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# EFFECT OF OXIDATIVE STRESS ON CATALASE INDUCTION AND OPTIMIZATION OF KINETIC PERFORMANCE OF ENZYME FROM *B. SUBTILIS* KIBGE HAS 1

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**ABSTRACT**

The Catalase ($H_2O_2$ oxidoreductase, EC.1.11.1.6) is an enzyme that catalyses the decomposition of hydrogen peroxide to oxygen and water to protect the cell against oxidative stress. The aim of current study is to observe the effect of increasing oxidative stress on the catalase synthesis. Results of the study shows that the increase in oxidative stress by adding 0.1 % cholesterol in medium raises the catalase production *by B. Subtilis* KIBGE HAS 1. The molecular mass of this partially purified catalase was found to be 210 kDa by native PAGE. The enzyme was purified up to 8.9 %. The enzyme exhibits high thermal stability and retain 80.2 % of its original activity when stored at 50°C for 120 min. with Km and Vmax of 41.89 mM and 77.97 U/mg of protein respectively. Among various metal ions, $Na^+$, $Ca^+$ and $Ba^+$ ions enhance the catalase activity however; $Hg^+$ causes an inhibitory effect. The findings of this study give advantage to industries regarding augmentation of catalase production by generating the oxidative stress and therefore indirectly increase the production of enzyme.

**Keywords:** *Bacillus*, enzymes, fermentation, characterization, reactive oxygen, purification

## INTRODUCTION

Catalases are oxidoreductase enzymes that found in almost all living organisms which are exposed to oxygen, substantially provokes defense mechanism against oxidative stress utilizing iron or manganese as a cofactor. It scavenges the hydrogen peroxide that is formed during different metabolic reactions as a by-product and converts it into oxygen and water (**Chelikani *et al*., 2004; John, G. S., 2002; Foyer and Noctor, 2000**) and maintains redox homeostasis of the cell as a part of the antioxidant response system. Several specific properties of catalase-peroxidases isolated from bacteria and fungi distinguished them from typical catalases. Multiple catalases are found in almost all bacterial species, including *E. coli*, *B. subtilis*, *streptomyces coelicolor* and are classified into three types on the basis of enzymological properties *viz.* heme containing mono functional catalases, heme containing bi-functional catalase-peroxidases and non-heme containing catalases (**Chelikani et al, 2004**).

Catalases wide range of usage in industrial and medical processes immensely increased its demand all over the world. For example robust post bleaching $H_2O_2$ eliminating action of bacterial catalases leaves the fabrics material free of peroxides in short time and therefore saves energy and water (Silgia **A. Costa et al, 2001; Weck, M. 1995**). Catalases are used in hair dyes and facial masks preparations, in treatment masks the enzyme is made reacted with $H_2O_2$ on the face to increase cellular oxygenation in the upper layers of epidermis, making them very useful in cosmetics industry. Besides, catalases have extensive use in medical and diagnosis field. For glimpse, its use as tumor metastasis inhibitor and in treatment of diabetes mellitus associated catalasemia makes it one of the favourite enzymes in pharmaceutical Industry (**Frlich and Riederer, 1995**). Catalase is used in medical technologies utilizing biosensors and biomarkers for analytical and diagnostic purpose (**Vatsyayan et al., 2010**). Stepping ahead, its high turnover rate, evolutionary diversity of origin and simple well-defined reaction mechanisms makes it worthy to be used in more advance technologies **(Mullineaux et al., 2006, Spirro et al, 1997**).

