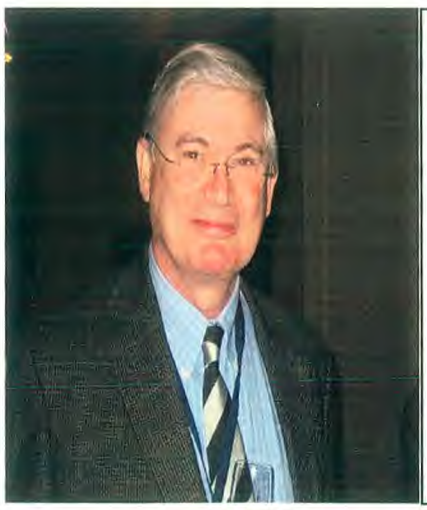


**Sir Alan Roy Fersht** (born 21 April 1943), often called father of protein engineering is a British chemist at the MRC Laboratory of Molecular Biology in Cambridge. He is distinguished for his pioneering work on protein folding. He developed a primary method for analysis of the structure, activity, stability and folding of proteins. He has developed methods for the high resolution of protein folding in the sub-millisecond time-scale and has pioneered the method of $\phi$ value analysis for studying the folding transition states of proteins.

# Chapter V

# THERMOSTABILIZATION OF AZOREDUCTASE USING POLYSACCHARIDE ADDITIVES IN AQUEOUS STATE


## ABSTRACT

The stability of proteins in aqueous solution is routinely enhanced by additives, which is known to shift the native protein to more compact states. Thermostability of intracellular azoreductase from *Pseudomonas oleovorans PAMD_1* was studied at 65 °C using various carbohydrate additives (starch, agar, and sucrose). The stability of the enzyme was enhanced with starch (10 %), agar (0.4 %), and sucrose (2 %). Thermostability of azoreductase with and without additives was studied at various temperatures using DSC. The Changes in enthalpies were observed and consequently, the thermal energy required for melting of crystal structures was be measured by DSC as $T_m$. The result shows the shifting of peak from 30.9 °C to 66.27 °C for enzyme and enzyme with starch additive respectively. Further, analysis of Arrhenius plot for stabilized enzyme proves the involvement of various additives in improving the thermostability of the azoreductase. The regression analysis in Arrhenius plot showed break point temperature ($T_{BP}$) of 45 °C for free enzyme with activation energy of 5.20 $KJmol^{-1}$. This break point shifted to 68 °C with activation energy of 10.35 $KJmol^{-1}$ for enzyme protected by the additive starch whereas for the additives, agar and sucrose the break point and activation energy was 59, 54 °C and 8.02, 6.64 $KJmol^{-1}$ respectively. Azoreductase has potential applications in the field of biosensing, bioremediation and biotechnological applications. Any improvement in the stability of the enzyme will greatly enhance its application in mentioned areas.

## 5.1 INTRODUCTION

Azoreductases have potential advantages in bioremediation of wastewater containing azo compounds (Zimmermann *et al.,* 1982). Recently, several studies describing the effluent treatment and degradation of azo dyes have been reported with the involvement of azoreductases. Various authors (Rafii *et al.,* 1990; Chung and Stevens, 1993) have emphasized the necessity of the involvement of azoreductase in the decolorization of azo dyes, chiefly assuming electron carriers (coenzymes), flavin nucleotides (FMN, FAD) or nicotinamide nucleotides (NADH, NADPH). The monomeric azoreductases (Zimmermann *et al.,* 1984; Mazumdar *et al.,* 1999) have important application in effluent decolorization and detoxification of azo dyes from textile industries.

Although enzymes have a wide range of applications in various fields, their applicability is limited due their lack of stability under extreme conditions, such as high temperature, pH, chemicals and other potentially denaturing chemicals (Renate *et al.,* 1999). Preventing their stability and the biological activity in commercial applications can pose serious challenges. Increasing the stability of enzymes under harsh pH and temperature by the addition of various additives has been reported previously (Padma *et al.,* 2008; Andrew *et al.,* 2000; Costa *et al.,* 2002).

Purified enzymes repeatedly necessitate being stored for the prolonged period of time with their unique structural integrity and activity. The extent of storage 'shelf life' can vary from a few days to more than a year and is dependent on the nature of the enzyme and the storage conditions used. Stability studies on the final product are

conducted to define the optimal storage conditions and expiration date. Typically a shelf life of at least 1.5 to 2 years at room temperature or under refrigeration is desirable (Schrier *et al.,* 1993).

Protein-stabilizing chemicals are referred as chemical additives, which needs for long term storage for proteins, can be broadly divided in to the following types: sugars and polyhydric alcohols, amino acids, amines, salts, polymers, and surfactants (Back *et al.,* 1979; Lozano *et al.,* 1994; Timasheff, 1995). The choice of the stabilizer also plays an important role in protein stabilization as it determines the isoelectric point of the protein (Kaushik *et al.,* 1998). Protein unfolding temperatures are usually in the range of 40-80 °C. Most proteins can be denatured by relatively small increases in temperature, and if the balance between folded and unfolded states is held by the equivalent of 5 to 8 hydrogen bonds (Perl and Schmid, 2002). This is due to the multiple and changing protein degradation pathways at different temperatures (Yoshioka *et al.,* 1994).

