Isolation, Fractionation and Characterization of Catalase from *Neurospora crassa* (InaCC F226)

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Abstract. Catalase from Indigenous isolate *Neurospora crassa* InaCC F226 has been isolated, fractionated and characterized. Production of catalase by *Neurospora crassa* was done by using PDA medium (*Potato Dextrosa Agar*) and fractionated with ammonium sulphate with 20-80% saturation. Fraction 60% was optimum saturation of ammonium sulphate and had highest specific activity 3339.82 U/mg with purity 6.09 times, total protein 0.920 mg and yield 88.57%. The optimum pH and temperature for catalase activity were at 40^0C and pH 7.0, respectively. The metal ions that stimulated catalase activity acted were Ca^{2+}, Mn^{2+} and Zn^{2+}, and inhibitors were EDTA, Mg^{2+} and Cu^{2+}. Based on K_m and V_{max} values were 0.2384 mM and 13.3156 s/ mM.

1. Introduction

Catalase (E.C.1.11.1.6) is classified as oxidoreductase enzymes that contribute to defense cells and oxidative stress because its ability to decompose hydrogen peroxide (H_2O_2) into oxygen and water [8]. Catalase can be applied for food industry to detect level of calcium in milk and water [1], in the medical field for therapy acatalasemia [10], inhibition of tissue damage and tumor metastasis [16], development biosensor for detection of bacterial infection mastitis [9] and degradation of industrial waste containing H_2O_2.

Catalase also can be used as a fuel cell enzymatic or Enzymatic Fuel Cell (EFC) that use enzymes as biocatalysts to change biochemical energy into electrical energy directly [4]. In this present research, catalase was investigated and planned in order to utilize oxydoreductase as Enzymatic Fuel Cell.

Some fungi produced catalase such as *Neurospora crassa*, *Saccharomyces cerevisiae*, *Aspergillus niger*, *Pichia pastoris*, and *Septoria tritici* [2], while catalase-producing bacteria including *Bacillus subtilis* 168 [13], *Bacillus* sp. [11], and *Lactobacillus sakei* [2]. Catalase from microorganisms source have many advantages, such as catalytic activity and high specific activity, wide range activity for pH and temperature, and high stability to acids and alkaline [16]. However, the range of potential application is increasing rapidly.

Catalase from *Neurospora crassa* (InaCC F226) have not been yet optimized and characterized. Therefore, in the present work catalase was isolated, fractionated ammonium sulfate saturation level of 20-80%, optimized pH and temperature and determined the kinetic parameter in order to utilize oxydoreductase as Enzymatic Fuel Cell for future work.

2. Materials and Methods

2.1. Preparation of Crude Extract catalase from N. crassa [12].
Neurospora crassa (InaCC F226) isolate were obtained from Indonesian Culture Collection Indonesian Institute of Sciences have been transferred to PDA medium and incubated at 37^0C for 10 days until fungus konidium ready to be harvested. The cells were treated with 50 mM phosphate buffer pH 7.0 with volume 200 mL and 3 mL 75% H_2O_2 were added until no bubbles as a result of catalysis H_2O_2. Cells were homogenized by centrifugation at speed of 9500 rpm for 15 min at 4° C. Supernatant is used as crude extract of catalase.

2.2. Fractionation by Precipitation Ammonium Sulphate $(NH4)_2SO_4$)
Supernatant as crude catalase was precipitated with different concentration of ammonium sulfate at saturation 20, 40, 60, and 80%. The addition ammonium sulphate was done at 4°C and stirred until dissolved then centrifuged at 3000 rpm for 10 min at 4°C and stored overnight at 4° C. Pellet were resuspended in 50 mM phosphate buffer pH 7 of 1-2 mL.

2.3. Determination of Catalase Activity Assay [22]
The enzymes that obtained from each step of ammonium sulphate fractionation were incubated for 3 minutes at 35°C and measured the absorbance by using spectrophotometer at wavelength 240 nm. The catalase activity calculated by using coefficient of the half-life of enzymes was 43.6 M^{-1} cm^{-1}.

2.4. Determination of Protein Concentration by Bradford Method [6]
Bovine Serum Albumin (BSA) is used as standard protein for determination of protein concentration with the range 0020-0120 mg / mL. The enzyme that obtained from precipitation with 60% of ammonium sulfate and crude extract with total volume 100 mL were added with 5 ml of Bradford reagent and homogenized by vortex. After incubated 5 minutes, absorbance was measured at 595 nm wavelength.

