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Research Article

PURIFICATION, PROPERTIES AND KINETIC STUDIES OF CATALASE FROM LEAVES OF *PHYLLANTHUS RETICULATUS*

K. Beulah[1*] and T. Ramana[2]

[1]Department of Biochemistry, Dr. L. Bullayya college, Visakhapatnam-530003, Andhra Pradesh, India.
[2]Department of Biochemistry, Andhra University, Visakhapatnam-530003, Andhra Pradesh, India.

ABSTRACT

Catalase (EC 1.11.1.6) an antioxidant enzyme has been screened in different medicinal plants, of which the leaf extracts of *Phyllanthus reticulatus* showed maximum activity of 784U/mg. Catalase from *P.reticulatus* has been purified 8 fold with 45% yield involving ammonium sulfate fractionation and gel filtration through SephadexG-150 column. The enzyme preparation is homogeneous on polyacrylamide gel electrophoresis and the molecular weight of the enzyme was estimated as 51.3kDa.The catalase exhibited optimum activity at pH 7.0 using 0.1M phosphate buffer and at temperature of 40°C.The enzyme was stable for 3 days at 4°C. The enzyme activity was slightly stimulated with $CuSo_4$ and $MnCl_2$, where as it was slightly inhibited with NaCl and citric acid. The apparent Km value for the enzyme was determined as 0.74 X 10^5moles l^{-1}

Keywords: catalase, *Phyllanthus reticulatus,* antioxidant enzyme, medicinal plants

1. INTRODUCTION

Phyllanthus reticulatus commonly known as potato bush is a small bushy shrub grows up to 5m in height and trunk upto15cm in diameter. It is used as folk medicine and antiviral agent in the treatment of genitourinary disorders, infections of kidney, intestinal infection and diabetes. This has therapeutic action in curing Hepatitis B infection. Leaf extract of *P.reticulatus* has antiplasmodial action against *Plasmodium falciparum*[1] and antioxidant activity[2].

Catalase (EC 1.11.1.6) is a heme containing enzyme that decomposes H_2O_2. In aerobic organisms, H_2O_2 is produced by various enzymatic reactions involving molecular oxygen by oxidases and the disproportion of superoxide by Super oxide dismutase (SOD). H_2O_2 is a toxic molecule as it can both oxidize and reduce organic substrates in cells. Catalase removes this toxicity by catalyzing the disproportion of H_2O_2 into molecular oxygen and water[3]. Plants have several antioxidant enzymes of which catalase is the most efficient[4]. Along with SOD, catalase plays an important role in defending the cell against oxidative stress and is distributed in almost all aerobic and facultative anaerobic organisms.

Increasing intake of antioxidant compounds has the potential to reduce the risk of free radical related health problems. There is extensive scientific evidence that links cardiovascular disorders, circulation problems, stroke, arthritis, inflammation, liver disorders, cancer and aging are due to elevated levels of free radicals within the body. Under normal environmental conditions antioxidants provide adequate protection against the deleterious effects of reactive oxygen species (ROS)[5].

Intake of medicinal plants in rats results in an increase in antioxidant enzyme activity and HDL cholesterol which may reduce the risk of inflammatory heart disease[6]. Leaves of *Hibiscus cannabinus* showed a hepato protective activity due to its antioxidative property[7].

The therapeutic use of an antioxidant enzyme complex containing SOD and catalase has been used for the treatment of several diseases due to

free radical damage. Plant catalases have been intensively studied and function mainly in the removal of excessive H_2O_2 generated during developmental processes or by environmental stimuli into water and oxygen in all aerobic organisms[8]. Catalase has been purified from germinating cotyledons of pumpkin seeds[9], *Zantedeschia aethiopica*[10], Pea leaf peroxisomes[11], Van apple (Golden delicious)[12], partial purification from Dill (*Anethum graveolens L*)[13] and characterisation of leaf type catalase in Sweet potato[14]. In view of the therapeutic application of catalase, a number of medicinal plants have been screened for the activity of this enzyme and *P.reticulatus* was observed to be a good source. Hence a systematic study on the purification and properties of catalase from *P.reticulatus* has been undertaken.

