

Research Paper

## Kinetic characterization of glucose aerodehydrogenase from *Aspergillus niger* EMS-150-F after optimizing the dose of mutagen for enhanced production of enzyme

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### Abstract

In the present study enhanced production of glucose aerodehydrogenase from *Aspergillus niger* has been achieved after optimizing the dose of chemical mutagen ethyl methane sulfonate (EMS) that has not been reported earlier. Different doses of mutagen were applied and a strain was developed basing upon the best production. The selected strain *Aspergillus niger* EMS-150-F was optimized for nutrient requirements in order to produce enzyme through fermentation and the results showed the best yield at 2% corn steep liquor (CSL), 36 hours fermentation time, pH 5, 30°C temperature, 0.3% KH<sub>2</sub>PO<sub>4</sub>, 0.3% urea and 0.06% CaCO<sub>3</sub>. The enzyme was then purified and resulted in 57.88 fold purification with 52.12% recovery. On kinetic characterization, the enzyme showed optimum activity at pH 6 and temperature 30°C. The Michaelis-Menton constants ( $K_m$ ,  $V_{max}$ ,  $K_{cat}$  and  $K_{cat}/K_m$ ) were 20 mM, 45.87 U mL<sup>-1</sup>, 1118.81 s<sup>-1</sup> and 55.94 s<sup>-1</sup> mM<sup>-1</sup>, respectively. The enzyme was found to be thermally stable and the enthalpy and free energy showed an increase with increase in temperature and  $\Delta S^*$  was highly negative proving the enzyme from *A. niger* EMS-150-F resistant to temperature and showing a very little disorderliness.

**Key words:** glucose aerodehydrogenase, mutagenesis, production, purification, thermal stability.

### Introduction

Microbial glucose aerodehydrogenase is currently receiving much attention due to its wide applications in chemical, pharmaceutical, food, beverage, clinical chemistry, biotechnology and other industries. It is also called as glucose oxidase and catalyzes the oxidation of  $\beta$ -D-glucose to gluconic acid by utilizing the molecular oxygen with simultaneous production of hydrogen peroxide. Of several microbial enzymes known for causing the oxidation of glucose, the best known is glucose aerodehydrogenase (Banker *et al.*, 2009). Glucose aerodehydrogenase is naturally produced enzyme by some insects as honey bee (Santos *et al.*, 2005) and grass hopper (Jacques, 2005) etc. It has been extracted from various micro-organisms as *Penicillium notatum*, *Penicillium chrysosporium*, *Penicillium amagasakiense* (Witt *et al.*, 2000), *Aspergillus niger* and *Botrytis cinerea* (Liu *et al.*, 1998) but majority of its commercial preparations are being obtained from *A. niger* (Hatzi-

kolaou *et al.*, 1996). It is successfully for industrial scale because it is metabolically versatile strain and is regarded as the mostly used and important species of fungus for production and extraction of catalytic proteins (Zia, 2007). It is stable on mutagenesis and does not cause problems on treatment with mutagens (Bosch *et al.*, 1995).

The main problems that are often complained about the enzyme production and stability are low productivity and loss of activity at increased temperature and fluctuating pH at industrial processes. To overcome such obstructions, mutated strains rather than native one are preferred to ferment such enzymes having better industrial effectiveness. *A. niger* is a versatile fungus for mutagenesis for increased enzyme production (Crognal *et al.*, 2006). Only a few reports are there that describe the mutagenesis and selection of *A. niger* for increased production of glucose aerodehydrogenase, but none has been reported yet about optimizing a chemical mutagen dose (EMS) with an increased and a stable activity of the enzyme. In the present

study enhanced production, kinetic and thermodynamic stability has been reported after optimizing the dose of ethyl methane sulfonate (EMS) *i.e.* a potent mutagen for microbes.

## Materials and Methods

All the chemicals and reagents used in this research work were of analytical grade and purchased from Oxoid, Sigma, Fluka and Riedel-de Haen etc. The organisms (parent *Aspergillus niger* EBL and mutant *Aspergillus niger* EMS-150-F) were procured from Enzyme Biotechnology Lab., Department of Chemistry and Biochemistry, University of Agriculture, Faisalabad. The parent strain was isolated from soil and was verified by Department of Pathology, University of Agriculture Faisalabad, Pakistan. Stock culture was maintained on Potato-Dextrose-Agar media (PDA) and refrigerated at 4 °C until its use.

