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EFFECTS OF STRAIN IMPROVEMENT ON THE PRODUCTION OF LIGNIN PEROXIDASE FROM SOIL ISOLATES THROUGH THE ETHIDIUM BROMIDE AND UV RAYS.

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ABSTRACT

Lignocellulosic biomass based fuels are also better in terms of controlling emission of greenhouse gasses which has been the centre of discussion worldwide. However, the physico-chemical techniques available to separate lignin from other components or the techniques for lignin modifications are costly and also not eco-friendly. Lignin oxidizing enzymes are produced in copious amount by fungi but the practical application of fungi in industries is not possible due to various factors. Lignin peroxidase producing bacteria C7 was isolated from soil near to the woody area and effects of physical and chemical mutagen on the production of lignin peroxidase was carried out in which. Later the small-scale optimization was performed and effect on activity and productivity by cloning was also studied.

1. INTRODUCTION

Peroxidase is an enzyme that catalyses the oxidations of wide range of organic and inorganic compounds involving peroxide as electron acceptor [1]. It is found in animals, plants, insects viz; termite, microorganisms including fungi and bacteria. The energy demand is continuously increasing with industrialization and advancements of societies and to meet these energy demand non-renewable fossil fuels are continuously burning that is continuously imposing threat to environment by emission of greenhouse gases and it is also causing the exhaustion of limited fossil fuel resources [2]. As an alternative starch and corn based fuel development has been focused but it cannot be long term practical solution as it will compete with limited agricultural land [3]. Lignocellulosic biomass based fuel is another but better alternative in terms of availability, reduced greenhouse gases emission etc. but the majority of present techniques for lignin separation and modification are costly and also not eco-friendly [4]. Lignin separation or oxidation is also needed in paper and pulp industry, textile industry, cosmaceuticals, development of various useful compounds by conversion of

lignin [5]. Fungi produces the copious amount of Lignin oxidizing enzymes but they can't be practically used in industries in an efficient way so researcher are now working on bacteria for lignin oxidizing enzymes [6]. Recently more and more studies on bacteria are coming for lignin oxidizing enzymes [7].

2. MATERIALS AND METHODOLOGY:

2.1. Bacterial isolation and screening:

Soil mixed with wood powder was collected from R.K Timber, factory, Lucknow, India. Ten fold, Serialdilution [8] was performed to isolate bacteria colony and it was spread on Nutrient Agar media. Different bacterial colonies were identified by observing their colony morphology. Mix cultures were converted into pure culture by streak plate methods [9]. The primary screening for lignin peroxidase producing bacteria was done by performing the catalase test of the cultures and secondary screening was carry out by using Methylene-Blue plate assay [10]. The culture was streaked over Methylene-Blue-LB plate and incubated at 37°C for 72 hours.

2.2. Strain improvement:

Chemical mutagens such as Ethidium Bromide and physical mutagen such as UV rays wereused for mutation [11]. The cultures in sterilized nutrient broth were inoculated with different concentration of ethidium bromide and incubated at 37°C for 24 hours in shaker. Then the OD was taken at 620 nm in UV-Vis spectrophotometer. Selected cultures were screened for LiPactivity by observing the clear zones, using Methylene-blue plate assay.

2.3. Media selection and its optimization:

The cultures were inoculated in two different sterilized media supplemented with lignin and incubated at 37°C for 48-72 hours. The bacterial growth was checked by taking optical density at 620nm with respect to blank. Further the modification in media was done by applying one factor at a time [12].

Table 1: Components of media for selection and optimization.

S no.	Factors	Modified media	Compositions	Quantity
1	Media 1	MM1	K_2HPO_4	6 g/l
			NaH_2PO_4	3 g/l
			NaCl	5 g/l
			NH_4Cl	2 g/l
			$MgSO_4$	0.2 g/l
			Dextrose	8 g/l
			Lignin	1 %
2	Media 2	MM2	Peptone	5 g/l
			NaCl	5 g/l
			Dextrose	3 g/l
			Lignin	4%
3	Carbon sources	MM3	Dextrose	3 g/l
		MM4	Fructose	3 g/l
		MM5	Sucrose	3 g/l
4	Nitrogen sources	MM6	Peptone	5 g/l
		MM7	Malt-extract	5 g/l
		MM8	NH_4Cl	5 g/l
5	Salts	MM9	NaCl	5 g/l
		MM10	$FeCl_3$	5 g/l
		MM11	$CaCl_2$	5 g/l
6	pH	MM12	pH	4
		MM13	pH	5
		MM14	pH	7
		MM15	pH	9
		MM16	pH	11

2.3.1. Effect of temperature:

The cultures were streaked in sterilized nutrient agar plates and incubated at different temperatures 4°C, room temperature, 37°C, 50°C for 24 hours [13].