2. MATERIALS AND METHODS

The leaves of *Jatropha curcas, Murraya koenigii, Carum copticum, Azadirachta indica, Phyllanthus niruri, Andrographis paniculata,* roots of *Asparagus racemosus* were collected from botanical garden of Dr.V.S.Krishna Government College, Visakhapatnam, A.P. Leaves of *Phyllanthus reticulatus,Centella asiatica,* rhizome of *Hedychium coronarium* were collected from the botanical garden of Aaraku valley, Visakhapatnam district, A.P.

Crystalline BSA (bovine serum albumin), SDS(sodium dodecyl sulfate), acrylamide, N,N'Methylene Bisacrylamide, Ammonium persulfate, Tris, Coomassie brilliant blue R_{250} were products of B.Genei, Banglore. Sephadex G-150 was procured from Amersham Pharmacia fine chemicals. Sweden. All other chemicals used were of analytical grade obtained through commercial sources.

2.1 Enzyme extraction: 25 gm of young leaves were washed thoroughly with double distilled water, cut into small pieces and homogenized using 0.1M phosphate buffer pH 7.5. The homogenate was centrifuged for 30 min at 12,000 rpm and the supernatant was used as the enzyme source.

2.2 Enzyme assay: Catalase activity was assayed by the titremetric method described by Radha Krishnan and Sarma[15]. The unit of catalase activity was expressed as ml of 0.1N Potassium permanganate equivalents of Hydrogen peroxide decomposed / min / mg of protein.

2.3 Protein content: Protein concentration of the enzyme extract was determined by the method of Lowry et al.,[16] using crystalline BSA as standard.

2.4 Purification of the enzyme: 25% (W/V) crude leaf extract of *P.reticulatus* has been subjected to fractionation with ammonium sulfate. To 25 ml of the extract, solid ammonium sulfate was added to 40% saturation with constant stirring, allowed to stand for 15 min and centrifuged at 12,000 rpm for 30 min at 4°C. The pellet containing the enzyme was dissolved in 10 ml of 0.1M phosphate buffer pH 7.5 and dialyzed for 12 h at 4°C, lyophilized and further purified by Sephadex G-150 gel filtration chromatography.

70mg of the dialyzed sample dissolved in 1.0 ml of the 0.1M phosphate buffer pH 7.5 was applied on the Sephadex G-150 column, which was pre equilibrated with the same buffer. 3ml fractions were collected at a flow rate of 20 ml/h with the same buffer. The fractions which exhibited catalase activity were pooled, dialyzed, lyophilized and used to study the properties of the enzyme.

2.5 Polyacrylamide gel electrophoresis: SDS-polyacrylamide gel electrophoresis was carried out by the method of Laemmli[17].

Gel dimensions are 10x10 cm with thickness of 1.0 mm. 12% separating gel (8cm) and stacking gel (2cm) was used. 0.05 M Tris-glycine buffer, pH 8.3 was employed as the reservoir buffer. Sample is prepared by adjusting the protein concentration using the sample buffer and heated in boiling water bath for 2 to 3 min.

30 µl of sample solution (crude extract, 40% ammonium sulfate fraction and enzyme fraction from Sephadex-150 column, Protein markers) were loaded in different wells. Electrophoresis was carried out for 3h using a current of 30 mA.

3. RESULTS AND DISCUSSION

Catalase is wide spread in nature having been found in all aerobic organisms studied to date. Most of the work has been performed on the enzyme obtained from mammalian and bacterial sources where it is present in high concentration[18, 19]. Among plants *Spinacia oleracea* was one of the earliest enzyme which was studied[20]. In aerobic organisms and plants,oxygen is an essential element but it can be reduced and form ROS to be a danger and limiting factor in growth and development of plants[21]. To minimize these ROS molecules, all aerobic organisms including plants have evolved various enzymatic and non enzymatic defense

mechanisms. Catalase is one of several antioxidant defense enzymes (SOD, Peroxidase, Glutathione peroxidase) that catalyses dismutation of Hydrogen peroxide to water and oxygen.

Catalase (EC.1.11.1.6) is a tetrameric heme containing enzyme found in all aerobic organisms is known to play a key role in protecting cells against oxidative stress[22]. In this paper catalase was purified from *Phyllanthus reticulatus* and its kinetic properties were studied.

