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Research Paper

ISOLATION AND OPTIMIZATION OF WHITE ROT FUNGI AND ITS APPLICATION IN ELECTROPLATING INDUSTRIES EFFLUENT

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Abstract

Fungal strains were isolated from the part of Western Ghats area, Ooty, Tamil nadu, India. Strains were identified and screened for ligninolytic activity. These isolates were tested to evaluate their applicability for heavy metal removal from electroplating industries effluent. The physico-chemical parameters of the samples were analyzed. The optimum conditions of pH, Temperature were determined for the microbial growth. The biosorption of *Ganoderma sp* was 39.58% of Cu, *Lenzites sp* was 60.19% of Zn. Results reveal the potential application of the isolate for the removal of certain heavy metal from electroplating industries effluent.

Key words: Heavy metal, Biosorption, Optimization, Fungal sorption.

INTRODUCTION

Wood is composed of three main constituents, lignin, cellulose and hemicelluloses. Lignins constitute the second most abundant group of biopolymers. Lignin is highly resistant towards chemical and biological degradation. Wood rotting fungi are an important component of forest ecosystems. Among the classified fungi, white rot is the most efficient degraders of the lignin. They belong to the order basidiomycetes that involves in biodegradation of lignin in nature which is important for global carbon recycling. For this current investigation three fungi were collected from decaying wood logs. Mostly fruit bodies were collected from the forest of Western Ghats area in Ooty, Tamilnadu, India.

Industrialization and various technological advancement vilely contaminating our environment by discharging the heavy metals into the effluents which cause severe health hazards to the living beings. Hence these fungi can degrade an extremely diverse range of persistent environmental pollutants. Due to their efficient enzymatic system these strains can degrade lignocellulosic materials. The present investigation mainly focused on the absorption of Cu and Zn, effluent from electroplating industry by using wild type white rot fungal strains to analyze its percentage efficiency over the absorption of Cu and Zn.

MATERIALS AND METHODS

Collection

The three fungi were collected from decaying wood logs. Mostly fruit bodies were collected from the forest of Western Ghats area in Ooty, Tamilnadu, India. The collection sight was situated in the latitude of -11.58^{o}S and longitude of 76.93^{o}E at 400 ± 50M MSL. It receives rain fall of about 300 mm per year with high humidity and temperature.

Isolation of the fungi

The portion of the fungi was cut, surface sterilized with 1 per cent mercuric chloride solution and then repeatedly washed with sterile distilled water. The fungi were then inoculated on 2 per cent malt agar medium in petriplates. Then the fungal growth which occurred on the plates was sub cultured on malt agar slants to obtain pure culture.

Preparation of spore suspension

The fungi were grown in malt agar plates for 6 days at 37˚C. Then the plates were flooded with sterile distilled water and brushed with camel hair brush smoothly without disturbing the mycelial growth. The suspension was filtered over a sterile filter system to remove the mycelia fragments and the concentration of the filtered spore suspension was adjusted to 10^5 spores/ml and inoculum is used for further studies.

Identification

The collected fungi were identified as *Polyporous grammocephallus, Ganoderma sp*.1 and *Lenzites sp*. The samples were identified based on the morphology of the fruit bodies and spores

Screening of white rot fungi for ligninolytic activity

The screening of white rot fungi for lingninolytic activity were done based on their ability to oxidize such dyes, Poly R and Remazol brilliant blue degradation of native lignin and the liberation of ethylene from KTBA (2-keto-4- thiomethyl butyric acid).

Oxidation of dyes

The ability of white rot fungi to oxidize Poly R and Remazol brilliant blue were tested in the C- limited medium.

