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Suganya D
Post Graduate student
Department of Microbiology,
Valliammal College for Women,
E9, Anna Nagar East,
Chennai, Tamil Nadu, India

Soundhari C
Associate Professor,
Department of Microbiology,
Valliammal College for Women,
E9, Anna Nagar East,
Chennai, Tamil Nadu, India

Correspondence
Soundhari C
Associate Professor,
Department of Microbiology,
Valliammal College for Women,
E9, Anna Nagar East,
Chennai, Tamil Nadu, India

Isolation and partial purification of laccase from *Calocybe indica* and its application in dye decolourization

Suganya D and Soundhari C

Abstract
Aim: To isolate laccase from Indian milky mushroom *Calocybe indica* and evaluated its potential in dye decolorization
Methods: Quantitative assay of laccase activity in broth was done spectrophotometrically using guaiacol broth as a substrate. Laccase enzyme was partially purified from *C. indica* by ammonium sulfate fraction. The fractions were collected and assayed for laccase activity and for protein concentration. Protein concentration was determined by the method of Brad-ford (1976) using bovine serum albumin (BSA) standard. After protein estimation, the purified sample subjected to SDS –PAGE analysis. Laccase is one of the major sources used for the dye decolorization. Decolorization of dye using laccase enzyme from *C. indica* was studied at different concentration (mg/ml) spectrophotometrically. The maximal toxic free concentration of laccase enzyme was evaluated on Vero cell line.
Result: The reddish brown color developed due to oxidation of guaiacol by laccase which was measured at 450 nm. The partially purified enzyme, when subjected to SDS PAGE analysis, revealed a molecular weight of 45kDa. HPLC analysis was performed which showed laccase at RT 4.572. The enzyme from *C. indica* was able to decolorize synthetic dyes malachite green to 83% and congo red 79% after 78 hrs of incubation. The maximal toxic free concentration evaluated on Vero cell line found it to be nontoxic at the concentration tested.
Conclusion: From the present study it can be concluded that the *C. indica* is a promising source for extracellular laccase production.

Keywords: Laccase, milky mushroom, guaiacol assay, dye degradation

Introduction
Synthetic dyes used in the textile industry dying and printing process are generally released into effluent waters. These dyes are non-biodegradable in nature and disrupt the whole wastewater ecosystem leading to serious environmental problems. Physical and chemical methods employed to remove these dyes are not effective due to low degradability of dyes and also it is not economically viable. Hence there is much interest in the enzymatic removal of colors.
Laccase is copper-containing oxidases that catalyze the oxidation of a wide variety of organic and inorganic compounds [1]. Laccase is an extracellular enzyme that can be easily isolated, purified and characterized by conventional methods and which due to their several applications has attracted increasing scientific attention in recent years. Laccase has a major role in the degradation of lignin and reported to have a wide range of application in effluent decolorization, detoxification of industrial effluents, removal of phenolics, used as a bioremediation agent to clean up herbicides, pesticides in soil etc [2].
Many microorganisms including fungi and their enzymes are reported to decolorize textile dyes. Enzyme-based dye decolorization method have the advantage of being eco-friendly and have low energy requirements [3]. Laccase is a common enzyme in nature. Laccase has been found in Ascomycetes, Basidiomycetes, Deuteromycetes fungi. Laccase of fungi is of particular interest because of their capability to oxidize a wide variety of industrial substrates. Solid state and submerged mode of fermentation have been used intensively for the production of laccase [4].
Calocybe indica is a white – rot fungus with great industrial importance [5]. This mushroom is getting popular by its snow white color with a long stipe and a robust pilus and is morphologically similar to *Agaricus bisporus*. In this context the present study was aimed to isolate and purify laccase from Indian milky mushroom *Calocybe indica*, to evaluate its

potential in dye decolorisation and study its cytotoxicity effect by MTT assay on cell lines.

Methodology

Collection of mushroom

The commercially available fruiting bodies of *Calocybe indica* was obtained from commercial outlets in Chennai.

