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Antioxidative enzymatic profile of mushrooms stored at low temperature

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Abstract Fruiting bodies of 6 mushrooms including *Agaricus bisporus, Hyspizygus ulmarius, Pleurotus florida* PF-01, *Pleurotus florida* PF-01 R$_5$, *Pleurotus platypus* and *Pleurotus sajor-caju* PSC-04 were analyzed for antioxidative enzymatic profile during low temperature storage. Colour, rehydration ratio and moisture were taken as indices of accessing their shelf-life/marketability, of which, colour contributed significantly while rehydration ratio and moisture did not change considerably during storage. Mushrooms were stored at 5 and 10 °C and activities and isozyme profile of antioxidative enzymes, superoxide dismutase (SOD) and peroxidase (POX) were analyzed after every 48 h interval till the fruiting bodies remained marketable. SOD activity increased generally at 5 and 10 °C while POX activity first increased and then decreased under similar conditions. Isozyme profile of SOD and POX did not show any new isozyme during storage, the only difference was in the intensity of bands.

Keywords Mushrooms · Superoxide dismutase · Peroxidase · Low temperature storage · Isozymes · Shelf-life

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Introduction

Mushrooms are now a days popularly known as functional foods (Liu and Wang 2009). World production of cultivated edible mushroom increased from 170 MT in 1960 to $10,995.5 \times 10^3$ MT in 2007 (Chang 2007). China has become a giant producer and consumer of mushrooms and a large share (70%) of the total world production comes from China (Chang 2007). India's share is estimated to be around 70,000 MT per annum in which major share is contributed by button mushroom (*Agaricus* spp.), while the rest being speciality one (Singh et al. 2003). Diversified agro-climatic conditions in India offer vast potential for growing different types of mushrooms. There are about 20 varieties of mushrooms being cultivated throughout the world for food. In India, only white button mushroom (*Agaricus bisporus*), oyster mushroom (*Pleurotus* spp.) and paddy straw mushroom (*Volvariella volvacea*) are grown commercially. Out of these, white button mushroom contributes ~90% of the total production. Mushrooms have been recognized as the alternate source of good quality protein and produce the highest quantity of protein per unit area from agro-wastes. Besides, they provide potentiality for generating employment, improving economic status of growers, help in checking pollution and earn foreign exchange (Rai and Arumuganathan 2005). Mushrooms contain ~90% moisture and are highly perishable in nature.

Edible mushroom suffers major post harvest loss due to short shelf-life mainly due to enzymatic browning, high metabolic rate and environmental stress factors (Beelman 1987). The tropical temperature being the major limiting factor in India for production as well as productivity of mushroom. The intrinsic antioxidative defense mechanism protects the produce from temperature induced stress to some extent. These include various antioxidative enzymes

like superoxide dismutase (SOD), peroxidase (POX) and antioxidants like glutathione and ascorbate. The changes in shelf-life and the defense mechanism countering them occur at molecular level (Misra and Fridovich 1972; Ravindranath and Fridovich 1975). Molecular markers are used for analyzing genetic variations in a number of organisms. Techniques include either DNA or enzyme based systems or both. But isoenzyme based system is the cheapest method yielding comparable results with DNA based system for genetic investigations (Packia et al. 2000). Many edible mushrooms are reported and thousands of unidentified strains are present especially in Aravali region of Rajasthan. The present paper describes the activity and isozyme profile of two antioxidative enzymes, SOD and POX along with other indices of marketability during low temperature storage till mushrooms remain marketable.

Materials and methods

Fruiting bodies and storage conditions Fruiting bodies of 6 strains of mushrooms, wild (*Pleurotus florida* PF-01, *Pleurotus florida* PF-01 R₅, *Pleurotus platypus* and *Pleurotus sajor-caju* PSC-04) and cultivated (*Agaricus bisporus, Hyspizygus ulmarius*), were obtained fresh just after harvesting from All India Co-ordinated Mushroom Improvement Project, Department of Plant Pathology of the College and stored at 5 and 10 °C in polypropylene bags of thickness 150 gauze. Activity and isozyme profile of antioxidative enzymes SOD and POX were investigated at 5 and 10 °C at every 48 h interval till the fruiting bodies remained edible/ marketable in order to know the shelf-life of mushrooms during storage.

