

Original Research Article

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## Screening, Localization and Activity of Mushroom Tyrosinase from Various Developmental Growth Phases of *Pleurotus florida*

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### ABSTRACT

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The present work is designed for the utilization of agricultural waste for mushroom production and extraction of tyrosinase through-out its developmental growth phases. Various edible mushrooms were screened for tyrosinase activity resulting highest activity in *Pleurotus* spectrophotometrically. The results also showed that Mushroom could be cultivated using wheat grains and wheat straw. Tyrosinase was observed throughout the growth phases from mycelium to mature stages revealing that maximum tyrosinase activity and highest protein content was observed in premature stage. Result for morphological parts evaluation of mushroom showed that maximum tyrosinase was present in stalk region of mushroom. This is first report about the screening of tyrosinase from both morphological parts and developmental phases of mushroom.

### Introduction

Tyrosinases are widely distributed in nature; they are found both in prokaryotic as well as in eukaryotic microbes, in mammals and plants. These enzymes are known as type 3 copper proteins having a diamagnetic spin-coupled copper pair in the active centre. The hydroxylation ability of the enzyme is also referred to cresolase or monophenolase activity (EC 1.14.18.1), and the oxidation ability to catecholase or diphenolase activity (EC 1.10.3.1). Monophenolase activity of Tyrosinases is known to be the initial rate-determining reaction.

Tyrosinases are involved in several biological functions. Presently there is an increasing interest in using tyrosinases in industrial applications. Traditionally tyrosinases have been exploited in plant-derived food products, e.g. tea, coffee, raisins and cocoa, where they produce distinct organoleptic properties. However, in fruits and vegetables, tyrosinases are also related to undesired browning reactions where upon, methods for controlling tyrosinase activity are constantly searched in the food industry Majidi and Aksöz, 2013.

Furthermore, tyrosinase has industrial applications such as its use as biosensor for oxygen and phenols, the stereospecific synthesis of quinones, phenols and phenolic polymers, and in the bioremediation of wastewater containing phenolic pollutants (Fenoll et al. 2002). Thus the present work was aimed to study tyrosinase from various edible mushroom.

The limit of these applications remains in the fact that native fungal tyrosinase are generally intracellular and produced in low quantity (Halaouli et al., 2006). The exploitation of tyrosinase from various parts of mushroom is considered, for *Pleurotus florida*.

Mushrooms are highly nutritive, low-calorie food with good quality proteins, vitamins and minerals. Mushrooms are an important natural source of foods and medicines. Traditional originals knew the medicinal importance of edible and wild mushrooms and these are now being screened for their bioactivity in various ailments. Mushroom represents a major and untapped source of potent new pharmaceutical products. They are also known to possess promising antioxidative, cardiovascular, hypercholesterolemia, antimicrobial, hepatoprotective and anticancer effects. The present paper aimed to discuss on mushroom cultivation as well as importance for tyrosinase extraction. *Pleurotus* mushroom cultivation is very popular and next to *Agaricus bisporus* mushroom in India in popularity and consumption. This together form bulk of edible mushrooms produced in country. In India *Pleurotus* has been successfully cultivated on banana pseudostem and paddy straw. Rice straw, wheat straw, ragi straw, hulled maize cob, waste paper were tried in different studies (Khatun et al., 2012). Use of costly substrate for growing oyster mushroom increases their cost of production. So there was need to

search for certain alternative materials which should be available in sufficient quantity at relatively cheaper price. This paper thus reports the cultivation of *Pleurotus* that has been reported to grow readily on a number of non-conventional substrates.

In the *Agaricus* strain, the epidermis, cap flesh, gill, and stalk tissue showed similar enzyme after isoelectric focusing, but the distribution of staining intensity appeared different for each type tissue. Younger developmental stages of *Agaricus* mushrooms showed a different enzyme profile than older ones. Mature mushrooms cut at the stipe showed a different distribution of enzyme staining than those not cut at the stipe, indicating possible activation of latent enzyme or new synthesis of specific tyrosinase isoenzymes. Till date as per our knowledge no such study is done on *Pleurotus florida*. The present study is the foremost study to locate tyrosinase in various morphological parts of *Pleurotus*.

