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Purification and Characterization of Catalase from Indigenous Fungi of *Neurospora crassa* InaCC F226

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***Abstract*— Microbial catalase is an important industrial enzyme that catalyzes the decomposition of hydrogen peroxide into water and oxygen. This enzyme posseses great potential of application in food, textile and pharmaceutical industries. In this paper, *Neurospora crassa* InaCC F226 was used to produce catalase. The aim of this research was to purify the catalase producing by *Neurospora crassa* InaCC F226 using gel filtration column chromatography and characterization of temperature, pH, Vmax, Km and molecular weight. In this study, the *Neurospora crassa* was cultured on Vogel's medium for 5 days. The cells were harvested in the 50 mM buffer sodium phosphate (pH 7) containing 3% hydrogen peroxide. Supernatant containing crude enzymes of Catalase was separated from cells by centrifugation at 15 minutes on 13000 x g and 4^{o}C. The purification result using gel filtration was specific activity 1464.9 U/mg, 10.7 fold and 21.7% yield. Characterization of the purified enzyme toward pH and temperature optimum was 7.0 (271.6 U/mL) and 40^{o}C (286.1 U/mL). The K_m and V_{max} found to be 8.8 mM and 5.7 s.mM^{-1}. The catalase activity increased by additiom of Fe^{+2}, Ca^{+2} and inhibited by EDTA, Cu^{+2} and Mn^{+2}. The molecular weight of the catalase was 59.4 kDa.**

***Keywords*— *Neurospora crassa*, Catalase, Pharmaceutical, Food, Textile,**

I. INTRODUCTION

Catalase (hydrogen peroxide oxidoreductase, EC1.11.1.6) is a group of oxidoreductase enzyme that catalyzes degradation of hydrogen peroxide into water and oxigen, and oxidizes electron donors such as ethanol, methanol and phenol [1],[2]. In the living organism, it is role to protect the cell from reactive oxygen spesies (ROS) [3]. This enzyme is classified into four groups based on their molecular weight: monofunctional heme (typical) catalases, catalase-peroxidases, manganese catalases, and catalase-phenol oxidases (CATPO). Monofunctional heme catalases is heme catalase that have iron-protoporphyrin IX as prosthetic group in their active sites and it have molecular masses of 200-340 kDa and also it was grouped into two classes based on their subunits constituent: small-subunit catalases (<60 kDa) and large-subunit catalases (>75 kDa) [4].

Today, monofuctional catalase have been applied in various industrial fields, such as food and textile [2],[5],[6], analytical fields as a component of different biosensor systems [6],[7],[9], medical [10] and also in energy [11]. Several researches reported that fungi could produce catalase effectively [12], such as: *Paracoccidioides brasiliensis* has been isolated from yeast cells grown in the presence of 15 mM of hydrogen peroxide and resulted the catalase yield and purity were 79% and 1.3-fold (aceton precipitation), respectively. The molecular weight from this fungi is 244 kDa [13]. The *Aspergillus niger* that tolerance to heavy metals and hydrogen peroxide have been reported by Buckova *et al* [14]. Catalase activity increased in *Thermoascus aurantiacus* when growth on medium containing 20 g/L dextrin and 1% ethanol and pepton [15].

Other fungi that also produced these enzyme are *Aspergillus oryzae* [16], *Penicillium marneffei* [17], *Blakeslea trispora* [18], *Neurospora crassa* [18-20], and *Scytalidium thermophilum* [21]. These fungi generally tetrametric enzyme that have subunits of molecular masses in the range of 61–97 kDa. Several patent of microorganism producing catalase has been reported too, Example *Geobacillus extremocatsoochus MTCC* 5873 [22], and *Helicobacter* [23].