The spores of *Aspergillus niger* EBL from PDA media were inoculated in a conical flask having 50 mL of Vogel's media (pH 5.5), which was kept in a rotary shaker at 120 rpm and 30 °C temperature for 24-36 hours (Zia *et al.*, 2010). To get the enhanced production of glucose aerodehydrogenase the organism was subjected to ethyl methane sulfonate (EMS) exposure (Khattab and Bazaraa, 2005) at dose of 100, 150, 200 and 250 mM. Each specific dose of 1 mL was added in 9 mL of Vogel's inoculum in a flask. The fractions of 1 mL were collected at different time intervals of 30, 60, 90, 120, 150 and 180 min. The fractions collected were centrifuged twice for 15 min. at 10,000 rpm so as to give out the mutagen from spores in the suspension (Khattab and Bazaraa, 2005; Zia, 2007). The EMS exposed culture was inoculated on PDA media containing 1% triton X-100 as colony restrictor (Rakariyatham *et al.*, 2006; Ifikhar *et al.*, 2010). In order to get the dose with best glucose aerodehydrogenase production, enzyme diffusion zone test was employed on agar media containing o-dianisidine and horseradish peroxidase, which gives brown color (Khattab and Bazaraa, 2005).

The best selected strain, *A. niger* EMS-150-F was used in submerged fermentation for optimization of glucose aerodehydrogenase production media. These parameters include carbon sources at various levels [corn steep liquor (CSL), molasses, rice polishing and sludge], fermentation period (18, 36, 45, 70 and 96 hrs.), pH (4, 4.5, 5, 5.5, 6, 6.5 and 7), temperature (4, 20, 30, 37 and 45 °C), urea (0, 0.1, 0.3, 0.5, 0.7 and 0.9%), KH<sub>2</sub>PO<sub>4</sub> (0, 0.1, 0.3, 0.5, 0.7 and 0.9%), CaCO<sub>3</sub> (0, 0.01, 0.03, 0.06, 0.09 and 0.12%) and MgSO<sub>4</sub>·7H<sub>2</sub>O (0, 0.01, 0.02, 0.03, 0.04, 0.5 and 0.06%). These trials were carried out in triplicate and after each step of optimization the enzyme was checked for activity (Worthington, 1988) based on the principle that the velocity of the reaction is determined by an increase in absorbance at 460 nm that is resulted by the oxidation of o-dianisidine through a peroxidase coupled system. One

unit causes the oxidation of one μ-mole of o-dianisidine in one minute at 30 °C and pH 6.0 under the specified conditions. Biuret method was used to estimate protein content of the samples (Rasul *et al.*, 2011).

## Purification and characterization

Crude extract of the enzyme was saturated at 60-80% ammonium sulfate then was subjected to DEAE-cellulose and Sephadex G-150 for purification that was analyzed by SDS-PAGE (Shin *et al.*, 1993; Sukhacheva *et al.*, 2004). Optimum pH was determined by assaying the mutant derived enzyme at 4-8.5 pH range and at different temperatures (4, 20, 30, 37 and 45 °C) to seek the optimal values (Sukhacheva *et al.*, 2004). Activation energy was determined by assaying at different temperatures ranging from 20-80 °C and entering data in Arrhenius plot (Lino and Teresa, 1998). The Michaelis-menton kinetic constant K<sub>m</sub> and V<sub>max</sub>, K<sub>cat</sub> and K<sub>cat</sub>/K<sub>m</sub> were determined by assaying the different concentrations of glucose (12, 15, 18, 21, 24%) as described (Leiter *et al.*, 2004).

For the determination of thermal denaturation of the enzyme the method described (Rajoka *et al.*, 2006) was followed. Data obtained was plotted in first order plot and was analyzed. The thermodynamic parameters for thermostability were calculated by rearranging the Eyring's absolute rate equation derived from the transition state theory.

## Statistical analysis

Data obtained was analyzed by statistical method described by Steel *et al.* (1997). MS Excel and slide write plus softwares were used to draw graphs.

