International Journal of Scientific Research and Management (IJSRM)
||Volume||5||Issue||08||Pages||6603-6612||2017||
Website: www.ijsrm.in ISSN (e): 2321-3418
Index Copernicus value (2015): 57.47 DOI: 10.18535/ijsrm/v5i8.05

Purification and characterization of thermo alkaliphilic catalase from haloarchaeal strain

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Abstract
In this study, Haloarchaeal strain was isolated from water samples collected from salt pan. The strain was further treated with 2% H_2O_2 and incubated in a water bath with shaker for 12 days. Supernatant was collected from early log phase and was used as enzyme source. Protein was precipitated by ice cold ethanol followed by purification with Sephadex G-150 column chromatography. SDS-PAGE report showed that that the molecular weight of catalase was found to be 111.2 KD using 12% gel. The results showed that the optimum pH of catalase in supernatant was found to be at 9.0 and optimum temperature was found to be at 65 °C. In case of purified sample the activity of catalase was found to be increased at pH 10 and optimum temperature was noticed at 65ºC. Activity of enzyme was increased in increasing concentration of enzyme upto 2.5ml, beyond that the activity declined. The enzyme has great affinity for the substrate $H_2O_2$and the Vmax and Km was calculated as 80mM and 40mM respectively by Michaelis menton plot. The activity of catalase from purified sample was also assayed in the presence of metal ions and the results obtained indicated that the activity was found to be increased significantly from 85.5 mM/mL and the activity was stable and constant up to 171 mM/mL of NaCl and at 0.024mM/mL of MgSO4 and 0.03mM/mL of MnSO4. The activity was found to be decreased when the concentration of ZnSO4 was increased.
Keywords: Halobacterium, Catalase, Zobell marine agar, SDS-PAGE

Introduction

There has been growing interest in recent years in utilizing hydrogen peroxide in industrial sectors such as food, dairy, textiles, pulp and paper [1] as a more environmentally friendly alternative to existing chemical treatments. As use of hydrogen peroxide in industrial settings grows, there will also be a need to remove hydrogen peroxide from process streams because it can interfere with subsequent process steps [2]. An example of hydrogen peroxide use is in the textile industry for bleaching of fabrics [3], where it has been shown that hydrogen peroxide interferes with subsequent dyeing steps and must be removed [2]. Current methods to remove the hydrogen peroxide either utilize extensive washing and result in the generation of large volumes of waste water [4] or utilize chemical treatments such as sodium bisulfite or hydrosulfite to reduce hydrogen peroxide and lead to high salt levels in the process streams [5]. It has been proposed recently that the enzyme catalase be used to remove residual hydrogen peroxide from the bleach water so that it can be reused in the subsequent dye steps [6]. However, since the hydrogen peroxide bleaching step occurs at elevated temperatures and pH (> 60 °C and pH 9), commercially available catalases that are optimally active at 20-50°C and at neutral pH require that the temperature and pH be adjusted prior to their use [7]. Catalase is a ubiquitous enzyme found in aerobic organisms, efficiently catalyzes the decomposition of hydrogen peroxide to oxygen and water and, together with other enzyme systems, protects cells against the harmful effects of reactive oxygen species such as superoxide anions, hydrogen peroxide, and hydroxyl radicals [8, 9, 10]. In addition to having activity at higher temperatures, the thermostable versions exhibit properties similar to those of their mesophilic counterparts. Many of the reported enzymes exhibited low thermal stability at temperatures above 60°C, several were rapidly inactivated in the presence of hydrogen peroxide,

and most of the enzymes had low activity and stability at elevated temperature and pH, making them unsuitable for industrial applications. By considering these major facts, this paper describes the isolation, purification, and characterization of an extremely thermo-alkali-stable catalase enzyme from *Haloarchaeal sp.*

Materials and methods

Collection of sample

Four water samples were collected from different locations of Puthalam salt pan in sterile polythene bags and were aseptically transported to the laboratory.

