

Bacterial Lignin Peroxidase in Biobleaching of Lignin-mimicking Indicator Dyes

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ABSTRACT

Lignin, a complex biopolymer and textile dyes are the major pollutants of industrial waste-water and responsible for its intense undesirable dark-brown color. Due to complicated structure and non-hydrolysable bonds, lignin and some lignin-mimicking dyes are resistant to degradation. Potential applications utilizing lignin-degrading bacteria and enzymes like lignin peroxidase have become attractive because they may provide environmental friendly method for waste-water treatment of various industries. The growth on polymeric lignin or lignin monomers is not necessarily only a measure of the ligninolytic potential of bacteria.

The present work represents isolation, screening, acclimatization and identification of lignolytic and dye-decolorizing bacteria. The isolates were screened for their lignolytic and dye decolorization activity quantitatively by spectrophotometry and qualitatively by solid-phase plate assay. The approach revealed biobleaching of various dyes by bacterial lignin peroxidase. The isolate *Corynebacterium jeikeium* was found to decolorize indicator dyes Methylene Blue, Toluidine Blue, Congo Red and Malachite Green up to 76.32%, 64.10%, 96.51% and 81.78% respectively while *Sphingomonas paucimobilis* efficiently removed dyes Malachite Green, Methylene Blue and Congo Red up to 90%, 60% and 96% respectively when applied as axenic culture. The complexity of the dye structure was found to have an influence on the decolorization rate by LiP. The diverse structures of dyes might have affected the approach of ligninase (Lignin Peroxidase) cation radicals towards dye molecules. Each dye molecule contained a chromophore and its color disappeared only after the chromophore structure was destroyed by many attacks of LiP. Thus, the bacterial LiP can be adopted as an efficient tool for biobleaching of indicator dyes for small as well as large scale applications.

Key words: Biobleaching, Dye-decolourization, Lignin Peroxidase, Lignin Mimicking Dye.

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INTRODUCTION

Some of the industries that discharge highly colored effluents are paper and pulp mills, textiles and dye-making industries. Each of these industrial effluents creates some specific problem producing aesthetically unacceptable intense coloring of soil and water bodies¹⁵. The intense color is due to the lignin and various dyes which are very stable and hard to degrade¹⁷. Lignin, the second most abundant renewable aromatic biopolymer on earth after cellulose is known to be one of the most recalcitrant materials. It is found in the secondary layers of the cell wall as well as in the middle lamellae of plant cells. Various dyes used in textile dyeing industries are carcinogenic, toxic and recalcitrant xenobiotic compounds which are difficult to treat by conventional waste water treatment methods¹¹. They block the passage of light to the lower depths of the aquatic system resulting in cessation of photosynthesis, leading to anaerobic conditions, which in turn result in the death of aquatic life causing foul smelling toxic waters⁸. There is a great need to find eco-friendly method to remove or mineralize these compounds in order to reduce water pollution.

Dye decolorization using microorganisms and their enzymes has gained great attention in recent years for its efficient application¹⁸. Bacterial oxidoreductase enzymes play an important role in degradation and transformation of polymeric substances. The partially degraded or oxidized products can easily be taken up by microbial cells where they are completely mineralized. Lignin-degrading enzymes are one such group of oxidoreductive enzymes, which have practical application in bioremediation of polluted environment. Lignin peroxidases are useful in the treatment of colored industrial effluents and other xenobiotics. For colored effluents, bioremediation is measured by estimating the reduction in color units of effluents.

The present investigation was aimed to exploit the biobleaching of lignin and decolorization of various lignin-mimicking dyes by LiP of axenic cultures of indigenous

bacteria as well as a mixed consortium for remediation purpose³. Bacteria were isolated, acclimatized and screened for decolorization of lignin specifically and dyes such as Malachite Green, Congo-Red, Methylene Blue, Toluidine Blue and Janus Green non-specifically¹⁴. Potency of the isolates for biobleaching of lignin and dyes was analyzed by spectrophotometry.

