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Production, Purification and Characterization of Catalase from *Aspergillus Fumigatus*

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Abstract

This study describes production, purification, and characterization of catalase enzyme from *Aspergillus fumigatus*. The crude enzyme extract was obtained from *A. fumigatus* on 7th day of cultivation of cells grown at 37 °C and 155 rpm in 1-liter YpSs medium containing 1% (w/v) glucose and 0.5 mM H_2O_2. Then, the enzyme was successfully purified 24-fold with 55% recovery. The molecular weight was found ~70 kDa by SDS-PAGE. The optimum reaction temperature of the enzyme was established as 60 °C and the pH was 7.0. Km and Vmax values were calculated as 7.4 mM and 1250 µM min^{-1}, respectively. Stability tests have shown that the enzyme can remain active in a wide range of pH (4.0-9.0). Thermal stability of catalase was between 30 °C and 50 °C. The enzyme also presented stability against various solvents including ethanol, methanol, acetone, and dimethyl sulfoxide depending on the concentration and incubation time. The biochemical properties of the enzyme (low Km value, stability against varying pH and organic solvents, etc.) indicate that it can function as a good biocatalyst in different industrial applications.

Keywords: Catalase; *Aspergillus Fumigatus*; Purification; Enzyme Characterization

Abbreviations: CAT: Catalase Activity; SDS-PAGE: Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis; DMSO: Dimethyl Sulfoxide.

Introduction

Catalases are one of the most studied enzyme groups. They show their antioxidant effects in the cell by dissolving the excessively accumulated hydrogen peroxide (H_2O_2) into molecular oxygen and water [1]. Some catalases (catalase-peroxidases) also show a peroxidase function (by oxidizing low molecular weight alcohols) when the appropriate organic compound is present, mostly as a secondary activity [2]. It was discovered that catalases isolated from *Amaranthus cruenthus, Bacillus pumilus, Scytalidium thermophilum, Thermobifida fusca* and mammalian catalase possess oxidase activity in the absence of H_2O_2 [3-7]. Catalase enzyme has been mainly used in the food, pharmaceutical, textile, paper industries and in various fields such as immobilization, enzymatic polymerization, and detoxification systems [4].

Mesophilic fungi are the most common eukaryotic microorganisms in nature. Examples of thermotolerant species include *A. fumigatus* and *A. niger*. Thermostable enzymes produced by these two *Aspergillus* species are industrially important [8]. Although *Aspergillus* is known as a good source of catalase enzyme, little is known about its kinetic and biochemical properties. Recently, the increase in the use of hydrogen peroxide in the industry has made it necessary to try to produce an economical and stable catalase

enzyme that converts H_2O_2 to water and oxygen. Here we report the production, purification, and characterization of the catalase enzyme from mesophilic fungus *A. fumigatus*.

Materials and Methods

Materials

All chemicals and biochemicals were of analytical grade and obtained from Sigma-Aldrich, Merck and AppliChem.

Microbial Strain, Maintenance, and Cultivation

Aspergillus fumigatus (IMI 385708) was grown on YpSs agar (Difco powdered yeast extract, 4.0 g; K_2HPO_4, 1.0 g; $MgSO_4.7H_2O$, 0.5 g; soluble starch, 15.0 g; agar, 20.0 g in 1 L distilled water) [9] at 30 °C for 7 days. Spores grown on agar were transferred into 30 mL sterile solution containing 0.01% (v/v) Tween 80 and spore counting was performed with a Petroff-Hausser slide [10]. Spore suspensions were diluted with a solution containing sterile Tween 80 to contain 1 million spores per mL, and then 80% (v/v) glycerol was added and stored at -80 °C. Inoculation and the preparation of precultures were conducted as described by Soyler [11]. Heat shock and oxidative stress treatments were performed to optimize catalase production. Cultures were grown at 30, 37, 42, 45, 50 and 55 °C to induce heat shock [12,13]. For oxidative stress, H_2O_2 was added to the main culture at 0.1 mM, 0.25 mM, 0.5 mM, 1 mM, and 2 mM concentrations [13] and then each culture was incubated for 7 days at 155 rpm.