C-limited Medium

D-Glucose	- 3.0 g
Diammonium tartrate	- 0.66 g
$MgSO_4 .7H_2O$	- 0.15 g
$CaCl_2$ $2H_20$	- 30.0 mg
$FeSO_4$ $7H_2O$	- 5.55 mg
H_3PO_4 (2N)	- 3.27 ml
Trace element solution	- 0.30 ml
Water	- 1000 ml

Trace element solution (per litre of distilled water)

Nitrilotriacetate	- 1.5 g
$MnSO_4.H_2O$	- 1.0 g
$CoCl_2.6H_2O$	- 1.0 g
$ZnSO_4.7H_2O$	- 3.0 g
$CuSO_4.5H_2O$	- 3.0 g
$AIK (SO_4)_2$	- 10.0 mg
H_3BO_3	- 10.0 mg
Na_2MoO_4	- 10.0 mg

Vitamin solution (1000ml)

Biotin	- 2.0 mg
Folic acid	- 2.0 mg
Thiamine HCl	- 50.0 mg
Riboflavin	- 5.0 mg
Pyridoxine HCl	- 10.0 mg
Cyanocobalamine	- 0.1 mg
Nicotinic acid	- 5.0 mg
Calcium pentothenate	- 5.0 mg
p- Amino benzoic acid	- 5.0 mg
Thioacetic acid	- 5.0 mg

The pH of the medium was adjusted to 4.5 with 4 N solution of NaOH or H_2SO_4.

The experiments were performed with 10 ml medium in 125 ml Erlenmeyer flasks inoculated with 10^5 spores/ml of medium (inoculums volume-10% v/v). At the beginning of the carbon limited phase (after 6 days), Guaiacol (dye 1.8 mM final concentration), remazol brilliant blue (3 mg/1) and poly R dye (200 mg/l) were added aseptically to the culture flasks. The presence of ligninolytic activity of the white rot fungi were indicated by the dye decolourization. At regular time intervals, the cultures were removed, filtered and the colour intensity of the culture filtrates were measured at 600 nm for remazol brilliant blue amended medium and 513 nm/360 nm for Poly R dye amended medium.

Lignin degradation

Alkaline extracted straw lignin was used as substrate for the lignin degradation studies (Janshekar *et al*., 1982).

Straw lignin preparation

The paddy straw was ground to about 250 mm mesh size and suspended in 4 per cent sodium hydroxide solution (10 g in 250 ml). It was heated for 1 h at 121° C and filtered. The filtrate was diluted with methanol (1:1 v/v) and kept overnight. The precipitate formed was discarded by centrifugation at 5000 rpm for 20 min and the supernatant was collected. The pH of the supernatant was reduced to 3.0 with hydrochloric acid and left overnight. The precipitate formed was collected by centrifugation at 20,000 rpm for 10 min, washed twice with distilled water of pH 3.0, dried under vacuum and stored in glass bottles in desiccator.

Lignin degradation assay

The ability of the white rot fungi to degrade lignin was assayed in the basal medium (Kirk *et al*., 1978).

Basal medium

Solution A (in 500ml of distilled water)

D-glucose	- 10.0 g
H_3PO_4 (2N)	- 10.9 ml
NH_4NO_3	- 46.9 mg
L-aspargine	- 89.0 mg
$MgSO_4.7H_2O$	- 0.5 g
$CaCl_2.2H_2O$	- 0.1 g
$FeSO_4$	- 10.0 g
Trace element solution	- 1.0 ml
Vitamin solution	- 1.0 ml

Solution B (Total volume 450 ml)

Lignin - 1.0 g
Potassium hydroxide - 0.86 g

The solutions were autoclaved separately. After cooling, solution A was added slowly with proper mixing to B. To the final solution, 50 ml of previously warmed 20 mM acetate buffer (pH adjusted to 4.5 with NaOH) was added via a sterile membrane filter. The medium was distributed in 50 ml quantities into 250 ml Erlenmeyer flasks fitted with rubber stoppers with glass tubes for gas-flushing. The flasks were inoculated with spore suspension (10^5 spores/ml) and incubated at 30°C with shaking (inoculums volume-10%v/v). Pure oxygen was flushed into the flasks on the third day of incubation. After six days, the fungal biomass was removed by filtration and washed with distilled water. Biomass was determined by drying at 105°C to a constant weight. Residual lignin was determined in the filtrate combined with biomass washing. To the combined filtrate, sodium hydroxide was added to a final concentration of 0.05 N and absorbance was recorded at 280 nm after appropriate dilution with 0.05 N NaOH (about 20, the original culture volume). Uninoculated medium served as control.

