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RANDOM MUTAGENESIS OF KOJI (*ASPERGILLUS ORYZAE*) TO ENHANCE THE CATALYTIC EFFICIENCY AND THERMOSTABILITY OF GLUCOAMYLASE

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Abstract

Current report explains the effects of γ-ray mutagenesis on kinetics and thermodynamics of stability-function of Koji glucoamylase (GA). It is generally believed that stability and function are inversely proportional. Our results are novel as it reports about the simultaneous improvement in stability and function of glucoamylase through random mutagenesis. *Aspergillus oryzae* strain mutated by 60 kRad γ-rays exposure was termed as M-60(5) and the mutant Koji showed enhanced (2.6 fold) production of GA. The pKa_1 & pKa_2 of active site residues (ASRs) evidenced about carboxyl and imidazole as ionizable groups. Thermostability of mutated GA increased ~2 fold at 55°C. The change in active site conformation due to γ-ray mediated random mutagenesis has improved kinetics of starch hydrolysis; constants for parent GA: K_m (% w/v), V_{max} /K_m & V_{max} (U mg^{-1} protein) for starch hydrolysis at 50°C and pH 5.0 were 0.022, 79.34 & 3606, whereas for mutant GA these were 0.065, 246 & 3785. The ΔH_i of ASRs ionization confirmed that the conformation of mutant "M-60(5)" GA was altered due to mutagenesis. The mutation altered the conformation of the GA active site that contributed to improve the functional energy (ΔG^*) from 99.35 kJ mol^{-1} for parent to 105.62 kJ mol^{-1} for mutant GA, resulted stabilization of the transition state (ΔG^* is the heat content a protein molecule has to resist against thermal unfolding), which made it thermostable and highly efficient in starch hydrolysis.

Key words: Enzyme kinetics, Thermodynamics, Gibbs energy, Half life, Ionization energy, Active site reidues.

Introduction

Glucoamylses are also known as amyloglucosidases (Pavezzi *et al*., 2008). Based on crystallographic analysis, revealing its three dimensional structure, glucoamylase is placed into Glycoside Hydrolase (GH) family 15 of amylases. The trasncribable number of amino acid in this enyzmes is 640. The larger catalytic domain (CD) of the enzyme is present at the N-terminal. The CD of the enzyme comprise of amino acids ranging from 1-470, whereas amino acid ranging from 509-640 is the C-terminal part. The C-terminal part is also known as the starch binding domain (SBD). The SBD and CD are linked by a linker region. A complete linker region is consisting of amino acids ranging from 471-508. The SBD has a bifurcated region that facilitate the strong binding of substrate to the enzyme (Riaz *et al*., 2012). The CD cleaves α-1,4 glycosidic linkages of substrate from the non-reducing side of the glycosidic chains thus releasing D-glucose (Wang *et al*., 2022). Whereas, this enzyme removes the D-glucose in the amylopectin part of substrate molecule, results into hydrolysis of α-1,6 linkage but at lower rate than reaction occurring at α-1,4 linkages (Ibrahim, 2018). No significant role has been reported for the SBD and linker region in the catalytic activity, however the linker region provides structural integrity to the enzyme (Chou *et al*., 2006).

At industrial scale saccharification, glucoamylases from fungal origin i.e. *Aspergillus* spp., are considered the most efficient enzymes (Karim *et al*., 2016). In recent era, *Aspergillus oryzae* has been a core focus in food industry to acquire commercial GA (Kumar & Satyanarayana, 2009). The *A. oryzae* considered as GRAS i.e generally recognized as safe for food and feed industry (Roth *et al*., 2019), however to resolve the issue of low thermostability, unstable pH and slow catalytic activity of fungal GA, generated an urgent demand to develop a novel strain for GA production suitable for food industry (Wang *et al*., 2020).

In order to make the commercial fermentation processes viable the strains improvement for the high yeild of industrial products is highly recomended (Aleem *et al*., 2018). The required strain improvement and modification can be achieved by mutations expecially by exposing the genetic material of the host strain to mutagens (Kumar *et al*., 2015) i.e., Ultra violet, X-rays & γ-rays. Ultra violet & X-rays exhibits much lower effect i.e pyrimidine dimerization (Amar *et al*., 2018). However, γ-rays are considered as highly ionizing and most energetic radiations (Kumar *et al*., 2003). Irradiation by γ-rays may cause some mutations through DNA repair mechanisms within cells (Tayyaba *et al*., 2012). The Koji strain mutagenized by γ-rays presented immense increase in production of α-amylase along with improvement in its thermostability, hence proven that γ-rays might have altered the enzyme structure at catalytic site (Aleem *et al*., 2018).

