 3). However, alpha amylase from fresh tigernut (*C. esculentus* seeds) had its optimum activity at a pH of 7 (neutral) and its activity declined with further pH increase. These results suggest that FTNE-α-amylase and DTNE-α-amylase are naturally not alkalinophilic and as such should mainly be applied in industrial processes in which acidic or neutral media are utilized (Saideh et al., 2016). Alpha-amylase from partially germinated mango seeds with optimum pH 6.0 (Afiukwa et al., 2009), pH 5.6 for α-amylase from mung beans (Tripathi et al., 2007), pH 5.5 for α-amylase from germinating soybean seeds (Kumari et al., 2010), and pH 7.0 for amylase from root of *Paederia foetida* Linn have also been reported in literature (Sottirattanapan et al., 2017). Results of pH stability also revealed that FTNE-α-amylase and DTNE-α-amylase generally retained more than 70% of their hydrolytic activities after pH treatment for 1 h at pH 6, 7 and 8 (Fig. 4) with FTNE-α-amylase exhibiting the highest pH stability at pH 6 (>90%). The results show that tigernut seeds α-amylases would be moderately pH stable when utilized in industrial processes under acidic, neutral and alkaline conditions (pH 6, 7 and 8) over a duration of 1 h. Such enzymes can find useful applications in brewing and confectionary industries where pH stable starch-degrading enzymes are desired (Emtenani et al., 2015).
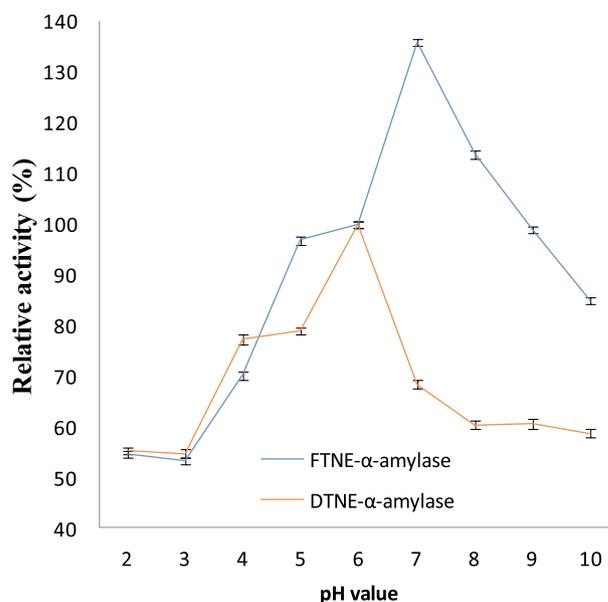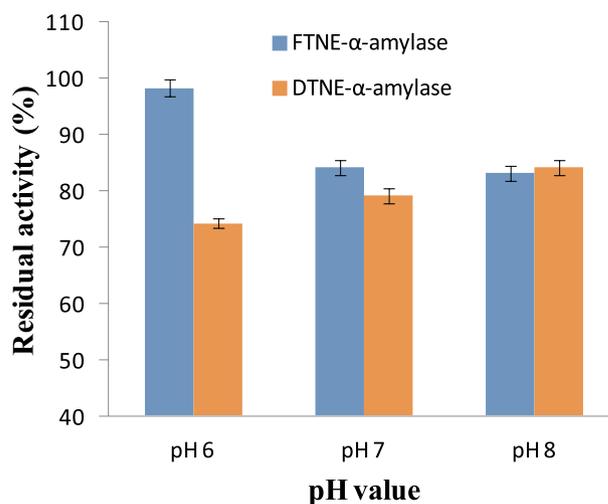

Fig. 4. pH stability of α-amylases from fresh and dried *C. esculentus* seeds after 1 h. α-Amylases from FTNE and DTNE were subjected to pH treatments for 1 h (pH 6, 7 and 8). Thereafter, the enzyme activities were determined following the assay protocol.

3.4. Effect of metal ions and chelating agent

Metal ions have been widely shown to participate in regulation of enzyme activities. According to Sinha and Khare (2013), metal ions could play key roles in enzyme stability and activity. The alpha-amylase activity was considerably impressible in the presence of metal ions and chelating agent. The activities of the alpha amylases from both fresh and dried tigernut extracts were more activated in the presence of divalent metal ions (Ca^{2+}, Mg^{2+}, Zn^{2+}, Cu^{2+}, Al^{3+}, Fe^{3+}, Ba^{2+}, Co^{2+}, Pb^{2+}, and Hg^{2+}) than the monovalent cations (Table 1), especially at a concentration of 10 mM. Apart from NH_4^+ which enhanced the enzymes' activities, K^+ mildly inhibited the activities of the amylases. Activation of

Table 1

Effect of metal ions and chelating agent on the activity of *C. esculentus* seed α-amylase.