These measurements have shown the difficulty in comparing the enzyme activity of different growth phases and thus in obtaining reproducible results in the comparative assay of the enzyme activity of mushroom (Boiret et al., 1985). Moreover, assays performed on a unique specimen of a given mushroom parts result on considerable differences in tyrosinase activity depending on the morphological part under study. Therefore we initiated a systematic approach of the enzyme's activity distribution in various morphological and different growth stages.

Much less information is available on the activity, location of tyrosinase in developing mushrooms. That tyrosinase activity is present in the pileus (cap) and stipe (stalk) of mushrooms at three developmental stages, it was reported that the skin

contained more enzyme than the flesh based on either a fresh weight or on a protein basis (Ingebrigtsen et al., 1989).

Thus the present paper was designed to study the activity of tyrosinase in various edible mushrooms, cultivation of the mushroom with maximum tyrosinase activity using agro waste, screening of various growth phases of mushroom for tyrosinase activity and localization of tyrosinase in morphological parts of the mushroom.

## **Materials and Methods**

### **Mushroom Sources**

Two edible mushrooms namely *Agaricus bisporus* and *Pleurotus florida* were purchased from food park Industrial area, Maneri, Mandla, M.P. While milky mushroom was purchased from mushroom farms, INA market, Delhi. Starting culture of *Pleurotus florida* was purchased from directorate of mushroom research (Solan).

### **Primary Screening of Mushroom for Tyrosinase Activity (Boiret et al., 1985)**

Qualitative imaging of mushroom was done to screen them for tyrosinase activity. The mushroom was cut into two longitudinal halves and immediately solution of 0.002 M of L-DOPA in 0.1MTrisHCl buffer (pH 7.0) containing 0.1M Sodium fluoride, pH 7.0 was sprayed in a homogenous manner, on its cross section surface and the appearance of red color along with time was recorded.

### **Secondary Screening of Mushroom for Tyrosinase Activity**

#### **Preparation of Enzyme Extracts (Zaidi et al., 2014)**

The sliced mushrooms were homogenized by warming blender. Enzyme extraction was

prepared with 500 mL of cold 100 mM phosphate buffer (pH 5.8) for 300 g of mushroom. The supernatant was used as a source of enzyme.

### **Determination of Tyrosinase Activity**

The method of Duckworth and Coleman, 1970, was followed to determine the tyrosinase activity. To 10 ml phosphate buffer (50m in pH 6.5) and 10 ml 1mMtyrosine was mixed and oxygenated by bubbling 99.9 % pure oxygen. (Fig.1) 0.1 ml enzyme extract was added to a cuvette containing 2.90ml of above prepared solution and was immediately mixed and the formation of dopachrome at 280 nm was recorded. One unit of enzyme was defined as 0.001 per min increase in absorbance at 280 nm of reaction mixture under standard assay conditions.

### **Determination of Protein Content (Lowry et al, 1951)**

To 0.1 ml of sample, 2.5 ml of alkaline copper sulphate reagent was added, mixed well and allowed to stand at room temperature for 10 min., then 0.25ml of 1:1 double diluted Folin-Ciocalteu's phenol reagent was added and after 30 min of incubation at room temperature, absorbance was measured at 660 nm. The protein content was calculated from a standard curve prepared with bovine serum albumin.

### **Determination of Specific Activity**

Specific activity (EU/ mg) was determined by dividing total activity (EU/ ml) with total protein concentration (mg/ ml).

### **Mushroom Production**

#### **Preparation of Mycelium Culture (Ramkumar et al., 2011)**

Potato dextrose agar medium was prepared and Starting culture was procured from

Solan was used to inoculate PDA plates. Forceps was disinfected with hypochlorite for 5 min and rinsed for 3 times in distilled water were placed on Potato Dextrose Agar in petridish and incubated at 25° C for 10 days.

### **Preparation of Spawn Culture (Ahlwat and Tiwari, 2007)**

Starting culture of *Pleurotus florida* was raised by following the method of Ahlwat and Tiwari, 2007. Small pieces of tissue mycelium (2mm) of *Pleurotus florida* were inoculated in plates of potato dextrose agar media and were incubated at 22 °C for 4 – 5 days. Small mycelium bearing portion was further used as inoculums.