2.5. Determination of Optimum Temperature and pH [12]
Optimum temperature and pH were determined by using the highest specific activity. Determination of optimum temperature were done at temperature range (20 - 60^oC). Determination of optimum pH were done with different buffer started at pH 4.0- 6.0 (in 50 mM sodium acetate buffer), pH 7.0-10.0 (in 50 mM sodium phosphate buffer). All assays were done by using 0.1 mM H_2O_2 3.0 mL and 0.100 mL enzymes. Absorbance was measured by spectrophotometer at 240 nm.

2.6. Effect of Metal Ion on Catalase Activity [12]
Some compounds were used to examine the effect of metal ions consisting of EDTA, $MnCl_2$, $MgCl_2$, $CuSO_4$, $ZnCl_2$ and $CaCl_2$ with concentration of 5 mM. A total of 3.0 ml of 0.1 mM H_2O_2 was added with 50 mL of metal ion and incubated 2 minutes. Enzyme was added and incubated back for 3 minutes. Inactivating of enzyme was done by heating 90°C and measured absorbance at wavelength 240 nm.

2.7. Determination of Kinetics Parameter of Catalase (Km and Vmax) [12]
Kinetics parameters (Km and Vmax) of catalase were determinated with varying susbtrate H_2O_2 in the range 0.02 – 0. 24 mM. Decomposition of H_2O_2 levels were measured by using spectrophotometer at 240 nm. The apparent of km value and Vmax of catalase was estimated by analysis data of Michaelis-Menten /Lineweaver-Burk plots.

3. Results

3.1. Catalase Results Isolation and Purification

The specific activity of catalase was showed presented in Table 1. Based on the results, total protein and specific activity of the crude extract obtained at 6325 mg and 548.47 U/ mg. After fractionated with ammonium sulfate obtained the highest total protein in the fraction with 60% saturation was 0920 mg.

Table 1. Specific Activity of Catalase.

Fraction	Total Activity (U)	Total Protein (mg)	Specific Activity (U/mg)	Purification (fold)	Yield (%)
Crude extract	3469.11	6.325	548.47	1.00	100.00
Ammonium sulphate 60%	3072.64	0.920	3339.82	6.09	88.57

Total protein and the highest specific activity obtained after ammonium sulfate saturation at 60% were respectively 0.920 mg and 3339.82 U mg. The total protein and specific activity fraction of 60% was lower compared to [7], total protein and specific activity of catalase-1 isolated from *Neurospora crassa* 35% ammonium sulphate precipitation were 8.2 mg and 292,980.00 U/mg, respectively. According to [5], the lower of total protein and specific activity due to amino acids has many hydrophilic group (56.20%). It was required high concentration of ammonium sulphate to change electrostatic force that effect to low solubility of proteins in water and then protein will be precipitated. Beside that, enzyme has more hydrophilic amino acids that required higher salt concentration for precipitation.

The fraction 60% of ammonium sulphate precipitation with highest specific activity 3339.82 U/ mg was used to determine optimum pH and temperature to hydrolysis H_2O_2 as substrate. The enzyme activity will increase by increasing temperature up to optimum temperature. The increasing of temperature above optimum temperature will decrease enzyme activity. Enzyme activity increased with the range of temperature 20-40^{o}C. Increasing of enzyme reaction will increase kinetic energy which accelerates vibrational motion, translational and rotational enzymes and substrates, thereby increasing intensity of collision between substrate and enzyme until the optimum temperature reached.

3.2. Optimum temperature and pH of catalase

Optimum temperature of catalase *N. crassa* (InaCC F226) was 40^{o}C with relative activity reached 100.00%. According to [21], catalase of *Vibrio rumoiensis* had optimum temperature 40^{o}C. At optimum temperature, collision between enzyme and substrate is most effective. Beside that, formation of complex between enzyme substrates are easily and also products formation will increase.

Increasing pH 4.0-7.0 will increase catalase activity and reached activity 100.00% at pH 7.0 (neutral) as shown at Figure 2 . Catalase activity decreased at range pH 7.0-10.0. Temperature and pH optimum catalase isolated from *N. crassa* (InaCC F226) was 40°C and pH 7.0, respectively.