Metal ions	Relative activity (%) of fresh *C. esculentus* seed α-amylase		Relative activity (%) of dried *C. esculentus* seed α-amylase	
	1 mM	10 mM	1 mM	10 mM
Control	100.0 ± 0.01	100.0 ± 0.03	100.0 ± 0.02	100.0 ± 0.02
Cations				
K^+	98.4 ± 0.87	$100.5 \pm 0.02^{\#}$	89.4 ± 0.09	90.5 ± 0.05
Na^+	$100.5 \pm 0.03^{\#}$	101.4 ± 0.01	92.5 ± 0.87	109.0 ± 0.65
NH_4^+	117.7 ± 0.4	308.6 ± 0.56	120.6 ± 0.94	169.8 ± 0.03
Ca^{2+}	115.1 ± 0.23	261.3 ± 0.76	106.5 ± 0.61	252.3 ± 0.55
Mg^{2+}	118.8 ± 0.31	252.2 ± 0.43	114.1 ± 0.04	261.3 ± 0.43
Zn^{2+}	118.3 ± 0.11	187.6 ± 0.72	106.0 ± 0.02	102.0 ± 0.05
Cu^{2+}	102.7 ± 0.51	280.1 ± 0.55	109.5 ± 0.07	156.8 ± 0.04
Al^{3+}	113.4 ± 0.14	216.1 ± 0.87	107.5 ± 0.02	162.8 ± 0.01
Ag^+	$99.5 \pm 0.03^{\#}$	252.7 ± 0.83	91.5 ± 0.04	176.9 ± 0.06
Fe^{3+}	184.2 ± 0.54	143.0 ± 0.05	141.4 ± 0.53	84.63 ± 0.04
Ba^{2+}	114.5 ± 0.21	233.9 ± 0.26	108.5 ± 0.08	196.5 ± 0.06
Co^{2+}	122.6 ± 0.14	116.7 ± 0.03	134.2 ± 0.76	127.1 ± 0.01
Pb^{2+}	116.1 ± 0.05	102.7 ± 0.04	107.5 ± 0.06	107.0 ± 0.04
Hg^{2+}	107.5 ± 0.04	210.8 ± 0.77	101.5 ± 0.02	117.6 ± 0.03
Chelating agent				
EDTA disodium	109.7 ± 0.01	225.8 ± 0.86	102 ± 0.02	206 ± 0.82

The enzyme activity assay was carried out under the standard experimental conditions of pH 6.0 and temperature 70 °C, in the absence and presence of 1 or 10 mM salt solution containing a cation or chelating agent. Values of relative amylase activities were expressed as mean $\pm$ SEM (n = 3). Values with superscripted hash tag (#) are not significantly different (p > 0.05) from the control, but those without it significantly differ (p < 0.05).

the FTNE-α-amylase and DTNE-α-amylase in the presence of Ba^{2+}, Cu^{2+} and Pb^{2+} disagree with the behavior observed for most alpha-amylases in which these heavy metals exhibited inhibitory effect (Bernhardsdotter et al., 2005; Wu et al., 2018). The chelating agent (EDTA), at concentrations of 1 and 10 mM, also enhanced the activities of the amylases significantly. This result is in agreement with a previous report on improvement of the activity of *Bacillus.* sp L1711 alpha-amylase in the presence of 5 mM EDTA (Bernhardsdotter et al., 2005). It has also been reported in a study that amylases vary in their responses to the chelator EDTA with some being unaffected (Arikan, 2008). Our findings indicate that the tigernut α-amylases reported in this study (FTNE-α-amylase and DTNE-α-amylase) require divalent metal ions for their hydrolytic activities and the binding of the metal ions to the enzyme was not hindered by the presence of EDTA. The observed differences in activities of FTNE-α-amylase and DTNE-α-amylase investigated may also be due to the different pretreatments given to the seeds. Studies on germinating seeds, involving seed soaking (wet seeds) have reported differences in amylolytic activities in both soaked (wet or germinating) and dry barley grains (Sheng et al., 2018). Enzymes in dormant seeds (dry seeds) sometimes appear to express relatively low activities due to their low moisture content than fresh (or soaked) seed samples (Penfield, 2017;

Sheng et al., 2018).