Ethylene production from 2-keto-4-thiomethyl butyric acid (KTBA)

C-limited medium was distributed as 50 ml aliquots in 250 ml Erlenmeyer flasks and inoculated with 10^5 spores/ml (inoculums volume-10% v/v).The flasks were incubated at 30°C for 6 days. After the incubation period, the fungal biomass was removed by filtration and the culture filtrate was centrifuged at 18000 rpm for 30 min at 4°C and the supernatant was used as enzyme source. Boiled culture filtrate was the enzyme blank.

To 2 ml of enzyme, 3 mM glucose and 1 mM KTBA were added in 10 ml serum bottles fitted with rubber stoppers and sealed. The bottles were incubated at 37°C. After one hour, 1 to 2.5 ml of gas was removed from the head space and ethylene content was analysed by gas chromatography (Column: Porapack N; column temperature 80°C; injection port temperature: 40°C; detector temperature: 120°C; carrier gas: nitrogen; flow rate of carrier gas: 30 ml/min; detector: flame ionization detector) (Glenn *et al.,* 1983).

Growth kinetics and ligninase production

Growth kinetics and ligininase enzyme production were studied in C- Limited medium (M14) of Janshekar and Fiechter (1988).

Growth

Aliquots (50ml) of medium were taken in 250 ml of Erlenmeyer flask and inoculated with 10^5 spores/ml (inoculums volume-10%v/v). The flask was incubated at 30°C. At regular time intervals, the fungal biomass was removed by filtration and the growth was determined as mycelial dry weight (mg) by drying the biomass at 105°C to a constant weight.

Enzyme production

The culture filtrate was centrifuged at 10,000 rpm for 20 min at 4°C and the clear supernatant was used as enzyme. The boiled culture filtrate served as enzyme blank.

Enzyme assay

Laccase

Laccase activity was measured by Evans method (1985). The reaction mixture consists of,

Culture filtrate - 0.5 ml
Guaiacol - 0.35 µl (0.035%v/v)

Sodium acetate buffer 0.1M; pH 5.0) - 2.0 ml

The enzyme activity was expressed as change in optical density (OD) at 440 nm and expressed as U/ml (1U=change in OD/min at 440 nm).

Effect of culture parameters on growth and enzyme production

For the determination of optimum culture conditions for growth and ligninase production, the fungi were grown in C-limited medium (Jansheker and Fiechter, 1988) in orbital shaker (120 rpm).

Incubation period

The white rot fungi were grown at various incubation periods at room temperature for 12 days and the growth and enzyme production were determined at the end of each incubation periods.

pH

The growth and enzyme production were determined at the pH range of 3.0 to 9.0 at room temperature.

Temperature

The effect of temperature was determined in the temperature range of 30^{o} C to 70^{o}C.

Biosorption of heavy metals

The biosorption of heavy metals was carried out in electroplating industries effluent containing C-limited medium. The medium was inoculated with fungal spore suspension (10^5 spores/ml) and incubated at 30^{o}C for 5-7 days in an orbital shaker. After 7 days of incubation, Copper (Cu) and Zinc (Zn) biosorption were analysed by using atomic absorption spectrophotometer. The sample was boiled and evaporated to 15 ml on a hot plate. Concentrated HCl (5ml) was added and boiled again. The solution was boiled until the sample became clear and brownish fumes were evident. Finally, it was cooled and diluted up to 50ml with distilled water. An aliquot of this solution was used for determination of the concentration of total chromium with the help of a flame atomic absorption spectrophotometer (Achal *et al*., 2011).

Calculation,

$$\text{Degradation (\%)} = \frac{\text{Control - Sample}}{\text{Control}} \times 100$$

RESULTS

Collection of fungi

Three fungi were collected from decaying wood logs. Mostly fruit bodies were collected from the forest of Western Ghats area in Ooty, Tamilnadu, India. The collection sight was situated in the latitude of -11.58^{o}S and longitude of 76.93^{o}E at 400 ± 50M MSL. It receives rain fall of about 300 mm per year with high humidity and temperature.