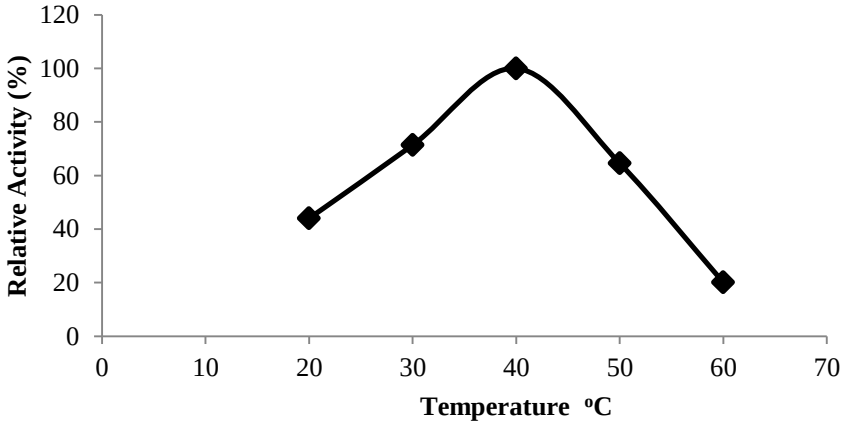

Figure 1. Relative activity (%) of catalase at different temperature.

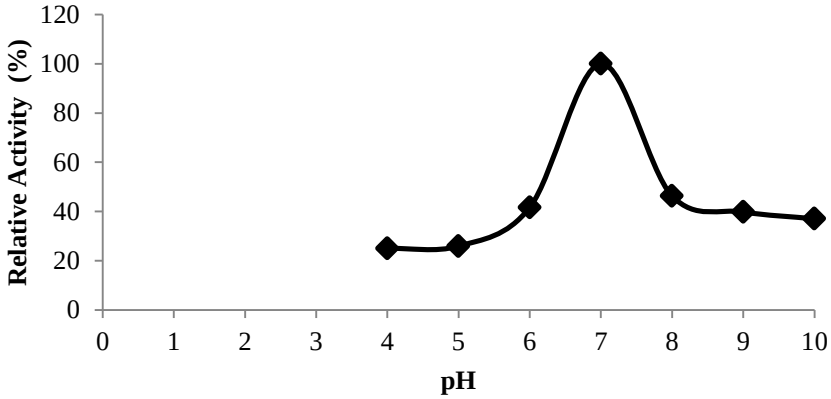

Figure 2. Relative activity (%) of catalase at different of pH.

One of factor that influence enzyme activity was pH, there was correlation between ionization of amino acids enzyme at active side. Optimum pH of *N. crassa* catalase (InaCC F226) was at pH 7.0 (neutral) with relative activity with 100.00%. Catalase from *Beauveria bassiana* has optimum at pH 7.0. At this optimum pH 7.0, active site of enzyme in maximum condition and most appropriate for binding to substrate and catalysis [18]. Catalase from *N. crassa* (InaCC F226) decreased relative activity in acidic and alkaline. pH of acids and bases will affect ionization changes and affect to catalytic ability that related to structure and enzyme active site [18].

3.3. Effect of Metal Ion on Catalase Activity

Some metal ions increased catalase activity were Ca^{2+}, Mn^{2+} and Zn^{2+} at concentration 5 mM. Ion Mn^{2+} and Zn^{2}+ increased relative activity up to 150.00% and 119.85%, and the highest activity catalase was obtained by adding Ca^{2+} with activity unit 266.63U/mL. Other research, [19] also reported that the addition of Ca^{2+} at level concentration of 0.5 mM can increase catalase activity by 111.2 % after purified by Sephadex G-75. Some metal ions gave inhibition effect of catalase activity i.e Cu^{2+}, Mg^{2+} and EDTA (Figure 3). EDTA and Mg^{2+} can reduced catalase activity became 86.03% and 77.94%, respectively. The highest of inhibition was Cu^{2+} with relative activity 44.78%.