3.5. Influence of effector molecules

The effect of 1,3-dinitrobenzene, 5,5′-dithiobis-(2-nitrobenzoic acid) [DTNB], urea, sodium dodecyl sulphate (SDS) and thiourea on the activities of FTNE-α-amylase and DTNE-α-amylase is as shown in Table 2. It was observed that at both concentrations investigated (1 and 10 mM), 1,3-dinitrobenzene, DTNB [5,5-dithiobis (2-nitrobenzoic acid)] and thiourea enhanced the activities of α-amylases from both fresh and dried tigernut seeds significantly. Whereas urea inhibited DTNE-α-amylase activity only at 10 mM, sodium dodecyl sulphate (SDS) significantly inhibited both FTNE-α-amylase and DTNE-α-amylase at 1 and 10 mM. A recent research on α-amylase from *Aspergillus* spp. revealed that the enzyme activity was mildly stimulated in the presence of thiourea and DTNB (Avwioroko et al., 2015). The results of the present suggest that FTNE-α-amylase and DTNE-α-amylase may be useful as an ingredient in industries that require enzymatic starch hydrolysis in the presence of thiourea and DTNB but could be used in industries where the inhibitory effect of SDS is not required.

3.6. Effect of organic solvents

The activities of FTNE-α-amylase and DTNE-α-amylase were drastically reduced in the presence of various organic solvents such as cyclohexanol, ethanol, ether and diethylamine investigated in this study at concentrations of 10 and 20%v/v. Although solvent's increasing concentration led to a decrease, activities of FTNE-α-amylase and DTNE-α-amylase were significantly increased above 100% in the presence of 10% (v/v) methanol (Table 3). Though further increase in concentration of methanol (organic solvent) to 20% (v/v) reduced the enzymes activities, the residual amylolytic activities expressed were still high (>100%). This is in contrast to the findings reported by Wu et al. (2018) who observed that some organic solvents did not have significant effects on activity of α-amylase from *Bacillus licheniformis* B4-423.

3.7. Enzymes' kinetic constants, turnover (K_{cat}) and substrate preference

The kinetic parameters of α-amylases isolated from fresh and dried *C. esculentus* seeds using soluble starch and other substrates are reported in Table 4 as obtained from their various Lineweaver–Burk plots (Supplementary data S1 and S2). The highest specific activity (V_{max}) of α-amylase from fresh *C. esculentus* seeds was found to be 25.58 U mg protein^{-1} when corn flour was used as substrate and 22.52 U mg protein^{-1} for α-amylase from dried *C. esculentus* seeds when cassava starch was used as substrate (Table 4). The Vmax values obtained for α-amylase from both and dried fresh *C. esculentus* seeds are, however, very low when compared to that for α-amylase isolated from wheat germ (700 U/mg) (Sharma et al., 2000). The highest catalytic efficiencies (K_{cat}/K_m values) of the enzymes when subjected to catalysis of various substrates were 2.71 and 4.44 mL·mg^{-1}sec^{-1} for FTNE-α-amylase and

Table 2

Influence of effector molecules on the activity of *C. esculentus* seed α-amylase.

Inhibitor	Relative activity (%) of fresh *C. esculentus* seeds α-amylase		Relative activity (%) of dried *C. esculentus* seeds α-amylase	
	1 mM	10 mM	1 mM	10 mM
Control	100.0 ± 0.01	100.0 ± 0.02	100.0 ± 0.02	100.0 ± 0.01
DTNB	265.59 ± 0.76	259.14 ± 0.65	264.82 ± 0.55	260.8 ± 0.47
1,3-Dinitrobenzene	237.63 ± 0.87	225.81 ± 0.51	247.74 ± 0.45	194.97 ± 0.23
Thiourea	118.8 ± 0.06	201.1 ± 0.32	117.6 ± 0.21	199.5 ± 0.18
Urea	107.5 ± 0.04	103.2 ± 0.03	105.5 ± 0.02	95.0 ± 0.02
SDS	13.2 ± 0.01	33.33 ± 0.02	14.0 ± 0.02	18.6 ± 0.03

DTNB, 5,5-Dithiobis (2-nitrobenzoic acid); SDS, Sodium dodecyl sulphate. Enzyme interaction with soluble starch (substrate) was carried out under the standard experimental conditions of pH 6.0 and temperature 70 °C, in the absence and presence of 1 or 10 mM solution of inhibitor or denaturant. Values of relative amylase activities were expressed as mean $\pm$ SEM (n = 3). Values with superscripted hash tag (#) are not significantly different (p > 0.05) from the control, but those without it significantly differ (p < 0.05).

