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Exploring the Kinetic and Thermodynamic Profiles of Amylase Thermal Inactivation Derived from *Bacillus sp.*

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Abstract: When it concerns optimising biochemical processes in food and its allied industries, evaluating the inactivation kinetic model of an enzyme is germane for their acceptable use. Thermal inactivation kinetics of amylase obtained from *Bacillus sp.* sourced from soil in a Cassava processing site were conducted in this study. Thermal inactivation of the amylase was examined in detail between 40 and 90°C within a specific time frame. This study suggests the possibility of the enzyme structure having a non-sensitive heat fraction, whose stability based on the enzyme activity was evident up to temperatures close to 70ºC. Denaturation of amylase was measured by loss in enzyme activity, which could be described as a first-order monophasic kinetic model, with k-values between 9 $\times 10^{-5}$ to 1.2 $\times 10^{-3}$ min^{-1}. Also, D- and k-values decreased and increased, respectively, with soaring temperature, indicating swift amylase inactivation at higher temperatures. Results suggested that amylase is a relatively thermostable enzyme with a Z-value of 47.39°C and Ea of 24.51 $kJmol^{-1}$. The high values obtained for activation energy (Ea), change in enthalpy (ΔH) which is 21.7 kJ/mol, indicated that a considerably large amount of energy will be needed to denature this amylase, possible due to its stable molecular conformation. Hence, the enzyme is relatively stable for biotechnological applications.

Keywords: Kinetic Studies, Thermodynamics, Thermal Inactivation, Amylase, *Bacillus sp.*, Cassava

1. Introduction

Starch an α-glucan polymer comprises of amylose (15–25%) and amylopectin (75–85%) [1] and has been shown to be recalcitrant and quite toughened to enzymatic cleavage at ambient temperature [2]. However, complete hydrolysis of starch can only be achieved by a cocktail of relevant enzymes working at high temperatures. This condition formerly limited the successful use of enzymes in many biotechnological processes [3], as starch previously, was disintegrated by acid hydrolysis into glucose. For the liquefaction starch, enzymatic degradation through amylases is been preferred over other chemical hydrolysis processes and these enzymes contributes about 25% of total enzymes presently used globally [4]. The major industries that utilize 75% of industrially provided enzymes, are detergents (37%), textiles (12%+), starch (11%), baking (8%), and animal feed (6%) [5, 6]. Amylase is a calcium metalloenzyme reported to originate and produced by several micro-flora and genetically modified *sp*ecies of microbes [6], and it catalyses the first step in the saccharification of starch. Amylase producing micro-flora includes archaea, actinomycetes, bacteria, and fungi [6]. The characterisation of several enzyme isolates has led to overcoming the previous constraint in starch hydrolysis after discovering thermostable amylases working near 100°C [7]. This thermostable enzyme has paved the way for new arenas in industrial enzyme applications [7-9].

It is noteworthy that amylase with higher thermostable properties is obtained from microbial sources, e*sp*ecially amylases from Bacillus *spp*. (B. *sp.*) [10]. B. *sp.* is a Gram-positive bacterium exhibiting morphological variability. It consists of small, plump, round-ended rods that typically occur in pairs or occasionally in short chains. Within slightly swollen sporangia, ellipsoidal endospores are present in central, paracentral, and sub-terminal positions. The colonies of B. sp. are smooth, flat, and butyrous, measuring approximately 1 mm in diameter. They often display irregular edges with pointed projections that can develop into rhizoid structures in mature cultures. The coloration of these colonies is creamy or off-white, featuring opaque centers. These

organisms possess facultative anaerobic characteristics and display positive catalase activity. Generally its optimal growth occurs at 30 and 40°C and between pH 6 and 10.5 [11].

With the growing industrial importance of amylase [12], their significant limitation remains that its activity and stability are primarily at ambient temperature; hence they are easily denatured. Also, naturally occurring thermostable amylases and their bio-engineered alternatives are expensive and not easily accessible to industries in most climes and nations with low GDP and low scientific and research interventions. Hence there are several efforts at finding a more thermostable source of this enzyme, e*sp*ecially from harsh environments where microbes still thrive, just as considered in this study - the soil of a scorched dump site of Cassava effluent waste.

