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# GLUCOAMYLASE PRODUCED FROM INDIGENOUS FUNGAL STRAIN HUMICOLA INSOLENS

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**Article Info**



***Abstract***

*In the present study, glucoamylase (GA) was produced from Humicola insolens by submerged fermentation. Maximum GA production (1.002 U/mL) and protein (0.12921mg/mL) was observed under optimum conditions of temperature (45 °C), pH (5.0), substrate concentration (4 % w/v) and inoculum density (5%). The enzyme was purified using ion exchange and gel filtration column with 10 fold purity and 7 % recovery. The apparent subunit molecular weight was 57 kDa. The number of isoforms of the GA was determined by running PAGE. The optimum temperature for GA activity was 45ºC at pH 5.4. An energy of activation (Ea ) of 26.52 kJ mol-1 was required for the formation of an activated enzyme- substrate complex at 45 ºC. KD at 65 ºC= 0.005423, ΔS = 0.273 kJ mol-1 K-1 , ΔG* = 70.95 kJ mol-1, ΔH=23.88 kJ mol-1 and its half-life =36 (min) at 55ºC. Enzyme was chemically modified by using succinic anhydride and aniline for the modification of amino group and surface carboxylic group. The data revealed that the modified forms of GA had deviation from the energy of activation than the native one. The amino group modified form has large value of Ea as compared to native enzyme whereas both forms showed the same optimum pH, 6.2 at optimum temperatures and showed stability in pH range of 5.4-7.2. The pH range of modified Ani-60 and succi-130 was higher than that of native. The values of Vmax, Km and kcat (s-1) for native were 34.78 U mg-1 protein, 4.76 mg starch ml-1 and 32.75 s-1 at 45 ºC respectively. The specificity constant, kcat/Km was 6.87. For amino group modified GA, the values of Vmax, Km and kcat were 76.92 U mg- 1 protein min-1, 2.17 mg starch ml-1 and 73.74 s-1 respectively. The specificity constant, kcat/Km was 31.74.*

**Keywords:** *Glucoamylase, chemical modification, characterization, Humicola insolens*

## Introduction

Amylases are the enzymes that hydrolyze starch and based on their mode of action are classified as Endo amylases, Ex amylases, debranching enzymes and transferases [4]. Starch is, after cellulose, the most abundant heterogeneous polysaccharide obtained by plants [1] mainly plant tuber and grains (Vanier et al.2017). It is present in the form of water insoluble granules and is the primary source of energy for most organisms on earth. It is composed of amylose and amylopectin, exclusively composed of D-glucose with α-(1→4) linkages in a linear amylose and α-(1→4) linkages and ∼5% α-(1→6) branch linkages in amylopectin[1]. As an important industrial feedstock, starch and its hydrolysates have many applications in food and non-food industries (Adewale et al., 2022) and in the paper, textile, pharmaceutical, cosmetics, and packaging industries (Hashim, 2020; Maniglia et al., 2021)

Glucoamylase is one of the three best known amylases, has particularly received industrial usefulness in starch degradation to yield soluble sugars widely used by many food industries for the production of glucose and fructose syrups and bioethanol. It is said to be the most important industrial enzyme because of its widespread uses together with α-amylases and debranching enzymes in the saccharification of starch [5]. Glucoamylase (α-1,4-glucan glucohydrolase, amyl glucosidase, EC 3.2.1.3) is used as a catalyst in different industries like food, textile and detergent industry to arbitrate the industrial process without being consumed in the whole practice [1]. Glucoamylase has the ability to degrade starch into β-D-glucose by attacking at 1-4 glycosidic or 1-6 glycosidic linkages from non-reducing ends of oligo and polysaccharides chains.(2) via an anomer-retaining reaction mechanism (Saburi et al., 2015).

These enzymes can be obtained from animals, plants and microorganisms. However, enzymes produced by microorganisms are more preferable to plant and animal-based ones owing to their high yield, reliability, higher stability, possibility of product modification and optimization, economic feasibility and regular supply because of the absence of seasonal fluctuations, a rapid growth of microbes on low cost media, ease of cultivation in large fermenters and greater catalytic activity [6]. Among fungal strains, *A. Niger* has been considered as most reliable and has got GRAS (Generally Recognized as Safe) status for many reasons for being fit for human usage, protein production and the most important one, the ability to produce large amount of enzyme for food and feed industry [7]. The major advantages of using microorganisms for production of amylases are in economical bulk production capacity and microbes are also easy to manipulate to obtain enzyme of desired characteristics. Alpha amylase can be derived from various sources such as plants, animals and microorganisms. Fungus produces α-glucosidases as Endo cellular and extracellular enzymes depending upon their ambient environment (Ichikawa et al., 2021)

*A. Niger*, filamentous fungus has ability to secrete hydrolytic enzymes, amylase, glucoamylase and- glucosidase which have the ability to hydrolyzes starch into glucose (Guo et al., 2021). Although fungal GAs are commonly employed for these purposes, they have limitations such as their low thermostability and an acidic pH requirement. Alternatively, GAs derived from prokaryotic organisms are a

good option to save costs as they exhibit greater thermostability compared to fungal GAs. Currently, commercial glucoamylases are mainly obtained from filamentous fungi, such as *Aspergillus Niger, Rhizopus niveous, and R. delemar*, displaying moderate thermostability and slow catalytic activities (3,4).