Table 3

Effect of various organic solvents on the activity of *C. esculentus* seeds α-amylase.

Organic solvents	Relative activity (%) of fresh C. esculentus seed α-amylase			Relative activity (%) of dried C. esculentus seed α-amylase	
	10% (v/v)	20% (v/v)		10% (v/v)	20% (v/v)
Control	100.0 ± 0.01	100.0 ± 0.01	100.0 ± 0.02	100.0 ± 0.01	
Cyclohexanol	88.17 ± 0.08	41.4 ± 0.03	99.5 ± 0.04	35.68 ± 0.01	
Ethanol	64.52 ± 0.06	74.19 ± 0.03	84.92 ± 0.02	79.9 ± 0.02	
Ether	11.29 ± 0.02	20.43 ± 0.01	34.67 ± 0.02	35.68 ± 0.01	
Diethylamine	13.98 ± 0.01	14.84 ± 0.01	14.57 ± 0.01	18.09 ± 0.02	
Methanol	150.54 ± 0.97	148.6 ± 0.11	234.67 ± 0.24	140.7 ± 0.04	

The enzyme catalyzed reaction was carried out under the standard experimental conditions of pH 6.0 and temperature 70 °C, in the absence and presence of 10 or 20% v/v of the organic solvents investigated. Values of relative amylase activities were expressed as mean ± SEM (n = 3). Values with superscripted hash tag (#) are not significantly different (p > 0.05) from the control, but those without it significantly differ (p < 0.05).

Table 4

Kinetic parameters of *C. esculentus* seed α-amylase using various substrates.

Substrate	V_{max} (U mg protein^{-1})	K_m (mg mL^{-1})	K_{cat} (s^{-1})	K_{cat}/K_m (mL·mg^{-1}s^{-1})
α-Amylase from fresh *C. esculentus* seeds				
Control (soluble starch)	9.36	6.49	0.76	0.12
Cassava starch	20.08	7.81	1.64	0.21
Cassava flour	21.88	3.16	1.79	0.56
Wheat flour	16.56	1.49	1.35	0.91
Yam flour	19.96	0.60	1.63	2.71
Corn flour	25.58	3.91	2.09	0.53
A-Amylase from dried *C. esculentus* seeds				
Control (soluble starch)	18.32	0.73	1.59	2.18
Cassava starch	22.52	1.02	1.96	1.92
Cassava flour	20.83	0.71	1.81	2.55
Wheat flour	16.37	0.57	1.43	2.50
Yam flour	19.38	0.38	1.69	4.44
Corn flour	18.45	0.66	1.61	2.43

Values of Michaelis constant (K_m) and maximum enzyme activity (V_{max}) were respectively deduced from intercepts on horizontal and vertical axes of various Lineweaver-Burk plots for the various substrates investigated (Supplementary data S1 and S2), while those of protein turn-over number (K_{cat}) were calculated.

DTNE-α-amylase, respectively in the presence of yam flour as substrate (Table 4). Values of K_{cat}/K_m are generally used as biochemical indicators of an enzymes preference for a substrate, with increased values indicating higher substrate preference by the enzyme (Kara et al., 2017). The values of K_{cat}/K_m obtained in this study suggest that yam flour is the most preferred substrate for both enzymes among other substrates investigated. Substrate specificity of α-amylase as well as rate of hydrolysis of substrates by enzymes, generally, is not only affected by the substrates' molecular sizes and structures but also by the types of bonds

in their chains (Avwioroko et al., 2015). Alpha amylases have been widely reported to catalyze hydrolysis of α-1,4-glycosidic bonds in polysaccharides and disaccharides like amylose (Sindhu et al., 2017; Kara et al., 2017; Tonukari et al., 2010). A study conducted by Aprianita et al. (2014) showed that yam flour has a higher amount of amylose than cassava flour, sweet potato and Konjac. The higher content of amylose in yam flour could therefore be responsible for its high preference over cassava starch by both FTNE-α-amylase and DTNE-α-amylase in this study, as that connotes the availability of more α-1,4-glycosidic bonds for the enzymes' catalysis.

