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Sequestration and purification of laccase, catalase and lipase from oyster mushroom (*Pleurotus florida*) compost waste and its application studies

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Abstract
Discarded oyster mushroom (*Pleurotus florida*) bed as tremendous source for isolation of industrial significant enzymes such as Amylase, Cellulase, Protease, Laccase, Lipase, Catalase, Xylanase, Lignin peroxidase, manganese peroxidase and pectinase. This study was conducted in order to achieve efficient extraction of lignocellulolytic enzymes Laccase (EC 1.10.3.2), Lipase (EC 3.1.1.3) and Catalase from spent oyster mushroom (*Pleurotus florida*) compost waste. Optimal enzyme recovery was achieved when spent oyster mushroom (*Pleurotus florida*) compost wastes were homogenized in 250ml of 50mM sodium acetate (pH 5.4) buffer at 5 ˚C for 2hr. The enzyme laccase released from oyster mushroom (*Pleurotus florida*) compost waste were showed activity of 0.962 U/ml. These were utilized in various industrial and environmental applications such as dye decolorization from textile industry waste water and Biosurfactant production. The synthetic dye Bromophenol blue and Cango red were showed high decolorization percentage (93.4% and 44%) in the presence of *Pleurotus florida* compost waste within 24 hrs. The crude extract of *Pleurotus florida* compost waste was successfully used in various other bioremediation applications.

Keywords: discarded oyster mushroom bed, industrial enzymes, application studies

Introduction
Approximately two million tons of spent mushroom compost waste is produced yearly by mushroom forms, with almost 1.2 million tons generated from oyster mushrooms. This high amount of spent mushroom compost waste is incompatible for reuse in mushroom production, therefore, it is used either as garden fertilizer or deposited in landfills, which pollutes the environment. Spent oyster mushroom (*Pleurotus florida*) compost wastes contain fungal mycelia, extracellular enzymes and unused lignocellulosic compounds [1]. The waste from oyster mushroom forms has been used in novel processes such as production of value-added products like biogas production, bulk enzyme extraction, production of organic fertilizer; animal feed supplements and used in degradation of pentachlorophenol [2, 3, 4]. The utilization oyster mushroom compost waste were benefit for human health and the environment because the agricultural waste from mushroom forms used as raw materials for various processes.
All plants are made up of lignocellulosic component; which will be produced by plant through the photosynthetic reaction. Which also known as photomass and representing the most aboundant renewable organic source in soil. Cellulose, Hemicelluloses and lignin are the major three types of polymers in lignocelluloses. These are strongly interconnected each other by non-covalent forces of chemical bonds and covalent cross linkages [5]. Wastes from agro-industries and forests contain high amount of lignin content, which produces lot of polycyclic aromatic hydrocarbon components like benzopyrene, hydroquinone, catechol, phenanthrene and naphthalene during burning of this in open environment [6]. All components from these cause lot of environmental and health problems in humans and animals like inhibition of DNA synthesis, Induction of cancerous tumours in lung, liver, larynx and cervix [7]. The fungal degradation of lignin from agro-industrial wastes is major key step to reduce these kind of environmental and health problems [8]. Oyster mushrooms have a major role in the biotransformation of lignocellulosic polymers into variable simple and soluble biomolecules through the secretion of lignocellulolytic enzymes like Cellulase, Amylase, Protease, Laccase, Lipase, Catalase, Xylanase, Lignin peroxidase and Manganese peroxidase [9].
The current investigation was performed to isolate and partial purification of various industrial enzyme from oyster mushroom (*Pleurotus florida*) compost waste and which utilized in various environmental applications like Synthetic dye decolorization and Biosurfactant production.

Materials and Methods
Mushroom bed preparation
Oyster mushroom (*Pleurotus florida*) bed was prepared by using rice straw waste from agro-industry in Tiruchengode, Namakkal (Dt) in Tamil Nadu. The autoclavable polypropylene bags containing layers of rice straw was inoculated with *Pleurotus florida* mushroom spawn, which was incubated at 25 ± 28 ˚C for 25 days in dark room. After 25 days of incubation the mycelia was fully covered in whole bed and produce mushroom flushes. After third or fourth yield of old mushroom bed was used as a source for crude industrial enzyme extraction.