2. Materials and Methods

2.1. Sample Preparation and Reagents

Purified amylases produced by B. *sp.* (which had undergone three-step purification - ammonium sulphate fractionation, ion-selectuve and gel permeation chromatography) were isolated from the soil of a cassava fermentation site in Akure, Nigeria. Geographical coordinates 7.2571° North, and 5.2058° East, were obtained from the Enzymology and Food Biotechnology Laboratory, Federal University of Technology Akure, Nigeria. Most reagents and chemicals used were purchased from Merck (Sigma-Aldrich, USA), while all other sourced chemicals were ensured to be of analytical grade.

2.2. Determination of Amylase Activity

The amylase activity was determined according to the method of Oboh [13] with slight modifications. 1 ml of purified enzyme solution was added to 1 ml of 1% soluble starch in 0.006 M NaCl dissolved in 0.2 M pho *sp.* hate buffer (pH 6.9) and then incubated at 30°C for 30 min. Dinitrosalicylic acid (DNSA), 2 ml was added to stop the reaction, then reaction vessel was incubated for 5 min at 100°C and then cooled by placing in ice bath. The absorbance was taken against a reagent blank using a UV/Vis spectrophotometer set at 540 nm. One unit of amylase activity was defined as the amount of enzyme in 1.0 ml of the purified amylase that produced 1.0 mg of reducing sugar from starch under this standard assay conditions.

2.3. Effect of Temperature on the Activity of Amylase

The enzyme optimum temperature was determined by estimating the amylase activity at pH 7.0 and temperature ranging from 30 - 90°C for 30 mins using the standard assay procedure.

2.4. Thermal Stability of Amylase

The thermal stability of the purified amylase was examined by measuring the residual activity of the amylase relative to its initial activity at 0 min. The purified amylase was incubated at different temperatures between 40 to 90°C at 10°C intervals for 3 hours. Aliquot 0.1 ml of the purified amylase was taken at an interval of 10 mins throughout the incubation period, and the residual activity was determined according to standard assay procedure. Residual activity (%) was calculated relative to the initial activity at 0 min [14].

2.5. Thermal Inactivation and Kinetics

2.5.1. Thermal Inactivation of Amylase

Amylase thermal inactivation was investigated by incubating the purified enzyme solution in Tris-HCl buffer, pH 8, at a temperature ranging from 30 - 90°C using a water bath, followed by the addition of KCN and $Na_2S_2O_3$. Residual activity was therefore calculated relative to the initial activity obtained after 0 min. The first order inactivation constant, (k) was determined from the slope of the first order enzyme inactivation equation:

$$\text{Residual activity (\%)} = \frac{C}{Co} \times 100\% \quad (1)$$

C and Co represent absorbance/minute after the heat treatment at time t and 0, respectively.

2.5.2. Estimation of Kinetic Parameters

The enzyme inactivation of amylase from B. sp. was obtained by Gouzi *et al*. [15].

$$\frac{lnC}{Co} = -kt \quad (2)$$

C denotes enzyme activity at the time, t, C_o is enzyme activity at time 0, k is the rate constant at the process temperature (min^{-1}), and t is time (min). The inactivation rate constant (k) was extrapolated from the slope of the logarithmic plot of residual activity against the treatment time, t.

Half-life is obtained from the equation given below [16].

$$t_{½} = \ln(2)/k \quad (3)$$

D-values show a relationship with the inactivation rate constant (k) and then give equation 4

$$D = \ln(10)/k \quad (4)$$

The Z-value, a temperature required for reduction of the D-value one log unit, is derived by plotting the log D value against the corresponding process temperature. The slope obtained gives the negative reciprocal of the Z-value.

2.5.3. Thermodynamic Analysis for Heat Denaturation

The temperature dependence of the rate constant is described by Arrhenius law in equation (3)

$$\ln(k) = \frac{Ea}{RT} + C \quad (5)$$

Thermodynamic parameters such as Gibbs free energy change [ΔG], the Enthalpy change [ΔH] and the Entropy change would be obtained from activation energy [Ea] and Arrhenius rate constant (k) considering the following

equations:

$$\Delta H = \Delta E - RT \quad (6)$$

$$\Delta G = -RTln(\frac{kh}{KbT}) \quad (7)$$

$$\Delta S = (\Delta H - \Delta G)/T \quad (8)$$

K_b is Boltzmann's constant (1.3806 × 1023 J/K), h is Planck's constant (6.6260×10^{-34} J.s).

