Journal of Advanced Scientific Research

Available online through http://www.sciensage.info

ISSN
0976-9595
Research Article

ASSESSMENT OF SUPEROXIDE DISMUTASE, CATALASE, AND PEROXIDASE ACTIVITIES IN *ASPERGILLUS SP.* AND *CLADOSPORIUM SP.*

Aqsa Alam, Prachi Kataria, Nethravathy. V*

P.G Dept of Life Science, Mount Carmel College Autonomous, Vasanth Nagar, Bengaluru, Karnataka, India

**Corresponding author: nethraprasad08@gmail.com*

ABSTRACT

Antioxidative enzymes are a family of powerful free radical scavenging proteins, naturally synthesized by all aerobic organisms. Family of antioxidant enzymes include superoxide dismutase (SOD), catalase (CAT), peroxidase (PRX), thioredoxins /thioredoxin reductase (TrxR), and glutaredoxins/glutathione reductase (GPx). These oxidative enzymatic machineries, sequentially sequester singlet oxygen (O_2^-) and Hydrogen Peroxide, which are the well-known reactive oxygen species. These enzymes can be intracellular as well as extracellular. In the present study, two most common soil fungi namely, *Aspergillus Sp.* and *Cladosporium Sp.* were cultivated by submerged cultivation on Potato dextrose broth and pellets were separated by filtration. Pellets were disrupted by detergent lysis method (0.5% Triton X 100, 1% CTAB, 1.5% Tween 80, 5% SDS). Culture media and cellular extracts were analyzed for intracellular and extracellular total proteins and their panel of antioxidative enzymes. Enzyme activity of SOD was carried out by Beauchamp and Fridovich method. Catalase and Peroxidase activities were determined by simple spectrophotometric method. In *Aspergillus Sp.*1.33 U of superoxide dismutase, 0.115 U catalase, 0.00097 U peroxidase activities per mg of protein was observed. Similarly, in *Cladosporium Sp.* 1.38 U Superoxide dismutase, 0.1460 U catalase, and 0.00150 U peroxidase activities per mg protein was recorded. Significant amount of antioxidative enzyme activity observed in both the test cultures. And comparatively, *Cladosporium Sp* has given promising results. Probably, with altered culture conditions and improved extraction procedures, still higher amounts of antioxidative enzymes would be expected.

Keywords: *Aspergillus Sp.*, *Penicillium Sp.*, Antioxidative enzymes, Spectrophotometry.

1. INTRODUCTION

Reactive Oxygen Species (ROS) are extremely reactive unsteady molecules and are formed as a by-product of aerobic respiration. These include peroxide, superoxide, hydroxyl radical, singlet oxygen. ROS are detrimental when found in concentrations above a certain threshold and can damage the DNA, proteins as well as the normal functioning and metabolism of the cell [1]. ROS can also be formed due to extrinsic factors such as nutrients deficiency, metal toxicity, UV radiation, stress and by the insufficient elimination of ROS by the antioxidants producing mechanism of the body [2]. When there is rise in ROS, molecules called as antioxidants come into play to bring down the levels of ROS. These antioxidants are also called as "free-radicle scavengers". Antioxidants include thiols, ascorbic acid (Vitamin C), glutathione and enzymes (superoxide dismutase, catalase, and Peroxidase) [3].

Superoxide dismutase (SOD) is an enzyme which breaks down superoxide (O_2^-) radical into molecular oxygen (O_2) and hydrogen peroxide (H_2O_2). Hydrogen peroxide is also damaging to the cell and is normally catalyzed by catalase and Peroxidase enzymes. Hence SOD is a first line defense mechanism in all aerobically respiring living cells. There are many types of SOD with different types of cofactors used for activation, Cu/Zn type SOD, Fe SOD, Mn SOD and Ni SOD and Cu only SOD, which is an extracellular SOD [4, 5].

Catalase (CAT) is a common enzyme, found in all aerobically respiring organisms, which catalyze the breakdown of hydrogen peroxide (H_2O_2) into water (H_2O) and oxygen (O_2) and not singlet oxygen (O^-).

Both peroxidase (PRX) and catalase (CAT) are heme containing enzymes that neutralize hydrogen peroxide [8].