### **Spawn Production (Royse and Sanchez-Vazquez, 2013)**

Wheat Grains were used as a substrate for *Pleurotus florida*. Substrate was soaked in 0.1% carbendazine solution overnight. The solution was drained completely and dried under shade. At 50% moisture level the substrate was mixed thoroughly with both artificial and precipitated CaCO<sub>3</sub>, to ¾ capacities of 500 ml glass bottles. Then the mouth of bottles was tightly plugged with non absorbent cotton and sterilized at 15lbs pressure for 2 hrs. The substrate was allowed to cool and inoculated with fungal stalk culture aseptically and incubated at 25°C for 15 days. 2mm mycelium were added to spawn with CaCO<sub>3</sub> (3%).

### **Extraction of Tyrosinase from each Growth Phase of Mushroom**

### **Preparation of Substrate (Ahlwat and Tiwari, 2007)**

The substrate (wheat straw) 150gm were chopped, soaked in water overnight and drained to remove excess water. 2% Urea

and calcium carbonate were then mixed (w/w), and autoclaved at 15psi, 121° C for 90 minutes.

### **Preparation of Spent Substrate Bags (Ahlwat and Tiwari, 2007)**

250 gm of Wheat straw was used as substrate. Substrate was then filled in thin polythene bags and soaked in water tank for 12 hrs and then bags were removed from water to drain excess of water, calcium carbonate (3%) were then mixed (w/w), and substrate was then autoclaved for 2 hrs at 121°C. After autoclaving, bags were opened and straw was spread on table for 5 hrs and then allowed to cool. Then 250 gm of substrate was put in bags of 12x 18 cm size, sterilized by autoclaving for 2 hrs at 121°C, and was inoculated with 5% spawn of *Pleurotus florida*.

### **Harvesting of Mushroom at Different Growth Phase (Ahlwat and Tiwari, 2007)**

After proper sterilization, inoculation, incubation, mycelium maturation and pigmentation of the tip of bump, all the packets were opened from the top side and soaked in water for 15–20 min. Then the packets were placed separately on the cemented floor of culture house.

The temperature, relative humidity and light were maintained at 18–22 °C, 60–70% and 10–20 lux, respectively. Sufficient water was applied and proper aeration was maintained in incubator to release CO<sub>2</sub> and to supply of proper oxygen for primordial initiation and fruiting body development.

### **Extraction of Enzyme at Different Growth Phase of Mushroom (Zaidi et al., 2014)**

At the end of each stage of development, crude enzyme from the fruiting body of

mushroom was extracted tyrosinase activity was determined according to standard assay method.

## Results and Discussion

### Screening of Mushroom for Tyrosinase Activity

All the four mushrooms were kept under similar condition and were screened qualitatively for tyrosinase activity (Fig-1) quantity substantial tyrosinase activity was determined in two mushrooms (Table-1) they are *Agaricus bisporus* and *Pleurotus florida* significant enzyme activity was observed in these isolates; *Agaricus bisporus* (6.112 Unit/mg), and *Pleurotus florida* (7.74 Unit/mg) followed by *Pleurotus dyjamore* (5.8 Unit/mg) and *Pleurotus oysterus* (3.63 Unit/mg).

### Quantitative Screening of Mushroom Revealed Non-homogenous Distribution of Mushroom Tyrosinase

Part of stem connecting cap revealed presence of tyrosinase in slightly more concentration. The distribution of tyrosinase activity seemed to be more homogenous in cap whereas other part showed even distribution of enzyme. The cap spin and lamellae are collected by peeling and scraping respectively. (Table2).

Stem region showed variation for the presence of tyrosinase with 9.0 Unit/mg, 7.1 Unit/mg and 6.0 Unit/mg respectively.

The result shows that mean mycelium fresh weight was 0.033gm of mycelium produced using 3% calcium carbonate. Mean mycelia extension was 0.161 cm of mycelium. Tyrosinase activity (Unit/mg protein) was maximum for mycelium is 0.68 Unit/mg protein (Table 3).

### Assesment of Mushroom Production, Tyrosinase Activity and Calcium Content

Primordial formation occurred after 20 days of inoculation of spent bags with 8 units of 0.05 gm each and 0.26% biological efficiency and 48.7 moisture content. It has 0.5 IU/3gm Tyrosinase activity and 2.13 mg/ 100 gm dry weight calcium.

Bud stage occurred after 21 days of primordial formation with 5 units of 1.31 gm each and 4.63% biological efficiency and 50.2 moisture content. It has 2.3 IU/3gm Tyrosinase activity and 5.2mg/ 100 gm dry weight calcium (Fig. 2)

Young stage occurred after 12days of bud stage with 3 units of 2.7gm each and 8.13% biological efficiency and 53 moisture content. It has 4.3 IU/3gm Tyrosinase activity and 8.9 mg/ 100 gm dry weight calcium.