Isolation of Haloarchaea

The water samples were serially diluted by taking 1 ml from each sample individually and a total of ten dilutions (10^{-1} to 10^{-10}) were made by sea water. The 10^{-6} dilution is used for plating on Zobell marine agar plates by spread plate method. The plates were incubated at 42°C for 12 days [11].

Media formulation for catalase activity (9%)

For the assay of catalase, 4.25gm) Zobell Marine broth contains NaCl(29gm), glucose (9%), fructose (9%), sucrose (5%), galactose (6%), maltose (4%), lactose (4%), starch (10%), cysteine (1.5%), histidine (1%), arginine (0.8%), glycine (0.8%), tryptophan (2%), tyrosine (5%), hydrogen peroxide (2%), $MgSO_4.7H_2O$ (20%), $MgCl_2.6H_2O$ (10%), $MnSO_4$ (10%) and pH (8.8). Alkaliphilic organisms grown at pH of 8-10 using carbonates to maintain the pH [12, 13].

Screening of catalase activity

The catalase activity was assayed by using dichromoacetic acid method [14].

Purification of enzyme

Precipitation by solvent

The cell free fermented media was collected and subjected to different steps of purification. The enzyme protein was partially purified using ice cold ethanol precipitation method [15]. Solvent was added to 100ml of culture filtrate to get 70% saturation and most proteins were precipitated out [16].

Purification by chromatography

Cultures were collected from early stationary phase and centrifuged at 7,500 × g for 10 minutes at 4˚C. Washed twice with 50 mM potassium phosphate (pH-7.0) containing 2M NaCl. Resuspended the cells in 50 ml of 50 mM potassium phosphate and then frozen at -70˚C for 15 minutes and thawed at 37˚C for 3 minutes (5 times). To achieve maximal cell disruption, cells were subjected to sonication at 70% on a Branson sonifier at 4˚C for 30 seconds, followed by cooling for 2 minutes inorder to prevent excessive heating. At the end of sonication, 1mg of DNase I was added to the homogenate and the mixture was stirred at room temperature for 1 hour. The extract was centrifuged at 10,000×g at 4˚C for 15 minutes and added solid polyethylene glycol (M.W=6000-8000) to the clarified supernatant. The suspension was stirred at room temperature for 15 minutes and then centrifuged at 7,500×g for 15 minutes. The final concentration of supernatant was made upto 15% (wt/vol) by adding polyethylene glycol and then stirred at room temperature. The mixture was recentrifuged and the resulting pellet was resuspended in 50ml of 50mM potassium phosphate (pH-7.0) containing 2M NaCl and dialyzed over night by using the same buffer. 2.5gm of solid hydroxylapatite was added to the solution, stirred at 4˚C for 15 minutes. The protein was eluted in batch wise fashion with 100mM potassium phosphate (pH-7.0) containing 2M NaCl. The eluant was assayed for catalase activity and then subjected to dialysis against 50mM potassium phosphate (pH-7.0) containing 2M NaCl. The preparation was concentrated to 1.0 ml by Amicon ultrafiltration and then loaded onto a Sephadex G-150 column purchased from sigma (0.75 by 79cm) equilibriated with 50mM potassium phosphate buffer of pH 7.0 containing 2M NaCl and the protein was eluted with the same buffer with the flow rate of 0.25ml/min. One unit of enzyme activity is defined as the amount of enzyme that catalyzes the oxidation of 1µmole of H_2O_2/min under standard assay conditions used here.

Molecular weight determination

Sample preparation

The extracted protein samples were subjected to one dimensional SDS-PAGE analysis according to Laemmli [17], by diluting the sample in 1X loading or cracking buffer containing 0.08M TrisHCl, pH-6.8, 2.7% SDS, 13.7% glycerol, 0.97M β-mercaptoethanol and 0.3% bromophenol blue, and kept at 100˚C for 3 minutes before loading.