Catalase activity in different medicinal plants is given in Table-1. High catalase activity has been observed in the leaf extract of *P.reticulatus* (784U/mg)and *A .paniculata* (660U/mg). Considerable activity has been noticed in the leaf extract of *P.niruri* and *M.koenigii.* In view of its high activity in *P.reticulatus*, preliminary studies have been carried out on the extractability, stability and properties of the crude enzyme. Maximum activity has been recovered in 25% (W/V)) leaf extract due to extraction of more protein. This crude enzyme has considerable activity at temperatures ranging from 5°C to 55°C. Activity was stable for one day. Activity is high at pH 7.5 with 0.1M phosphate buffer (data not given)

The purification profile of catalase from *P.reticulatus* leaves is given in Table-2. The enzyme is purified 8 fold with 45% yield. The crude extract when precipitated with 40% ammonium sulfate saturation purified to 4.3 fold with 71% yield and 8 fold with yield of 45% by gel filtration chromatography through Sephadex G-150 column. Two minor and one major protein peaks were observed on gel filtration. Catalase activity was distributed only in the major peak (Fig-1).Catalase was partially purified from Van apple (Golden delicious) to 8.7 fold with an yield of 11%[12].

3.1 Homogeneity: Purity of the catalase during different stages of purification was examined by SDS--PAGE in 12% polyacrylamide gels. Single distinct protein band was noticed with the purified enzyme preparation, indicating its homogeneity (Fig-2).

3.2 Molecular weight: Approximate molecular weight of the purified catalase was estimated as 51.3kDa on comparison with electrophoretic mobilities of marker proteins of known molecular weight. The catalase purified from cotyledons of germinating pumpkin seeds had molecular weight 55kDa[9]. The catalase purified from *Zantedeschia aethiopica* also had a molecular weight 54 kDa[10].

3.3 Optimum pH: The enzyme activity, when studied at different pH values ranging from 3.0 to 10.0 is found to be maximum at pH 7.0 (Fig-3) The optimum pH of catalase from Van apple is *pH* 5.0[12].

3.4 Optimum temperature: The effect of temperature on catalase activity was studied by assaying the enzyme activity at different temperatures ranging from 20°C to 80°C. The catalase activity increased gradually from 20°C to 40°C and then declined due to the denaturation of enzyme. The results obtained indicate that the catalase is maximally active at 40°C (Fig-4).The catalase of *Zantedeschia aethiopica* also showed similar optimum temperature and it was stable between 0°C to 50°C[10].

3.5 Effect of buffer concentration on catalase activity: The enzyme activity was studied using 0.01M to 0.5M phosphate buffers, pH 7.0. The activity was maximum at 0.1M concentration. (data not given)

3.6 Stability of catalase: Stability of catalase activity was studied by assaying the enzyme activity up to 10 days by storing the enzyme at 4°C.The results presented (Fig-5) indicate that the enzyme was stable up to 3 days and then the activity gradually decreased and completely inactivated on 9th day.

3.7 Effect of different compounds on catalase activity: The effect of $MnCl_2$, NaCl, $NaNO_2$, $CuSO_4$, Benzoic acid and citric acid on catalase activity was studied at a final concentration of 5mM. $CuSO_4$, $MnCl_2$ showed slight stimulatory effect, NaCl, citric acid exhibited slight inhibitory effect, whereas $NaNO_2$ and Benzoic acid did not influence the enzyme activity.

3.8 Kinetic studies of the catalase: Effect of different concentrations of substrate on the catalase activity was studied using H_2O_2 as substrate in 0.1M phosphate buffer pH 7.0 using 10 mg of enzyme/ml. The results obtained are depicted as Line weaver Burk double reciprocal plot for H_2O_2 as substrate in Fig-6. The apparent Km value calculated for H_2O_2 as substrate is 0.74×10^{-5} moles l^{-1}. It has been concluded that *P.reticulatus* is a good source of antioxidant enzyme catalase. Its therapeutic role as an antioxidant and as ROS defense system is further being evaluated.