Premature stage occurred after 8 days of young stage with 3 units of 4.3gm each and 12.9% biological efficiency and 53.8 moisture content. It has 4.8 IU/3gm Tyrosinase activity and 12.5 mg/ 100 gm dry weight calcium.

Mature stage occurred after 6 days of premature stage with 2 units of 5.2 gm each and 6.93% biological efficiency and 57.2 moisture content. It has 5.6 IU/3gm Tyrosinase activity and 18.1 mg/ 100 gm dry weight calcium (Fig 3).

Mushrooms are appreciated, not only for texture and flavor but also for their chemical and nutritional characters (Manzi et al., 1999). Some recently isolated and identified compounds, originating from mushrooms, show other quite significant medical properties, such as immunomodulatory, cardiovascular, liver protective, anti-fibrotic,

anti-inflammatory, anti-diabetic, anti-viral, and antimicrobial activities (Mata and Salmones, 2003). The success of the commercial mushroom cultivation is largely depending on the quality of components used for its production (Ramkumar et al., 2011) thus we tried to develop the substrate as agrowaste for cultivation of *Pleurotus florida*.

Tyrosinase (monophenol, 3-4 dihydroxy L-phenylalanine (L-DOPA): oxygen oxidoreductase, EC 1.14.18.1) is a bifunctional copper containing enzyme that uses molecular oxygen to catalyze the oxidation of mono - phenols to their corresponding o- diphenols (monophenolase activity) and their subsequent oxidation to o-quinones (catecholase activity) (Faria et al., 2007). The resulting highly reactive o-quinones auto-polymerize to form polyphenolic catechol melanins, which are an important constituent of humus and help

in soil conditioning. Mushrooms are important source of this enzyme and many researchers are exploiting mushrooms for the production of Tyrosinase enzyme (Decker et al., 2000). It also play an important role in the conversion of phenolic compounds to organic derivatives and correspondingly to humus.

Enzyme and their application in different industrial sectors have gained importance during the present industrial age; the present study also exploited this function of tyrosinase (Ikehata and Nicell, 2000).

Results from the study have clearly shown that mushroom species used in the study contained considerable amount of tyrosinase which are vital in supplementing nutrition to mankind and could be used for various biotechnological applications

**Table.1** Tyrosinase Activity of Mushroom

| S.No. | Mushroom                        | Total activity (Units) | Total protein (mg) | Specific activity (U/mg) |
|-------|---------------------------------|------------------------|--------------------|--------------------------|
| 1-    | <i>Agaricus bisporus</i>        | 30.56                  | 5.01               | 6.112                    |
| 2-    | <b><i>Pleurotus florida</i></b> | <b>38.57</b>           | <b>4.98</b>        | <b>7.74</b>              |
| 3-    | <i>Pleurotus dyjamore</i>       | 36.23                  | 6.23               | 5.81                     |
| 4-    | <i>Pleurotus oysterus</i>       | 42.89                  | 11.6               | 3.69                     |

**Table.2** Tyrosinase Activity Measured in the Morphological Parts of Mushroom

| S.No. | Parts of mushroom | Weight (gm) | Total activity (Unit/mg) | Total protein (mg/ml) | Specific activity (Unit/ml) |
|-------|-------------------|-------------|--------------------------|-----------------------|-----------------------------|
| 1-    | Stem              | 3.01        | 39.78                    | 4.36                  | 9.12                        |
|       |                   | 3.12        | 35.79                    | 4.86                  | 7.36                        |
|       |                   | 3.03        | 33.26                    | 4.98                  | 6.67                        |
| 2-    | Cap               | 3           | 28.01                    | 3.84                  | 7.29                        |
|       |                   | 3.05        | 30.89                    | 5.16                  | 5.98                        |
| 3-    | Lamellae          | 2.89        | 27.85                    | 4.87                  | 5.71                        |
| 4-    | Skin              | 2.57        | 20.54                    | 4.85                  | 4.23                        |
| 5-    | Fruit body        | 3           | 40.56                    | 4.12                  | 9.84                        |