2.4. Growth curve study:

Growth study [14] was made for selected culture sample with medium that had been optimized. The media with optimized composition and pH was made, autoclaved, inoculated and incubated for overnight at 37°C, 120rpm in shaker incubator. Inoculation was made directly from pure culture plate. The culture growth was measured by observing absorbance at 620nm.

2.5. Fermentation:

The wild potential isolates and mutated strains were used for shake flask [15] and submerged fermentation [16] insterilized optimized media with same composition. Further the purification of enzyme was performed by salt precipitation and dialysis [17].

2.6. Estimation-Methylene-Blue Assay:

Enzyme activity can be estimating the percentage decolorization of methylene blue dye [18, 19, 20]. Reaction mixture includes 1ml of 50mM Sodium potassium tartrate buffer (pH 4), 0.1ml of 0.1mM H_2O_2as inducer, methylene blue as substrate and enzyme preparation. The reaction mixture is then incubated at RT followed by measuring OD at 650nm. Control will remain without enzyme. %decolorization of methylene blue dye canbe calculated by:

$$\%\ De - \boldsymbol{colorization} = \left(\frac{A_{\text{control}} - A_{\text{test sample}}}{A_{\text{control}}}\right) \times 100$$

3. Results

3.1. Sample Collection and Bacterial Isolation:

Five samples were collected from which 23 bacterial isolates after serial dilution and streaked over sterilized nutrient agar plates.

Figure1: Pure bacterial cultures after streaking

3.2. Screening for lignin peroxidase:

3.2.1. Peroxidase test:

Initially the cultures were screened for the peroxidase test to check the presence of peroxidase.

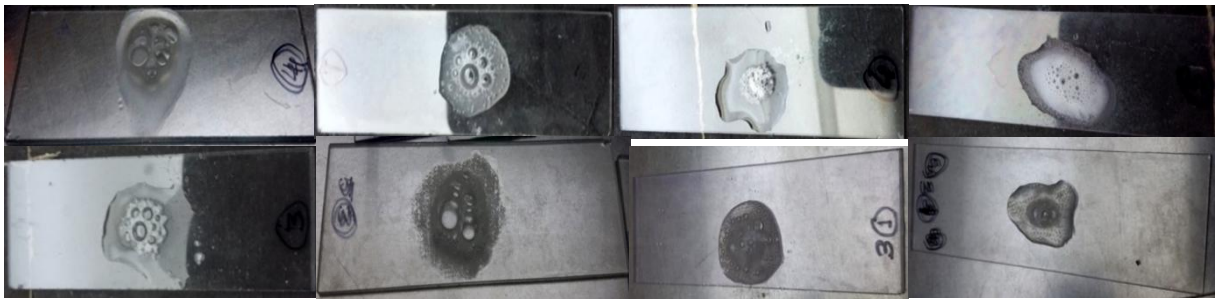


Figure 2: Presence of bubbles shows positive results.

3.2.2. Methylene blue plate test:

The presence of lignin peroxidase was checked by taking methylene blue as indicator. The culture C7 (sample3) were shortlisted as the positive for the production of lignin peroxidase.

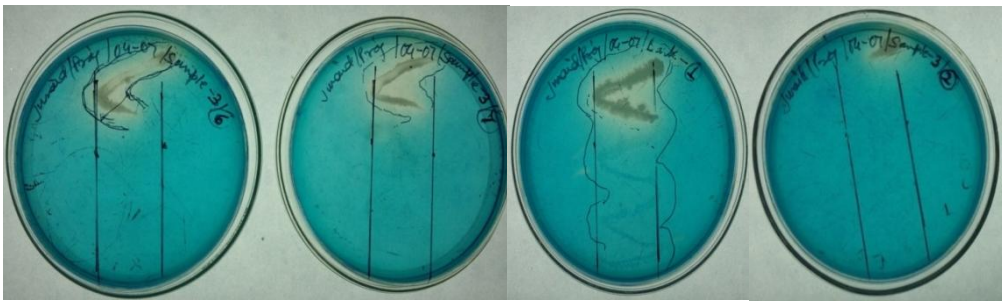


Figure 3: The clear zones indicate the positive result.