## MATERIALS AND METHODS

A fungal strain, *Humicola insolens* was collected from the Environmental Biotechnology Division, National Institute of Biotechnology and Genetic Engineering, Faisalabad, Punjab, Pakistan. *Humicola insolens* culture was maintained by Potato-dextrose agar medium (PDA) slants and Petri plates. PDA was prepared by mixing 1.5 g agar, 25 g peeled potato and 1g glucose (%w/v) medium in test tubes and Petri plates then autoclaved for 15 min at 121 ℃ and left them until solidified at room temperature. The strain was shifted to PDA in aseptic conditions and kept at 45℃ for propagation for 7 days. As brownish black mature spores of *Humicola insolens* were seen, collected and stored at 4℃ and refreshed fortnightly.

### Inoculum reparation

For inoculums preparation, 250 mL Erlenmeyer flasks were taken and filled to one third part with Eigen and Push medium maintained at pH 5.0. Eigen and Push medium was prepared by mixing weighed amount of 0.5g/L KCl, 0.5. g/L $(NH_4)_2SO_4$, 0.2g/L $MgSO_4$, 0.1g/L $CaCl_2$, 1g/L $KH_2PO_4$, 0.5g/L yeast extract , 20% urea and 2% glucose, pH was maintained at 5.0 by using 1M NaOH/ 1M HCl. It was transferred to 250 mL Erlenmeyer flasks (80 mL). For uniform fungal growth and breakage of mycelia, five glass beads were also added in each flask. The flasks were cotton plugged, covered with aluminum foil and autoclaved at 121℃ for 20 min to avoid contamination factors. 2% (w/v) glucose solution was prepared in distilled water and autoclaved at 120℃ for 5 min. 20% urea was also prepared and autoclaved separately at 120℃ for 15 mi n. One loop full of spores from mature slants was added to each flask under aseptic conditions containing 2% glucose and 20% urea. Flasks were incubated in orbital shaker at 45℃ at 120 rpm for four days. Then flasks were harvested, and inoculum was shifted to growth medium.

### Glucoamylase assay

Glucoamylase activity, µmol of glucose produced per unit mL per min was calculated by the method described by Iqbal *et al*. (2003). Calculated amount of enzyme was taken in test tube and mixed
it with soluble starch solution 1% (w/v) in acetate buffer and incubated at 40 ℃ for 40 min. After that the

reaction of the enzyme with starch was quenched by putting test tubes in boiling water for only 5 min and cooled them in ice cold water. From this reaction mixture, 100 µl was taken and allowed it to react with 2mL from glucose peroxidase kit (Biomega, Germany). Total volume of reaction mixture (2.1mL) was kept constant during all the experimentation. The color intensity was noted at 500 nm.

**Production of GA at optimum conditions**

Production of glucoamylase (GA**)** was done by optimizing the growth conditions in Erlenmeyer flasks of
250 mL in Eigen and Push medium (80 mL per flask) at 45℃, pH 5.0, 5% (w/v) inoculum size, 5% (w/v)

wheat bran by placing in orbital shaker. All the flasks were harvested at once after 72 h of incubation and combined in one-liter flasks.

**Isolation of enzyme from growth medium**

GA was isolated from the growth medium by filtration through muslin cloth and filtrate was collected for further purification process.

**Purification of Glucoamylase**

Partial purification of GA was done by using ammonium sulphate. For this purpose, ammonium sulphate saturation concentration was also optimized. Different concentrations of ammonium sulphate (0-100%)
were added in 1mL crude enzyme in Eppendorf, mixed gently and stored at 4℃ overnight. Centrifuged at

12,000 rpm for 15 minutes. Pallet and supernatant were separated and supernatant was assayed for residual enzyme activity. The concentration which completely precipitated the enzyme protein was selected for. The optimized concentration of ammonium sulphate was added crystal by crystal in crude enzyme by keeping
the flask at ice bath with continuous shaking then kept it over night at 4℃. Next day the crude enzyme

was centrifuged, pallets and supernatant were collected in separate flasks. Pallets were dissolved in small quantity of distilled water and dialyzed against distilled water in cellulose dialyzing tubing of 2 inch diameter at 4℃ for 15 h. Dialyzed enzyme was collected.

**Characterization of glucoamylase**

Physiochemical, kinetic and thermodynamic studies of partially purified GA were carried out as mentioned below.

**Temperature, Energy of activation and pH optimization for glucoamylase assay**

GA assay temperature was optimized at variable pH ranging from 3.0-8.2 with increment of 0.4. Enzyme

was incubated at various temperatures, 30-65 ℃ to optimize the enzyme assay temperature. Assay

procedure was same as described earlier. Energy of activation of GA for starch hydrolysis was determined using Arrhenius plot (Siddique *et al*., 1997; Rashid & Siddique, 1997). Optimum pH for GA was determined at variable temperature in a range of pH 3.0-8.2 with increment of 0.4. Different combinations of buffer were used; glycine-HCl buffer (3.0-3.8), Na-acetate buffer (4.2-5.8) and Phosphate buffer (6.2- 8.2). Dixon plot was used for the determination of pKa for active site of the enzyme (Hakamada *et al*., 2000; Jabbar *et al*., 2008).

**Effect of substrate**

Stock solution of 1% soluble starch was prepared in Na-acetate buffer of pH 5.0. 100-1300 µl of starch were taken in test tubes with 200 µl of GA and final volume was maintained to 2.1mL by adding ΔG*E-T(free energy of substrate binding)= -RTlnKa ---5 Where,

acetate buffer and same enzyme activity was calculated. Michelis-Menten constants (Vmax, Km, kcat) were determined as described by Siddique *et al*. 1997a.

