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EFFECT OF METAL SALTS ON EXTRACELLULAR MELANIN AND LACCASE PRODUCTION BY *PLEUROTUS DJAMOR* (Fr.) BOEDIJN. AND *PLEUROTUS CITRINOPILEATUS* SINGER

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ABSTRACT
Melanins are among the most stable, insoluble and resistant of biochemical materials and melanins have a strong binding affinity for metal ions. Present study was carried out on isolation and characterization of melanin from an edible mushroom *Pleurotus djamor* and *P. citrinopileatus* at different concentration of metal salts such as Cu, Zn, Ni, were used as the induction of melanin pigment. Both the culture was inoculated into the medium, and then the cultures were kept under continuous light illumination for high amount of pigments production. Colour change was observed. The extracellular melanin were extracted and purified by alkaline and acid treatment. The black colour pigment was confirmed as melanin based on melanization assay, chemical tests, UV-visible and IR spectrum.

KEYWORDS: Melanin, Melanization, Metal salts, *Pleurotus djamor* and *P.citrinoplieatus*.

INDRODUCTION

Most life forms produce melanins, which are dark (usually black), complex, poorly characterized pigments, synthesized enzymatically or auto-oxidatively from a variety of cyclic, heterocyclic, phenolic or other resonance stabilized precursor molecules. One type of melanin found in fungi is eumelanin, derived from tyrosine or the L enantiomer of 3, 4-dihydroxyphenylalanine (DOPA). Oxidation of this precursor by phenoloxidase enzymes initiates the melanization pathway (Butler & Day, 1998). They serve as energy transducers, affect cellular integrity, used for sexual display and camouflage, colouration in black and red hair, major role in innate immune system of insects which synthesize the polymer to damage and entomb microbial intruders, UV irradiation associated with virulence for a variety of pathogenic microbes etc. Isolation and characterization of melanin pigment from *Pleurotus cystidiosus* was done by Selvakumar *et al*. (2008). The pigment was confirmed as melanin based on UV, IR and EPR spectra. In all fungi DHN is synthesized in the polyketide pathway which involves the condensation and cyclization of acetate to form 1,3,6,8, tetra hydroxynapthalene (THN) followed by alternating reduction and dehydration reactions to form 1,8,-DHN which finally undergoes oxidative polymerization to form melanin (Kubo and Furusawa, 1991). Melanin is the natural protection against the harmful effects of ultraviolet rays or high frequency light from the sun (Tae joon *et al.,* 2008). Melanin can breakup water molecule and give up energy suggesting an additional behavior for melanin. Melanin exhibit electrical conductivity and photoconductivity in the condensed phase. It has been speculated that melanin may be a bio-organic semiconductor (Arturo Solis *et al.,* 2007). In view of the need for documenting the efficiency of mushroom fungi in extracellular melanin and laccase production for their biotechnological potential, the present work is designed to compare the efficiency of two *Pleurotus* (Oyster mushroom) species *viz*., *Pleurotus djamor* and *Pleurotus citrinopileatus* in extracellular melanin and laccase production.

MATERIALS AND METHODS

The slant culture of *Pleurotus djamor* (Fr.) Boedijn. were procured from Tamil nadu Agricultural University, Coimbatore. The cultures were maintained on Potato Dextrose agar (PDA) plates and stored under refrigeration. Zinc sulphate, Nickel sulphate, Copper sulphate, L-Dopa used for the regulation of melanin production were of analytical grade and were purchased from SD (fine chemicals), India Pvt.Ltd, Chennai. The composition of the Sabouraud Dextrose broth (g/l) as follows viz., Peptone-10, Dextrose (or) Maltose- 20, Distilled water-1000ml, pH-5.6.

Culture growth and biomass in the presence of metal salts

The selected mushrooms were grown by inoculating a single mycelilal agar plug into the medium. The plates were incubated at room temperature (28±2°C). The radial growth of the mushroom colonies and change in colour of the medium were noted. Sabouraud Dextrose Agar (SDA) medium was prepared and taken in Erlenmayer flasks (250ml). The flasks with the medium were sterilized at 15 pounds pressure and 121°C for 15 minutes. Then the medium was cooled and poured into petri plates (9cm diameter). For the treatments testing the effect of various metal salts on the induction of melanin production by the selected mushroom species, different concentrations of sterilized solutions of the salts (0.005% to 06% w/v) were added to the medium just before pouring it into petri plates (Angeetha ana Eyini, 2010).