In our previous study 52 *Aspergillus oryzae* mutants were developed by γ-rays mutagenesis for the hyper production of thermostable α-amylases (Aleem *et al*., 2018), which were further screened for the GA production. Where, Koji mutant M-60(5) was found to be the potent strain for the hyper production of thermostable GA (data not shown). In the present study, mutant M-60(5) strain of *A. oryzae* (super Koji) was exploited to produce thermostable GA. The current report is focused on the kinetic and thermodynamic characterization of GA produced by mutant M-60(5). The report has novelty as it deals with the influence of γ-rays mediated random mutagenesis on the catalytic site of Koji GA, kinetics and thermodynamics of the stability-function.

Materials and Methods

Koji cultures maintenance: The *Aspergillus oryzae* (koji) and its mutant derivative M-60(5) hyper producer of thermostable GA were provided by Industrial Enzymes & Biofuels Group (IEB), Industrial Biotechnology Division, NIBGE (National Institute for Biotechnology and Genetic Engineering), Faisalabad. The cultures were sustained on liquid fungal growth media/potato dextrose agar (PDA) medium at 30°C for 12-15 days until the growth of golden brown spore formation, and stored at 4°C.

Production of glucoamylase: The parent *A. oryzae* and mutant M-60(5) strains were propagated in liquid broth at 30°C, pH 6.5 and Rafhan maize starch was used as substrate (2% w/v) in 10L bioreactor. The cell density of inoculum was measured based on wet packed cells and transferred 0.3% (weight/volume) under aseptic conditions. The biomass (cell mass) was separated by filtering the media by muslin cloth-multipurpose and centrifuged at 13,000 rpm (25,900×*g*) for 25 min at 4°C. The cell free extracts was lyophilized to concentrate the enzyme by method described (Bhatti *et al*., 2007).

Glucoamylase assay: The GA enzyme was tested to determine its catalytic activity. The total reducing sugars was calculated as glucose equivalents where dinitrosalicyclic acid (DNS method) was used to calculate total released sugars (Aleem *et al*., 2018). Briefly, 1 ml of DNS reagent was mixed with 0.4-1.10 ml of quenched reaction mixture (QRM), total volume was made up to 2.1 ml and incubated for 10 min at 100°C. Once cooled, the change in absorbance (ΔA°) was determined at 550 nm. Where, 1 unit of GA activity was considered as the total amount of enzyme required to release 1 μ mol of glucose equivalent min^{-1} from soluble starch under defined assay conditions of temperature and pH. The activity units of GA enzyme were calculated by using the formula:

$$\text{Units/ml/min} = \frac{\Delta A° \text{ x G.S. factor (2.02) x DF x TRM (2.1 ml)}}{\text{Enzyme (0.1 ml) x Time (30 min) x QRM for DNS assay (0.5 ml)}}$$

Where,
ΔA° = Change in optical density (OD) at 550 nm = Experimental 'OD' – Blank 'OD'.
Glucose Standard Factor = G.S. factor = 1.0 OD = 2.02 μmole glucose
QRM = Quenched reaction mixture
DF = Dillution factor
TRM = Total reaction mixture

Protein assay: The total extracellular released proteins were estimated by using Bradford assay. The standard solution in this method was bovine serum albumin (Bonjoch & Tamayo, 2001).

Ammonium sulfate precipitation of GA: The GA from both Koji strains was partially purified by fractional precipitation using Ammonium sulfate as a salt. Briefly, equal volume of GAs (1 ml) produce of both strains were pipetted out in various microfuge tubes ,ammonium sulfate was gradually added to achieve the saturation at 0°C from 20% to 100%. The tubes were then placed in chopped ice for about 4 hours. Afterwards, centrifuged for 15 min and the supernatants were analyzed for the residual GA activity.

