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DECOLORIZATION OF KRAFT LIGNIN BY LIGNINOLYTIC BACTERIAL ISOLATES FROM TIMBER SOILS OF RAIPUR, CHHATTISGARH

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ABSTRACT

Black liquor, the dark brown color of the effluent generated in the process of wood chips digestion, contains kraft lignin which is a toxic liquid and it contaminates the aquatic ecosystems. The proper disposal of this black liquor has gained momentum in the last five years across the world. Two bacterial strains TSB2 and TSB5 were isolated from soil. After isolation and purification of the bacterial isolates, they were tested for the decolorization of kraft lignin (KL) using sterile mineral salt medium (MSM) containing KL 600 mg l^{-1} (designated here after L-MSM) and supplemented with 1.0% glucose and 0.3% peptone (w/v) and incubated for six days under aerobic conditions at 30 °C and 120 rpm. Samples were withdrawn periodically at 1-day intervals for six days and analysed for pH and reduction of color. It was observed that TSB2 and TSB5 reduced color by 50% after 3rd and 1st day of incubation respectively.

Key words- *Decolorization, Kraft lignin, LigninolyticBacteria, Timber Soil,*

I. INTRODUCTION

The pulp and paper mill is a major industrial sector utilizing wood and non-wood materials and water during manufacturing process [1]. Pulp manufacturing involve two main processes i.e. kraft pulping and bleaching. Kraft lignin is polymer by product of this process. In the kraft pulping process, wood chips are cooked in the solution of sodium hydroxide and sodium sulfide at elevated temperature and pressure. Under these conditions the semisolid pulp is collected and washed. At this point the pulp is dark brown in color and known as black liquor [2]. The black liquor contains lignins, cellulose, phenolics, resins, fatty acids and tannins [3]. Discharge of untreated effluent from the pulp and paper industry caused serious pollution and loss of aesthetic beauty in the environment. They also increase the amount of toxic substance significantly in the water causing death to the zooplankton and fish [4].

Although several chemical and physical methods are available for lignin depolymerization. Thermochemical method is cost effective, energy consuming and harmful to environment hence the researchers have focused on environmental friendly technologies for the depolymerization. Therefore the degradation can be advantageouslydirected by applying selective and more effective ligninolytic microbes and enzymes[5].

Bacterial breakdown of lignin of lignin is studied because of their adaptability toward extreme environmental conditions versatility in substrate utilization. Bacteria can display alternate molecular pathways and enzymes. Enzymes involved in degradation are phenol oxidases such as copper dependantlaccase, hemedependantlignin peroxidases and manganese peroxidases.

Bacteria such as *Aneurinibacillusaneurinilyticus*, *Paenibacillus* sp. and *Bacillus*sp. have been confirmed by identifying intermediate product through GC-MS analysis [6][7]. A comparative analysis of *Rhodococcus* sp. RHA1 and *Pseudomonas putida* for lignin degradation was studied through spectrophotometric assays [8].The involvement of extracellular peroxidases and laccase enzymes were reviewed [9]. Evidences for lignin degradation were obtained by indigenous tropical estuarine *Sreptomyces* sp. from Lagos [10]. Anaerobic bacterial strain, *Acetoanaerobium* species WJDL-Y2 was isolated and their ligninolytic activity has been confirmed by identifying degradation intermediates and products [11].Lignin degrading potential was reported by hemophilic bacterial strains i.e. *Bacillus subtilis* and *Bacillus licheniformis* isolated from soil, water and sludge[12]. Marine sediment bacterial isolates *Bacillus subtilis*, *Bacillus endophyticus* and *Bacillus* sp. were selected for high potential in the degradation and decolorization of lignocelluloses compounds of pulp and paper mill effluent.Degradation and decolorization studies by axenic and consortium were also reported. It was found that consortium made by these bacterial strains enhances the degradation and decolorization of lignin [13].