Isolation and Identification

The collected fungi were isolated and identified as *Polyporous grammocephallus, Ganoderma sp*.1 and *Lenzites sp.* The fruit body was cut in to small pieces and sterilized with 1 per cent mercuric chloride solution, repeatedly washed with sterile distilled water (Roy Watling, 1971) and inoculated on 2 per cent malt agar medium in petriplates. Plates were incubated at 37°C for six days. The fungal growth occurred on the plates were sub cultured and maintained on malt agar slant.

Inoculation

The fungi were grown in malt agar plates for 6 days at 37°C. Then the plates were flood with sterile distilled water and brushed with camel-hair brush smoothly without disturbing the mycelial growth. The spore suspension was filtered over a sterile filter system to remove the mycelial fragments. Concentration of the filtered spores suspension was adjusted to 10^5 spores/ml and used as inoculums for further studies.

Screening of white rot fungi for ligninolytic activity

Primary screening of white rot fungi for ligninolytic activity was carried out by dye reduction method. Poly R dye (0.02%) and Remazol brilliant blue (3 mg/ml) dye were used as test dyes. The ability of the white rot fungi to degrade these dyes were studied in the C-limited medium (M14) of Janshekar and Fiechter (1988) and the results were presented in table 1.

The results revealed in Poly R dye, maximum degradation (31.7%) was brought by *P. grammocephallus*. The fungus *Ganoderma sp*.1 degraded 30.6 per cent of the dye and the minimum degradation (18.7 %) was observed in *Lenzites sp* treatment.

In Remazol brilliant blue maximum degradation up to 70.6% and 60.0% was observed in *P. grammocephallus* and *Ganoderma sp*.1 treatment respectively. Minimum degradation of remazol brilliant blue (16.4%) was observed by the fungus *Lenzites sp*.

Degradation of lignin by white rot fungi in synthetic medium

For confirmation of ligninolytic activity of the fungi, the ability of the fungi to grow and to degrade lignin was studied in lignin amended basal medium. The growth was measured in terms of increase in mycelial dry weight (mg) per day. The results revealed that (Table 1) all the three fungi were capable of utilizing lignin as growth substrate. The mycelial growth was observed in the range of 2.91 to 2.98 mg/day for *P. grammocephallus* and *Ganoderma sp*.1 respectively. The growth rate of the fungus *Lenzites sp* was 2.78 mg/ day.

The results showed that all the fungi were capable of degrading lignin synthetic medium. Among the fungi *P. grammocephallus* was the weakest lignin degrader, the per cent of lignin degradation was only 26.3 per cent; whereas *Ganoderma sp*.1 and *Lenzites sp* exhibited maximum degradation up to 55.4 and 51.1 per cent respectively.

Production of ethylene from 2-keto-4-thiomethyl butyric acid (KTBA)

The ligninolytic activities of fungi were further confirmed by their ability to release ethylene from KTBA. The data is presented in the Table 1. Once again confirmed that all the test fungi were lignin degraders, but the efficiency differ from fungus to fungus. For instance the ethylene production from KTBA was maximum (2.621 ppm) in *Ganoderma sp*.1 whereas in *P. grammocephallus* very low level of ethylene was produced (1.426 ppm). The fungus *Lenzites sp* produced 2.233 ppm of ethylene.

Table: Screening of ligninolytic activity of white rot fungi by dye reduction, lignin degradation and ethylene production methods

Methods	*P.grammocephallus*	*Ganoderma Sp.*1	*Lenzites sp*
1.Dye reduction method			
a. Poly R dye			
Degradation/ hour	0.111	0.106	0.099
Percent degradation	31.7	30.6	18.7
b.Ramazol brilliant blue			
Degradation/ hour	0.159	0.135	0.027
Percent degradation	70.6	60.0	16.4
2. Lignin degradation			
Growth on lignin (mg/day)	2.91	2.98	2.78
Percent degradation	26.3	55.4	51.1
3.Production of ethylene from KTBA(ppm)	1.426	2.621	2.233

Values are mean of the three replicates

Optimization of culture condition on growth and ligninase production
Incubation period

The effect of incubation period on culture condition and laccase production by *P. grammocephallus, Ganoderma sp*.1*, Lenzites sp* were presented in table 2. The results showed that maximum growth was observed by *P. grammocephallus* (17.0 mg mycelial dry weight), on the first day, the laccase enzyme production was 0.39 U/ml, the maximum production (1.34 U/ml) was achieved on fourth day, after that the enzyme production was reduced.