**Thermodynamic studies for starch hydrolysis**

Thermodynamic parameters (ΔH, ΔG, ΔS) were determined by rearranging the Erying's absolute rate equation (Eyring & Stearn, 1939).

$k_{cat}=(k_bT/h)e^{(-\Delta H/RT)}.e^{(\Delta S^*/R)}$ ---------- 1

$\Delta H = E_a - RT$---------- 2

ΔG (Free energy of Activation) = -RT ln (kcat h/kb. T) _____(3) ΔS = (ΔH* - ΔG*)/ T ____(4)

Substrate free energy of binding and transition state formation was calculated by using following

derivation

$K_a = 1/K_m$

$\Delta G^*_{E-T}$ (free energy for transition state formation) = $-RT \ln/k_{cat}/K_m)$ ---------- (6)

$K_d=(k_bT/h)e^{(-\Delta H/RT)}.e^{(\Delta S^*/R)}$ (7)

Few modifications were carried out in equation 3 and 4, $E_a$ was replaced by $E_d$, $k_{cat}$ by $k_d$ and other thermodynamic parameters (ΔS*, ΔG*, ΔH*) were calculated by using the equation 2, 3 and 4.

## Chemical modification of glucoamylase

Glucoamylase was coupled with aniline and succinic anhydride for chemical modification of carboxyl and amino group modification respectively. Carboxylic group of GA was modified by using 25 mM aniline hydrochloride as nucleophile (Bhatti *et al*., 2007; Rashid *et al*., 1998). The purified enzyme was dissolved in 20 mM $KH_2PO_4$ buffer at pH 5.5. 50 mM glucose was added as competitive inhibitor, used for the protection of active site carboxylic groups in the presence of 50mM EDC. Aliquots were taken after various time periods (0, 5,10,15,20,25,30,35,40 and 65 minutes) and reaction was quenched by adding equivalent volume of 0.5M Na-acetate buffer having pH 5.5. Aliquots were dialyzed against distilled water using dialyzing tube and enzyme activity was determined. Succinic anhydride as an electrophile was used for the modification of amino groups of GA (Klotz, 1967). 30mM succinic anhydride solution was prepared, added to the enzyme at pH 7.0 with 2.0 M NaOH and incubated at room temperature. Various time course aliquots (30, 50, 70, 90, 110, 130 min) were taken out. Then dialyzed against distilled water by using dialysis tubing. Volume of chemically modified GA was measured after dialysis. Modified enzyme having abrupt change in activity was selected and prepared for determination of stability function

relationship.

## RESULTS AND DISCUSSION

### Purification and molecular mass determination

Glucoamylase was produced by *Humicola insolens* at optimized conditions, 45 ℃ temperature, 5.0 pH, 4%

inoculum density under submerged fermentation conditions for 72 hours. It was processed for partial purification by using ammonium sulphate precipitation. The precipitation of glucoamylase was started at
30% saturation of ammonium sulphate at 0 ºC and completed at 80% saturation at 0℃. Purification factor

after partial purification procedure was 1.0 and % recovery was 76, while specific activity was 18.6 U $mg^{-}$

[1]. Then it was processed through ion exchange column and gel filtration column by using DEAE-Cellulose and Sephadex G-100 for purification . GA was purified with 10 fold purity and 7 % recovery by three steps of purification. The molecular mass of GA produced from *Humicola insolens* was determined by native-PAGE. The apparent sub unit molecular weight was 57 kDa. The number of isoforms of the GA was determined by running SDS-PAGE. The position of band at the native gel given clue that the enzyme was polymeric in nature. The apparent sub unit

molecular weight was 57 kDa. Optimum temperature for GAs
from *Humicola insolens* was found 45°C for best enzyme activity. The Arrhenius plot for *Humicola insolens*

was monophasic to make ES* complex and GA required 23.52 kJ/mol energy. Low energy of activation reflected that the enzyme conveniently convert the substrate into product and more efficient than produced from *Thermocycles lanuginose* and *Arachnoids sp.* The result of the study was very close to the previous
study as 21.09 kJ/mol energy of activation for ES* complex was reported for *Humicola sp.* at 55°C by Riaz

*et al.*(2007). GA produced by *Humicola insolens* had pH optimum of 5.4 at 45°C. It showed stability in pH

range of 4.6-6.6. Most of the GAs extracted from fungal sources have optimum pH values ranging from 4- 6 (Marlida *et al*., 2000; Bhatti *et al.*, 2007; Niaz *et al*., 2004).

**Characterization of Glucoamylase**

Optimum temperature for GA activity was 45 °C at pH 5.4. Biphasic trend was observed in Arrhenius plot
for determination of activation energy (Fig. 1) $E_a$ of 26.52 kJ.mol$^{-1}$ was required for the formation of activated enzyme-substrate complex. Optimum pH was obtained 5.4 at 45°C . Enzyme showed stability in

pH range of 4.6-6.6. The p*K*a of ionizable groups of active site residues was found by Dixon plot (Fig. 2). The values of p$K_{a1}$ and p$K_{a2}$ at optimum conditions were 4.98 and 6.68 respectively. Arrhenius plot was

used for the measurement of activation energy for thermal inactivation (Fig. 3). $K_d$ was maximum at 65°C,

i.e. 0.005423. At 55 ºC, the value of $\Delta S^*$ = 0.273 J mol$^{-1}$ K$^{-1}$, $\Delta G^*$ = 70.95 kJ mol$^{-1}$ and its half-life 36 (min)(Table 1).