3. Results and Discussion

Effect of temperature on the activity of amylase from B. *sp.* isolated from the cassava processing site is described in figure 1. The amylase showed optimum activity between 30 - 40ºC with a very high relative activity of 96 and 95% at 30 and 40ºC, re*sp*ectively.

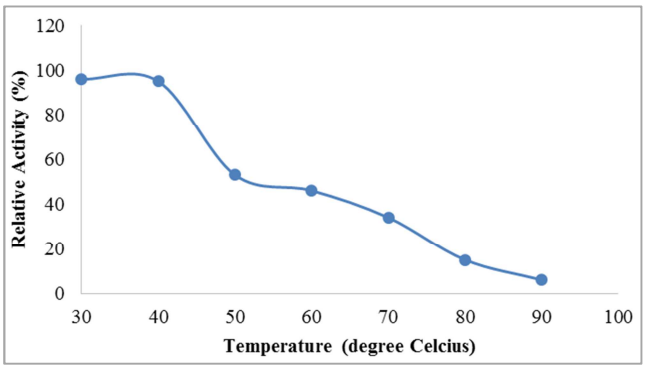

Figure 1. *Effect of temperature on the activity of the purified amylase.*

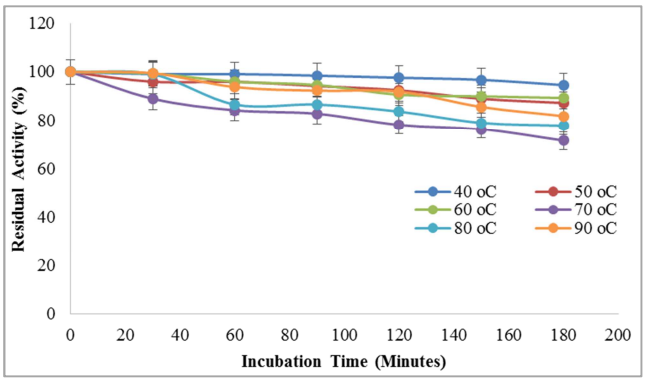

Figure 2. *Thermostability of the amylase isolated from B. sp..*

Further incubation of the amylase with a continual increase in temperature showed a sharp loss of enzyme activities. Several authors have reported that many bacterial amylases have an optimum temperature in the range of 30 – 100°C; however, the activity of this enzyme is observed to significantly decrease as the temperature goes beyond 40°C [17, 18]. Mahdavi et al. [19], in their work on amylase from acid neutralizing Bacillus cereus strain, reported that the optimum temperature of the amylase was identified as 50°C; however, low sensitivity to temperature was observed between 10 – 50°C and a rapid loss of activity over 70°C. However, the effect of temperature on the stability of amylase from *B. sp.* (figure 2) showed the maximum enzyme stability was observed at 40ºC with over 95% residual activity. In contrast, over 80% of activity remained at all other studied temperatures observed after 60 minutes. These findings indicate that amylase from B. *sp.* was thermostable and retained about 70 – 90% residual activity at 40 – 60°C.

These findings were corroborated by Mahdavi et al. [19] studies on B. cereus strain amylase thermostability showed that at 60°C, the amylase retains more than 80% of its activity after 60 mins of incubation but lost 50% of its original activity after 10 mins of incubation at 70°C. After 10 mins, complete inactivation of *B. cereus* amylase was witnessed.

The graph of the log of residual activity against the time of incubation at all temperatures investigated (figure 3) describes the thermal inactivation kinetics of amylase.

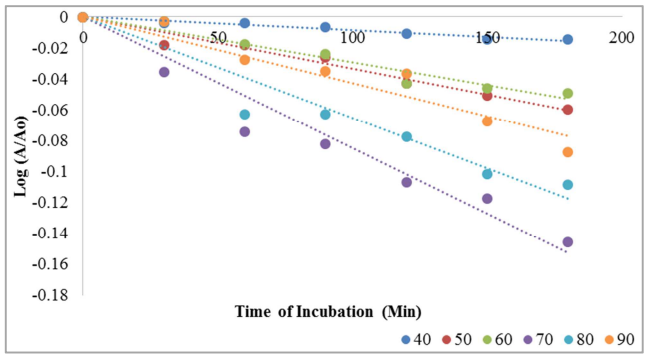

Figure 3. *Thermal inactivation of amylase isolated from B. sp..*

Thermal inactivation kinetics of the studied enzyme indicated that catalysis followed a first-order monophasic kinetic model. This model implies that the rate of this enzymatic reaction depends on the molar concentration of one reactant, and this reaction rate is proportional to the concentration of the reactant. Also, the calculated thermal inactivation rate constant (k_d) values indicate a gradual inactivation of the studied amylase with an increase in temperature, as was observed by the increase in the values of k_d. The lower the value of k_d at higher temperatures, the more stable the enzyme (table 1).