Indices of marketability Moisture content was determined by oven drying method (Rangana 2000) while for re-hydration ratio, method of Kumar et al. (2007) was followed where pre-weighed samples were soaked in ample amount of water for 30 min at room temperature (32 °C). The ratio of mass of re-hydrated to normal samples was used to find the re-hydration ratio whereas the colour observations were done visually.

Preparation of crude extract After preliminary studies of standardizations with respect to buffer, pH and extraction conditions, the extractions were done by following procedure: 5 g of fresh tissue (mushroom fruiting body) was homogenized with 10 ml of 0.1 M Tris-HCl buffer (pH 7.5) containing 1 mM EDTA, 3% polyvinyl pyrrolidone and 1 mM CaCl₂ in a pre-chilled pestle and mortar using acid washed sand as an abrasive. The homogenate was filtered through 4 layers of cheesecloth and the filtrate centrifuged

at 12,000 rpm for 15 min in a refrigerated centrifuge (Remi, India) at 4 °C. The supernatant obtained was used as crude preparation for enzyme assays and other biochemical estimations. Soluble protein was estimated as described by Bradford (1976) using bovine serum albumin as standard.

Assay of SOD activity SOD was assayed by measuring its ability to inhibit the photochemical reduction of nitroblue tetrazolium (NBT) adopting the method of Beauchamp and Fridovich (1971). The reaction mixture 3 ml contained 0.05 M Tris-HCl (pH 7.8), 14 mML-methionine, 60 μM NBT, 3 μM riboflavin, 0.1 mM EDTA and 0.2 ml of enzyme extract. Riboflavin was added at the end. The tubes were properly shaken and placed 30 cm below light source consisting of two 100 W fluorescent lamps (Phillips, India). The reaction was started by switching on the light and terminated after 20 min of incubation by switching off the light. After terminating the reaction, the tubes were covered with black cloth to protect them from light. A non-illuminated reaction mixture that did not develop colour served as the blank. The reaction mixture developed maximum colour without enzyme extract and its absorbance decreased with the addition of enzyme. The absorbance was recorded by Spectrophotometer (Elico, India) at 560 nm. The enzyme activity was calculated by the formula of Gianopolitis and Ries (1977) and the activity expressed in units/mg protein.

$$\text{SOD activity} = \frac{A_{560}(Control) - A_{560}(Sample)}{A_{560}(Sample)}$$

where Control = Rate of assay reaction without SOD, Sample = Rate of assay reaction with SOD.

Assay of POX activity POX activity was assayed by the modified method of Seevers et al. (1971). The reaction mixture contained 0.1 ml of 2.0% o-dianisidine in methanol, 0.1 ml of 10 mM H₂O₂, 2.7 ml of 0.1 M sodium acetate buffer (pH 5) and properly diluted aliquots of enzyme extract (0.1 ml). The reaction was initiated by the addition of H₂O₂ and change in absorbance was followed at 470 nm spectrophotometrically (Systronics, India) up to 3 min. The linear change in absorbance was taken to calculate enzyme activity. One unit of enzyme activity is defined as 0.001 change in OD per min (Neves 2002) and the activity is expressed in units/mg protein.

Specific enzyme staining of SOD Specific enzyme staining of SOD was done by the method of Beauchamp and Fridovich (1971). SOD was localized by soaking the native PAGE gel in 2.45×10⁻³ M NBT for 20 min followed by immersion for 15 min in a solution containing 0.028 M

TEMED, 2.8×10^{-5} M riboflavin and 0.036 M Tris-HCl buffer (pH 7.8). The gel was then illuminated under fluorescent light for 1 h. During illumination, the gel became uniformly faint blue except at positions containing SOD. Illumination was discontinued when maximum contrast between the achromatic zones and the general blue colour had been achieved.

Specific enzyme staining of POX After electrophoresis, the gels were stained for specific enzyme activity of POX with 100 ml benzidine reagent containing equal amounts of 0.3% benzidine in 25% acetic acid and 0.5% H_2O_2 for 20–30 min (Guikema and Shermen 1980). Blue coloured bands appeared which turned brown after 10–15 min. These brown coloured bands represented the POX activity.

Statistical analysis The statistical analysis of the data was done by using complete randomized design (CRD). The 6 accessions during storage were studied replicating each observation thrice. The critical differences of stages, varieties and interactions were calculated at $p \leq 0.05$ using the table 'ANOVA'.