The outstanding performance and uses of catalases in almost all advance fields and its escalating demand in industry compelled us to design current study aiming to find new techniques and strategies to obtain the maximum production of Catalase enzyme from previously isolated, multi-enzyme producing strain of *Bacillus subtilis.* By generating the oxidative stress through addition of cholesterol into the medium, extracellular cholesterol oxidase ($C_{OX}$) is stimulated in cholesterol-enriched broth (**Kumari, L. and Kanwar, S.S. 2016**), a microbial enzyme that belongs to the oxidoreductase family and contains Flavin adenine dinucleotide (**Vrielink A. 2010**) that decomposes cholesterol and cholesterol-derived compounds in the presence of oxygen and gives rise to the hydrogen peroxides (**Lashgarian, et al.2016**), it is observed that yield of Catalase Enzyme is increased significantly. The degradation of cholesterol by Mycobacterium, Rhodococcus, Brevibacterium, Streptomyces, and some other Gram positive as well as Gram negative genera including Comamonas, Burkholderia, Pseudomonas, and Chromo bacterium has also been reported (**Doukyu,N. 2009, Bholay, B D. 2013**).

## MATERIALS AND METHODS

### *Bacterial strain*

Previously isolated strain of *B. subtilis* KIBGE HAS was selected for evaluating the catalase potential, which was also reported for production of multiple enzymes like α-amylase, protease, β-endoglucanase and alkaline phosphatase (**Bano et al, 2011; Anwar et al, 2009; Bano et al, 2013 and Qader et al, 2008**).

### *Bacterial growth conditions*

*B. subtilis* KIBGE HAS was grown at 37°C in liquid medium consisting of (g/L): glucose 20.0; yeast extract 5.0; $MgSO_4$ 0.3; peptone 5.0; $K_2HPO_4$ and cholesterol 1.0. The pH of the medium was adjusted to 7.0 with 1.0 M NaOH before autoclaving.

### *Purification of catalase enzyme*

For purification of crude enzyme solid ammonium sulphate $(NH_4)_2SO_4$ was added slowly to the crude enzyme up to 40 % saturation , with constant stirring at 4.0°C and then kept for 18 hours at same temperature. Followed by, proteins precipitation and then separation by centrifugation at 35,000g and temperature was kept 0°C. Precipitates were dissolved in minimal volume of 0.05 M phosphate buffer (pH 7.0) to get homogenous mixture. The enzyme solution was dialyzed (Molecular porous membrane tubing-Spectrum Labs.) and stored at -20°C for further studies.

### *Enzyme assay and Units*

Catalase activity was estimated by method of Patterson et al. (**1984**). The decomposition of $H_2O_2$ was measured at 240 nm taking Δε at 240 nm as 43.6 mM $cm^{-1}$ (Miyagawa *et al.* 2000). Reaction mixture (3.0 ml) was consisted of 10.5 mM $H_2O_2$ in 0.05 M potassium phosphate buffer (pH 7.0) and reaction was commenced after addition of 0.1 ml of enzyme extract at 25°C. The decline in absorbance at 240 nm was used to calculate the activity. "One unit of CAT activity is defined as the amount of enzyme that catalyzes the conversion of 1.0 mM of $H_2O_2 min^{-1}$ at 25°C".

### *Protein estimation*

Protein content of the enzyme samples were estimated by Lowry et al. (**1951**) method and bovine serum albumin was used as standard.

***In-situ electrophoresis of enzyme***

Zymography of enzyme was performed by native polyacrylamide gel electrophoresis according to the method of Laemmli (**1970**). A polyacrylamide gel (6.0 %) was prepared with 0.5% soluble starch. For CAT activity, protocol of Thorup et al. (**1961**) was followed. Gel was washed with deionized water and soaked in substrate for 30 seconds to 1.0 min, then washed with deionized water and incubated in potassium iodide solution until gel retained blue color.

***Optimum temperature and Thermal stability***

To know the optimum temperature for catalase activity; enzyme activity was performed at different temperatures (10°C to 45°C). The thermal stability profile of extracellular catalase enzyme was determined by pre-incubation at various temperatures (from 30°C to 70°C) in the absence of substrate for 0.0 to 120 minutes. Enzyme activity was checked at different time interval.