The values of $V_{max,}$ $K_m$ and catalytic events ($k_{cat}$) were 34.48 Umg$^{1}$ protein, 4.76 mg.mL$^{-1}$ and 32.75 s$^{-1}$, respectively. The value of specificity constant ($k_{cat}/K_m$) was 6.87. The results are closer to the earlier studies. Riaz *et al.* (2007) studied the kinetics of GA from *Humicola sp*. and reported $V_{max}$ = 69 U mg$^{-1}$ protein and $k_{cat}$ = 69 s$^{-1}$ value. $K_m$ and $E_a$ values are in line with calculated for GA extracted from *Fusarium solani* ( Bhatti *et al., 2007c).*

Thermodynamic parameters determined for native GA , ($\Delta H^*$, $\Delta G^*$, $\Delta S^*$ and $\Delta G^*_{(E-T)}$) were lower than determined for GA extracted from *Fusarium solani* but value for ($\Delta G^*_{E-S}$) was greater (Bhatti *et al,*. 2007c). The lower value of enthalpy depicted that the formation of activated complex between the enzyme and substrate is more feasible. Less enthalpy value of GA i.e. 23.88 kJ mole$^{-1}$ produced by *Humicola insolens* than produced from *Gymnoascella citrine*, i.e. 35.5 kJ mole$^{-1}$ showed that formation of activated complex between enzyme and substrate was very feasible. Riaz *et al*., (2007) reported thermodynamics parameters, ($\Delta H^*$, $\Delta G^*$, $\Delta S^*$, $\Delta G^*_{E-S}$ and $\Delta G^*_{E-T}$ for GA from *Humicola sp.* 36.72, 61.27,- 73.73, -1.12 and -21.71 respectively.

A B

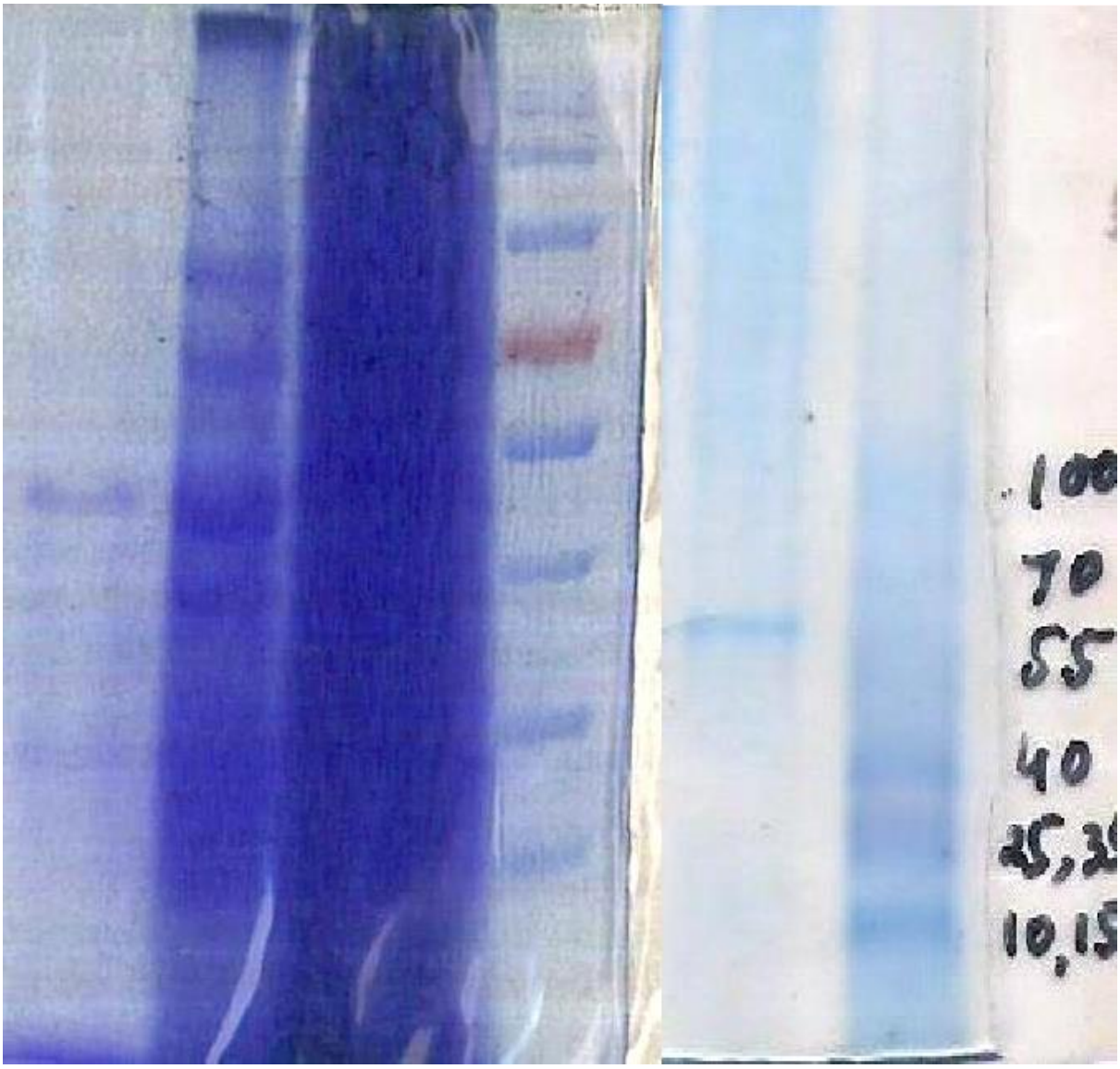



**Fig. A:** Native PAGE lane A marker protein, Lane B crude enzyme Lane C ammonium sulphate purified, Lane D purified

**Fig. B:** SDS PAGE Lane A protein marker, Lane B purified GA

**Characterization of chemically modified forms of Glucoamylase**

The cross-linking of aniline nucleophile to carboxyl groups present at the surface of GA shows no effect at optimum temperature for GA as compared to the native enzyme but the amino group modification