The mechanism behind the facilitation of structural stability of proteins is retention of surface water activity of the protein. By adding polyhydric alcohols the surface water activity is modified relative to the absolute concentration of the additive (Costa *et al.,* 2002). The poly alcohol may include sugars and sugar alcohols which modify the water environment surrounding a protein thus replacing and competing for free water within the system (Poonkuzhali *et al.*, 2011). This modified hydration shell confers protection to the protein maintaining 3D structure and biological activity. This enables storage of biological materials both in solution and in the dehydrated state. In other hand, polyelectrolyte includes numerous polymers of varying charge

and structure. The interactions between proteins and these polymers are electrostatic and form large protein-polyelectrolyte complexes which retain the complete biological activity (Guido Drago, 2001).

The stability of a protein and the thermodynamic parameters that control the noncovalent bond formation in proteins can be directly studied by differential scanning calorimetry (DSC). Differential scanning calorimetry is a thermo-analytical technique used for assessing protein thermal behavior and to obtain thermodynamic parameters of folding-unfolding transitions (Bruylants *et al.,* 2005). DSC are characterized not only by high sensitivity but by the high stability of their baseline and the ability to scan aqueous solutions up to and above 100 °C under excess pressure and by supercooling down below 0 °C. The wide operational range is important because changes of many macromolecules take place over a very broad temperature range. DSC scans are summed to give an accurate measure of enthalpies (ΔH) of protein unfolding. In a single DSC experiment we can determine the transition midpoint ($T_m$) and enthalpy (ΔH).

Arrhenius plots are often used to analyze the effect of temperature on the rates of chemical reactions. An Arrhenius plot displays the logarithm of kinetic constants (lnk), ordinate axis plotted against inverse temperature (1/T, abscissa). For a single rate limited thermally activated process, an Arrhenius plot gives a linear or non linear graph, from which the activation energy and the pre-exponential factor can both be determined (Arrhenius, 1889). Observations of the azoreductase activity at higher temperature depict the high temperature stability and its applications in biodegradation of azo dye toxicants.

In this context, the aim of this study was: (i) to stabilize the azoreductase enzyme in the soluble state using additives at different temperature, (ii) to assess the thermal inactivation pattern of azoreductase from *Pseudomonas oleovorans PAMD_1* (iii) to determine the midpoint of the transition for the enthalpy change ($T_m$) for azoreductase with and without additives by DSC method (iv) to determine the break point of azoreductase in free form and with additives by Arrhenius plot.

## 5.2 MATERIALS AND METHODS

### 5.2.1 *Azoreductase production and purification*

Kindly refer chapter II and III.

### 5.2.2 *Azoreductase assay*

The activity of Azoreductase was determined based on the procedure described by (Zimmermann *et al.,* 1982) with minor modifications as described in previous chapters. Protein was determined by the method of Lowry *et al.* (1951) with BSA as the standard.

### *5.2.3 Decolorization assay*

The percentage decolorization of the Orange II was determined based on the calculation described by Chen *et al.,* 2003 (detail in third chapter).

### *5.2.4 Stability assays*

Stability assays were carried out in 100 mM phosphate buffer pH 7.0. Sealed tubes containing 5 U of azoreductase in a total volume of 3 ml were incubated up to 48 hrs at 50, 55, 60, 65, and 70 °C respectively and the activity was measured in

terms of percentage decolorization periodically. The effects of various additives after 48 hrs incubation at pH 7.0 were tested at the following concentrations: starch (2.5 %, 5 %, 7.5 %, and 10 %), agar (0.1 %, 0.2 %, 0.3 %, and 0.4 %), and sucrose (0.5 %, 1.0 %, 1.5 %, and 2.0 %).

### *5.2.5 Differential scanning calorimetry (DSC) analysis*

DSC experiments were performed in a DSC calorimeter (Shimadzu DSC-60), which has the cell volume and temperature resolution of 0.2 ml and 0.1 °C respectively. In order to obtain a reproducible baseline, at least three scans with 100 mM phosphate buffer pH 7.0 on both the cells were performed. The highest activities of the sample with respect to the percentage of the additives were alone subjected to DSC. The sample cell was loaded with samples and equilibrated for 20 mins at room temperature. Heating scans has been done from – 40 °C to 120 °C with raise of 10 °C per hour. The temperature was then decreased to 20 °C before rescanning the sample. The data were analyzed by using the Micro Cal Origin software package 5.0. The result of a DSC experiment is a curve of heat flux (molar heat capacity) versus temperature or versus time. It can be shown that the enthalpy of transition can be expressed as $\Delta H$ and transition temperature ($T_m$).

### *5.2.6 Arrhenius Plot*

The stabilization of azoreductase by different polysaccharide additives followed the model of first order kinetics (Mutafov *et al.*, 2006). The kinetics of thermal inactivation of free and stabilized enzyme at different temperature was analyzed by Arrhenius plot. From a semilogarithmic plot of residual activity versus concentration of additives, the inactivation rate constant *k* was calculated (Stiller, 1989).

$$\ln k = \ln A - \left(\frac{E}{R}\right)\frac{1}{T}$$

Where:

$k$ is the rate constant of a reaction at temperature $T$ kelvin.
$A$ is a constant for a particular reaction with the same units as $k$;
$Ea$ is the Arrhenius activation energy (in J $mol^{-1}$) of the reaction.
$R$ is the gas constant (8.3145 J $mol^{-1}$ $K^{-1}$).
ln means log to the base $e$.