Biomass and Total Protein Determination

From culture media, 50 mL samples were taken, filtered using Whatmann No. 1 filter paper, and then washed with sterile water. By drying the samples at 100 °C until a consistent weight was attained, the dry weight of the fungus biomass (gL^{-1}) was calculated. The total protein was determined by the method described by Bradford [14] using bovine serum albumin as a standard.

Enzyme Assay

For catalase activity (CAT), the reaction mixture was obtained by adding 2.9 mL of a 10 mM H_2O_2 solution and 0.1 mL (pH 7.0) of the enzyme sample to a final volume of 3 mL using samples obtained from the supernatant. The substrate solution (H_2O_2) prepared in 100 mM sodium phosphate buffer solution was incubated for 1 min in a water bath at 25 °C. After pre-incubation, the solution was transferred to the reaction cuvette and the enzyme sample was added. After the reaction temperature was adjusted to 25 °C, the absorbance value of the device was adjusted to 240 nm and the measurement was performed. The enzyme activity was determined by using the initial reaction rate and the extinction coefficient value of hydrogen peroxide (39.4 M^{-1} cm^{-1}) [15]. 1 unit of enzyme activity corresponds to the enzyme that catalyzes the decomposition of 1 µmole H_2O_2 in 1 min [7].

Enzyme Purification

Catalase enzyme was purified by GE AKTA Prime plus FPLC System (USA) in two steps including anion exchange and size exclusion chromatography. Anion exchange was carried out using 20 mL prepacked HiPrep 16/10 Q XL column (Cytiva, USA) with a flow rate of 2 mL/min and 50 mM Tris-HCl (pH 8.0). Size exclusion was performed using HiPrep 16/60 Sephacryl S-100 HR column (Cytiva, USA) equilibrated with same buffer at a flow rate of 1 mL/min [16].

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The purity of protein sample eluted from column was checked by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) according to the method described by Laemmli [17] on Bio-Rad Mini-Protean gel electrophoresis unit (Bio-Rad Laboratories, Richmond, CA). Protein bands were visualized by Coomassie blue staining.

Thermal Effect and Thermostability

To determine the effect of reaction temperature on enzyme activity, activity assays were performed at temperatures between 30 °C and 80 °C at 10 °C intervals. The concentration of enzyme and the reaction pH were kept stable. By plotting percent relative activity values against temperature, the optimum temperature at which the catalase enzyme exhibited its highest activity was determined. Relative activities were calculated by dividing enzyme activities observed at each temperature by maximum enzyme activity. For thermal stability, the purified enzyme was incubated at 30-80 °C for 2 h and the activity assay was performed under standard assay conditions. Plotting percent residual activity values against temperature gave the temperature range at which the enzyme remained stable. Residual activities were determined from the ratio of enzyme activity after heat treatment to its original activity measured at each temperature.

Reaction pH Effect and pH Stability

To determine the effect of pH on catalase activity, activity assays were measured at different pHs ranging from 4.0 to 11.0 at 1.0 pH intervals. The concentration of enzyme and the reaction temperature were kept stable. The buffers used were 100 mM citrate buffer solution (for pH 4.0-5.0), 100

mM sodium phosphate buffer solution (for pH 6.0-7.0), 100 mM tris buffer solution (for pH 8.0), and 100 mM glycine sodium hydroxide buffer solutions (for pH 9.0-11.0). The optimum pH value of the enzyme was determined from the plot of percent relative activity against reaction pH. To test the effect of pH on catalase stability, enzyme solution was incubated at same pH values given above 2 h and then activity assay was performed under standard assay conditions [16]. The pH range at which enzyme maintained its stability was determined from the plot of percent residual activities versus different pH values ranging from 4.0 to 11.0 as stated above.

Km and Vmax Determination

The Km and Vmax values of the catalase enzyme were established by measuring enzyme activity in the presence of 0-300 mM H_2O_2 concentrations in 0.1 M sodium phosphate buffer (pH 7.0, 25 °C) [18] using a Lineweaver–Burk plot [19].

Organic Solvent Effect and Stability

The effect of organic solvents including ethanol, methanol, acetone, and dimethyl sulfoxide (DMSO) on catalase activity was tested. The concentrations used for each solvent were 2.5, 5, 7.5, 10, 15 and 20 (v/v) [20]. The stability tests were also performed by incubating the enzyme with solvents at stated concentrations for 1 h in ice, and standard activity assay was carried out every 15 min. Percent relative activity values were calculated as given above.