***Substrate maxima***

Hydrogen peroxide concentration was varied from 10 mM to 100 mM 0.05M phosphate buffer pH 7.0 for the optimum extracellular catalase activity. The kinetic parameters of enzyme were estimated by linear regression from double reciprocal plots, according to *Lineweaver and Burk* (**1934**).

***Impact of metal ions***

Effect of different metal ions on the efficacy of extracellular catalase was observed. For this purpose enzyme was incubated with the chloride salt of the different ions in 1:1 ratio at 30°C for 15 min and then enzyme activity was determined. The activity of enzyme in the absence of ions was taken as control (100%).

***Surfactant consequence on catalase activity***

Effect of different surfactants such as Tween-20, Tween-80, Brij-35, Triton X-100, sodium dodecyl sulphate (SDS) and sodium tauroglycocholate was observed on catalase activity in a concentration of 1mM and 5.0 mM that were added to the enzyme solution and incubated at 30°C for 30 min. Consequences of surfactants presence was checked by determining the relative activity by taking control as 100 %.

***Organic solvents outcome on catalase activity***

To understand the effect of organic solvent on catalase activity 20-100% solutions of ethanol, dimethyl sulfoxide (DMSO), methanol and iso-propanol were prepared and mixed with enzyme solution in 1:1 ratio and incubated at 30°C for 1.0 hr. Residual activity of enzyme was determined by taking control as 100%.

**RESULTS**

Six different reported media were initially used to achieve the good yield of catalase enzyme and maximum CAT production was obtained in suggested medium, with enzyme activity of 8.6 U/ml. While different component of the medium were varied to optimize, it was found that enzyme production (Table 1) was increased when cholesterol and yeast extract were used at concentration o f 0.1% and 0.5% respectively at pH 7. 0.

**Table (1)** Effect of Cholesterol on Catalase Production from *Bacillus subtilis* KIBGE HAS1

| Cholesterol Concentration (g/l) | Enzyme Activity (U/ml/min) |
|---|---|
| 0 | 23 |
| 1 | 40 |
| 2 | 33 |
| 3 | 28 |
| 4 | 21 |
| 5 | 19 |

It was also noticed that enzyme production start decreasing at cholesterol concentration of 0.5 % within the same environmental conditions and concentration of other components of medium. After getting good yield of enzyme, crude enzyme was precipitated and partially purified (Figure.1).Thus, partially purified catalase exhibited improved specific activity of 214.1 U/mg of protein and purified up to 8.99 fold after dialysis (Table 2).

**Table (2)** Purification steps of Catalase from *Bacillussubtilis* KIBGE HAS

| Purification Step | Total Volume (ml) | Enzyme Activity (U) | Total Protein (mg) | Specific Activity (U/mg) | Fold Purification |
|---|---|---|---|---|---|
| Crude | 100 | 4000 | 168.0 | 23.80 | 1.00 |
| PartiallyPurified | 1.2 | 1516 | 7.08 | 214.1 | 8.99 |