In *Ganoderma sp*.1 maximum growth (18.0 mg mycelial dry weight) and laccase production (1.42 U/ml) was observed on fourth day later it was decreased.

In *Lenzites sp* maximum growth (21.0 mg mycelial dry weight) and laccase production was observed maximum (1.61 U/ml) on fourth day; but after the optimum period the enzyme productions were gradually decreased.

Table: Optimization of incubation period on growth and ligninase production by white rot fungi

Incubation period (days)	Growth (mycelial dry weight in mg)			Laccase(U/ml)		
	Pg	G	L	Pg	G	L
1	5.0	5.1	6.2	0.39	0.46	0.57
2	5.4	5.4	8	0.44	0.57	0.93
3	5.6	5.6	11	0.81	0.67	0.99
4	7.5	7.1	18	1.34	1.42	1.61
5	10	10	14	1.26	1.16	1.45
6	12	13.5	14.5	1.17	1.79	1.19
7	15	14	15	1.11	1.03	1.76
8	17	16	17	1.08	0.99	0.77
9	17	18	18	0.99	0.86	0.66
10	16	17	19	0.78	0.75	0.48
11	16	17	21	0.40	0.68	0.40
12	16	17.5	19	0.35	0.61	0.38

Pg:*Polyporous grammocephallus*; G:*Ganoderma sp.*1; L:*Lenzites sp*.

Values are mean of the three replicates

pH

Effects of pH on culture condition and laccase production were studied in the pH range from 3.0 to 9.0. The results in table 3 revealed that, *P. grammocephallus* (24.0 mg dry weight) exhibited maximum level of laccase production (1.05 U/ml) at pH 7.0.

In *Ganoderma sp*.1 and *Lenzites sp* maximum growth (24.0 mg and 32.0 mg dry weight) and laccase production (1.16 U/ml and 1.22 U/ml) were observed at pH 7.0.

Temperature

The effect of temperature on culture condition and ligninase production was studied in the temperature ranges from 30 to 70°C. The results in table 4 showed that, in *P. grammocephallus* maximum growth (22.0 mg dry weight) and laccase enzyme production were optimum at 40°C and the productions was 1.85 U/ml.

In *Ganoderma sp*.1 and *Lenzites sp* maximum growth (21.0 mg and 24.0 mg) and laccase production (1.82 U/ml and 1.89 U/ml) were favoured at 40°C.

Table: Optimization of pH on growth and ligninase production by white rot fungi

pH	Growth (mycelial dry weight in mg)			Laccase (U/ml)		
	Pg	G	L	Pg	G	L
3.0	14	15	16	0.63	0.67	0.65
3.5	15	17	18	0.68	0.75	0.71
4.0	16	18	19	0.70	0.80	0.77
4.5	16	18	21	0.76	0.83	0.83
5.0	18	19	24	0.80	0.86	0.91
5.5	19	20	25	0.86	0.89	0.94
6.0	20	23	28	0.96	0.92	0.96
6.5	22	24	30	0.97	1.01	1.07
7.0	24	26	32	1.05	1.16	1.22
7.5	20	20	27	0.72	0.95	0.55
8.0	18	18	25	0.45	0.65	0.33
8.5	17	17	24	0.38	0.40	0.30
9.0	16	16	24	0.26	0.32	0.28

Pg:*Polyporous grammocephallus*; G:*Ganoderma sp.*1; L:*Lenzites sp*.