MATERIALS AND METHODS

Lignolytic bacteria produce extracellular enzymes like Lignin peroxidase which acts on lignin and thus are able to degrade lignin¹⁸. For isolation of lignolytic bacteria, samples from different sources were collected, enriched and isolated using synthetic media containing lignin.

Lignin preparation

Crude Lignin was extracted from bark sample by alkaline delignification method. Removal of unwanted material other than lignin from lignocellulosic material was carried out by physical grinding to enhance specific surface area and pretreatment with dilute sulfuric acid at 140⁰C for 20 min. Alkaline delignification using 4% sodium hydroxide solution at elevated temperature efficiently removes lignin as dark brown colored liquid from the lignocellulosic biomass. The sources used for extraction of lignin and pretreatment were : Dried bark sample grinded to powder, 1% sulfuric acid (H₂SO₄), 4% sodium hydroxide (NaOH). In 50 gm of dried bark powder added 200 ml of 1% sulfuric acid and heated for 20 mins as acid pre-treatment and the mixture was allowed to cool to obtain the lignocellulosic mass. To the above pre-treated lignocellulosic mass was added 200 ml of 4% sodium hydroxide and heated for 30 mins. These steps precipitated all cellulosic material in the dried bark sample except lignin. The dark brown colored liquid obtained was filtered, autoclaved, refrigerated and used as crude lignin for further experimental work.

Enrichment, isolation and acclimatization of the lignolytic bacteria

Enrichment and isolation of lignolytic bacteria were done by using various sample sources

like Rhizosphere soil with cow dung, Lake water containing decomposing plant material, Compost feedstock, Decaying bark sample-scrap of decaying bark with crushed termites and industrial effluent. The soil extract used for enrichment, isolation and acclimatization of the lignin degrading bacteria contained g/L, soil (40), D/W (960), settled for 1 hour. Clear supernatant (extract) was collected. 1% Lignin soil extract agar medium containing (ml/100ml): soil extract (99); lignin (1); H₂O₂ (0.1 mM), antifungal- Fungidid B and agar agar, (2.5g) was prepared, pH adjusted to 7, autoclaved at 15 lbs for 15 min. and stored in sterile bottle for further study. Similarly, for acclimatization of the isolates, 2.5%, 5% and 10% v/v soil extract lignin agar media were prepared using 2.5 ml, 5ml and 10 ml crude lignin in 97.5ml, 95 ml and 90 ml soil extract. All the other components of the media remained same.

The five enriched samples were poured on lignin agar medium. The plates were incubated at 30°C for 4-5 days. Each plate was examined for number of isolates and single isolated colonies were sub-cultured to obtain pure culture of selected isolate. All the isolates were then inoculated in 100 ml of 1% lignin broth separately to check the decolourization of lignin. All the flasks were incubated at 30°C, 120 rpm for 3-4 days in a shaker incubator and checked for biobleaching/ colour removal visibly by comparing with un-inoculated control. The isolates were acclimatized at higher concentration of lignin to enhance their lignolytic activity on the same media with increasing concentration of crude lignin like 1%, 2.5%, 5% and 10% v/v and checked for qualitative lignolysis.

Screening of lignolytic bacteria

The qualitative nonspecific action of lignin peroxidase on dyes and indicators was correlated with lignin decolourization using Phenol Red test. Growth of the isolates was monitored on media which contained g/100ml: peptone (1), meat extract (0.3), NaCl (0.5), agar agar (2.5) and a few drops phenol red indicator. The pH was adjusted to 7 at which phenol red is yellow in colour. The lignolytic

activity was checked by streaking the isolates on nutrient agar plate containing phenol red and observed for color change.

Characterisation and identification of potent isolates

Potent isolates were identified by using morphological and biochemical characterization methods at lab scale and using recent tools like API system (Vitek2).

Qualitative dye decolourization

Decolourization of lignin-mimicking dyes was assessed both in liquid and in solid-phase assays. The following dyes were selected: Malachite Green (MG), Congo Red (CR), Methylene Blue (MB), Toluidine Blue O (TB) and Janus Green (JG). For qualitative solid phase dye decolourization assay, bacteria were streaked onto dye-containing agar plates with various media. Davis Minimal medium was used, supplemented with a carbon source. Dyes were added to 50 mg/L (MG, CR) or 25 mg/L (MB, JG and TB)³. The plates were monitored daily over a period of 120 hrs for growth and the development of decolourization.