When a reaction has a rate constant that obeys the Arrhenius equation, a plot of ln$k$ against $1/T$ gives straight line, whose gradient and intercept can be used to determine Ea and A (Siva Sai Kumar *et al.,* 2006).

## 5.3 RESULTS AND DISCUSSION

### 5.3.1 *Effect of additives on enzyme stability*

Fig 1 shows the stability of the purified azoreductase which decreases with increasing temperature, which clearly depicts the thermolabiality of azoreductase from *Pseudomonas oleovorans PAMD_1.* The enzyme activity decreases greatly above 40 °C, which may due to the temperature that could interfere with the stability of the enzymes along with intrinsic mechanisms like unfolding or depletion of a prosthetic group (Leandro Papinutti *et al.,* 2008). The loss of enzyme activity was prevented at high temperature by using various stabilizers. Most proteins need a stabilizer for long term storage; which are referred as chemical additives (Allison *et al.,* 1998).

The additives when added to the azoreductase at different temperatures (50, 55, 60, 65 and 70 °C), increased the enzymatic activity when compared to the control (enzyme without additives) as shown in Fig 2. The increase of additives concentration was generally associated with an increase in azoreductase activity. The gelling nature of the polymer additives at high concentration may be the major reason for increasing thermostability. Fig 2 (a, b, and c) shows the action of starch, agar and sucrose at different concentrations in increasing the thermostability of azoreductase. The enhanced stability was observed above 65 °C in response to starch might be useful for wide range of industrial applications.

Similar results were observed in thermal stability of enzymes by using various additives like sugars, salts (Baptista *et al.,* 2000), and polyols (Costa *et al.,* 2002). The stabilization of multimeric enzymes by polymers had been used to prevent sub-unit dissociation in ribonuclease (Roberto Fernandez-Lafuente, 2009). Polysaccharide like starch was used to stabilize enzymes like trypsin (Fernandez *et al.,* 2004).

To the best of our knowledge this may the first report demonstrating the ability of various studied additives to stabilize the azoreductase efficiently in solution state.

### *5.3.2 Differential scanning calorimetry (DSC)*

Enzymes were used to assess their melting temperature ($T_m$) in response to temperature (Bruylants *et al.*, 2005). Fig 3a, b, and c, shows the DSC thermogram result of enzyme with and without additives. In DSC, heating the control (without additives) sample initially produces a slightly increasing baseline but as heating

progresses, heat is absorbed by the protein and causes it to thermally unfold over a temperature range characteristic for that protein, giving rise to an endothermic peak. During the unfolding process, water molecules around the protein reorganize and restructure as more non-polar side chains are exposed (Shinoda, 1997). Once unfolding is complete, heat absorption decreases and a new baseline is established. After blank subtraction, the data can be analyzed to provide a complete thermodynamic characterization of the unfolding process (Bruylants *et al.*, 2005).

The calorimetric enthalpy (ΔH) of the control and enzyme with additives such as starch, agar and sucrose were -9.5, -10.20, -11.21 and -8.79 J respectively. It is an absolute measurement of the heat energy uptake, given by the area under the transition peak. It depends on the total amount of (active) protein in the calorimeter cell, which may due to endothermic events such as the breaking of hydrogen bonds, and exothermic processes such as the disruption of hydrophobic interactions (Bruylants *et al.*, 2005).

The temperature midpoint of the transition for the enthalpy change ($T_m$) occurs when the protein goes from native to denatured form. The transition midpoint $T_m$ is the temperature where 50 % of the protein is in its native conformation and the other 50 % is denatured. Tm is an indicator of thermostability, and in general, the higher the $T_m$, the more stable the protein (Schrier *et al.*, 1993; Remmele *et al.*, 1998). Taking into account the results of DSC, half-life time ($T_m$) value for each treatment was estimated from the fitted curve. The highest $T_m$ value was obtained in the presence of 10 % starch ($T_m$- 66.27 °C) while 0.4 % agar ($T_m$- 52.89 °C) and sucrose 2.0 % ($T_m$- 47.63 °C) also shows stabilization effect significantly when compared to

control enzyme ($T_m$- 30.12 °C). The thermal profiles of azoreductase with different additives are characterized by sharp endotherms. This sharp endotherm is characteristic for proteins and is due to less internal heterogeneity or dehydration in contrast with broad endotherm peak of such molecules (Fan and Cooper, 1994; Chan and Gonda, 1998). Similar kind of stabilization results were demonstrated for different enzymes like lysozyme (Elkordy *et al.,* 2004) and DNase I (Elkordy *et al.,* 2008).

### *5.3.3 Arrhenius plot*

The Arrhenius plot for enzyme in absence and in presence of additives were derived from the slopes of the plot of log % residual activity versus additives concentration as shown in Fig 4. The non-linear plot depicts two distinct lines with different slopes that were used to analyze the break point for azoreductase. Nonlinear Arrhenius plots have been previously reported for many enzymes, because of the behavior of the enzyme with temperature. Nonlinearity appears as curve (Allen *et al.,* 1990), a break (Massey *et al.*, 1966) or a jump (Moosavi-Nejad *et al.*, 2001; Poonkuzhali *et al.,* 2011).