SDS-PAGE Electrophoresis

Proteins were resolved by SDS PAGE. 0.75 mm thick gels were cast in a gel apparatus using separating and stacking gels. The separating gel mixture was poured into a glass plate cassette assembly, overlaid with water saturated with isobutanol (1:1) and allowed to polymerize for 2 hours at room temperature. After polymerization water saturated isobutanol layer was removed and the separating gel was overlaid with 0.1% SDS and stored over night at 4˚C. After removing 0.1% SDS, top of the gel was rinsed with water and the stacking gel was then poured on the top of the separating gel. Comb was then inserted into the stacking gel giving 1cm space between the bottom of the well and the top of separating gel. Then the stacking gel was allowed to polymerize for 45 minutes at room temperature. The comb was removed carefully and the wells were cleaned by rinsing with electrophoresis buffer. Protein samples were loaded on to the wells. 1X electrophoresis buffer was then added in the upper and lower chamber of the gel casting unit and the gel was run at a constant voltage of 50 volt until the proteins reach the separating gel. After that the voltage was raised to 150 volt.

Characterization of enzyme activity

The activity of catalase from crude as well as purified sample was assessed by using various pH, temperature, enzyme concentration and substrate concentration. The activity of catalase was also analysed in the presence of metal ions such as $MnSO_4$, $MgSO_4$, NaCl and $ZnSO_4$ by dichromoacetic acid method [14].

Results

Screening of catalase producing strain

Table 1: Activity of catalase in different strains

Strain	Catalase Activity U/ml
I (Red)	2.826
II (yellow)	0.716
III (White)	0.458

Figure 1: Growth of Haloarcheal strain on Zobell marine agar plate

All the three isolates were screened for catalase activity. Among the three strains, strain 1 red pigmented bacteria has the highest catalase activity (2.826 U/mL), shown in table (1). Hence the red pigmented strain (RHA) was used for further study.

Estimation of protein

Protein was estimated by Lowry's method [18].

Enzyme production in optimized medium

The catalase activity was determined from control and optimized medium. The results were recorded and shown in table 2.

Table 2: Enzyme production in optimized medium

Particulars	Enzyme activity (U/ml).
Control medium	3.12
Optimized medium	14.36.

The production of catalase was enhanced by cultivating the isolated strain in optimized medium and the observed results indicate that the production of catalase was found to be more (14.36 U/ml) in optimized optimized medium than the control. Similar results published by Higa *et al.*, [19] suggested that the enzyme production was high in optimized medium. **S**everal researchers reported that fermentation medium has an important role in enzyme production and microbial enzyme production was enhanced under optimized medium conditions [20, 21]. So media formulation is an important prerequisite to enhance enzyme production.

Purification of catalase

The crude extract collected from early stationary phase cultures of test strain was subjected to centrifugation. The supernatant was

concentrated by ice-cold ethanol precipitation followed by dialysis and gel filtration chromatography.

Table 3: Activity of Catalase in ice cold ethanol solvent fraction

Five fractions were collected, among the five fractions, fraction IV showed highest enzyme activity (70 U/ml) and specific activity of 8.64U/mg as shown in Table 3. Various methods have been used for recovery of extracellular proteases from a mixture of different proteins. Commonly used methods are ammonium sulphate precipitation followed by affinity chromatography and gel filtration [22, 23]. In the present study, best precipitation yield was noticed in ice cold ethanol and purified by dialysis and gel filtration chromatography. This is because haloarchaeal proteases do not usually precipitated by saturating with ammonium sulphate because of the presence of high salt in the medium. Studdert *et al.,* [24]; Giménez *et al.,* [25]; Vidyasagar *et al.,* [26], reported that ethanol precipitation method instead of ammonium sulphate is one of the best methods for precipitating haloarcheal proteins. May and Dennis, [27] used ammonium sulphate precipitation, Ion exchange chromatography and Gel filtration Chromatography in their purification studies of Superoxide dismutase.

Activity of catalase

Activity of catalase was estimated from culture supernatant, crude fraction and Sephadex G-150 purified fraction. The results are encapsulated in table 4.

