KAVAKA 56 : 98-104 (2021) 10.36460/Kavaka/56/2021/98-104

Isolation and quantification of biotechnologically important enzymes from *Podaxis pistillaris* and *Termitomyces heimii* from Andhra Pradesh

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(Submitted on April 16, 2021; Accepted on June 11, 2021)

ABSTRACT

In the present study crude extracts of two wild mushrooms, *Podaxis pistillaris* (L.) Fr. and *Termitomyces heimii* Natarajan in different buffers and their precipitates were used for the assessment of activity of three biotechnologically important enzymes: tyrosinase, laccase, and superoxide dismutase (SOD). Study of enzyme activity in gel revealed the presence of all three enzymes in *Podaxis pistillaris* with prominent bands whereas *Termitomyces heimii* showed activity only for tyrosinase and laccase. The spectrophotometric analysis of enzymes revealed that the crude extract of *Podaxis pistillaris* possesses significant amount of tyrosinase, laccase, and SOD on a dry weight basis as compared to *Termitomyces heimii*. Maximum activity was observed for tyrosinase followed by SOD and laccase. Furthermore, buffers of different pH were seen to play a crucial role in the isolation of these enzymes. The activity of tyrosinase and laccase enzyme was found maximum in the extracts isolated in the buffer of pH 7.0 or 6.5 whereas SOD showed enhanced activity in the extracts isolated in the buffer of pH 5.0 or 6.5.

Keywords : *Podaxis pistillaris, Termitomyces heimii,* tyrosinase, laccase, superoxide dismutase

INTRODUCTION

Wild edible mushrooms are an integral component of the forest ecosystem. Being saprophytic, mushrooms play an important role in the decomposition of organic molecules. By virtue of being rich sources of diverse groups of enzymes and acids they breakdown natural polymers like keratin, chitin, lignin, pectin, cellulose, and hemicellulose (King *et al.,* 1997; Lamar and white, 2001). Enzymes derived from mushrooms are considered as green biocatalysts and have found use in the industrial biotechnological sector. Hence, identifying wild mushroom species and quantifying their enzymes would raise the scope of mushrooms as an alternative source of biotechnologically important enzymes.

Immobilized laccase and tyrosinase has been used for various industrial and biotechnological applications in bioremediation, wastewater treatment, detoxification of industrial effluents, must, and wine stabilization (Duran *et al.,* 2002). Laccases comparatively have wider applications in the decolorization of dyes, pulp and paper industry, xenobiotic degradation, food industry, textile industry, organic synthesis, pharmaceutical industry and nanobiotechnology (Kunamneni *et al.,* 2008; Virk *et al.,* 2012; Viswanath *et al.,* 2014). Superoxide dismutase (SOD) is the antioxidant enzyme that catalyzes the conversion of superoxide anion to oxygen and hydrogen peroxide, playing a key role in the cellular antioxidant defense system. Due to its rapid scavenging ability of free radicals, it is being extensively researched and used in various therapeutic applications as anti-inflammatory, antitumor, cancer radiation therapy and antisenility (Prasad *et al.,* 2015; Luisa *et al.,* 2002; Morikawa and Morikawa, 1996). In the present study, three important enzymes namely, tyrosinase, laccase, and superoxide dismutase (SOD) were isolated and quantified from two wild edible mushrooms, *Podaxis pistillaris* and *Termitomyces heimii* from Andhra Pradesh, India.

MATERIALS AND METHODS

Samples: Two wild edible mushrooms, *Podaxis pistillaris* (L.) Fr. and *Termitomyces heimii* Natarajan (**Fig.1**) were collected from Anantapur, Andhra Pradesh, India. These were taxonomically identified and authenticated based on their morpho-anatomical and ITS sequenced based molecular characteristics. The fruiting bodies of mushrooms were cleaned, sliced into thin pieces, and were air-dried in an oven at 40°C. Dried mushrooms were further reduced to fine homogeneous powder and used for analysis.