by succinic anhydride shifted the optimum temperature 10 ℃ for GA and it was 55℃. Energy of

activation for the modified Ani-60 and Succi-130 was 147.24 kJmol$^{-1}$and 34.05 kJmol$^{-1}$, calculated by Arrhenius plot. Carboxyl group modified i.e. Ani-60 GA and Succi-130 showed the same pH optimum of
6.2 at optimum temperatures and showed stability in pH range of 5.4-7.2. The values of p$K$a$_1$ and p$K$a$_2$ for native at optimum conditions were 4.52 and 6.68, respectively while Ani-60 GA had p$K$a$_1$ and p$K$a$_2$ values of 4.3 and 7.3 respectively and Succ-130 had 5.8 and 7.8. (Table 1 ). For native, the values of $V_{max}$, $K_m$ and
$k_{cat}$ (s$^{-1}$) were 34.78 U.mg$^{-1}$ protein, 4.76 mg starch ml$^{-1}$ and 32.75s$^{-1}$ at 45℃. The specificity constant,

$k_{cat}/K_m$ was 6.87 (Table 2). For amino group modified succinylated GA, the values of $V_{max}$, $K_m$ and $k_{cat}$ were

76.92 Umg$^{-1}$ protein min$^{-1}$, 2.17 mg SS mL$^{-1}$ and 73.74 s$^{-1}$ respectively. The specificity constant, $k_{cat}/K_m$ was 31.74 (table 3). After carboxylic group modification with aniline (Ani-60) the values of $V_{max}$ =33.33 U.mg$^{-1}$ protein, $K_m$ =8.33 mg starch ml$^{-1}$, $k_{cat}$ =31.74 s$^{-1}$ , $k_{cat}/K_m$ =3.81.

Thermodynamic studies of native and chemically coupled GA was carried out. For native Δ$H$* =

23.88 kJ mol$^{-1}$, Δ$G$* = 37.37 kJmol$^{-1}$, Δ$S$*= *0-0.04* J mol$^{-1}$ K$^{-1}$, Δ$G$*$_{E-S}$ = 4.13 kJ mol$^{-1}$, Δ$G$*$_{E-T}$ = -6.07kJ

mol$^{-1}$.Thermodynamic para meters value for carboxylic group coupled Ani-60 were, Δ$H$* = 21.33kJ mol$^{-1}$, Δ$G$* = 37.70 kJmol$^{-1}$, Δ$S$*= *0.336* J mol$^{-1}$ K$^{-1}$, Δ$G$*$_{E-S}$ = 5.51 kJ mol$^{-1}$, Δ$G$*$_{E-T}$ = -4.28kJ mol$^{-1}$.
Thermodynamic para meters value for amino group coupled Succi-130 were, Δ$H$* = 31.32kJmol$^{-1}$, Δ$G$* =

37.53 kJmol$^{-1}$, Δ$S$*= -0.02 J mol$^{-1}$ K$^{-1}$, Δ$G$*$_{E-S}$ = 2.11 kJ mol$^{-1}$, Δ$G$*$_{E-T}$ = -10.035 kJ mol$^{-1}$.

Half-life,t½ of native was greater than Ani-60 and Succi-130. Δ$H$* of native was greater than all modified forms of GA, i.e. 160.45 kJ mol$^{-1}$. All modified forms of GA showed higher values of Δ$G$* as compared to native form of GA. Δ$S$* for all modified forms of GA was lower as compared to native, showing least value for succinic anhydride as compared to native. The results revealed that thermos stabilization involved conformational changes. Decrease in Δ$S$* values showed that conformation of active site becomes highly ordered by aniline and succinic anhydride coupling. Therefore, it was revealed that thermos stabilization of aniline and succinic anhydride coupled GA at higher temperatures (66℃) was due

to rise in free energy and was also entropically driven.

The cross-linking of aniline nucleophile to carboxyl group present at the surface of GA had shown no effect at optimum temperature (45℃ for GA as compared to the native enzyme (Table1) Kinetic

parameters, $K_m$ value in line with chemical coupling of GA with aniline where as $K_{cat}$ and $K_{cat}/K_m$ values are lower . $\Delta H^*$ and $\Delta G^*$ values are lower whereas $\Delta S^*$ ,$\Delta G^*_{(E-S)}$ and $\Delta G^*_{(E-T)}$ greater than obtained from GA of *Fusarium solani* (Bhatti, *et al.*, 2007c). Cross linking of amino group with succinic anhydride (Succi-
130) shifted optimum temperature at 55 ℃. This temperature was in line with determined by Pavezzi *et al.*,

2008 whereas $E_a$ was higher. $V_{max}$ $K_{cat}/K_m$ and $\Delta G^*_{E-T}$ value increased after modification but $\Delta H^*$, $\Delta G^*$ and $\Delta S^*$ remained almost same whereas little decreased was observed in $\Delta G^*_{E-S}$.

