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Research Article

MOLECULAR DYNAMICS OF MORPHOGENESIS IN *Volvariella volvacea* (BULL. EX FR.) SING

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Abstract- Mannitol dehydrogenase, tyrosinase and water activity (a_w) were found to be variously expressed during morphogenesis of *V. volvacea*. Very less enzymatic activity was recorded at pinhead stage, which increased with progression of growth. In different parts of mushroom maximum activities of mannitol dehydrogenase, tyrosinase, browning and water activity were found in pileus followed by volva and stipe. Mannitol dehydrogenase was found to be involved in morphogenesis whereas tyrosinase was found to be involved during senescence and browning of the sporophores of *V. volvacea*. Browning degree, water activity and tyrosinase activities are presumably conjugated and are responsible for the senescence of the mushroom. Further studies involving the molecular interaction of these enzymes in the developmental stages is most warranted for commercial marketing of mushrooms.

Keywords- Browning, enzymes, mannitol dehydrogenase, tyrosinase, water activity.

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Introduction

Mushroom fungi are major chunk of nutritious healthy foods, which are eulogized throughout the world both as food and as medicine for thousands of years. Among the edible mushrooms, gourmet species such as *Volvariella volvacea* (Bull. Ex Fr.) Sing, known as paddy straw mushroom belonging to the family Pluteacea (Kolt and Pouz) of Phylum Basidiomycetes, ranks sixth amongst the cultivated mushrooms; accounting to about 5 per cent of the total world mushroom production. Cellulases play a critical role during substrate colonization while, laccase dominates during sporophore development. These enzymes were found to be expressed differently with different strains of *V. volvacea* [1]. Mannitol is the most common polyol, found in large quantities in spores, fruiting bodies, sclerotia and mycelia contributing to about 20 per cent of the mycelium. This sugar alcohol increases dramatically to 30-50 per cent in differentiating sporophores [23, 27].

In mushroom fungi, mannitol was purported to function as an osmoregulator, which encouraged influx of water from the environment to develop turgor pressure thereby helped in fruiting body development [3, 12]. It also acted as a respiratory substrate during the post-harvest storage and senescence of mushroom [10]. Earlier, mannitol-1-phosphate dehydrogenase (M1PDH), a zinc-containing long-chain alcohol dehydrogenase was thought to be limited to Zygomycetes and Ascomycetes. However, their activities were reported in Basidiomycetes such as, *Pleurotus ostreatus* and *Cryptococcus neoformans* [4, 28].

Sporocarps of Volvariella are perishable and tend to lose their appearance due to short shelf life, a ravaging impediment to the distribution and marketing of the fresh mushrooms. Extension of the quality and shelf life is therefore a scientific, technical and economical challenge. Tyrosinase, a glycosylated cytosolic copper-containing monooxygenase belonging to poly phenol oxidase family, is the principal enzyme for the synthesis of melanin pigment from tyrosine, which is responsible for enzymatic browning during development and postharvest storage [6, 22]. Keeping this information, the present study is thus aimed to emphasize an arrant knowledge on enzymatic dynamics involved in the morphogenesis of *V. volvacea*.

Materials and Methods

V. volvacea strain CBE TNAU 1505 obtained from the germplasm bank of Mushroom Research Laboratory, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India was used in this experiment. It was maintained on 90 mm petri dishes with potato dextrose agar medium at 32± 2^{0}C.

Spawn preparation and crop rising

Paddy straw-based spawn was prepared by following the method suggested by Krishnamoorthy *et al.* [14]. Fresh, good quality paddy straw bits (1-2 inches) were soaked in water for 6 h and dried under shade up to 65% moisture content. The paddy straw substrate was supplemented with horse gram powder @ 2% (dry weight basis) and the contents were packed in polypropylene bags and autoclaved at 1.46 Kg/cm^2 for 90 min. On the next day 90 mm mycelial mats of 7 days old culture was propagated and the bags were incubated at room temperature. Fifteen days old spawn with well-developed chlamydospores were used to seed the beds. For bed preparation, circular compact bed method was followed [24]. In which, golden yellow, well-dried, good quality paddy straw was made into small twists of 2.5 m length and 5 to 8 cm diameter; each twist weighing 1.25 kg. After draining the excess water, the twists were pre-soaked in cold water for about 24 h and steam sterilized at 1.46 kg/cm^2 for 1 h. Later, the paddy straw twists were shade dried to get 65 to 75% moisture. The twists were compactly placed clockwise in a circular fashion as close as possible on a wooden plank to make the first layer. Eight paddy straw spawn bits each weighing 25 g were placed at the periphery of the first layer of the bed leaving equal distance between them. Over the spawn bits 20 g of pre sterilized horse gram powder was sprinkled. Second layer was formed over the first layer following the same procedure but the twist was placed compactly in the anticlockwise direction. Similarly, the third and fourth layers of bed were formed. The size of the bed measured 30 cm diameter