Indonesia is a mega biodiversity country and second biggest biodiversity after Brazil in the world. These biodiversities include microbial diversity in soil and water. Up to now, InaCC (Indonesia Culture Collection) have isolated more than 10,000 isolates of species of bacteria, *Actinomycetes*, fungi, yeast and microalgae. The potential of these microorganisms can be used as a source of various

types of enzyme potential [24]. According to that, there are great potencies to obtain catalase producers. One of the fungi that have potential to produce the hydrogen peroxide oxidoreductase is *Neurospora crassa* [18-20]. It has three monofunctional catalases, two large-subunit catalases, CAT-1 and CAT-3, and a small-subunit catalase, CAT-4. It also has a catalase peroxidase, CAT-2, five peroxidases, and four peroxiredoxins. CAT-1 (AY027545.2) is associated with non-growing cells and is accumulated particularly in asexual spores (conidia). CAT-3 (AY027544.2) is associated with growing cells and is induced under different stress conditions [20]. To date, The utilization of this mold that isolated from Indonesia have not been reported.

Ema (2015) used *Neurospora crassa* InaCC F226 that be grown on potato dextrose agar medium and precipitated by 60% ammonium sulphate resulted in specific activity, fold and yield, 3338.6 U/mg, 6.1 fold and 88.6%, respectively (unpublished data). Diaz *et al* reported that *Neurospora crassa* which be grown on the Vogel's medium had specific activity 1,4040.9 U/mL and yield 48% [25]. Therefore, In the Current study we focus on production of catalase on the Vogel's medium and purification by filtration gel. The characterization of catalase, including temperature, pH, metal ions, K_m and V_{max} were also investigated. The current study also focus on determination of molecular weight using SDS PAGE.

II. Material and Methods

A. Material

All of the chemical were from Merck chemical unless otherwise stated.

B. Strains, culture conditions and preparation of crude enzyme

Neurospora crassa (no. F226) were obtained from the Indonesia Culture Collection. This mold were isolated from the leaf litter at the Salak mountain. It was grown from stocks of conidia to slant agar medium. Conidia were inoculated on solid minimal-medium of Vogel [26], supplemented with 1.5% sucrose (w/v) and the cultures were incubated for 3 days in the dark at 30°C followed by 2 days in the light at 25°C [*20*]. Cell extract was centrifuged 15 minutes at 13000 x g and 4°C. The supernatant was joined and it was used as the crude enzyme solution.

C. Catalase Purification

The 60% ammonium sulfate precipitate was stirred an hour and centrifuged. The precipitate was resuspended in 5 mL of 50 mM sodium phosphate buffer, pH 7, after that the enzyme was dialysis. And than 15 mL of Sephadex G-75, equilibrated in the same buffer, was added [25]. The recovered Sephadex G-75 sludge was stirred for 3 h at 90°C, washed with 100 mL of 50 mM sodium phosphate buffer, and then loaded on a small column (1.5 cm x 15 cm). The column was washed with 100 mL the same buffer and 500 µL of the 60% precipitated enzyme eluted with 50 mM sodium phosphate buffer (pH 7). The highest activity fractions were collected and concentrated using a nanosep 10K centrifugal concentrator omega (Pall, life biosciences) [27].

D. Enzyme assays

The commonly used procedure to investigate catalase activity conducted from modification of Didem's method [27]. The hydrogen peroxide was used as substrate and decrease of hydrogen peroxide concentration was measured spectrophotometrically at 240 nm. The catalase activity was measured in controlled temperature (35°C) in 50 mM sodium phosphate buffer (pH 7.0). The 0.03 mL free enzyme were mixed on 0.97 mL 20 mM hydrogen peroxide in buffer solution. The decrease of absorbance concentration hydrogen peroxide at 240 nm was monitored. Enzyme activity was determined using the initial rate of the reaction and the extinction coefficient for H_2O_2 of 39.4 M^{-1} cm^{-1} [28]. One enzyme unit was defined as the amount of enzyme that catalyzes the decomposition of 1 µmol H_2O_2 per 3 minute at 35°C.

E. Measurement of protein concentration

To measure of protein concentration on solution was used the Bradford's method [29]. A sample of 0.01 mL was added into 0.99 mL of Bradford solution in the test tube. It were incubated at room temperature for 10-15 min. The quantity of protein in solution was determined by spectrophotometer at wave length 595 nm using bovine serum albumin as standard protein.