Optimum pH, p*K*a & heat of ionization (ΔH_I) of ASRs: To analyze the effect of pH on the GA enzyme activity, the purified enzyme was isolated from both parent and mutant Koji were determined in buffers ranging from pH 3.2−10.4 (Citrate buffer with pH 3.0−6.2, Sorenson's buffer with pH 5.8−7.9 and Glycine-NaOH buffer with pH 8.5−10.7). The experiment was performed at various temperatures (40−55°C). The p*K*a values of ASRs controlling maximum velocity were calculated by Dixon analysis (Dixon & Webb, 1979) by plotting V_{max} versus pH. The lines of 0, +1 and −1 slopes were drawn and pKa_1 and pKa_2 values were obtained from the points where +1 and −1 slope lines intersect on the slope line of 0. To calculate ΔH_I following equation was applied:

$$\Delta H_I = -\text{Slope} \times 2.303R$$

Temperature optimum, temperature quotient (Q_{10}) & activation energy: To determine the optimum temperature, the Koji GA were incubated with 1% soluble starch at temperatures 40°C- 60°C with the difference of 5°C, pH 5.0 (50 mM sodium-acetate buffer) for 40 min. The activation energy (E_a) of GA and it's temperature quotient (Q_{10}) were calculated by Arrhenius plot as defined (Dixon & Webb, 1979).

$$Q_{10} = \text{Antilog}_{\varepsilon} (E \times 10/ RT^2)$$

$$E = E_a = \text{Activation energy}$$

Kinetics of Soluble Starch Hydrolysis: The Michaelis Menten kinetics constants (V_{max}, K_m, and V_{max}/K_m) for soluble starch hydrolysis were determined by heating the mutant and parental GA with substrate concentration from 0.025% to 0.25% (w/v) at 50°C, pH 5.0 as defined (Riaz *et al*., 2012).

Thermodynamics of irreversible thermal stability: The irriversible thermal inactivation was determined by hearting GAs at various temperatures ranging from 45-65°C in a water bath. The aliquots were taken after every 5 min and placed in chopped ice immediately for 30 min. Afterwards, the withdrawn aliquots were examined for the residual GA activity and denaturation rate constant (K_d) was calculated by applying pseudo first order plot. The activation energy for denaturation [$E_{a(d)}$] of GAs was deteremined by Arrhenius plot analysis.

Thermodynamic parameters for irriversible thermal inactivation were determined by the Eyring's absolute rate equation (Eyring & Stearn, 1939).

$$k_{cat} = (k_b T/h)\, e^{(-\Delta H^*/RT)} \cdot e^{(\Delta S^*/R)}$$

where k_b Boltzmann's constant (R/N) = 1.38×10^{-23} J K^{-1}, T (absolute temperature, K), h (Plank's constant) = 6.626×10^{-34}J s, N (Avogadro's number) = 6.02×10^{23} mol^{-1}, R (gas constant) = 8.314 J K^{-1} mol^{-1}, ΔH^* (enthalpy of activation), ΔS^* (entropy of activation).

ΔH^* (Enthalpy of activation) = $E_a - RT$

ΔG^* (Gibbs free energy of activation) = $-RT \ln (k_{cat}\, h/k_b \cdot T)$

ΔS^*(Entropy of activation) = $(\Delta H^* - \Delta G^*)/T$

Results and Discussion

Production & purification of GA enzyme: The Glucoamylase (GA) from parent *A. oryzae* and its mutant named: M-60(5) strain were produced in submerged conditions on maize starch in 10L bioreactor. The GA production of M-60(5) was approximately four times higher (27.2 U ml^{-1}) than parental strain (6.32 U ml^{-1}), while specific activity of mutated GA was increased to 3.4 fold (136.5 U mg^{-1}). The crude GAs from *A.oryzae* parent and Mutant M-60(5) were purified by ammonium sulphate precipitation as described (Riaz *et al*., 2007; Wingfield, 1998). Both GAs were fully soluble upto 30% saturation of ammonium sulfate ($(NH_4)_2SO_4$) at 0°C. Hence, onset of precipitation occurred at 30%, while complete precipitation of both GAs occurred at 80% saturation (Fig. 1). The specific activity of purified GAs from parent and mutant M-60(5) Koji strains was 55.9 and 246.9 U mg^{-1}, however ammonium sulphate precipitation has resultant into 1.54 and 1.81 folds enzymes purification, respectively (Table 1).

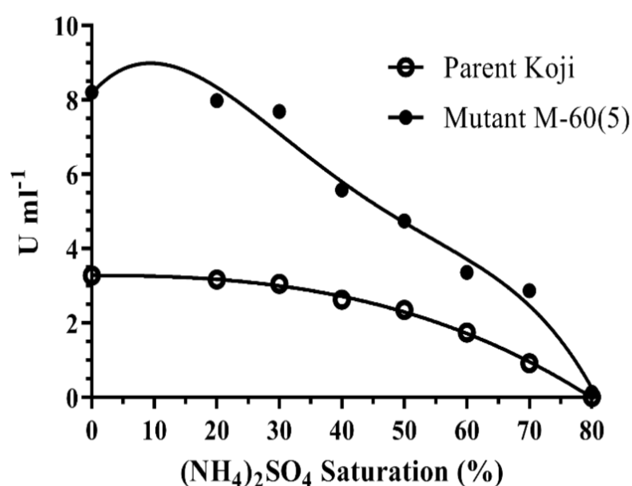

Fig. 1. Partial purification of glucoamylases (GA) produced by parent and mutant M-60(5) Koji strains by ammonium sulfate precipitation.