Results and discussion

Indices of marketability Greater shelf-life was observed at 5 °C than at 10 °C (Table 1). Mushroom respiratory rate and decay increased with the increase in storage temperature. Mushrooms at 10 °C have 3.5 times higher respiratory rate than at 0 °C (Hyun et al. 1996). Wijewardane and Guleria (2009) reported shelf life of 150 days in case of pre-treated apple fruits stored at 2±1 °C and 85–90% RH by lowering the incidence of fruit softening and spoilage. Fresh colour of *A. bisporus*, *P. florida* PF-01, *P. florida* PF-01R₅ and *P. platypus* were whitish or creamish white while colour of *P. sajor-caju* PSC-04 was bluish grey and that of *H. ulmarius* was blue and had excellent appearance (Table 2). As the time elapsed, colour started deteriorating.

Table 1 Shelf-life (h) of various accessions of mushrooms at 5 and 10 °C

Strains	5 °C	10 °C
A. bisporus	192	144
H. ulmarius	240	192
P. florida PF-01	240	192
P. florida PF-01 R5	192	144
P. platypus	240	192
P. sajor-caju PSC-04	240	192

The deterioration was more at 10 °C than at 5 °C. Results indicated that colour changes reduced the marketability of mushrooms stored at 10 °C.

Activity profile of SOD and POX As the temperature stress increased, SOD activity increased (Table 3). There had been 8–10 times increase in specific activities with respect to fresh SOD activity in all the fungal accessions tested. Also, SOD activities were more at 10 °C storage as compared to 5 °C. This suggested that increased stress conditions might have created more SOD activity either at the level of expression or activity to counter temperature stress, as it is the very first enzyme involved in countering oxidative stress conditions. Among the accessions, maximum variation in SOD activity was observed in *H. ulmarius* while minimum in *P. platypus*. POX activity (Table 4) showed a mixed type of response. Generally, specific activity of POX increased during first sampling and then decreased both at 5 and 10 °C sampling, but, under certain conditions, activity again aggravated during second or third sampling. In all the accessions, POX activity decreased with respect to sampling time and high temperature stress at 10 °C. However, highest POX activities were observed with *P. platypus*. Rabinowitch et al. (1982) found role of SOD in supplementing the protective action of carotenoids against photo-oxidative injury in ripening tomato fruit. SOD activity increased with increased photo-oxidative damage while POX activity was not changed much. Neves (2002) while working on peach fruit, reported that at mature green stage, POX activity was low and increased at ripened stage. After harvest, POX activity first increased up to certain period and then decreased at senescence. Kumar (2009) reported higher ascorbate peroxidase and glutathione reductase activities at 4 °C than at 12 °C in case of potato tubers during storage at low temperature. However, Flurkey et al. (1994) observed low or undetectable POX activity in *Agaricus*, oyster and Shiitake mushrooms. Control of enzymatic browning in different mushroom types may depend upon the distribution of different oxidases in each of them. Gullen and Eris (2004) reported that heat stress enhances POX activity in strawberry plants indicative of countering stress condition. However, total protein content decreased at elevated temperature. POX activity also increased with increase in temperature. Our results are in conformity with Rabinowitch et al. (1982) and Neves (2002) while contradictory to Flurkey et al. (1994) and Gullen and Eris (2004). Gupta and Gupta (2005) incubated leaf discs of 15 day old seedlings of wheat genotypes 'C-306' (temperature tolerant) and 'HD-2329' (widely adapted) at 25, 35 and 45 °C. The genotype, 'C-306' exhibited lower accumulation of malondialdehyde and H_2O_2 due to increased activities of SOD, POX and catalase under high temperature conditions. SOD activity enhanced continu-

Table 2 Colour pattern for all strains during sampling at 5 and 10 °C

	At 5 °C					At 10 °C			
	48 h	96 h	144 h	192 h	240 h	48 h	96 h	144 h	192 h
A. bisporus	+++	+++	+++	+	–	+++	+++	++	–
H. ulmarius	Blue	Blue + Brown	Blue + Brown	Brown	Brown	Blue	Blue + Brown	Blue + Cream	Light yellow + Brown
P. florida PF-01	+++	+++	++	+	Dark cream	+++	+++	+	Dark cream
P. florida PF 01 R₅	+++	+++	Light white + Cream	Light white + Brown	–	+++	+++	+	Brown
P. platypus	+++	+++	+++	++	+	+++	+++	++	+
P. sajor-caju PSC-04	Bluish grey	Bluish grey	Light brown	Light brown	Light brown + Cream	Bluish grey	Bluish grey	Light brown	Light brown

+++ excellent white, ++ good white, + creamy white (–) indicates samples spoiled

All samples spoiled at 240 h at 10 °C

ously with increasing temperature and POX also followed similar trend.