Table 1. *Kinetic values for the inactivation of amylase form B. sp..*

Temp (°C)	K $(min)^{-1}$	$T_{1/2}$ (min)	D (min)	R^2	Log D
40	0.00009	7701.64	25584.3	0.9606	4.407973
50	0.0003	2310.49	7675.28	0.964	3.885094
60	0.0004	1732.87	5756.46	0.9535	3.760156
70	0.0007	990.21	3289.41	0.9165	3.517118
80	0.00095	729.629	2423.77	0.9436	3.384492
90	0.0012	577.623	1918.82	0.9256	3.283034
Z-value (40 - 90°C) = 47.39°C and Ea = 24.51 KJ/mol					

Half-life ($t_{1/2}$) and decimal reduction time (D value) are essential economic parameters in many industrial application enzymes because the higher its value, the higher the enzyme thermostability. The $t_{1/2}$ and D values for amylase obtained from *B. sp.* isolated from the cassava processing site were 2310.5 mins and 7675.3 mins, respectively, at 50 °C (figure 4). These values were relatively higher than PV8891 amylase

studied by Apostolidi *et al*. [20], which reported the $t_{1/2}$ and *D* values of 108.3 min and 359.8 min, respectively at 50 °C. Asoodeh *et al*. [21] also reported $t_{1/2}$ of 138 min at 45 °C for the amylase produced by *Bacillus sp.*. DR90. amylase of *A. awamori* had $t_{1/2}$ and *D* values of 289 min and 959 min, respectively, at 50 °C. In contrast, comparable values were reported for the *A. pullulans* amylase ($t_{1/2}$ 900 mins, *D* value 3096 mins) at the same temperature [22, 23]. This high thermal stability observed by the studied amylase qualifies this *B. sp.* as an extremophile as this enzyme, amongst other molecules they produce, allow them to sustain life in their peculiarly harsh environment.

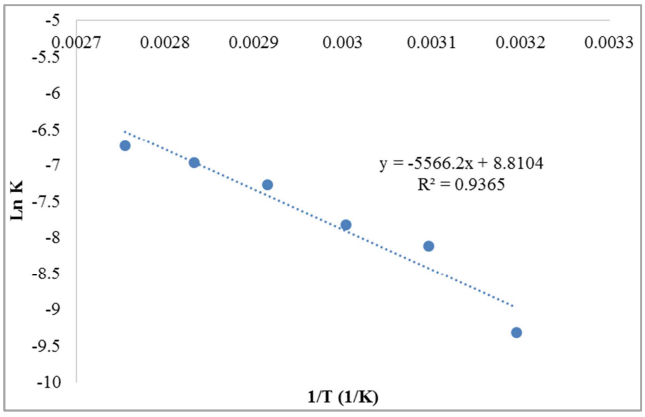

Figure 4. *Temperature dependence of the decimal reduction (D-value) for the amylase.*

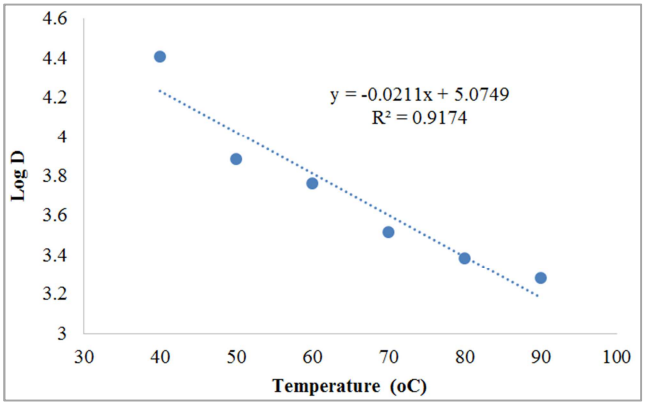

Figure 5. *Arrhenius plot for heat inactivation of the amylase.*

Arrhenius plot obtained from this study (figure 5) calculates the enzyme's activation energy (*E*a) for starch hydrolysis, was 24.5 kJ mol^{-1}. From this studies, the result obtained showed that the dependence of the inactivation rate constants on temperature was fitted into the Arrhenius equation.