Results and Discussion

Time Course of Catalase Production

A. fumigatus extracellular catalase and biomass production during 10 days of incubation on 1% (w/v) glucose-containing medium is presented in Figure 1. Accordingly, the lag phase occurred in the first two days, and then the logarithmic phase started and continued until the sixth day. *A. fumigatus* entered the stationary phase on the 6th day. Catalase production was the highest on 7th day at which *A. fumigatus* was still in the stationary phase. This indicates that enzyme production is the result of secondary metabolic activity.

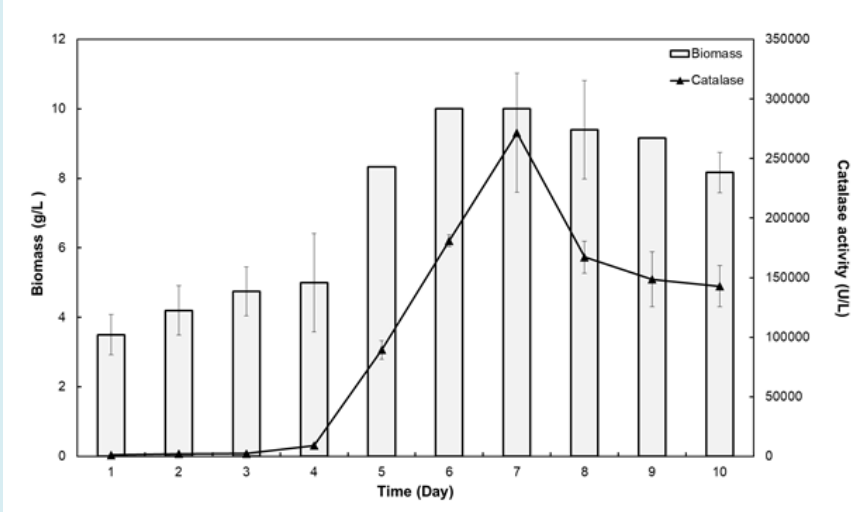

Figure 1: Biomass (g/L) and catalase (U/L) activity graph of *A. fumigatus* versus time.

Catalases have been isolated from various fungi and fermentation time required for optimum production varied between 3 and 6 days. For example, a catalase enzyme from *Septoria tritici* (ATCC 48507) was isolated after 3-day incubation [21]. Similarly, a catalase was obtained from *A. niger* on day 3 of its cultivation [22]. On the other hand, *S. thermophilum* was reported to produce extracellular catalase on day 5 at optimum level [16,20]. The difference in fermentation time primary depends on the source, the growth medium used, medium pH and growth temperature [23].

Effect of Heat Shock and Oxidative Stress on Catalase Production

It's been reported that cellular catalase production in several *Aspergillus* species increases in response to increase in H_2O_2 concentration after treatment of heat shock or

oxidative stress [24-26]. For this reason, the effects of H_2O_2 and temperature on catalase enzyme production were investigated in *A. fumigatus*. Optimum growth temperature of *Aspergillus* generally varies from 28 to 30°C [23], and it has been reported that some can tolerate up to 55 °C [12]. Therefore, in this study, *A. fumigatus* was grown at different temperatures (28, 30, 35, 37, 42, 45, 50 and 55 °C) and the enzyme activity was measured using the cell free growth medium collected on the seventh day of cultivation when catalase activity was the highest. As shown in Table 1, the optimum temperature for enzyme production was 37 °C. Above 37 °C, enzyme production presented a sharp decrease and at 50 °C and 55 °C it was totally inhibited indicating that heat shock application presented negative effect on catalase activity. On the other hand, the activity value (213 U/mL) measured in *A. fumigatus* grown at 37 °C was 3 to 3.6 times higher than the reported values (59-65 U/mL) for the catalase enzymes of *Aspergillus* species grown at lower temperatures [23].

Growth temperature (°C)	Catalase activity (U/mL)
28	115±8.9
30	186±9.6
37	213±9.4
42	23±6.1
45	16±4.4
50	0
55	0

Table 1: Effect of temperature on catalase production.

