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Research Article

IN VITRO EVALUATION OF PARTIALLY PURIFIED ANTIOXIDANT ENZYMES FROM LICHEN *LEPTOGIUM PAPILLOSUM*

JEYA PRAKASH SUNDARARAJ, AYYAPPADASAN GANESAN*, DEEPAK KUMAR PURUSOTHAMAN, PONMURUGAN PONNUSAMY

Department of Biotechnology, K.S. Rangasamy College of Technology, Tiruchengode - 637 215, Namakkal, Tamil Nadu, India.
Email: ayyappadasan07@gmail.com

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ABSTRACT

Objective: In this study, the enzyme activity of the partially purified six different antioxidant enzymes (AEs) of *Leptogium papillosum* such as superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), polyphenol oxidase (PPO), glutathione transferase (GST), and glutathione peroxidase (GPx) was carried out.

Methods: Ammonium sulfate precipitation, dialysis, and DEAE-cellulose column chromatography were the three different methods performed to purify the six different AEs from *L. papillosum*. The antioxidant enzyme activity of the purified extracts was determined *in vitro* by the following standard procedure of SOD, CAT, POD, PPO, GST, and GPx.

Results and Conclusion: The results revealed that there was a significant increase ($p<0.001$) in the specific activity of purified fractions of all the enzymes with a corresponding increase in the purification fold. The comparable activity of PPO and GST were determined using cluster analysis using short linkage distance. Principal component analysis indicated that $SOD_{DEAE\text{-}cellulose}$ contributed primarily to the total variation in the AEs among all the other fractions with the specific activity of 21.70 U/mg by attaining the purification fold of 5.91. Thus, our findings suggested that the purified AEs of *L. papillosum* possess potent antioxidant defense machinery by scavenging free radical population. Moreover, SOD was played a major role of capturing free radical by having highest enzyme activity followed by GST and CAT.

Keywords: Antioxidant enzymes, Cluster analysis, DEAE-cellulose column chromatography, Lichen, *Leptogium papillosum*, Principal component analysis.

INTRODUCTION

Free radicals were highly reactive atom having one or more unpaired electron produced from the oxidation reaction that takes place in the transfer of electrons capable of attacking the stable biomolecules. These free radicals initiate cascades of events which ultimately lead to cellular damage [1]. However, physiologically these free radical preserve homeostatic action by signal transduction process [2]. The formation of these highly reactive free radical occurs, which has a tendency to break the stable bonds present in the molecules by the pair of electrons [3]. Oxidative stress occurs due to the alteration in the antioxidant level, which leads to several disorders such as neurodegenerative diseases, cancer, diabetes mellitus, coronary heart ailments, and aging process [4,5]. The potent role of many antioxidant compounds possesses anti-cancerous, anti-inflammatory, anti-carcinogenic, anti-bacterial, or antiviral activities [6-9].

The sequential event of metabolic processes was carried out with the aid of enzymes. Some of antioxidant enzymes (AEs) systems catalyze reaction to neutralize the free radicals and reactive oxygen species (ROS) which includes superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), glutathione transferase (GST), and ascorbate oxidase [10]. These form the body's endogenous defense mechanism to protect against free radical-induced oxidative damage. In our system of natural antioxidants are present such as vitamins, which balance the redox reaction in the biological molecule [11]. Today's and tomorrow's pharma industry depends on the naturally derived pharmaceutical products for the development of active molecules against chronic diseases.

Lichens were symbiotic partners of photobiont (algal partner) and mycobiont (fungal partner) which exhibits a wide range of biological activities. Lichens occur in different forms such as crustose, foliose, and fruticose. Traditionally, lichens have been used to treat various ailments, viz., diabetes, bronchitis, leprosy, tuberculosis, dyspepsia, etc. The metabolites produced by the lichen possess antimicrobial, antioxidant, anthelmintic, anticaries, cytotoxic, insecticidal, larvicidal, wormicidal, etc. [12-17]. The objectives of this study were aimed to evaluate AE of different purified extracts of *Leptogium papillosum.* Because it grows on the hills, rocks and also in unsupported climatic conditions. These foliose lichens belong to Collemataceae family according to the study done by Nayaka and Upreti [18]. Thus, the study was planned to evaluate the various defense enzymes which support the lichen from different environmental factors such as temperature, pH, stress, pollution, and free radicals.