Plate assay for various enzymes
Oyster mushroom (*Pleurotus florida*) was screened for various industrial enzymes using agar plate method with the following compositions.

Catalase test medium
Catalase test agar medium (Malt extract, 20g/l; Agar 15g/l) were inoculated with *Pleurotus florida* mycelia stage and incubated at 28 ˚C for 8 days. The presence of catalase was determined by flooding of agar plate with a freshly prepared 0.4% H_2O_2. The bubble formation around the fungal mycelia indicates presence of catalase activity [10].

Lipase test medium
The presence of lipase activity from *Pleurotus florida* mycelia were analyzed by using lipase test medium. Which contain Peptone, 10g/l; Sodium chloride, 5g/l; Calcium chloride 0.1g/l; Agar 20 g/l; Tween 20 10 ml/l. The lipase test medium was inoculated with *Pleurotus florida* mycelia and incubated at 28 ˚C for 8 days. The presence of visible precipitate of calcium salts of lauric acid on agar plate indicates lipase activity [11].

Medium for laccase test
Laccase activity was determined by the oxidation of 1-Naphthol method. Laccase medium contain Malt extract, 20g/l; Agar, 15g/l; 1-Naphthol 0.05g/l, which was inoculated with *Pleurotus florida* mycelia and incubated at 28 ˚C for 8 days. The growth of *Pleurotus florida* mycelia can change the colorless laccase medium into brownish-blue colour due to oxidation of l-Naphthol by laccase enzyme [12].

Collection of oyster mushroom (*Pleurotus florida*) compost waste
The oyster mushroom (*Pleurotus florida*) compost waste was collected from Mushroom farm, Vivekanandha College of Engineering for Women, Thiruchengode, Namakkal (Dt) in Tamil Nadu.

Preparation of crude enzyme extract
The fresh oyster mushroom (*Pleurotus florida*) compost waste (25g~75 to 85% moisture) was taken and homogenized by using marten pestle in 250ml of 50mM sodium acetate buffer, pH 5.4, and filter through muslin cloth. The filtrate was clarified by centrifugation at 4000 rpm for 15 min. The obtained supernatant was used for further studies [13].

Protein estimation
The total protein content of crude enzyme extract from Oyster mushroom (*Pleurotus florida*) compost waste was determined by Lowry's method. To 1000µl of sample, 4.5ml of alkaline copper sulphate reagent was added, mixed well and allowed to stand at room temperature for 10min., then 0.5ml of diluted Folin-Ciocalteus phenol reagent was added and after 30min of incubation at room temperature, absorbance was measured at 660nm. The presence of protein content in sample was calculated from a standard curve prepared with bovine serum albumin [14].

Estimation of enzyme activities
Laccase assay
The presence of laccase activity in crude enzyme extract was determined by reddish brown color developed due to oxidation of guaiacol in reaction mixture. Laccase enzyme activity was measured at 450nm. The reaction mixture contain 0.5ml of distilled water, 1ml of sodium acetate buffer (pH 4.5), and 0.5ml of substrate solution (46mM guaiacol) to 0.5ml of crude enzyme extract. Which was incubated at 37 ˚C for 3hrs.U/ml of laccase activity is calculated by this formula [15].

$$E.A=A\times V/t\times e\times v$$

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)

Acetone precipitation
The enzymes in crude extracts were further precipitated by gradual addition of chilled 60% (v/v) acetone with continuous stirring and kept in a cold temperature at 4 ˚C for 24 h. The precipitated protein was collected by centrifugation at 12,000 rpm at 4 ˚C for 30min and dissolved in Tris-Hcl buffer (pH 8.0). The sample was dialyzed against the same buffer using acetylated dialysis bag [16].

Purification of laccase
The purification of laccase enzyme was done by using silica gel chromatography column (1 by 3). This was equilibrated with 50mM potassium phosphate buffer (Buffer A-pH 7). 30µl of the dialyzed laccase enzyme solution was applied to the column and washed with 4ml of Buffer A. Elution was done by distilled water. The active fraction of laccase 420nm respectively.