The estimated low activation energy (Ea) of the studied amylase suggests that the formation of the activated complex during starch hydrolysis requires less energy, emphasizing its sufficient hydrolytic capacity. A similar *E*a value of 22.8 kJ mol^{-1} was obtained for *B. cereus* [24], while other much lower values of 5.82 and 14.56 kJ mol^{-1} were obtained for *A. awamori* and *A. pullulans*, respectively [20]. amylase in this study also presented a *z* value of 47.39 °C, indicating the degree of temperature that needs to be increased to achieve a tenfold (i.e. 1 log10) reduction in the D-value.

The inactivation of enzymes through thermal processes occurs as a result of the disturbance of non-covalent connections, which includes hydrophobic interactions. The Gibbs free energy (ΔG*) serves as the energy barrier for enzyme inactivation. The enthalpy change (ΔH*) quantifies the number of non-covalent bonds that are broken during the formation of a transition state for enzyme inactivation. Moreover, the entropy change (ΔS*) reflects the extent of disorder in the enzyme caused by structural disruptions [27]. This study calculated the thermodynamic parameters of amylase in the temperature range of 40 – 90°C (table 2). The results obtained for free energy (ΔG) were in the range of 63 - 69 kJ/mol, while the average value of enthalpy change (ΔH) was 21.7 kJ/mol and entropy change (ΔS) was 130.8 J/mol.K.

Table 2. *Thermodynamic parameters of the thermal inactivation of purified alpha amylase.*

	Temp. (°C)	ΔH (J/mol)	ΔS (J/mol.K)	ΔG (J/mol)
	40	21907.7	-131.67	63121.05
	50	21824.6	-131.41	64270.18
	60	21768.9	-129.85	65010.32
	70	21658.3	-130.91	66560.78
	80	21575.2	-130.67	67702.41
	90	21492	-130.44	68841.69
Mean		21704.5	-130.83	65917.74

In the studied temperature range, there was a slight decrease in the ΔH* values (from 21.9 to 21.5 kJ $mole^{-1}$) as the temperature increased. This decrease suggests that less energy was needed to denature the enzyme. At 50 °C, the amylase exhibited a ΔH* value of 21.8 kJ $mole^{-1}$, which is significantly lower than the values reported for the PV8891 amylases [20]. According to Pace [25], the energy required to break a hydrophobic bond by removing a -CH2 group is approximately 5.4 kJ $mole^{-1}$. Based on this estimation, the transition state leading to the inactivation of the studied amylase involved the disruption, on average, of 4.04 non-covalent bonds.

When the structure of an enzyme is disrupted, there is typically an increase in disorder and randomness, as indicated by significant and positive values of entropy change (ΔS*) [26]. However, in the current study, negative ΔS* values were observed, ranging from -131.7 to -130.8 J mol^{-1} K^{-1}, suggesting an even higher level of orderliness in the transition state. Gummadi [27] noted that negative entropy changes are commonly observed in biological systems, particularly in proteins, and that these changes align with the compaction of the enzyme molecule. Nevertheless, such changes could also arise from the formation of charged particles and the associated ordering of solvent molecules. For comparison Δ*S** values of amylases from *A. awamori*, *L. sacchari* TSI-2R, *A. beppuensis* TSSC-1, *A. pullulans* and *Bacillus sp.*. TSSC-3 were reported in the -249.7 $Jmol^{-1}$ $K^{-1} \leq \Delta S^* \leq -118.9$ J mol^{-1} K^{-1} [22, 23, 28, 29].

