International Journal of Science and Research (IJSR)
ISSN: 2319-7064
SJIF (2019): 7.583

Isolation and Characterization of Laccase Producing Bacteria from Soil Used for the Degradation of Malachite Green

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Abstract: ***A Soil sample was collected from rhizosphere and rhizosphereic zone of Vertak Garden, Vasai west and Mangroove soil from Vasai creek (Maharashtra) for microbial and enzymatic study. Twenty-five bacterial strain from Vertak Garden and Twenty-nine bacterial strain from Vasai creek were isolated and after further screening five bacterial strain were isolated from the soil sample and all they are gram positive and gram negative in nature. Five bacterial strains were selected as laccase producers from both the samples. The bacterial isolates were examined for Morphological, Physiological and Biochemical characteristics. The Bacterial isolates were having potential to produce the bacterial laccase enzyme were analyzed respectively.***

Keywords: ABTS, DMP, Guaiacol, Laccase, Microbial and Enzymatic analysis

1. Introduction

Laccases are monomeric, dimeric or tetrameric glycoproteins with four Copper atoms (belonging to three types: 1, 2 or 3) per monomer located at the catalytic site. Type 1 (Ti) Copper is responsible for the oxidation of the substrate and imparts the blue colour to the enzyme. Laccases use molecular oxygen to oxidize a variety of aromatic and non-aromatic hydrogen donors via a mechanism involving radicals. These radicals can undergo further laccase-catalysed reactions or non-enzymatic reactions such as polymerization, hydration or hydrogen abstraction. For phenolic substrates, oxidation by laccase results in formation of an aryloxy radical, an active species that is converted to a quinone in the second stage of the oxidation. Quinone intermediates can spontaneously react with each other to form soluble or insoluble coloured oligomers, depending on substrate and environmental parameters [Walker, 1988].

Laccase can decarboxylate phenolic and methoxyphenolic acids [Agematu et al., 1993], and also attacks methoxyl groups through demethylation [Chhabra et al., 2009; Gomare et al., 2009; Leonowicz et al., 2001]. Dehalogenation of substituents located in the ortho or para position may also occur in the case of substituted compounds [Schultz et al., 2001].

Laccases play an important role in several industry, paper and pulp industry, textile industry, synthetic chemistry, cosmetics, soil bioremediation and biodegradation of environmental phenolic pollutant and removal of endocrine disruptors. Recently laccases have been efficiently applied to Nano biotechnology due to their ability to catalyze electron transfer reactions without additional cofactor (Shraddha et al., 2011).

In future, laccase is a useful enzyme for biotechnological application in decolourization and biodegradation of contaminating environmental pollutants. Laccases have many biotechnological applications because of their oxidation ability towards a broad range of phenolic and non-phenolic compounds (Mohammadian et al., 2010).

Laccase was first discovered by Yoshida [1883] in the sap of lacquer tree Rhusvernicifera and the enzyme has been characterized in great detail later by Huttermann et al., [2001]. However, the report of laccase in other plant species is more limited and partially characterized.

A few years later after the discovery of the plant laccase by Yoshida [1883], The first report on bacterial laccase was from the rhizospheric bacterium *Azospirillumlipoferum* [Givaudan *et al.,* 1993].

Laccase and laccase-like activity has been reported from some of the bacteria, these are *Sphingobacterium*sp., *Pseudomonas* sp., *Brevibacilluslaterosporus*, *Agromycessalentinus* and *Sinorhizobiummorelense*, *Comamonas*sp, *Ralstoniasolanacearum*, *Bacillus subtilis*, *Streptomyces lavendulae*, *Streptomyces griseus*, *Sinorhizobiummeliloti*, *Bacillus subtilis*, *Marinomonasmediterranea*, *Pseudomonas syringae*, *Bacillus sphaericus* and *Escherichia coli*.

This study is an attempt to isolate and characterize laccase enzyme producing bacterial strains from the Vertak Garden and Vasai creek, Mumbai, India.

2. Materials and Methods

2.1 Sampling & Isolation of Bacteria

Soil samples were collected from rhizosphere and rhizospheric zone of Vertak Garden, Vasai West and mangrove soil from Vasai creek (Maharashtra). The soil was collected from the surface to a depth using sterile spatulas and transported to the laboratory and stored at – 20^{o}C.