3.3. Strain improvement by chemical and physical mutation:

The C7 was mutated by using chemical mutagen Ethidium Bromide (EtBr) at different concentrations and the growth absorbance was taken at 620 nm.

Table 2: Effects of different concentration of ethidium bromide on culture C7 growth.

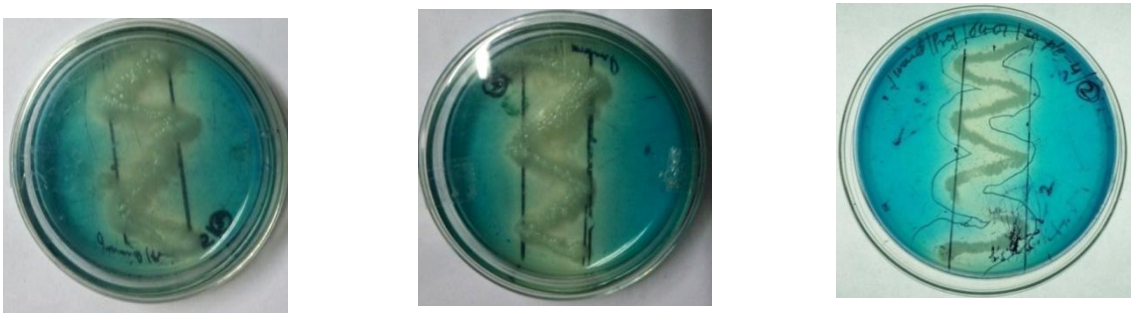
S no.	EtBr Concentration	OD at 620 nm
1	2 µg	0.21
2	4 µg	0.32
3	6 µg	0.14
4	8 µg	0.24
5	10 µg	0.03

The agar plates of C7 was exposed in UV rays at different time interval (2 min, 4 min, 6 min, 8 min and 10 min) and the growth result was observed after 24 hours incubation at 37°C.

Table 3: Effects of UV rays exposure on C7 growth.

S no.	Time (UV exposure)	Remarks
1	Control	Lawn formation
2	2 min	Lawn formation
3	4 min	Lawn formation
4	6 min	Lawn formation
5	8 min	165colonies
6	10 min	90 colonies

The cultures selected after chemical and physical mutationswere also screened for the presence of lignin peroxidase with respect to wild (control).



a. wild C7 **b. EtBr mutated C7** **c. UV mutated C7**

Figure 4: The screening for the production of lignin peroxidase from wild C7, EtBr mutated C7 and UV mutated C7.

3.4. Media selection and its optimization:

The media was selected and modified for all three cultures such as wild, EtBr mutated, UV mutated and the growth was observed after taking the absorbance at 620 nm.

Table 4: The growth of wild, EtBr mutated, UV mutated C7 on the modified media.

Modified Media	OD at 620 nm		
	Wild	EtBr mutated	UV mutated
MM1	0.44	0.40	0.29
MM2	0.53	0.63	0.52
MM3	0.12	0.09	0.13
MM4	0.18	0.16	0.23
MM5	0.10	0.09	0.01
MM6	0.30	0.25	0.32
MM7	0.11	0.11	0.05
MM8	0.02	0	0.1
MM9	0.33	0.29	0.35
MM10	0.13	0.1	0.18
MM11	0.26	0.24	0.19
MM12	0	0.01	0
MM13	0	0.03	0.05
MM14	0.59	0.6	0.39
MM15	0.56	0.58	0.22
MM16	0.07	0.09	0