F. Determination of molecular weight of Neurospora crassa catalase

The purity of catalase was conducted from modification of Kandukuri *et al* [30] by SDS-PAGE using 30% polyacrylamid gel. The crude, 60% ammonium sulfate, and gel filtration were treated with 30% SDS and heat denatured at 70°C for 20 min before loading on the gel, the prepared samples were loaded on to the gel and electrophoresis was carried out at 200 V, 40 A, for 140 min. After the electrophoresis the gel was subjected to Coomassie Brilliant Blue R-250 staining for 24 h and molecular weight was estimated with the help of a medium range (20-250 kDa) protein ledde (Bio-Rad).

G. Determination of optimum pH and temperature on catalase activity

The optimum pH for catalase was determined using various pH buffer solutions ranging from 5.0 to 9.0 using 50 mM of the following buffers: citrate-sodium phosphate (pH 5.0-5.5), sodium phosphate (pH 6.0-8.0), and Tris-HCl (pH 8.5-9.0). Enzyme concentration and temperature were kept constant as stated in standard assay condition. The residual activities were calculated by taking the ratio of the enzyme activities at different pH values compared to the maximum enzyme activity [27].

The optimum temperature of *Neurospora crassa* catalase was determined by measuring the activity at pH 7.0 in a water bath. The temperatures ranging from 30°C to 70°C at 5°C intervals. Enzyme concentration and pH were as stated in standard assay condition. The residual activities were calculated as the ratios of enzyme activities at different temperatures to the maximum enzyme activity.

H. Effect of inhibitor and metal ions on catalase activity

To determination of inhibitor and metal ions, the enzyme was incubated in the various concentrations (0.5 to 1.5 mM) of inhibitor EDTA and metal ions (Fe^{+2}, Cu^{+2}, Mg^{+2}, Mn^{+2}, and Ca^{+2}) [30]. The concentrated eluate obtained from 60% ammonium sulfate was incubated for 15 min at water bath (40^{o}C) with inhibitor and metal ions made in 50 mM phosphate buffer, pH 7.0 [20]. The percentage of activity relative was calculated. For all inhibitor and metal salt concentration the enzyme (U/mL) used for incubation was kept constant.

I. Determination of K_m and V_{max} on catalase enzyme

The kinetic parameters K_m and V_{max} were determined for catalase activity. Different concentrations of H_2O_2 (0.5 to 40 mM) were prepared in 50 mM phosphate buffer, pH 7.0. Hydrogen peroxide with various concentration was incubated for 2 min at water bath (40^{o}C), than addition 30 µL of enzyme from 60% ammonium sulphate, after that incubated for 3 min at water bath (40^{o}C). The rate of H_2O_2 decomposition was estimated spectrophotometrically at λ= 240 nm (UV-Mini 1240, Shimadzu). The data obtained were plotted according to the method of Lineweaver–Burk to determine K_m and V_{max} graphically [30].

III. Result and Discussion

A. Precipitated of enzyme by Ammonium Sulphate

Neurospora crassa InaCC F226 grown in the Vogel's medium and incubated 5 days (3 days in the dark at 30^{o}C followed by 2 days in the light at 25^{o}C). In the range of the time, the enzyme resulted the highes of activity and it useful in the production of enzymes in the cell. Biomass harvested from solid medium and it was collected as much as 25 Gram. The cells were destroyed using an sonicator (Q-Sonica CL-188) and dissolved in the 100 mL of Na-phosphate buffer (50 mM, pH 7.0) containing 3% hydrogen peroxide. The supernatant containing crude enzymes of catalase was separated from cells using centrifugator at 15 minutes, 13000 x g and 4^{o}C.