Fig. 1: A) *Podaxis pistillaris* and B) *Termitomyces heimii*

Enzyme isolation: Enzyme in crude form was extracted from mushroom samples according to the modified method of Alici and Arabaci (2016). Dried mushroom samples were soaked overnight in three different extraction buffers of pH 5.0 (100mM sodium acetate), pH 6.5 (100mM sodium phosphate) and pH 7.0 (100mM sodium phosphate) each containing 1mM ascorbic acid and 0.5% polyvinylpyrrolidone (PVP). Samples were homogenized using mortar and pestle until no fibrous residue could be seen. The obtained homogenate was centrifuged at 12000 rpm for 15min at 4°C. The supernatant was collected and the residue was re-extracted and the pooled supernatant was filtered and was referred to as crude enzyme extract and stored at 4°C.

Enzyme precipitation: Salting out of protein/enzyme was done using ammonium sulfate precipitation where ammonium sulfate was added incrementally to mushroom extract to precipitate enzymes. Different percentage solution [10%-70% (w/v)] of ammonium sulfate was prepared. Ammonium sulfate percentage solution and crude mushroom

extract were taken in an equal ratio (1:1 v/v) and centrifuged at 5000 rpm at room temperature for 20min. Obtained protein pellets were dissolved in 50mM Tris-HCl buffer (pH 8.0) and were stored at 4°C.

In-gel activity assay: The presence of tyrosinase, laccase, and SOD enzymes in crude extracts and in pellets of both the mushrooms was assessed by in-gel activity based on the principle of enzyme visualization using standard enzyme solution (U/µl). Native polyacrylamide gel electrophoresis was performed using the Biorad's Mini-PROTEAN Tetra Cell system with 8% (for tyrosinase) and 12% (for laccase and superoxide dismutase) resolving gel. Power of constant voltage was applied and the samples were run through stacking gel in electrophoresis buffer at 81V at 4°C for ~1hour. After the dye front had reached the resolving gel, the constant voltage was increased to 99V and the gel was run until the bromophenol blue (BPB) reaches the bottom of the resolving gel (~2hrs). After electrophoresis, gels were subjected to activity staining for the presence of enzymes.

Tyrosinase activity: The rapid detection of catecholase activity of tyrosinase on slab gels, after non-denaturing electrophoresis has been carried out using the modified method of Rescigno *et al.* (1997) wherein the gel was incubated in ddH_2O for 3min, then 10ml of 25mM solution of 4-tert-butyl-catechol (tBC) in 10mM acetic acid was added to the gel. After the appearance of the canary yellow spots corresponding to the conversion of tBC to tBQ, 5ml of 25mM solution of coupling agent, 4-amino-N,N-diethylaniline-sulphate (ADA) in 10mM HCl was added to the gel. Tyrosinase enzyme activity appeared as a prominent blue-colored band.

Laccase assay: The laccase activity gel assay was carried out according to the method of Srinivasan *et al.* (1995) based on oxidation ABTS in an acidic medium. After electrophoresis, the gel was fixed for 10min in a 50ml fixative solution containing 10% (vol/vol) acetic acid and 40% (vol/vol) methanol. The staining was done by soaking the gel in 50ml of 2.7mg/ml concentration of ABTS solution. Intense bluish-green color bands appeared within few minutes indicating the presence of laccase enzyme in the samples. Photographs were taken after the appearance of bluish-green color bands.

Superoxide dismutase (SOD) assay: The SOD activity gel assay was carried out according to the method of Weydert and Cullen (2010) based on the inhibition of the reduction of NBT by SOD. SOD native gel stain was prepared freshly by adding 40ml of ddH_2O, 80 mg NBT (2.43mM), 170µl of TEMED (28mM) and 8µl of stock riboflavin-5'-phosphate (0.14M (53mg/ml) in 50mM phosphate buffer (pH 7.8). The gel was placed in SOD native gel stain for 20 min on gel rocker in the dark. After incubation, the gel was gently rinsed twice with ddH_2O. Sufficient ddH_2O was added to cover the gel and placed under a fluorescent light for 15-20min. The gel began to turn purple and clear bands appeared gradually. The achromatic bands were intensified over the next 4-24 hrs, indicated the presence of SOD. The gels were then photographed.