and 20 cm height. Total weight of each bed was 5 kg on dry weight basis. The perfectly prepared bed was pressed tightly and placed in a poly house for cropping. Appropriate room temperature inside the cropping room ranged from 32 to 35^{0}C and the relative humidity of 80 to 85% was maintained to prevent desiccation of young buttons. The sporocarps of the fungus at different growth stages were collected separately as described by Chakraborty *et al.* [5] and used for morphogenesis related enzyme assay.

Assay of mannitol dehydrogenase (MtDH) (EC 1.1.1.138)

Mannitol dehydrogenase was assayed as suggested by Chakraborty *et al.* [4] with some modifications. The fresh mushroom tissue was extracted with 20 mM HEPES (Hydroxyethyl piperazin ethane sulfonic acid) KOH buffer (pH 7.5) containing 1 mM EDTA, 2 mM 2- mercapto ethanol and 2 mM PMSF (Phenyl Methyl Sulfonyl Fluoride). Unbroken cells, and cell debri were removed by centrifugation at 32,000 rpm for 60 min at 4°C and the supernatant was used as the cell extract. MtDH activity was determined by incubating the cell extract at 25°C in 20 mM HEPES-KOH (pH 7.5) - 500 mM fructose - 0.25 mM NADPH. The enzyme activity was monitored by recording the change in absorbance at 340 nm.

Assay of tyrosinase (EC 1.14.18.1)

The tissue was macerated with phosphate buffer (pH 6.8) at 1:1 (w/v) ratio. The extract was collected and centrifuged at 15,000 rpm for 30 min at 4°C. The supernatant was used as enzyme source. Assay was conducted with the reaction mixture, total volume of 3.0 ml: 3 mM-L-tyrosine, 47 mM-sodium phosphate buffer (pH 6.8) and 25 µl of enzyme source. The rate of formation of dopachrome was measured at 475 nm. The control was kept by adding the same reaction mixture without enzyme source. The enzyme activity was expressed as change in absorbance at 470 nm.

Determination of water activity

The harvested mushrooms were sorted into six stages of maturity *viz.,* pinhead, tiny button, button, egg, elongation and maturation stages. Sample of 3 g of freshly harvested mushroom tissue was placed in cups of Aqua lab series water activity meter for 1 min at 30°C and the water activity was determined and expressed in a_w.

Measurement of browning

The browning degree of *V. volvacea* was evaluated by a spectrophotometric method described by Zhou *et al.* [29] with some modification. A sample containing 4 g of mushroom sample was ground with 8 ml of 0.2 M phosphate buffer solution (pH 6.8) in an ice bath. After centrifugation at 10000 rpm for 20 min at 4^{o}C, the browning degree was determined by measuring the absorbance of the supernatant at 420 nm using a UV-vis spectrophotometer (GS5703AT).

Statistical Analysis

In order to avoid experimental errors the enzyme assays were carried out for five times and the results presented are mean values ± standard deviations. Statistical software AGRES was used for the data analysis. In case of zero values the data was log transformed (X+0.5) before statistical analysis.