Quantitative dye decolorization

For liquid assay, the individual strains were grown in LB supplemented with 0.1 % synthetic lignin w/v to an OD at 600 nm to approximately 0.7 -0.9 (mid-exponential growth) which is used as an inoculum³. Dyes were added to the broth medium at concentration of 25 mg/L in separate set and different concentrations of Janus Green (0.01-0.05 mM) were used for the study. Cultivation was monitored for 4-5 days in 250-ml amber Boston bottles at 30°C with shaking at 200 rpm. Medium containing dye and without inoculum were included as controls for spontaneous dye decolourization. Samples were withdrawn at various time intervals and centrifuged for 15 min at 15,000 g. Dilutions of the supernatant up to 10⁻¹ and 10⁻² with sterile distilled water were done as per the requirement. Absorbance was recorded at λ_{max} for each of the dyes. The decolourization of a specific dye was calculated as a percentage of the initial absorbance at λ_{max} . The colour of the pellet was also visually inspected to

establish whether the dye had adsorbed to the cells rather than being degraded.

For spectrophotometric analysis of the various dyes, a universal micro plate spectrophotometer (Bio-tech) was used. The absorbance spectra of the dyes between 200

nm to 800 nm were measured to establish λ max for each dye (MB-665 nm, TB-635 nm, MG-615 nm, CR-470 nm JG- 660 nm). The percent dye decolorization was estimated using formula¹⁸:

$$\text{Colour removal (\%)} = \frac{\text{Abs Control} - \text{Abs Test}}{\text{Abs Control}} \times 100$$

RESULTS AND DISCUSSION

Alkali lignin preparation

The crude alkali lignin was successfully extracted from natural sources of lignin by alkaline delignification method.

Enrichment, Isolation and Acclimatization

After enrichment and sub-culturing of the lignolytic bacteria, pure cultures were obtained on lignin agar plates (Fig.1). Some of the isolates were acclimatized on higher concentration of lignin and termed as potent isolates.

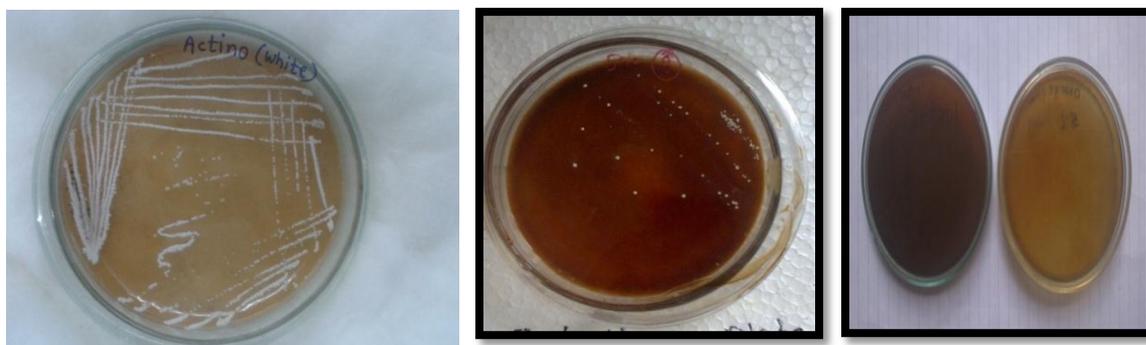


Fig. 1: Growth of bacteria on lignin-agar and lignin decolorization

Total 16 isolates were obtained from various sample sources (Table 1) and their lignolytic activity was confirmed by decolourization of lignin agar plates after incubation (Fig 1).

Table 1: Isolates and their sample sources

Source	Isolates
Lake water	L1,L2,L3,L4,L5
Rhizosphere soil with cow dung	and White C1,C2,C3,Red and
Bark sample with termites	Orange T1,T2,T3,T4 and 11(Actino).