These antioxidative enzymes are found to have various commercial applications. Superoxide Dismutase has powerful anti-inflammatory and detoxification activities, it is also used in cosmetics and personal care products as an anti-ageing ingredient. Mainly it helps boost immunity and detoxifies the body, protect against mutagenesis and degenerative diseases [9]. Promising applications of catalases are in wastewater treatment, therapeutics, baking and brewing, pulp and paper industry [7, 10]. Peroxidase has a potential for bioremediation of soil and wastewater contaminated with phenols, cresols, chlorinated phenols. Peroxidase has significant application in diagnostic kits, such as quantitation of uric acid, glucose, cholesterol, lactose, Enzyme linked immunosorbent assay (ELISA) and so on [11].

Many researchers are exploiting microorganisms for the commercial production of these antioxidative enzymes for their significant commercial importance. Hence the main aim of this study is to spectrophotometrically analyze antioxidative enzymes from fungal species commonly found in soil.

2. MATERIAL AND METHODS

2.1. Isolation of fungi from soil

Soil sample collected from the botanical garden, Mount Carmel College campus was serially diluted and spread plated on sterilized Potato dextrose agar medium. The plates were incubated at room temperature for 5 days. From the mixed culture plates, randomly *Aspergillus Sp.* and *Cladosporium Sp.* isolates were selected for anti-oxidative enzyme production.

2.2. Submerged cultivation of selected fungi on potato dextrose broth

The identified and selected fungal species (*Aspergillus Sp* and *Cladosporium Sp*) were inoculated into 50 ml of sterile PDB, under aseptic conditions. The test samples were incubated on shaker incubator at room temperature (28±4°C) up to 5 days under submerged cultivation. The test samples were cultured in triplicates. The uninoculated PDB was used as control.

2.3. Filtration and separation

The submerged cultures were filtered using Whattman filter paper No. 1 and the culture filtrates of triplicates were pooled and refrigerated for further assays to be performed. The pellets were blot dried using blotting paper. The wet weights of the cell biomass were recorded.

2.4. Cell disruption

Cell lysis was performed by detergent lysis method. The composition of detergent lysis buffer is Triton X 100 (0.5%), CTAB (1%), Tween 80 (1.5%), SDS (5%), all these chemicals were prepared in pH 7.8 sodium phosphate buffer. The pellets of *Aspergillus Sp* and *Cladosporium Sp* were suspended in detergent solutions for 40 mins in shaker incubator at 30°C. The disrupted pellets were centrifuged (REMITM SKBM 80 plus) at 12,000 RPM for 15 mins at 4°C. The cellular extract (supernatant) was collected. The cellular extracts of the triplicates were pooled for further analysis [12]. Protein estimation was performed according to Lowry's method for *Aspergillus Sp.* and *Cladosporium Sp.* crude samples. Culture filtrate and cellular extracts were analyzed for protein production. The bovine serum albumin (BSA) standard curve was plotted to determine the amount of secretory and intracellular protein.

2.5. Antioxidative enzyme assay

2.5.1. Estimation of superoxide dismutase (SOD)

SOD assay was carried out by Nitro blue tetrazolium (NBT) method [13]. The assay was performed as follows: Blank (B); 2.5ml methionine+0.3ml riboflavin+0.2 ml phosphate buffer. Test (T); 2.5ml methionine+0.3ml riboflavin+0.1 ml NBT+0.1 ml culture filtrate. Standard (S); 2.5ml methionine+0.3ml riboflavin+0.1ml NBT+0.1ml phosphate buffer. Control (C); 2.5ml methionine+0.3ml riboflavin+0.1 ml phosphate buffer+0.1ml culture filtrate. The control tube is without the substrate (NBT), the standard tube is without the enzyme that is the culture filtrate, the test is with the enzyme and substrate and the blank has no enzyme and no substrate in it. All the tubes were incubated in illumination chamber for exact 10 mins after which immediately absorbance was taken at 560nm [14]. The substrate used for the assay consists of nitro blue tetrazolium chloride (NBT) which reacts with superoxide anions produced upon illumination of riboflavin in the presence of methionine as an electron donor, to produce formazan which is a blue colored complex. The decrease in the formation of formazan is directly proportional to the amount of SOD in the sample. A decline of 50% in the formation of formazan is taken as one unit of SOD.

The reaction was initiated and terminated by turning the light on and off. The specific activity of the enzyme is reported as U/mg protein.