Table 4: Purification of catalase

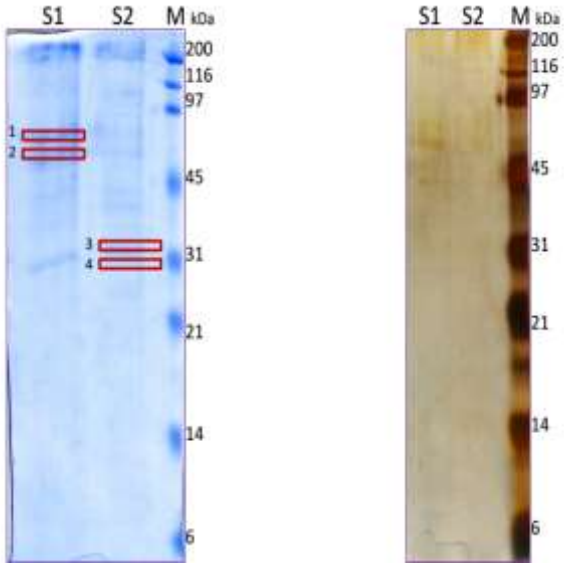
Sample	Enzyme Activity (U/ml)	Protein Content (mg/ml)	Specific Activity (U/mg of Protein)
Culture Supernatant	48	6.0	8.0
Crude fraction	120	8.8	13.63
Sephadex G-150	322	1.6	201.255

The results clearly indicate that the purified fraction shows highest catalase activity (201.25 U/mg) compared with culture supernatant (8.0 U/mg) and crude fraction (13.63 U/mg). The crude enzyme (SOD) isolated from the seeds of *Amaranthus spinosus* showed specific activity of 0.54 Units/mg [28].

Molecular weight determination

Fractions	Activity of catalase (U/ml)	Protein content (mg/dl)	Specific activity (U/mg)
I	28	7.0	4.0
II	47	7.6	6.18
III	64	7.6	8.42
IV	70	8.1	8.64
V	48	6.0	8.0

SDS-PAGE analysis yielded two identical subunits having a molecular weight of 55.6 Kda. From the results obtained, catalase of the present study is a dimer composed of two identical subunits and the total molecular weight of catalase was found to be 111.2 Kda. Similar reports suggested that catalase containing two subunits have been identified in prokaryotes such as *Streptomyces venzuelae, Comamonas compransoris, Klebsiella pneumonia Kpa, Mycobacterium tuberculosis* and *Bacteroides fragilis* respectively. Catalase having six and four subunits has been reported in *Haemophilus influenza* and *E.coli* respectively. But catalase



isolated from *D. gigas* seems to have three subunits with low heme content per molecule, which are the remarkable characteristics of this catalase [29]. Typical catalases such as mammal

type catalases are commonly isolated from animals, plants, fungi and bacteria are composed of four subunits of equal size with molecular mass range of 225 to275 Kda [30].

Characterization of enzyme

The supernatant collected from crude broth and purified sample were used as enzyme source and were subjected to find out the effect of pH, temperature, enzyme concentration and substrate concentration. The activity of catalase was also analysed in the presence of metal ions such as $MnSO_4$, NaCl, $MgSO_4$ and $ZnSO_4$ by dichromoacetic acid method [14] with hydrogen peroxide as substrate.

Effect of pH

To determine optimum pH both the supernatant and purified enzyme fractions were incubated in phosphate buffer of varying pH ranging from 7.0 to 10.5 and the results are shown in fig- 2 and 3.