## Results and Discussion

Development of the superior strains through mutagenesis has become a recurrently used practice. Strain developers have searched for improved strains among random survivors of mutagenesis. Microbiologist and biochemists often try to apply some practices that may be through physical or chemical means so as to improve certain required characteristics in a strain and to decrease or eliminate the factors that are undesirable (Gadgil *et al.*, 1995). Although chemical and physical mutagenesis induce mutations non-randomly causing the mutations that are not site or gene specific and may have their effect more at some sites than has been expected but it is still cost effective procedure for strain development with desired characteristics (Rowlands, 1984). Ethyl methane sulfonate is a carcinogen which has been documented and reported to cause mutagenesis in microorganisms (Lino and Teresa, 1998; Rakariyatham *et al.*, 2006).

Kill curve has been used to know the best time of mutagenesis for each dose. It helps to determine the best mutant with the capacity to produce more glucose aerodehydrogenase (Zia, 2007). To obtain an instant mutation a high

dose of mutagen is required as the results of kill curve (Table 1) have shown an increase in killing with time and increase in dose. The best dose was found to be 200 mM with killing %age of 79.4 at 150 min EMS exposure. The dose with 250 mM EMS resulted in killing %age less than that of 200 mM dose which can be attributed to the development of resistance against the chemical mutagen developed with time. Khataab and Bazaraa (2005) also used EMS as mutagen but have used only a single dose of 200 mM that also showed in an increase in activity after mutagenesis and resulted in an augmented resistance with further exposure to the mutagen.

For the selection of mutant, after mutagenesis the dilutions were plated on agar media containing 1% triton X-100 to restrict the colonies that was also used earlier (Elkeles *et al.*, 1994; Khattab and Bazaraa, 2005). Plate screening method was used to check the potential of the colonies to grow and then enzyme diffusion zone test was employed to screen and to identify the mutant strain. The zone size of the EMS-150-F was found to be 16 mm as compared to the control which was 9 mm (Figure 1). The zone size analyzed for EMS treated strain was the maximum with 14 mm (Khattab and Bazaraa, 2005), which clearly indicates the dominance of enzyme obtained in the present study, as there is a strong relation between the activity and zone size in enzyme diffusion zone test.

**Production of enzyme**

As the mutant strain *A. niger* EMS-150-F may has a modified genotype so optimization of culture condition is necessary to reveal the actual potential. Therefore a series of consecutive experiments were performed in shaken flasks to determine the effect of medium composition on glucose aerodehydrogenase from this mutant strain (Petrucchioli *et al.*, 1997). The effect of using different carbon sources is very imperative for the fermentation process. There is always a quest for an economical but with better production substrate. During the fermentation process of microbes, the carbon source acts as the most important nutrient for building of the material required for the synthesis of the cells and is also a vital as energy provider. Out of four carbon sources used, CSL at 2% was found to be the best with optimum activity.

Fermentation time is an important factor for the synthesis of the enzyme from the microorganism, as there comes a time when running down of nutrients in the medium

or amassing of some auto-toxic products in the medium may bound the enzyme production rate. The present study has resulted in highest activity at 36 hours of fermentation while Banker *et al.* (2009) have observed an optimum growth at 96 hours after which a drastic decline was observed, while in a study of glucose aerodehydrogenase production from mutant *Penicillium funiculosum*, the maximum yield of enzyme was observed at 52 hours of cultivation. Thus an improved enzyme production has been obtained without wasting too much time in the present study.



**Table 1** - Optimization of mutagen dose, formulated from kill curve.

Dose	Killing % age (% ± SEM)	Optimum dose (min.)
100 mM	65 ± 0.362	120
150 mM	71.64 ± 0.147	150
200 mM	79.4 ± 0.132	150
250 mM	78.3 ± 0.255	150

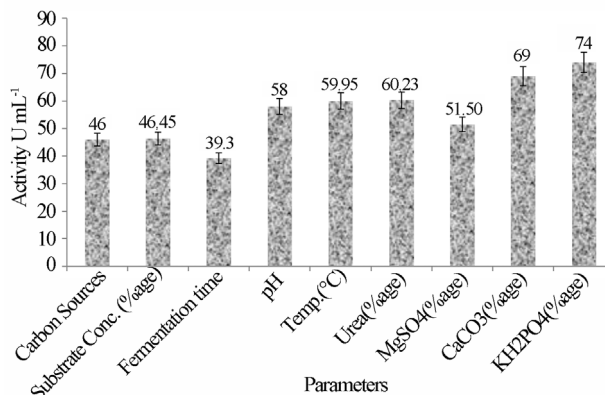
**Figure 1** - Enzyme diffusion zone test for control and EMS-150-F.