2.5.2. Estimation of catalase activity

Catalase activity was measured by following the decline in absorbance as H_2O_2 ($\varepsilon = 36M^{-1}cm^{-1}$) was catabolized, according to the method of Aebi 1984. The reaction mixture contained 30 µl crude enzyme extract in 50 mM sodium phosphate buffer (pH 7.8) and 2970 µl of 20 mM H_2O_2, making the total reaction volume to 3ml. The reaction was started by the addition of 2970 µl of 20 mM H_2O_2 and its consumption was measured at 20 Seconds intervals up to 3 minutes at 240 nm. The absorbance was read at 240 nm at 20 seconds time interval for 3 minutes [15, 16].

2.5.3. Estimation of peroxidase activity

Extracellular peroxidase activity was determined by modified method as described by Wariishi et al., [17]. Reaction mixture was prepared using 0.5ml cellular extract in 50mM phosphate buffer (pH 7.4)+0.1ml 25mM lactate+0.1ml M Manganese sulphate+0.1ml BSA+0.1 ml phenol red indicator. The reaction was started by the addition of 0.1ml of 0.1 mM H_2O_2 to the reaction mixture. Decrease in the absorbance of these complexes was read at 610 nm at time intervals of 1 minute up to 3 minutes. The reaction was stopped by adding 50µl of 10% NaOH. The blank was prepared without manganese sulphate and the control without the phenol read indicator [18].

3. RESULTS AND DISCUSSION

3.1. Cell lysis and total protein estimation

Aspergillus Sp. and *Cladosporium Sp.* biomass were harvested by filtration after 5 days of incubation using Whatman filter no 1(GE Healthcare UK limited). Amount of biomass produced was determined. The wet weight of *Aspergillus Sp.* was 1.74 g and *Cladosporium Sp.* Was 0.82 g. In this study, shake flask cultivated test organisms have shown higher extracellular protein than the intracellular protein. The shaking conditions, 50 RPM at room temperature (28°C±4°C) would have influenced the extracellular protein production. However, with optimization of cell lysis methods, we would extract increased amounts of intracellular protein. Protein estimation was performed according to Lowry's method for *Aspergillus Sp.* and *Cladosporium Sp.* crude samples. The amount of secretory protein (extracellular protein), cellular protein (intracellular protein) and total protein were calculated using standard BSA graph and tabulated in [Table 1]. Lowry's method of protein estimation is sensitive to low concentrations of protein estimation. In this study, shake flask cultivated test organisms have shown higher extracellular protein than the intracellular protein. The shaking conditions, 50 RPM at room temperature (28°C±4°C) would have influenced the extracellular protein production. However, with optimization of cell lysis methods, we would extract increased amounts of intracellular protein.

Table 1: Amount of total protein produced by *Aspergillus Sp.* and *Cladosporium Sp.*

Sl. No	Sample	Amount of Intracellular protein (mg/ml)	Amount of extra cellular protein (mg/ml)	Amount of total protein (mg/ml)
1	*Aspergillus Sp*	2.76	26.32	29.08
2	*Cladosporium Sp*	5.18	17.58	22.75

3.2. Estimation cellular and secretory SOD

Superoxide dismutase activity was analyzed by *Beauchamp* and *Fridovich* (1971) method. It is a photo-chemical assay consisting of methionine, riboflavin, and p-nitro blue tetrazolium. Crude extracts of the sample were analyzed for SOD isoenzyme activity at 550nm. It includes both secretory (extracellular) enzyme as well as intracellular enzyme assays. Secretory SOD, intracellular SOD and total SOD activity of *Aspergillus Sp.* and *Cladosporium Sp.* are summarized in [Table 2]. SOD activity was calculated using the following formula.