For the enrichment of the soil bacteria, 250 gm soil samples were added to 250 ml of distilled water. The solution was stirred vigorously and then autoclaved at 15 psi for 45 min. Then collect the supernatant by centrifuging the soil sample

and now supernatant called as soil extract. Prepare minimum salt media in 100 ml soil extract and add 5 gm of soil in this medium. 1 g of the soil sample added into the medium was stirred vigorously and placed in incubator shaker for 2 days at 30°C. [Shikha Yadav et al, 2014].

Take 1 ml of sample from Minimum Salt Media and perform serial dilution in 0.9 % Saline (NaCl). 100 of the liquid mixture were serially diluted until a dilution of 106. Then 100 of this from each dilution were plated on Minimum Salt Media (Phase I & Phase II) by using spread plate technique. The plates were incubated at 30°C for 48 to 72 hrs. [Demissie A. G et al 2014].

2.2 Screening

Individual bacterial colonies from the plates will screened on Petri plates containing nutrient agar supplemented with 0.5 mMguaiacol to detect laccase activity. The plates will be incubated at 25–33°C for 96 h. Colonies showing brown colonies or brown colour zone around bacterial colony indicate the presence of laccase activity. Positive selected bacterial colonies stored (maintained) at Nutrient Agar Plate containing 0.5 mMGuaiacol.

2.2.1 Microbiological Analysis

Different Morphological, Physiological and Biochemical test like Gram's staining, colony morphology, motility test and oxidation and fermentation test were studied according to

2.2.2 Enzyme production and enzyme activity:

For this, 0.1 % of overnight-grown bacterial culture was inoculated in M162 broth containing 0.2 % yeast extract, 0.2 % tryptone, and 0.1% CuSo4. After 48 hours, the culture supernatant was obtained by centrifugation at 10,000 rpm for 15 min
and used as extracellular enzyme. For Intracellular Laccase enzyme, cells obtained were washed twice, resuspended in 5 ml of 0.1 M Tris–HCl buffer (pH 8.0), disrupted by ultrasonication, or centrifuged at 10,000 rpm for 10 min at 4°C. The supernatant obtained from the cell extract was used as intracellular enzyme (Sonica Sondhi et al 2014).

The enzyme assay was performed at 90°C for 5 min in 0.1 M phosphate buffer (pH 8.0) using 2,6 – dimethoxy phenol (2mM) (DMP) as Substrate. The change in absorbance due to the oxidation of DMP was monitored at 470 nm (extinction coefficient = 14,800 M-1cm-1) in a UV – Visible spectrophotometer.

The laccase activity was calculated by the formula: -

$$E.A = \frac{A \, x \, V}{t \, x \, e \, x \, v}$$

Where, E.A.: Enzyme Activity, A: Absorbance at 470 nm, V: Total mixture volume, t: incubation time, e: Extinction coefficient for DMP (14,800 M-1cm-1), v: Enzyme Volume,

2.2.3 Optimization of Enzyme Activity

In the characterization the laccase enzyme involved determination of effect of different factors such as temperature, pH on its activity and stability was studied. Effect of temperature and pH was determined on laccase activity.

2.2.4 pH

To find out the optimum pH for laccase enzyme activity, a pH range of 4.0-10 was investigated. DMP was used as a substrate in the following four buffers: acetate (pH: 4.0-5.6); citrate phosphate buffer (pH: 6-7); Sodium phosphate buffer (pH: 7-9) and glycine- NaOH (pH: 9-10) at 32°C. The activity was measured using standard DMP assay as above.

2.2.5 Temperature

The effect of temperature on enzyme activity was investigated within the range of 25- 90°C at a pH of 4.5 + 0.2. The laccase activity was measured using standard DMP assay as above.

2.2.6 Partial Purification of Enzyme

All steps of purification were performed at a temperature of 4oC using 100 mM sodium phosphate buffer, pH 6.5. Techniques used for the purification of laccase enzyme were Ammonium sulphate precipitation, dialysis. The enzyme preparations at various stages of laccaseenzyme purification were analyzed for protein concentration and enzyme activity.

2.3 Application

2.3.1 Dye decolourization by partially purified laccase: -

Partially purified enzyme extracts were investigated for dye decolourization of Malachite Green. The dye was solubilised in distilled water to the final concentration of 50 mg/l. The reaction mixture (6.0 ml) contained 2.0 ml acetate buffer (pH: 4.6), 2.0 ml of dye solution and 2.0 ml of crude enzyme extract/ laccase enzyme followed by incubation at 37°C for 3-12 hrs.