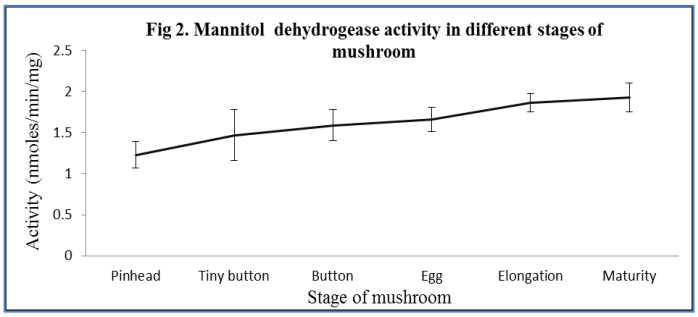
Results

As the enzymes like mannitol dehydrogenase and tyrosinase are related to morphogenesis and shelf life at different growth stages of mushrooms *viz.,* stage 1 (pin head), stage 2 (tiny button), stage 3 (button), stage 4 (egg), stage 5 (elongation) and stage 6 (maturity) [Fig-1], the present study was undertaken to find out their level of expression at different growth stages of *V. volvacea* strain CBE TNAU 1505.

Expression of mannitol dehydrogenase (MtDH)

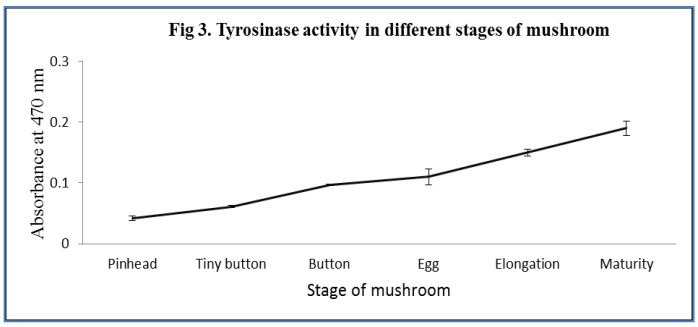
Mannitol dehydrogenase activity was spectrophotometrically assayed at all the growth stages of mushroom. Enzyme activity was expressed as change in absorbance at 340 nm. The results indicated that stage 6 and stage 5 are on par and expressed more activity of MtDH (1.97, and 1.88), followed by stage 4 (1.66). Whereas, stage 1 recorded poor activities of MtDH (1.23) [Table-1]. The rate of increase in activity of MtDH. was higher from stage 1 to 4 when compared to stage 5 and 6 [Fig-2]. Pileus recorded the maximum activity of the enzyme (1.80), followed by stipe (1.74) and volva (1.66) [Table-2].

Fig-1 Morphogenesis of Volvariella



Expression of Tyrosinase

Tyrosinase is an enzyme that belongs to PPO family and its expression was significantly different at all stages of morphogenesis. It is expressed as change in absorbance at 475 nm. Stage 6 registered significantly high levels of tyrosinase activity of 0.19, followed by stage 5 recording 0.156 and stage 4 (0.11). Whereas, stage 1 showed less tyrosinase activity (0.042) as shown in [Table-1] and [Fig-3]. When tested with different parts of the mushroom, high tyrosinase activity was recorded in pileus (0.13) followed by volva (0.10) and stipe (0.091) [Table-2].