Decrease in OD= (S-B)-(T-C)

SOD activity = Decrease in OD X 2/S-B

SOD activity/mg of protein = SOD activity X 10/Total protein (mg/100µL) U/mg protein

SOD being the first line of defense against oxygen toxicity, higher SOD isoenzyme activity was expected. Previous research revealed, SOD protect cells from exogenous as well as endogenous reactive oxygen radicals. Hence all aerobic organisms have been observed to produce extracellular and intracellular SOD. In the present study, both secretory and cellular SOD isoenzyme activity was analyzed at room temperature 28±4°C. Interestingly, *Aspergillus Sp* and *Cladosporium Sp* have showed almost equal extracellular SOD activity. Whereas, the intracellular SOD isoenzyme activity is higher in *Cladosporium Sp* compared to *Aspergillus Sp*. Indicating that, *Cladosporium Sp* has a higher potential of super oxide scavenging activity (Fig. 1). However highest intracellular SOD isoenzyme activity was observed and explained in the literature by S. Tosi et al., 2010. These values are promising, with the *Aspergillus Sp* cultivated at 25°C was found to be 4.22U/mg protein and *Cladosporium herbarum* has showed 3.79 U/mg protein [19]. Improved extraction procedures and purification techniques would reveal the actual potency of reactive oxygen scavenging activities in both the test organisms.

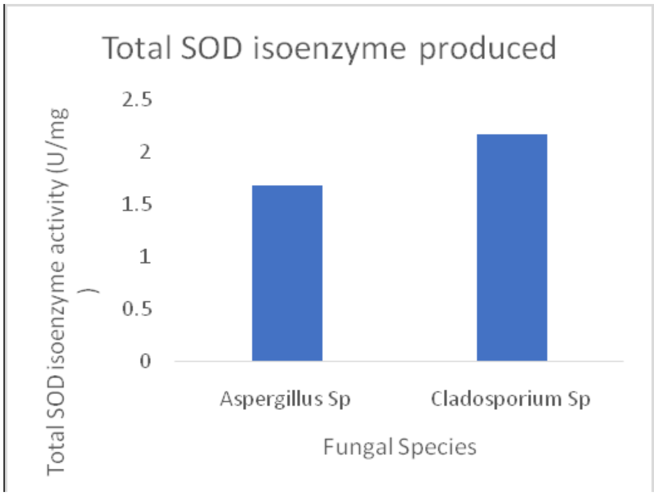

Fig. 1: SOD isoenzyme activity of *Aspergillus Sp.* and *Cladosporium Sp.*

3.3. Estimation of catalase activity

Total CAT activity was determined spectrophotometrically by following the decline in absorbance with H_2O_2 $(E = 36\ M^{-1}\ cm^{-1})$ catabolized, according to the method of Beers and Sizer (1952) at 240 nm. Enzyme activity (Unit) is defined as the amount of enzyme that oxidized 1µmol of substrate/min. Catalase activity is calculated using the below formula:

Enzyme activity (Units/L) = (ΔAbs × Total assay volume)/(Δt x ε x l x Enzyme sample volume). Where, ΔAbs is the change in absorbance, ε (extinction coefficient) for catalase is 39.4 mM-1 Cm-1. l is cuvette diameter, it is 1 cm.

Catalase activity of *Aspergillus Sp.*and *Cladosporium Sp.* crude cellular extract is summarized in [Table 3]. Catalase being an intracellular enzyme, the cellular extract was analyzed for catalase activity. *Aspergillus Sp.* and *Cladosporium Sp.* have shown considerably good amount of catalase activity in the crude extract. Results indicated,*Cladosporium Sp.* could produce slightly higher amounts of Catalase compared to *Aspergillus Sp.* against H_2O_2 toxicity (Fig. 2). Purified cellular extract might show significantly higher Catalase activity. However, highest Catalase activity was observed and explained in the literature by S. Tosi et al., 2010. These values are promising, with the *Aspergillus Sp.* cultivated at 25°C was found to be 19.07 U/mg protein and *Cladosporium herbarum* has showed 12.65 U/mg protein at 25°C [19]. These findings suggest the improvement of cell disruption protocol and optimizing the growth temperature.

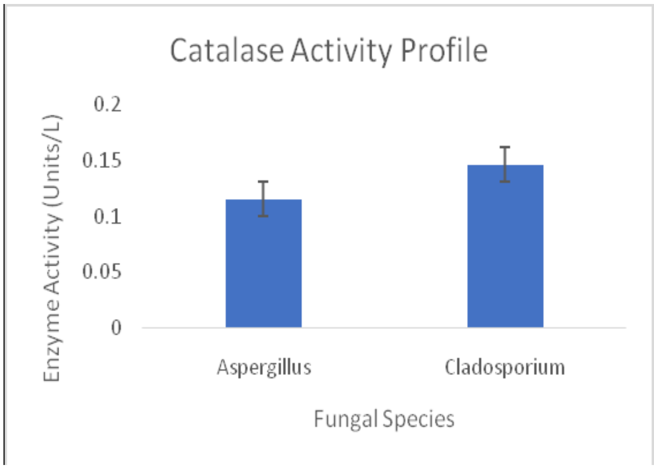

Fig. 2: Catalase activity in cellular extracts on *Aspergillus Sp.* and *Cladosporium Sp.*