To introduce oxidative stress, H_2O_2 was added as described in Materials and Methods section. Figure 2 revealed that H_2O_2 at all concentrations resulted in higher catalase activity in comparison to the control (no H_2O_2 present). The highest activity was observed in the presence of 0.5 mM H_2O_2 at second hour of H_2O_2 treatment. This result was consisted with previous reports [13,25,27].

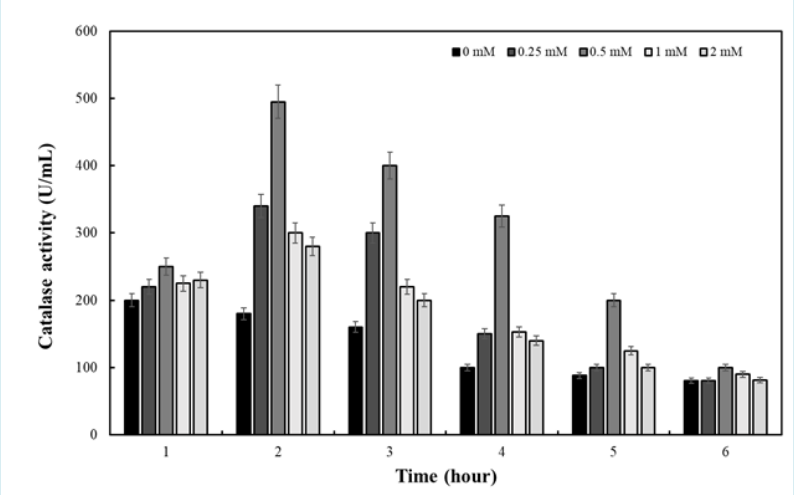

Figure 2: Effect of H_2O_2 concentration on catalase production. *A. fumigatus* was grown in 1-liter YpSs broth containing 1% (w/v) glucose as the carbon source. H_2O_2 was added to the growth medium at 30-minute intervals on the seventh day when enzyme production was the highest.

Catalase Purification

Catalase enzyme was purified by anion exchange chromatography and size exclusion chromatography. Purification fold and activity recovery values were calculated as 24 and 55%, respectively Table 2.

	Volume(mL)	Total activity (U)	Total protein (mg)	Purification fold	Activity recovery (%)
Crude extract	25	12375	75	1	100
AEC	30	8910	5.4	10	72
SEC	9	6806	1.7	24	55

Table 2: Purification results for two-step chromatography of catalase from *Aspergillus fumigatus*.
AEC: Anion exchange chromatography, SEC: Size exclusion chromatography

Table 3 compares the purification parameters revealed by different fungal catalases. Accordingly, the activity recovery value (55%) obtained in this study is the highest among the reported values (13.6-48%). The purification fold value is higher than that reported for catalases from *S. thermophilum* and *Thermoascus aurantiacus*. On the other hand, it is slightly lower than that given for catalase from *Septoria tritici*, while quite lower than *Neurospora crassa* catalase.

Microorganism	Chromatographic method	Purification fold	Activity recovery (%)	Reference
Scytalidium thermophilum	AEC, SEC	10	45	Sutay Kocabas, et al. [16]
Thermoascus aurantiacus	Ethanol precipitation, DEAE (DE-52), Sep (CL6B)	5.1	29.9	Wang, et al. [28]
Septoria tritici	Dialysis, DEAE-C, Phenyl Sep	36	13.6	Levy, et al. [21]
Neurospora crassa	Acetone precipitation, ASF, Phenyl Sep column	170	48	Diaz, et al. [35]
Aspergillus fumigatus	AEC, SEC	24	55	This study

Table 3: Comparison of purification parameters of catalase enzymes isolated from different fungi.
Sep: sepharose; ASF: ammonium sulfate fractionation; SEC: size exclusion chromatography; DEAE-C: DEAE cellulose; AEC: anion exchange chromatography

The purity of catalase enzyme eluted from size exclusion column was checked using SDS-PAGE. As shown in Figure 3, a single band corresponding to approximately 70 kDa was detected. Catalases from other fungi have been reported to possess 61–97 kDa subunit size. For example, the subunit of *S. thermophilum* catalase is 80 kDa [16] and that of *T. aurantiacus* is 75 kDa [28].