Synthesis of laccase enzyme from mushroom

Synthesis of laccase from mushroom was carried as follows. The mushroom was cleaned thoroughly. Potato dextrose agar was prepared, autoclaved and sterilized. The PDA was supplemented with chloramphenicol 150mg/L to reduce the number of bacterial contaminants. The spawn region of mushroom was taken using sterile inoculation needle and spatula and kept in the PDA agar plate. The plates were incubated at room temperature around 27°C for the growth of fungi. The growth of mycelia was observed after 12 days. The fungi were identified by their morphological and mycelial growth. From the developing mycelium, mycelial tissues were transferred using inoculation needles to fresh PDA plates and slants and subcultured routinely [5]. Chaudhary *et al.,* 2016 [5]

Mycelia growth in PDB broth

100 ml of Potato dextrose broth (PDB) was prepared in Erlenmeyer flask. The broth was autoclaved for 30 min. And the broth was allowed to cool to hand bearable temperature. Approximately four to five mycelial discs from growing edges from PDA was inoculated into the broth aseptically. The inoculated flask was incubated for 12 days at room temperature around 27°C in static conditions with intermittent shaking [5].

Visual confirmation of laccase:

The mycelia on PDA were screened for visual confirmation for the presence of laccase enzyme by using guaiacol in the media. Guaiacol is one of the substrates of the laccase enzyme and the degradation product of guaiacol is reddish in color. Various authors reported that the laccase enzyme catalyzes the oxidative polymerization of guaiacol to form reddish brown zones in the medium. A reddish color zone around mycelial culture in the medium indicates the laccase production by the mushroom. The plates inoculated with mycelial cultures of *C. indica* were observed for the development of a red color during the incubation period.

Guaiacol assay method:

Twelve days old PDB grown culture was centrifuged at 10000 rpm for 15 mins. The supernatant was collected separately and the pellet was saved for further process. Collected supernatant was used for the assay method. Laccase activity was assayed using guaiacol as a substrate [1]. The reaction mixture was prepared as follows. To one test tube (2Mm) of 1ml guaiacol solution, (10mM) 3ml of sodium acetate buffer, 1ml of enzyme source (fungal supernatant) was added. To another test tube, (2Mm) of 1ml guaiacol solution, (10mM) 3ml of sodium acetate buffer and 1ml of distilled water was added instead of enzyme which served as blank. The mixture was incubated at 30°C for 15 min. After 15 min reddish brown color developed due to the oxidation of guaiacol and the absorbance was read at 450nm using UV spectrophotometer. Enzyme activity was expressed as International Units (IU), where 1 IU is the amount of enzyme required to oxidize 1 µmol of guaiacol per min. The laccase activity in U/ml is calculated by this formula:

E.A = A x V/t x e x v

Where, E.A = Enzyme activity, A =Absorbance, V =Total mixture volume (ml), v = enzyme volume (ml) t =incubation time, e = extinction coefficient for guaiacol (0.6740 µM/ cm).

Ammonium sulfate precipitation:

A crude extract of *C. indica* laccase was fractionated by ammonium sulfate precipitation in an ice bath using the finely ground ammonium sulfate. The power was weighed (100 ml of mushroom extract and 31 gram of ammonium sulfate) and added slowly to the extract by constant stirring to ensure complete solubility, and the solution was centrifuged at 10,000 rpm for 15 min at 4°C. The supernatant was collected in a separate beaker. Pellet was collected in an Eppendorf tube and 200µl of sodium acetate buffer was added and vortexed.

Dialysis

Each precipitated fraction was resuspended in a small volume of 0.1 M sodium acetate buffer and subjected to dialysis to remove salt before laccase activity assay and protein determination.

Estimation of protein: **Bradford Assay Procedure**

Two blank tubes were set. For the standard curve 60µl water was added instead of the standard solution. For the unknown protein sample, 60µL protein preparation buffer was added. The protein solution was assayed in triplicate. 3ml of Bradford reagent was added to two test tubes and were incubated at room temperature (RT) for 5 min. Absorbance was measured at 595nm. A standard graph of concentration Vs absorbance was plotted.

Sodium Dodecyl Sulphate-Polyacrylamide Gel

The purity of the enzyme was checked by SDS-PAGE as per standard procedure two microfuge tubes were taken and 2.40µl of sample and 40µl of sample solubilizing buffer was added to one microfuge tube it was boiled at 100°C for 5-10 min in boiling water bath. 40µl of the sample was loaded in the first lane and the protein molecular weight was added to the next nearest lane.

HPLC analysis

The HPLC for laccase was carried out based on the method followed by Bagewadi, 2017.[6] a sample volume of 25 µL was injected into the system, and the detection was carried out at a wavelength of 255 nm.