The optimum production was obtained at pH 5 and 30 °C temperature, while Sabir *et al.* (2007) observed the optimum pH to be 6 and Hamid *et al.* (2003) found it to be 3. The difference can be attributed to the mutated strain having pre-requisite different from that of natural one. The concentration of urea as nitrogen source in growth medium is of extensive importance for the production of enzyme from fungi. The microorganisms require an adequate supply of nitrogen source for various metabolic activities. The optimum activity of enzyme was observed at 0.3% of urea, which is in accordance with that of (Rasul *et al.*, 2011).

Different concentrations of  $MgSO_4 \cdot 7H_2O$  were tested out for their consequence on activity of glucose aerodehydrogenase from *A. niger* EMS-150-F that resulted in a downfall effect with increase in concentration of  $MgSO_4$ . It has been demonstrated the same conclusion suggesting that addition of  $Mg^{+}$  strongly inhibits production of glucose aerodehydrogenase (Petruccioli *et al.*, 1997).  $CaCO_3$  besides acting as buffering agent provides support for growth and also substantially induces glucose aerodehydrogenase activity. Results depict an abrupt increase in activity of the enzyme from *A. niger* EMS-150-F at 0.06% of calcium carbonate. The increase in activity by adding calcium carbonate is due to the fact that it causes a transfer of metabolism from glycolytic pathway to direct oxidation of glucose by enzyme (Liu *et al.*, 2001). Potassium and phosphate salts were supplemented to the medium to enhance the production of enzyme in growth culture.  $KH_2PO_4$  as potassium and phosphorus source was found to bestow an optimum production at 0.3%, showing cost-benefit ratio of the production in the present study. All of these results are statistically significant and have been shown in Figure 2.

### Purification of glucose aerodehydrogenase

After ammonium sulfate precipitation and desalting, specific activity was increased to  $45.07 \text{ U mg}^{-1}$ . The enzyme produced by the mutant strain *A. niger* EMS-150-F had attained 2.58 fold purification and 94.15% recovery as compared to the crude enzyme. Ion exchange chromatography exploits differences in signs and magnitude of net electric charge of protein at given pH. DEAE-cellulose resulted in 73.12 fold purification and %age recovery was obtained to be 74.59. After the purification of the enzyme through ion exchange chromatography it was further purified by gel filtration chromatography that isolates the proteins according to molecular size. It resulted in the highest specific ac-



**Figure 2** - Change in activity ( $\text{U mL}^{-1} \pm \text{SEM}$ ) of the enzyme with gradual optimization of nutrients for *A. niger* EMS-150-F.

tivity of  $1962.59 \text{ U mg}^{-1}$  having 112.59 fold purification and 67.07% recovery (Table 2). The purity of the enzyme was analyzed by SDS-PAGE that indicated that enzyme is a dimer and both monomers have the molecular weight near 67 kDa (Figure 3).

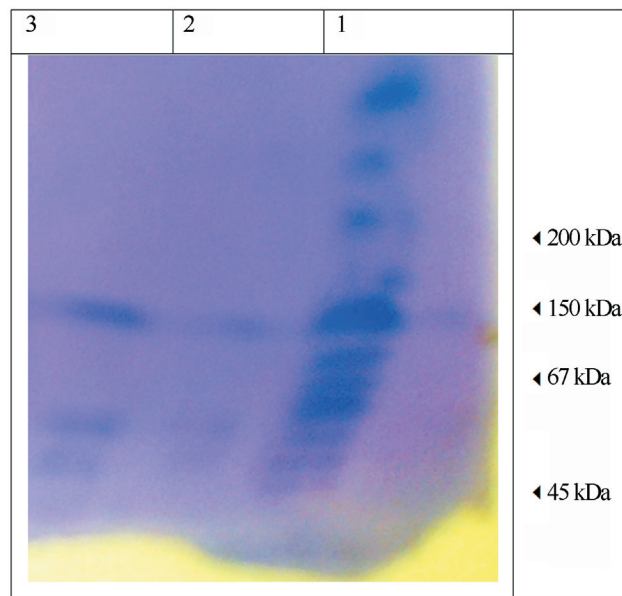
Such findings are in accordance with the results of various researchers (Sukhacheva *et al.*, 2004; Bhatti and Saleem, 2009; Rasul *et al.*, 2011) who found an increase of specific activity of glucose aerodehydrogenase after each purification step. Bhatti and Saleem (2009) have obtained a specific activity of  $280 \text{ U mg}^{-1}$ , with 16.47 fold purification and 15% yield after complete purification of the enzyme from *Penicillium notatum* that is far less than that is observed in the present study.