Table 1: Catalase activity in medicinal plants

Medicinal plant	Catalase activity (U/mg)
Andrographis paniculata	660.3
Asparagus racemosus	281.8
Azadirachta indica	146.13
Carum copticum	94.43
Centella asiatica	26.03
Hedychium coronarium	92.5
Jatropha curcas	173.2
Murraya koenigii	555.2
Phyllanthus niruri	618.6
Phyllanthus reticulatus	784.2

Table 2: Purification of Catalase from leaf extract of P.reticulatus

Fraction	Volume (ml)	Protein (mg/ml)	Activity (U/ml)	Specific activity (U/mg)	Purification fold	Yield %
Crude extract	25	0.85	633.3	745	1	100
Ammonium sulfate precipitation	10	0.35	1133.3	3237	4.3	71.5
Gel filtration chromatography	12	0.1	600	6000	8.05	45.40

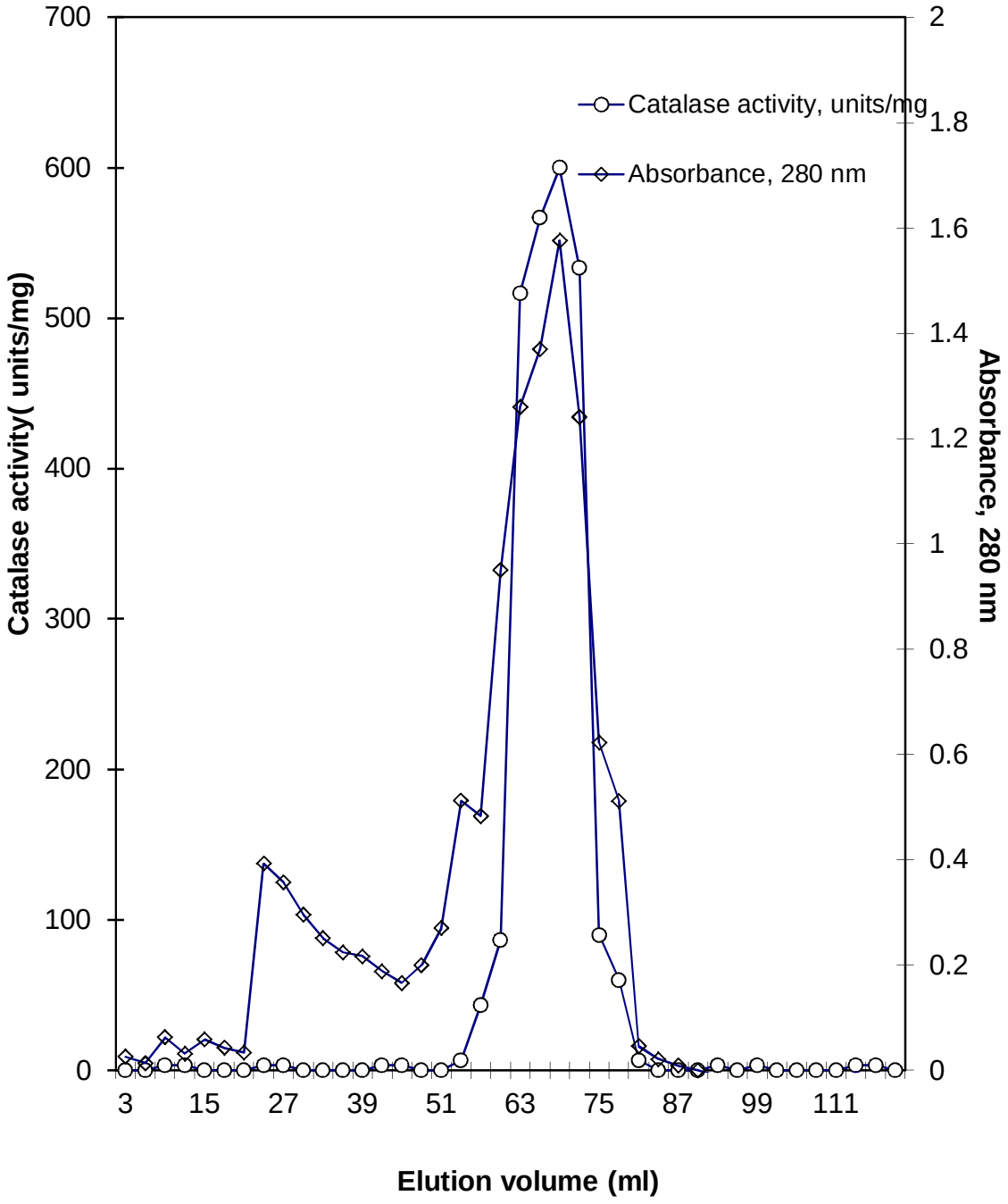

Fig. 1: Elution profile of catalase through Sephadex G-150 Gel filtration column

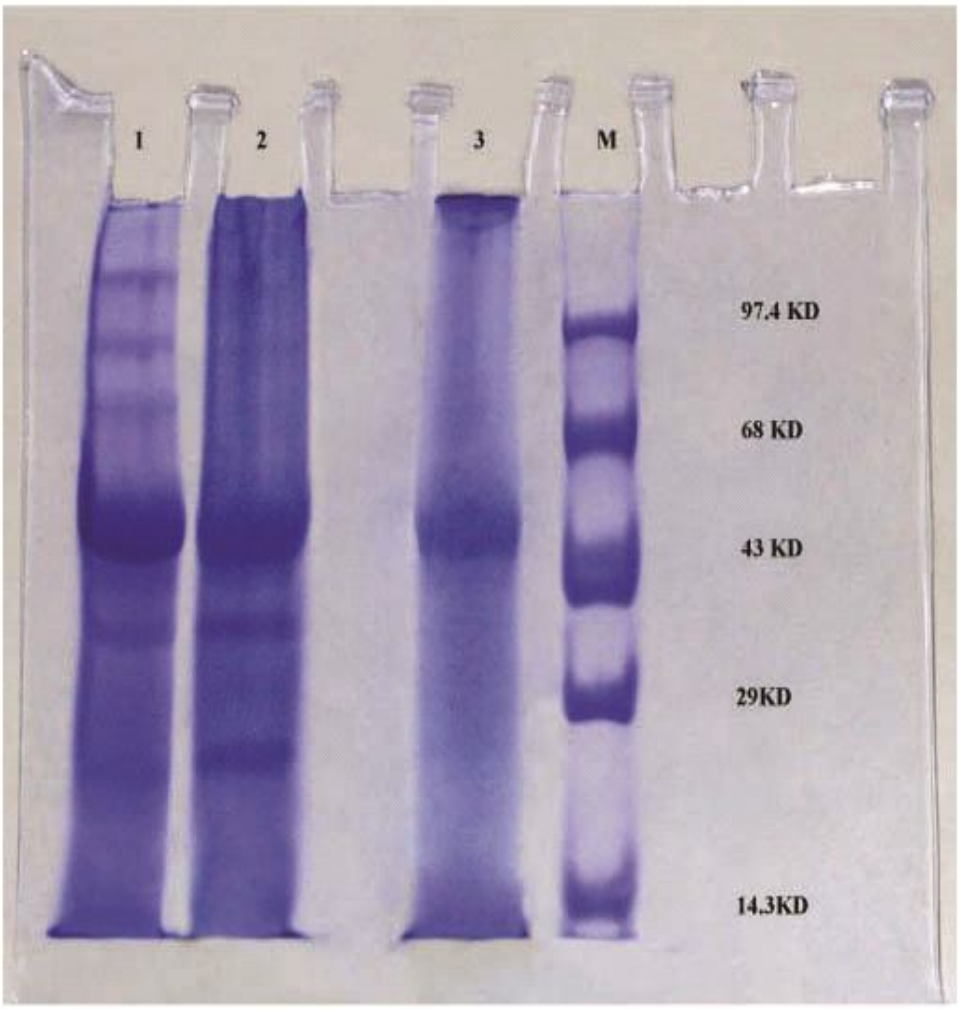

Fig-2

Fig. 2: SDS-Polyacrylamide gel electrophoresis

Lane-1Crude extract,Lane-2 Ammonium sulfate fraction, Lane-3 Fraction purified by Sephadex-150 gel filtration,Lane-4 Protein markers.

1. Phosphorylase-b 97.4 kDa
2. Bovine serum albumin 68kDa
3. Ovalbumin 43kDa
4. Carbonic anhydrase 29kDa
5. Lysozyme14.3kDa.