II. MATERIALS AND METHODS

2.1 Chemicals

All reagents were usedof analytical grade. Synthetic Kraft Lignin (KL) was purchased from Sigma Aldrich (USA).

2.2 Sample collection, Isolation and Purification of potential Bacterial strains

The soil samples were collected from timber area of Raipur (C.G.). The soil sample collected in sterile polythene bags were brought to the laboratory and immediately stored in 4°C until used for further analysis. Alkali Kraft lignin (KL) degrading bacteria was isolated from soil by enrichment culture technique [14]. One gram of soil sample was inoculated to 100 ml sterile mineral salt medium (MSM) containing KL 200 mg $^{-1}$(designated here after L-MSM). MSM (pH 7.6) consisted of (g/l) Na_2HPO_4, 2.4; K_2HPO_4, 2.0; NH_4NO_3, 0.1; $MgSO_4$, 0.01; $CaCl_2$,0.01: D- glucose, 10.0 g: peptone, 3.0g and trace elements solution 1.0 ml. The latter solution composed of (mg/l): $ZnCl_2$, 70; $MnCl_2.4H_2O$,100; $CoCl_2.6H_2O$, 50; $NiCl_2.6H2O$, 50; $CuCl_2.2H_2O$, 25; $NaMoO_4.2H_2O$, 50; $NaSeO_3.5H_2O$, 26; $NaVO_3.H2O$, 10;$NaWO_4.2H_2O$, 30 and HCl 25%, 1.0 ml. Flasks were incubated for 6 days on rotary shaker 120 rpm under aerobic conditions at 30°C. Samples fromflasks exhibiting decolourization were serially dilutedand spread on L-MSM agar plates and incubated in darkat 30^{o}C for 6 days. Two phenotypically differentbacterial colonies were picked and purified by repeatedsub-culture in order to obtain pure isolates. The purity ofisolates was checked by microscope and these isolateswere designated TSB2 and TSB5.

2.3 Screening of potential bacterial strains for ligninolyticactivity

The isolated and purified bacterial strains were screened for ligninolytic enzyme activity by plate assay method. For screening,different substrate containing agar plates were used. The substrate usedfor manganese peroxidase (MnP) phenol red, for lignin peroxidase was Azure B 0.002%. While laccase activity was detected in presence of guaiacol (Sigma, USA) as a substrate in B&K agar medium containing dextrose 1%, peptone 0.5%, NaCl 0.5%, beef extract 0.3% and $CuSO_4$ (1 mM). The disappearance of blue color of the media confirmed the presence of LiP activity whileconversion of dark pink color to yellow indicated the presenceof MnP and brown color halos indicated the presence of laccaseactivity [15].

2.4 Kraft Lignin de-colorization and degradation

Decolorization and degradationexperiment was carried out in 250ml flask containing 100 ml sterile L-MSM (600 mg l^{-1} KL) at pH 7.6, which is greater than that found in pulp mill effluent. Culture suspension 2% (v/v) was inoculated into flasks. The flasks were incubated for six days on rotary shaker incubator under aerobic conditions at 30^{o}C and 120 rpm. Un-inoculated medium was used as control in all cases. Samples were withdrawn periodically at 1-day intervals for six days and analyzed for pH, reduction of KL color.

2.5 Analytical techniques

2.5.1 Color reduction

The intensity of the KL color, before and after incubation was determined according to (Morii*et al*., 1995). The samples were centrifuged at 10000 rpm for 30 min. to remove the suspended particles.1ml supernatant was diluted by adding 3ml phosphate buffer (pH 7.6) and absorbance measured at 465 nm for color reduction. The absorbance at 465 nm against distilled water was measured using a spectrophotometer [16]. The absorbance values were then transformed into color units (CU) according to the followingformula.

CU (PtCo)= 500 × (A2/A1)

Where, A1 =A465 of a 500-CU platinumcobalt standard solution

A2 =Absorbance of the sample.