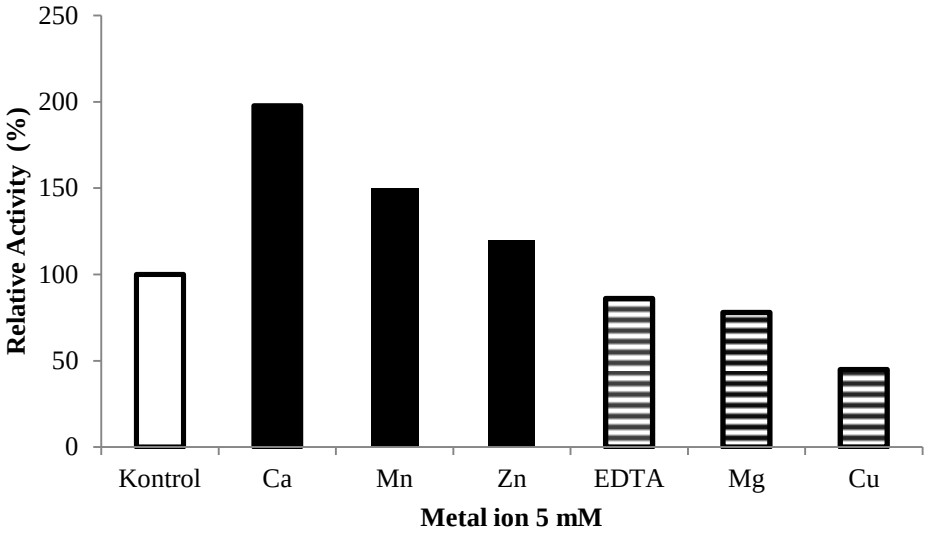

Figure 3. Relative activity of catalase at concentration 5 mM of metal ions.

Ions have ability to increase (activator) or decrease (inhibitors) enzyme activity after interaction with substrate [17]. Metal ions has important role for catalytic reaction by binding substrate to active site of enzyme, to stabilize active conformation or induce formation of binding or active site. In this present research, metal ions Ca^{2+}, Mn^{2+} and Zn^{2+} with concentration 5 mM had ability to increase catalase activity *N. crassa* (InaCC F226) and highest activity of catalase reached with Ca^{2+} as metal ion (relative activity 197.79%). Catalase has active site on the binding of Ca^{2+}, and supported for binding of to the substrate [14]. Mn^{2+} and Zn^{2+} increased catalase activity by enhancing stability of enzyme structure and also by changing conformation of catalase and enzyme active site of enzyme. Therefore, hydrolysis of substrate of catalase will increased [23].

Cu^{2+}, Mg^{2+} and concentration of 5 mM EDTA reduced catalase activity (Figure 3). EDTA inhibited catalase activity by chelating ions Fe^{3+} as structure component of catalase. Ion Mg^{2+} and Cu^{2+} inhibited catalase activity due to reduce reaction of oxidation reduction during decomposition of H_2O_2. Therefore, in this present research, metal ion Ca2+, Mn2+ and Zn2+ were the activator and metal ion Cu^{2+}, Mg^{2+} and EDTA were inhibitor of catalase activity from *N. crassa* (InaCC F226). The results of this research are in accordance with Kandukuri *et al*. that found metal ions Mn^{2+} and Ca^{2+} could stimulated catalase activity, while EDTA, Mg^{2+} and Cu^{2+} concentrations of 5 mM inhibited catalase activity from *Vigna mungo* [17]. Catalase had metal ion in active site. Addition of other metal ions at certain concentrations could affect stability of catalase structures or affect oxidation reduction reaction to active site enzyme, therefore addition of metal ion will affect activity.

3.4. Kinetics Parameter of Catalase

Kinetic properties of enzyme can be determined by maximum catalytic velocity (Vmax) and substrate concentration when kinetic rate reached half maximum (Km) [15]. Kinetics catalase *N. crassa* (InaCC F226) were determined through the Michaelis-Menten equation and Lineweaver Burk. Michaelis-Menten equation was used to determine concentration of H_2O_2 on the catalase activity. The rate of hydrogen peroxide decomposition as function of hydrogen peroxide concentration was given in Figure 4 Catalase activity at concentrations 0.02 up to 0.10 mM H_2O_2 increased significantly against substrate concentration, but at higher concentration 0.10 mM the increasing of enzyme activity was not significantly against substrate concentration.

Based on this curve, the increasing of catalase activity significantly different at concentrations of H_2O_2 from 0.02 up to 0.10 mM and started from 0.16 to 0.24 mM concentrations of H_2O_2 increasing in

activity catalase are ramps (not significant). Based on Lineweaver Burk curve, value of Km and Vmax for catalase *N. crassa* (InaCC F226) obtained were 13.3156 0.2384 mM and s/mM, respectively (Figure 5).