The evaluation of enzyme stability is better accomplished using the Gibbs free energy of inactivation (ΔG*), which

considers both enthalpic and entropic factors, providing a more accurate and reliable assessment [23]. In the context of thermal inactivation, smaller or negative ΔG* values indicate a more spontaneous process, suggesting reduced enzyme stability and increased susceptibility to thermal inactivation. Conversely, an increase in ΔG* indicates enhanced resistance to thermal inactivation. In the present study, the determined ΔG* values ranged from 63.1 to 68.8 kJ mole^{-1}. As temperature increased from 40 to 90°C, the ΔG* values also increased, signifying that the thermal stabilization of the amylase resulted from the higher usable energy, enabling the enzyme to withstand the unfolding of its transition state [26]. Notably, the amylase produced by *Bacillus sp.* TSSC-3 exhibited a significantly higher ΔG* value (930 kJ mole^{-1}) at 50°C [28] compared to the amylase under investigation. Comparable ΔG* values were reported for the amylase derived from *A. beppuensis* TSSC-1, while lower values were observed for *L. sacchari* TSI-2R, *A. pullulans*, and *A. awamori* amylases [22, 23].

4. Conclusion

The kinetic analysis of the purified amylase demonstrated its stability and spontaneity in catalyzing starch hydrolysis. Furthermore, thermal stability investigations indicated the potential for the purified enzyme to withstand high temperatures, making it suitable for future applications. With its sufficient hydrolytic capacity for starch hydrolysis and promising performance at temperatures comparable to those required in industrial-grade amylases, this enzyme source holds promise for utilization in the starch processing industry.

References

[1] Sarian, F. D., van der Kaaij, R. M., Kralj, S., Wijbenga, D. J., Binnema, D. J, van der Maarel, M. J. & Dijkhuizen, L. (2012). Enzymatic degradation of granular potato starch by *Microbacterium aurum* strain B8.A. *Applied Microbiology and Biotechnology* 93 (2), 645-654.

[2] Bauer, M. W., Driskill, L. E. & Kelly, R. M. (1998). Glycosyl hydrolases from hyperthermophilic microorganisms. *Current Opinion of Biotechnology* 9 (2), 141-145.

[3] Apar, D. K. & Ozbek, B. (2005). alpha-Amylase inactivation during rice starch hydrolysis. *Process Biochemistry* 40 (3–4), 1367-1379.

[4] Adrio, J. L. & Demain, A. L. (2014). Microbial enzymes: Tools for biotechnological processes. *Biomolecules* 4: 117-39.

[5] Das, K., Doley, R. & Mukherjee, A. K. (2004). Purification and biochemical characterization of a thermostable, alkaliphilic, extracellular amylase from Bacillus subtilis DM-03, a strain isolated from the traditional fermented food of India. *Biotechnol Appl Biochem* 40 (Pt3), 291-8.

[6] Savaner, S. & Sohani, S. (2020). Review on Microbial amylase, Types and their Industrial application. *Kala Sarovar* (*UGC Care Group-1 Journal*), 23 (4), 1-8.

[7] Atalah, J., Caceres-Moreno, P., E*sp.*ina, G. & Blamey, J. M. (2019). Thermophiles and the applications of their enzymes as new biocatalysts. *Bioresources Tech*. 280, 478-488.

[8] Ozdemir, S., Fincan, S. A., Karakaya, A. & Enez, B. (2018). A novel raw starch hydrolyzing thermostable amylase produced by newly isolated *Bacillus mojavensis* purification, characterization and usage in starch industries. *Brazilian Archives of Biology and Technology* 61, 1-16.

[9] Sanni, D. M., Jimoh M. B., Fatoki, T. H., Ekundayo, F. O. & Omotoyinbo, O. V. (2017). Isolation of phytate degrading Activity from African giant land snail (*Archachatina marginata*). *J Agric Food Tech*. 7, 1-9.

[10] Dou, S., Naiyu, C., Xinshang, Z., Qingtang, Z., Fei P. & Zhilong, X. (2018). Molecular cloning, expression and biochemical characterization of a novel cold active alpha amylase from *Bacillus sp.p*. Dsh 19-1. *Extremophiles.* 22, 739-749.

[11] Logan, N. A., Lebbe, L., Verhelst, A., Goris, J., Forsyth, G., Rodriguez-Diaz, M., Heyndrickx, M. & De Vos, P. (2004). *Bacillus shackletonii sp.*. nov., from volcanic soil on Candlemas Island, South Sandwich archipelago. *Int. J Systemic Evolutionary Microbiol*. 54, 373-376.

[12] Sarethy, I. P., Saxena, Y. & Kapoor, A. (2013). Amylase produced by *Bacillus sp.*. SI-136 isolated from sodic-alkaline soil for efficient starch desizing. *J Biochemistry Tech.* 4, 604-609.