Melanization Assays

Growth on minimal and starvation media

For *in vitro* melanization assays, the selected mushroom cultures were inoculated in the defined Minimal agar medium (MMA).The Composition of the Minimal medium (mM) as follows**,** Glucose – 15, $MgSO_4$- 10, KH_2PO_4 - 29.4, Glycine- 13, Thamine- 3, Dopa – 1, Distilled water-1000ml, pH- 5.5.A mycelial agar plug from 6 days old fungal culture was inoculated into the centre of the plate. The plates were incubated in the dark for several days at room temperature (28± 2°C). Plates were examined daily to monitor growth and pigment production. A mycelial agar plug from 7 days old fungal culture from the minimal medium was inoculated on the starvation medium. The composition of the Starvation medium (g/l) as follows, K_2HPO_4-0.02g, KH_2PO_4- 0.01g,Agar- 1.5g,Distilled water-100ml, pH -7. The plates were incubated at room temperature (28 ± 2°C) for 3 days. Drops of a solution of L -DOPA (0.3% w/v) were pipetted directly on to the culture. Pigment formation was monitored visually over a time of 5-6 minutes (Chaskes *et al.,* 1981).

Biomass

Biomass fresh weight was measured by weighing the mycelial mat after blotting it dry on coarse filter paper.

Extraction and purification of melanin

The extraction and purification of melanin pigment was measured by using Gadd method (1982). In that supplementation treatments in which the colour of the cultutre fluid of *Pleurotus djamor,* change to dark brown or black were chosen for extraction and purification of the pigments.

Qualitative confirmative test for melanin

Qualitative confirmative test for melanin was measured by the method of Babitskaya *et al*., 2000.

Chemical tests for melanin

The isolated melanin pigments were treated in 9 different chemical tests and observations were noted (Fava *et al.,* 1983**).**

Immobilization of melanin

The samples were suspended in sterile tap water and mixed with 50 ml of 4% sodium alginate. Then the suspension was passed through a narrow tube and was dropped into a 50 µm calcium chloride solution (pH 6.8) in the flasks. The beads obtained were inoculated in the $CaCl_2$ solution at 20ºC-22ºC for 2 hours. The cross section of the beads were taken and observed under the microscope.

Laccase activity

Laccase activity was measured by using Steffen methods (2003).

Spectroscopic studies

The pigment obtained from the mushroom fungal culture broth was dissolved in 3 ml of borate buffer (pH 8.0) and its UV-Visible spect recorded in Burker 72 FTIR Spectrophotometer (Selvakumar *et al*., 2008).

RESULTS

Two species of edible oyster mushroom *Pleurotus djamor* (Fr.) Boedijn and *Pleurotus citrinopileatus* Singer (Plate 1) were studied for the present investigation.

Culture growth and biomass in sabouraud medium

The selected mushroom species were grown in Sabouraud broth supplemented with copper sulphate, zinc sulphate or nickel sulphate in the concentration range between 0.02%-0.06% (w\v). Both *P. djamor* and *P. citrinopileatus* could not initiate mycelial growth in the presence of supplemented nickel sulphate and zinc sulphate in the selected concentration. The mycelial growth of *Pleurotus djamor* was progressively inhibited with an increase in concentration of 0.01% to 0.06% copper sulphate compared to the control the colour of the broth changed from bluish green to brown through yellow brown in the range of concentration between 0.005% to 0.015% copper sulphate. At higher concentrations (0.02% to 0.06%) the broth was dark brown in colour.

The biomass of *P. djamor* was not affected by copper sulphate at 0.005% concentration, while the threshold level of copper toxicity to *P. citrinopileatus* was observed from 0.005% concentration itself. The mycelial growth of *P. citrinopileatus* was more sensitive to copper sulphate concentration, mycelial growth was insignificant at 0.06% copper supplementation and color change could not detected at or above 0.02% copper sulphate supplemented sabouraud broth. Mycelial biomass of *P. citrinopileatus* gradually decreased control to 0.06% copper sulphate supplementation.

