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Full length Research Paper

Purification and characterization of thermo-alkali stable catalase from *Bacillus* sp

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Thermo-alkali stable catalases are promising enzymes in biotechnological applications as H_2O_2-detoxifying systems. Catalase has been purified and characterized in this study from *Bacillus* sp. The purification was performed with a three step procedure consisting of ammonium sulfate precipitation, ion exchange chromatography on DEAE- cellulose and gel filtration chromatography on Sephacryl-S-300 and finally achieved a 16.6-fold-purifying over the crude extract with 50% overall yield. The purified catalase evidenced an estimated molecular weight of 65 kD. The enzyme also exhibited a broad optimal pH (6.0-10.0), and remained stable (100%) in alkaline pH (8.0-10.0),also it was most active at 70℃ and stable within a range of 50-80℃. 2- mercaptoethanol, sodium azide, and potassium cyanide are known protein inhibitors, inhibited catalase activity by 70, 50 and 35% respectively.

Keywords: Purification, characterization, thermo-alkali stable, catalase, *bacillus* sp.

INTRODUCTION

Catalases (H_2O_2 oxidoreductase; E.C. 1.11.1.6) are widely spread in aerobic (Facultative or not) bacteria such as *Escherichia coli* and *Bacillus subtilis* (Rochat *et al.,* 2005). Catalases are one of the central components of the detoxification pathways that prevent the formation of highly reactive hydroxyl radical by catalyzing the decomposition of H_2O_2 into water and oxygen by two electron transfer (Shin *et al.,* 2008). Based on their enzymological properties, bacterial catalases have been classified into three types: heme-containing monofunctional catalases, heme containing bifunctional catalase-peroxidases that have a peroxidative activity as well as the catalatic activity, and non-heme- containing catalase (Chelikani *et al.,* 2004). Typical catalases have been isolated from numerous animals, plants, and microorganisms and have similar properties regardless of the source (Thompson *et al.,* 2003).

Catalase has found its application in various biotechnological processes.. There are several studies on catalases from agents that cause human disease in relation to protection against the oxidative bursts of macrophages (Archibald and duong, 1986; Bishai *et al.,* 1994; Yumoto *et al.,* 2000), the enzyme also used in diagnostical centres, and for polyenzime antioxidant drugs obtaining in the form of water – soluble conjugates with polymers or soimmobilized proteins on carriers. Such drugs are necessary for purification of biological liquids from O_2 and H_2O_2, for many diseases and especially for radiotherapy in different pathologies (Popovici, 2001; Sirbu, 2011). On the other hand, the enzyme is applied in scientific research, in environmental monitoring, in biosensoring technologies for determination of hydrogen peroxide and ethanol number containing (Borisov, 2000).

Hydrogen peroxide (H_2O_2) is a powerful oxidant, and is used as bleaching or microbiocidal agent in the paper, food, textile and seminconductor industries. However, due to its toxicity to environment and human health, hydrogen peroxide needs to be eliminated after industrial processes. Thus, catalases can be utilized in these industrial sections (Zeng *et al.,* 2010). Since the hydrogen peroxide bleaching step occurs at elevated temperatures and pH (> 60 ℃ and pH 9), commercially available catalases that are optimally active at 20-50 ℃ and at neutral pH require that the temperature and pH be adjusted prior to their use. Availability of a catalase enzyme able to operate at higher temperatures and pHs would be attractive for the above applications (Spiro and Griffith, 1997). However, research on catalases is still going on and this class of enzymes continues to surprise us (Loewen *et al.,* 2000). On the other hand, there is very little known about catalases from alkalithermophilic

Microorganisms, although enormous progress has been made over the last few years in the research area of extremophiles (Michaudsoret *et al.,* 1998; Takahashi *et al.,* 2000).

MATERIALS AND METHODS

Microorganism and culture conditions

The isolate that we examined was *Bacillus* sp*.,* which exhibits catalase activity. The organism was cultivated aerobically up to the late-logarithmic-growth phase (10^8 cells per ml) at 37°C in LB medium (pH 7) containing (per liter of deionized water) 10 g of tryptone, 5 g of yeast extract, and 10 g of NaCl. The organism was cultured in 1L liter of the above medium with an agitation speed of 200 rpm. The cells were harvested by centrifugation at 10,000 rpm for 20 min at 4°C and disrupted by sonication. Sonic disruption at 4°C was performed for 20 s and was followed by 2 min of cooling in order to prevent excessive heating. At the end of 5 min (total) of sonication,Cell debris was removed by centrifugation (10,000 rpm for 30 min) and the supernatant was collected.