Degradation of dye

Two synthetic dyes such as malachite green and congo red were studied for decolorization by the method of Majolagbe *et al.,* 2013 [4]. The dyes were tested at various concentrations for a period of 72 hrs. The reaction mixture containing dye solutions (25µl, 50µl, 75µl, 100µl) were incubated with purified laccase (100µl) at 30°C. The readings were taken according to the time interval in the UV- spectrometer at 618nm for malachite green, and 485 nm for Congo red. The percentage of decolorization was calculated according to the formula:

$$\% \text{ Dye Decolorization} = \frac{\text{Initial absorbance (Ac) - Final Absorbance (As)} \times 100}{\text{Initial absorbance (Ac)}}$$

Where Ac is the absorbance at the maximum absorption without enzyme, as is the absorbance with the enzyme.

Estimation of *in vitro* cytotoxicity activity on Vero cell line
The vero cell line was obtained from National Centre for Cell Sciences, Pune (NCCS). The cells were maintained in DMEM supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 µg/ml) in a humidified atmosphere of 50 µg/ml CO2 at 37 °C. *In Vitro* assay for Cytotoxicity activity was done by MTT assay[7]. The % cell viability was calculated using the following formula:
% Cell viability = A570 of treated cells / A570 of control cells × 100

Result
The mushroom C. indica was cultured in PDA plates and later in PDA slants and was incubated at room temperature at 30°C for 12 days to obtain mycelial culture. The mycelia growth was observed for their colony characteristics and stained by lactophenol cotton blue for mycelial characteristics. A white mycelial growth was obtained confirmed to be *C. indica* (fig.4). Observed similar characteristics on par with the present investigation.

Visual confirmation of laccase
The mycelia on PDA supplemented with guaiacol were screened for visual confirmation for the presence of laccase enzyme. Results of the screening test of laccase activity showed a large red-brown zone around the mycelia after four days of incubation and revealed that strong laccase activity (fig.1)

Laccase assay by Guaiacol method
Laccase enzyme was assayed using guaiacol solution in two different test tubes one test tube for the sample and other for control. After 15 mins reddish brown color developed in the sample due to the oxidation of guaiacol by laccase and it was used to measure enzyme activity at 450nm using UV spectrophotometer. (Fig: 2).