P. citrinopileatus biomass was significantly inhibited by copper sulphate at 0.02% and above, bringing about nearly 90% inhibition of biomass at 0.02% copper sulphate supplementation. While 90% inhibition of *P. djamor* biomass was observed at 0.06% copper sulphate supplementation. Higher radial growth were observedin *P.djamor* when compare to *P.citrinopileatus.*

Melanization Assays

Both *P. djamor* and *P. citrinopileatus* grew to in the minimal medium. When Dopa was added to the minimal medium a brown zone was observed below at the reverse of the culture in both the organisms, both under light and dark incubation. The Z/C ratio showing the extent of Dopa utilization was higher for *P. djamor* than for *P. citrinopileatus*

Growth of both *P. djamor* and *P. citrinopileatus* was less on starvation medium compared to their growth on minimal medium. Pigment formation was observed when Dopa was added to both the cultures in drops. Initially a pink pigment formed within 15 minutes and over time within 1 hour, a brown pigment formed in both the *Pleurotus* cultures.

Identification parameters for melanin

The dark brown pigment in the extracellular broth of 14 days old *P. djamor* and *P. citrinopileatus* was extracted, partially purified and it was subjected to several tests to prove its melanin nature. The pigment was insoluble in water and organic solvents but was soluble in 1M NaOH at 100°C for 2 hours. The pigment was dark brown in colour and it precipitated readily in the presence of 3N HCL. Oxidation agents like H_2O_2 decolorized the pigment while to brown precipitate was formed in response to polyphenols, Sodium dithionite decolorized the pigment while addition of potassium ferric cyanide solution turned the pigment into brown colour. The pigment formed a grey coloured silver precipitate lining the sides of the test tubes. Based on these parameters, the pigment was identified as melanin**.**

The dilution of extracted melanin pigment was subjected to 1% potassium permanganate and allowed to stand for 15

mins. The colour change from green to brown indicates the conformation of the melanin pigment. Thus the melanin pigments were qualitatively analysed.

Extracellular laccase activity

Extracellular laccase activity of *P. djamor* was significantly higher than that of *P. citrinopileatus*. Laccase activity increased over the control at 0.005% copper sulphate concentrations in the sabouraud broth. Copper sulphate at and above 0.01% in the nutrient medium significantly inhibited the laccase activity of both the organisms

Spectroscopic Analysis of partially purified pigments

The UV – Visible spectrum of the pigment isolated from sabouraud broth containing copper sulphate showed strong absorbance in the UV region. The absorbance progressively decreased at the visible wavelength. The absorption spectra showed characteristic absorbance peaks in the UV – Visible region from 460 – 490 (Figure 1).

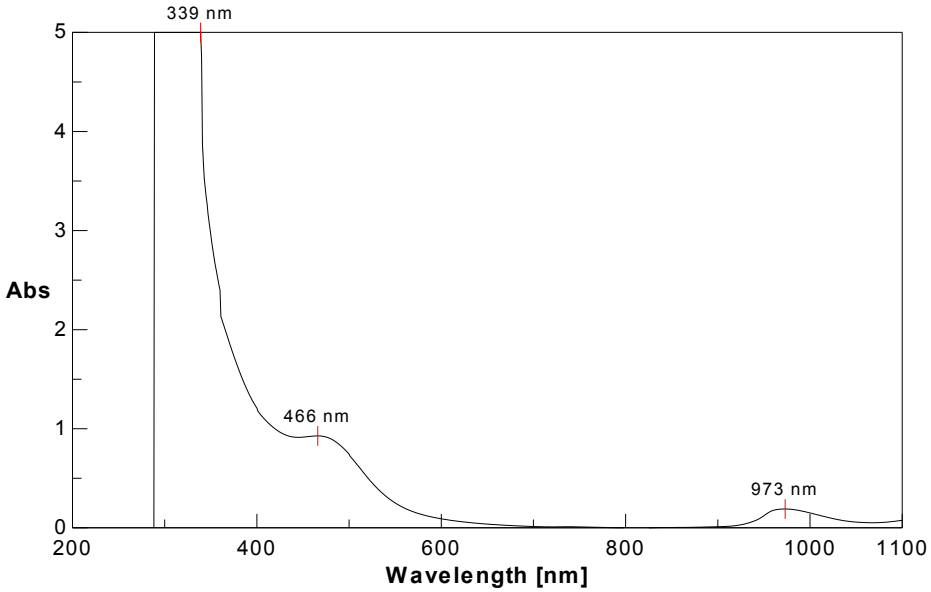

FIGURE 1: UV – Visible Spectrum of extracellular melanin pigment of *Pleurotus djamor* growing in sabouraud broth

The extra cellular pigment of *P. djamor* and *P. citrinopileatus* growing in nutrient media containing copper sulphate was extracted partially purified and characterized by FTIR. The FTIR spectrum of the pigment showed several peaks from 400 to 4000 $cm^{-1.}$ A broad absorbance band near 3424.96 cm^{-1} or 3411. 46 cm^{-1} was commonly present in all the spectra. Common bands were observed near 2923-2925 cm^{-1}, 2853-2857 cm^{-1}, 2358-2360 cm^{-1}, 1731-1740 cm^{-1}, 1454-1456 cm^{-1}, 1024-1028 cm^{-1}.