### Characterization of enzyme

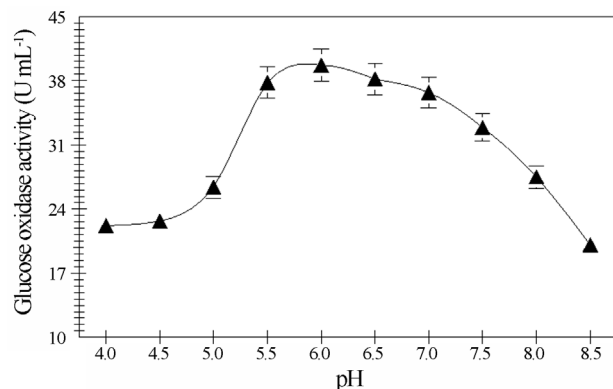
In view of the fact that pH shows a prevailing task in the activity, as glucose aerodehydrogenase from *Aspergillus niger* was found to lose its activity by 12% when kept at pH higher than 8 in a stability test performed earlier (Ferreira *et al.*, 2005). So a pH range from 4-8.5 was checked for optimum enzymatic activity. The results obtained have depicted that enzyme showed a good activity between the pH range of 4-8.5, with activity of  $39.7 \text{ U mL}^{-1}$  at pH 6 (Figure 4). These results are exactly same where optimum pH was obtained at 6 for *A. niger* glucose aerodehydrogenase (Ko *et al.*, 2002). In contrast the enzyme produced from *A. niger* EMS-150-F in the present study is stable as compared to obtained from *Penicillium pinophilum* that was found unstable in pH range of 2-4 and

**Table 2** - Purification summary of glucose aerodehydrogenase from *A. niger* EMS-150-F.

Purification Step	Activity ( $\text{U mL}^{-1}$ )	Protein ( $\text{mg mL}^{-1}$ )	Specific activity ( $\text{U mg}^{-1}$ )	Fold purification	% age recovery
Crude	79	4.53	17.43	1	100
Ammonium sulfate desalted	74.38	1.65	45.07	2.58	94.15
DEAE-cellulose	58.93	0.046	1274.63	73.12	74.59
Sephadex G-150	52.99	0.041	1962.59	112.59	67.07



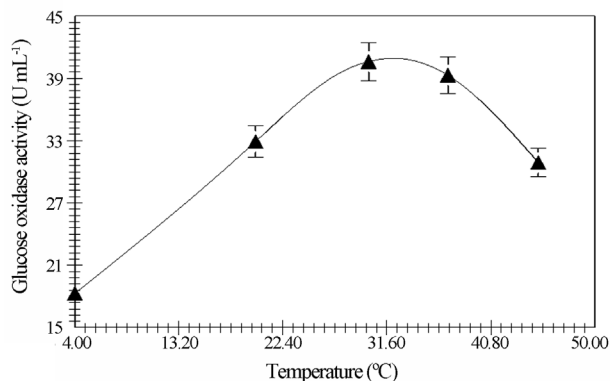
**Figure 3** - SDS-PAGE of glucose aerodehydrogenase (Lane 1 is marker and purified enzyme loaded in duplicate Lanes 2 & 3).