Table 1 reveals the difference in activation energies (Ea) among starch, agar and sucrose when compared to control. The break point temperature in the Arrhenius plot for azoreductase in the absence of additives was 45 °C, and it shifted to 68 °C, with the addition of starch, whereas for agar and sucrose it was 59 and 54 °C respectively. The possibilities of non-linear Arrhenius plot are there due to change in the rate-limiting step, as the temperature is varied (Massey *et al.*, 1996). The non-linearity may due to purely kinetic phenomena without any changes in the active site

conformation (Allen *et al.,* 1990). Another reason may be due to temperature induced conformational change in a soluble enzyme or phase change in a membrane associated enzyme (Biosca *et al.,* 1983). The sudden alteration of the activation energy with temperature change is a well known phenomenon in microbial physiology. But the reasons for sudden change in activation energy are still unclear (Poonkuzhali *et al.*, 2011).

Similar kind of two-part linear Arrhenius plot for azo bond reduction by microorganism was reported previously by Blaga Angelova *et al.,* (2008). Ceuterick *et al.,* (1978) also reported the thermal characteristic of enzymes which is temperature dependent. Results of our present study will be used to understand the effect of temperature on the activity of azoreductase and its employability in bioremediation.

The general structural stabilities afforded by sugar polymers and poly alcohols warrant more discussion. The main driving force in stabilization by sugars (polysaccharides) is via preferential hydration of proteins in the presence of these osmolytes (Arakawa *et al.,* 1982; Santoro *et al.*, 1992). Therefore, the apparent volume of the protein decreases leading to a more stable conformation. It has been proved that trehalose has a larger hydrated volume than other related sugars and therefore concomitant increase in the specific viscosity. This property of the sugar molecules correlated with the ability to protect the structure and function of proteins against temperature (Back *et al.*, 1979). Back *et al.,* also proposed the stabilization by sugars due to the strengthening of hydrophobic interaction that reinforce the hydrogen-bonded organization of water. Therefore the active sites of enzymes are more flexible that the molecule as a whole (Tsou, 1993).

Palvannan *et al* (Palvannan *et al.,* 1998) reported that thermostabilization of aspartyl protease from *Rhizomucor pusillus* was attained by sugars like trehalose, sucrose, sorbitol etc. Polysaccharide like modified starch was used to stabilize enzymes like trypsin (Fernandez *et al.,* 2004).

## 5.4 CONCLUSION

Results of the present study indicate that the stabilization of azoreductase using starch provide certain advantages as compared to agar and sucrose. This observation hopefully contributes to the elucidation of the mechanisms of temperature effect on stabilization of azoreductase. The stabilization of enzyme by starch gave higher activity, improvement of break point to higher temperature and showed better thermal stability. It is clear that the approach is much simpler than the other sophisticated strategies like immobilization on carriers or protein engineering. This will enable proper biochemical analysis of industrial effluent in order to identify and detoxify their toxic constituents.