Effect of pH & enthalpy (ΔH_i) of ASRs ionization: The GAs from *A. oryzae* parent and mutant strains indicated slight difference in pH range i.e 4.0−6.8 and 3.1−6.8, respectively. However, pH 6.0 was recorded as optimum pH for GAs of both strains. The slightly acidic pH range in mutant GA indicated the modifications in microenvironment of the active site. The GA isolated from novel fungus *Gymnoascella citrina* had shown the optimum activity range as 3.4 to 5.5 (Yasin & Rashid, 2019). Jørgensen, *et al*., repoted the fungal GA remains more active at acidic pH (Jørgensen *et al*., 2008). The maximum catalytic activity of this enzyme has also been reported at pH 5 to 6, whereas in *A. Niger* the optimum reported as pH 4.8 (Jafari-Aghdam *et al*., 2005).

The dependence of a chemical shift or enzyme activity upon pH of the reaction is described by *pKa* values (ionization constant). The p*K*a values were determined by Dixon plot (Fig. 2A, B) in order to make *ES**-complex. The values of pKa_1 (proton-donating ionizable group) of mutant and parent GA were 4.68 and 5.4, respectively, while pKa_2 (proton-receiving ionizable group) was 6.8 and 7.3. According to already published studies, GA has one Asp and three Glu residues in the active site (Lee & Paetzel, 2011), and we consider the p*Ka* values of mutant GA are those of the base, Glu 424 and acid, Glu 203. The different p*Ka* values of both parent and mutant GA indicated the alteration in microenvironment around the Glu 203 and Glu 424 as a result of random mutagenesis. Where, imidazole and carboxylic acid were acting as basic and acidic limbs, respectively at 50°C. In previous studies, the presence of one Aspartic acid and three Glutamic acid (Glu) residues as the electron donor in GA active site was reported (Lee & Paetzel, 2011).

Furthermore, the Dixon method (Dixon & Webb, 1979) was applied to determine the effect of temperature on p*K*a values and heat of ionization (ΔH_i) of ionizable ASRs (Fig. 3A, B). The heat of ionization (ΔH_i) for proton donating residue of parent GA was calculated as 1321 cal mol^{-1} and decreased to 1071 cal mol^{-1} for mutant GA; while for proton receiving residue, ΔH_i of parent GA (8178 cal mol^{-1}) was decreased in mutant GA (7574 cal mol^{-1}). The decrease in heat of ionization of ASRs indicated a noticeable change in conformation of ASRs due to random mutagenesis. The ionization energies α-L-amino acids were evaluated previously (Dehareng & Dive, 2004) and reported 3 to 5 conformations for the lysine, arginine, isoleucine, tryptophan and tyrosine, depicting that nature and numbering of ionized molecular orbitals can affect the conformation and values of ionization energies of related amino acids.

Table 1. Purification of glucoamylase isolated from both parent and mutant m-60(5) koji strains by ammonium sulfate pericipitation.

Strain	Treatment	Total activity (Units)	Total protein (mg)	Specific activity (U mg^{-1})	Purification (fold)	Recovery (%)
Parent Koji	Crude	493	13.61	36.2	1.00	100
	Purified	397	7.10	55.9	1.54	81
M-60(5)	Crude	598	4.38	136.5	1.00	100
	Purified	521	2.11	246.9	1.81	87

Where, the crude GA was concentrated by freeze drying before purification. The GA (enzyme) and protein assays were done after dialyzing the samples against distilled water