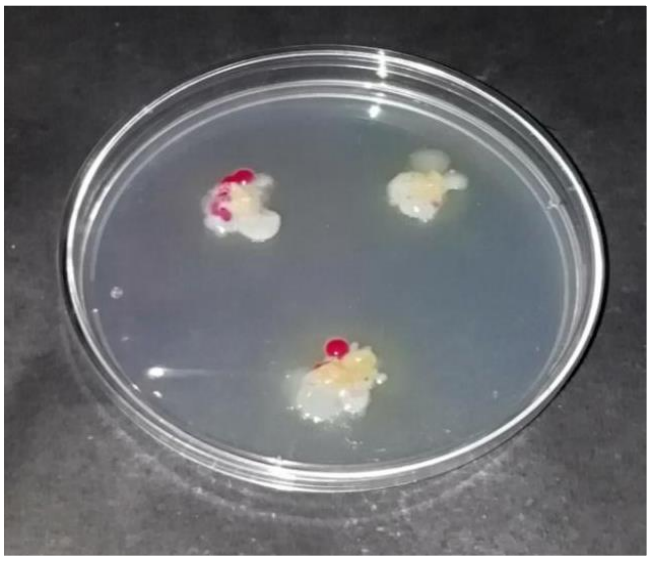


Fig 1: Extracellular Laccase Production on PDA

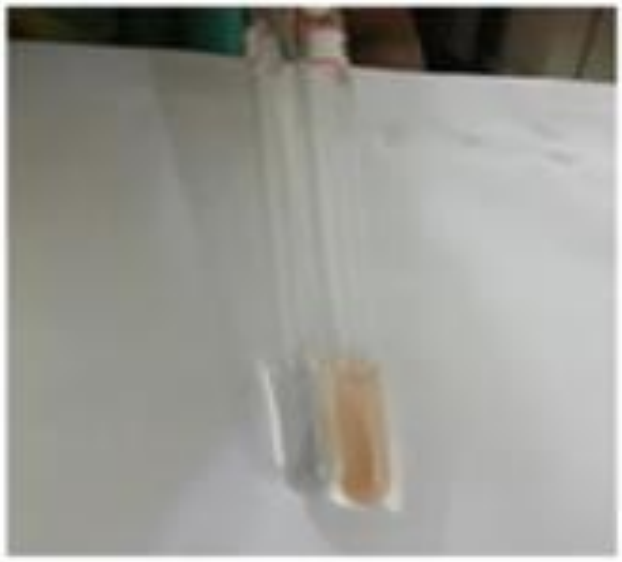


Fig: 2 Laccase assay

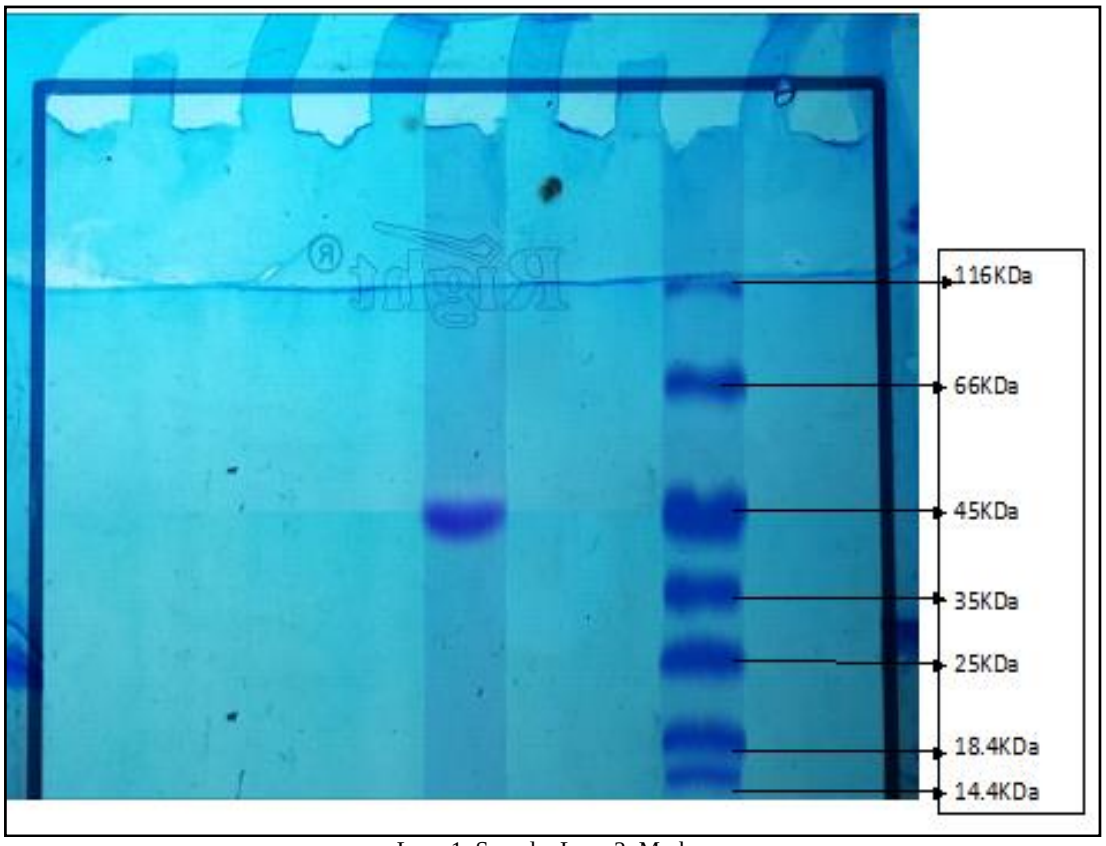
Purification of laccase from *Calocyba indica*
Laccase enzyme was partially purified from *C. indica* by dialysis and SDS-PAGE. The sample was dialyzed against sodium acetate buffer using dialysis membrane.

Protein estimation
Protein concentration was determined by the method called Bar-ford (1976) using bovine serum albumin (BSA) standard as observed in (Fig: 3).