3.8. Activation energies, substrate and transition state binding energies

The activation energy (Ea), minimum energy required for catalysis to occur, of α-amylase from fresh *C. esculentus* (FTNE-α-amylase) was found to be 3.56 kJ mol^{-1} and that of dried *C. esculentus* α-amylase (DTNE-α-amylase) was 6.81 kJ mol^{-1} (Fig. 5; Table 5), thus the energy barrier

Table 5

Activation energy (Ea), enzyme-substrate (ΔG_{E-S}) and transition state (ΔG_{E-T}) binding energies for *C. esculentus* seeds α-amylases using soluble starch as substrate.

Enzyme source	Ea (KJ mol^{-1})	ΔG_{E-S} (KJ mol^{-1})	ΔG_{E-T} (KJ mol^{-1})
FTNE-α-amylase	3.56	5.34	6.12
DTNE-α-amylase	6.81	−0.90	−2.22

FTNE-α-amylase, α-Amylase from fresh *C. esculentus* seeds; DTNE-α-amylase, α-Amylase from dried *C. esculentus* seeds. Value of activation energy (Ea) was deduced from the slope of the Arrhenius plot in Fig. 5, while the free Gibb's binding energies (ΔG_{E-S} and ΔG_{E-T}) were calculated from Arrhenius equation using K_m and K_{cat} values obtained for soluble starch at the assay temperature (70 °C). The enzyme samples used here were not subjected to heat stress.
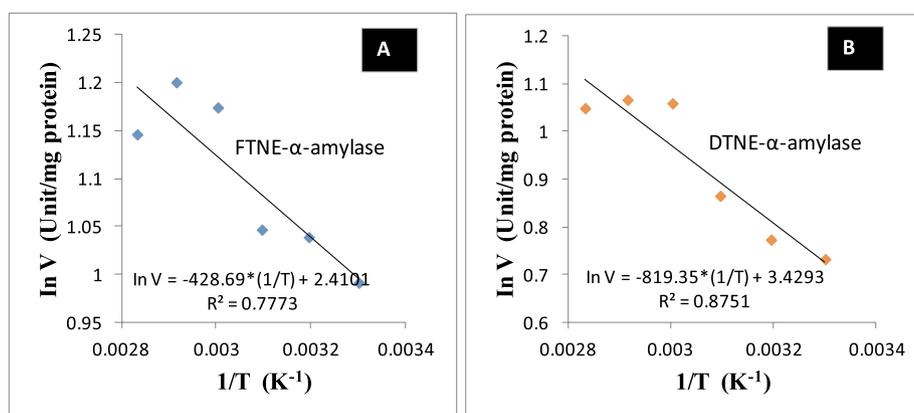

Fig. 5. Activation energies of α-amylase of *C. esculentus* from fresh seeds (A1) and dried seeds (A2). The plots are based on the Arrhenius equation using data obtained from the assay for effect of temperature on enzyme activity at the experimental pH of 6.0. FTNE-α-amylase and DTNE-α-amylase were not subjected to heat stress.

for reactions catalyzed by FTNE-α-amylase would be 1.91 times more easily overcome than those catalyzed by DTNE-α-amylase (Pal and Khanum, 2011; Ferreira et al., 2018; Saleem et al., 2005).

However, the enzyme-substrate free binding energy (ΔG_{E-S}) and enzyme-transition state formation free energy (ΔG_{E-T}) for FTNE-α-amylase were higher/positive (5.34 and 6.12 kJ mol^{-1}, respectively) compared to those of DTNE-α-amylase which were lower/negative (-0.90 and -2.22 kJ mol^{-1}, respectively) (Table 5). The small/negative values of ΔG_{E-S} and ΔG_{E-T} for DTNE-α-amylase show that, once initiated, its catalytic process occurs spontaneously at the temperature investigated (70 °C) without further need for thermal/energy drive as opposed to FTNE-α-amylase with high/positive binding energies (Ferreira et al., 2018). Also, as reported in Table 4, it could be inferred that the high catalytic efficiency (K_{cat}/K_m) of DTNE-α-amylase (for all the substrates tested) was due to its transition state stabilization whereas the low catalytic efficiency of FTNE-α-amylase was due to its transition state destabilization since DTNE-α-amylase required less free energy to form the transition state (ΔG_{E-T}) as well as less amount of energy for substrate binding (ΔG_{E-S}) (making it more stable) than FTNE-α-amylase (Table 5) (Ferreira et al., 2018; Saleem et al., 2005).