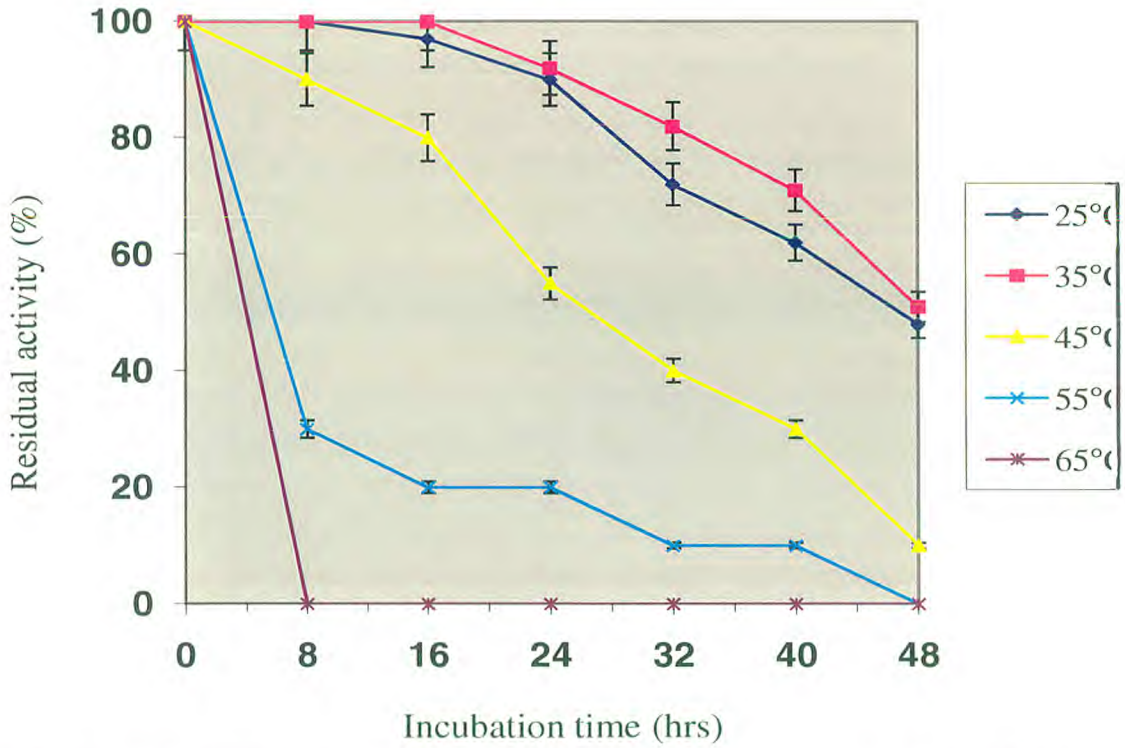
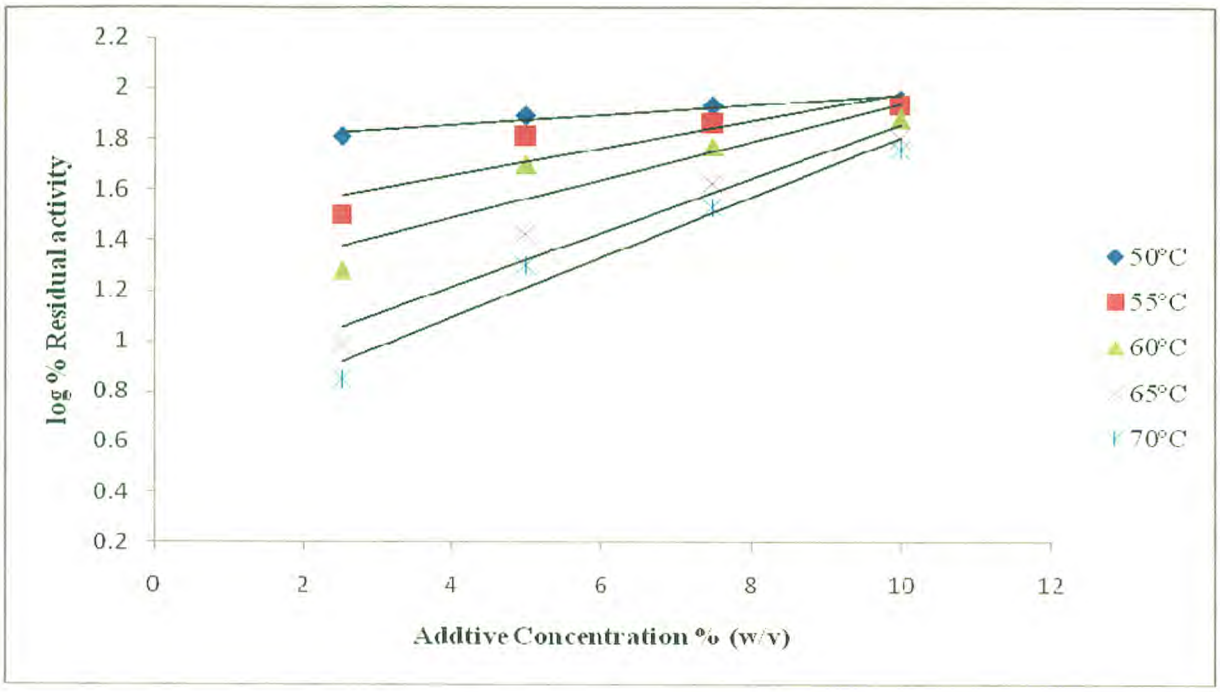
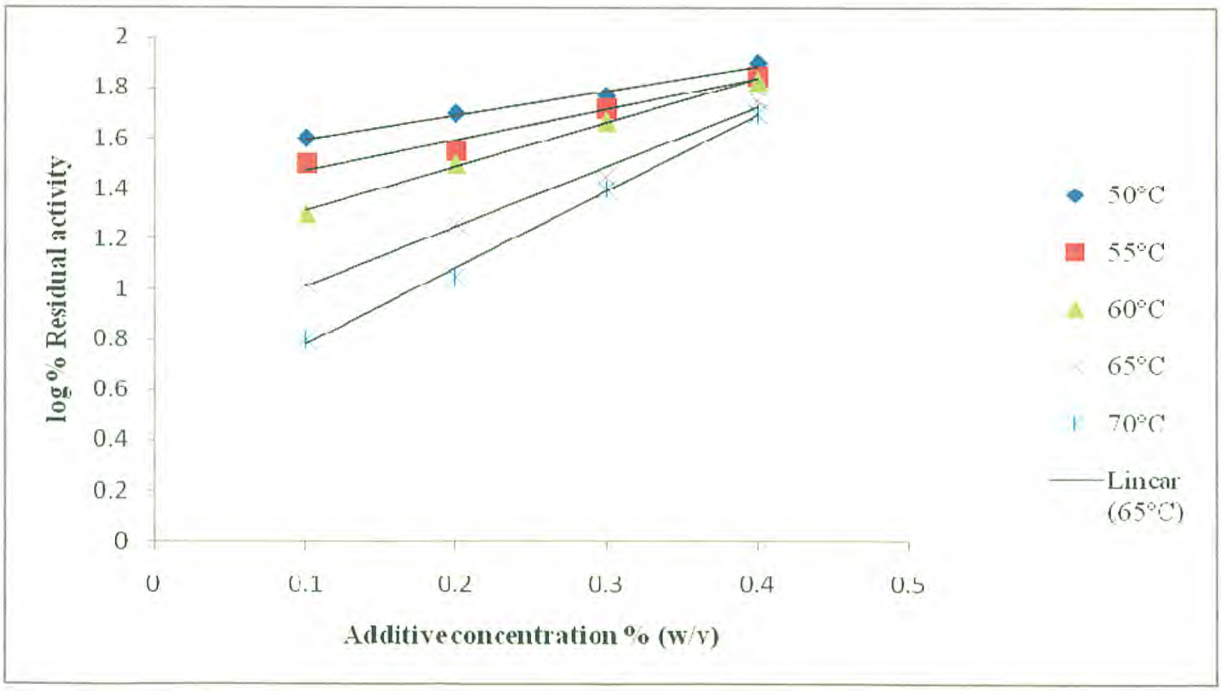