Phenol Red Test

After incubation at 30°C for 24 hrs, the inoculated phenol red plates with the isolates separately showed colour change from yellow to red. The plates were compared with uninoculated control.

Identification

One of the potent Gram positive isolate (Orange) with excellent dye decolorization activity was identified as *Corynebacterium jeikeium* (Table-2) while the second potent Gram negative isolate (White Tp) which showed growth on all the lignin concentrations and efficient lignolytic and dye decolorization activity was identified as *Sphingomonas paucimobilis* (Table-3) as per the analysis report of Bactest Lab using Vitek 2 System tool.

Table 2: Identification Report of the Isolate 'Orange' by Bactest Lab, Vitek 2 System

Bac-test laboratory		Printed :Apr 13, 2015,01:51 IST		Printed by Bactest													
bioMerieux Customer: System #		Laboratory Report		Bench WATER													
Bionumber: Bionumber: 2320000400001																	
Selected Organism: <i>Corynebacterium jeikeium</i>																	
Identification Information	Card: ANC		Lot Number: 244242910		Expires: Aug 8, 2015 12:00 IST												
	Completed: Apr 13, 2015 01:51 IST		Status: Final		Analysis Time: 6.00 hours												
Selected Organism		96% Probability <i>Corynebacterium jeikeium</i>		Bionumber: 2320000400001													
				Confidence: Excellent													
Biochemical Details:																	
4	dG	-	5	LeuA	+	6	ELLM	(7	PheA	+	8	ProA	+	10	PyrA	-
1	dC	-	13	TyrA	+	15	APPA	-	18	dGLU	-	20	dMN	-	22	dMA	-
2	SA	-	30	ARB	-	33	NAG	-	34	BGLU	-	36	URE	-	37	BGU	-
3	BG	-	41	AARA	-	42	AGALi	-	43	BMA	-	44	ARG	-	45	PVA	+
5	MT	-	53	ESC	-	54	BdFUC	-	55	BNA	-	56	AMA	-	57	AlFU	-
5	PH	-	60	IARA	-	61	dRIB2	-	62	OPS	-	63	AAR	-	64	dXY	-
	GR	+		MORP	?		AERO	?									
Installed VITEK 2 Systems Version: 06.01						Therapeutic											
Interpretation Guideline:						AES Parameter											
MIC Interpretation Guideline:						AES Parameter											
Last Modified:																	

Table 3: Identification Report of the Isolate 'White' by Bactest Lab, Vitek 2 System

Bac-test laboratory		Printed Jun 8, 2015 18:53 IST		Printed by Bactest													
bioMerieux Customer: System #		Laboratory Report		Bench WATER													
Bionumber: Bionumber: 1410121100220401																	
Selected Organism: <i>Sphingomonas paucimobilis</i>																	
Identification Information	Card: GN		Lot Number: 241255140		Expires: June 8, 2015, 12:00 IST												
	Completed: Jun 8, 2015 18:53, IST		Status: Final		Analysis Time: 5.00 hours												
Selected Organism		89% Probability <i>Sphingomonas paucimobilis</i>		Bionumber: 1410121100220401													
				Confidence: Good identification													
Biochemical Details:																	
2	APPA	+	3	ADO	-	4	PyrA	-	5	lARL	-	7	dCEL	-	9	BGAL	+
10	H2S	+	11	BNAG	-	12	AGLTp	-	13	dGLU	-	14	GGT	-	15	OFF	-
17	BGLU	+	18	dMAL	-	19	dMAN	-	20	dMNE	-	21	BXYL	+	22	BAlap	-
23	ProA	+	26	LIP	-	27	PLE	-	29	TyrA	+	31	URE	-	32	dSOR	-
33	SAC	-	34	dTAG	-	35	dTRE	-	36	CIT	-	37	MNT	-	39	5KG	-
40	lLATk	-	41	AGLU	+	42	SUCT	-	43	NAGA	-	44	AGAL	+	45	PHOS	-
46	GlyA	-	47	ODC	-	48	LDC	-	53	lHISa	-	56	CMT	-	57	BGUR	+
58	O129R	-	59	GGAA	-	61	lMLTa	-	62	ELLM	+	64	lLATa	-			
Installed VITEK 2 Systems Version: 06.01						Therapeutic Interpretation											
Guideline:						AES Parameter Last											
MIC Interpretation Guideline:						AES Parameter Last											
Modified:																	