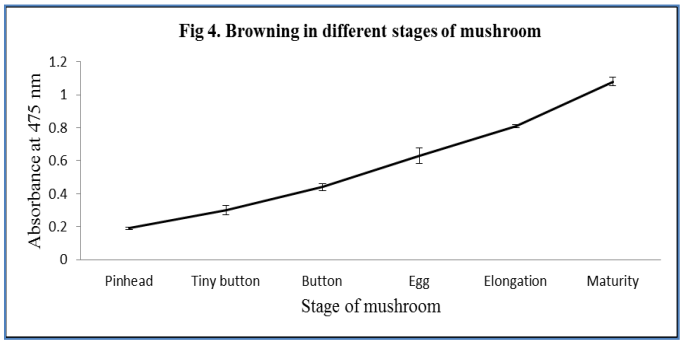
Browning degree

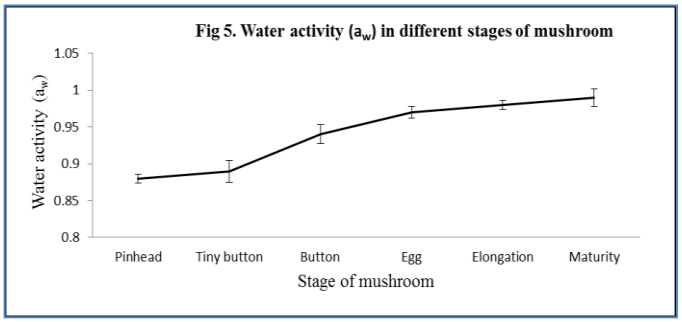
Browning of mushroom is an important senescence related factor that causes economical loss. In this study, degree of browning at different growth stages was evaluated by spectrophotometric method. Increased level of browning was noticed at stage 6 (1.08) that was statistically different from all other stages. This was followed by stage 5 (0.81) and stage 4 (0.63). Stage 1 recorded very less browning activity (0.19). Browning is recorded at all stages of maturity and it was found to be accelerated at late stages *i.e.,* after opening of the pileus [Table-1] and [Fig-4]. When tested with different parts of mushroom, the degree of browning was found to be significantly high in pileus (0.98) followed by volva (0.49) and stipe (0.34), respectively [Table-2].

Water activity

The water activity was found to be significantly higher at stage 4 to 6 (0.976, 0.983

and 0.997 a_w), respectively. This was followed by stage 3 (0.947 a_w). Minimum water activity was recorded at stage 1 (0.881 a_w) [Table-1] and [Fig-5]. There was no significant difference between the water activity of pileus, volva and stipe [Table-2].





Discussion

Volvariella volvacea is a large pileate fungus with dark grey basidiocarps, which are roughly divided into six stages of growth namely, pinhead, tiny button, button, egg, elongation and mature stages; each stage having its own morphological and anatomical characteristics [7].

Table-1 *Enzymatic dynamics during the morphogenesis of V. volvacea*

Stage	Mannitol dehydrogenase activity*	Tyrosinase activity*	Browning degree (OD_{420})*	Water activity (a_w) at 30^{0}C*
Pinhead	1.23 ± 0.28^d	0.042 ± 0.0060^f	0.19 ± 0.012^f	0.88 ± 0.010^c
Tiny button	1.47 ± 0.54^c	0.061 ± 0.0035^e	0.30 ± 0.047^e	0.89 ± 0.026^c
Button	1.59 ± 0.33^b	0.097 ±0.0024^d	0.44 ± 0.039^d	0.94 ± 0.022^b
Egg	1.66 ± 0.25^b	0.11 ± 0.063^c	0.63 ± 0.080^c	0.97 ± 0.014^a
Elongation	1.88 ± 0.19^a	0.15 ± 0.030^b	0.81 ± 0.014^b	0.98 ± 0.011^a
Maturity	1.97 ± 0.30^a	0.19 ± 0.060^a	1.08 ± 0.048^a	0.99 ± 0.021^a
CD (P = 0.05)	0.10	0.0030	0.024	0.024

*Data are expressed as mean ± Standard deviation (n=5). Mannitol dehydrogenase and tyrosinase activity; browning degree are expressed as change in absorbance at 340, 470 and 420 nm, respectively. Water activity is measured at 30^{0}C. Means in a column followed by the same letter are not significantly different at P = 0.05 by one way ANOVA.

Table-2 *Enzymatic activities in the morphological parts of V. volvacea*

Part of mushroom	Mannitol dehydrogenase activity*	Tyrosinase activity*	Browning degree*	Water activity (a_w)*
Volva	1.66 ± 0.28c	0.10 ± 0.021b	0.49 ± 0.031b	0.96 ± 0.025
Stipe	1.74 ± 0.41b	0.091 ± 0.007c	0.34 ± 0.053c	0.95 ± 0.051
Pileus	1.80 ± 0.17a	0.13 ± 0.014a	0.98 ± 0.028a	0.99 ± 0.020
CD (P = 0.05)	0.048	0.0036	0.031	NS

*Data are expressed as mean ± Standard deviation (n=5). Mannitol dehydrogenase and tyrosinase activity; browning degree are expressed as change in absorbance at 340, 470 and 420 nm, respectively. Water activity is measured at 30^{0}C. Means in a column followed by the same letter are not significantly different at P = 0.05 by one way ANOVA.