Storage stability of crude enzymes
The storage stability of crude laccase enzyme were analysed by using 1ml of crude extract was mixed with 1ml of 50mM sodium acetate buffer (pH 5.8) and stored at the 4 ˚C. The crude laccase activity were analysed every day up to 5 days.

Application studies
Decolorization of synthetic dyes
Preparation of dyes stock solution
Methyl red, Congo red, Bromophenol blue and Coomassie brilliant blue were prepared separately by dissolving 0.01M concentration in 100ml of distilled water. From this stock solution 10ml of dyes were diluted in 90ml distilled water for further decolorization studies.

Decolorization of Synthetic dyes by the *Pleurotus florida* compost waste
Dye-decolorization capability of Oyster mushroom (*Pleurotus florida*) compost waste was tested at 37 ˚C, in static condition of sterile glass flasks. Each 25 g of fungal mycelia contain *Pleurotus florida* compost waste substrate was submerged in 100ml of diluted dyes containing flaks. Two set of flasks were prepared for each dilution. One set of flasks were act as a control, other sets of flask was inoculated with 25g of compost waste substrate. This was incubated at 30 ˚C for 24hrs. After 24hrs of incubation the supernatant from substrate containing flasks were collected,

it was centrifuged at 10,000rpm for 10min.The absorbance of various dyes were analyzed by using various OD range (Methyl red-530nm; Bromophenol blue-600nm; Congo red-595nm; Coomassie brilliant blue-585nm) based on the dyes had been used. The decolorization percentage of dyes was calculated by using this formula:

$$\text{Decolorization percentage (\%)} = (A_0\text{-}A)/A_0 \times 100$$

Where,
A_0= Initial absorbance of dye
A = Absorbance of dye at after treatment

Biosurfactant production

Lactose fermentation

Lactose fermenting character of *Pleurotus florida* mycelia was determined by inoculation mycelia on MacConkey agar (Oxoid) plate. Plate was incubated at 28 ˚C for 15 days. The growth of mycelia on agar plate indicates lactose fermentation capability of *Pleurotus florida* mushroom [17].

CTAB agar plate method

Oyster mushroom (*Pleurotus florida*) mycelia was inoculated on blue agar plate containing Cetyltrimethylammonium bromide (CTAB), 0.78 (g/l); glucose, 20 (g/l); peptone, 10(g/l); beef powder, 1(g/l); methylene blue, 0.002(g/l); yeast extract, 0.5 (g/l); agar, 17(g/l). This medium was used to detect extracellular glycolipid production from *Pleurotus florida* mushroom mycelia. Biosurfactant productions from mycelia were observed by the formation of dark blue halos around the mycelia [18].

Emulsification index test

Emulsifying capacity of crude sample extract from oyster mushroom (*Pleurotus florida*) compost waste was evaluated by an emulsification index (E24) for crude oil. To do so, 1.5ml of crude oil was added to 1.5 ml of crude extract in a test tube, which was vortexed at high speed for 2 min and allowed to stand for 24h. The percentage of the emulsification index in crude extract was calculated using the following equation [19]

$$E24 = \text{Height of emulsion formed} \times 100/\text{Total height of solution}$$

Result and Discussion

Mushroom bed preparation

Oyster mushroom (*Pleurotus florida*) bed were prepared by using rice straw as substrate. After 25 days of incubation at dark condition the first stage of mushroom flush was formed. After 3rd and 4th yield of mushroom the compost was used as a good source for various industrial enzyme extractions.

Enzymatic screening of oyster mushroom (*Pleurotus florida*) using agar plates

After incubation of various Agar plates for 8 days at 28 ˚C for the industrial enzymatic tests, the following results were obtained

Table 1: Screening of various industrial enzymatic activity of oyster mushroom (*Pleurotus florida*)

Enzymes	Substrates	Test reagent	Plate appearance	Zone size (mm)
Lipase	Tween 20	-	Formation of visible precipitation	-
Catalase	-	H_2O_2 solution	Formation of Bubbles around the mycelium	-
Laccase	l-Naphthol	-	Colorless medium changes to brownish color	5mm

It was seen that the oyster mushroom (*Pleurotus florida*) showed that the best industrial enzymatic activities.