The crude enzyme in the supernatant was precipitated by ammonium sulphate. The precipitation enzyme using ammonium sulphate can stabilize the protein from denaturation, proteolysis and contamination of bacteria. In our study, the supernatant (100 mL of crude enzyme) was precipitated by 60% ammonium sulphate at 4^{o}C and centrifugation at 10000 x g and 4^{o}C. The precipitated enzyme was dissolved with Na-phosphate buffer (pH 7.0) untill the volume being 10 mL and it dialysis at over night (4^{o}C) in the Na-phosphate buffer (pH 7.0) to eliminate the ammonium sulphate and other ions that can contamination of enzyme. The 60% ammonium sulphate precipitation resulted a specific activity 305.2 U/mg (Table 1). This result showed that the purity of the enzyme is quite good when compared of crude enzyme (136.7 U/mg). It value was less than reported by Diaz *et al* [25] and Ema. Ema (2015) reported that the specific activity of catalase *Neurospora crassa* InaCC F226 is 3338.6 U/mg while Diaz *et al* reported the specific activity of catalase from *Neurospora crassa* with combination precipitation (ammonium sulphate and aseton) was 1,4040.9 U/mg. These combination selected based on catalase stability in organic solvents and denaturing agents and also took advantage of catalase hydrophobicity. Fang *et al* [15] reported that addition of 20 g/L dextrin and 1% ethanol and peptone in the medium culture *Thermoascus aurantiacus* can improve of catalase activity (1594 u/mL). It was concluded that dextrin might act as a major carbon source in the complex, while ethanol was rather a stimulator than a carbon source. The stimulation effect of ethanol on catalase production was postulated to be two aspects; catalase-dependent alcohol metabolism is activated by acute alcohol, thus more catalase need to be synthesized for that use, named direct induction. As for indirect induction, which may result from little amount of H_2O_2 generation in process of NADH regeneration in respiratory chain.

The yield of the purification in this process were lower than several reported (Table 2). It might be caused the concentration of nitrogen, carbon source and effect of inducer that used in growth medium [31-34]. Gromada and Fiedurek [35] reported that replacing of $NaNO_3$ and peptone with KNO_3 and yeast extract can increase of catalase activity 1.5 and 1.3 fold, respectively.

Futhermore, increasing of production the catalase was observed by effect of stress condition by adding inducer. Zhenxiao Yu [2] reported that addition of 2% to 4% ethanol can decreased catalase activity from 33,966±22.78 to 18,564±20.16 U/mL. In this study, there is effect of the ethanol that produced during fermentation process. It was indicated by alcohol odor from fermented culture after 3 days. Addition of 0.5% Triton X-100 can also improve the production of catalase (12,760±20.45 U/mL).

B. Purification catalase by Sephadex G-75

In our study, we used the matric of chromatography using Sephadex G-75 to increasing the purity of catalase. This matric can separate peptida and protein with range of molecular weight between 3000 to 80000. The final result of separation is affected by the type of a column that used. Column used should be free of air bubbles, free of impurities, and the flow rate of column relatively constant.

The purification enzyme using chromatography resulted one peak at 240 nm (Fig 2). The fraction of 13-19 resulted catalase activity from 4.3-4.1 U/mL. The specific activity of purified enzyme using filtration gel (1464.9 U/mg) was high compared to crude extract (136.7 U/mg) and ammonium sulphate (305.2 U/mg). It is because of the protein constituent of catalase has been separated to protein of catalase and the contaminant. Therefore, the enzyme afinity toward substrate was high (table I).

C. Determination of molecular weight

Based on the results of SDS PAGE (Fig 1), obtained two bands on purification using chromatography. These results indicate that the enzyme still relies not pure. It is caused the use of column chromatography is not appropriate. The length, diameter, and the flow rate in the column chromatographic that used to separation of the enzyme greatly affect the outcome. The longer of the columns used in the analysis process can separate the enzyme perfectly. The SDS PAGE result show that molecular weight of catalase is 50, and 59.4 kDa, approximately. Xinhua Li *et al* [4] reported that the molecular weight of *Acinetobacter* sp

YS0810 with 57.2 kDa. *Serratia marcescens* SYBC08 with 58 kDa [34] and *Vibrio salmonicida* with 57 kDa [36].