METHODS

Collection and identification of lichens

The lichen sample was obtained from the Yercaud (Shevaroy hills), Tamil Nadu state, India. The collected lichen was identified based on its anatomical, morphological, and chemical tests. Color tests were performed in the cortex and the medulla region using three reagents, namely, 10% potassium hydroxide (K), Steiner's stable paraphenylenediamine solution (P), and calcium hypochlorite solution (C). Species confirmation was done by performing thin layer chromatography to identify the presence of appropriate secondary metabolites [19]. Then, the voucher specimen has been kept on the KSTCT/BT herbarium, Tiruchengode.

Preparation and purification of the enzyme extract

The lichen thalli were dried and grounded with a mortar and pestle using liquid nitrogen containing 100 mM sodium phosphate buffer (pH 7.4), 0.05% polyvinylpolypyrrolidone for extraction. The

samples were centrifuged at 10,000 g at 4°C for 15 minutes, and the supernatant was collected and kept at 4°C for further use [20]. The crude extract was subjected to ammonium sulfate precipitation, which was done in an ice bath using finely powdered 40% ammonium sulfate. The powder was added slowly to the extract by continuous stirring for complete solubilization and it was kept at 4°C for overnight. Then, the sample was centrifuged at 15 000 g for 20 minutes. After discarding the supernatant, the pellet (precipitate) was suspended in 100 mM sodium phosphate buffer (pH 7.4). Activation of dialysis membrane by adding 2% sodium carbonate. Followed by washed it with double distilled water. Then, the precipitate was dialyzed in the magnetic stirring condition using a dialysis membrane for 24 hrs at 4°C by changing the buffer for every 1 hr. After that, the sample was stored at 4°C for further use. The dialysate was next subjected to ion exchange chromatography using DEAE-Cellulose column (20×1 cm). The dialyzed fraction was loaded onto the DEAE - cellulose column at 4°C previously equilibrated with 0.1 M of sodium phosphate buffer (pH 7.4). Initially, the column was washed with equilibrated buffer and the proteins bound to the column is to be eluted out with a linear salt gradient of 0-100mM NaCl (in 100mM sodium phosphate buffer) at a flow rate of 1mL/minute. 2.5 ml of the fraction was collected using the fraction collector and aliquots were assayed for different antioxidant enzyme activity as well as protein concentration. The fractions with the highest enzyme activity and also protein concentration taken out for further assays.

Protein concentration

Protein concentration of the above three purified extracts was determined by Lowry *et al*. method [21] using bovine serum albumin as a standard protein.

Enzyme assays

SOD [EC 1.15.1.1] assay was performed according to the method of Das *et al.* [22] using 20 mM L-Methionine, 1% (v/v) Triton X-100, 10 mM Hydroxylamine hydrochloride and 50 mM EDTA and incubated at 30°C for 5 minutes. 50 µM of riboflavin was added, and the tubes were exposed to 200 W-Philips fluorescent lamps for 10 minutes. After the exposure, add 1 ml of Greiss reagent (a mixture of equal volume of 1% sulfanilamide in 5% phosphoric acid) and the absorbance was read at 543 nm. One unit of enzyme activity was measured as the amount of SOD capable of inhibiting 50% of nitrite formation under the assay conditions.

CAT (EC 1.11.1.6) activity was assayed according to the method of Sinha [23] using 0.01 M phosphate buffer (pH 7.0) along with 0.5 ml of 0.2 M H_2O_2. The reaction was terminated by adding 2 ml of the acid reagent (1:3 volume ratio of 5% potassium dichromate and glacial acetic acid). Read the absorbance at 610 nm by incubate all the tubes at 50°C for 10 minutes. The activity of the enzyme was calculated based on consumption of H_2O_2/min/mg of protein.

POD (EC 1.11.1.7) assay was done by 0.05 M of phosphate buffered pyrogallol and 1% H_2O_2 [24]. Change in the absorbance was measured at 430 nm for every 30 seconds up to 2 minutes. The POD activity was reflected in terms of mole of pyrogallol oxidized/min.

Polyphenol oxidase (PPO) (EC 1.10.3.1) activity was assayed in accordance with the procedure of Sadasivam and Manickam [25]. 0.1 ml of extract was mixed with 3.0 ml of distilled water and 1.0 ml of catechol solution (0.4 mg/ml) reactants were quickly mixed. The enzyme activity was measured as a change in absorbance/min at 490 nm.