3.9. Fourier Transformed infra-red (FTIR) spectra of the enzymes

The FTIR spectra of α-amylases from fresh and dried *C. esculentus* seed in the absence and presence of soluble starch (substrate) are shown in Fig. 6. The N–H stretching vibrations for proteins were observed at 3430 and 3434 cm^{-1} in the α-amylases from fresh (FTNE-α-amylase) and dried (DTNE-α-amylase) *C. esculentus* seeds, respectively (Fig. 6a and b) both in the absence and presence of the substrate. However, the peak corresponding to amide's –N-H bending (1594 cm^{-1}) in FTNE-α-amylase

decreased in intensity due to the interaction with soluble starch followed by a shift from 1594 to 1634 cm^{-1} in the presence of the substrate (FRSM). A similar trend was obtained for DTNE-α-amylase, with an amide –N-H bending peak of 1588 cm^{-1} in the native enzyme increasing to 1640 cm^{-1} in the presence of the substrate (DRSM); a resultant decrease in the intensity of peak was also observed.

The peak corresponding to C–O stretch (1123 cm^{-1}) in the native amylase from the fresh *C. esculentus* seed increased to 1152 cm^{-1} with a concomitant decrease in sharpness in the presence of soluble starch. This trend was also maintained for the interaction between the native amylase from dried *C. esculentus* seed and the substrate, with C–O stretch peak increasing from 1118 to 1177 cm^{-1} plus decline in peak sharpness. The FTIR results reported in this study (Fig. 6) reveal changes in C–O stretching and -N-H bending corresponding to amide vibrations of the peptide linkages in the enzymes (Ji et al., 2018). With the addition of soluble starch, the absorption bands of the enzymes in the infrared spectra shifted when compared with the native enzyme. These results indicate that there was indeed change in the structure of the alpha amylases (FTNE-α-amylase and DTNE-α-amylase) due to their catalytic interaction with soluble starch, the enzymes' substrate (Ji et al., 2018; Abhijeet et al., 2013).

3.9.1. Thermal stabilities of α-amylases from fresh and dried Cyperus esculentus seeds

The deactivation constant (K_d) of both α-amylases from fresh *C. esculentus* seeds (FTNE-α-amylase) and dried *C. esculentus* seeds (DTNE-α-amylase) increased as temperature increased from 60 - 80 °C (Table 6). This suggests that the stability of the enzymes was subject to temperature. The D-value of α-amylase from fresh *C. esculentus* seeds at 80 °C (D value = 71.73 min) suggests that it is more thermally stable compared to α-amylase from dried *C. esculentus* seeds at that temperature (D = 68.12 min) (Fig. 7; Table 6). The D-value of any biocatalyst at a particular temperature provides an idea of the duration of time it would take for that biomolecule to lose 90% of its catalytic activity at that temperature (Savvidou et al., 2018). Other the hand, Z-value refers to a change in temperature in degree Celsius required to induce a 10% decrease in the D-value of an enzyme. The Z-value gives an overall information of an enzyme's thermal resistance or stability; the higher the value the more thermally stable the biocatalyst is and vice versa (de Oliveira et al., 2018). In the present study, α-amylase obtained from fresh *C. esculentus* seeds was more resistant to heat compared to that from dried *C. esculentus* seeds as it has a higher Z-value (Fig. 7; Table 6).

Values of half-lives ($t_{1/2}$) of both enzymes measured also showed that FTNE-α-amylase has a higher half-life value than that of DTNE-α-amylase at 70 and 80 °C. The half-life of an enzyme refers to the interval of time required for half of the enzyme's activity to be lost. The $t_{1/2}$ values reported in this study are in agreement with the D- and Z-values, affirming that FTNE-α-amylase is more thermally stable than DTNE-α-amylase. Hence, although DTNE-α-amylase has a higher catalytic efficiency, FTNE-α-amylase would be more applicable as a starch hydrolyzing enzyme than DTNE-α-amylase in industrial processes where enzyme thermal resistance/stability is of utmost interest.