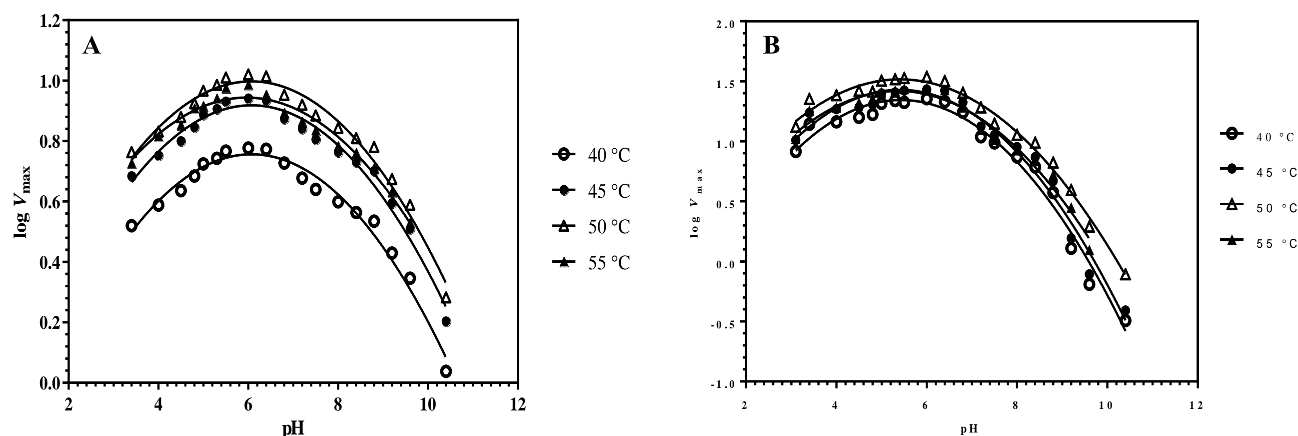

Fig. 2. Determination of p*k*a values of ASRs of GAs by Dixon plots, (**A**): *Aspergillus oryzae* parent, (**B**): *Aspergillus oryzae* M-60(5) indicating maximum velocity for soluble starch hydrolysis at various temperatures.

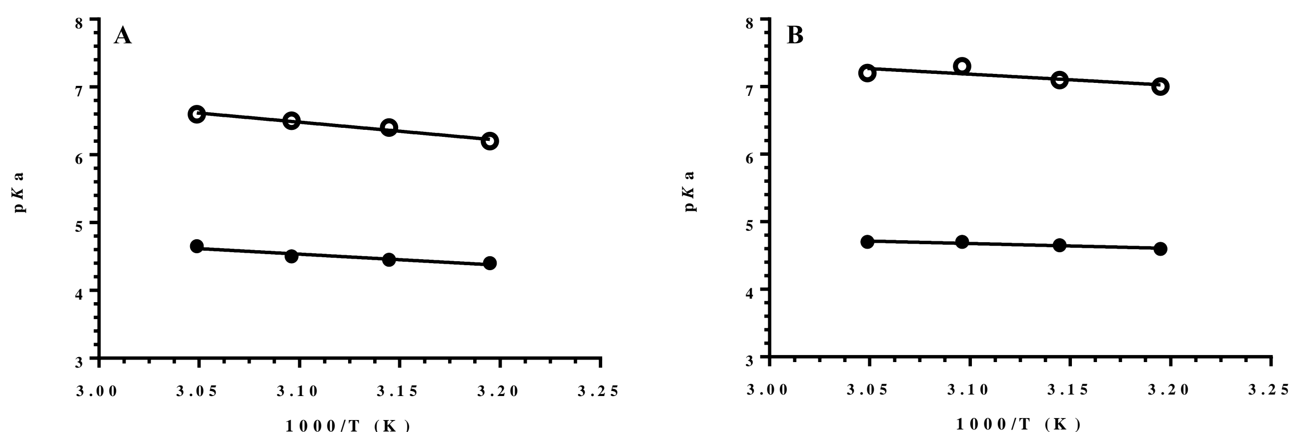

Fig. 3. Dixon plot for the calculating heat of ionization (ΔH_i) of the ASRs of GAs from *Aspergillus oryzae* (A) Parent strain, (B) Mutant: M-60(5).

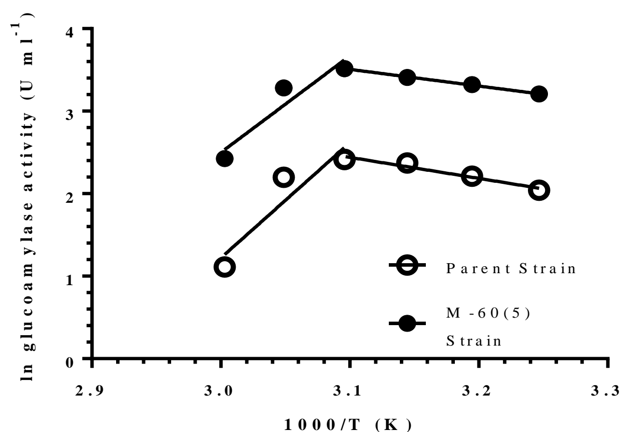

Fig. 4. Determination of activation energy for soluble starch hydrolysis by GA from *Aspergillus oryzae* parent and mutant M-60(5) strains by Arrhenius plot.