In supernatant

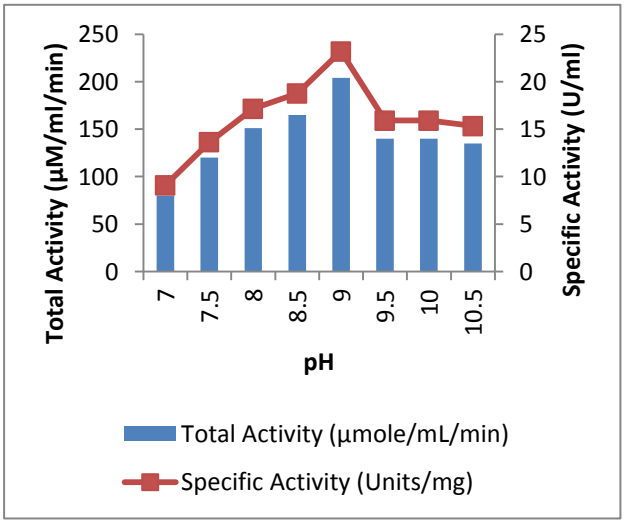


Figure 2: Effect of pH in catalase activity from supernatant In purified sample

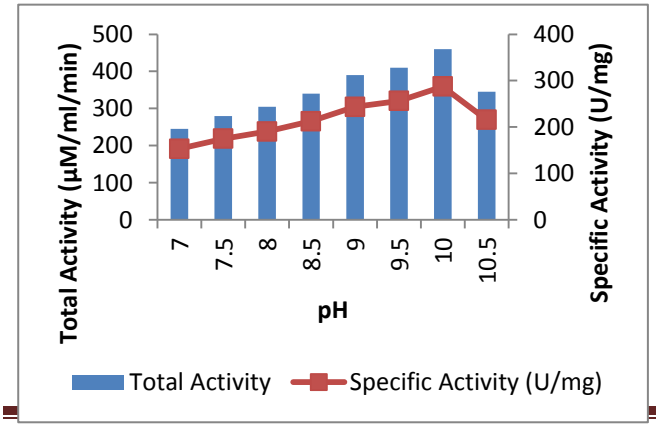


Figure 3: Effect of pH in catalase from purified sample

The activity of catalase was found to be increased from pH 7.0 and reached its maximum at pH 10.0 with specific activity of 287.5 U/mg and very less activity was noticed at pH 7.0 with specific activity of 153.125 U/mg. The results pointed out that the optimum activity of catalase was found to be at pH 10.0 in purified sample and about 23.181 U/mg at pH 9.0 (optimum pH) in supernatant. The activity of catalase in purified sample was significantly increased to about 12.4 folds at pH 10.0 compared with activity at pH 7.0. Comparative study with supernatant, the activity of catalase was found to be high in purified sample. Catalase isolated and purified from *Vibrio rumoiensis* S-1T showed optimum pH range from pH 6.0 to 10.0, while the activity was completely eliminated below pH 3.0 [30]. Similar study with catalase isolated from *Rhizobium radiobacte*r Strain 2-1was stable at pH 5-11 and optimum pH was 6-11 [31]. A catalase-peroxidase isolated from a thermoalkaliphilic *Bacillus sp.* was stable at high pH with a half life of 104 h at pH 10 at 25˚C and 14 h at 50˚C. Because of its high pH stability and thermostability, this enzyme has potential for the treatment of textile bleaching effluents [32].

Effect of temperature In supernatant

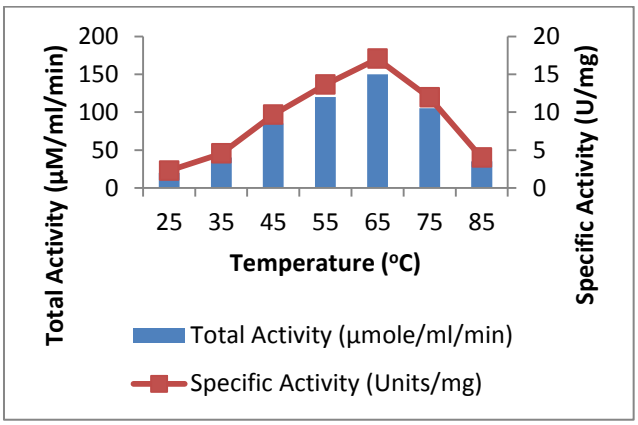


Figure 4: Effect of temperature in catalase from supernatant

The results indicate that highest activity of catalase was found to be noticed at 65˚C (17.04 Units/mg) whereas activity was significantly decreased at temperature below and above 65˚C (Fig.4).