After succinylation of GA with succinic anhydride the values of $V_{max}$ increased two fold and $K_m$ value decreased to half as compared to the native, reflecting that conversion of substrate into product was more convenient in case of Succi-130. The modified forms showed lower value of $K_m$ as compared to control which means that affinity of enzyme for the substrate increased after succinylation. Higher $k_{cat}$ ($s^{-1}$) values of Succi-130 as compared to control showed that amino group modification made the enzyme more efficient, hence increased the rate of product formation. The specificity constant values of succinylated GA were higher as compared to native, which showed more capability of the enzyme to form product when the substrate is in extremely low concentration.

It has been stated that chemical modification of surface carboxylic and amino groups could significantly increase the thermostability of the enzymes (Rashid & Siddiqui, 1998; Siddiqui *et al*., 2000; Bhatti *et al*., 2007b; Bhatti *et al*., 2007c). Kinetics and thermodynamics of irreversible thermal inactivation of native, Ani-60, Succi-130 GA showed increase in value of $K_d$ with increase in temperature. $K_d$ of native was greater than both modified forms of GA. Half-life, $t_{½}$ of native, Ani-60 and Succi-130 showed decreasing trend with rise in temperature of incubation. $\Delta H^*$ of control was greater than both modified forms of GA. $\Delta H^*$ showed decreasing trend with increase in time of incubation in case of native as well as modified forms of GA. Coupling of aniline and succinic anhydride with GA resulted in increase of $\Delta G^*$ value which in turn was used to resist against thermal unfolding. It is evident from the above results that coupling of aniline and succinic anhydride improved the thermal stability of GA by increasing the $\Delta G$. There was significant increase in $\Delta S^*$ values by coupling of GA with aniline as compared to native form and slight increase with succinic anhydride coupling as compared to native. The results revealed that thermostabilizing involved conformational changes. Increase in $\Delta S^*$ values showed that conformation of active site becomes highly disordered by aniline and slightly in succinic anhydride coupling. Therefore, it was revealed that the stabilization of aniline and succinic anhydride coupled GA at higher temperatures (66°C) was due to rise in free energy and entropically not stabilized.

Irreversible thermal denaturation of soluble and immobilized endoglucanases

revealed that entropy and enthalpy of deactivation were significantly decreased by immobilization justifying the thermodynamical stability of immobilized endoglucanase (Saleem *et al*., 2005).

## Conclusion

Glucoamylase from *Humicola insolens* was extracted, characterized and chemically coupled with aniline and succinic anhydride. Aniline coupled GA showed maximum activity at 45 C like native enzyme but succinic anhydride had 55 °C temperature for activity. modified forms had same value of Gibbs free energy like native but high energy of activation in case of succinic anhydride coupled GA. Succinic anhydride coupled enzyme can tolerate high temperature which is the industrially favourable and worked in almost acidic to neutral pH range (5.2-72).

**Table. 1:** Comparison of kinetics and thermodynamics for soluble starch hydrolysis by native GA, aniline and succinic anhydride coupled GA of *H. insolens* at 45°C, pH 6.2

| **Properties** | **Native GA** | Aniline coupled (ani-60) | Succinic anhydride coupled (succi-130) |
|---|---|---|---|
| pH optimum | 5.4 | 6.2 | 6.2 |
| pH range | 4.6-6.6 | 5.2-7.2 | 5.2-7.2 |
| p*K*a$_1$ | 4.52 | 4.3 | 5.8 |
| p*K*a$_2$ | 6.68 | 7.3 | 7.8 |
| Temp. optima (°C) | 45 | 45 | 55 |
| $V_{max}$(Umg$^{-1}$protein min$^{-1}$) | 34.48 | 33.33 | 76.92 |
| $K_m$ (SS = mg.mL$^{-1}$) | 4.76 | 8.33 | 2.17 |
| $K_{cat}$ (s$^{-1}$) | 32.75 | 31.74 | 73.74 |
| Kcat / Km | 6.87 | 3.81 | 33.98 |
| $E_a$ (kJ mol$^{-1}$) | 26.52 | 147.24 | 34.05 |
| Δ*H** (kJ.mol$^{-1}$) | 23.88 | 21.33 | 31.32 |
| Δ*G** (kJ.mol$^{-1}$) | 37.37 | 37.70 | 37.53 |
| Δ*S**( J mol$^{-1}$ K$^{-1}$) | -0.04 | 0.336 | -0.02 |
| Δ*G**$_{E-S}$ (kJ mol$^{-1}$) | 4.13 | 5.51 | 2.11 |
| Δ*G**$_{E-T}$ (kJ mol$^{-1}$) | -6.07 | -4.28 | 10.035 |

**Table 2:** Kinetics and thermodynamics of irreversible thermal inactivation of GA native, aniline coupled and succinic anhydride coupled