Dye decolourisation was expressed in terms of percentage calculated according to the equation.

$$Decolourisation\ (\%) = \frac{Ao - At}{Ao}\ X\ 100$$

Where, A_o is an absorbance at an appropriate wavelength of the dye immediately measured after adding the enzyme solution and A_t is an absorbance after each time intervals.

3. Result and Discussion

The Soil samples were collected from rhizosphere and rhizospheric zone of Vertak Garden, Vasai West and Mangrove soil from Vasai creek (Maharashtra). 1 gm of soil sample was inoculated in 100 ml of MS Media Broth for 48 hrs at 30°C in incubator shaker. The enriched sample was used for isolation of Laccase producing bacteria. The enriched sample was inoculated onto sterile minimum salt medium (Phase I & Phase II).

Plates were incubated at 30°C for 24–72 h and 25 positive colonies from sample 1 and 29 positive colonies from sample 2 were chosen for first phase screening and purified in stepwise screening techniques. Colonies showing brown colour zone around bacterial colony indicate the presence of laccase activity. From the samples, many bacterial strains

were isolated and screened; it was found some of those bacterial strains showed positive result for laccase production.

The better zone and better colour formed and five bacterial strains were considered for further study. Five bacterial isolates were shown in Image: 1.

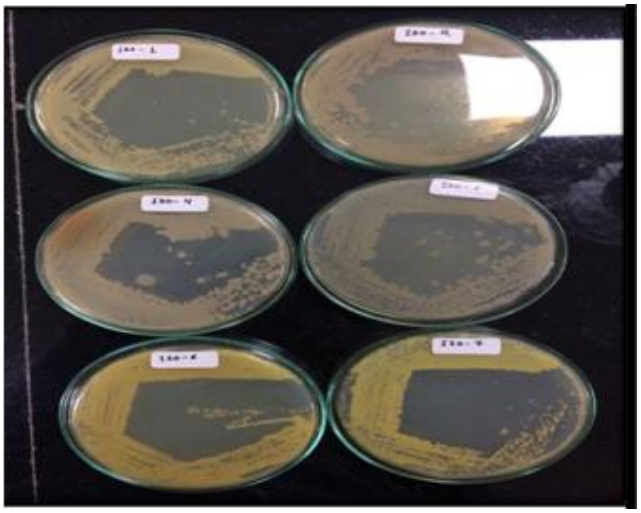


Image 1

The isolated five samples were characteristic analyzed shown in Table – 1, Sugar Fermentation shown in Table – 2 & biochemically analyzed and showed variable characters shown in Table - 3.

Table 1

Test \ Isolate	IR 1	IR 2	IR 4	IR 5	IR 6
Sugar Fermentation:					
1% Glucose	A	A	A	A	A
1% Lactose	A	-Ve	-Ve	-Ve	-Ve
1% Sucrose	A	A	A	-Ve	A
1% Mannitol	A	-Ve	-Ve	-Ve	-Ve

Table 2

Character \ Isolate	IR 1	IR 2	IR 4	IR 5	IR 6
Shape	Circular	Circular	Circular	Circular	Circular
Colour	White	Brown	Brown	White	Yellow
Margin	Regular	Irregular	Regular	Regular	Regular
Elevation	Convex	Convex	Convex	Convex	Convex
Opacity	Opaque	Opaque	Opaque	Opaque	Opaque
Consistency	Sticky	-	-	-	Sticky
Gram Nature	-Ve, Rods	+Ve, Coccus	+Ve, Coccus	-Ve, Rods	+Ve, Rods

Table 3

Test \ Isolate	IR 1	IR 2	IR 4	IR 5	IR 6
Indole	+Ve	-Ve	-Ve	-Ve	-Ve
Methyl Red	-Ve	+Ve	+Ve	+Ve	+Ve
Voges – Proskauer	-Ve	-Ve	-Ve	-Ve	-Ve
Citrate utilization	+Ve	+Ve	+Ve	+Ve	+Ve
Urease production	+Ve	-Ve	-Ve	+Ve	-Ve
Catalase	+Ve	+Ve	+Ve	-Ve	-Ve
Oxidase	+Ve	-Ve	-Ve	+Ve	-Ve
Triple Sugar Iron Agar: -					
Slant	Alk	Acid	Acid	Acid	Acid
Butt	Alk	Acid	Acid	Alk	Acid
Gas production	+Ve	-Ve	+Ve	+Ve	+Ve
H2S production	-Ve	+Ve	-Ve	+Ve	-Ve