Fig 3: Estimation of Protein

SDS-PAGE

The purified laccase was analyzed by SDS-PAGE. The proteins were separated and bands were visualized under UV Transilluminator. The purified laccase demonstrated a single band in SDS-PAGE analysis. The molecular weight was found to be 45KDa (fig. 4)



Lane 1: Sample; Lane 2: Marker

Fig 4: SDS-PAGE Analysis of sample laccase

***In vitro* cytotoxicity on Vero cell lines**

The mushroom extract was evaluated for cytotoxic effects on Vero cell line. The morphology of the cells was inspected daily and observed for microscopically detectable alterations. Cell viability was monitored after 48hrs and was recorded as micrographs. The extracts were nontoxic from the highest concentration tested 1000µg/ml (table 1)

Table 1: Cytotoxicity Effect of Sample *on Vero* Cell Line

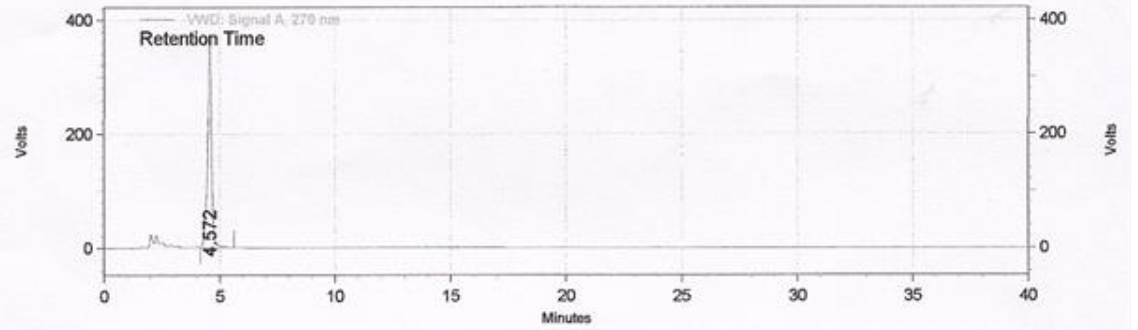
S. No	Concentration (µg/ml)	Dilutions	Absorbance (O.D)	Cell viability (%)
1	1000	Neat	1.072	**52.26**
2	500	1:1	1.210	58.99
3	250	1:2	1.371	66.84
4	125	1:4	1.492	72.74
5	62.5	1:8	1.612	78.59
6	31.2	1:16	1.786	87.07
7	15.6	1:32	1.924	93.80
8	7.8	1:64	2.043	99.60
9	Cell control	-	2.051	100

HPLC for laccase

To confirm the presence of laccase from *C. indica*. We subjected to HPLC analysis. The concentration of laccase in the sample were compared against the literature reference at a retention time and peak area. The laccase showed similar peak area and retention time as reported earlier which indicates the presence of laccase in the isolated sample. Chromatography and analysis of *C. indica* indicate the peak of laccase with the retention time of 4.572(RT). The retention time was much similar to the time obtained in the reference (Graph 1)

Area % Report

Data File: C:\EZChrom Elite\Enterprise\Projects\Default\Data\23-02-2019\P SAMPLES\5 LACCASE
Method: C:\EZChrom Elite\Enterprise\Projects\Default\Method\FLAVONOIDS.met
Acquired: 23-02-2019 21:46:51
Printed: 25-02-2019 12:06:50