The initial pH of the sabouraud broth is 5.6, but the pH changed to 4 after 28th day of incubation period. When the initial pH of the sabouraud medium was kept at pH 4 growth of *Pleurotus citrinopileatus* was inhibited while growth of *P. djamor* was not affected. But copper sulphate supplementation at broth pH 4, completely inhibited the biomass and melanin production in both the organisms.

Antimicrobial activity of melanin solution

Aqueous melanin extracted from both *P. djamor* and *P. citrinopileatus* was characterized for its antimicrobial activity against *Escherichia coli* and *Bacillus subtilis*, the diameter of inhibition zone was higher against *Bacillus subtilis* than the inhibition zone produced against *E.coli* .

DISCUSSION

Melanins are high molecular weight pigments that are ubiquitous in nature (Hill 1992). They are dark in dark in colour, insoluble in aqueous or organic solvents, resistant to concentrated acid, susceptible to bleaching agent (Nicholus *et al.,* 1964; Prota, 1992; Butler and Day, 1998). Melanins are negatively charged, hydrophobic pigments (White 1958, Nosanchuk and Casadevell, 1997, Nosanchuk *et al.,* 1999, Jacobson, 2000). Melanins also chelate metal ions (Hong and simon 2007) a property that might be useful in bioremediation strategies whereby melanized microorganisms such as bacteria or fungi are used to collect and immobilize heavy metal pollutants (Turick *et al.,* 2003).

The present investigation was set to the extra cellular melanin production in *Pleurotus djamor* and *Pleurotus citrinopileatus.* The objective of the present investigation was set to investigate the extra cellular laccase and melanin production in two different mushroom species during their life cycle and to study the effect of different metal ions like copper sulphate, nickel sulphate, zinc sulphate on the extracellular melanin production.

Many fungi bind or sequester metals and could be exploited as heavy metal bioremediators. Some fungi secrete chelating compounds that sequester metal ions extracellularly. Others secrete organic acids, such as oxalic acids, that form extra cellular complexes or crystals with metal ions. Metal ions are also actively transported into fungal cells, where they may bind specific proteins, such as the metallothioneins, or possibly become sequestered in organelles (Kosman, 1994). *Saccharamyces cerevisiae* generate H_2S that traps metal ions as insoluble sulfides either intra cellularly or in periplasm (Ashida and Higashi, 1963; Gutierrez, 1994).