Key: -
A: - Acid Production AG: - Acid & Gas Production
-Ve: - Negative Test +Ve: - Possitive Test

Enzyme Activity: -

Table 4: Enzyme activities of selected isolates

Sr No.	IR 1	IR 2	IR 4	IR 5	IR 6
Enzyme Activity U/ml	0.009054	0.007567	0.013783	0.008783	0.005945
Enzyme Activity U/l	9.0540	7.5675	13.783	8.7837	5.9459

Table 5: pH

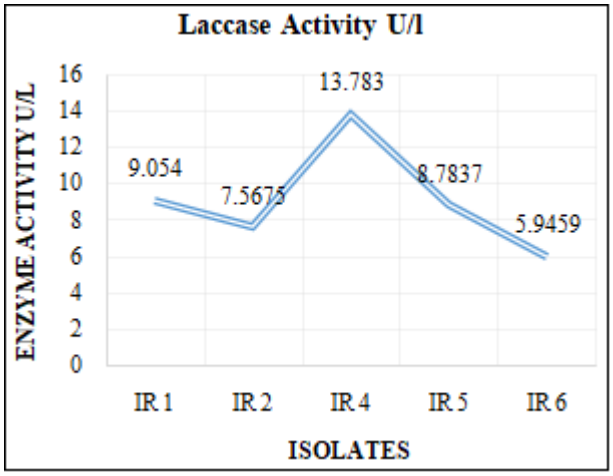
Sr No.	pH	Enzyme Activity of IR 1	Enzyme Activity of IR 1	Enzyme Activity of IR 4	Enzyme Activity of IR 4
		U/ml	U/l	U/ml	U/l
1	4	0.004729	4.7297	0.009729	9.7297
2	5	0.00581	5.8108	0.007972	7.9729
4	6	0.000581	0.8108	0.002567	2.5675
5	7	0.00581	5.8108	0.007027	7.027
6	8	0.009054	9.054	0.008783	8.7837
7	9	0.009054	9.054	0.009324	9.3243
8	10	0.007837	7.8378	0.008378	8.3783