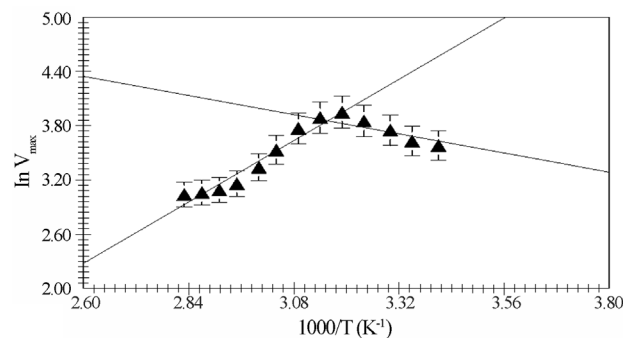
**Figure 4** - Effect of pH ( $\pm$  SEM) on glucose aerodehydrogenase from *A. niger* EMS-150-F.

above 7 (Rando *et al.*, 1997). In another study on glucose aerodehydrogenase, the results had shown that the rate of reaction augmented with raise in pH of the medium, however as the pH became immense, the rates started to decline and showed a greatest at pH 6.5 (Lino and Teresa, 1998).

Change in temperature causes a change in the activity of the enzyme, so the enzyme from *A. niger* EMS-150-F was observed for its stability on the basis of temperature. It was observed that enzyme had an optimum temperature of 30 °C (Figure 5). The energy of activation derived from the Arrhenius plot (Figure 6) was 7.45 kJ mol<sup>-1</sup>. The enzyme is active over a wide range depicting it a stable for industrial scale production and to accept the higher temperatures, if employed. Similarly glucose aerodehydrogenase from *P. amagassakiense* showed a wide range of the temperature for enzyme performance (Witt *et al.*, 1998). Glucose aerodehydrogenase catalyzed reaction was deliberated at di-



**Figure 5** - Effect of temperature ( $^{\circ}$ C  $\pm$  SEM) on glucose aerodehydrogenase from *A. niger* EMS-150-F.

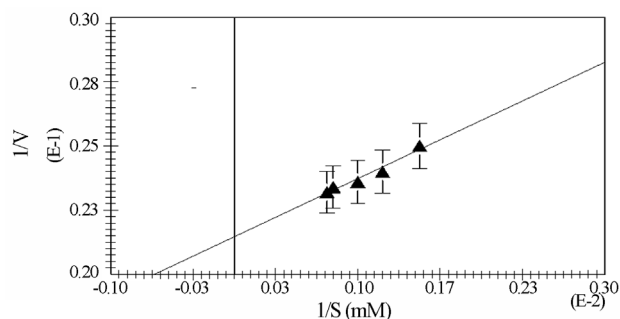


**Figure 6** - Arrhenius plot for energy of activation of glucose aerodehydrogenase.

verse temperatures from 25-60 °C that resulted in increased reaction velocities with increase in temperature (Lino and Teresa, 1998). It further obtained activation energy of 28.9 kJ mol<sup>-1</sup>, showing higher activation energy as compared to the present study. So, it can be concluded that glucose aerodehydrogenase from *A. niger* EMS-150-F is superior as compared to earlier studied reports as it necessitates a lesser amount of energy to start up the reaction.

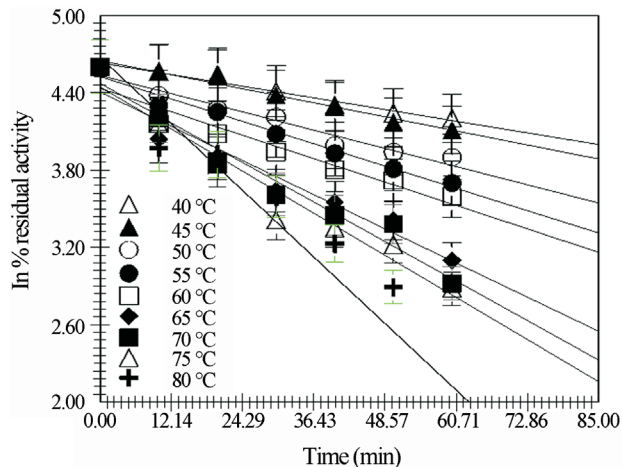
Glucose aerodehydrogenase from fungal sources (*Aspergillus niger*) has a low  $K_m$  ranging from 0.11-33 mM. The values observed in the present study have clear indication of the above described results and are in accordance with it (Figure 7). The values obtained are 20 mM, 45.87 U mL<sup>-1</sup>, 1118.81 s<sup>-1</sup> and 55.94 s<sup>-1</sup> mM<sup>-1</sup> for  $K_m$ ,  $V_{max}$ ,  $K_{cat}$  and specificity constant, respectively, evaluating the enzyme perfect for the production and exploit as catalyst at industrial level. It is further reported that enzyme from mutant *Aspergillus niger* have  $K_m$  and  $V_{max}$  as 28 mM, 60 U mL<sup>-1</sup>, respectively (Zia, 2007). A low  $K_m$  value in the present study as compared to the earlier ones depicts its higher affinity and specificity for glucose as substrate.