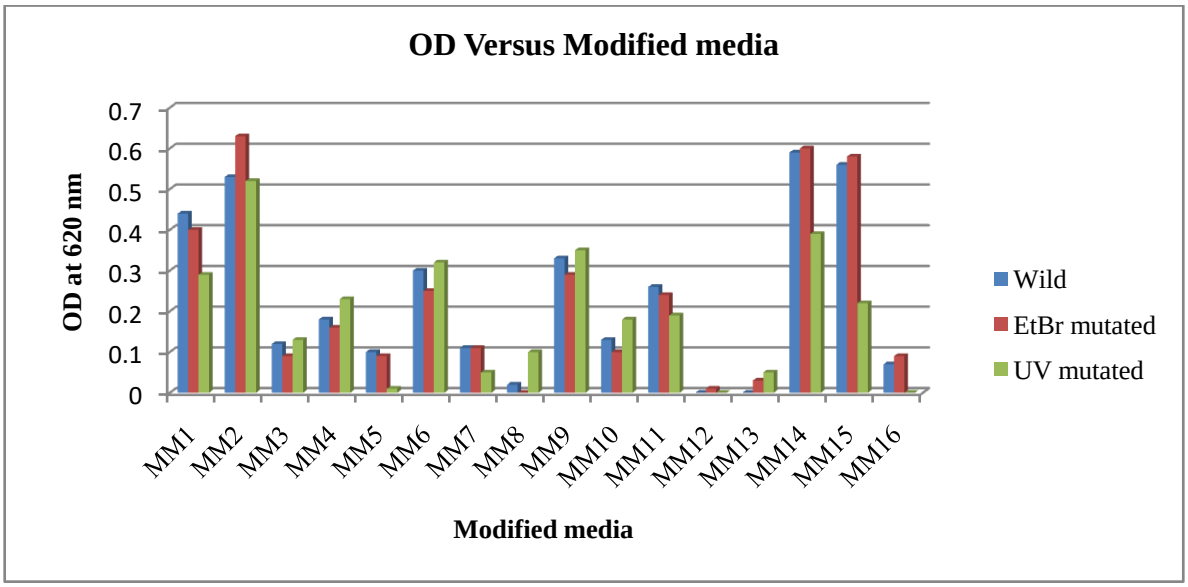


Figure 5: Growth of wild, EtBr and UV rays mutated C7 in modified media.

3.5. Growth curve:

The growth of all cultures such as wild C7, EtBr mutated C7 and UV mutated C7 was studied on optimized media. The OD was taken at 620 nm after one hour of time interval.

Table 5: The growth of wild C7, EtBr mutated C7 and UV mutated C7 at different time intervals.

S no.	Hours	OD at 620 nm		
		Wild	EtBr mutated	UV mutated
1	**0**	0.01	0.01	0.01
2	**1**	0.02	0.02	0.03
3	**2**	0.04	0.03	0.03
4	**3**	0.07	0.04	0.04
5	Overnight incubation			
6	**1**	0.14	0.19	0.17
7	**2**	0.21	0.25	0.23
8	**3**	0.22	0.27	0.25

9	**4**	0.26	0.29	0.29
10	**5**	0.29	0.35	0.28
11	Overnight incubation			
12	**1**	0.35	0.35	0.30
13	**2**	0.38	0.43	0.31
14	**3**	0.41	0.45	0.37
15	**4**	0.49	0.52	0.40
16	**5**	0.50	0.52	0.38
17	Overnight incubation			
18	**1**	0.45	0.47	0.44
19	**2**	0.39	0.26	0.35
20	**3**	0.20	0.23	0.10