Isozyme profile of SOD and POX Isozyme profiles of SOD and POX (Fig. 1), as on second day of storage, have been reported. However, during storage, no new isozyme was reported. Isozyme profile of SOD in 6 accessions shown in Fig. 1a indicated 4 isozymes of SOD in *A. bisporus* and 6 each in *H. ulmarius*, *P. florida* PF-01, *P. florida* PF-01 R₅, *P. platypus* and *P. sajor-caju* PSC-04. Most of the species had monomorphic isozymes and only 2 loci of isozymes (in case of *A. bisporus*) showed polymorphism on the basis of specific enzyme staining of SOD. The difference observed in SOD isozymes was in terms of intensity and size of bands. Less number of isozymes in *A. bisporus* as compared to other strains might be responsible for lesser shelf-life of *A. bisporus* and susceptibility to high temperature stress during fruiting body development as well as

during storage. Two bands of each accession were more towards cathodic side, possibly having net positive charge and high molecular weight, while rest bands were towards anode indicating net negative charge and corresponding low molecular weights. The number of isozymes of SOD had been reported to vary, viz., 2 in roots and needles of *Picea abies* (Kroniger et al. 1992), 3 in *Spirulina* (Lumsden and Hall 1974), kidney bean leaves (Kono et al. 1979), Brussels sprouts (Walker et al. 1991) and *Fagopyrum tataricum* cv. Wutai leaves (Wang et al. 1993), 4 in maize (Gianopolitis and Ries 1977) and tomato (Kumar et al. 2006) and 5 in maize (Baum et al. 1983). However, when studied the polymorphism of 15 species of the genus *Pleurotus* (oyster mushroom) at genetic level using electrophoretic separation of the isozymes esterase, POX, malate dehydrogenase and SOD, the differences observed in enzyme profiles between species were found to be adequate analyzing the extent of genetic variability at interspecific

Table 3 Superoxide dismutase activity (units/mg protein) profile of different accessions of mushroom during sampling at 5 and 10 °C

	At 5 °C						At 10 °C			
	0 h	48 h	96 h	144 h	192 h	240 h	48 h	96 h	144 h	192 h
A. bisporus	1.69	1.98	12.11	16.48	29.20	–	2.52	15.10	20.51	–
H. ulmarius	12.27	28.87	35.02	80.92	109.50	140.96	31.63	66.67	96.62	138.56
P. florida PF-01	8.33	10.71	12.44	15.80	16.14	17.02	12.81	15.46	15.88	16.32
P. florida PF-01 R₅	2.55	3.25	11.20	14.89	31.86	–	4.72	13.75	17.13	–
P. platypus	2.68	4.39	6.53	12.12	16.79	32.21	5.01	11.56	14.46	16.65
P. sajor-caju PSC-04	19.06	19.93	24.73	33.21	37.81	40.63	23.78	32.32	34.01	38.45
SEm ±	0.0194	0.1234	0.4363	0.3684	1.1014	0.5832	0.1234	0.3684	0.4673	0.9945
CD (p=0.05) (n=3)	0.0598	0.3803	1.3443	1.1352	3.3937	0.7970	0.3803	0.1352	1.4400	3.0644

– samples spoiled

Table 4 Peroxidase activity (units/mg protein) profile of different accessions of mushroom during sampling at 5 and 10 °C

	At 5 °C						At 10 °C			
	0 h	48 h	96 h	144 h	192 h	240 h	48 h	96 h	144 h	192 h
A. bisporus	317	702	1,358	521	657	–	2,038	589	974	–
H. ulmarius	622	533	667	444	311	356	800	400	622	667
P. florida PF-01	360	1,216	675	626	653	–	360	1,463	1,689	405
P. florida PF-01 R_5	777	1,554	691	669	583	–	820	2,331	820	–
P. platypus	1,990	2,674	1,244	1,306	435	470	653	870	777	404
P. sajor-caju PSC-04	257	486	400	229	771	800	286	1,143	343	514
SEm ±	8.01	19.604	18.073	9.243	12.136	2.5586	19.4222	26.0800	12.8156	4.8237
CD (p=0.05) (n=3)	24.68	60.405	55.687	28.482	37.394	7.8838	59.8456	80.3605	39.4889	14.8634