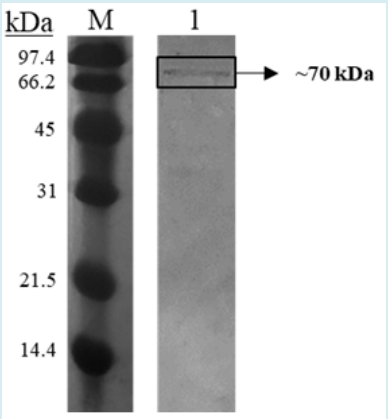

Figure 3: SDS-PAGE gel image of catalase purified from *A. fumigatus*. M, protein marker (10 µg) (Bio-Rad Unstained SDS-PAGE Standards, USA); 1, catalase sample isolated from size exclusion column (10 µg).

Effect of Temperature on Catalase Enzyme and Stability

How enzyme reacted in response to change in reaction temperature was presented in Figure 4A. The optimum reaction temperature of *A. fumigatus* catalase was found to be 60 °C. Although above 60 °C the catalase enzyme activity gradually decreased, percent activity values were 80% and 70% at 70 °C and 80 °C, respectively. Relatively high activity values observed at higher temperatures indicate that the catalase enzyme can still function at elevated temperatures. Similar to our results, catalases from different sources including *Bacillus sp*., *Desulfovibrio gigas* and *S. thermophilum* have been reported to reach its optimum level at 60 °C [16,29,30].

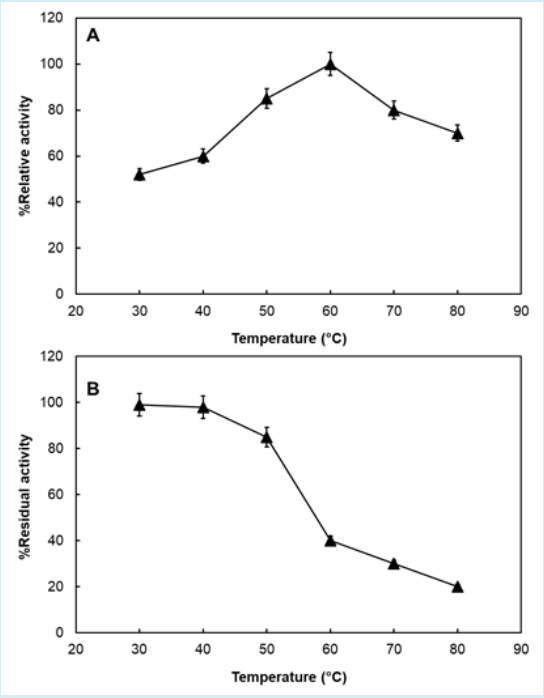

Figure 4: Percent relative and residual activities measured at varying temperatures.

In terms of thermal stability, catalase enzyme considerably maintained its original activity up to 50 °C as shown in Figure 4B. 99%, 98% and 85% of its initial activity were remained at 30 °C, 40°C and 50 °C, respectively. On the other hand, 60% (60 °C), 70% (70 °C) and 80% (80 °C) of activity was lost at higher temperatures. The stability presented at 50 °C by *Aspergillus* catalase would be an advantage for its use in industrial applications. There are examples of catalases revealed stability at lower temperatures including *Serratia marcescens* FZSF01 and *Escherichia coli* catalase [31,32].

Effect of pH on Catalase Enzyme and Stability

The effect of reaction mixture prepared at different pH values (4.0-11.0) on catalase activity was also investigated. The highest activity was measured at pH 7.0 Figure 5A. On the other hand, percent relative activity values were closest to 100% at pH 6.0 (88%) and pH 8.0 (92%). Even at acidic pH values activity values were 60% (pH 4.0) and 75% (pH 5.0) with respect to its highest observed at pH 7.0. However, at pH values above 9.0, more than 50% decrease was observed. The results reveal that *Aspergillus* catalase can actively work in a wide range of pH. The optimum reaction pH value is similar to that reported for various catalases including *Beauveria bassiana*, *Rhodospirillium rubrum* S1 and *S. thermophilum* catalases [16,33,34].