Temperature optimum, activation energy & temperature quotient: The working ability of an enzyme at high temperature in the presence of a substrate is termed as thermophilicity whereas working capacity of an enzyme in the absence of a substrate, to resist the thermal unfolding is called thermostability (Georis *et al.*, 2000). The accociation of GA activity with temperature was determined at 40°C to 60°C. The GAs isolated from both parent and mutant strains demonstrated optimum activity at 50°C. The activation energy (*E*a) of parent and mutant GA was 21.2 kJmol^{-1} and 16.7 kJ mol^{-1}, respectively (Fig. 4). The plot confirmed that GAs from parent and mutant strains have similar conformation up to 50°C, whereafter it exhibited a decline. In comparison to parent GA, the mutant enzyme showed lower energy requirement that indicated better efficiency in mutant GA to form the transition-state complex (ES*). The optimum temperature GA from *Lactobacillus amylovorus* ATCC 33621 was reported at 45°C (James *et al.*, 1997). However, it was reported that *Aspergillus niger* NCIM-1248 worked at 60°C to its optimum level whereas the glucoamylase from *Aspergillus niger* hydrolyze starch into reducing sugars at temperature range of 40-60°C and pH 4.5 (Wee *et al.*, 2011). The GA from *Bacillus subtilis* worked optimally for starch hydrolysis at 55°C (Kunamneni & Singh, 2005).

Kinetics & thermodynamics of substrate hydrolysis: To calculate Michaelis Menten kinetic constant, both linear and non-linear regressions determined for soluble starch hydrolysis (Fig. 5). However, the comparisons presented below are based on the constants obtained from non-linear plot (Table 2). The kinetics constants K_m (% w/v), V_{max} (U mg^{-1} protein) &

V_{max}/K_m for starch hydrolysis in parent GA were 0.022, 79.34 & 3606, while the values for mutant GA were 0.065, 246 & 3785, respectively. Hence, the kinetic properties of mutant M-60(5) were improved as compare to those of the parent. The V_{max} of mutant enzyme was increased 3.1 folds than parent.

Previous studies has proved that γ-rays mediated mutagenesis in *A. niger* has improved the kinetic properties of mutant strains where, the V_{max} (U mg^{-1} protein), k_{cat} (s^{-1}) and K_m (mg mL^{-1}) for the *Aspergillus niger* parent GA enzyme were 283, 343 and 0.25, however for mutant GA were 606, 727 and 0.16, respectively (Riaz *et al*., 2012). Similar patterns of improved kinetic properties as a result γ-rays mediated mutagenesis in *A. niger* for lignocellulose hydrolysis by β-glucosidase was published by Javed *et al* (Javed *et al*., 2018). The K_m and V_{max} values for starch hydrolysis by GAs isolated from *Aspergillus flavus* NSH9 were 5.84 mg ml^{-1} and 153.85 U mg^{-1} (Karim *et al*., 2016). The kinetic properties of our mutant M-60(5) GA were observed significantly higher than high salt tolerant *Aspergillus flavus* with K_m & V_{max} values 0.72 mg ml^{-1} and 12.48 μmol/min/mg respectively (Ayodeji *et al*., 2017). The comparison of kinetic properties of GAs from *A. Oryzae* parent and mutant M-60(5), indicated that the mutant GA might be utilized in the industry proficiently.

The enthalpy (ΔH^*) of mutant GA for activated *ES**-complex formation was decreased than that of the parent GA (Table 2). The current finding has indicated that the mutant GA required comparatively low energy to hydrolyze the starch than parent GA. As a result of γ-rays mutagenesis in *A. niger*, the thermodynamics of starch hydrolysis was improved to a significant level with the value ΔH^* for parent as 41.50 kJ mol^{-1} and for mutant as 46.12 kJ mol^{-} (Riaz *et al*., 2012). Hence, we concluded that the mutated GA was thermodynamically more efficient in conversion of soluble starch into final product.

Irreversible thermostability kinetics and thermodynamics: The mutant M-60(5) GA exhibited 4 folds high thermostable behavior than the parent enzyme at 55°C, 2.3 folds at 50°C, 10 folds at 60°C whereas parent enzyme has lost the complete activity completely at 65°C. (Fig. 6 A, B). The increase in thermal stability of M-60(5) GA was due to higher Gibbs free energy. The higher Gibbs free energy facilitated the mutated GA to resist against thermal unfolding of its transition state (U*) into an inactive enzyme.