In Purified Sample

DOI: 10.18535/ijsrm/v5i8.05

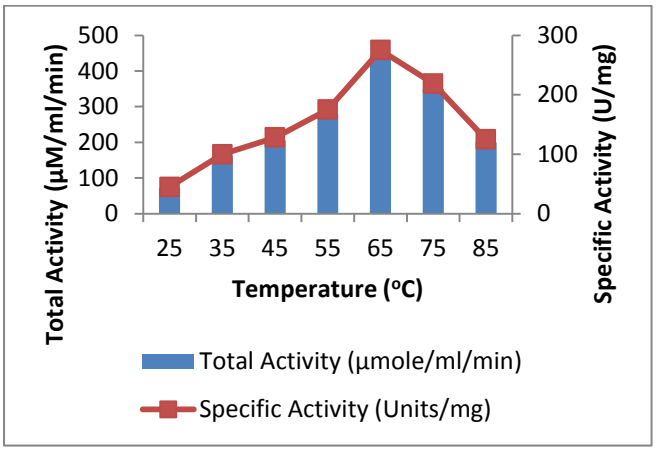


Figure 5: Effect of temperature in catalase activity

The activity of catalase showed optimum at temperature 65°C (275 U/mg) (Fig.5). It explained that the activity was markedly increased to about 6.1 folds at 65°C, compared the activity at 25°C (45 U/mg). Comparative analysis revealed that the enzyme was stable and active when temperature was raised from 25°C upto 65°C, apart from the activity decreased drastically. Similarly catalase derived from *Rhizobium radiobacter* strain 2^{-1} was more stable at high temperature, which is widely used for industrial catalase production. The activity remained stable at approximately 30% after incubation for 15min at 65°C [31]. Because of its high pH stability and thermostability, this enzyme has potential for the treatment of textile bleaching effluents [32].

Effect of enzyme concentration

Activity of catalase from purified sample in various concentrations of enzyme ranging from 0.5 mL to 2.5 mL and the observed results are summarized in fig (6).

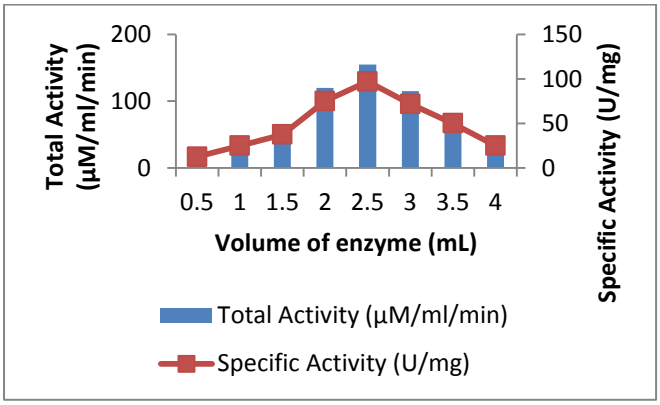


Figure 6: Effect of enzyme concentration in catalase activity

The obtained results revealed that the activity of catalase was found to be increased upto 2.5ml of enzyme with specific activity of 96.87 U/mg of protein, superior to that there was no improvement was remarked in the activity of catalase instead the activity declined. Similar works suggested that the enzyme activity in purified sample was gradually increased with increase in concentration of enzyme and after saturation level there was no increase in the activity, instead it declined [33, 34, 35].

Effect of substrate concentration

Activity of catalase in the presence of various concentration of substrate was assessed and the results are given in table (5).

Table 5: Effect of substrate concentration in catalase activity

Concentration of Substrate [S] (mM)	Optical Density at 470 nm [V_0]
020	0.05
40	0.10
60	0.18
80	0.24
100	0.24

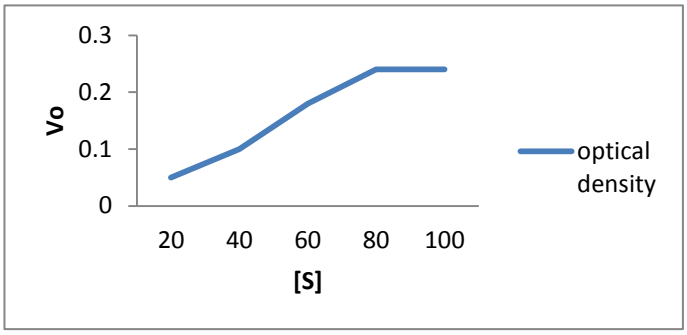


Figure 7: Michaelis-Menton plot

Based on the observations, the Vmax of catalase was calculated as 80mM and the Km value was found to be 40mM, presented in fig (7). This indicates that the enzyme has strong affinity with the substrate H_2O_2. Vicki *et al.*, [36] reported that the catalase isolated from *Thermus brockianus* showed a Km value of 35.5 mM and a Vmax of 20.3 mM/min/mg protein.

