Research Article

Optimization of Cultural Conditions for the Treatment of Pulp and Paper Industrial Effluent by *Pleurotus ostreatus* (L.)

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Abstract | The efficacy of different *Pleurotus ostreatus* (L.) isolates to treat pulp and paper industrial effluent on a shake flask were studied. In the shake flask studies, *Pleurotus ostreatus* (L.) decolorized the effluent 69.68 %, on 7th day of incubation. Various cultural conditions including different glucose concentrations, nitrogen sources, pH, temperature and incubation period influence on biomass and different enzymes viz; xylanase, CMCase and laccase production by *Pleurotus ostreatus* were optimized in shake flask cultures. The results indicated that the optimum fermentation medium in shake flask studies contained a carbon (glucose 4%), nitrogen (0.5% ammonium chloride), inoculation level (three disks, 0.5 cm in diameter), temperature (30 °C), incubation period (at 7 days) and initial pH 5.0. Under these culture conditions, the maximum level of xylanase activity (9.29 Uml^{-1}), CMCase (7.37 Uml^{-1}), laccase (13.57 Uml^{-1}) and effluent decolorization (69.68 %) were observed on 7th day during shake flask studies.

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Introduction

The pulp and paper processing industries annually produce several colored mostly toxic and harmful wastewaters in huge amount as billion liters all over the world (Pokhrel and Viraraghavan, 2004). Amount of water utilized by these pulp mills reappear as an effluent containing huge amount of various organic compounds. The color of the effluent is generally associated with compounds having higher molecular weight such as lignin along with its derivatives. (Milestone et al., 2007). The high toxicity of some organic pollutants has, however, caused inhibition and death of preventing the degradation process. (Cohen et al., 2002). For this reason, several studies have been devoted to find new organisms capable of degrading organic pollutants and, at the same time, to implement technologies to protect these microorganisms from the competition by indigenous microorganisms in contaminated soils and wastewaters.

White-rot fungi (WRF) are the kind of organisms capable to tolerate and degrade higher concentrations of diverse persistent organic compounds as has been reviewed by Pointing (2001) and (Tortella et al., 2005). The degrading action of WRF has been credited to the non-specific extracellular ligninolytic enzyme systems, composed principally of laccases, Xylanases, lignin peroxidases (LiP) and manganese peroxidases (MnP). On the other hand,

the filamentous multicellular colonial form of these organisms provides both a high cell to substrate ratio and a mechanical adjunct to substrate breakdown (Pointing, 2001).

Several white-rot fungi are being tried to treat these colored effluents. Among these, white-rot basidiomycetous fungi are the most effective groups organisms for this purpose. These can degrade the lignin extensively under aerobic conditions.

The main purpose of this work was to develop a biological way of treatment for pulp and paper mill effluent by optimization of cultural condition for by using white rot fungi *P. ostreatus*.

Materials and Methods

Collection, isolation and purification of Pleurotus ostreatus (L.)

The *Pleurotus ostreatus* was collected in sterilized polythene bags present on decaying wood in and around the Khanspur, Abbottabad District. Two standard methods were used for separation of fungal spores. The first one is known as drop spore/shoot spore technique in which spore shooting is concerned directly from fresh samples of mushrooms onto medium (Tien and Kirk) containing antibiotics. The second method is involved in cutting of fresh samples of mushrooms into small pieces. These small pieces are heated roughly on fire flame and then placing on Tien and Kirk medium containing antibacterial. Medium and mushroom samples containing agar plates incubated for 24 h at room temperature.

The spores obtained in result of the first technique were observed in groups, therefore needed to be isolate. Isolation of spores was done under microscope using needle. After this, spores were again placed on Tien and Kirk medium containing antibacterials. The growth of the fungal mycelium was verified in the second technique. Small piece of agar containing fungal mycelium was cut and placed on Tien and Kirk medium containing antibacterials.

The sub-culturing of fungal mycelium resulting from both methods was done again and again until the purified fungal strains were obtained.

Maintenance of mushroom culture

The collected mushroom isolates were grown on Tien and Kirk media. The media was dissolved in 250 ml conical flask and cooked for 15 minutes. The cooked media was autoclaved at 121 °C for 20 minutes and allowed to cool but not solidify. The media solution was poured in sterilized Petri plates and allowed to solidify.