Spectrophotometric assays: The quantification of enzymes was measured (unit of enzyme in per milligram of mushroom on a dry weight basis) only in the crude extracts using the spectroscopic method based on respective activity assay.

Tyrosinase activity: The diphenolase activity of tyrosinase enzyme in mushroom extracts was determined spectrophotometrically by measuring the oxidation of 3,4-dihydroxyphenylalanine (L-DOPA) according to Zhang and Flurkey (1999). The assay mixture contained 10mM sodium phosphate buffer pH 7.0, 0.3mM L-DOPA, and mushroom crude extracts (50-300µl). The mixture was incubated for 45min at room temperature (32±2°C). Tyrosinase activity was monitored by observing the increase in absorbance at 475nm. The unit of enzyme/µl of mushroom extracts was determined by the linear activity assay of standard tyrosinase enzyme of different units/µl.

Laccase activity: The activity of the laccase enzyme was determined using the modified method of Pardo and Camarero (2018) based on direct oxidation of syringaldehyde at 370nm to a yellow product, 2,6-dimethoxy p-benzo-quinone. The assay reaction the mixture contained 100mM sodium acetate buffer pH 5.0, 2mM syringaldehyde in 0.1M sodium acetate buffer (pH 5.0) and mushroom crude extracts (50-300 µl). The reaction mixture was incubated for one hour. Finally, the enzymatic oxidation of the substrate was measure at 370nm. The unit of enzyme/µl of mushroom extracts was determined by the linear activity assay of standard laccase enzyme from *Trametes versicolor* of different units/µl.

Superoxide dismutase activity: The modified method of Giannopolitis and Ries (1977) was used to determine the SOD activity in the crude extracts of mushroom samples. The reaction mixture was composed of 1.3µM riboflavin-5-phosphate, 13mM methionine, 6.5µM NBT (Nitro-tertrazolium Blue chloride), 0.05M sodium carbonate buffer (pH 10.1), and the different volumes (50µl - 300µl) of mushroom extracts. The mixtures were taken in glass tubes and illuminated under a fluorescent lights rack. Blanks were the identical solutions kept in dark. The enzymatic reaction was initiated by turning the light on and terminated by putting it off. After 15min of illumination, the absorbance was measure at 560nm. In the presence of the SOD enzyme, the reaction was inhibited and the amount of inhibition was used to quantitate the enzyme. Each extract was assayed thrice. The unit of enzyme/µl of mushroom extracts was determined by the inhibition activity assay of standard SOD enzyme from bovine erythrocytes of different units/µl.

Statistical analysis: All the experimental analyses and assays were performed in triplicate. The results were expressed as mean value ± standard deviation (S.D.). The statistical significance difference between the means was tested using a one-way analysis of variance (ANOVA). After ANOVA, multiple comparisons amongst the samples were also

performed using the Tukey's-test where every mean is compared with every other mean of the samples. Results represented in the form of graphs and line diagrams were prepared using GraphPad Prism 8 software.

RESULTS AND DISCUSSION

Enzyme isolation and precipitation: All three enzymes were extracted in crude form using three different buffers (0.1M sodium acetate pH 5.0, 0.1M sodium phosphate pH 6.5, and 0.1M sodium phosphate pH 7.0) from both mushrooms. Enzyme precipitation of each extract was carried out in different increment percentages of ammonium sulfate **[**10 to 70% (w/v)**]**. The precipitation in ammonium sulfate solutions resulted in the formation of pellets. Extracts from *Podaxis pistillaris* at pH 5.0 produced pellet only in 70% ammonium sulfate solution whereas extracts at pH 6.5 produced pellets in 40%, 50%, 60%, and 70% ammonium sulfate solutions as well respectively. The extracts at pH 7.0 produced pellets in 30%, 40%, 50%, 60% and 70%. However, the crude extracts of *Termitomyces heimii* showed precipitation in none of the percentage solutions of ammonium sulfate.

In-gel activity assay: Presence of all three enzymes in crude extracts and in the precipitates of both mushrooms were detected using respective in-gel activity after Native-PAGE.