Effect of NaCl

DOI: 10.18535/ijsrm/v5i8.05

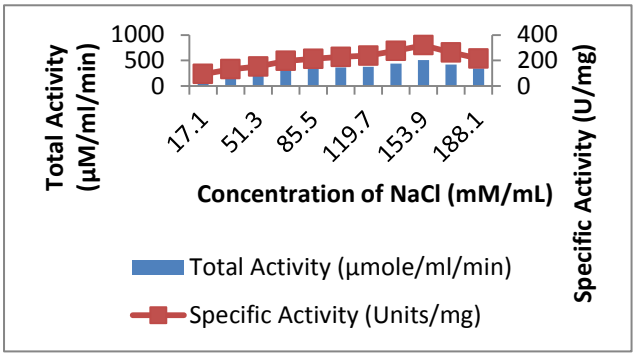


Figure 8: Effect of NaCl in catalase activity

The results denote that catalase showed maximum activity of at 153.9 mM of NaCl (318.75 U/mg), and further step up in NaCl indicates no improvement in catalase activity as shown in fig (8).

Effect of metal ions

Activity of catalase in control

Activity of catalase from control sample was determined and the results indicates that the total activity of catalase was found to be 40 µM/ml/min with the specific activity of 25 U/mg.

Effect of $MnSO_4$ in catalase activity

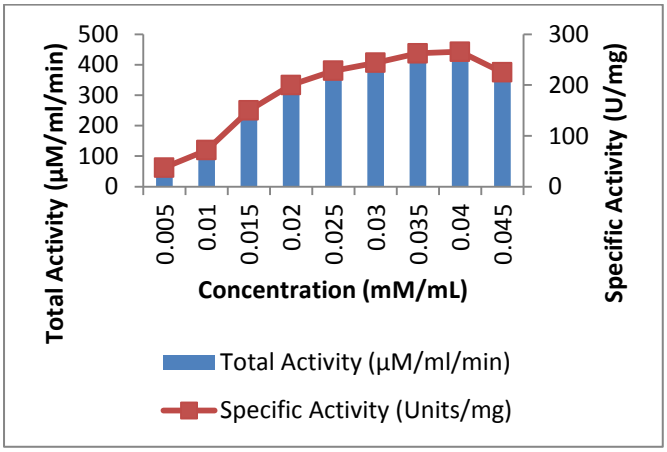


Figure 9: Effect of $MnSO_4$ in catalase activity

The graphical representation in fig (9) shows the activity of catalase in response to increasing concentration of $MnSO_4$. The enzyme exhibited to amplify its activity from 0.005 mM to 0.045 mM of $MnSO_4$. Maximum activity was noticed at 0.04 mM (265.625 U/mg) of $MnSO_4$, greater than that the activity showed gradual diminution.