– samples spoiled

level. The isozyme pattern of SOD was unique in such a way that it was monomorphic and found in all the species studied including *P. sajor caju* PSC-04, *P. platypus* and *P. florida* (Pradeep et al. 2002).
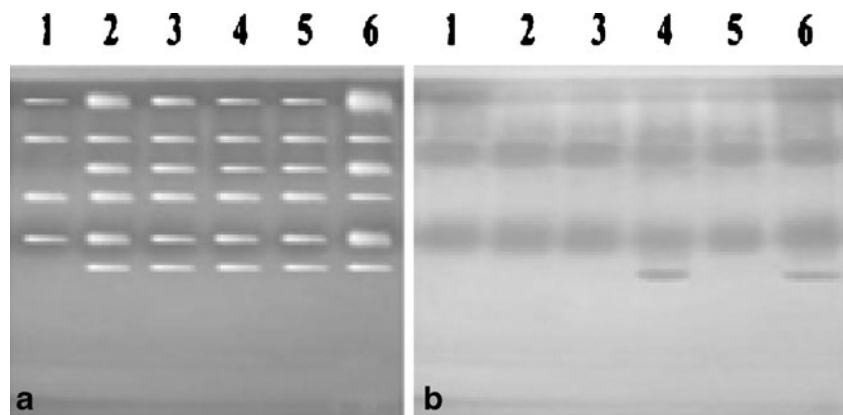
Isozyme profile as observed for POX presented in Fig. 1b indicate 3 isozymes of POX each in *A. bisporus* and *P. platypus*, 2 of each in *P. florida* PF-01, *P. florida* PF-01 R_5 and *P. sajor-caju* PSC-04 and 5 isozymes in *H. ulmarius*. Two bands were monomorphic while 3 bands were polymorphic; band 2 of *H. ulmarius* was exclusively present in this mushroom species. Two bands were towards cathodic side while most of the POX were of low molecular weight and towards anodic side. Three POX isozymes had been characterized in bean leaves (Racusen and Foote 1966), while 7 in horseradish roots (Shannon et al. 1966). Seven isozymes may be segregated into 2 groups on the basis of their chromatographic behaviour, electrophoretic migration, spectrophotometric properties and amino acid and carbohydrate composition. However, while studying genetic variation of 7 mutant lines of *Pleurotus citrinopileatus*, out of the 7 enzymes clearly resolved, 19 loci were ascertained for genetic analysis. Esterase, SOD, polyphenol oxidase and malate dehydrogenase were polymorphic, while

acid phosphatase, lactate dehydrogenase and POX resolved to only one locus (Packia et al. 2000). A total of 6 isozymes of POX in *Agrocybe aegerita* had been observed and out of that, 2 fractions of the enzyme, Aap I and Aap II, had identical molecular masses (46 kDa) and isoelectric points of 4.6–5.4 and 4.9–5.6, respectively. Hence, POX are found in plants, fungi, bacteria and animals and have been grouped on the basis of sequence similarity into 2 superfamilies: animal POX form one superfamily and plant, fungal, and bacterial POX form another superfamily. POX of fungal superfamily are involved in white rot formation and degradation of ligno-cellulosic wastes (Ullrich et al. 2004).

Conclusion

As the temperature stress increases, shelf-life decreases *i.e.* more shelf-life at 5 °C as compared to 10 °C. SOD activity increased correspondingly while POX activity first increased and then decreased with respect to temperature stress and time. This may possibly be the major cause of fungal decay. Out of the total loci tested for both isozymes, 2 of SOD and 3 of POX showed polymorphism.

Fig. 1 Isozyme profile of (**a**) Superoxide dismutase and (**b**) Peroxidase during sampling 1. *A. bisporus*, 2. *P. florida* PF-01, 3. *P. florida* PF-01 R5, 4. *P. platypus*, 5. *P. sajor-caju* PSC-04, 6. *H. ulmarius*

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