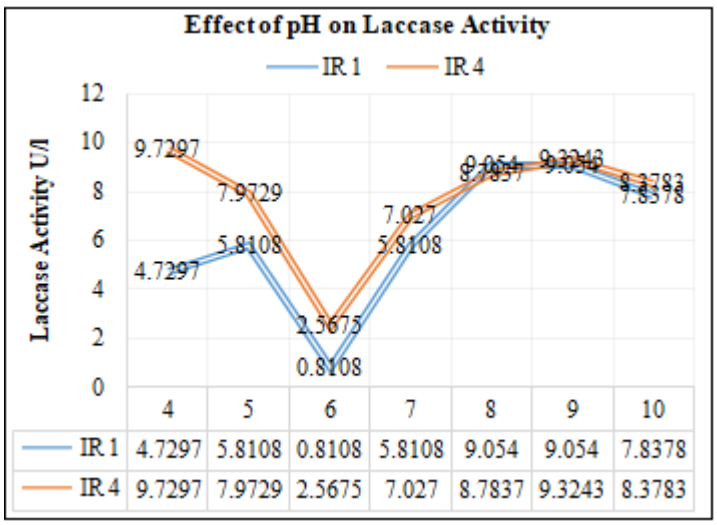
Table 6: Temperatures

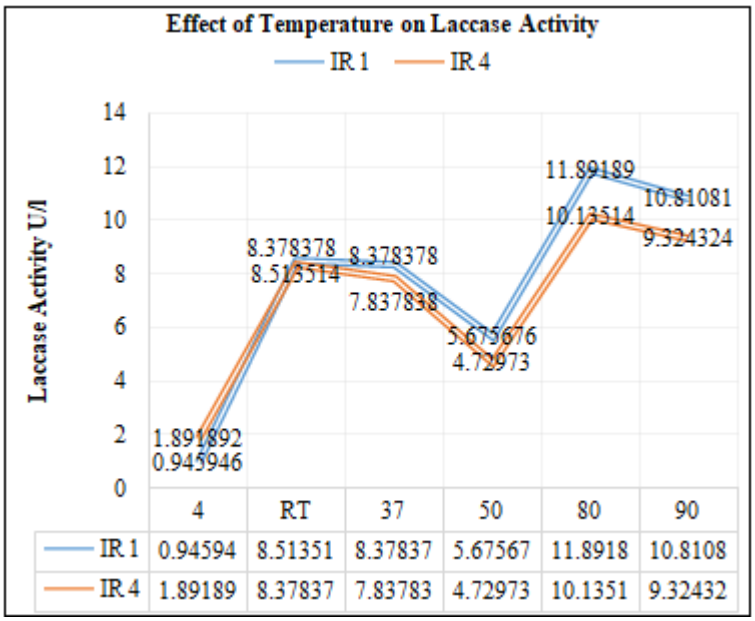
Sr No.	Temp	Enzyme Activity of IR 1	Enzyme Activity of IR 1	Enzyme Activity of IR 4	Enzyme Activity of IR 4
		U/ml	U/l	U/ml	U/l
1	4	0.000946	0.945946	0.001892	1.891892
2	RT	0.008514	8.513514	0.008378	8.378378
4	37	0.008378	8.378378	0.007838	7.837838
5	50	0.005676	5.675676	0.00473	4.72973
6	80	0.011892	11.89189	0.010135	10.13514
7	90	0.010811	10.81081	0.009324	9.324324

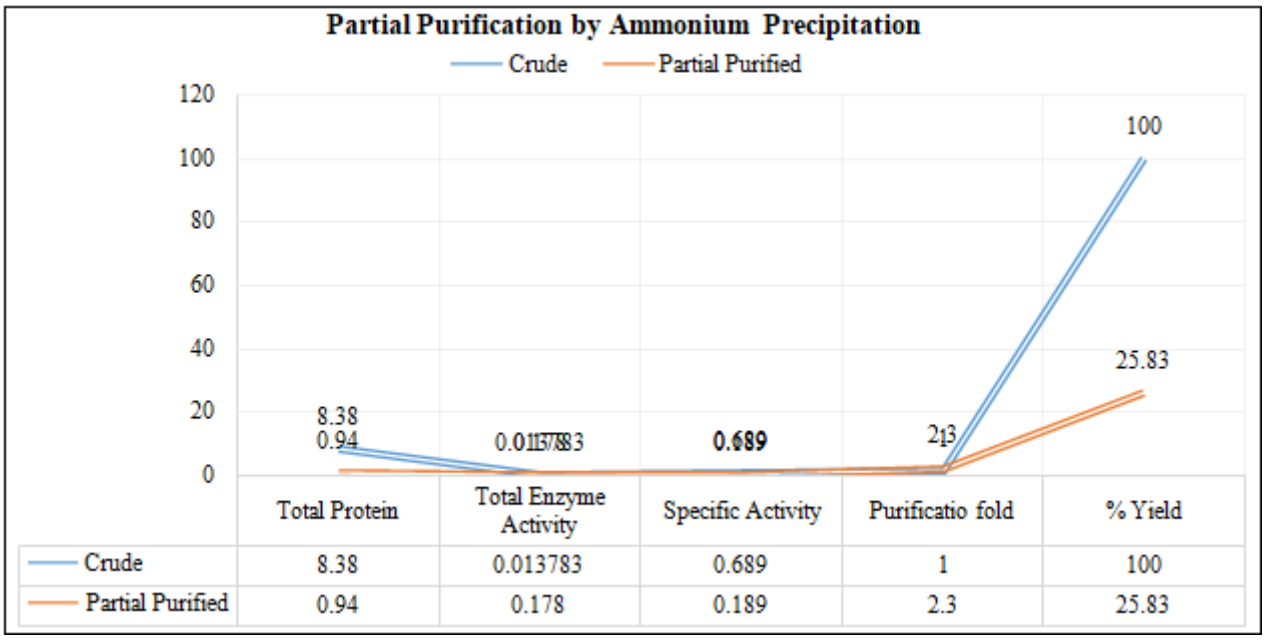
Laccase activities of the crude enzyme of selected five bacterial isolates were screened quantitatively by 2,6 – dimethoxy phenol (DMP). Laccase activities were found out 10 minsof incubation of all five bacterial isolates and it was found out that a total of 5 bacterial isolates were found to produce laccaseextracellularly.