Effect of $MgSO_4$

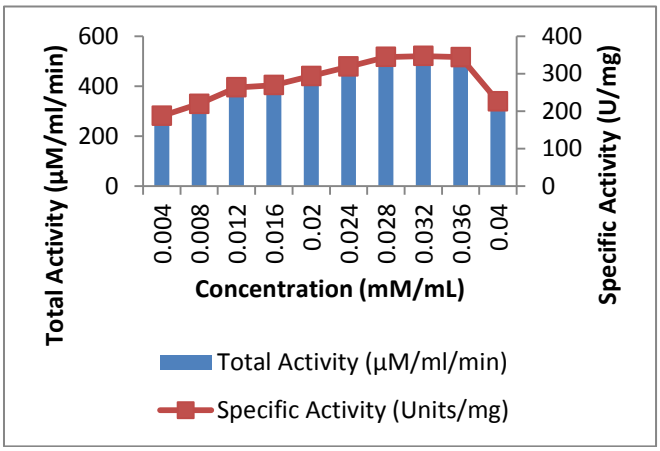


Figure 10: Effect of $MgSO_4$ in catalase activity

The activity of catalase in different concentrations of $MgSO_4$ ranging from 0.004 mM to 0.04 mM was represented in fig (10). The graphical representation indicates that the activity of catalase was found to be increased upto 0.032 mM of $MgSO_4$ (346.875 U/mg), moreover gradual decrease was noticed in the activity of catalase.

Effect of $ZnSO_4$

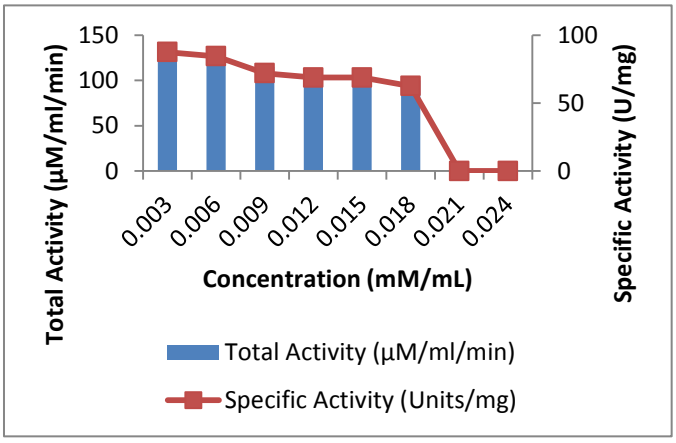


Figure 11: Effect of $ZnSO_4$ in catalase activity

The results reveal that activity of catalase was found to be decreased gradually as the concentration of $ZnSO_4$ was increased, given in fig (11). Catalase activity was decreased to about 3.5% when the concentration of $ZnSO_4$ was increased from 0.003mM to 0.006mM, 17.8% at 0.009mM, 21.4% at 0.012mM, 21.4% at 0.015mM and 28.5% at 0.018mM of $ZnSO_4$ respectively. The enzyme lost its catalytic activity completely at 0.021mM of $ZnSO_4$. Metal ions

have been proposed to prevent autolysis of protease enzymes or protect their 3D structures. Metal ions such as Mg^{2+}, Ca^{2+}, Cu^{2+} and Ag^{2+} enhanced protease activity [37]. Metal ions apparently protect the enzymes from thermal denaturation and played a key role to continue the active conformation of the enzyme at extreme temperatures [38]. Increased intracellular concentration of oxy radicals is the reason for triggering the activity of antioxidant enzyme in cells [39]. The present fact finding coincides the above report and indicates that $MnSO_4$, NaCl and $MgSO_4$ influenced the activity of catalase to certain concentration, over that the activity was declined slowly, whereas in case of $ZnSO_4$ the activity was found to be decreasing when doubling the concentration of $ZnSO_4$. Moreira *et al.*, [40] observed that K^+, Mg^{2+} and Na^+ stimulated protease activity of *Nocardiopsis sp.* Similar report published by Ahmad *et al.*,[41], found that Mn^{2+} as stimulatory for protease from *Streptomyces avermectinus* but Zn^{2+},Fe^{2+} and Cu^{2+} were found inhibitory.

Conclusion

The catalase isolated and purified from haloarchaeal strain showed stability at extreme temperature and optimum activity was found to be at alkaline pH compared to other reported industrially important catalases. Due to its thermostability, it can be used in textile industries for bleaching effluents. In addition, this catalase has a number of unusual characteristics compared to other catalases. The activity of catalase was found to be increased exponentially when increasing the concentration of NaCl, $MnSO_4$ and $MgSO_4$. But compared to other reported catalases the activity of this catalase was found to be decreased gradually as the concentration of $ZnSO_4$ was increased.

Acknowledgement

The authors thank Dr. N.C.J. Packia Lekshmi for her kind assistance during the study.

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