Tyrosinase activity: The results of in-gel activity revealed that crude extracts of *Podaxis pistillaris* at pH 5.0, pH 6.5, and pH 7.0 of buffers showed distinct blue bands in the gels showing the presence of tyrosinase. The extracts of *Termitomyces heimii* from all the three buffers showed no prominent band but instead gave diffuse light blue color to the gel indicating the low amount of tyrosinase in the mushroom (**Fig.2**). The precipitates of *Podaxis pistillaris* extracts at pH 5.0 in 70% ammonium sulfate solution, at pH 6.5 in 40% and 50% ammonium sulfate solutions and at pH 7.0 in 30%, 40%, 50% ammonium sulfate solutions showed no in-gel activity whereas precipitates in 60% and 70% ammonium sulfate solutions at pH 6.5 and pH 7.0 showed distinct blue color activity bands in the gels as compared to the standard tyrosinase (**Fig.2**). Similar activities were also reported by Wichers *et al.* (1996) in fruiting bodies of *Agaricus bisporus* U1 and by Zaidi and Ali (2015) in *Agaricus bisporus* and *Pleurotus ostreatus*.

Laccase activity: Detection of laccase in the gel was visualized as intense bluish-green color bands. The results revealed that crude extracts at pH 5.0, pH 6.5, and pH 7.0 from *P. pistillaris* and *T. heimii* showed bluish-green color bands in the gels whereas no gel activity was detected in the precipitates of *P. pistillaris* extracts in 70% ammonium sulfate solution at pH 5.0, in 40%, 50%, 60%, 70% at pH 6.5 and in 30%, 40% at pH 7.0 whereas precipitates in 50%, 60% and 70% ammonium sulfate solutions at pH 7.0 showed bluish-green colored activity bands in the gel (**Fig.3**). The in-gel activity of purified laccase enzyme was also been reported in the culture supernatant of *Magnaporthe grisea* by Iyer and Chattoo (2003); in copper-induced *Ganoderma lucidum* MDU-7 under liquid fermentation condition by Kumar *et al.* (2015); in *Lentinula edodes* culture by Niku-Paavola *et al.* (1990); in the mycelial culture of *Flammulina velutipes* by Saito *et al.* (2012); in the culture of *Pleurotus* sp. by More *et*

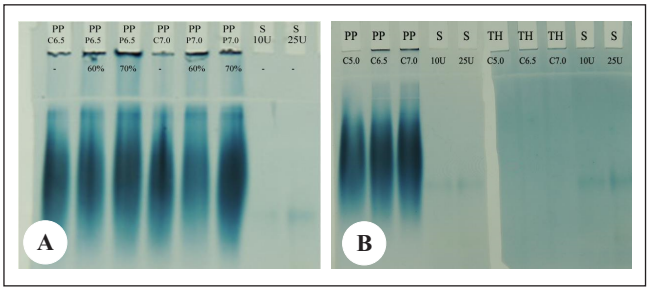

Fig. 2: In-gel activity of tyrosinase: A). Lane number 1, 2, 3, 4, 5, 6 from left to right showing prominent blue color bands for crude extract (C) of *Podaxis pistillaris* at pH 6.5, precipitates (P) of extracts at pH 6.5 in 60%, 70% ammonium sulfate solutions, crude extract (C) at pH 7.0 and precipitates (P) of extracts at pH 7.0 in 60%, 70% ammonium sulfate solutions. Lane no. 7 and 8 showing faint blue color activity bands standard (S) tyrosinase at 10U and 25U. B).Lane number 1, 2 and 3 from left to right showing distinct intense bands for crude extracts (C) of *Podaxis pistillaris* at pH 5.0, pH 6.5 and pH 7.0. Lane no. 4 and 5 showing faint blue color bands of tyrosinase standard (S) at 10U, and 25U. Lane no. 6, 7 and 8 showing diffuse color for the extracts of *Termitomyces heimii* at pH 5.0, pH 6.5 and pH 7.0. Lane no. 9 and 10 showing activity faint band of tyrosinase standard (S) at10U and 25U.