Enzyme assay

Catalase activity was determined spectrophotometrically by monitoring the decrease in absorbance at 240 nm caused by the disappearance of hydrogen peroxide (Beers and Sizer, 1952). The assay was initiated by addition of enzyme solution to 20 mM hydrogen peroxide in 20 mM Tris buffer, pH 8.

The initial absorbance change (typically the first 30 s) was used to calculate the rate of hydrogen peroxide decomposition. The molar absorption coefficient for hydrogen peroxide at 240 nm was assumed to be 43.6 M^{-1} cm^{-1} and one unit (U) of catalase activity was defined as the amount of enzyme required to degrade 1 μmol of hydrogen peroxide per minute.

Determination of protein

Protein amount for catalase was done according to method of Bradford with bovine serum albumin as standard (Bradford, 1976).

Catalase Purification from *Bacillus* sp

Step 1: Ammonium sulfate fractionation

The crude cell extract is brought to 55% saturation in $(NH_4)_2SO_4$, then allowed to stand 1 h at 4°C and centrifuged at 6000 rpm for 20 min. The residue containing catalase activity is dissolved in the minimum amount of 5 mM sodium phosphate buffer, pH 7.0 and dialyzed against the same buffer until the ammonium sulfate was totally removed.

Step 2: DEAE-cellulose chromatography

The dialyzed material from step 2 is centrifuged (5 000 × g, 4°C) and applied to a column of DEAE-cellulose (1.5 cm x 30 cm) equilibrated with 50 mM sodium phosphate buffer pH 7.0 The column is washed with three column-volumes of the same buffer. Elution of the enzyme activity is performed using a linear gradient 0-1 M of the same buffer. Throughout the process the flow is held constant at 30 ml/h and 3ml fractions were collected.

The protein peak containing enzymatic activity was collected and loaded onto a Sephacryl S-300 column.

Step 3: Sephacryl S-300 chromatography

The samples containing the highest specific activity from step 3 are applied to a Sephacryl S-300 column (1x 75cm) equilibrated with 50mM sodium phosphate buffer pH 7.0, with a flow constant of 30 ml/h and 3ml fractions were collected.

The column is washed with two column volumes. A280 was monitored, and protein peaks were collected and assayed for catalase activity, and stored at 4°C as purified enzyme for characterization study.

Sodium dodecylsulfate-polyacrylamide gel (SDS-PAGE) electrophoresis of purified catalase

Subunit molecular weight of *Bacillus* sp. Catalase was estimated electrophoretically with SDS-PAGE by the method of Laemmli (1970) using 5% stacking gel and 12% running gel. Electrophoresis was performed at a constant 125V (8.0 mA) for 4 h in Tris-HCl buffer pH 8.3. After electrophoresis, proteins in the separating gel were made visible by staining with Coomassie Brilliant Blue R250. Phosphorylase b (97 kDa), Plasma albumin (66 kDa), Ovalbumin (43kDa), Carbonic anhydrase (30 kDa),and Trypsin inhibitor (20 kDa) were used as molecular weight markers.

Effect of pH on catalase activity and pH stability

The effect of pH on catalytic activity was determined in 50mM K_2HPO_4-citric acid buffer (pH 3.0-8.0) and 50mM glysine-NaOH buffer (pH 9.0-11.0) which had been distributed evenly into aliquots. Catalase assays were performed and the activity of catalase was plotted against

the pH values. While the effect of pH range of 3.0-11.0 on catalase stability was assayed by incubating the purified enzyme with the buffer solutions in a water bath at 35ºC for 30 min. then transferred immediately into an ice bath. Enzymatic activity for each one was measured and the highest remaining activity is expressed as 100% of the activity.

Effect of temperature on catalase activity and thermal stability

The effect of temperature on catalase activity was measured at temperature from 30 to 100℃. For thermal stability determination, the enzyme was incubated at 30-100℃ for 30 min in the assay buffer, and the highest activity was defined as 100% of the activity.

Effect of some compounds on enzyme activity

The effect of inhibitors (2-mercaptoethanol, Sodium azide, Potassium cyanide) and metal ions ($MnCl_2$, $CuSo_4$, $FeSo_4$) on catalase activity was determined by measuring the enzyme activity in various concentrations of each one in 50 mM phosphate buffer pH 7.0. Enzymatic activity was measured and the remaining activity was expressed as 100% of the activity.

RESULTS AND DISCUSSION

Catalase purification

A three-step purification procedure consisting of ammonium sulfate precipitation, Ion exchange chromatography, and gel filtration chromatography was used to obtain a highly purified catalase from *Bacillus* sp. The effectiveness of each purification step is given in Table1.