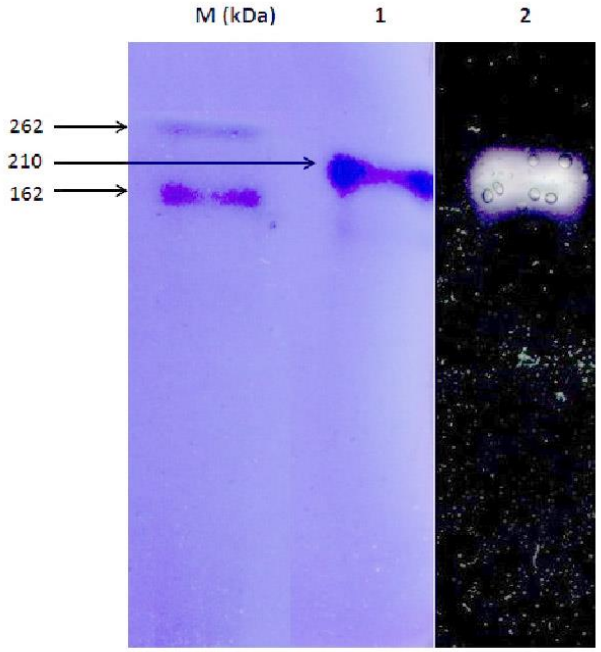


**Figure 1:** Electrophoretic Pattern and Zymogram of Purified Catalase
**Lane: M:** (Marker) Urease: Hexamer, 545 kDa; Trimer, 272 kDa
**1:** Purified Catalase from *Bacillus subtilis* KIBGE HAS
**2:** Zymograpic Analysis of Catalase

This partially purified enzyme showed catalytic activity at wide range of temperatures and maximum activity was noted at 25°C (Figure. 2).

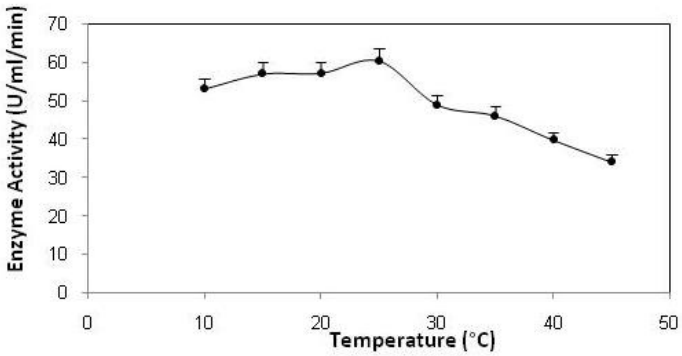


**Figure 2** Effect of temperature on Catalase Activity

The substrate maxima for CAT activity was achieved 53.6 U/ml/min of enzyme at 80.0mM concentration of $H_2O_2$ (Figure. 3).

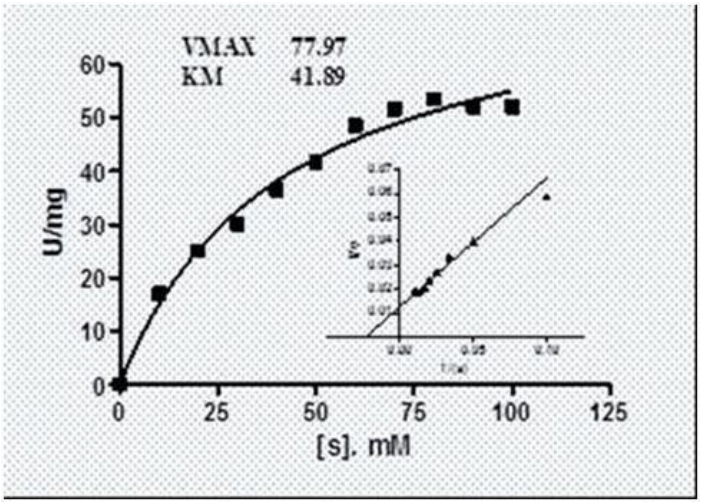


**Figure 3** Effect of substrate concentration on catalase activity

**Std. Error**

VMAX= 4.961
KM= 6.446
$R^2$= 0.985

Activation profile of this enzyme was checked in the presence of different metal ions, solvents and surfactant, it was observed that the enzyme was activated by $Na^+$, $Ca^{+2}$ and $Ba^{+2}$ ions at 5.0mM concentration (Table. 3).

**Table 3** Effect of Metal Ions on Catalase Activity

| Metal Ions* | % Relative Activity | |
|---|---|---|
| | 5mM | 10mM |
| control | 100 | 100 |
| $Al^{+3}$ | 110 | 130 |
| $Ni^{+2}$ | 94 | 95 |
| $Fe^{+2}$ | 103 | 99 |
| $Na^{+1}$ | 125 | 103 |
| $Ca^{+2}$ | 124 | 117 |
| $Hg^{+2}$ | 13 | 43 |
| $K^{+1}$ | 113 | 106 |
| $Ba^{+2}$ | 127 | 97.1 |
| $Mn^{+2}$ | 106 | 103 |
| $Co^{+2}$ | 111 | 113 |
| $Mg^{+2}$ | 96 | 112 |

Metal ions used as chloride salt

When observing yhe effect of organic solvents on the enzyme activity it was noticed the activity was slightly increased in the presence of DMSO and ethanol (Table 4.)