3.9.2. Thermal deactivation thermodynamics

Gibb's free energy of thermal inactivation ($\Delta G^{\#}$) increased from 91.88 to 97.04 kJ mol^{-1} in FTNE-α-amylase and 93.26 to 96.89 kJ mol^{-1} in DTNE-α-amylase as temperature increased from 60 - 80 °C, whereas enthalpy of deactivation ($\Delta H^{\#}$) decreased for both enzymes. Values of $\Delta H^{\#}$ and $\Delta S^{\#}$ for thermal inactivation of both enzymes are all less than zero (Table 6). Since $\Delta G^{\#}$ is positive ($\Delta G^{\#} > 0$), it suggests that the thermal inactivation processes are not spontaneous, thus additional energy must be inputted for deactivation to occur (Pal and Khanum, 2011). The decreasing values of $\Delta H^{\#}$ during denaturation process with increasing temperature indicate that less energy is required to denature the enzymes at high temperature (Ferreira et al., 2018). The negative values of $\Delta S^{\#}$ in this study, as similarly observed by Kumari et al. (2016)
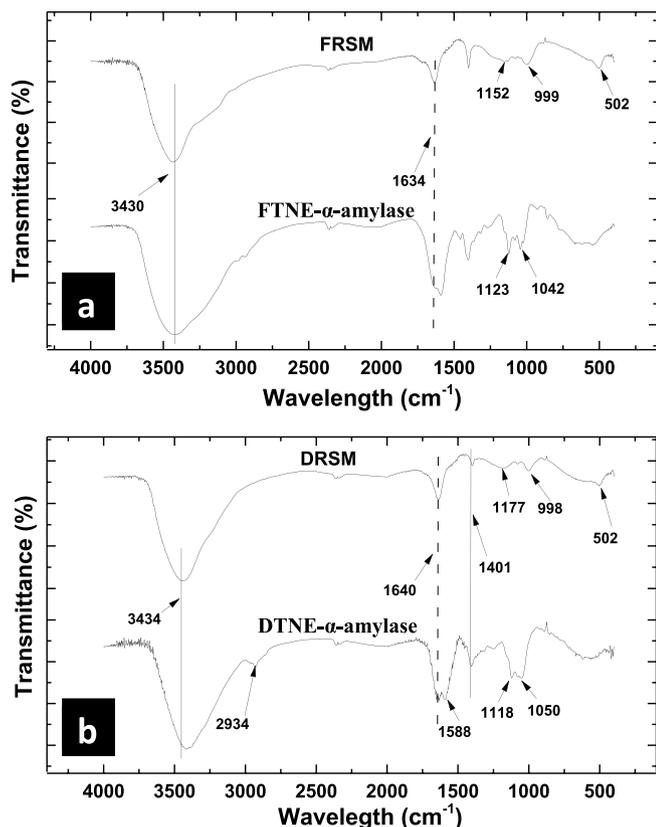
Fig. 6. FTIR spectra showing interaction between *C. esculentus* seed α-amylases and soluble starch used as substrate. (a) Native α-amylase from fresh *C. esculentus* seed (FTNE-α-amylase) and its substrate-bound form (FRSM); (b) Native α-amylase from dried *C. esculentus* seed (DTNE-α-amylase) and its substrate-bound form (DRSM).

Table 6

Thermal stability and thermodynamic properties of fresh and dried *C. esculentus* seeds α-amylases.

Temp. (°C)	K_d (min^{-1})	$t_{1/2}$ (min)	D-value (min)	Z-value (°C)	$\Delta H^{\#}$ (kJ mol^{-1})	$\Delta G^{\#}$ (kJ mol^{-1})	$\Delta S^{\#}$ (kJ mol^{-1})
α-Amylase from fresh *C. esculentus* seeds							
60	0.0268	25.86	85.92	256.41	−2.64	91.88	−0.28
70	0.0287	24.15	80.23		−2.73	94.53	−0.28
80	0.0321	21.59	71.73		−2.81	97.04	−0.28
α-Amylase from dried *C. esculentus* seeds							
60	0.0163	42.52	141.26	63.29	−2.25	93.26	−0.29
70	0.0309	22.43	74.52		−2.33	94.32	−0.28
80	0.0338	20.51	68.12		−2.42	96.89	−0.28