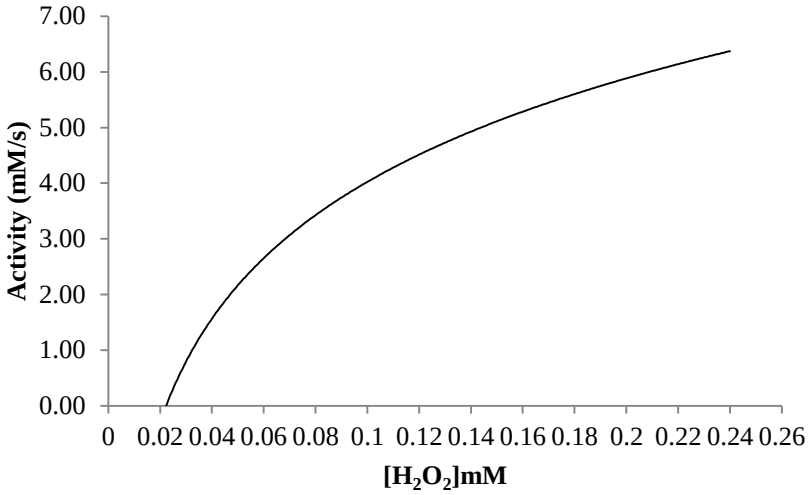

Figure 4. Michaelis Menten Curve : Correlation between Catalase Activity at Different Concentration of H_2O_2.

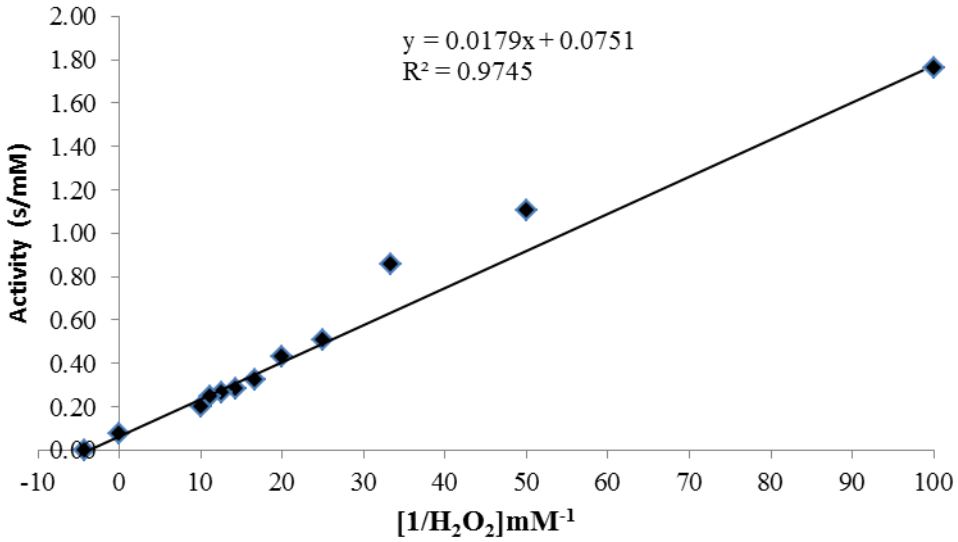

Figure 5. Lineweaver Burk curve : correlation between catalase activity at different concentration H_2O_2.

Compared to other research [7] that isolated *N.crassa*, Km value obtained from this study is lower. The lower of Km value was considered as high affinity of catalase from *N. Crassa* to H_2O_2 as the substrate. But, Vmax value obtained from this research is lower than the Vmax obtained from research [7].

4. Conclusions

Isolation of catalase from indigenous isolate *Neurospora crassa* (InaCC F226) by fractionation with 60% ammonium sulphate saturation showed highest specific activity. Optimum temperature and pH for catalase activity were 40^{o}C and pH 7.0, respectively. The metal ions Ca^{2+} , Mn^{2+} and Zn^{2+} stimulated catalase activity , while EDTA, Cu^{2+} and Mg^{2+} inhibited activity of catalase. The value of Km for Catalase was 0.2384 and Vmax values was 13.3156 s mM / mM.

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