**Table.3** Spawn Production of *Pleurotus Florida*

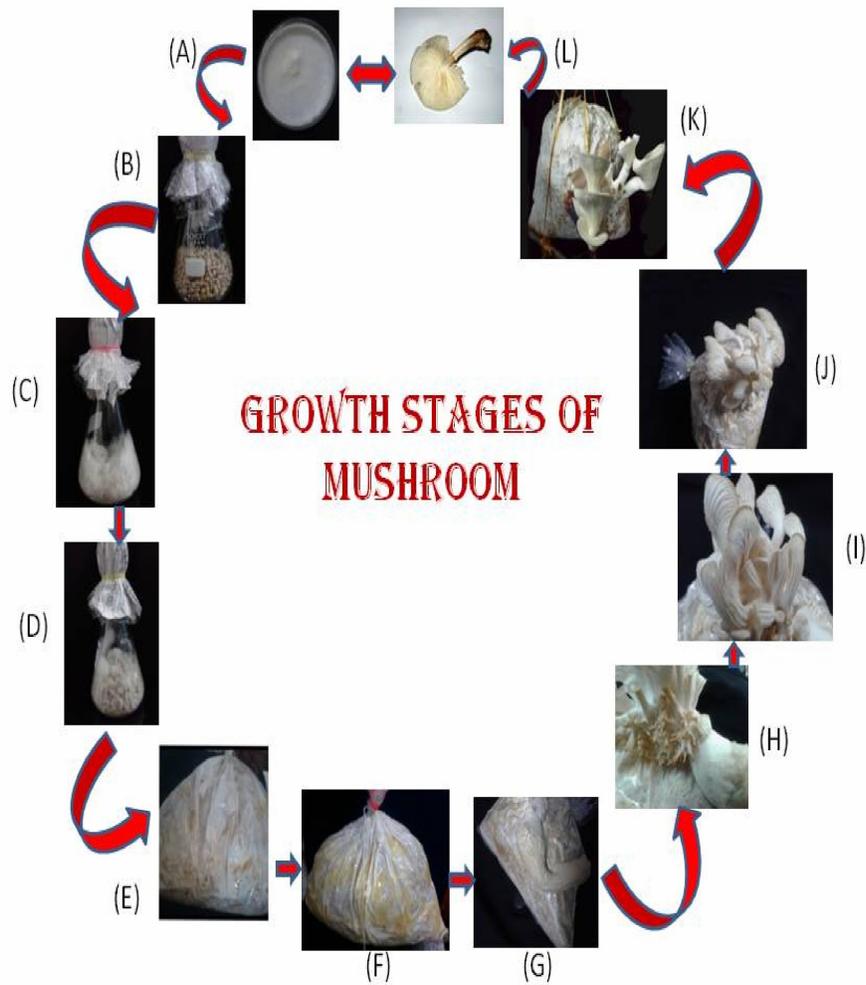
|   |              |
|---|--------------|
| Weight of flask (gm)                    | 97.825       |
|   | 95.216       |
|   | 96.431       |
| Dry weight of grains (gm)               | 82           |
|   | 145.322      |
| Weight of grains (gm)                   | 147.591      |
|   | 143.616      |
| Dry weight basis CaCo <sub>3</sub> (gm) | 2.46         |
|   | 0.012        |
| Weight of mycelium (gm)                 | 0.053        |
|   | 0.034        |
| mean                                    | <b>0.033</b> |
|   | 0.132        |
| Height of mycelium (cm)                 | 0.21         |
|   | 0.141        |
| mean                                    | <b>0.161</b> |
|   | 0.32         |
| Tyrosinase Activity (Unit/mg)           | 0.37         |
|   | 0.38         |
| Mean                                    | <b>0.35</b>  |

**Fig.1** Qualitative Imaging of mMushroom (A= *Pleurotus florida* and B=*Agaricus bisporus*) was Done to Screen it for Tyrosinase Activity



Fig 1. Qualitative imaging of mushroom (A= *Pleurotus florida* and B= *Agaricus bisporus*) was done to screen it for tyrosinase activity. The mushroom was cut into two longitudinal halves and immediately solution L-DOPA in Tris HCl buffer containing Sodium fluoride, was sprayed in a homogenous manner, on its cross section surface and the appearance of red color was recorded.