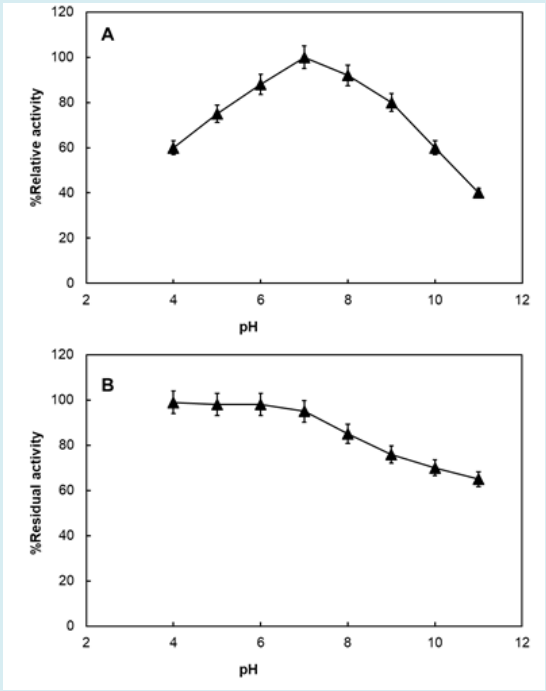

Figure 5: Percent relative and residual activities measured at varying pH.

The results of the pH stability studies are given in Figure 5B. Accordingly, catalase enzyme remained stable up to pH 7.0 and then a gradual decrease was detected up to the final pH value (11.0) tested. At pH 11.0, 65% of original activity was maintained. Consistent with our results, catalase from *T. aurantiacus* has been reported to remain stable at pH values from 5.0 to 13.0 [28]. The stability exerted over a wide pH range is important especially for enzymes exposed to ambient pH for a long time in industrial applications.

Determination of Km and Vmax

The Michaelis Menten graph and Lineweaver-Burk diagram to calculate the Km and Vmax values of the *A. fumigatus* catalase enzyme are given in Figure 6. According to the graph, the Km and Vmax values were determined as 7.4 mM and 1250 µmole min^{-1} L^{-1}, respectively. The Km value of *A. fumigatus* catalase was lower than *Archaeoglobus fulgidus* catalase (8.6 mM), *N. crassa* catalase (21.7 mM), *B. subtilis* 168 catalase (39 mM), and *Serratia marcescens* SYBC08 catalase (78 mM), but higher than *Septoria tritici* catalase (3.4 mM) and *Klebsiella pneumoniae* (6.5 mM) catalase [35-39].

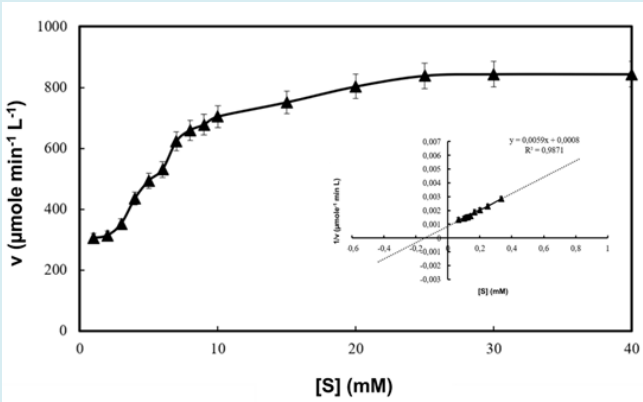

Figure 6: Michaelis-Menten plot showing the effect of H_2O_2 concentration on *A. fumigatus* catalase activity (Substrate [S]: Hydrogen peroxide). Inset, Lineweaver-Burk diagram of catalase enzyme

Effect of Organic Solvents on Catalase Activity and Stability

The effect of organic solvents on catalase enzyme activity is shown in Figure 7. Organic solvents can change the physicochemical properties of proteins. In general, non-covalent bonds in the protein change properties such as dipoles and ionic groups, hydrophobic interactions, and hydrogen bonds [40]. The enzyme stability was in the order of Ethanol>Acetone>Methanol>DMSO. It always remained 55-70% of its original activity (no solvent present) in ethanol up to 20% (v/v).