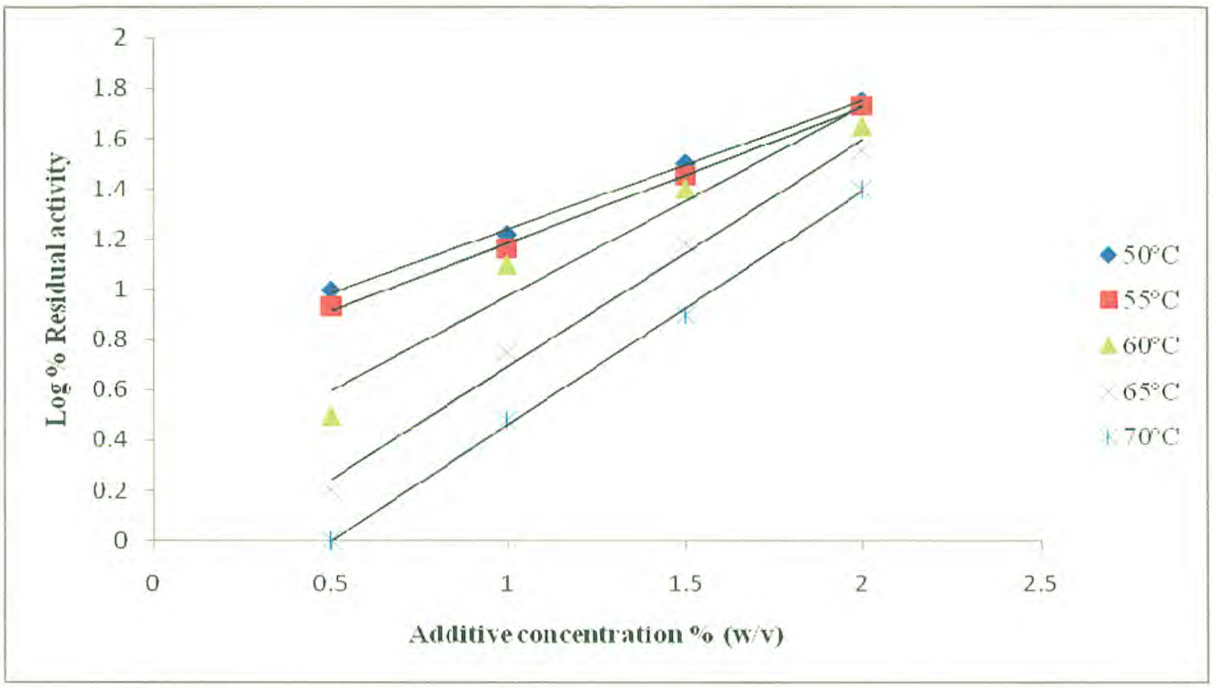

**Figure 1:** Thermostability of azoreductase from *Pseudomonas oleovorans PAMD_1* shows decrease in enzyme activity at different temperature at different time intervals.


log % Residual activity
2.2
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
2
4
6
8
10
12
Addtive Concentration % (w/v)
50°C
55°C
60°C
65°C
70°C

(a)

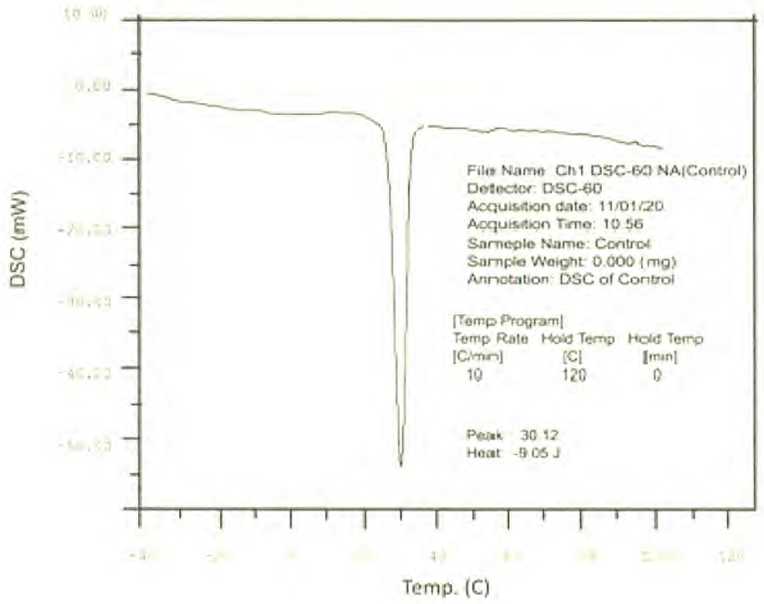

log % Residual activity
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
0.1
0.2
0.3
0.4
0.5
Additive concentration % (w/v)
50°C
55°C
60°C
65°C
70°C
Linear (65°C)