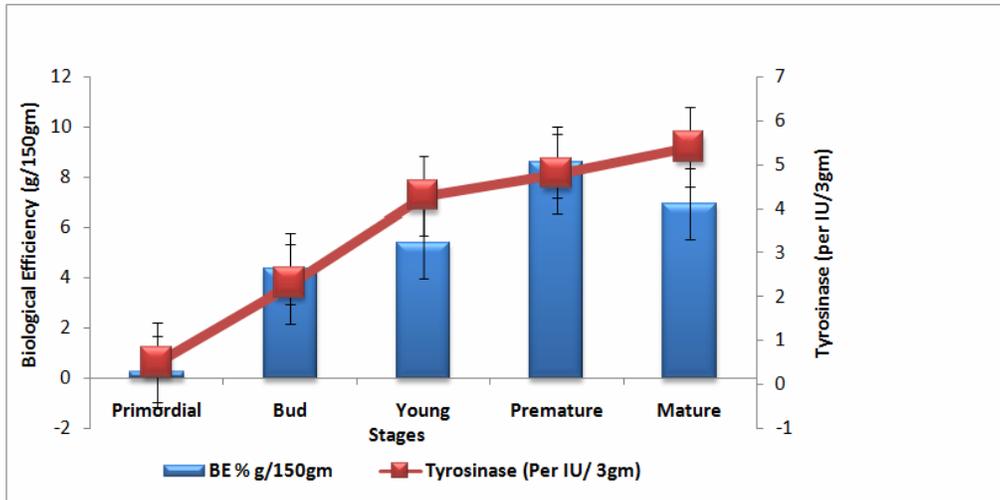
**Fig.2** Different Growth Phases of Mushroom Production



**Fig 2** Different growth phases of mushroom production

- A. Mycelium grown in potato dextrose agar medium
- B. Wheat Grains used as substrate for spawn production
- C. Spawn running
- D. Mother Spawn used for inoculation of spent bag
- E. Spent bag prepared from wheat straw
- F. Spent bag with fully grown mycelium culture
- G. Appearance of bud in spent bag
- H. Appearance of mushroom bud
- I. Mushroom in young stage
- J. Mushroom in premature stage
- K. Mushroom in mature stage
- L. Fully grown mushroom

**Fig.3** Tyrosinase Activity (IU/3gm) and Biological Efficiency (g/ 150gm ) of Mushroom at Different Growth Stages



Mushroom Tyrosinase has been examined extensively with regard to physical and enzymatic characteristics. Many of these studies have used commercial enzymes which were not pure, uncharacterized, lacked all the enzymes present in growing mushrooms, and may have been derived from mixtures of mushrooms strains. In contrast, little information is available on the enzyme in mushrooms during development, post-harvest treatment, and storage. A few studies have been directed toward examining the enzyme during aging, in processed mushrooms, in post-harvest treatments, and in different strains of mushrooms. Even less information is available on the enzyme during specific development stages although tyrosinase was studied in the M8 strain of *Agaricus*, in the D649 and U3 *Agaricus* strain, in *Agaricus bisporus*, and in white *Agaricus bisporus* no such study is reported with *Pleurotus*. The objective of our study was to examine tyrosinase in different strains of Mushroom. We observed that *Pleurotus* has maximum enzyme activity. Distribution of tyrosinase appeared to change with development and activity of some tyrosinase appeared to

decrease with development of the mushroom. This observation was in accordance with Adenipekun and Lawal 2012 who also reported the same. Deliberate damage to the mushrooms changes the distribution of enzyme activity compared to undamaged tissues. Maximum enzyme activity was primarily screened in stem of *Pleurotus* which was confirmed by secondary screening with 9.12 U/mg protein. Similarly highest enzyme activity was observed in premature growth phases of *Pleurotus* which was in accordance with Royse and Sanchez-Vazquez, 2003 who also screened activity in various growth stages of *Lentinus edodes*.

Reason for the above finding could be that during mushroom development many changes occur as the mushroom expands from small pins to the mature mushroom. Tyrosinase was monitored in several developmental stages selected by size and/or maturity. Results have shown that the various developmental stages contained different amounts of protein as well as different amounts of tyrosinase. The enzymes were present in all developmental

stages but showed much less staining intensity in immature and mature mushrooms.

*Pleurotus*, second most used mushroom after *Agaricus* could be grown using agricultural waste. Tyrosinase, enzyme which has lots of biotechnological application could be successfully extracted from various morphological parts of *Pleurotus florida* throughout the developmental cycles of mushroom growth. Thus, this work shows the exploitation of wheat straw for cultivation of *Pleurotus*, which could be consecutively, used for extraction of tyrosinase.

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