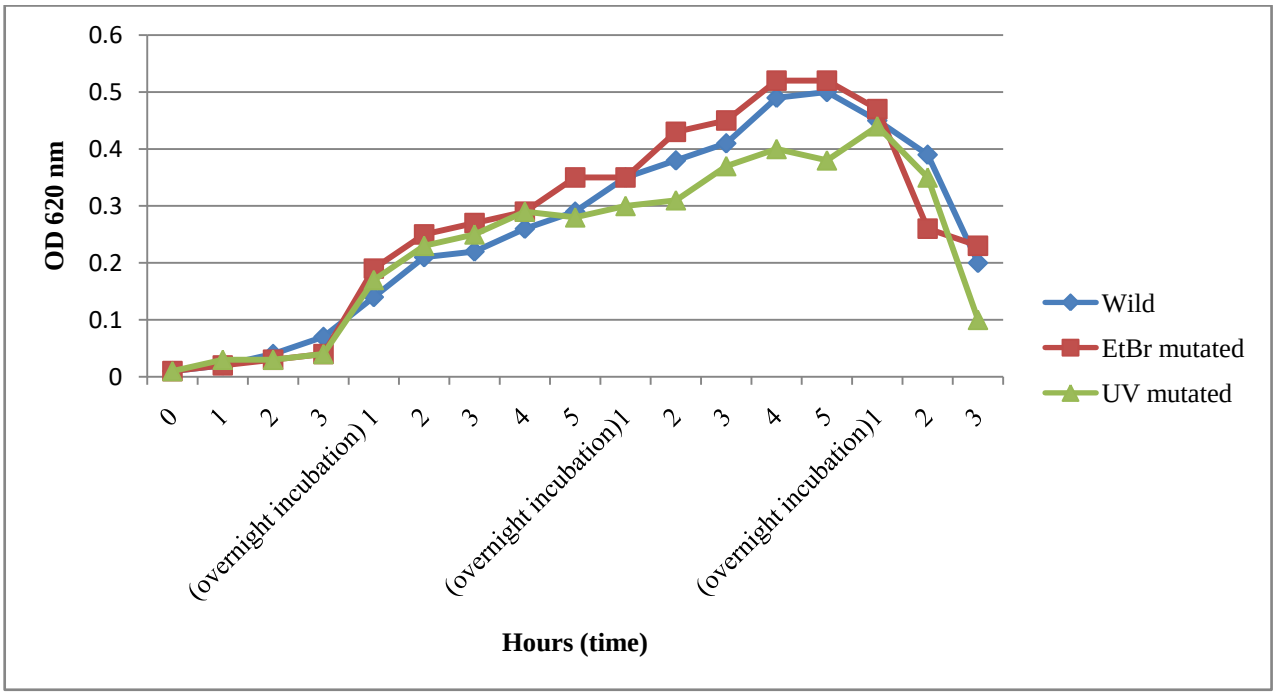


Figure 6: Growth curve study ofwild C7, EtBr mutated C7 and UV mutated C7

3.6. Fermentation and downstream processing:

By using growth curve study it was found that the stationary phase occurs after 72 hours. Sterilized media for the fermentation was prepared and all cultures wild, EtBr mutated and UV mutated was inoculated and incubated for 72 hours at 37°C. Further the downstream processing was performed by taking 40% ammonium sulphate for salt precipitation and then dialysis was done. The enzyme estimation was carrying out from the dialysis product.

3.7. Estimation of Enzyme Activity- Methylene Blue Assay:

Table 7: % De-colorization of Methylene Blue by wild C7, EtBr mutated C7 and UV mutated C7

S no.	**Sample**	**A 620 nm**	**% De-colorization**
Crude enzyme			
1	**Wild**	0.210	4.55%
2	**EtBr mutated**	0.204	7.27%
3	**UV mutated**	0.201	8.36%
Partial pure enzyme			
4	**Wild**	0.197	9.09%
5	**EtBr mutated**	0.199	9.545%
6	**UV mutated**	0.200	10.45%

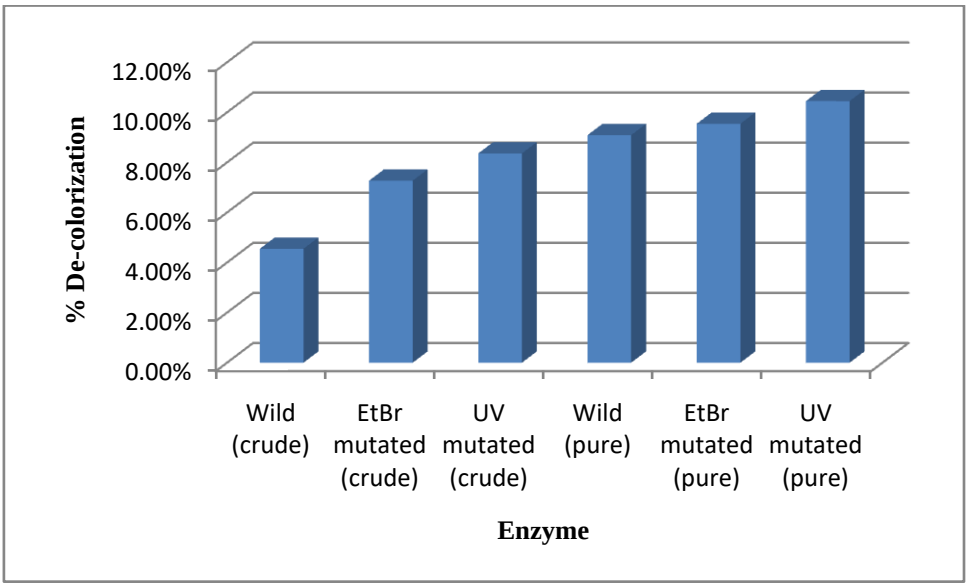


Figure 7: Studies on % de-colorization of Methylene Blue by wild C7, EtBr mutated C7 and UV mutated C7.