(b)

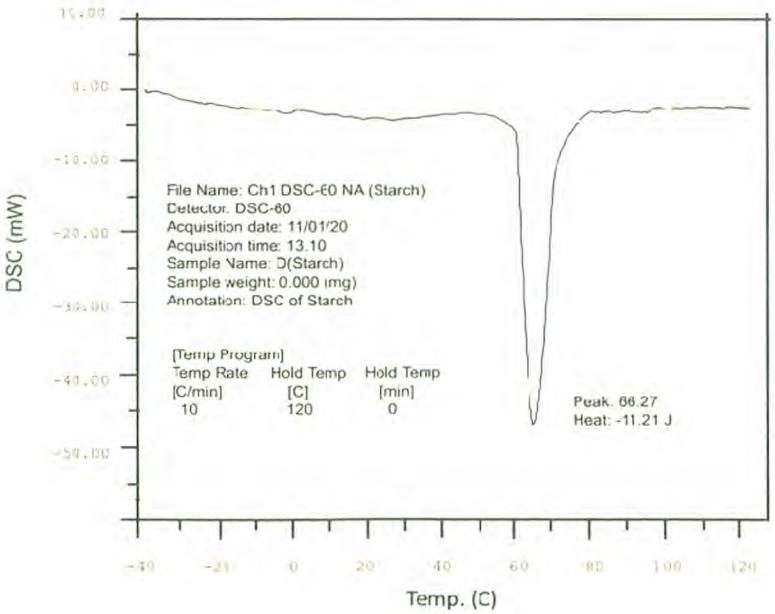



(c)

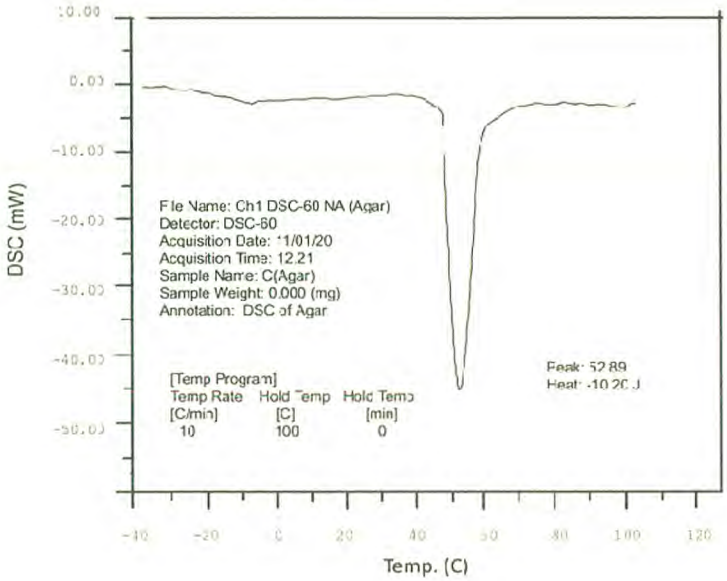
**Figure 2:** Thermal inactivation kinetics of azoreductase with additives (a) Starch (b) Agar (c) Sucrose. For determination of thermal inactivation parameters, enzyme was incubated with and without additives for 48 hrs at pH 7.0 at different temperatures.


DSC (mW)
Temp. (C)
File Name: Ch1 DSC-60 NA(Control)
Detector: DSC-60
Acquisition date: 11/01/20
Acquisition Time: 10:56
Sarneple Name: Control
Sample Weight: 0.000 (mg)
Annotation: DSC of Control
[Temp Program]
Temp Rate [C/min] 10
Hold Temp [C] 120
Hold Temp [min] 0
Peak 30.12
Heat -9.05 J

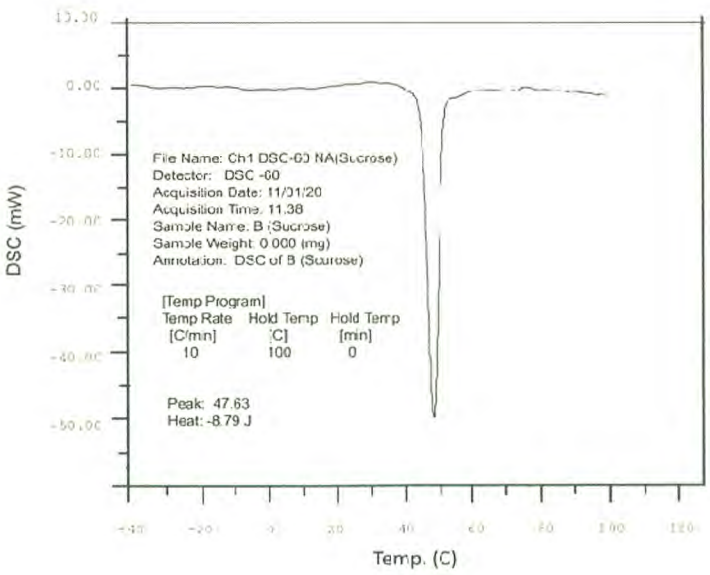
(a)