During ion exchange chromatography on DEAE-cellulose, four protein peaks appeared, and the second peak contained most catalase activity (Figure1).

In this step, the enzyme showed a specific activity of 566.6 U/mg protein and the purification fold was 6.2. The catalase active fractions was further purified on Sephacryl-S300, result in figure 2 showed that there was only one peak of catalase activity suggesting that the microorganism possessed only one kind of catalase. On the other hand the enzyme was purified approximately 16.6 fold purification with a 50 % yield. The purified catalase showed a high final specific activity of approximately 1500 U/mg of protein.

A survey of catalase literature showed a range of purification levels achieved: from 40-fold for a catalase from *Escherichia coli* (Claiborne *et al.,* 1979) to 400-fold for a catalase from *Streptomyces coelicolor* (Kim *et al.,* 1994). Also An alkaline catalase has been purified and characterized from a slightly halophilic and alkaliphilic bacterium *Bacillus* sp. F26. The purification was performed with a four step procedure consisting of ammonium sulfate precipitation, ion exchange, gel filtration and hydrophobic interaction chromatography, and finally achieved a 58.5-fold-purifying over the crude extract (Zhang *et al.,* 2005).

Molecular weight

A number of proteins of known molecular weights were electrophoresed alongside the purified *Bacillus* sp. catalase on denaturing SDS- Polyacrylamide gels (Figure 3). An apparent size of 65 kDa was determined, which was very similar to the subunit size of most other catalases. It was reported that the subunit and native sizes for this enzyme are significantly smaller than those reported for other tetrameric catalase enzymes (i.e., *Bacillus* sp. with 70.5 and 282 kDa, *E. coli* with 84.3 and 337 kDa, *Rhodobacter capsulatus* with 59 and 236 kDa, and *Neurospora crassa* with 80 and 320 kDa subunit and native molecular mass, respectively) (Thompson *et al.,* 2003).

Optimal pH and pH stability

The activity versus pH profiles for the catalase activity of purified *Bacillus* sp. catalase was studied in a pH range of 3.0 to 11.0 (Figure 4). A broad optimum pH range was observed from pH6.0 to 10.0 with the maximum activity at pH 9.0. This pH range of activity is typical of monofunctional catalases whose pH activities usually range from 4 to 10, this is in contrast to catalaseperoxidases that have sharp pH optima (Yumoto *et al.,* 1990).

When the enzyme was incubated in a buffer solution (pH range, 3.0 to 11.0) at 35℃ for 30 min, the enzyme showed maximal stablility 100% in the pH range from 8.0 to 10.0 and more than 80% of the activity remained at pH 7.0 (Figure 5). Kang et al. (2006) recommended that monofunctional catalase purified from photosynthetic bacterium *Rhodospirillum rubum S1*exhibited a broad optimal pH (5.0~9.0), and Kandukuiri et al. (2012) detected that catalase purified from *Vigna mungo* seed was stable over a broad range of pH 6.0-10.0.

Optimal temperature and thermal stability

Catalase activity was assayed at various temperatures (30.0-100.0℃) using the enzyme purified from *Bacillus* sp. A broad optimum temperature

Table 1. Purification steps of *Bacillus* sp. Catalase

Purification step	Volume (ml)	Protein conc. (mg/ml)	Activity (U/ml)	Specific Activity (U/mg)	Total activity (U)	Purification (fold)	Yield (%)
Crude extract	50	0.5	45	90	2250	1	100
$(NH4)_2SO_4$	20	0.7	90	128.5	1800	1.42	80
Ion exchange on DEAE-cellulose	15	0.15	85	566.6	1275	6.2	56.6
Gel filtration on Sephacryl S-300	15	0.05	75	1500	1125	16.6	50

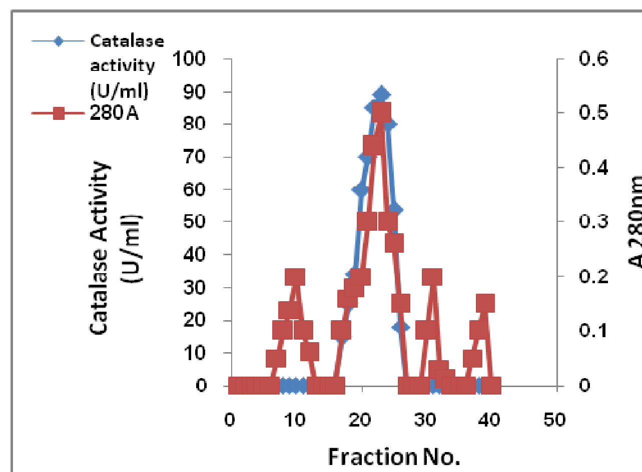

Figure 1. Ion exchange chromatography of *Pseudomonas* sp. AlPase on DEAE-cellulose (1.5 x 30 cm) equilibrated with 50 mM sodium phosphate buffer pH 7.0 containing 0-1 M NaCl; 3 ml fraction at a flow rate of 30 ml/h.