GST (EC 2.5.1.18) activity using 2, 4 dichloronitrobenzene as substrates was assayed spectrophotometrically by Habig *et al.* [26]. 3 ml reaction mix consists of 0.1 M phosphate buffer (pH 6.5), 1 mM GSH and 1 mM of chlorodinitrobenzene and add 100 µl of enzyme extract. Change in the absorbance at 340 nm was read against blank containing all reagents except the enzyme. Specific activity was expressed as µmol conjugate formed/min/mg protein.

Glutathione peroxidase (GPx) (EC 1.11.1.9) activity was assayed according to the procedure of Rotruck *et al*. [27]. The reaction mix consisting of 0.4 M sodium phosphate buffer (pH 7.0), 10 mM sodium azide, 4 mM reduced glutathione, 2.5 mM H_2O_2 and 0.1 ml of enzyme. The reaction was terminated by adding 10% TCA and after centrifugation, the addition of 2 ml of the supernatant to 0.1 M phosphate buffer and 1 ml of DTNB reagent (0.04% DTNB in 1% sodium citrate). The absorbance was read at 412 nm, and the enzyme activity is expressed in terms of µg of glutathione utilized/min/mg protein.

Statistical analysis

Analysis of mean±standard deviation was determined using Graph Pad Prism 5.0 Software. Cluster analysis was performed to determine the close relationship between the different components using STATISTICA 10.0 Software. Principal component analysis (PCA) was performed using PAST 3.06 and ORIGINPRO 9.0, used to evaluate the important component and identify the relationship between the variables.

RESULTS AND DISCUSSION

Extraction and purification of AE from *L. papillosum* were performed. The total protein content, specific activities, and purification fold of diverse AEs in four different purified fractionation were summarized in Table 1.

In this work, partial purification and activities of six different AEs such as SOD, CAT, POD, PPO, GST, and GPx were studied. The total protein content of the homogenate was 22.45 mg having the specific activities of AEs in order were found to be 3.67, 2.26, 1.45, 1.09, 0.92, and 0.67 units/mg, respectively. In this context, it was reported that the activities of SOD, CAT, POD, PPO, and GST were 4.645; 0.069; 0.137; 0.017; 0.454, 3.418; 0.113; 0.829; 0.019; 0.333, and 4.631; 0.042; 0.139; 0.003; 0.169 units/mg, respectively, for three different lichen species (*Sticta weigelii, Dermatocarpon vellereum* and *Heterodermia boryi*) [28]. Moreover, studies on lichen *L. saturninum* stated the POD activity at 0.5 units/mg of the crude extract [29]. In above mentioned two studies, our result had far better activity for all the six AEs of *L. papillosum.* It indicated that *L. papillosum* had more defense mechanism against the stress condition due to the morphological behavior and appearance.

Homogenate of *L. papillosum* undergone for partial purification phase using 40% ammonium sulfate precipitation and dialysis. At these stages, purification fold in six AEs increased with increased specific activity of an enzyme. For the precipitation step, the specific activity (U/mg) 4.34, 3.14, 2.23, 1.99, 1.24, 1.04 and purification fold 1.18, 1.39, 1.54, 1.83, 1.35, 1.56 for six AEs, respectively, with the yield % of around 27.26, 32.77, 35.63, 42.14, 31.27, 38.59. Correspondingly, the purification fold tends to increase slightly to 1.49, 1.81, 2.38, 2.33, 2.25 and 2.44 for the next fractionation process (i.e., dialysis) with the specific activity (U/mg) of 5.47, 4.09, 3.45, 2.53, 2.07 and 1.63, respectively, for six AEs. The specific activity of catalase for the dialysate fraction was to be 0.89 U/mg in *Agaricus bisporus* by attaining the purification fold of 3.1 [30] and it was better in *L. papillosum.*

Finally, dialyzed ammonium sulfate precipitate was loaded onto a DEAE - cellulose column and 25 fractions of the extract were collected by changing the concentration of NaCl as shown in Fig. 1. The total protein and the specific activity of enzymes were calculated for the collected fraction to determine which fraction had the maximum activity. SOD was the effective, intracellular, tumor-suppressor that catalyzes the dismutation of oxygenated free radicals (O_2^-) to less-reactive species. Fraction 10 showed the maximum enzyme activity of about 21.70 U/mg of protein with the fold-purification of about 5.91. It was reported that the SOD activity of *Zizyphus mauritiana* Lamk was 30.9 U/mg having the purification fold of 4.5 [31] which was in-line with our studies. Consecutively, a significant increase ($p<0.001$) in $CAT_{DEAE\text{-}Cellulose}$ enzyme activity occurs (17.73 U/mg and purification fold-7.85) at fraction number 7. In *A. bisporus,* the specific activity of $CAT_{DEAE\text{-}Cellulose}$ was 1.67 U/mg with 5.9 purification