**Table 4** Effect of Organic Solvents on Catalase Activity

| **Organic Solvents %** | **% Relative Activity** | | | | |
|---|---|---|---|---|---|
| | **DMSO** | **Ethanol** | **Acetone** | **Methanol** | **Iso-Propanol** |
| 20 | 135 | 133 | 103 | 105 | 97 |
| 40 | 127 | 135 | 122 | 102 | 97 |
| 60 | 122 | 137 | 103 | 103 | 101 |
| 80 | 122 | 139 | 108 | 105 | 97 |
| 100 | 126 | 142 | 107 | 108 | 97 |

However, none of the surfactant had a pronounced inhibitory effect on enzyme activities while triton X-100 (non-ionic detergent) and sodium tauroglycocholate at a concentration of 5.0 mM stimulate activity 10 and 13% respectively (Figure 4).

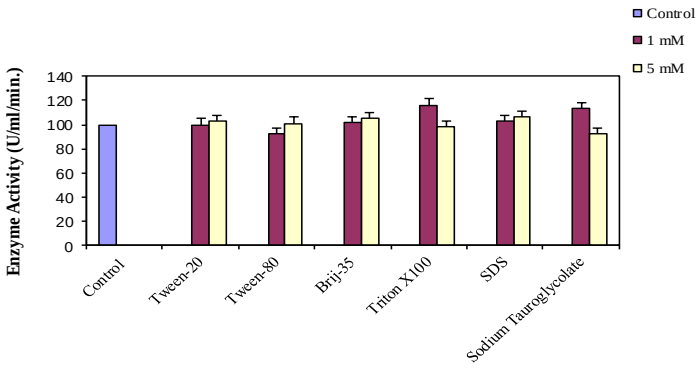


**Figure 4** Effect of surfactant on Catalase Activity

**DISCUSSION**

This study showed that partially purified enzyme is quite stable at the temperature ranging 10°C to 50°C as compared to higher temperature similar to most of the reported catalases .A gradual decline in enzyme activity was observed after 25°C and this decline may be attributed to the enzyme denaturation at high temperature. As reported prior the conformational changes in the three dimensional structures of protein molecule may occur at higher temperatures that may lead to defective enzyme and substrate binding **(Katsuya et al, 1998**). Although, it is observed that the optimum temperature of *B. subtilis* KIBGE HAS is comparatively high to other reported CAT from *Bacillus* sp. N2aA and *Proteus mirabilis;* however, it is lower than *B. subtilis* (**Wang et al, 2008; Lorentz et al, 2006; Lowen & Switala, 1987**). The enzyme was much stable at 50°C and retained 80.2% of its original activity even after 120 min of incubation while at 70°C the enzyme showed activity for 5minutes and completely inactivated after 120 minutes of storage (Figure. 5).