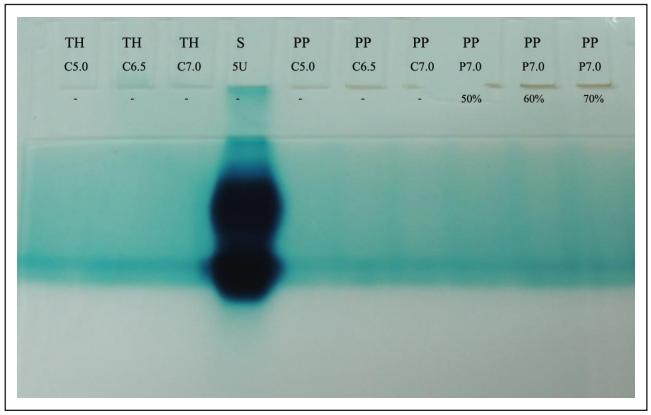

Fig. 3: In-gel activity of laccase: All the lanes in the gel showing visualization of bluish-green color as laccase activity. Lane number 1,2 and 3 from left to right are the crude extracts (C) of *Termitomyces heimii* at pH 5.0, pH 6.5 and pH 7.0, lane 4 is laccase standard (S) at 0.84U with a distinct intense band, lane number 5, 6 and 7 are crude extracts (C) of *Podaxis pistillaris* at pH 5.0, pH 6.5, pH 7.0 and lane no. 8, 9 and 10 are precipitates (P) of *Podaxis pistillaris* at pH 7.0 in 50%, 60% and 70% ammonium sulfate solutions.

al. (2011) and in the cultures of *Phanerochaete chrysosporium* by Srinivasan *et al.* (1995).

SOD activity: The results of in-gel activity for SOD revealed the presence of enzyme as prominent achromatic bands in crude extracts of *P. pistillaris* at pH 5.0, pH 6.5, and pH 7.0 of buffers whereas extracts of *T. heimii* from all three buffers showed no band indicating the absence of SOD in the mushroom (**Fig.4**). Achromatic bands were also detected in the precipitates of *P. pistillaris* extracts at pH 5.0 in 70%, at pH 6.5 in 50%, 60%, 70% and at pH 7.0 in 50%, 60%, 70% ammonium sulfate solutions whereas no band appeared in precipitates at pH 6.5 in 30%, 40%, and in precipitates at pH 7.0 in 30% and 40% ammonium sulphate solutions in the gels (**Fig. 4**).

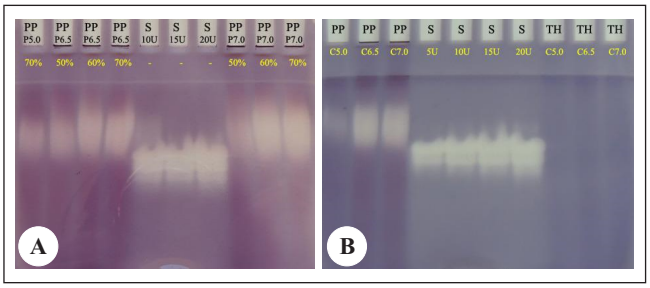

Fig. 4: In-gel activity of superoxide dismutase (SOD): A). Lane number 1, 2, 3 and 4 showing achromatic bands for precipitates of *P. pistillaris* extracts at pH 5.0 in 70%, of pH 6.5 in 50%, 60%, 70%. Lane No. 5, 6 and 7 - achromatic bands of standard SOD (S) at 10U, 15U and 20U and Lane No. 8, 9, and 10 showing activity bands for precipitates (P) at pH 7.0 in 50%, 60%, 70% ammonium sulfate solutions. B). Lane 1, 2 and 3 showing achromatic bands of crude extracts of *P. pistillaris* at pH 5.0, pH 6.5 and pH 7.0. Lane No 4, 5, 6 and 7 showing SOD standard (S) at 5U, 10U, 15U and 20U. Lane 8, 9 and 10 showing no activity band in crude extracts (C) of *T. heimii* at pH 5.0, pH 6.5 and pH 7.0

Spectrophotometric assay: The amount of all three enzymes was determined by using the linear regression equation obtained from the calibration curve of standards. In all the extracts of both the mushrooms at different pH, the amount of each enzyme was found to increase with the increase in the volume of extract from 50µl to 300µl.