Table 2: SOD isoenzyme activity profile of *Aspergillus Sp.* and *Cladosporium Sp.* crude extracts

Sl. No	Sample	Intracellular SOD activity (U/mg protein)	Extra cellular SOD activity (U/mg protein)	Total SOD isoenzyme activity (U/mg protein)
1	*Aspergillus Sp.*	1.33	0.350mg/ml	1.68mg/ml
2	*Cladosporium Sp.*	1.38	0.792 mg/ml	2.167mg/ml

Table 3: Catalase activity in cellular extracts on *Aspergillus Sp.* and *Cladosporium Sp.*

Sl. NO	Sample	Catalase activity (U/mg protein)	Time interval 20 S/3 min
1	*Aspergillus Sp.*	0.115	20sec
2	*Cladosporium Sp.*	0.146	20sec

3.4. Estimation of peroxidase activity

Extracellular Mn peroxidase was analyzed spectrophotometrically at 240 nm. Mn peroxidase is an extracellular enzyme. Crude culture filtrate is analyzed for Mn peroxidase activity in *Aspergillus Sp.* and *Cladosporium Sp.* Peroxidase activity was determined using the below formula:

Enzyme activity (Units/L)=(ΔAbs×Total assay volume)

/(Δt x ε x l x Enzyme sample volume).

Where, ΔAbs is the change in absorbance, ε (extinction coefficient) for peroxidase is 26.6mM^{-1} cm^{-1}, l is cuvette diameter, it is 1 cm.

Extracellular Peroxidase activities for both the samples are summarized in table 4. Peroxidase is a large family of isoenzyme. It includes both intracellular and extra cellular, we tried to estimate extracellular peroxidase activity. Results have indicated that, both the test organisms produce extracellular peroxidase isoenzyme. The enzyme activity is considerably low in the test fungal species. However, extracellular peroxidase activity is higher in *Cladosporium Sp.* compared to *Aspergillus Sp.* (Fig.3). With further purification processes and improved assay protocols, we would get improved extracellular peroxidase activity. It has been reported previously by Tamara vares et al., 1995, in *Phlebia radiata*l ignolytic filamentous fungi, an increased extracellular Mn peroxidase activity 0.068 U/mg protein at pH 5.08 [20]. It is observed that pH of the culture media greatly influences extracellular Peroxidase activity. With optimized culture conditions and modified assay methods, we would get higher amounts of extracellular peroxidase activity.

Table 4: Extracellular peroxidase activity in *Aspergillus Sp.* and *Cladosporium Sp.*

Sl. No	Sample	Peroxidase activity (Units/L)	Time of exposure	Total time
1	*Aspergillus Sp.*	0.00097	1 minutes	3 minutes
2	*Cladosporium Sp.*	0.00150	1 minutes	3 minutes

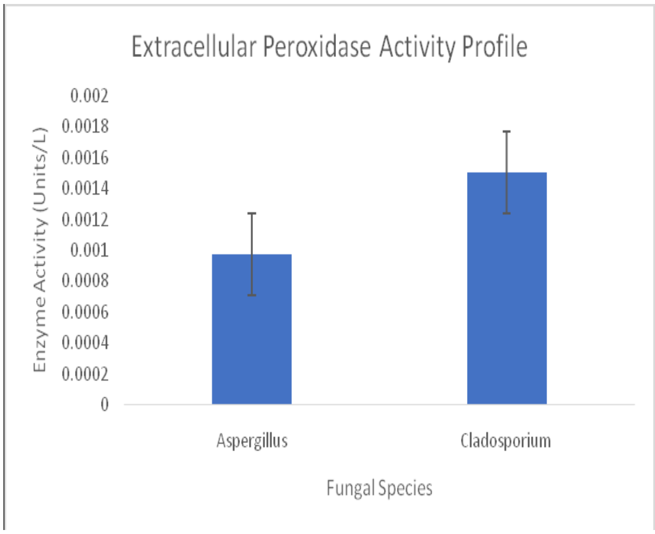

Fig. 3: Extracellular peroxidase activity in *Aspergillus Sp.* and *Cladosporium Sp.*

Previous research reveals that many cultural conditions such as type of carbon source, type of nitrogen source, dissolved oxygen conditions, mutations, pH, temperature, and other stresses increases the synthesis of SOD, CAT and PRX. We anticipate optimizing culture conditions and extraction protocols to enhance the production, purity, and efficacy of these anti-oxidative enzymes.