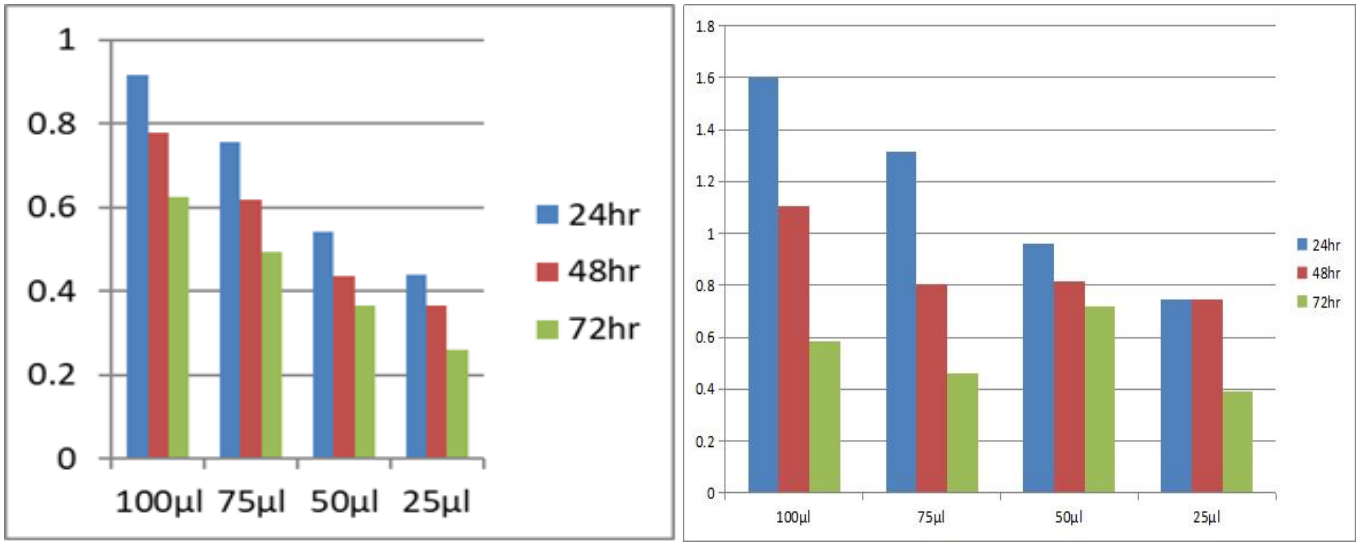


Retention Time	Area	Area %	Height	Height %
4.572	76928431	100.00	6271612	100.00
Totals	76928431	100.00	6271612	100.00

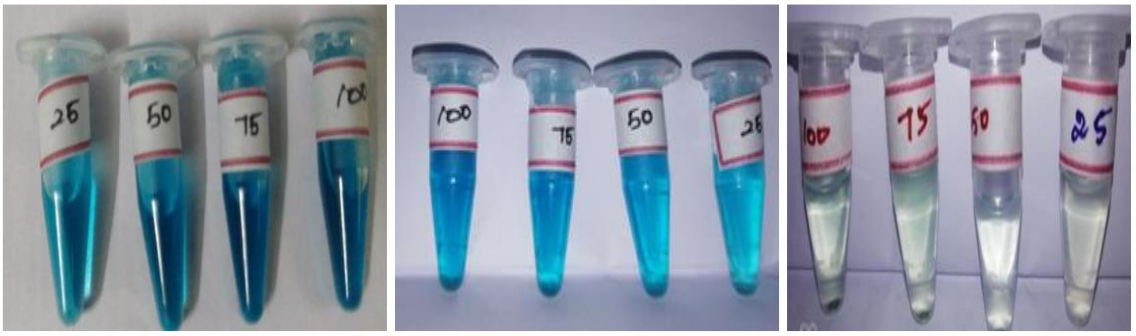
Graph 1: Graphical representation of HPLC for Laccase.

Dye Degradation

The laccase enzyme was monitored for its ability to degrade dye decolorization and its concentration of dye degrading property of malachite green and congo red was recorded (Graph 2 a, 2b). The partially purified enzyme decolorized synthetic dye such as malachite green 83% and congo red 79%. (fig. 5a, 5b)

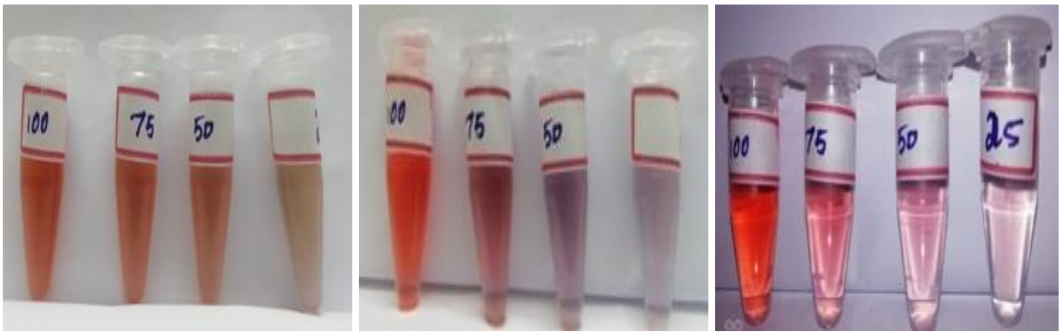


Graph 2: Dye Reduction by Laccase a) Malachite green b) Congo red



a) 24 hrs b) 48 hrs c) 72 hrs

Fig 5a: Degradation of malachite green



a) 24 hrs b) 48 hrs c) 72 hrs

Fig 5b: Degradation of congo red

Discussion

Laccase was first reported in 1883 from the Japanese lacquer trees. Since then laccases from several sources have been studied and reported based on their biological functions, substrate specificity, copper binding structure and industrial applications. Laccase is extracellular enzymes produced by different groups of fungi, of which basidiomycetes fungi secrete substantial amount of the enzyme. Earlier studies on production of laccase *from Pleurotus sajor caju, Agaricus bisporus, Ganoderma lucidum* have been reported. Hence the present study was aimed in isolating laccase from milky mushroom *C. indica* which has not been reported earlier.