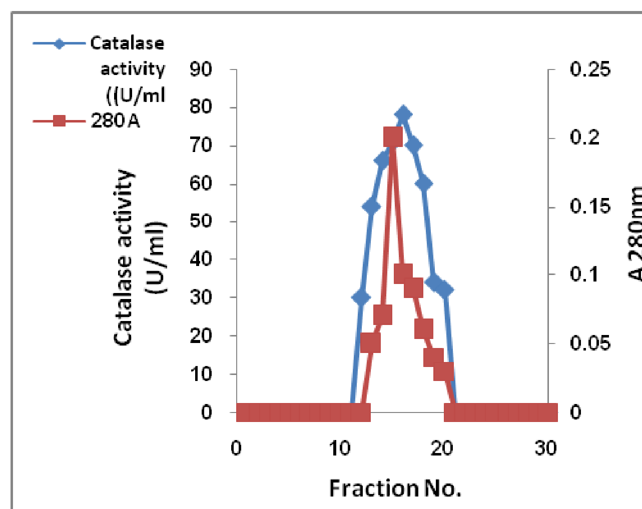

Figure 2. Gel filtration chromatography of *Bacillus* sp catalase. on Sephacryl S-300 column (1x 75cm) equilibrated with 50mM sodium phosphate buffer (pH 7.0). 3 ml fraction at a flow rate of 30 ml/h.

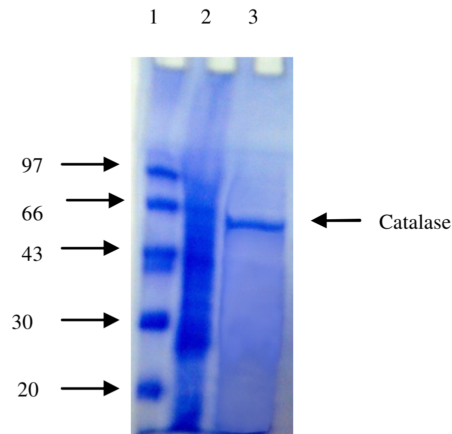

Figure3. SDS-PAGE chromatograph (12%) of purified Bacillus sp. catalase where: 1- Medium molecular weight marker 2- Ammonium sulfate precipitate, 3- Purified catalase, 125V, 4hr.