Table 1: Purification steps of AEs (SOD, CAT, POD, PPO, GST and GPx) from *L. papillosum*

Type of fraction	Total protein (mg)	Specific activity (units/mg)	Total activity (units)	Yield (%)	Purification fold
SOD					
T1	22.45±0.012	3.67±0.155	82.21±0.812	100	1
T2	5.23±0.005	4.34±0.127	22.41±0.911	27.26	1.18
T3	1.35±0.002	5.47±0.163	7.33±0.614	8.92	1.49
T4	0.546±0.009	21.70±0.184	11.86±0.338	0.14	5.91
CAT					
T1	22.45±0.012	2.26±0.108	50.29±0.947	100	1
T2	5.23±0.005	3.14±0.093	16.48±0.833	32.77	1.39
T3	1.35±0.002	4.09±0.078	5.02±0.313	9.98	1.81
T4	0.546±0.009	17.73±0.095	9.72±0.345	0.19	7.85
POD					
T1	22.45±0.012	1.45±0.026	32.25±1.099	100	1
T2	5.23±0.005	2.23±0.119	11.49±0.458	35.63	1.54
T3	1.35±0.002	3.45±0.137	4.12±0.298	12.76	2.38
T4	0.546±0.009	14.89±0.107	8.48±0.526	0.26	10.27
PPO					
T1	22.45±0.012	1.09±0.083	24.68±0.736	100	1
T2	5.23±0.005	1.99±0.098	10.40±0.377	42.14	1.83
T3	1.35±0.002	2.53±0.058	3.44±0.319	13.94	2.33
T4	0.546±0.009	11.63±0.077	6.71±0.626	0.27	10.67
GST					
T1	22.45±0.012	0.92±0.129	20.72±0.518	100	1
T2	5.23±0.005	1.24±0.088	6.48±0.334	31.27	1.35
T3	1.35±0.002	2.07±0.068	2.73±0.329	13.18	2.25
T4	0.546±0.009	10.13±0.052	5.69±0.323	0.27	11.01
GPx					
T1	22.45±0.012	0.67±0.091	15.73±0.391	100	1
T2	5.23±0.005	1.04±0.067	6.07±0.406	38.59	1.56
T3	1.35±0.002	1.63±0.109	2.27±0.316	14.43	2.44
T4	0.546±0.009	6.97±0.048	3.78±0.301	0.24	10.40

T1=Homogenate; T2=40% $(NH_4)_2SO_4$ precipitation; T3=Dialysate; T4=DEAE - Cellulose column chromatography. AEs: Antioxidant enzymes, SOD: Superoxide dismutase, CAT: Catalase, POD: Peroxidase, PPO: Polyphenol oxidase, GST: Glutathione transferase, GPx: Glutathione peroxidase, *L. papillosum: Leptogium papillosum*

fold [31]. Since SOD and CAT are interrelated one, they both perform consistently by abolishing the free radical (O_2^-) into H_2O_2 and into water naturally.

The role played by POD was similar to catalase, in which removal of H_2O_2 and the oxidation of organic substrates occur [32,33]. But compare to CAT, the enzymatic activity of POD was somewhat lower of about 14.89 U/mg. Scavenging of H_2O_2 in chloroplast occurs by one of the major AE systems is PPO [34], attained the maximum specific activity at fraction number 10 of 11.63 U/mg and the purification fold of 10.27. The study reported that the specific activity of POD in the DEAE - cellulose purified *Lactuca sativa* was 2.90 U/mg [35]. By comparing this, our study shows the better POD activity.

The major antioxidant defense detoxifies active electrophilic carcinogens was the glutathione S-transferase [36] and GPx acts as a free radical scavenger by stabilizing the cell membrane [37]. Compared to all AEs there was a higher amount of purity of 11.01 purification fold conquered at fraction 13 with the activity of 10.13 U/mg for GST. During the purification of *Mucor mucedo*, the specific activity of $GST_{DEAE\text{-}Cellulose}$ was 13.5 U/mg with the purity of 8.04 [38], and there was a significant increase ($p<0.001$) in the GPx activity of about 6.97 U/mg. By analyzing the results of all the six AEs, SOD showed maximum activity for all kinds of purification. It directly says that the lichen *L. papillosum* had the better defense mechanism to overcome the stress conditions. It might be due to their morphological appearance.