Xylanase activity

The suspended materials and fungal biomass were separated by centrifugation at 6000 × *g* for 15 min and the clarified supernatant used as the source of the crude enzyme. Activity of Xylanase (1,4-d-xylanase) was evaluated by using the diluted extract of enzyme with 1% aqueous solution (w/v) of oat spelt xylan in 50mM Na_3PO_4 buffer. The pH 7 was maintained and reaction was done for 15 min at 50°C. The released reducing sugars (as d-xylose) were assayed by adding 2ml of 3,5-dinitrosalicylic acid (DNS) reagent, boiling for 10 min, cooling and adding 5ml distilled water, then measuring the absorbance at 540 nm by Miller (1959).

One unit of xylanase activity was defined as the amount of enzyme necessary to produce 1 umol of reducing sugars (d-xylose) hydrolyzing xylan substrate, per min at 50 °C.

Laccase activity

The suggested procedure of Coll et al. (1993) was used to test the laccase activity. For this purpose, the enzyme form source was mixed with 50 mM Na-acetate buffer with pH 4.5. 1mM guaiacol (Sigma Co.) was added as substrate to make the volume up to 5 ml. The blank one contained the substrate only as the source enzyme that was inactivated by boiling. These tubes were then incubated for 15 min at 37°C. The OD (optical density) of the tubes was determined against blank one at 465 nm wavelength using Jenway 6105 spectrophotometer.

One-unit relative enzyme activity was described as the amount of enzyme causing a 0.1unit increase in the optical density of the reaction mixture under the experimental conditions.

Cellulase (CMCase) activity

The Ghose method (1987) was used to conduct the cellulase (CMCase) activity. Diluted enzyme (1 ml) along with 1 ml of 0.05 M citrate buffer was taken in test tube. One ml of 1% carboxymethyl cellulose (CMC) was added. For blank, sample was replaced

Table 1: *Effect of various incubation periods to evaluate enzymes level and decolorization by Pleurotus ostreatus in pulp and paper industrial effluent.*

Incubation period (days)	Decolourization (%)	Biomass (g/L)	Enzymes (U/ml)		
			Xylanase	Laccase	Cellulase
4	25.41±2.25c	1.95±0.02f	2.36±0.04f	2.45±0.12e	1.25±0.05e
5	33.56±2.95b	2.14±0.01e	3.56±0.03d	3.91±0.03d	2.89±0.02c
6	50.25±4.87a	2.91±0.01c	5.24±0.05b	6.54±0.07b	4.12±0.04b
7	62.58±6.02a	4.23±0.07a	7.98±0.04a	8.65±0.09a	5.48±0.07a
8	59.36±4.92a	3.52±0.03b	4.28±0.02c	4.56±0.04c	2.61±0.01d
9	55.68±3.85a	2.65±0.02d	2.78±0.01e	2.13±0.09f	1.04±0.04f

±: Standard error; Treatment mean, followed by different letters in the same column are significantly different at P = 0.05 according to Duncan's multiple range test (Steel and Torrie, 1960) were calculated from the data obtained from various parameters during present studies by using software package Co-stat Version 3.03.

Table 2: *Effect of various temperatures on pulp and paper industrial effluent to check enzymes level and decoloization by Pleurotus ostreatus.*

Temperature (°C)	Decolourization (%)	Biomass (g/l)	Enzymes (U/ml)		
			Xylanase	Laccase	Cellulase
20	18.98±2.52c	2.96±0.01d	2.18±0.02d	3.01±0.06b	1.86±0.001d
25	55.93±4.01a	3.38±0.07b	5.15±0.09b	3.04±0.12b	2.31±0.02c
30	61.35±5.03a	3.79±0.03a	8.89±0.12a	6.48±0.09a	4.42±0.05a
35	30.73±3.36b	3.24±0.02c	3.12±0.06c	2.71±0.02c	3.41±0.03b
40	8.21±1.02d	0.93±0.01e	1.00±0.001e	0.42±0.001d	1.02±0.02e

±: Standard error; Treatment mean, followed by different letters in the same column are significantly different at P = 0.05 according to Duncan's multiple range test (Steel and Torrie, 1960) were calculated from the data obtained from various parameters during present studies by using software package Co-stat Version 3.03.

with distilled water. Test tubes containing reaction mixture were incubated in water bath for 30 min at 50°C. DNS of 3 ml was added in each test tube and boiled for 10 min to terminate the reaction. A blank known as spectrozero containing substrate and buffer was used to set the spectrophotometer. Reading was taken at 540 nm from spectrophotometer.