A B C

Fig 1: Plate Assay for Different Industrial Enzymes A) Plate test for lipase, B) Plate test for catalase, C) Plate test for laccase

From these results, the formation of a visible precipitation around the mycelia was indicates +ve lipase activity (Figure1A). The activity of catalase was evaluated by hydrogen peroxide solution. Formation of bubbles around the mushroom mycelia were indicates catalase enzyme activity (Figure 1B). The laccase enzyme activity was analyzed by visualization of brownish blue color changes in laccase medium (Figure 1C).

Many reports suggested that the secretions of industrial enzymes in mushrooms are used for its fruiting body formation [20]. Lipase enzyme from fungi is widely used in the food and detergent industries [21]. Lot of research reported that the production of laccase enzyme from white-rot fungi helps to degrade of its substrate through the oxidative process [22, 23]. In this investigation high level of laccase activity was identified in *Pleurotus florida* mushroom. Previous studies had shown there several forms of catalase enzymes are exist from many plants like tobacco,

saffron, cotton, etc. [24]. The present study indicates high level of catalase enzyme activity from *Pleurotus florida* like other plants.

Quantification of laccase enzyme

Oyster mushroom (*Pleurotus florida*) compost waste contain industrially very important ligninolytic enzyme. The oxido reductive enzyme laccase from crude extract of *Pleurotus florida* compost waste were showed the total activity of 0.962 U/ml and specific activity of 1.17 U/mg. From this result, the oyster mushroom (*Pleurotus florida*) compost waste as a good source for extraction of industrially important ligninolytic enzyme such as laccase (Table 2).

Table 2: Enzyme Activities of Oyster Mushroom (*Pleurotus florida*) compost waste extract

Enzymes	Total activity (U/ml)	Specific activity (U/mg)
Laccase	0.962	1.17

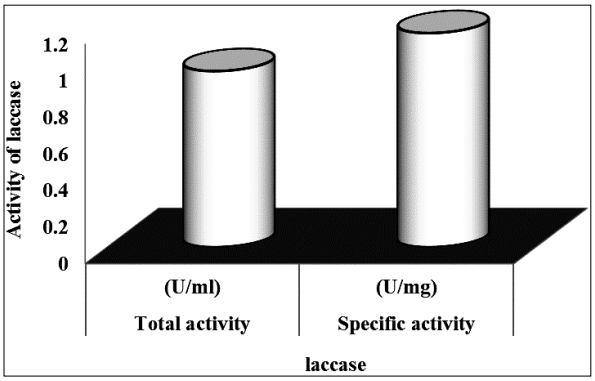


Fig 3: Quantification of laccase

Similar to this observation, the waste produced from oyster mushroom forms for extraction of industrially important enzymes have previously been studied [25]. The reutilization of oyster mushroom compost waste were reduce the lot of environmental problems such as disposals of bulk density of waste in to the soil, stability, surface crust, temperature changes, pH changes in soil, aeration and water retention capacity of soil [26]. Many investigation reported that, the spent oyster mushroom compost have a plenty of extracellular enzymes. Lccase is the main enzyme in spent mushroom substrate from *A. bisporus*, *P. sajor caju*, *P. ostreatus*, *L. edodes*, *Flammulina velutipes* and *Hericium erinaceum* [27]. From this investigation the low level of laccase enzyme produced from *Pleurotus florida* compost waste when compare to other enzymes. The lowest laccase specific activity (1.17 U/mg) was detected in 50mM sodium acetate (pH 5.8) buffer. By this study, it was concluded that oyster mushroom (*Pleurotus florida*) compost waste as a good source for extraction of industrially important enzymes.