Color removal % = (A-B)\A × 100

Where, A =colorunits of uninoculated

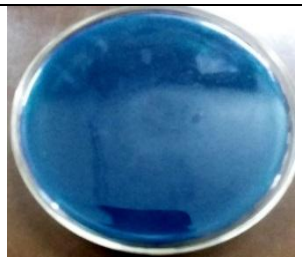
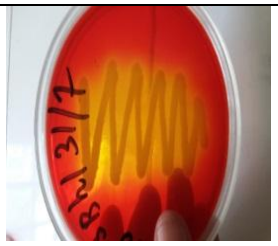
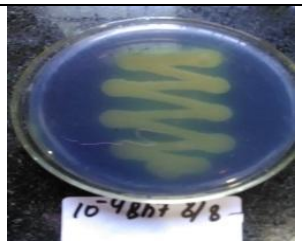
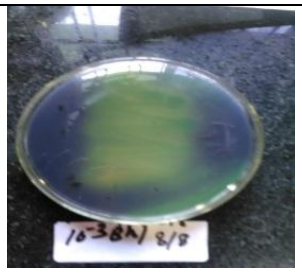
B = color units of inoculated sample.

III. RESULTS AND DISCUSSION

3.1 Isolation and screening of bacterial strains

Two bacterial strains i.e. TSB 2 and TSB 5appeared on the plates were isolated based on morphological difference of individual colonies from soil sample. TSB 2 and TSB 5 have capability to produce ligninolytic enzymes. TSB 2 producesManganese peroxidases (MnP), Lgignin Peroxidase (LiP) while TSB 5 produces Linin Peroxidase (LiP), Laccase, Table 1.

Table 1.Ligninolytic activities of TSB2 and TSB5

Bacterial Strain	MnP	LiP	Laccase
Control			
TSB2			Negative
TSB5	Negative		

3.2 Kraft LiginDecolorization

Degradation and decolorization of KL indicated by the decrease of absorbance at 465 nm is shown in Fig.1. It shows that the initial absorbance of the culture medium containing 600 ppm KL was 0.14nm, 0.13nm by TSB2 and TSB5 on day 1respectively. It was dropped to 0.13nm on day 2 by TSB2 and remains 0.13nm by TSB5. The initial color unit of KL was 985 PtCo but after treatment through TSB2 and TSB5 the color unit reduced to490 at 2nd and 1rdday of incubation. Low decolorization at initial phase in spite of bacterial growth might be due to the utilization in growth media. Subsequent starvation of easily available nutritional source in media insisted the bacterial culture to utilize lignin as co-substrate. A significant decrease in pH 5.73and 6.54 was noted on day 1 followed by gradual increase till the six day by TSB 2 and TSB 5 due to bacterial metabolic activity during biodegradation Fig.2. The shift in pH toward acidic condition was noted within initial 24h of bacterial growth which indicated the formation of acidic compound.