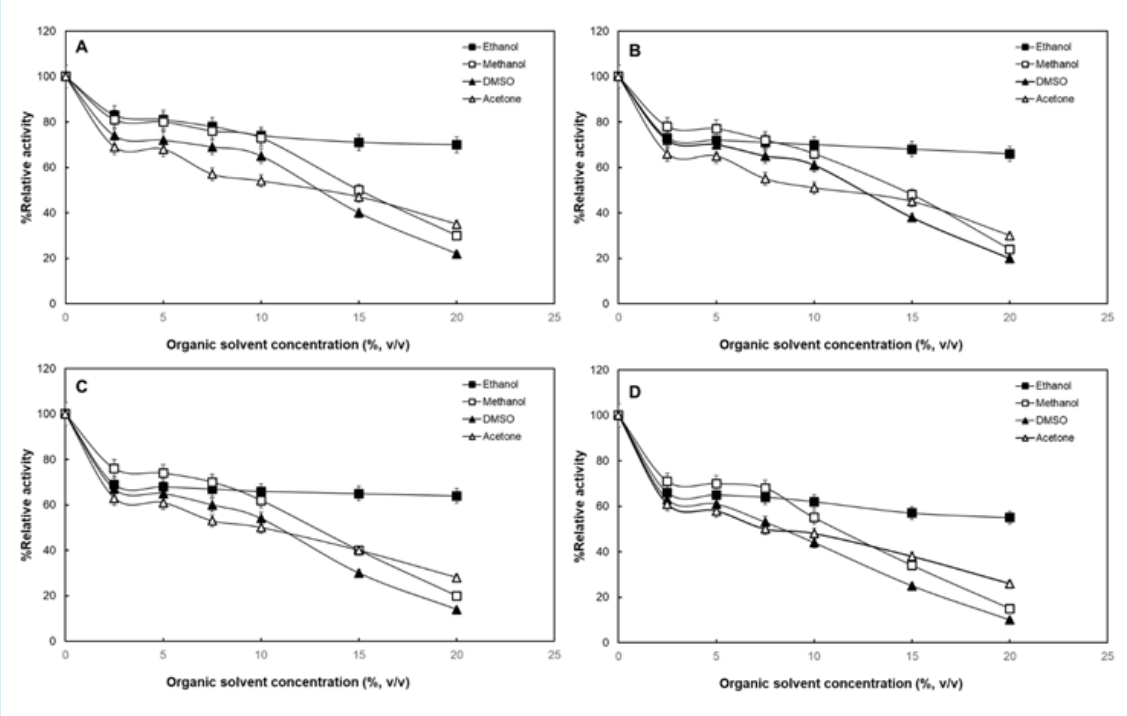

Figure 7: Effect of different organic solvents on *A. fumigatus* catalase activity after 15 min (A), 30 min (B), 45 min (C) and 60 min (D) incubation.

Conclusion

In this study, catalase production from *A. fumigatus* was optimized. Time course of catalase production has shown that enzyme production reaches its optimum value on the 7th day of cellular growth when *A. fumigatus* was in the stationary phase. This indicates that enzyme production occurs as a result of secondary metabolism. Additionally, the catalase activity was increased when 0.5 mM H_2O_2 (as a source of oxidative stress) was added to the culture medium. Catalase enzyme was purified from *A. fumigatus* for the first time. The purification factor and activity recovery values were calculated as 24-fold and 55%, respectively. The purity was checked by SDS-PAGE, where a single band was observed. The molecular weight was calculated as approximately 70 kDa. The optimum temperature and pH of the pure enzyme were determined as 60 °C and 7.0, respectively. It was also observed that the enzyme remained stable for about 2 h between 30°C-50°C and pH 4.0-9.0. With the help of Lineweaver-Burk diagram (1/S vs 1/V) Km value was calculated as 7.4 mM and Vmax value was calculated as 1250 µmole min^{-1} L^{-1}. Finally, the resistance of the enzyme to organic solvents was determined as Ethanol>Acetone>Methanol>DMSO at concentrations ranging from 2.5% to 20%. The stability of *Aspergillus* catalase against varying temperature and pH values can provide an advantage to the enzyme for its use in long lasting processes during industrial applications. In addition, the exerted resistance of the enzyme against different organic solvents at various concentrations is another remarkable characteristic supporting enzyme use in industry where organic solvents are predominantly used.

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