Quantitative dye Decolorization

The percent dye decolorization for each isolate were given in Fig. 2 to 6. *Corynebacterium jeikeium* was found to be efficient in decolorization of indicator dyes Methylene Blue, Toluidine Blue, Congo Red and Malachite Green and found to decolorize 76.32%, 64.1%, 96.5% and 81.78% (Fig. 4, 5, 6 and 3) respectively. *Sphingomonas paucimobilis* efficiently decolorized dyes Malachite Green, Methylene Blue and Congo Red up to of 90%, 60% and 96% (Fig. 3, 4 and 6) respectively. Maximum decolorization of the dye Malachite green i.e. 99.65% was obtained for the isolate Red(SM) (Fig.3). The maximum percent decolorization of Congo Red obtained for the isolate orange i.e. 96.5

(Fig. 6) was in accordance with the results obtained by Sivakalai. The percent decolorization of dyes Methylene Blue (76.32%), Toluidine Blue (64.1%) and Malachite Green (99.65%) for the isolates *Sphingomonas paucimobilis* and Red (SM) (Fig. 4, 5 and 3) were significantly greater as compared to the results obtained by Bandounas³, who got decolorization of 53%, 8% and 18% respectively. The difference for significant increase in the percent decolorization of MG for the isolate *Sphingomonas paucimobilis* might be attributed to MG’s antimicrobial properties that are particularly effective against gram positive microorganisms but ineffective against Gram negative microorganisms³.