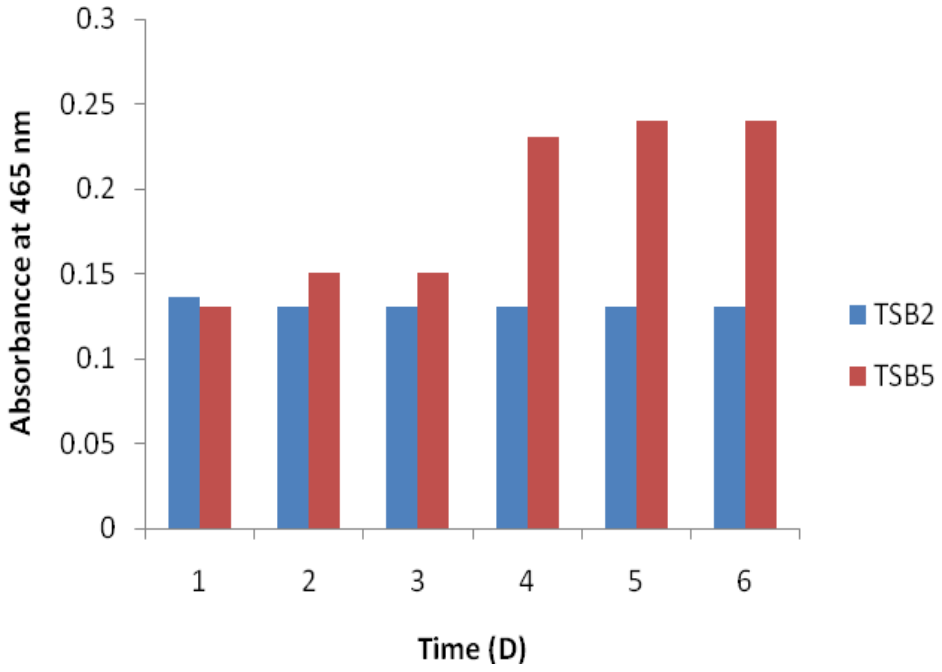


fig.1.Time course of kraft lignin decolorization by TSB2 and TSB5.

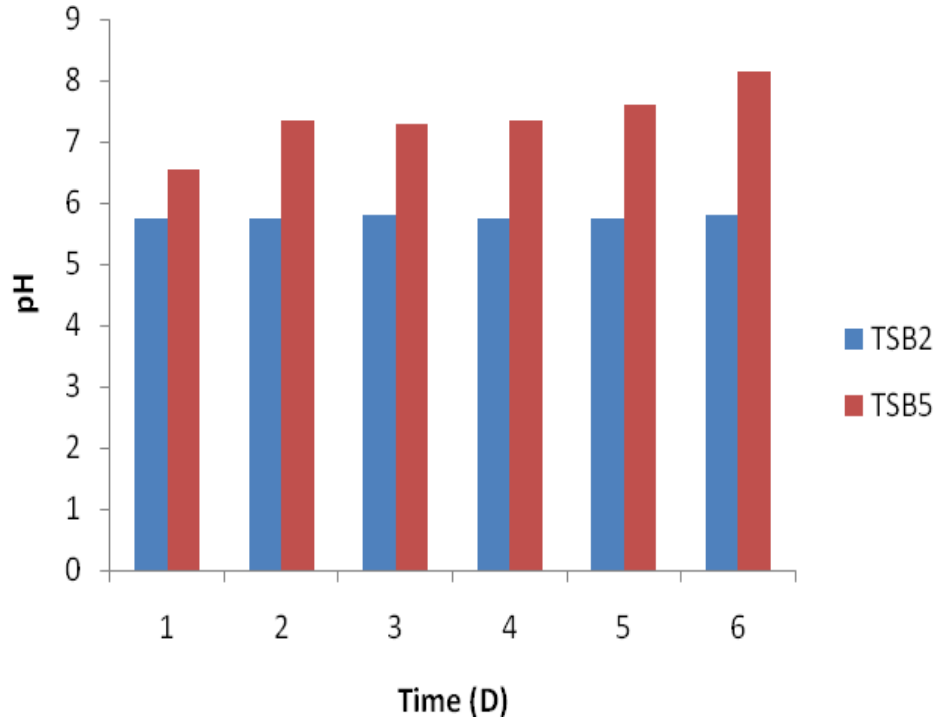


fig. 2. Effect on pH by TSB2 and TSB5 during KL decolorization.

|IV. CONCLUSION

This study highlights that Up to 50% of color was reduced with in 48h and 24h of incubation at 30±1ºC and 120 rpm by TSB2 and TSB5 when compare to it with control sample (0.26nm: OD). TSB5 has high potential to decolorize the KL. Nevertheless, the ability of these bacterial strains has potential application in the bioremediation of lignin contaminated pulp paper mill wastewater.

V. ACKNOWLEDGEMENTS

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