TABLE I
PURIFICATION RESULT OF CATALASE FROM NEUROSPORA CRASSA INACC F226

Fraction	Volume (mL)	Total activity (U)	Total protein (mg)	Spesific activity (U/mg)	Purification (fold)	Yield (%)
Crude extract	100	6155.3	45	136.7	1.0	100.0
60% $(NH_4)_2SO_4$	10	3392.6	11.1	305.2	2.2	55.1
Sephadex G-75 Column	5	1335.6	0.9	1464.9	10.7	21.7

Therefore, we can predict the molecular weight of catalase *Neurospora crassa* InaCC F226 is 59.4 kDa and we can conclude that the catalase *Neurospora crassa* InaCC F226 is monofunctional heme (typical) catalases.

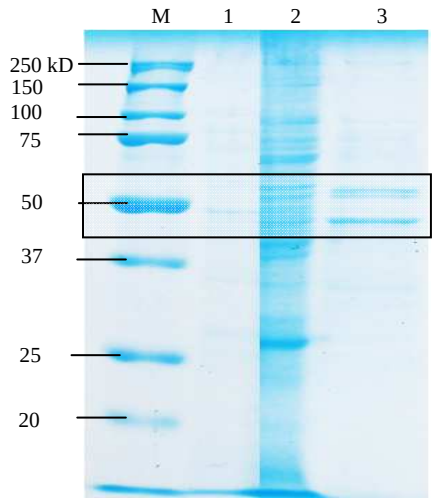

Fig 1. SDS–PAGE (30%), lane M: low molecular weight protein marker, Lane 1: crude enzyme (45 mg of protein concentration), lane 2: 60% Ammonium sulphate precipitated (11.1 mg protein concentration), Lane 3: concentrated elute of gel filtration (0.9 mg protein concentration).

D. Optimum pH and temperature on catalase activity

The aim of pH and temperature optimization is to know on the optimum condition in catalytic process at substrate degradation. The range of pH that used in this study is 5.0-9.0. In our study, the catalase was found to be stable in broad pH range of 5.0-9.0 (fig 4) and optimum pH obtained from *Neurospora crassa* InaCC F226 were 7.0 (fig 3) with catalase activity 264.8 U/mL and the relative activity 100%. In the pH 5.0; 6.0; 8,0;9.0 the catalase activity were 211.7 U/mL (77.9%); 237.3 U/mL (87.3%); 249.6 U/mL (91.9); and 234.9 U/mL (86.5%), respectively. Generally, catalase that isolated from fungi stable at range pH 6-9 [12]. Daniel *et al* [37] reported that catalase-I and catalase-II isolated from *Trigonopsis variabilis* active in the pH range 3.0 and 10.0 and optimum pH at 5.0 and 8.0, respectively. Dindem Sutay *et al* [*27*] also reported that catalase from *Scytalidium thermophilum* active at pH 7.0. The same result also reported by Kandukuri [30] that isolated catalase from sprouted black gram (*Vigna mungo*) seeds, while Tian [33] reported that *Hansenula polymorpha* active at pH 2.6. These results indicated that the stability of pH enzyme in the microorganism each other not same. It is maybe caused condition of the place where is the fungi isolated, species and metabolism of the fungi.

The condition of temperature catalase optimum contained in 40^{o}C (100%) with unit activity 286.1 U/mL (fig 4) and it stable at 35^{o}C, retained >85 % of the activity up to 2 h (data not showed).