Tyrosinase enzyme: The amount of tyrosinase activity in *P. pistillaris* in the buffer at pH 7.0 was observed to be significantly ($p<0.05$) higher in the buffer at pH 5.0 but it was almost the same in the buffer at pH 6.5 (**Table 1; Fig. 5**). However, there was no significant difference observed in the amount of tyrosinase activity evaluated in the extracts of *T. heimii* in buffers at pH 5.0, pH 6.5 and pH 7.0. The amount of tyrosinase activity in 100µl of crude extract of *P. pistillaris* showed a significantly higher amount of tyrosinase (85.25 ± 0.93U/mg) at $p<0.05$ compared to *T. heimii* and also in the developmental stages of *Agaricus bisporus* ranging from 15U/mg to 30U/mg reported by Ingebrigtsen *et al.* (1985). Even, Zaidi and Ali (2015) reported lower tyrosinase activity

Table 1: Tyrosinase activity (U/mg) in crude extracts of mushrooms with buffers of different pH

Mushroom Sample	pH	Amount of Tyrosinase (U/mg)* Crude Extract (µl) 50	100	150	200	250	300
Podaxis pistillaris	5.0	20.49± 0.54^a	67.19± 0.54^c	105.79± 0.93^b	140.04± 1.94^b	166.19± 1.94^b	187.05± 0.93^b
	6.5	32.32± 2.85^b	85.25± 0.93^e	126.96± 2.16^c	163.08± 2.16^c	199.19± 3.24^c	225.97± 1.08^c
	7.0	36.37± 2.85^b	82.13± 0.54^d	127.28± 4.28^c	168.99± 1.94^d	203.55± 1.94^c	230.95± 0.93^d
Termitomyces heimii	5.0	0.00± 0.00	0.02± 0.01^a	0.43± 0.00^a	0.84± 0.01^a	1.17± 0.03^a	1.56± 0.04^a
	6.5	0.00± 0.00	0.38± 0.01^b	0.83± 0.01^a	1.26± 0.03^a	1.75± 0.03^a	2.27± 0.00^a
	7.0	0.00± 0.00	0.32± 0.03^a	0.79± 0.02^a	1.23± 0.01^a	1.69± 0.06^a	2.12± 0.02^a

*Each value is expressed as mean ± standard deviation (n = 3). Different superscript letters within each column indicate significant difference between mean values ($p<0.05$)

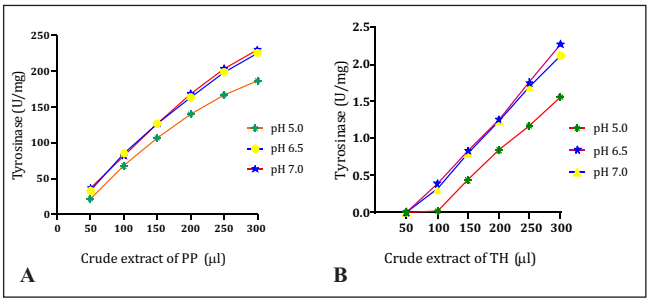

Fig. 5: Tyrosinase activity (U/mg) in the crude extracts of A) *Podaxis pistillaris* (PP), and B) *Termitomyces heimii* (TH) with buffers of different pH.

of 52.19U/mg in *Agaricus bisporus* and 46.4U/mg in *Pleurotus ostreatus* compared to presently studied *P. pistillaris*.