Optimization of cultural conditions for the treatment of pulp and paper industrial effluent

Following parameters were optimized to achieve the treatment of Pulp and Paper industrial effluent in submerged conditions; Temperature, Initial pH, Incubation period, Carbon sources, Nitrogen sources etc.

Statistical analysis

Treatment mean and standard error were calculated from the data obtained from like (Temperature, Initial pH, Incubation period, Carbon sources, Nitrogen sources) of present studies using software package Costat version 3.03.

Results and Discussion

Incubation period

For color reduction, biomass and enzymatic activities cause effect of various incubation periods in days (4 to 9) were studies. The best incubation period at while *Pleurotus ostreatus,* IMPP-02 their maximum growth was seven days. For this purpose, harvesting was done after every 24 hours to determined best incubation period.

Incubation temperatures

In shake flask fermentation different temperatures, ranging from 20 °C to 40 °C for 7 days. The maximum decolorization of effluent and various enzymes by *Pleurotus ostreatus,* IMPP-02 were maximum at 30 °C at constant pH 5.0.

Effect of pH

In shake flask fermentation different pH, ranging from 3.0 to 8.0 for 7 days. The pH of the effluent medium was adjusted by using 1M HCl/NaOH before sterization at 121 °C for 15 min. The sterilized

Table 3: *Effect of various pH on decolourization and lignolytic enzyme production by Pleurotus ostreatus.*

pH	Decolourization (%)	Biomass (g/L)	Enzymes (U/ml)		
			Xylanase	Laccase	Cellulase
3.0	14.03±1.05e	2.71±0.09b	4.54±0.05b	1.02±0.01f	1.72±0.01d
4.0	38.53±2.59c	3.05±0.12a	4.55±0.03b	2.25±0.02c	1.85±0.02c
5.0	57.28±4.01a	3.35±0.10a	5.23±0.09a	3.02±0.01a	2.25±0.01a
6.0	49.32±3.25b	3.14±0.03a	4.36±0.07c	2.81±0.03b	2.02±0.02b
7.0	22.95±2.05d	3.21±0.02a	3.15±0.03d	2.12±0.01d	1.46±0.03e
8.0	11.65±1.03f	2.04±0.05c	1.86±0.01e	1.20±0.02e	0.98±0.001f

±: Standard error; Treatment mean, followed by different letters in the same column are significantly different at P = 0.05 according to Duncan's multiple range test (Steel and Torrie, 1960) were calculated from the data obtained from various parameters during present studies by using software package Co-stat Version 3.03.

Table 4: *Effect of various glucose concentrations as a carbon source to check enzymes level and decolorization by Pleurotus ostreatus in pulp and paper industrial effluent.*

carbon source Glucose (%)	Decolourization (%)	Biomass (g/L)	Enzymes (U/ml)		
			Xylanase	Laccase	Cellulase
Control	62.19±3.59b	4.82±0.09e	7.90±0.07c	8.51±0.05f	2.91±0.01f
1	64.25±4.01a	4.99±0.07d	6.25±0.02e	9.48±0.07e	3.87±0.02e
2	65.35±4.52a	5.68±0.09c	7.13±0.05d	10.84±0.09c	5.28±0.05d
3	67.25±3.92a	6.56±0.07b	8.16±0.07b	11..30±0.07b	6.09±0.07b
4	69.68±4.01a	7.37±0.05a	9.29±0.09a	13.57±0.09a	7.37±0.05a
5	52.39±3.25c	4.23±0.03f	4.67±0.03f	10.25±0.09d	5.96±0.03c
6	33.36±2.51d	2.37±0.01g	2.15±0.01g	7.63±0.07g	2.90±0.01f

±: Standard error; Treatment mean, followed by different letters in the same column are significantly different at P = 0.05 according to Duncan's multiple range test (Steel and Torrie, 1960) were calculated from the data obtained from various parameters during present studies by using software package Co-stat Version 3.03.

flasks were inoculated with fungal discs under aseptic conditions and the flasks were incubated at 25 °C with continuous shaking at 125 rpm for 7 days. Decolorization, biomass and various enzymes such as Xylanase, Laccase and Cellulase were estimated and find out that pH 5 shows maximum activity in treated effluent broth after harvesting.