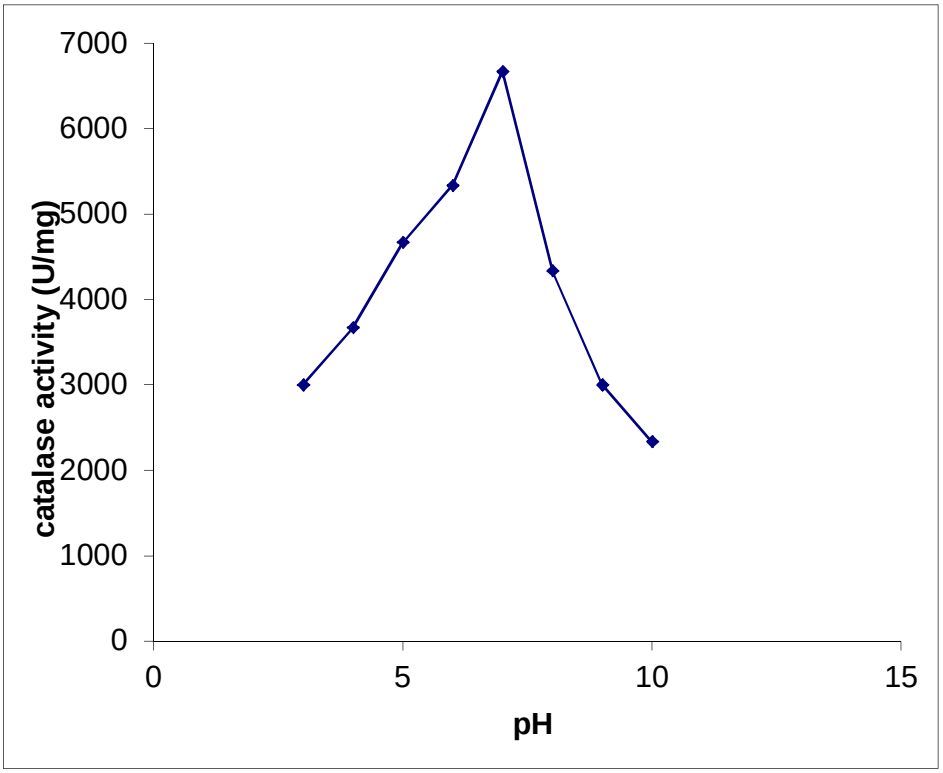

Fig. 3: Effect of pH on Catalase activity in *Phyllanthus reticulatus*

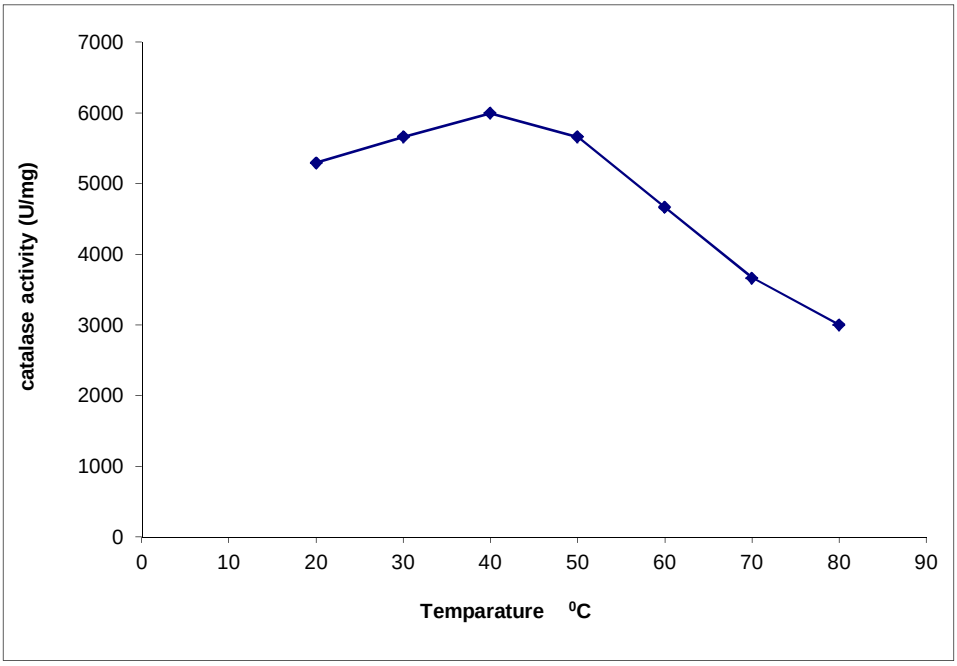

Fig. 4: Effect of temperature on catalase activity

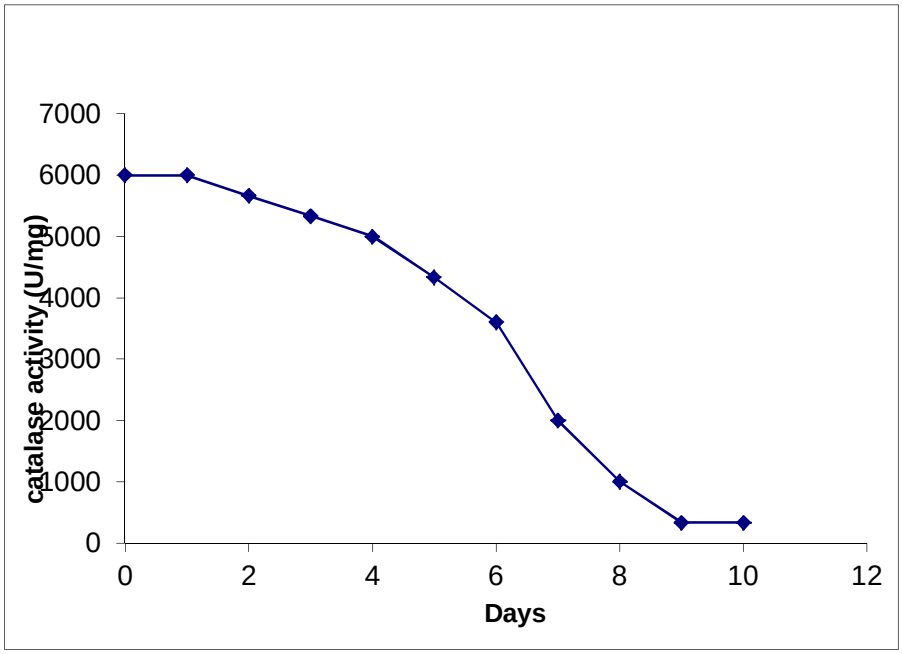