The characteristic feature of genus Volvariella is opening of basidiocarp before full maturity (hemi-angiocarpous) with the universal veil. The aerial hyphae aggregate into knots on mycelial cultures and are known as pinheads. Under favourable conditions, these primordia (pinheads) would grow into button stage and then to commercially marketable egg stage. Mannitol, a six carbon polyol accumulates in growing sporophores *i.e.* in both pileus and stipe during and between the flushes of fruiting body development and is also responsible for salt tolerance of *A. bisporus* [11, 27]. In accordance with Bonner *et al.* [3] and Jennings [12] mannitol was indeed supposed to function as an osmoregulator by encouraging the influx of water from the environment to develop turgor pressure thereby, helps in fruiting body development. In edible mushrooms such as *A. bisporus* and *Lentinulla edodes,* fructose is reduced to mannitol and the reaction is catalysed by MtDH in the presence of NADPH, obtained from pentose phosphate pathway [8]. In the present study, MtDH activity was recorded in pinhead stage and showed a gradual uptick in subsequent stages of maturity, which is positively, compared to accumulation of mannitol in developmental stages of the fruiting bodies of *A. bisporus* and *L. edodes,* as reported by Hammond and Nichols [11] and Kulkarni [15]. In contrast, cytoplasmic non glycosylated MtDH activity was reported to be abundant in vegetative cells and early developmental stages of *A. bisporus* which dwindled progressively during sporophore growth and expressed at very low level after sporulation [19, 21]. Perhaps, the increase in MtDH activity in developmental stages had been attributed to the increase in fructose-6-phosphate by Hexose monophosphate (HMP) activity [9]. However, the depletion of mannitol concentration during mycelial growth might be due to unavailability of NADPH. According to Hammond and Nichols [11] the levels of enzymatic activities change with considerable change in carbohydrate metabolism during flushing. This fascinatingly insinuates that greater participation of metabolic pathways is certainly responsible for enzymatic activities during the morphogenesis of *V. volvacea*.

In mushrooms, browning is the kenspeckle quality factor and economically ascertained phenomenon in which phenols are enzymatically processed into quinone's that evolve eventually into melanins [6, 26]. Concentrations of Water activity, pH, temperature, active PPO and phenolic compounds determine the browning degree of the tissue [17]. Tyrosinase, a multifunctional, glycosylated copper containing oxidase is responsible for enzymatic browning in fresh fruits, vegetables, beverages and mushrooms [16]. It catalyzes the rate limiting steps in melanogenesis *i.e.* oxidation of both monophenols and o-diphenols into o-quinones, which finally leads to melanins [6, 13]. The results presented in the [Table-1 and 2] cogently imply that tyrosinase activity increased with subsequent stages of maturity, which showed positive correlation with browning degree and water activity. In fungi, tyrosinases are associated with formation, stability of spores and also for melanin production, which constitute a mechanism of defence to stress such as dehydration, UV radiation, extreme temperatures and free radicals. Moreover, they also contribute to the fungal cell-wall resistance to hydrolytic enzymes, avoiding cellular lysis [2, 20, 25]. Meng *et al.* [18] reported that the polyphenoloxidase (PPO) activity of *Agaricus bisporus* increased gradually with storage time. The increased activities of tyrosinase and browning in ensuing developmental stages may be featured to the two distinct mechanisms of phenol oxidation *i.e.,* by the activation of tyrosinase that belongs to PPO family and by spontaneous oxidation [16, 26].

The compelling results of present investigation [Tables-1 and 2] envisaged that the browning degree gradually accreted with the developmental stages of *V. volvacea i.e.,* from pin head to flattening and final maturity. Noticeably, the water activity (a_w) showed positive correlation with the browning degree; a_w increased significantly with morphogenesis of fungi towards final maturity. Similar results were obtained in *A. bisporus* in which, browning degree showed negative and positive correlation with firmness and respiration rate, respectively due to its thin and porous epidermal structure [16]. This explicitly indicates that browning and water activity are partly responsible for firmness and respiration rate of *V. volvacea.* Further, this information univocally explains that browning degree, water activity and tyrosinase activities are concatenated thereby, amenable for accelerating senescence in fruiting bodies of *V. volvacea*. Further studies in this context are very much warranted for commercial marketing of Volvariella.

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Conflict of Interest: None declared

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