Each enzyme is thermodynamically stable within a particular temperature and pH range and can transform its activity from higher to lower at some transition temperature. The development of methodologies that can increase



**Figure 7** - Line-weaver Burk plot for glucose aerodehydrogenase from *A. niger* EMS-150-F.

enzyme stability is an important goal in enzyme technology. The enzyme from EMS-150-F was thermally stable as at 40 °C with half life of 93.64 min which gradually decreased with increase in temperature to 80 °C (Figure 8). Protein molecules show only a certain degree of stability especially at elevated temperatures. Thermal denaturing of multimeric enzymes is paralleled by the distraction of non-covalent bonds leading to subunit dissociation with an affiliated augmentation in the enthalpy of activation (Rodriguez-Nogales, 2004; Srivastava *et al.*, 2005). The unfolding of enzyme configuration causes a raise in mess, uncertainty or entropy of activation (Vieille and Zeikus, 1996). The results obtained in the present study (Table 3; Figure 8) have portrayed that enthalpy has increased in the range of 0.541-0.611 kJ mol<sup>-1</sup> with increase in temperature from 40-80 °C (313-353 K). The Gibb's free energy calculated had also a constant trend to increase with rise in temperature, with its maximum value at 80 °C *i.e.* 96.36 kJ mol<sup>-1</sup> presenting that the enzyme has exhibited the resistance against thermal unfolding at higher temperatures. The entropy of the system was found to be -271.24 J mol<sup>-1</sup> K<sup>-1</sup> at 80 °C. The decidedly negative value of entropy of inactivation ( $\Delta S^*$ ) observed for glucose aerodehydrogenase in the present case has suggested that there was negligible disorderliness, indicating that enzyme is thermodynamically much stable. In contrast to the results in the present study Bhatti and Saleem (2009) and Zia (2007) have observed



**Figure 8** - Irreversible thermal denaturation of glucose aerodehydrogenase from *A. niger* EMS-150-F.

higher entropy with -96.12 and -11.92 J mol<sup>-1</sup> K<sup>-1</sup> respectively showing more randomness with increase in temperature as a result of opening of the enzyme structure. Comparison with the other studies clearly indicates the present enzyme as more ordered in transition state and more resistant and stable at higher temperatures.

## Conclusion

On the basis of the above described results it can be concluded appropriately that mutagenesis with 200 mM EMS has resulted in such glucose aerodehydrogenase from *Aspergillus niger* EMS-150-F that has a higher activity. This modified novel enzyme is kinetically and thermodynamically much stable enzyme which can resist at higher temperature (40-80 °C) and has a wide pH range (4-8) required for production at industrial scale. It has lower activation energy to avoid the losses of energy just to start up the working of the enzyme. The enzyme is unique with its higher half life and resistance against thermal unfolding at higher temperatures that are usually required for its production processes. Moreover, it has a negligible randomness

**Table 3** - Kinetic and thermodynamic parameters for irreversible thermal inactivation of glucose aerodehydrogenase from *A. niger* EMS-150-F.

Temperature (K)	$K_d$ (min <sup>-1</sup> )	$t_{1/2}$ (min)	$\Delta H^*$ (kJ mol <sup>-1</sup> )	$\Delta G^*$ (kJ mol <sup>-1</sup> )	$\Delta S^*$ (J mol <sup>-1</sup> K <sup>-1</sup> )
313	0.007	93.64	0.541	89.65	-284.69
318	0.008	77.52	0.549	90.64	-283.31
323	0.116	59.54	0.558	91.42	-281.31
328	0.014	48.53	0.567	92.32	-279.93
333	0.015	46.32	0.576	93.61	-279.38
338	0.022	31.76	0.584	94.01	-276.41
343	0.025	27.44	0.593	95	-275.23
348	0.026	25.77	0.602	96.27	-274.91
353	0.042	16.29	0.611	96.36	-271.24

depicting its ability to survive at the higher temperatures that are often complained by the enzyme users as biocatalyst and its production and availability at assorted levels.

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