DSC (mW)
Temp. (C)
File Name: Ch1 DSC-60 NA (Starch)
Detector. DSC-60
Acquisition date: 11/01/20
Acquisition time: 13.10
Sample Name: D(Starch)
Sample weight: 0.000 img)
Annotation: DSC of Starch
[Temp Program]
Temp Rate [C/min] 10
Hold Temp [C] 120
Hold Temp [min] 0
Peak. 66.27
Heat: -11.21 J

(b)




(c)




(d)

**Figure 3:** Differential Scanning Calorimetry thermogram result of azoreductase (a) without additives (b) with starch (c) with agar (d) with sucrose

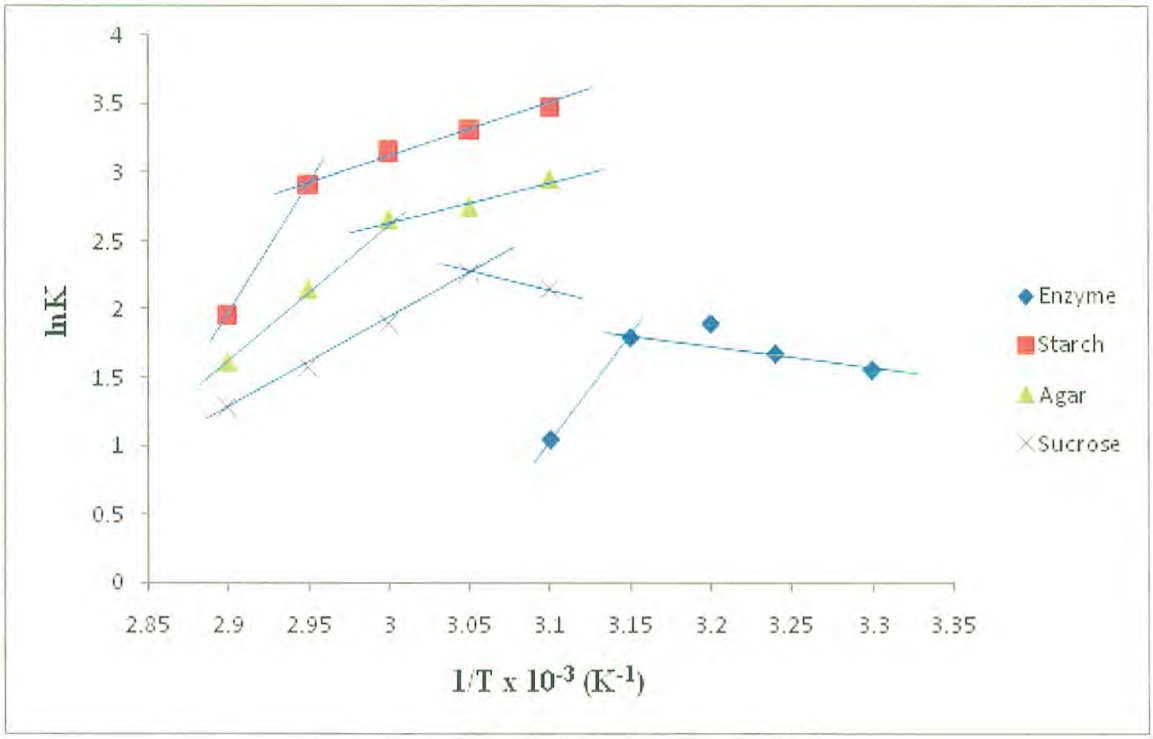



**Figure 4:** Arrhenius plot for free and stabilized azoreductase with different additives

**Table 1:** Results of the two-part linear regression analysis from the experimental data.

| **Parameters** | **Enzyme** | **Starch** | **Agar** | **Sucrose** |
|---|---|---|---|---|
| Distribution of the experimental points | Four points to the left, one point to the right | Four points to the left, one point to the right | Three points to the left, two points to the right | Two points to the left, three points to the right |
| Left side | | | | |
| Temperature range (°C) | 30-50 | 50-70 | 50-70 | 50-70 |
| $E_a$ (kJ $mol^{-1}$) | 5.20 | 10.35 | 8.02 | 6.64 |
| A | 6.05 | 9.59 | 8.09 | 7.61 |
| $r^2$ | 0.678 | 0.98 | 0.95 | 0.99 |
| Right side | | | | |
| Temperature range (°C) | 30-50 | 50-70 | 50-70 | 50-70 |
| $E_a$ (kJ $mol^{-1}$) | 4.15 | 52.58 | 28.78 | 18.26 |
| A | 17.88 | 53.14 | 45.45 | 28.53 |
| $r^2$ | 1.0 | 1.0 | 0.99 | 0.99 |
| Break point temperature (°C) | 45 | 68 | 59 | 54 |