Values are mean of the three replicates

Table : Optimization of temperature on growth and ligninase production by white rot fungi

Temperature (°C)	Growth (mycelial dry weight in mg)			Laccase (U/ml)		
	Pg	G	L	Pg	G	L
30	19	16	18	0.84	0.85	0.86
35	21	19	20	1.66	162	1.65
40	22	21	24	1.85	1.82	1.89
45	17	18	21	0.80	0.76	0.83
50	15	13	16	0.72	0.63	0.68
55	16	18	15	0.71	0.62	0.65
60	17	19	16	0.69	0.60	0.63
65	18	20	19	0.67	0.59	0.60
70	17	18	18	0.64	0.55	0.58

Pg :*Polyporous grammocephallus*; G:*Ganoderma sp.*1; L:*Lenzites sp.*

Biosorption of heavy metals by using white rot fungi

In the present study biosorption of Copper (Cu) and Zinc (Zn) by white rot fungi were analyzed by atomic absorption spectrophotometer and the results were tabulated in table 10.

In *P. grammocephallus* the maximum growth of the mycelium was found to be 28.0 mg. In *Ganoderma sp.*1 was found to be 24.0 mg and in *Lenzites sp* it was reported to be 22.0 mg.

The biosorption of Cu by *P. grammocephallus* was observed to be 26.67 per cent. Similarly, for *Ganoderma sp.*1 and *Lenzites sp* the biosorption of Cu was 39.38 and 37.66 per cent respectively. The biosorption of Zn by *P. grammocephallus* was observed to be 58.13 per cent. Similarly 56.49 and 60.19 per cent biosorption of Zn was achieved by *Ganoderma sp.*1 and *Lenzites sp* respectively.

Table : Biosorption of heavy metals by white rot fungi

Fungi Degradatioon	Incubation period (Days)	Mycelial dry weight (mg)	Percent Copper (Cu)	Zinc (Zn)
Control	7	-	-	-
P.grammocephallus.	7	28	26.67	58.13
*Ganoderma sp.*1.	7	24	39.38	56.49
Lenzites sp.	7	22	37.66	60.19

Values are mean of the three replicates

DISCUSSION

Biosorption of heavy metals was studied using biomass obtained from *G. sepiarium* with an initial Cr (VI) concentration of 3.4mg of soil. Even though the Cr (VI) reduction rate increased with an increase in incubation time period, 94 per cent reduction of Cr (VI) was at observed at six months. Reduction of Cr (VI) to Cr (III) is a microbially mediated process and provision of suitable electron donor to contaminated soils shall greatly speed up this reaction, thereby decreasing Cr (VI) toxicity and mobility The biotransformed Cr (VI) remained in the soil as Cr (III). A significantly higher soil nutrient distribution of 3.7 per cent organic matter, 2 per cent carbon, 0.25 per cent nitrogen and 10.3 per cent phosphorus was observed in soil contaminated with chromium after six months of incubation with fungal biomass compared to control. The nutrient content in the control contaminated soil was least 2.1 per cent organic matter, 1.3 per cent carbon, 0.21 per cent nitrogen and 7.9 per cent phosphorus. Similar kind of results was also observed. biosorption of heavy metals (Copper and Zinc) were degraded by white rot fungus was analyzed by atomis absorption spectrophotometer. The biosorption of Copper 26.67, 39.38 and 37.66 per cent by *P. grammocephallus, Ganoderma sp.*1 and *Lenzites sp.* In *P. grammocephallus, Ganoderma sp.*1 and *Lenzites* sp. The Biosorption of Zinc were 58.13, 56.49 and 60.19 per cent. The mycelial dry weight were found to be 28.0, 24.0 and 22.0 mg for *P. grammocephallus, Ganoderma sp.*1 and *Lenzites sp* on seventh day of incubation.

CONCLUSION

From the present investigation it was confirmed that white rot fungi can be used for the biosorption of Copper (Cu). The biosorption of Cu by *P. Grammocephallus* was 26.67 Per cent then 39.38 and 37.66 Per cent was exhibited by *Ganoderma sp.*1 and *Lenzites sp* on 7th day of incubation. The biosorption of Zinc (Zn) of *P. grammocephallus* was 58.13 Per cent then 56.49 and 60.19 Per cent was exhibited by *Ganoderma sp.*1 and *Lenzites sp* respectively on 7 th day of incubation.

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