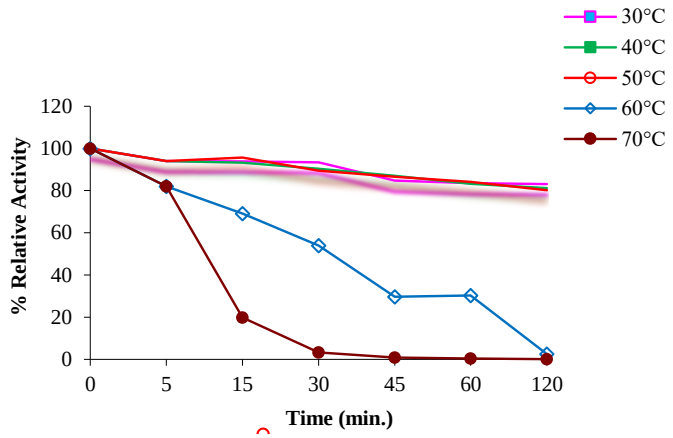


**Figure 5** Thermal stability of Catalase

It has been reported that catalase from *streptomyces coelicolor* (ATCC 1047) was remained stable only up to 40°C for 5 min and enzyme completely inactivated at 60°C (**Kim et al, 1994**) On the other hand enzyme isolated from *Serratia mareescens* SYBC01 maintained 75% of its initial activity at 60°C for 60 min. (**Zeng et al, 2010**). The Km and Vmax value of this enzyme for hydrogen peroxide were calculated as 41.89 mM and 77.97 U/mg of protein respectively at 25 °C. It indicates that the enzyme has high affinity to its substrate and it is similar to other reported catalases (Table 5).

**Table 5** Km value of catalase from different *Bacillus* species

| ***Bacillus* species** | **Optimum temperature (°C)** | **Km (mg/ml)** | **Reference** |
|---|---|---|---|
| *B. subtilis* KIBGE HAS | 25 | 41.8 | Present work |
| *Bacillus* sp. N2aA | 25 | 41.5 | Wang et al, 2008 |
| *B. subtilis* | 37 | 40.1 | Loewen& Switala,1987 |
| *Serratia marcescens* | 20 | 29.7 | Zang et al, 2010 |

| | | | |
|---|---|---|---|
| *Proteus mirabilis* | 20-25 | 127 | Lorentzen et al, 2006 |

Further study revealed that the metal ions may stimulate the enzyme activity by acting as a binding link between enzyme and substrate so the substrate firmly clasp to active site of the enzyme (**Afifi et al., 2008**). It has also been reported that improvement of activity in the presence of $Mn^{+2}$, $Ca^{+2}$, $Co^{+2}$, $Fe^{+3}$ and $Ba^{+2}$ ions may be attribute to its ability to interact with negatively charged amino acid residues of enzyme such as aspartic and glutamic acid (**Linden et al., 2003**). However, $Hg^{+2}$ proved to be inhibitors for catalase probably due to the presence of SH group at the active site of the enzyme that oxidizes them by $HgCl_2$. Moreover, the presence of $HgCl_2$ in substrate solution may form a complex with the enzyme and intercepted its binding to substrate and consequently cease the product formation. It has also been reported that the inhibition of enzyme activity in the presence of mercuric ions indicates the association of indole amino acid residues in the enzyme function (**Ramirez-Zavala et al, 2004).**

This purified enzyme stands long enough in the presence of organic solvent which may be attributing to either its rigid structure or its lowered hydration level (**Jaiswal, et al, 2013**). The enzymes that naturally remains stable in the presence of organic solvents without the need for special stabilization considered very useful for biotechnological applications in which organic solvents are used repeatedly (**Ogino et al, 2000**). Enzymatic catalysis in the presence of organic solvents offers many advantages and increases the solubility of hydrophobic substrate, catalytic efficiency and decrease the microbial contamination (**Y. Sardessai et al, 2004**).

Purified catalase enzyme also exhibited good activity when exposed to different surfactants such as SDS, Triton X-100 tauroglycocholate and Tween 80. The enhanced stability of this enzyme could be attributed to the glycosylation, similar to those observed in recombinant phytase (**Guo et al.2007; Gebert et al. 2015).**

## CONCLUSION

The increasing concentration of cholesterol builds oxidative stress in the cell that indirectly increases the catalase enzyme production, thus the generation of oxidative stress within the cell could be exploited for the production of catalase enzyme. This enzyme was purified up to 8.99 fold after dialysis and showed specific activity of 214.1 U/mg of protein.

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**Conflict of interest:** None of the authors have conflicts on the publication of this paper.

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