Storage stability test for industrial enzymes

From this study the storage stability of crude amylase enzyme from oyster mushroom (*Pleurotus florida*) compost waste were decreased after five days of incubation at 4 ˚C in pH 5.6 (Table 3). In day1 the activity of crude laccase was 0.962 U/ml, after 2nd, 3rd and 4th days it was decreased about 0.87 U/ml, 0.32 U/ml and 0.12 U/ml. There is no activity was found at 5th day of storage at 4 ˚C in pH 5.6.

Table 3: Storage stability of laccase

Days	Enzyme activity (U/ml)
	Laccase
Day 1	0.962
Day 2	0.87
Day 3	0.32
Day 4	0.12

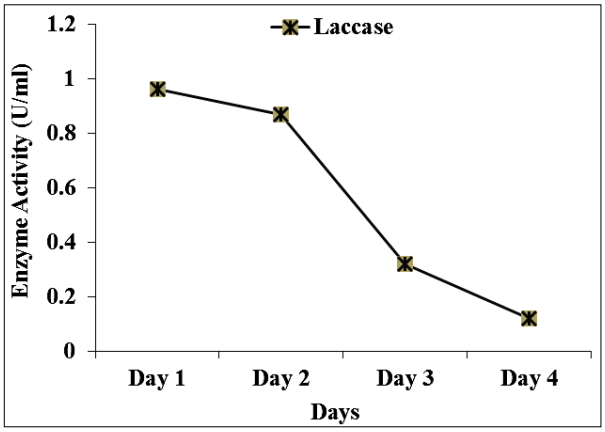


Fig 4: Storage stability of laccase

Purification of different enzymes

Table 4 shows the result of each step of laccase purification. Activity of purified laccase enzyme using gel filtration chromatography was 0.256 U/ml respectively.

Table 4: Purification of industrial enzymes

Purification step	Activities of enzymes (U/ml)
	Laccase
Crude extract	0.962
Acetone precipitation	0.544
Gel filtration chromatography	0.256

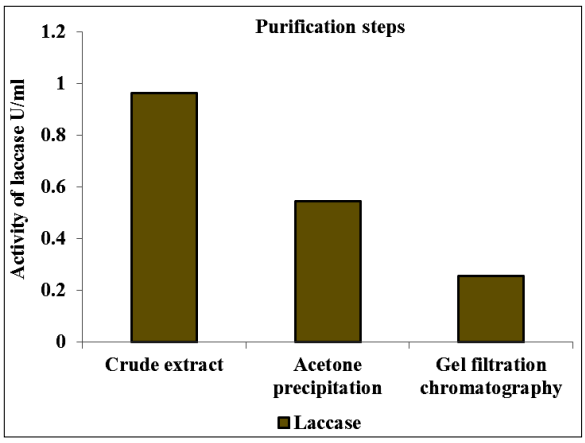


Fig 5: Purification of laccase

Application studies

Decolorization of synthetic dyes

In order to evaluate the decolorization capability of oyster mushroom (*Pleurotus florida*) compost waste were analyzed in incubation of compost waste in diluted various synthetic dyes containing flasks. There are four different synthetic dyes were selected for evaluation of the dye decolorization potential of compost waste from oyster mushroom

(*Pleurotus florida*) cultivation. The four industrial synthetic dyes are Methyl red, Bromophenol blue, Cango red and Coomassie brilliant blue; represent Azo dyes, Phenolsulfonphthalein dye and Triphenylmethane dye, respectively and they were used as decolorization substrates for evaluation of the dye-decolorization efficacy of laccase in *Pleurotus florida* compost waste. Decolorization values of four different dyes were varied based on their structural and redox potential differences. Before measurement of decolorization%, the full wavelength absorption spectra of the dyes were recorded. Then sample from dyes incubated with *Pleurotus florida* compost waste were measured at different wavelength based on the dyes. From this investigation the dye bromophenol blue have high decolorization percentage (93.4%) in the presence of *Pleurotus florida* compost waste and the dye Coomassie brilliant blue have low decolorization percentage (3.4%) (Table 5).