Fig. 5: Stability of catalase activity

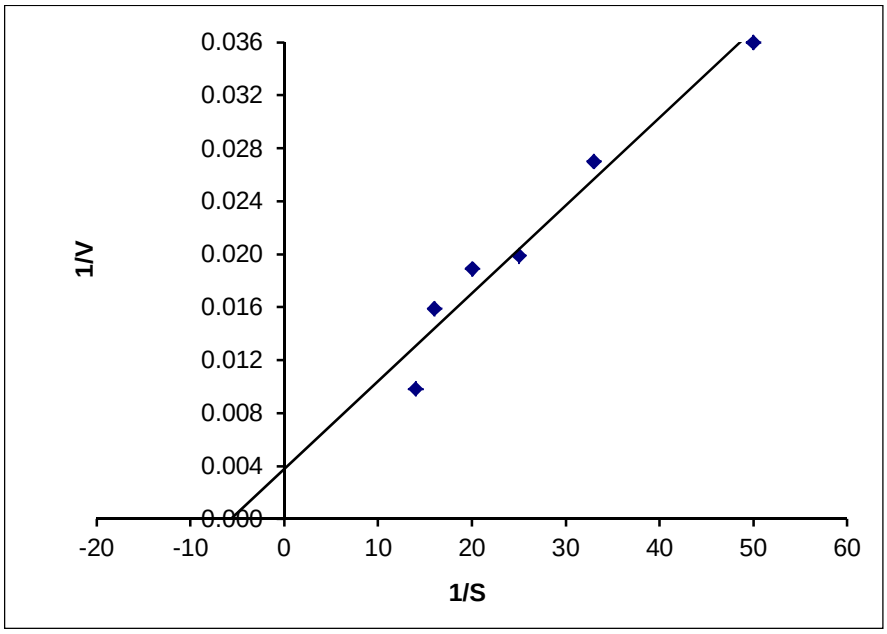

Fig. 6: Effect of H2O2 (substrate) concentration on catalase activity

4. ACKNOWLEDGEMENT

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