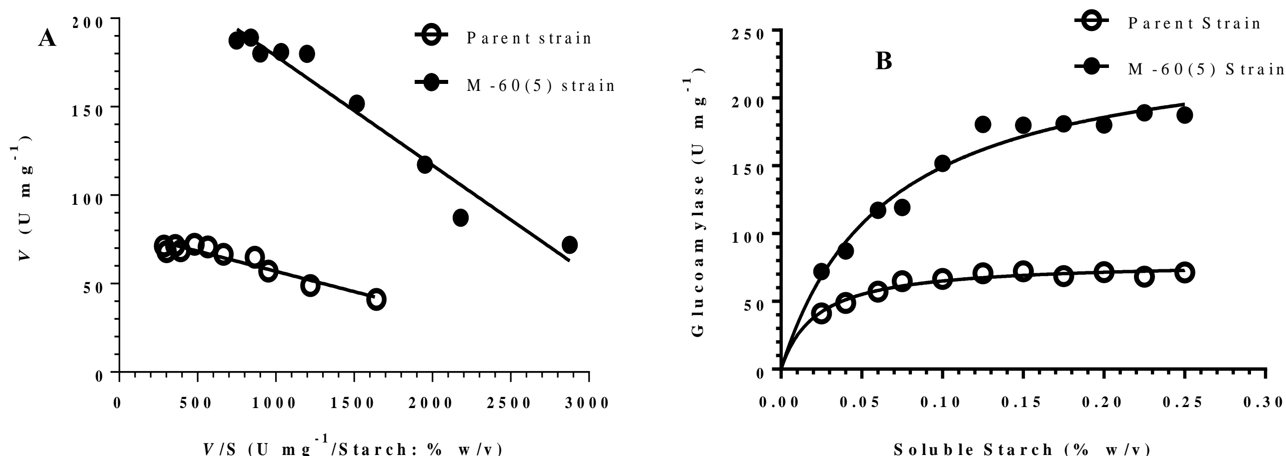

Fig. 5. Michaelis-Menten kinetic (V_{max}, K_m) constants determination for the soluble starch hydrolysis into total reducing sugars by GA from *Aspergillus oryzae* parent and mutant M-60(5) at 50°C, pH 5.0. (**A**) Eadie-Hofstee plot for linear regression (**B**) Non-linear plot - Direct fit model.

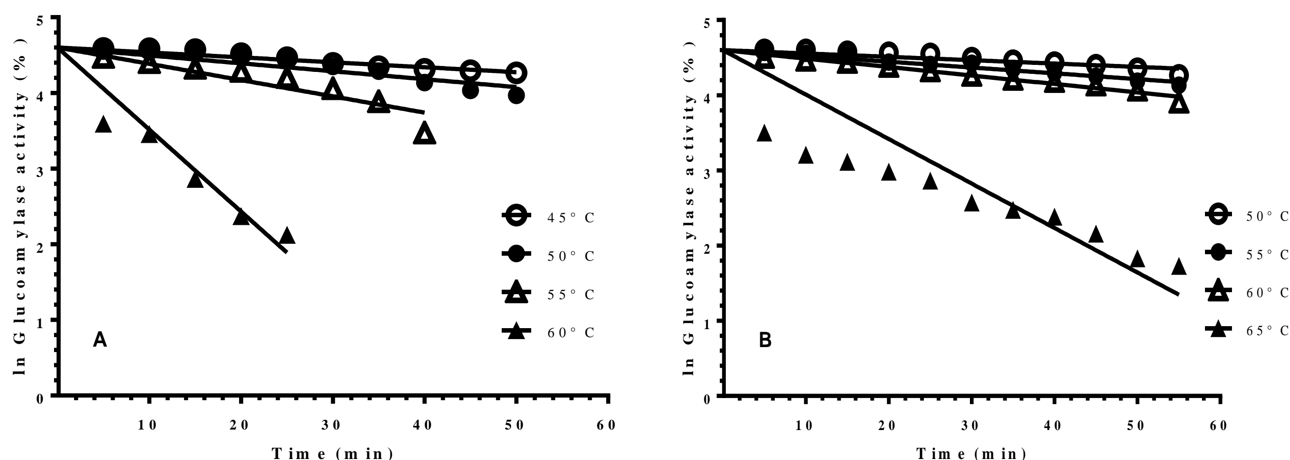

Fig. 6. Irreversible thermal inactivation of GA from (**A**) *Aspergillus oryzae* parent (**B**) *Aspergillus oryzae* mutant M-60(5) at variable temperatures by Pseudo first order plots.