The hierarchical cluster analysis done for *L. papillosum* on the different kinds of purification process based on the result obtained from the specific activity of the antioxidant enzyme system. This cluster analysis allows grouping of different AEs based on the intimate relationship of activity of an enzyme. The dendogram for the four purified fractions of six AEs were illustrated in Fig. 2 shows that SOD, CAT, POD, PPO, GST, and GPx are linked by branches. In this smaller the linkage distance, greater is the similarities in the AEs. PPO and GST were greatly clustered with short linkage distance shows comparable in its activity. Since the linkage distance for CAT, POD was to be high, but in some way show similar activity between them. Since SOD, GPx were linked at a greater distance, the results of both were considered as an exception.

Table 2: Proportion of variance by PCA and associated with four PC axes of characters associated with partial purification of six different antioxidant enzymes

PCA Parameters	PCA-1	PCA-2	PCA-3	PCA-4
Eigen values	3.93	0.50	0.01	0.004
Proportion of variance (%)	98.39	1.23	0.30	0.09
Cumulative proportion of variance (%)	98.39	99.62	99.91	100.00
Eigen vectors				
SOD	3.224	0.226	0.0301	−0.011
CAT	1.259	−0.132	−0.047	0.042
POD	0.042	−0.321	0.0645	−0.075
PPO	−0.841	−0.048	−0.180	0.023
GST	−1.522	0.008	0.139	0.078
GPx	−2.162	0.267	−0.008	−0.058

SOD: Superoxide dismutase, CAT: Catalase, POD: Peroxidase, PPO: Polyphenol oxidase, GST: Glutathione transferase, GPx: Glutathione peroxidase, PCA: Principal component analysis

Principal components analysis (PCA) was performed to know how six AEs such as SOD, CAT, POD, PPO, GST, and GPx contribute to the four different purified fractions of *L. papillosum* and also the component scores of the first and second component were depicted in Fig. 3. According to Table 2, 98.39%, 1.23%, 0.30%, and 0.09% were the