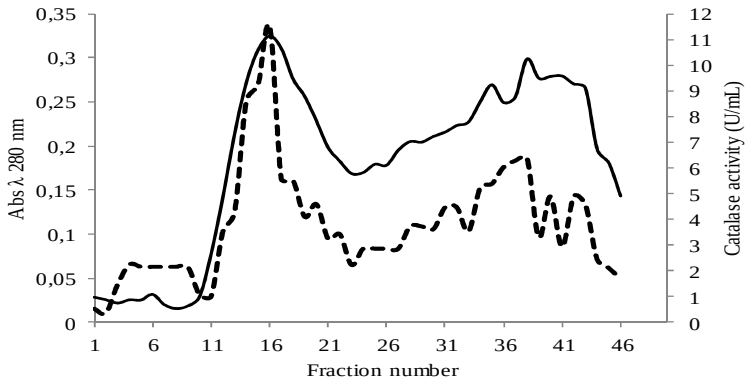

Fig. 2 The chromatographic profile of catalase (mL/min) by Sephadex G-75 column (——Absorbance of protein at 280 nm , ... catalase activity), the protein concentration in the load: 1.1 mg/mL, column was equilibrated with 50 mM sodium phosphate buffer (pH 7). The 60% $(NH_4)_2SO_4$ (v/v) fraction was applied to a 1.5 cm x 15 cm column (the load of volume enzyme was 500 µL) . The graph show: Fraction number 13-19 (4.3-11.5 U/mL

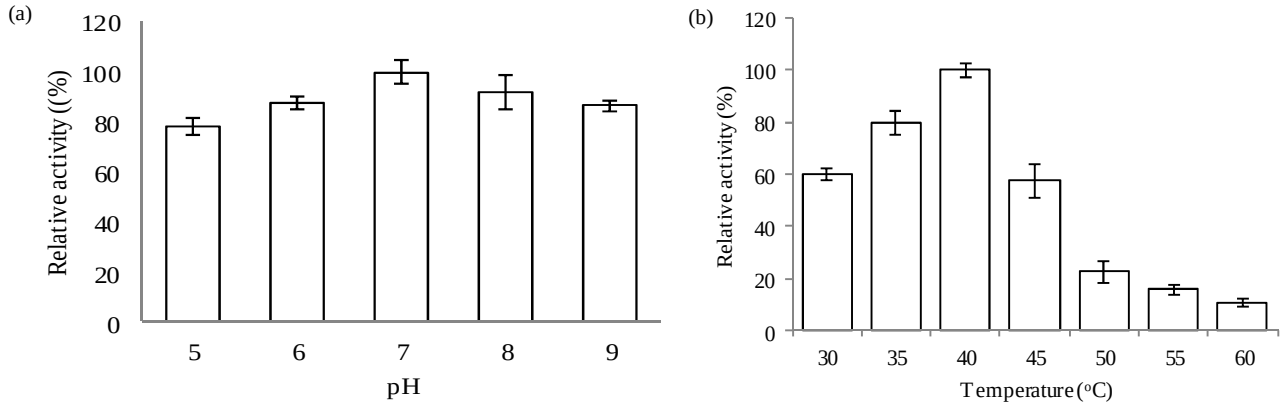

Fig 3(a) pH-dependence on catalase activity (under standart assay conditions at 35°C and different pH values), the pH optimum contained in pH 7.0 (264.8 U/mL) with relative activity 100% (mean ± SE, n=3). (b) Temperature dependence on catalase activity (under standart assay conditions at different temperatures), the optimum of temperature contained in 40°C (286.1 U/mL) with relative activity 100% (mean ± SE, n=3).

E. Effect of inhibitor and metal ions on catalase activity

The effects of inhibitor and various metal ions (0.5-1.5 mM) on the activity of purified catalase were studied at pH 7.0 and 35°C by addition of respective cation to the reaction mixture (Table 3). The addition of Fe^{+2} at level concentration of 0,5 mM increased catalase activity by 102.8% and 144.7%, respectively, while the activity decreased at the addition of 1.5 mM (89.6%). The increasing of activity on catalase caused addition of iron also reported by Tian (enhanced 60%). The addition of Ca^{+2} at level concentartion of 0.5 mM can increase catalase activity by 111.2% and declined at concentration of 1-1.5 mM (91.8% and 82.5%) of the control (control of enzyme without addition metal ions). These result similarly reported by Kandukuri that addition of Ca^{+2} till 1 mM can increase the activity of catalase (130%) and there was decrease in the activity at 1.5 mM and 2 mM (125%, 89%). The declined catalase activity in the present of EDTA, Cu^{+2}, and Mn^{+2} showed at table 3. EDTA known to be a metal chelator would have chelated the Fe^{+2} metal ions from the active site of catalase there by inhibiting the activity [30].