[13] Oboh, G. (2001). Isolation and characterization of amylase from fermented cassava (*Manihot esculenta* Crantz) wastewater. *Afr J Biotechnology* 4, 1117-1123.

[14] Prakash, O., Jaiswal, N. & Kumar, P. R. (2011). Effect of metal ions, EDTA and sulfhydryl reagents on soybean amylase activity. *Asian J Biochemistry*. 6, 282-290.

[15] Gouzi, H., Depagne, C. & Coradin, T. (2011). Kinetics and Thermodynamics of thermal inactivation of polyphenol oxidase in an Aqueous Extract from Agraricu bi*sp.*horus. *J. Agric. Food Chem.* 60 (1), 500-6.

[16] Deylami, M. Z., Rahman, R. A., Tan, C. P., Bakar, J. & Lasekan, O. O. (2014). Thermodynamics and Kinetics of Thermal Inactivation of Peroxidase from Mangosteen Pericarp (Garcinia mangostana). J. Eng. Sci. Tech. 9 (3), 374-383.

[17] Gupta, R., Gigras, P., Mohapatra, H., Goswami, V. K. & Chauhan, B. (2003). Microbial amylases: a biotechnological per*sp.*ective. *Process Biochem* 38, 1599-1616.

[18] Divakaran, D., Chandran, A. & Pratap, C. R. (2011). Comparative study on production of amylase from bacillus licheniformis strains *Brazilian J. Microbiol* 42, 1397-1404.

[19] Mahdavi, A., Sajedi, R. H., Rassa, M. & Jafarian, V. (2010). Characterization of alpha amylase with broad temperature activity from acid neutralizing *B. cereus* strain. *J. Biotechnology* 103-111.

[20] Apostolidi, M. E., Kalantzi, S. Hatzinikolaou, D. G., Kekos, D. & Mamma, D. (2020). Catalytic and thermodynamic properties of an acidic alpha amylase produced by fungus *paecilomyces variotii* ATHUM 8891. 3 *Biotech* 10 (7).

[21] Asoodeh, A., Alemi, A., Heydari, A. Akbari, J. (2013). Purification and biochemical characterization of an acidophilic amylase from a newly isolated *Bacillus sp.*. DR90. *Extremophiles* 17, 339-348.

[22] Ademakinwa, A. N., Agunbiade, M. O., Ayinla, Z. A. & Agboola, F. K. (2019). Optimization of aqueous two-phase partitioning of *Aureobasidium pullulans* amylase *via* re*sp.*onse surface methodology and investigation of its thermodynamic and kinetic properties. *International J Biological Macromolecoules.* 140, 833-841.

[23] Karam, E. A., Wahab, W. A., Saleh, S. A., Hassan, M. E., Kansoh, A. L. & Esawy, M. A. (2017). Production, immobilization and thermodynamic studies of free and immobilized *Asp. ergillus awamori* amylase. *Int. J Biological Macromolecoules.* 102: 694-703.

[24] Sugumaran, K. R., Ponnusami, V. & Srivastava, S. N. (2012). Partial purification and Thermodynamic analysis of Thermostable amylase from *Bacillus cereus* MTCC 1305. *Int J Pharm Bio Sci* 3, 407-413.

[25] Pace, C. N. (1992). Contribution of the hydrophobic effect to globular protein stability. *J Molecular Biology* 226 (1), 29-35.

[26] de Oliveira, R. L., da Silva, O. S., Converti, A. & Souza, P. T. (2018). Thermodynamic and kinetics studies on pectinase extracted from *Asp.ergillus aculeatus*: free and immobilized enzyme entrapped in alginate beads. *Int. J. Biological Macromolecoules* 115, 1088-1093.

[27] Gummadi, S. (2003). What is the role of thermodynamics on protein stability. *Biotech. and Bioprocess Engineering* 8 (1), 9-18.

[28] Kikani, B. A. & Singh, S. P. (2012). The stability and thermodynamic parameters of a very thermostable and calcium independent alpha-amylase from a newly isolated bacterium, *Anoxybacillus Beppuensis. Process biochemistry* 12, 1791-1798.

[29] Shukla, R. J. & Singh, S. P. (2015). Characteristics and thermodynamics of alpha amylase from thermophilic action bacterium*, laceyella sacchari*. *Process biochemistry* 50 (12).