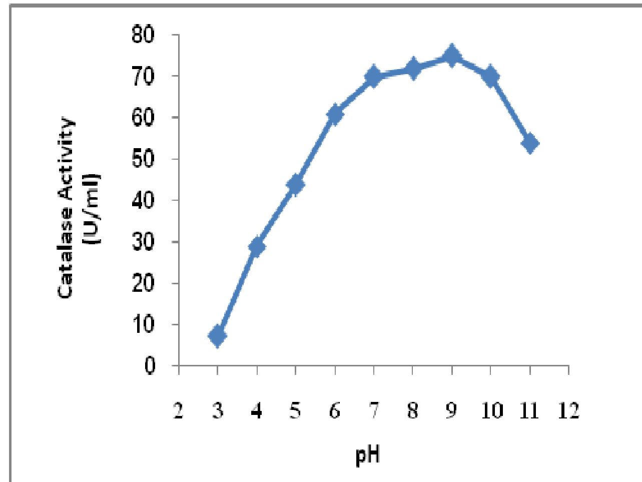

Figure 4. Effect of pH on purified catalase activity

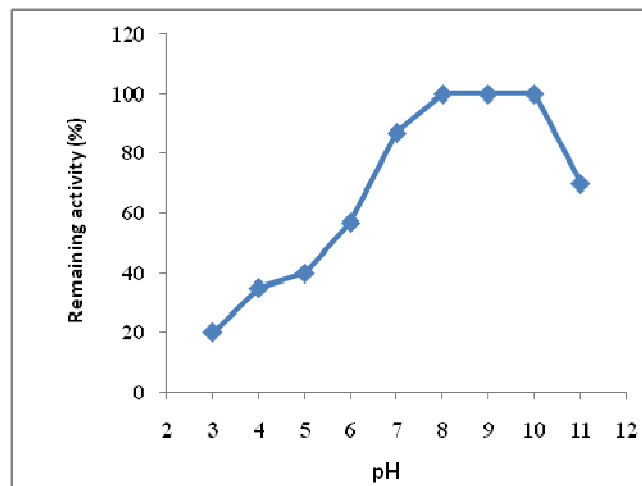

Figure 5. pH stability of purified catalase

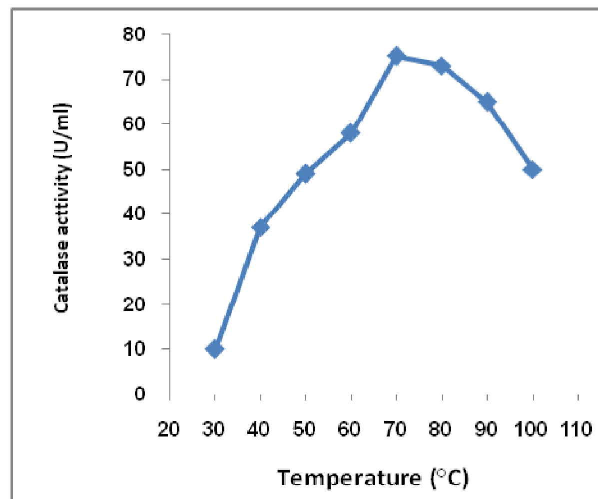

Figure 6. Effect of temperature on purified catalase activity

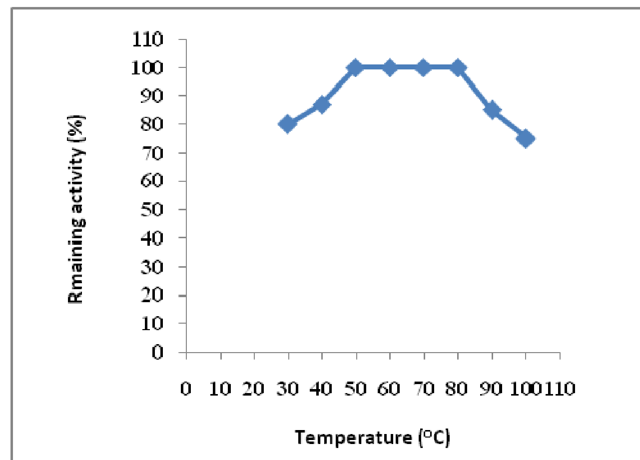

Figure 7. Thermal stability of purified catalase

range from 50 to 90℃ was observed with highest activity at 70℃ (Figure 6).

On the other hand, the thermostability profile (Figure 7) shows that the enzyme was stable at a range from 50-80℃ and more than 80% of activity was remained at 90℃. These activity and stability are similar to that reported for a thermostable catalase from *Escherichia coli* whose maximum activity occurred at 84 ℃ (Wang *et al.,* 2002). A Mn-catalase from Pyrobaculum *calidifontis* VA1 had maximum activity at 70 ℃ (Taku *et al.,* 2002).

Inhibitors and compounds effect

Table 2 summarizes the effect of various substances on catalase activity. Thiols had a strong inhibitory effect than other inhibitors, since 2-mercaptoethanol, sodium azide and potassium cyanide inhibited 70%, 50% and 35% of enzyme activity, respectively. Whereas the cations Mn^{+2}, Cu^{+2} and Fe^{+2} had weak or no inhibitory effect on the enzyme.

Upon purification of catalase from *Bacillus* sp., Loewen and Switala (1987) indicated that the enzyme was inhibited with both azide and cyanide and various sulfhydral compounds. Aydem and Kuru (2003) studied the effect of various inhibitors on catalase activity and they were founded that the activity of purifed catalase was inhibited by azide, cyanide, 2-mercaptoethanol, dithiotreitol (DTT) and iodoacetamide**,** in other study potassium cyanide and sodium azide also showed strong inhibitory effect on catalase purified from Van apple (Yoruk *et al.,* 2005).

Table 2. Inhibitors effect on purified *Bacillus* sp. catalase

Compound	Concentration (mM)	Remaining activity (%)
2-mercaptoethanol	20	30
Sodium azide	5	50
Potassium cyanide	5	65
MnCl2	0.5	97
CuSO4	0.5	100
FeSO4	0.5	100

CONCLUSION

The properties of catalase purified from *Bacillus* sp. has several advantageous features for industrial applications, the enzyme showed exceptional stability at elevated temperatures and pH compared to that of many other reported catalase enzymes. On the other hand, most commercial catalases would hardly withstand the conditions used during textile bleaching. Thus, new thermo-alkali-stable enzymes acting at temperatures above 60 °C and pH values above 9 are required.

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