Table 5: decolorization of synthetic dyes by utilization of oyster mushroom (*Pleurotus florida*) compost waste

Synthetic dyes	Absorbance wavelength (nm)	Control OD at (nm)	Incubation time	Test OD at (nm)	Decolorization (%)
Methyl red	410 nm	0.433	1 day	0.304	29.8
Bromophenol blue	590 nm	1.964	1 day	0.129	93.4
Cango red	497 nm	0.772	1 day	0.432	44
Coomassie brilliant blue	595 nm	2.323	1 day	2.245	3.4

Many repots says that, Most of the recent process used in dye decolorization in textile, food, pharmaceutical and other chemical industries are very cost-consuming step, and which are ineffective and uneconomical process [28, 29]. Therefore, the development of industrial dye waste water treatment based on laccase enzyme appears to be an attractive solution for synthetic dye decolorization, because laccase enzymes from various plant sources are degrade lot of structurally different chemical dyes from unbreakable dyes by current waste water treatment process [30, 31]. In this study revealed that, the compost waste from oyster mushroom (*Pleurotus florida*) cultivation have ability to decolorize the chemically different synthetic industrial dyes with documented toxic effect for the environment. The utilization of organic waste from *Pleurotus florida* cultivation in industrial dye decolorization was suitable for cost effective waste treatment method and profitable for management of the disposal of oyster mushroom compost as an organic waste.