Laccase is synthesized during secondary metabolism as inducible or constitutive enzyme [2]. In accordance with this research, the extracellular laccase activity was visually confirmed by using guaiacol in the media. The mycelial showed strong laccase activity with the development of a red zone around the mycelium. This is in conformity with earlier reports Debnath *et al.*, 2018 and Ferdes *et al.*, 2018 [8, 9] who also have reported similar red zone around the growth as visual confirmation of extracellular laccase activity.

Both solid state and submerged state fermentations have been employed in various studies for extracellular production of laccase. The current study used submerged fermentation. Laccase yields reported earlier were similar in both shaken and static cultures. Hence the cultures were incubated in static condition. Quantitative assay of laccase activity in broth was done spectrophotometrically using guaiacol broth as a substrate. The reddish brown color developed due to oxidation of guaiacol by laccase was measured at 450 nm. The highest enzyme activity was measured to be 3.23 U/ml with the reports according to Monssef *et al.*, 2016 [1].

The crude enzyme-protein solution was saturated by ammonium sulfate fractionation. The protein recovered by centrifugation at 10,000 rpm for 15 mins was dissolved in sodium acetate buffer and dialyzed. The dialyzed product was tested for its purity by SDS-PAGE which showed the molecular weight to be about 45kDa. The molecular mass of laccases from this study was in a similar range as the molecular masses obtained by Majolagbe *et al.*, 2013[4] and also by Suwannawong 2010 [3] who have reported extracellular laccases having a molecular weight of 45kDa in *Lentinus subnudus.*

The decolorization of synthetic dye was investigated by using partially purified laccase. Dyes were used in a different concentration from 25µl, 50µl, 75µl, 100µl mg per ml. The concentration of enzyme was 100µl. There was a gradual fading of the dye color from 24 hrs, and maximum decolorization was observed after 72 hrs. The corresponding values of decolorization percentage were as follows. The decolorization percentage for malachite green after 72 hrs of incubation was 66%, 57%, 76%, 83% for 100µl, 75µl, 50µl 25µl per ml of malachite green respectively and the dye decolorization percentage for congo red after 72 hrs of incubation was 74%, 70%, 76%, 79% for 100µl, 75µl, 50µl, 25µl per ml respectively. The currently existing dye treatment process being ineffective and not economical this development of the decolorization process based on laccase, definitely an alternative solution due to the potential of laccase in degrading synthetic dyes. Decolourization of synthetic dye by laccase of *C. indica* was found to show significant activity the result of the study came to a conclusion that laccase from *C. indica* can be used in synthetic dye decolorization.

It is important to estimate cytotoxicity for products purified from microorganisms and fungi. The cytotoxicity assay of crude laccase was estimated on Vero cell lines. Different concentration of crude laccase enzyme was tested by MTT assay and the CC_{50} calculated revealed that the Vero cells to be viable at the highest concentration tested. The crude extract hence did not exhibit any cytotoxicity. This result is in agreement with EI Fakharany *et al.*, 2010 [10]. Who reported laccase from *Pleurotus osteratus* had no cytotoxicity effect of HepG 2cells.

Conclusion

From the present study, it can be concluded that the *Calocybe indica* is a promising source for extracellular laccase production. The crude enzyme is partially purified by ammonium sulfate precipitation and dialysis. The molecular mass of laccase was determined to be 45Kda and HPLC elution profile showed laccase activity at a single peak at the retention time 4.572. The partially purified enzyme decolorized synthetic dye such as malachite green 83% and congo red 79%. This reasonably high decolorization efficiency makes this fungal enzyme a potential interest in the treatment of real effluents containing dyes. To best of our knowledge, this is the first report of laccase from *Calocybe indica*.

Acknowledgment

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