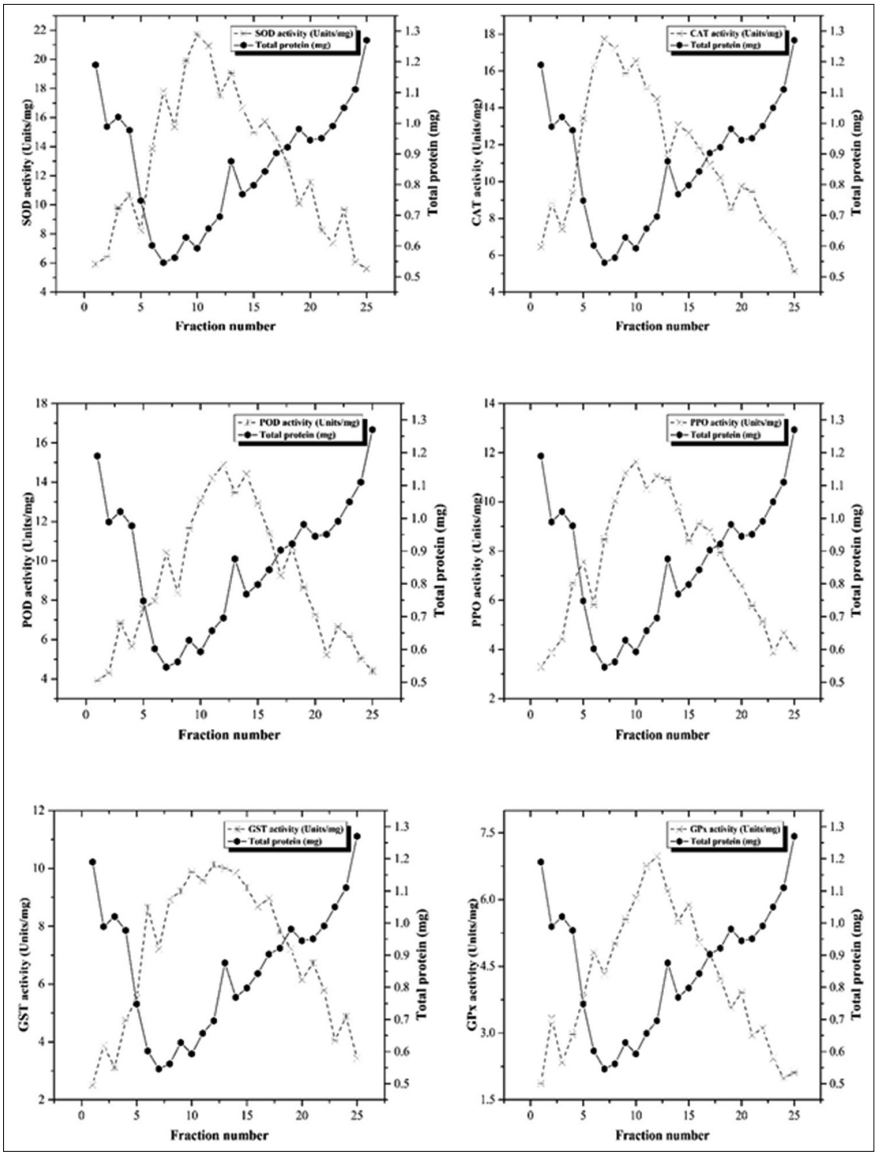

Fig. 1: Elution profile of DEAE- cellulose chromatography for antioxidant enzymes (superoxide dismutase, catalase, peroxidase, polyphenol oxidase, glutathione transferase, and glutathione peroxidase)

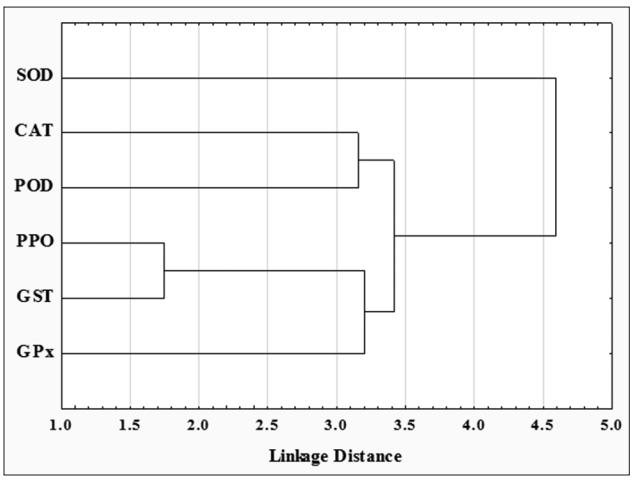

Fig. 2: Hierarchical cluster analysis of *Leptogium papillosum* based on antioxidant enzymes (superoxide dismutase, catalase, peroxidase, polyphenol oxidase, glutathione transferase, and glutathione peroxidase) in different purified fractions

proportion of variance explained in the first four components. The result revealed that DEAE - cellulose fraction of *L. papillosum* was highly loaded on the PC1 axis which made a strong influence toward SOD, CAT, POD, PPO, GST as well as GPx. Among these SOD showed a major contribution to the activity of all kinds of purification. Remaining three fractions were loaded on PC2 axis. Factor analysis revealed that the number of principal components involved in the enzymes and SOD was mainly contributing to the activity of all fractions of *L. papillosum.*

CONCLUSION

This study was concluded that lichen species had the considerable level of defense as well as for stress enzyme to protect themselves for several factors. It was identified by the lichen *L. papillosum* with six different enzymes. Among that SOD, CAT, and GST have more influence toward the enzyme activity. Three different purification processes were studied and it was successful for the separation of lichen-based enzyme. Further these enzymes have to be processed for various industrial application for the development of purified enzyme powder. Moreover, characterization of above enzymes has to be studied to understand the kinetics of enzymes.

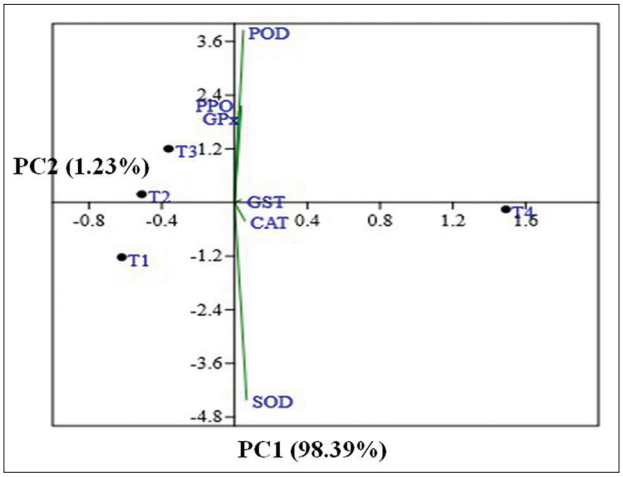

Fig. 3: Analysis of principal components in antioxidant enzyme activity against different purified fractions

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