TABLE II
EFFECT OF INHIBITOR AND METAL IONS ON THE CATALASE ACTIVITY
(MEAN ± SE, N=3)

Inhibitor/metal ions	Relative activity (%)		
	0.5 mM	1 mM	1.5 mM
EDTA	81.4	33.2	13.5
Cu+2	51.4	25.8	19.5
Ca+2	111.2	91.8	82.5
Mn+2	83.7	76.1	14.4
Fe+2	102.8	144.7	89.6

F. Determination of V_{max} and K_m

To determination of V_{max} and K_m in the catalase catalytic activity, the enzyme was incubated in various concentration of hydrogen peroxide (0.5-40 mM) at pH 7.0, and 40°C to 3 min. In this study, the kinetic parameters for catalase *Neurospora crassa* InaCC F226 was analyzed by Lineweaver Burk plot (Fig 4). Based on the Michaelis's curve, the concentration of 10 mM hydrogen peroxide was a concentration that needed of catalase to get a half of catalase maximum velocity. The value of K_m and V_{max} catalase *Neurospora crassa* InaCC F226 from Lineweaver Burk is 8.8 mM and 5.7 s.mM^{-1}, respectively.

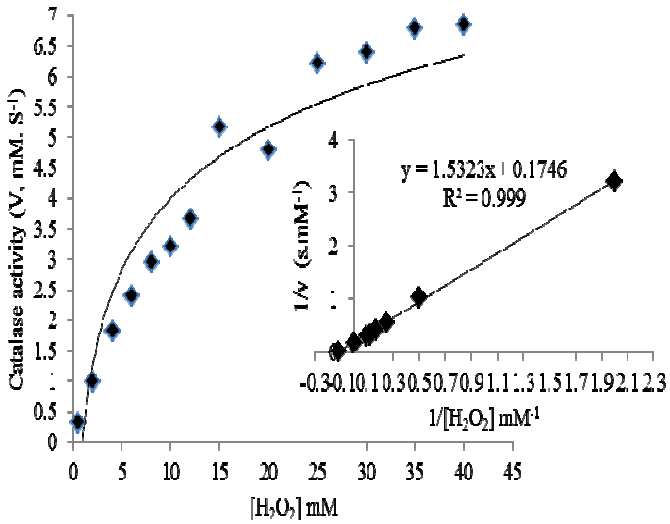

Fig 4. The curve of Michaelis Menten and Lineweaver Burk plot of catalase *Neurospora crassa* InaCC F226 using H_2O_2 as substrate

The value of K_m indicate the affinity of enzyme to the substrate. The higher of the value K_m, the affinity of enzyme to the substrate is low. Several journal reported that the K_m of various microrganism are differences. The K_m of *Pigmentiphage* sp DL-8 is 9.48 mM and 81.2 s.mM^{-1}(V_{max}). Tian [33] also reported that The K_m and V_{max} of catalase *Hansenula polymorpha* is 0.95 mM and 25 s.mM^{-1}, respectively.

IV. CONCLUSIONS

In this study, the purified catalase was characterized as a monofunctional catalase with molecular weight of catalase is 59.4 kDa, approximately. The enzyme was stable at pH 7.0 and 35^{o}C (2 h). The activity was increas by addition of Fe^{+2} and Ca^{+2} while inhibited by EDTA, Cu^{+2} and Mn^{+2}. The K_m and V_{max} was obtained 8.8 mM and 5.7 s.mM^{-1}. The stability and thermostability (2 h) of catalase demonstrated good application prospect in medical, environment, and industrial fields.

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