It was found out that IR 4 & IR 1 bacterial isolate showed maximum extracellular laccase activity i.e. 13.783 U/l &9.0540 U/l after 10 minutesof incubation. Highest laccase activity was observed for IR 1 and IR 4 at pH 8 to 9 i.e. 9.0540 U/l &9.3243 U/l but IR 4 also showed maximum activity at pH 4 i.e. 9.7297 U/l. Highest laccase activity was observed for IR 1 and IR 4 at temperature 80^{o}C i.e. 11.89189 U/l for IR 1 &10.13514 U/l for IR4.



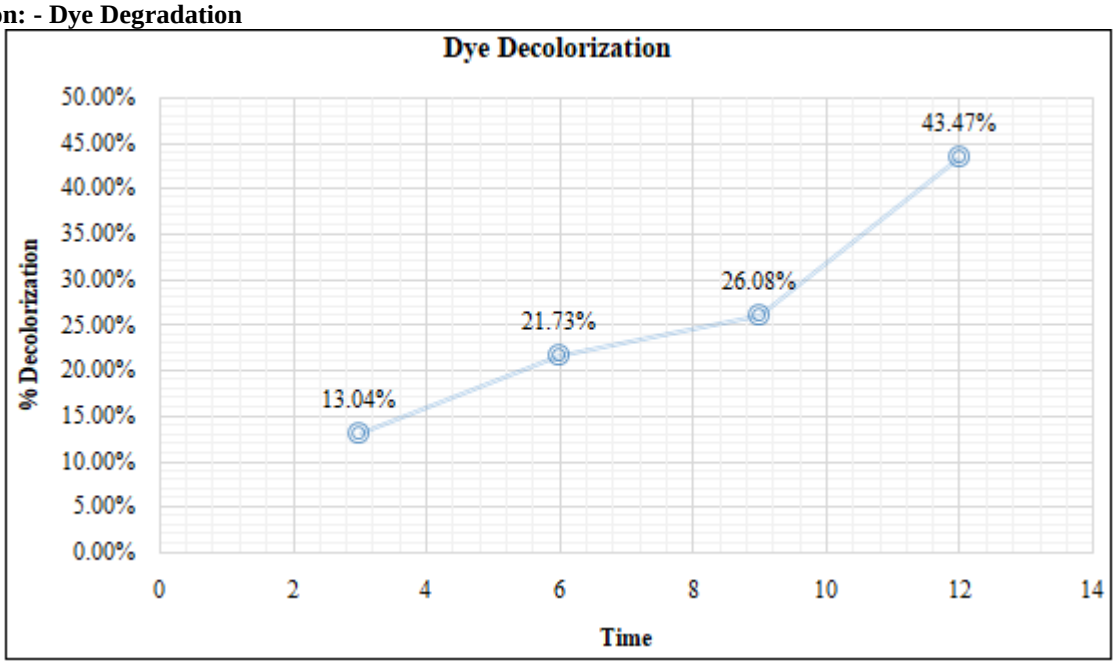






Total Laccase Activity in sample (10 ml) was 0.178U with 25.83% recovery and 2.30 purification fold with specific activity of Laccase was 0.189U/mg Protein.

Application: - Dye Degradation



The medium supplemented with 50 mg/l of dye, percent decolorization of dye was determined after a regular interval of time. Significant decolorization of 43.47% was observed after 12 hrs in case of malachite green dye.

4. Conclusion

Laccases are exceptionally versatile enzymes and ubiquitous in nature being produced by wide variety of plants, fungi and bacteria. The functions of enzymes differ from organism to organism and it has been observed during the perusal of literature that these enzymes have a wide range of applications because of their potential to curtail different types of the pollutions caused by toxic chemicals, xenobiotics and industrial effluents. ABTS and 2,6 – dimethoxy phenol used as substrate for laccase activity. After studying the effect of temperature and pH on laccase activity. Partial purification step was carried out by ammonium sulphate method for isolate IR 4 because it showed the highest enzyme activity. The total protein (mg) after purification was found to be 0.94 mg and total enzyme activity after partial purification was found to be 0.178 U. After partial purification, degradation of dye i.e. Malachite Green was done by using IR4 and the degradation was seen from three hours to twelve hours and the degradation of dye was estimated to be 43.47% after twelve hours.

Isolate IR 4 was found to be high activity and it is said efficient strain for laccase production. The ability of the isolated strain and laccase in the degradation of dyes was successfully proved.

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