3.8. Protein Estimation- Bradford Assay:

Table 8: Concentration of protein extracted from wild C7, EtBr mutated C7 and UV mutated C7

S no.	Sample	A 680 nm	Concentration (mg/ml)
Crude enzyme			
1	**Wild**	0.15	0.04
2	**EtBr mutated**	0.16	0.05
3	**UV mutated**	0.10	0.03
Partial pure enzyme			
4	**Wild**	0.25	0.09
5	**EtBr mutated**	0.25	0.09
6	**UV mutated**	0.40	0.15

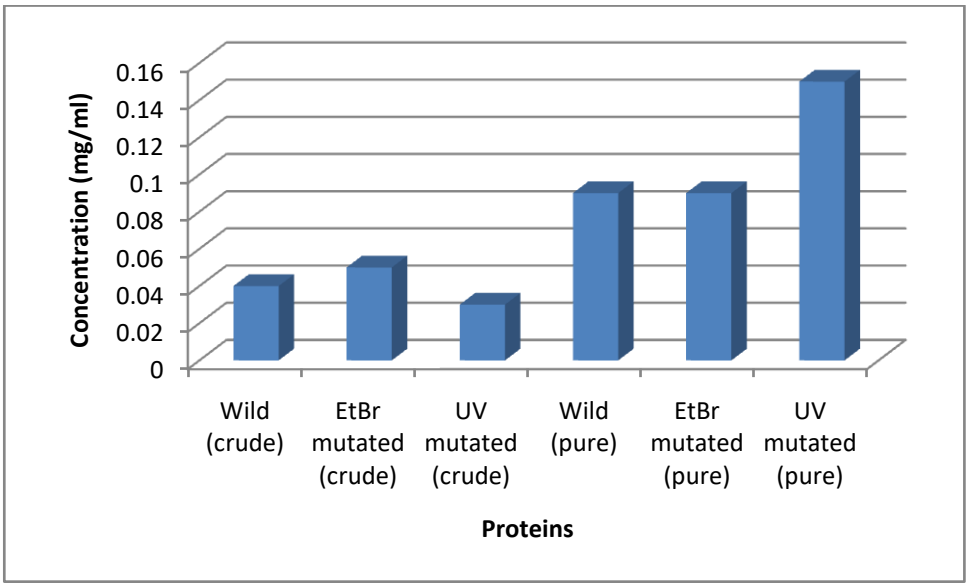


Figure 8: Studies on the concentration of protein extracted from wild C7, EtBr mutated C7 and UV mutated C7.

3.9. Biochemical test for Bacteria:

Table 9: Biochemical tests for the identification of bacteria.

Property	Culture 7
Gram stain	Negative
Shape	Bacillus
Endospore	Negative
Catalase	Positive
Glucose fermentation (Acid formation)	Positive
Mannitol test	Negative
Colony Color	Yellow translucent
LiP Activity (Zone on Methylene blue plate)	Positive
Growth on methylene blue plate	Positive
Laccase Activity (halo zone on bromophenol blue plate)	Positive

4. DISCUSSION:

Five samples were collected from different area rich in lignin sources. From which 23bacterial cultures isolated after serial dilution method and spread plate technique. These 23 isolates were further subjected to peroxidase test, through which total of 21 isolates were obtained that were peroxidase positive. The peroxidase positive isolates were further screened for lignin peroxidase on methylene blue plate through which 9 isolates were selected as Lignin peroxidase positive but only one culture C7 was selected, based on zone of de-colorization and growth, for further process. That means 42.86% of the peroxidase positive isolates were lignin peroxidase positive on Methylene-Blue-LB plate in other words just 39.13% of total isolates obtained after serial dilution were lignin peroxidase on methylene blue LB plate.

The selected isolate was also positive for Laccase activity on Bromophenol Blue. The strain improvement was done by chemical and physical mutation was carryout. Ethidium bromide and UV rays was selected as the mutagens for the enhancement of the production of lignin peroxidase from C7 culture. The sterilize optimized media was The inoculation with the all three types of cultures such as wild, UV mutated and Ethidium bromide mutated in sterilized fermentation media, then incubated for 48 hours to 72 hours at 37°C in shaker incubator.

After that the downstream processing was performed by salt precipitation (40% ammonium sulphate) and dialysis. The enzyme activity and the concentration was achieved and calculated by percent de-colorization of the methylene blue and Bradford assay. Where the UV mutated culture shows maximum activity by showing extreme de-colorization.

5. CONCLUSION:

It was concluded that the culture C7 after the mutation with UV rays enhances the production of the lignin peroxidase as compare to Ethidium bromide mutation with respect to wild C7. Further the activity and performance of the enzymes can be enhanced by treating the sample with the activators, chelators etc.

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