| native | **Temp.(°C)** | **Temp.(K)** | **$K_d$ ($min^{-1}$)** | **$t_{1/2}$ (min)** | **$\Delta H^*$ ($kJmol^{-1}$)** | **$\Delta G^*$ ($kJmol^{-1}$)** | **$\Delta S^*$( $J mol^{-1}K^{-1}$)** |
|---|---|---|---|---|---|---|---|
| | 45 | 318 | 0.009 | 73.00 | 160.45 | 70.72 | 0.282 |
| | 50 | 323 | 0.014 | 49.43 | 160.40 | 70.74 | 0.278 |
| | 55 | 328 | 0.019 | 36.28 | 160.36 | 70.95 | 0.273 |
| | 60 | 333 | 0.115 | 6.04 | 160.32 | 67.03 | 0.28 |
| | 65 | 338 | 0.325 | 2.13 | 160.28 | 65.06 | 0.28 |
| Aniline coupled | 45 | 318 | 0.034 | 20.38 | 62.28 | 73.46 | 0.035 |
| | 50 | 323 | 0.040 | 17.19 | 62.25 | 74.21 | 0.037 |
| | 55 | 328 | 0.069 | 9.91 | 62.21 | 73.90 | 0.035 |
| | 60 | 333 | 0.097 | 7.14 | 62.16 | 74.15 | 0.036 |
| Succinic anhydride coupled | 60 | 333 | 0.025 | 27.72 | 99.46 | 77.91 | 0.065 |
| | 63 | 336 | 0.035 | 19.8 | 99.44 | 77.69 | 0.064 |
| | 66 | 339 | 0.04 | 17.32 | 99.41 | 78.04 | 0.063 |
| | 69 | 342 | 0.070 | 9.82 | 99.39 | 77.14 | 0.065 |

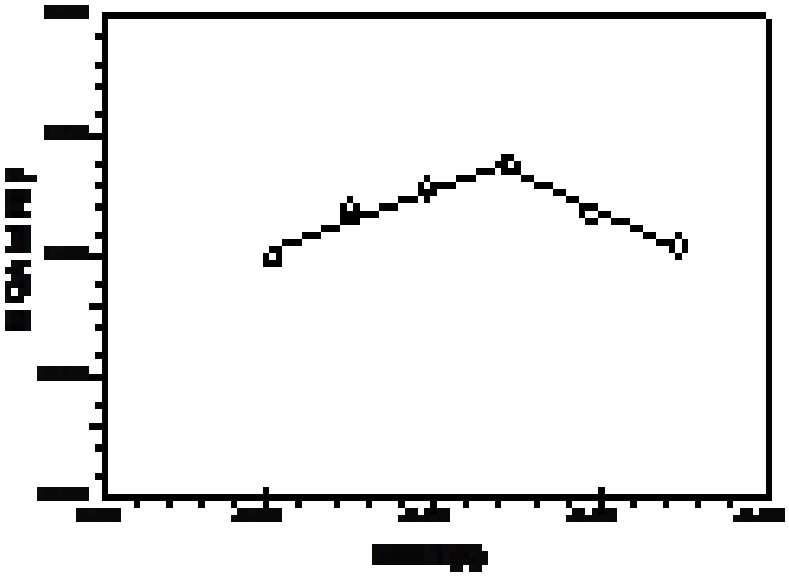


**Fig. 1:** Arrhenius plot for the determination of activation energy for starch hydrolysis by *Humicola* insolens

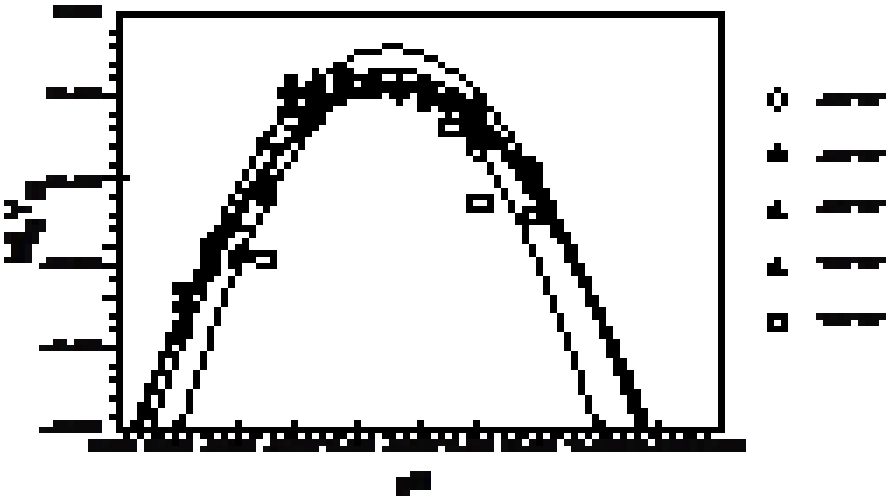


**Fig. 2:** Dixon plot for the determination of p*K*a values of active site residues of *Humicola* insolens. Data presented are average of n = 3 experiments

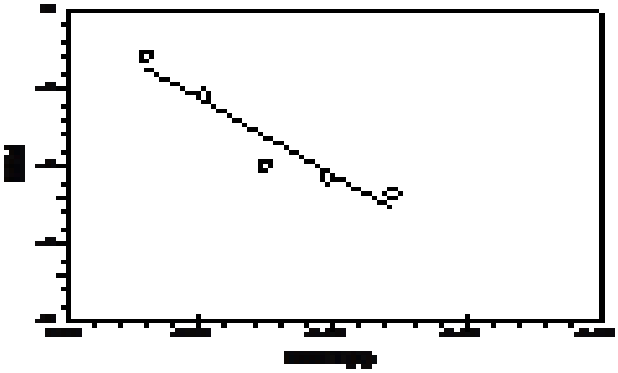


**Fig 3:** Arrhenius plot for determination of $E_a$ of irreversible thermal inactivation [$E_{a(D)}$] of GA from *H.insolens*