Laccase enzyme: Laccase quantification in both mushrooms revealed that buffers of pH 6.5 and pH 7.0 have almost the same effect in the isolation of this enzyme when compared to pH 5.0. The enzyme activity in *P. pistillaris* was found significantly ($p<0.05$) higher than the *T. heimii* with respect to the extraction buffers. Activity in 100µl of crude extract of *P. pistillaris* showed $47.31\pm1.22\times10^{-3}$ U/mg of laccase and $7.81\pm0.38\times10^{-3}$ U/mg in *T. heimii* on a dry weight basis (**Table 2; Fig. 6**). Results of laccase activity in the present study are corroborated by the reports of other mushrooms using fermentation technology for laccase production. Iyer and Chattoo (2003) found laccase activity of 21.0U at 24hrs in liquid culture of *Magnaporthe grisea* at a biomass level of 0.57g/L. Kumar *et al.* (2015) reported laccase activity of

Table 2: Laccase activity (U/mg) in crude extracts of mushrooms with buffers of different pH

Mushroom Sample	pH	Amount of Laccase (x10^{-3} U/mg)*					
		Crude Extract (µl)					
		50	100	150	200	250	300
Podaxis pistillaris	5.0	0.00± 0.00	9.55± 1.22^{c}	21.62± 1.22^{d}	32.32± 0.34^{d}	42.83± 0.89^{c}	52.96± 1.01^{c}
	6.5	10.13± 1.78^{a}	34.07± 0.34^{d}	60.35± 0.67^{e}	85.27± 0.89^{e}	108.63± 1.35^{d}	136.47± 1.75^{d}
	7.0	18.31± 1.88^{b}	47.31± 1.22^{e}	81.57± 0.58^{f}	120.12± 1.01^{f}	149.71± 3.80^{e}	191.36± 6.58^{e}
Termitomyces heimii	5.0	0.00± 0.00	2.83± 0.44^{a}	9.31± 0.07^{a}	15.78± 0.40^{a}	21.30± 0.53^{a}	27.36± 0.26^{a}
	6.5	0.00± 0.00	5.93± 1.03^{b}	14.88± 0.46^{b}	22.96± 0.54^{b}	30.55± 0.21^{b}	38.98± 0.82^{b}
	7.0	0.00± 0.00	7.81± 0.38^{b}	16.94± 0.67^{c}	26.15± 0.06^{c}	35.11± 0.26^{b}	45.15± 0.58^{b}

*Each value is expressed as mean ± standard deviation (n = 3). Different superscript letters within each column indicate significant difference between mean values ($p<0.05$)

Table 3: Superoxide dismutase (SOD) activity (U/mg) in crude extracts of mushrooms with buffers of different pH

Mushroom Sample	**pH**	**Amount of SOD (U/mg)***				
		Crude Extract (µl)				
		50	100	150	200	250
Podaxis pistillaris	5.0	18.87± 0.71^{b}	39.83± 0.86^{c}	45.75± 0.59^{c}	52.81± 1.04^{c}	56.57± 0.52^{d}
	6.5	18.42± 0.90^{b}	37.21± 1.03^{c}	43.82± 1.20^{c}	51.22± 0.34^{c}	54.86± 1.04^{c}
	7.0	17.51± 0.52^{b}	32.65± 7.11^{b}	38.80± 1.54^{b}	45.41± 1.49^{b}	51.10± 0.86^{b}
Termitomyces heimii	5.0	3.40± 0.22^{a}	4.18± 0.07^{a}	4.50± 0.09^{a}	4.88± 0.12^{a}	5.24± 0.09^{a}
	6.5	3.08± 0.14^{a}	3.86± 0.07^{a}	4.20± 0.12^{a}	4.48± 0.10^{a}	4.89± 0.14^{a}
	7.0	2.97± 0.26^{a}	3.71± 0.10^{a}	4.05± 0.07a	4.32± 0.05^{a}	4.67± 0.05^{a}

*Each value is expressed as mean ± standard deviation (n = 3). Different superscript letters within each column indicate significant difference between mean values ($p<0.05$)

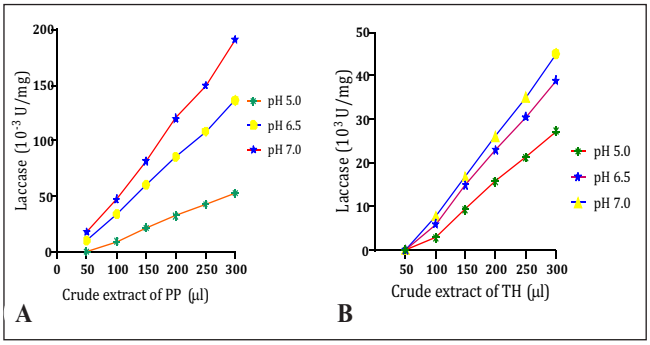

Fig. 6: Laccase activity (U/mg) in the crude extracts of A) *Podaxis pistillaris* (PP), and B) *Termitomyces heimii* (TH) with buffers of different pH.