Effect of carbon source

The glucose was taken as constant carbon source and then was an optimized different concentration of glucose ranging from 1 % to 6 %. Each flask contains 50 ml of media, autoclaved as already described and then inoculated under sterilized conditions.

Effect of nitrogen source

In shake flask studies different inorganic nitrogen sources were selected like Ammonium sulphate $(NH_4)_2SO_4$, Ammonium hydrophasphate (NH_4HPO_4), Ammonium nitrate (NH_4NO_3), Ammonium chloride (NH_4Cl) and urea (CH_4N_2O) in the effluent medium for efficient decolorization, biomass and enzymatic activity. Ammonium chloride (NH_4Cl) was selected best for decolorization, biomass and enzymatic activity by using different concentrations ranging from (0.2 % to 0.7 %).

Fungi are well known to produce variety of organic acids, extracellular proteins and other important metabolites. These are capable to survive in severe environment (Tripathi et al., 2005). Therefore, it appears to be most suitable system to treat the colored and metallic effluents. Fungal mycelium not only produces important metabolites as homogeneous and heterogeneous proteins, citric acid and peroxidases, but it is more effective in reduction, detoxification and removal of industrial effluents ingredients. Therefore, *Pleurotus ostreatus* (L.), a white-rot fungus was tried for bioremediation of industrial effluents to bring out the capabilities of fungi. Bioremediation is the use of different microorganisms to detoxify the contaminants, which are real threat to the public health, especially of soil, water and other sediments. Organic matter as well as toxic compounds discharged

Table 5: *Effect of screening of various nitrogen sources to check enzymes level and decolorization by Pleurotus ostreatus in pulp and paper industrial effluent at 30 °C.*

Nitrogen sources	Decolouriza-tion (%)	Biomass (g/L)	Enzymes (U/ml)		
			Xylanase	Laccase	Cellulase
Ammonium sulphate $(NH_4)_2SO_4$	63.19±2.51a	6.52±0.12b	8.35±0.17b	10.82±0.12c	6.01±0.09b
Ammonium hydro- phosphate (NH_4HPO_4)	65.46±1.95a	5.23±0.09d	6.23±0.15d	11.62±0.17b	5.26±0.07c
Ammonium nitrate (NH_4NO_3)	66.58±1.05a	6..35±0.09b	8.29±0.16b	9.25±0.16e	3.95±0.02e
Ammonium chloride (NH_4Cl)	68.29±2.01a	7.34±0.08a	9.01±0.19a	12.51±0.10a	6.26±0.05a
Urea (CH_4N_2O)	58.35±1.02b	5.96±0.07c	6.94±0.15c	10.29±0.09d	4.59±0.03d

±: Standard error; Treatment mean, followed by different letters in the same column are significantly different at P = 0.05 according to Duncan's multiple range test (Steel and Torrie, 1960) were calculated from the data obtained from various parameters during present studies by using software package Co-stat Version 3.03.

Table 6: *Effect of nitrogen source to check enzymes level and decolorization by Pleurotus ostreatus in pulp and paper industrial effluent at initial pH 5, agitation speed 125 rpm at 30 °C for 7days.*

Nitrogen source Ammonium chloride (%)	Decolorization (%)	Biomass (g/L)	Enzymes (U/ml)		
			Xylanase	Laccase	Cellulase
Control	63.19±2.05b	6.62±0.12b	7.52±0.14b	9.32±0.15c	4.65±0.16e
0.2	34.26±1.52e	1.18±0.06f	3.15±0.09g	4.26±0.09g	1.52±0.05g
0.3	46.54±3.59c	2.84±0.01e	5.95±0.10d	7.62±.12e	3.65±0.08f
0.4	53.26±1.92c	4.74±0.12d	6.92±0.10c	10.29±0.10b	5.56±0.11c
0.5	68.29±2.12a	7.05±0.17a	8.42±0.21a	11.84±0.11a	7.04±0.12a
0.6	50.81±4.56c	5.65±0.09c	4.95±0.09e	8.95±0.07d	6.84±0.10b
0.7	41.25±1.21d	2.81±0.03e	3.62±0.01f	5.62±0.05f	4.95±0.09d

±: Standard error; Treatment mean, followed by different letters in the same column are significantly different at P = 0.05 according to Duncan's multiple range test (Steel and Torrie, 1960) were calculated from the data obtained from various parameters during present studies by using software package Co-stat Version 3.03.

from sources i.e. domestic and industries for many years, have been removed using microorganisms (Tripathi et al., 2005).