Table 2. Kinetics and thermodynamics of soluble starch hydrolysis by GA produce of *A. oryzae* parent and mutant M-60(5) at 50°C and pH 5.0.

Description	Parent		Mutant M-60(5)	
	Direct fit	Eadie-hofstee	Direct fit	Eadie-hofstee
V_{max} (U mg^{-1})	79.34	80	246	240
K_m (% w/v)	0.022	0.023	0.065	0.062
V_{max}/K_m	3606	3478	3785	3871
ΔH^* (kJ mol^{-1})	18.5	18.5	14.1	14.1

Table 3. Kinetics and thermodynamics of irreversible thermal stability of GA from parent and mutant M-60(5) *A. oryzae*.

Temp. (°C)	Temp. (K)	K_d (min^{-1})		t½ (min)		ΔH^* (kJ mol^{-1})		ΔG^* (kJ mol^{-1})		ΔS^* (J mol^{-1}K^{-1})	
		P	M	P	M	P	M	P	M	P	M
45	318	0.0065	ND	106	ND	157.98	ND	102.19	ND	175.46	ND
50	323	0.0104	0.0045	66	155	157.94	146.72	102.56	104.85	171.45	129.63
55	328	0.0321	0.0077	32	90	157.90	146.68	102.23	105.02	169.74	127.00
60	333	0.1256	0.011	6	62	157.86	146.62	99.35	105.62	175.71	123.17
65	338	ND	0.059	ND	12	ND	146.59	ND	102.58	ND	130.20

Where, P = Parent GA; M = Mutant M-60(5) GA. The $Ea_{(d)}$ of parent and mutated GAs was 160 and 149 kJ mol^{-1}, respectively.
The ND = Not determined

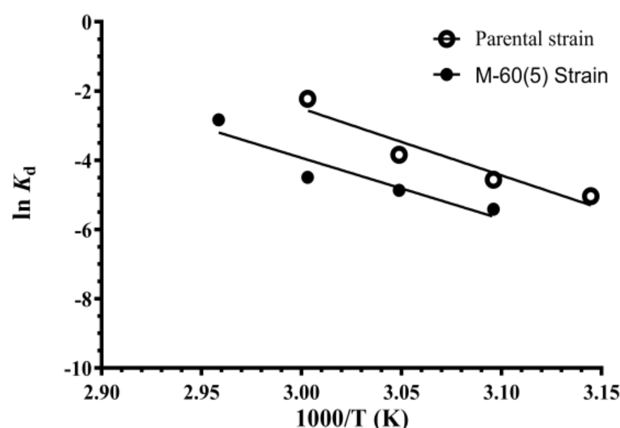

Fig. 7. Activation energy determination for irreversible thermal denaturation of GA enzyme from *Aspergillus oryzae* parent and mutant M-60(5) strains by Arrhenius plot.

Moreover, mutant GA required comparitively low activation energy $E_{a(d)}$ to make the transition state 'U*' than parental enzyme, indicated higher flexibility of the mutated GA for thermal unfolding (Fig. 7). The mutant GA required 149 J.mole^{-1} of activation energy (E_{ad}) whereas parent GA required 160 kJ mol^{-1} to achieve transition state between folded and unfolded state. The mutant GA exhibited low activation energy than parent GA, indicated that the mutation has created conformational change in active site of enzyme and made it more flexible. The change in entropy (ΔS^*) of mutated GA at temperatures ranging from 45°C- 65°C was lower than the parental GA, proved that its active site was more ordered (Table 3). It was concluded that an increase in thermostability of mutant GA was due to higher ΔG^* and entropically driven.

Conclusions

γ-Ray-mediated mutagenesis of *A. oryzae* resulted in enhanced thermostability, catalytic efficiency and productivity, indicating that mutation changed the microenvironment of GA active site, resulting into modification in active site conformation of mutant M-60(5) GA. The improved p*K*a values and heat of ionization (ΔH_i) of basic & acidic limbs of the ASRs of mutated GA confirms the alteration of active site conformation. The increase in thermostability of mutant GA is due to its higher ΔG^* and is entropically driven. We concluded that stability-function of enzymes may be simultaneously enhanced by strain improvement through γ-Ray treatment. The mutated GA, due to its high catalytic efficiency and thermostability, proved that it has high potential for application in food industry such as beverages, baking and starch saccharification, etc.

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