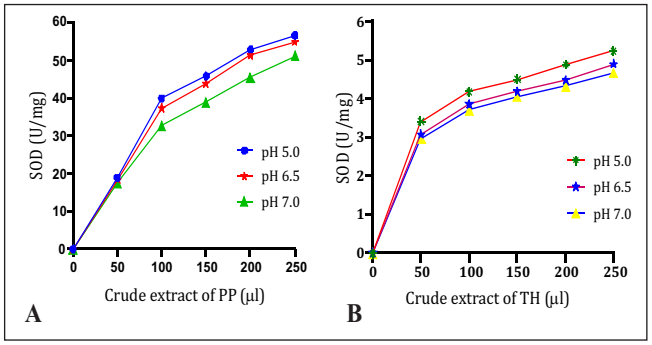

Fig. 7 : SOD activity (U/mg) in the crude extracts of A) *Podaxis pistillaris,* and B) *Termitomyces heimii* with buffers of different pH.

771U/ml in copper-induced *Ganoderma lucidum* MDU-7 under liquid fermentation conditions in 336h. Similarly, Niku-Paavola *et al.* (1990) reported laccase activity of 12.7nkal/ml in 15µl of *Lentinula edodes* in culture. Ning *et al.* (2016) reported purified laccase with a recovery rate of 19.8% from fermentation broth of *Leucoagaricus naucinus* LAC-04. More *et al.* (2011) reported laccase activity of 112.88u/ml at pH 6.5 on the 19th day of culture of *Pleurotus* sp.

Superoxide dismutase enzyme: The effect of different buffers was not significant on the isolation of SOD from both the mushrooms at pH 5.0, pH 6.5, and pH 7.0. The results also revealed that the amount of SOD activity was significantly ($p<0.05$) higher in ***P. pistillaris*** (32.65±7.11 to 39.83±0.86 U/mg, DW) than the *T. heimii* (3.71±0.1 to 4.81±0.07 U/mg, DW) for 100 µl of crude extracts (**Table 3; Fig. 7**). On mushrooms, there are no earlier reports on SOD enzyme isolation and its activity.

CONCLUSION

The results of the study revealed that buffers play a crucial role in the isolation of enzymes from both the mushrooms. The activity of tyrosinase and laccase enzyme was maximum in the extracts isolated in the buffer of pH 7.0 or 6.5 whereas SOD enzyme showed enhanced activity in the extracts isolated in the buffer of pH 5.0 or 6.5. In-gel activity of all the three enzymes was observed for *P. pistillaris* with prominent bands whereas *T. heimii* showed diffuse activity for tyrosinase and distinct bluish-green bands for laccase but no achromatic bands were observed for SOD. In a spectrophotometric analysis of enzymes, *P. pistillaris*, in 100µl of the crude extract showed a significant amount of tyrosinase (85.25±0.93U/mg), laccase (47.31±1.22x10-3U/mg), and

SOD (37.21±1.03U/mg) enzymes on a dry weight basis as compared to *T. heimii*. Therefore, these mushrooms would be useful as an alternative source of enzymes in the biotechnological sector of the paper and pulp industry, textile industry, food industry, pharmaceutical industry, organic synthesis, and wastewater treatment. Mushroom has wide scope for the investigation of other industrially important enzymes.

ACKNOWLEDGEMENTS

Authors would like to acknowledge UGC-SAP (DRS) and DST-FIST, Govt. of India for the infrastructural support to the Department of Biosciences, Sri Sathya Sai Institute of Higher Learning.

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