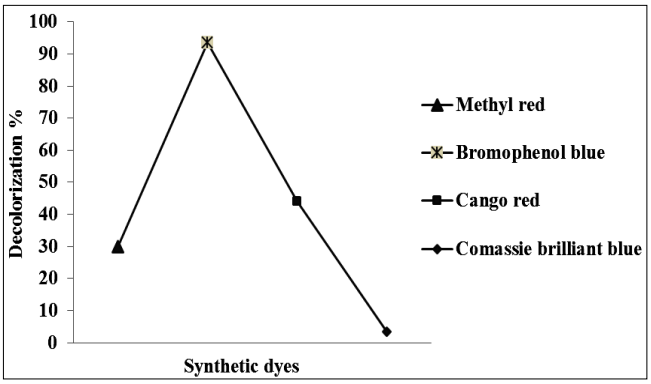


Fig 6: Decolorization % of Synthetic Dyes

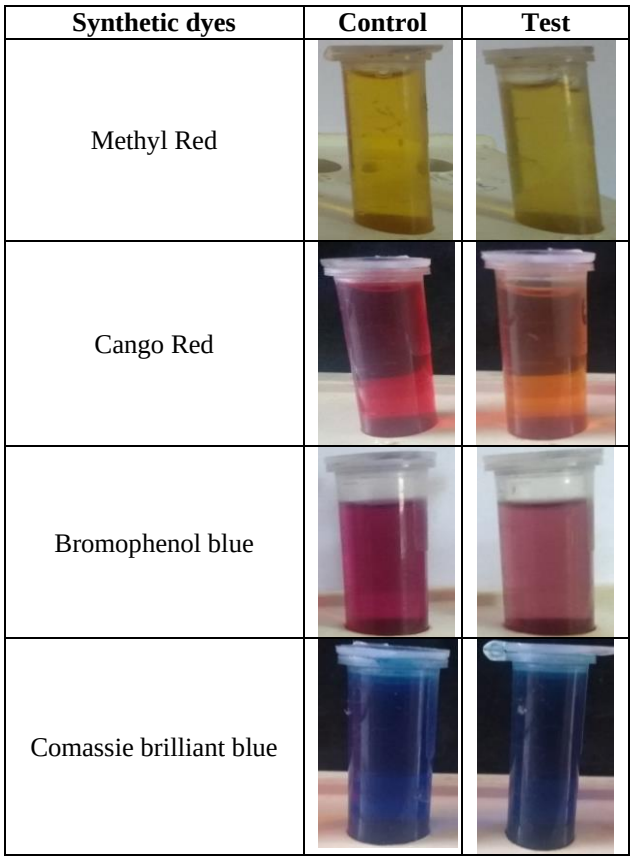


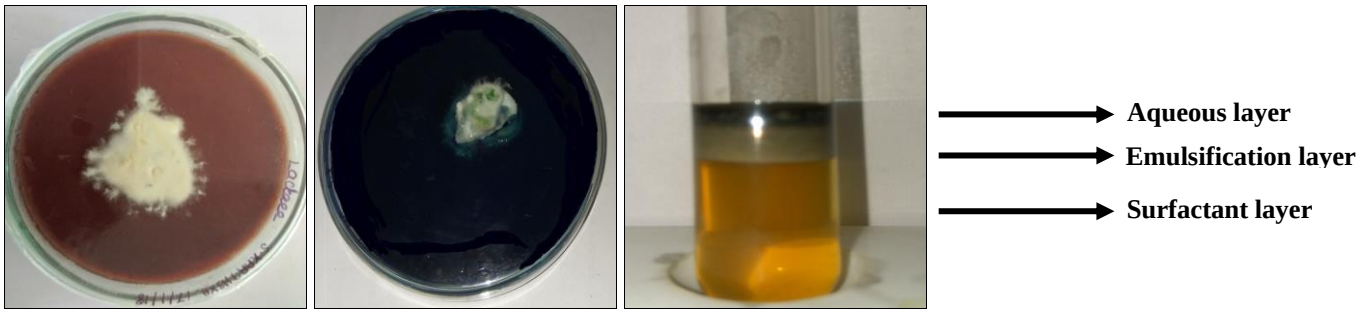
Fig 7: Decolorization of synthetic dyes

Biosurfactant production

In this investigation the production of Biosurfactant from oyster mushroom (*Pleurotus florida*) was analysed by lactose fermentation, CTAB agar plate method and Emulsification index test. The presence of mycelia growth on MacConkey agar plate was indicates the production of extracellular glycolipid (Biosurfactant) from *Pleurotus florida*. The productions of extracellular glycolipid were determined by semi quantitative assay of CTAB agar plate method, it was introduced by [18]. From this investigation, the appearance of light blue zone around the *Pleurotus florida* mycelia determine the positive result for Biosurfactant production capability of oyster mushroom. The production of bioemulsifier was analysed by emulsifying activity test [32]. In the present study, the emulsifying nature of Biosurfactant from *Pleurotus florida* was analysed by emulsion index test. Here crude extract from *Pleurotus florida* compost waste were produced 16.67% of emulsion layer on crude oil.

Table 6: Screening of biosurfactant production

Test	Result
Lactose fermentation	+ +
CTAB agar plate method	+ +
Emulsification index test	16.67% of emulsification index



A B C

Fig 8: Screening Method for Biosurfactant Production A) Lactose fermentation test, B) CTAB agar plate method C) Emulsification index test

Conclusion

The investigation of present work concluded that, the oyster mushroom (*Pleurotus florida*) compost waste as a good source for extraction of extracellular industrially important ligninolytic enzymes such as Laccase, Lipase, Catalase, Xylanase, Lignin peroxidase, manganese peroxidase and pectinase. The enzymatic activity of oyster mushroom *Pleurotus florida* mycelia was screened by using different agar media. Higher activities of Laccase, Lipase and Catalase were observed. The total protein content of crude extract from compost waste and acetone purified sample were analysed by Lowry's method. The protein content of crude sample were 0.82 mg/ml and acetone precipitated sample were 1.25 mg/ml. The activity of crude industrial enzymes from *Pleurotus florida* compost waste extract was analysed by various quantitative enzyme assay. The laccase activities of crude sample were 0.962 U/ml. The presence of extracellular laccase enzyme in *Pleurotus florida* compost waste was utilized in decolorization of various industrial dye effluents. When compare to other dyes (such as Cango red, Methyl red, Coomassie brilliant blue) bromophenol blue showed high decolorization percentage about 93.4%. The production of Biosurfactant from *Pleurotus florida* compost waste extract were analysed by emulsification index test. The percentage of emulsion layer on crude extract containing crude oil was 16.67%. In summary in this work, the various industrial enzymes were extracted from oyster mushroom (*Pleurotus florida*) compost waste and which was utilized divers environmental applications such as decolorization of industrial dye effluents and Biosurfactant production.

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