White-rot fungi are well known mostly due to production of important cellulose hydrolysing and lignin biodegrading enzymes in result of various cellular metabolic processes i.e. cellulases, laccases, lignin peroxidases, manganese peroxidases and xylanases. Degradation of several dyes and xenobiotic compounds is due to this ligninolytic system of white rot fungi. Dye degradation ability of WRF (white-rot fungi) is directly linked with its lignin degradation ability as dyes are degraded along with lignin. Now a day, the most exclusive exclusive is use of this most important WRF as these have abilities to degrade various organo pollutants such as xenobiotic (Christian et al., 2005). *Pleurotus ostreatus* is a well-studied specie of genus *Pleurotus*. It is the 3rd most important cultivated mushroom. The specie of genus *Pleurotus* use agricultural wastes and convert it into valued food products due to activities of ligninolytic enzymes (Cohen et al., 2002).

To assess the remediation potential of *Pleurotus ostreatus* (L.), were collected in sterilized polythene bags from in and around the Khanspur, Abbottabad District, a pulp and paper industrial effluent was treated on two scales. since the different efficiencies in the treatment are shown at diverse scales. Furthermore, color, BOD and COD in the effluents are considered as important factors to evaluate the water quality. To evaluate these parameters, a biological airlift fermentor using *Pleurotus ostreatus* on shake flask was used. In a shake flask treatment with *P. ostreatus* the color was reduced maximally by 69.68% by day 7 of the incubation.

In the present investigations, the efficacy of *Pleurotus ostreatus* (L.) to treat pulp and paper industrial effluent on a shake flask was studied. The mushroom was cultivated on Tien and Kirk media containing lignin from effluent at room temperature for 7 days in order to confirm their capability of growth

in lignin. Furthermore, the isolate IMPP-02, shown the maximum decolorization capacity and enzymatic activity was selected for further shake flask culture studies. Similar results were reported by Apiwatanapiwat et al. (2006) Various cultural conditions including different glucose concentrations, nitrogen sources, pH, temperature and incubation period influence on biomass and different enzymes viz; xylanase, CMCase and laccase production by *Pleurotus ostreatus* IMPP-02 were optimized in shake flask cultures. The results indicated that the optimum fermentation medium in shake flask studies contained a carbon (glucose 4%), nitrogen (0.5% ammonium chloride), inoculation level (three disks, 0.5 cm in diameter), temperature (30 ˚C), incubation period (at 7 days) and initial pH 5.0. Under these culture conditions, the maximum level of xylanase activity (9.29 Uml^{-1}), CMCase (7.37 Uml^{-1}), laccase (13.57 Uml^{-1}) and effluent decolorization (69.68%) were observed on 7th day during shake flask studies. Furthermore, the optimum pH and temperature were found to be 5.0 and 30˚C, respectively in fermentor studies. The maximum color reduction of 66.66 %; biological oxygen demand (BOD) and chemical oxygen demand (COD) reduction of 65.35 % and 61.3 % respectively, were determined during fermentor studies. In another study, Santos et al., (2002) reported that *Pleurotus ostreatus* was used for the treatment of pulp and paper mill effluent and glucose was added. Thus, the use of co-substrates improved the decolourization potential of fungi (Mehna et al., 1995).

Pulp and paper industrial effluent cause environmental pollution and effect on health of biodiversity. This study help us to reduce this pollution by using biological methods (White rot fungi) rather than chemical.

Conclusions and Recommendations

The present studies indicate that the treatment of a pulp and paper industrial effluent by *Pleurotus ostreatus* (L.) is a competent and cost-effective method. Biological treatment of pulp and paper industrial effluent using *Pleurotus ostreatus* to decolorize effluent has yielded results (66-70%) similar to some of the best decolorizing activities reported in the literature (in the range of 55.1–66.7%).

Authors Contribution

Azeem Haider and M Mohsin Alam conceived the idea and managed the whole project. Methodology and analysis were conducted by Azeem Haider. Write up and project management was done by Mohsin Alam. References were organized by Muhammad Asif Zulfiqar and Azhar Ali Khan.

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