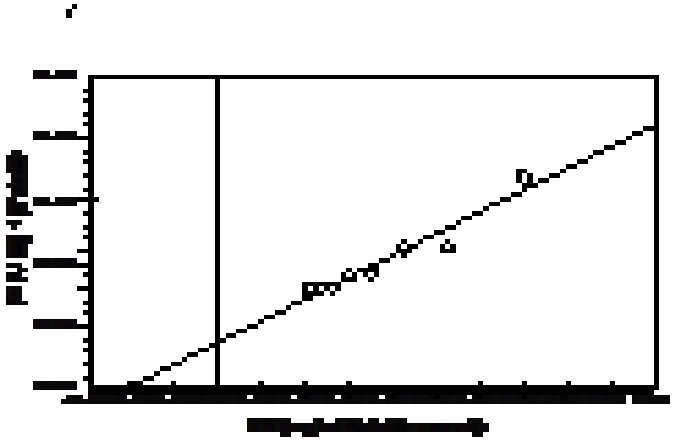
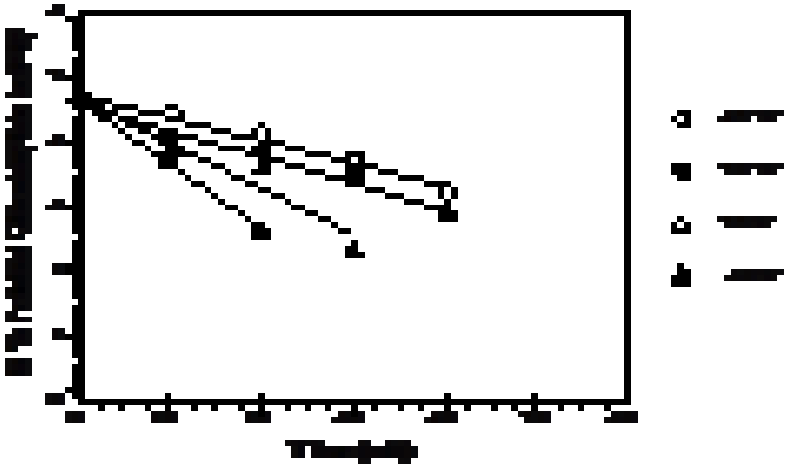


**Fig. 4:**Line weaver-Burk plot for the determination of Michaelis-Menten kinetic constants ($V_{max}$, $K_m$)



for soluble starch hydrolysis at 45 °C, pH 5.4 by GA. Data presented are average of n = 3 experiments. The R-value of the graph was 0.965

**Fig. 5:** Pseudo first order plots for the determination of irreversible thermal denaturation of Aniline coupled GA from H. insolens.

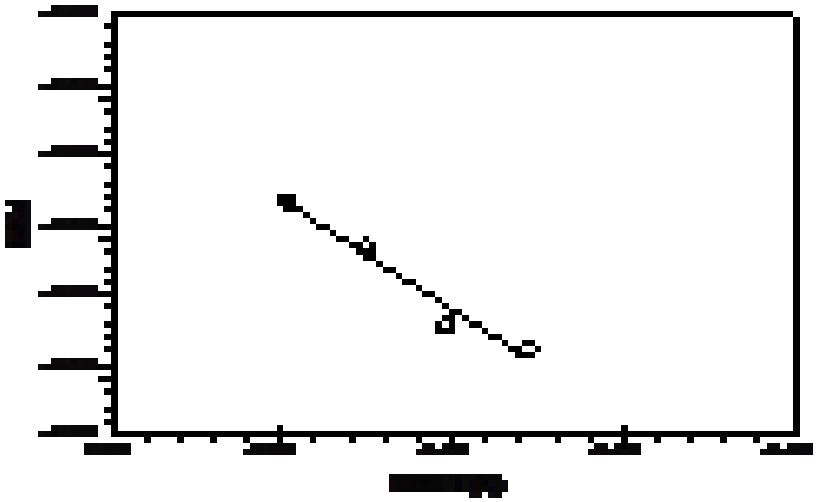


**Fig. 6:** Arrhenius plot for the determination of $E_{a(D)}$ for irreversible thermal inactivation of aniline coupled GA. Data presented are average of n = 3 experiments.

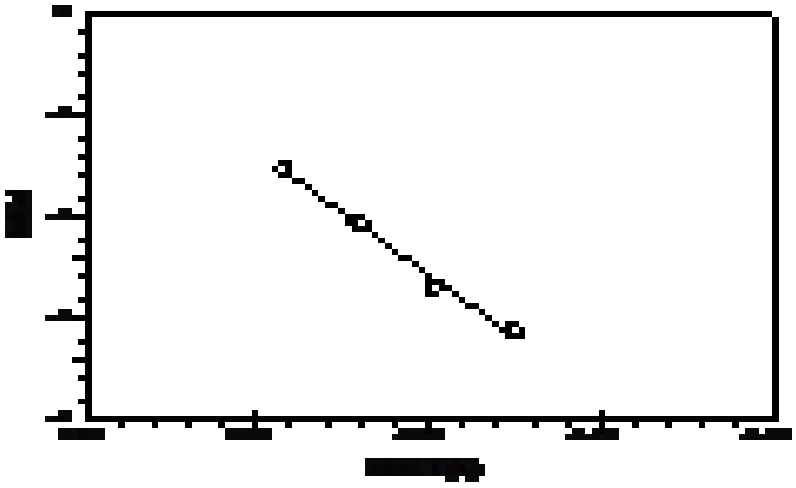


**Fig. 7:** Arrhenius plot for the determination of $E_{a(D)}$ for irreversible thermal inactivation of succinic

anhydride coupled GA. Data presented are average of n = 3 experiments

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