It is possible to make use of these fungi for pharmaceutical grade (SOD, CAT) and industrial grade (Peroxidase) enzymes with improved cultural conditions and strain improvement too. As already many fungal species are being used extensively in the industrial production of many biological substances, antioxidative enzyme production would greatly benefit the detoxification therapeutic medicines.

4. CONCLUSION

The present study clearly demonstrated the antioxidant enzymatic defense in *Aspergillus Sp.* and *Cladosporium Sp.* Both intracellular and extracellular enzyme activity was detected using UV visible spectroscopy. According to the results obtained, *Cladosporium Sp.* is showing good amount of Catalase and Peroxidase activity when compared to *Aspergillus Sp*. Spectrophotometric analyses revealed, both *Cladosporium Sp.* and *Aspergillus Sp.* are showing almost equal amount of extracellular and intracellular SOD activity. It is possible to produce increased amounts of these antioxidative enzymes in fungal species.

5. ACKNOWLEDGEMENT

The authors are thankful to Dr. Suba Manuel, Head of Life Science Department of Mount Carmel College, for providing all the necessary requirements and constant encouragement to complete this project.

Conflict of interest

The authors declare no conflict of interest.

6. REFERENCES

1. Nethravathy V, Anisa Athhar, Mrinalini Devi UG. *Asian Jr. of Microbiol. Biotech. Env. Sc.*, 2018; **20**:230-236.
2. Nethravathy V, Anisa Akhthar, Jayaveera KN. *J. Pharm. Chem.,* 2016; **10**:25-30.

3. Baba KY. *Inflammation Research.*, 2006; **55**:359-363.
4. Cadenas AB. *FEBS Letters*, 2012; **54(3)**:311-314.
5. Nethravathy V, Anisa Akhthar, Jayaveera KN. *J.Pharm. Chem.,* 2013; **7**:22-29.
6. Kirkman HN, Gaetani GF. *Proc. Natl. Acad. Sci. USA*, 1984; **10**:4343-4347.
7. Mondal P, Ray M, Kar M, Sabat SC. *Biotechnology Letters*, 2008; **30**:563-568.
8. Okohab AO. *Biotechnology Reports*, 2017: **48**:12-17.
9. Isabel A, Abreu D. *Biochimica et Biophysica Acta*, 2010; **18**:263-274.
10. Hadwan MH. *BMC Biochem.*, 2018; **19**:173-182.
11. Regalado C, Garcia-Almendarez BE, Duarte - Vazquez MA. *Phytochemistry Reviews*, 2004; **3**:243-256.
12. Magdalena Klimek-Ochab M. *Folia Microbiologica,* 2011; **56**:469-475.
13. Beauchamp C, Fridovich I. *J. Biol. Chem.*, 1970; **24**:4641.
14. G Loschen AA. *FEBS Lett*, 1974; **15**:68-72.
15. Nagesh Babu R, Devaraj VR. *Australian Journal of Crop Science,* 2008; **2**:40-48.
16. Pashova ST. *Antarctica. Polar Biol.*, 2010; **18**:1227-1237.
17. Wariishi H, Valli K, Gold MH. *J Biol Chem.,* 1992; **267**:23688-23695.
18. Preety Vatsyayan A, Christian Iffelsberger A,Carmen C, Mayorga-Martinezb, Frank-Michael Matysik. *Anal. Methods,* 2016; **8**:6847-6855.
19. Tosi S, Kostadinova N, KrumovaE,Pashova S,Dishliiska V, Spassova B, et al. *Polar Biol.*, 2010; **33**:1227-1237.
20. Tamara Vares MK. *Applied and Environmental Microbiology*, 1995; **15**:3515-3520.