Both *P. djamor* and *P. citrinopileatus* could not initiate mycelial growth in the presence of supplemented nickel sulphate and zinc sulphate. The mycelial growth of *P. djamor* was progressively inhibited from concentration of 0.01% to 0.06% (2.27g to 0.15g/ml) compared to the control. Several research papers have reported the melanization of hyphae of ascomycetes or basidiomycetes fungi in the presence of several melanin inducers (Hill 1992). Copper and iron complexation by soil humic material derived from fungal melanins had been reported by Saiz-Jiminez and Shafizadesh (1984). Rizzo *et al*., (1982) reported the binding of metals to the mycelial melanin of *Armillaria* spp. Found that the melanized rhizomorph mycelia concentrated Al, Zn, Fe and Cu ions. Some fungal melanins are efficient bio absorbents of copper (Gadd and De. Rome, 1998) and some fungi produce more melanin in response to copper (Caesar-Tonthat *et al.,* 1995). Extra cellular production of brown or black coloured pigments in the culture fluid has been reported by Butler and Day (1998) and Langfelder *et al.,* (2003). The present investigations indicating melanization of hyphae did not occur either in *Pleurotus djamor* and *P. citrinopileatus* in the presence of copper sulphate but these melanin inducers were found to influence extra cellular melanin production by these two organisms. Melanization plate assay shows the colonization of *P. djamor* and *P. citrinopileatus* on minimal agar media supplemented with the 1mM DOPA. Colony diameter of both the organisms was increased significantly over the control when supplemented with L-DOPA (1mM). Colony diameter of *P. djamor* was found to increase with the corresponding increase in concentrations of copper sulphate, in contrast with its biomass production which decreased from the control and the decrease was inversely related to the copper sulphate concentrations. The extra cellular melanin pigments in the culture fluid of *P. djamor* receiving copper sulphate (0.005% -0.06%) supplementation were extracted partially purified and subjected to the 9 different chemical tests as described by Thomas (1955). The pigment could be extracted with 1M NaOH or 1M KOH with a standard procedure used for the extraction of melanin by Gadd (1982). Autoclaving the KOH or NaOH washed black coloured mass of arthospores at 120°C for 20 min., facilitated the dissolution of the pigment in KOH or NaOH. In the present study, the partially purified pigment confirms the characteristic of melanin and the pigment answered positively to all the chemical tests that are diagnostic for fungal melanin as described by Thomas (1955). The black or brown pigments of *P. djamor* and *P. citrinopileatus* could not be extracted with organic solvents acetone, benzene, methanol, hexane and petroleum ether. The pigment was black coloured and insoluble in water and ethanol benzene, chloroform and acetone. The pigment was soluble in alkaline solution and in phenol. The dissolved pigment was decolorized by oxidizing and resulting reagents such as NaOCl, H_2O_2, H_2S and $Na_2S_2O_4$. The pigmented tested positive for polyphenols with $FeCl_3$, producing a flocculent brown precipitate and reduced ammonical silver nitrate (Bell and Wheeler, 1986).

There was a significant difference in the laccase enzyme activities of *P. djamor* and *P. citrinopileatus* in copper sulphate supplemented sabouraud broth. *P. djamor* lactase enzyme activities were significantly higher than that of *P. citrinopileatus*. The granules of melanin were immobilized and observed under the microscope. They were measured to be in the size range of 1 µm. The size of the melanin granules were reported to be having a diameter of less than 800 nm.

UV Visible spectrum was typical adsorption profile of melanin. Melanin adsorbed strongly in the UV region and progressively less at the wavelength increased (Bell and Wheeler 1986). The extra cellular pigment of *P. djamor* and *P. citrinopileatus* growing in nutrient media containing copper sulphate was extracted partially purified and characterized by FTIR. The FTIR spectrum of the pigment showed several peaks from 400 to 4000 $cm^{-1.}$ A broad absorbance band near 3424.96 cm^{-1} or 3411. 46 cm^{-1} was commonly present in all the spectra. Common bands were observed near 2923-2925 cm^{-1}, 2853-2857 cm^{-1}, 2358-2360 cm^{-1}, 1731-1740 cm^{-1}, 1454-1456 cm^{-1}, 1024-1028 cm^{-1}. The IR spectrum of the pigment was characteristic of fungal melanin. Suryanarayan *et al*. (2004) have described the IR spectrum of melanin with peaks near 3352.5cm^{-1} ascribed to – OH and – NH bonds in melanin (Baner and Dunken 1962), The IR spectrum of present study exhibited a broad absorption band near 3424.96 cm^{-1} or 3411.46 cm^{-1} which revealed the presence of hydroxyl groups. The broadening of the band might be due to the hydrogen bonding of OH with the amine groups in melanin. The peak at 2923 or 2925 cm^{-1} which appeared as a minute projection was assigned to N-H stretch absorption appeared at 1024 or 1028 cm^{-1} was attributed to aromatic ring or CH stretching. Aqueous melanin extracted from both *P. djamor* and *P. citrinopileatus* was characterized for its antimicrobial activity against *Escherichia coli* and *Bacillus subtilis*, the diameter of inhibition zone was higher against *Bacillus subtilis* than the inhibition zone produced against *E.coli*. Similar results were reported by several scientists (Sanmee, *et al*., 2003; Manzi *et al.*, 1999). Smania *et al*. (2001) demonstrated antimicrobial activity of *Ganoderma applanatum* against *Bacillus cereus, Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa*.

In that present investigation both the organisms were inoculated in the specific medium. Regular colour were observed. Extracellular melanin was produced in both the organisms at different concentrtation. High amount of extracellular melain were observed in 0.005% to 0.01% in P.djamor than the P.citrinopileatus. Extracellular melanin were centrifuged and qualitatively analysed.

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