Values of deactivation rate constant (K_d), D-value, and Z-value were deduced from the slopes of the Arrhenius plots in Fig. 7, while the half-lives ($t_{1/2}$), enthalpy of inactivation ($\Delta H^{\#}$), entropy of inactivation ($\Delta S^{\#}$) and Gibb's free energy of inactivation ($\Delta G^{\#}$) were calculated from Eqs. (7) and (11)–(13). The activation energies of deactivation (E_d) of FTNE-α-amylase and DTNE-α-amylase were 1.27×10^{-1} and 5.19×10^{-1} kJ mol^{-1}, respectively (Supplementary data S3).
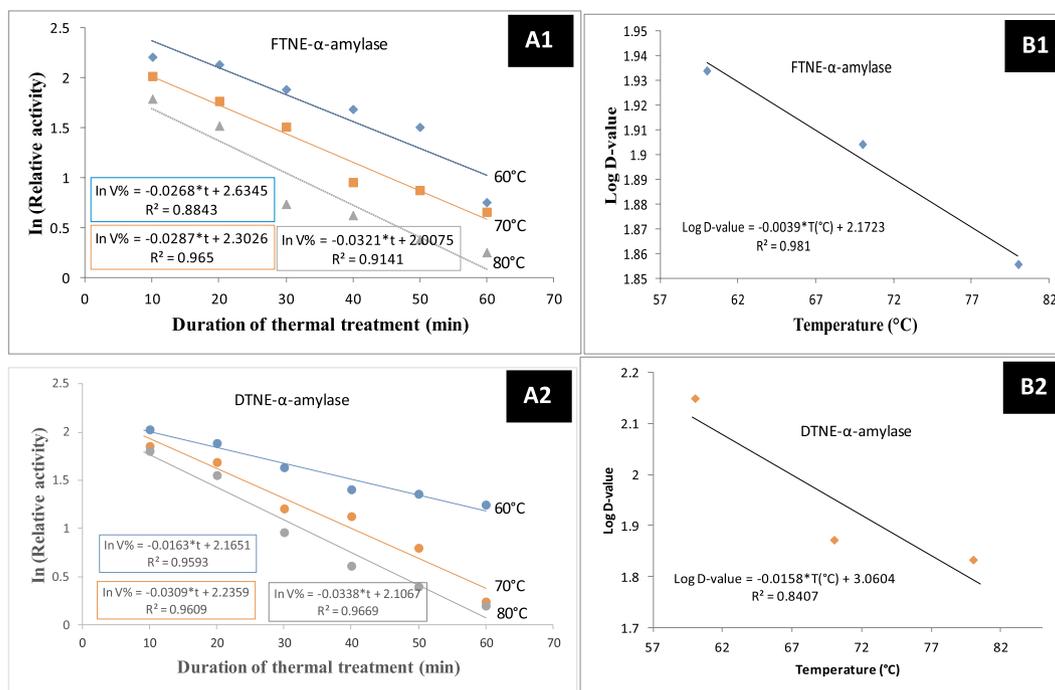

Fig. 7. Thermal deactivation of fresh and dried *C. esculentus* seeds α-amylases and estimation of deactivation rate constant (K_d) (A1,A2); plots of log D values against temperature (°C) for determination of Z value (B1,B2). The plots are based on Arrhenius equation. Here, the enzyme samples were subjected to heat stress at different temperatures (60, 70 and 80 °C) for various durations (10–60 min) before measurement of enzyme activity.

and Pal and Khanum (2011) indicate that there are significant processes of protein aggregation. $\Delta G^{\#}$ values are greater than $\Delta H^{\#}$ values due to the negative entropic contribution in total free energy change of the inactivation process.

4. Conclusion

The α-amylases obtained from dried and fresh *C. esculentus* seeds investigated in this study had their optimum activities in acidic (pH 6.0, DTNE-α-amylase) and neutral pH (pH 7.0, FTNE-α-amylase) conditions, respectively. The activity of alpha amylase from fresh and dried *C. esculentus* seed was optimum at high temperatures between 60 – 70 °C. This suggests that the isolated alpha amylases from both fresh and dried *C. esculentus* seeds are mildly thermophilic in nature. Both enzymes were modulated by metal ions and organic solvents amidst others. Fourier Transformed-Infrared (FTIR) spectra revealed changes in C–O stretching and -N-H bending peaks in enzymes during interactions with starch. Thermal inactivation of the amylases followed a first-order rate kinetics. Alpha amylases from both fresh and dried *C. esculentus* seeds have remarkable thermal inactivation thermodynamic parameters; the thermal inactivation process was non-spontaneous ($\Delta G° > 0$). FTNE-

α-amylase half-lives (24.15 and 21.59 min) and D-values (80.23 and 71.73 min) at 70 and 80 °C, and Z-value (256.41 °C), were higher than in DTNE-α-amylase (Z-value = 63.29 °C) at same experimental conditions. Hence, FTNE-α-amylase is more thermally resistant/stable than DTNE-α-amylase, an implication of its higher Z-value. From the findings of the study, it could be concluded that even though DTNE-α-amylase has a relatively higher catalytic efficiency (K_{cat}/K_m) during starch hydrolysis, FTNE-α-amylase would be more applicable as a starch hydrolyzing enzyme in industrial applications where an enzyme's stability at high temperature is desired.

Conflicts of interest

None.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bcab.2019.101298.

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