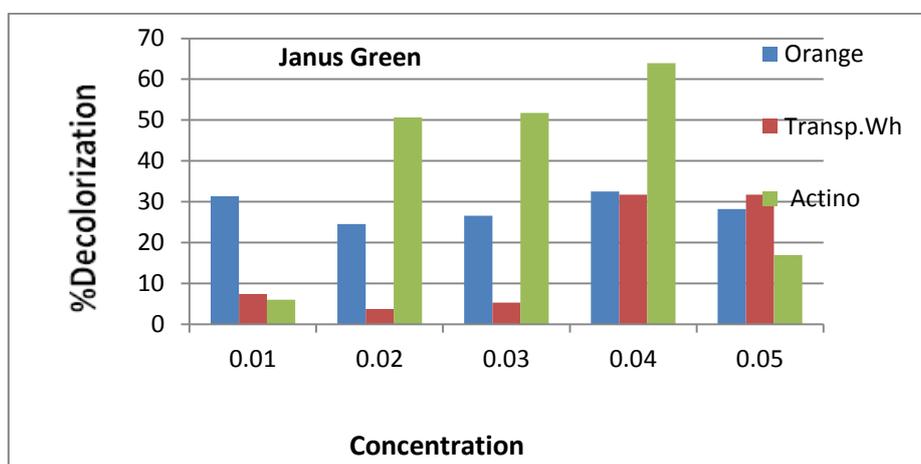


Fig. 2: % Decolorization of Janus Green

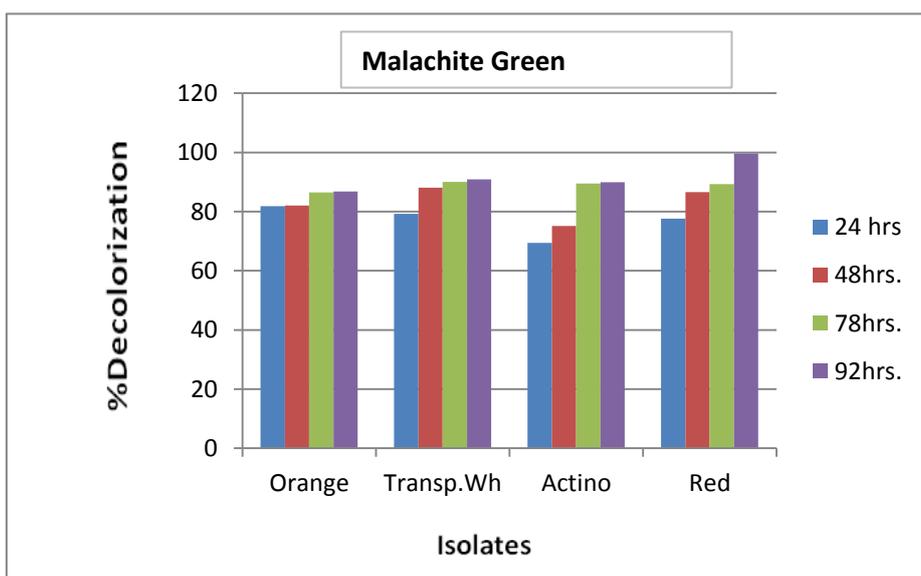


Fig. 3: % Decolorization Malachite Green

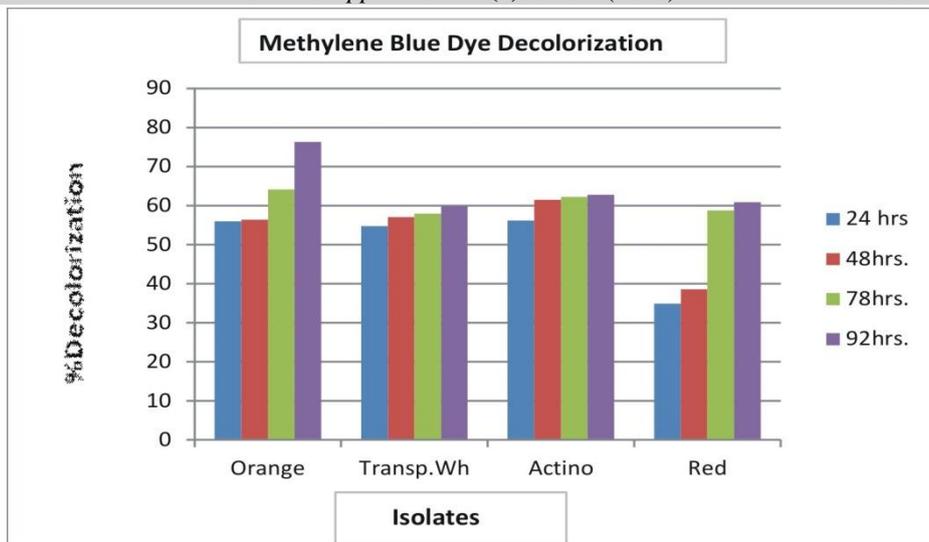


Fig. 4: % Decolorization Methylene Blue

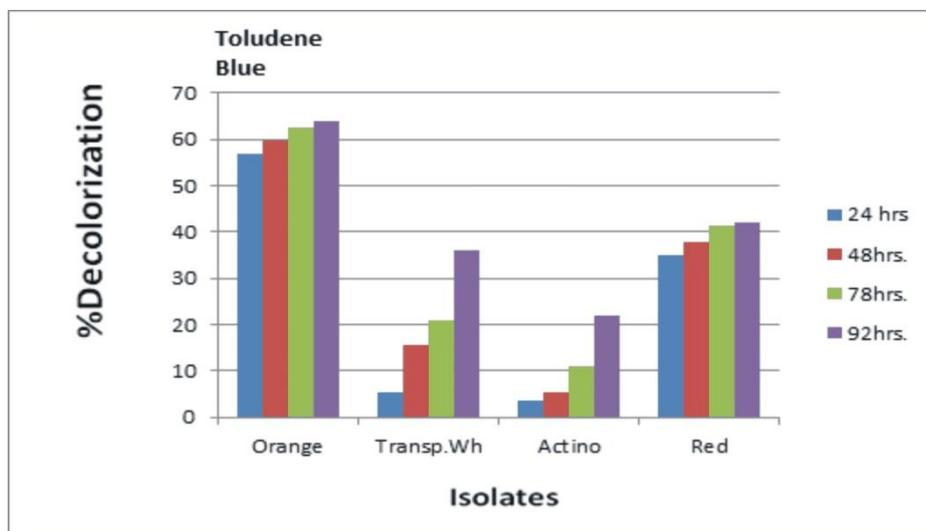


Fig. 5: % Decolorization Toluidine Blue

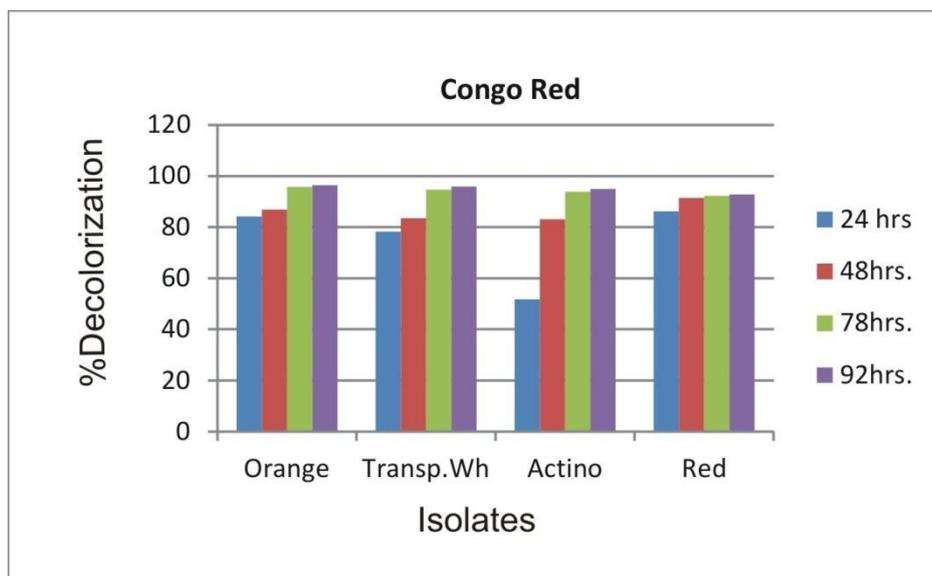


Fig. 6: % Decolorization Congo Red

CONCLUSIONS

Lignolytic bacteria were successfully isolated and acclimatized to enhance their decolorization potential. Positive phenol red test indicated the oxidizing nature and the non-specific action of LiP on Phenol red which can be correlated with its role in lignolysis and dye decolorization. Orange isolate obtained from rhizosphere soil with cow dung sample identified as *Corynebacterium jeikeium* was found to be efficient in decolorization of indicator dyes Methylene Blue, Toluidine Blue, Congo Red and Malachite Green i.e. 76.32%, 64.1%, 96.5% and 81.78% respectively. It was found to be the most efficient bacteria in dye decolorization study as compared to the other isolates. The isolate 'white(Tp)' obtained from lake water sample was identified as *Sphingomonas paucimobilis* and efficiently decolorized dyes Malachite Green, Methylene Blue and Congo Red i.e. 90% , 60% and 96% respectively. It was found to be the second most efficient bacterium among the remaining isolates. Isolate Actino was found to decolorize dyes Congo Red, Malachite Green, Methylene Blue and Janus Green up to 94.91%, 90%, 61.41% and 63.93% respectively but the percent decolorization as compared to time for isolate Actino was moderately lesser than the isolates Orange and white except for the dye Janus Green. The isolate Red(SM) was found to degrade all the selected dyes but the range was least as compared to the other isolates except for the dye malachite green which was 99.65%.

The application of lignin peroxidases as advanced biological method for biobleaching of lignin and various dyes showed significant color removal. Therefore, this treatment can be adopted and applied at industrial level for decolourisation of various colored effluents especially by using the two identified isolates i.e. *Corynebacterium jeikeium* and *Sphingomonas paucimobilis*. The crude extract of the enzyme from the culture broths of these isolates may be used directly or in the purified form for the treatment. The enzyme may also be immobilized and used repeatedly on larger

scale for the treatment of waste water from paper and pulp mill or dye- stuff industries.

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