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Evaluation of Decolorization and Lignin Degrading Potentiality of Ligninolytic *Bacillus aryabhattai* Isolated from Pulp and Paper Mill Waste Water

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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. Total five bacterial strains PMB1-PMB5 were isolated from pulp and paper mill waste water. 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 Samples were withdrawn periodically at 1-day intervals for six days and analyzed for pH and reduction of color and lignin content . Biochemical and 16S rDNA gene sequence analysis suggested that strain PMB3 belonged to the Bacillus aryabhattai. It was observed that this bacterial strain reduced color by 47% and lignin content 17%.

Key words- Bacillus aryabhattai Decolorization, Kraft lignin, , pulp and paper mill waste water.

I. INTRODUCTION

As food wood is almost as important to humanity and have enormous environmental value as natural forests from which most of it is harvested [1]. However, continually growing demand for paper is putting pressure on the world's forests, and resulting in the loss and degradation of forest. The alternative raw materials are Agricultural remains which could meet global paper making demand five times over [2]. One kind of such remains is wheat straw and because of wheat straw can make high quality paper than other agricultural residues it is often used to make paper pulp [2, 3]. Wood digestion and bleaching are two main processes during pulping in pulp paper industries. In the process of wood digestion, wood chips are cooked in the solution of sodium hydroxide and sodium sulphate at elevated temperature and pressure to break chips into fiber mass., all the depository materials which are hard to degrade are dissolve by The chemical reaction with wood fibers and these derivatives are washed away from the fiber during washing and dewatering process. The extracted products such as lignins, cellulose, phenolics, resins, fatty acids and tannins during washing processes mixed together and make dark black viscous alkaline waste known as black liquor. The color of the effluent is mainly due to the presence of lignin and its derivatives. Alkaline effluent is of high pH, BOD, COD and color which make it significantly toxic to the environment [4]. Lignin is a heterogeneous, three dimensional polymer, composed of oxyphenylpropanoid units [5]. Processes based on physical (adsorption, microfiltration and

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photoionization etc.) and chemical (sedimentation, coagulation, oxidation and ozonation etc.) methods for treatment of pulp and paper mill effluent suffer from operational and secondary pollution problems with high cost of treatment further affecting their suitability. Biological methods involving fungi, bacteria and actinomycetes have become important because of their environmental friendliness. Two dominant bacteria *Bacillus subtilis* and *Micrococcus luteus*, and one fungus *Phanerochaete crysosporium* was isolated from pulp and paper mills effluent soils. These microbes has potential to reduce COD up to 94.7% , BOD up to 87.2 %, and lignin content up to 97% after 9 d under shaking conditions and brought down pH of raw PPME to neutral [6]. A bacterial strain and a fungal strain were applied to effectively degrade the toxic substances in the waste water released from pulp and paper industry. It was observed that *Pseudomonas* sp. which was able to degrade the pollutants more efficiently than the fungal strain. The bacterial isolate reduced 20.3% color, 70.7% biochemical oxygen demand 60.3% chemical oxygen demand, , 20.3% color and total suspended solids by 39.2% and total dissolved solids by 10.3% in 72hrs of incubation at 35°C and pH7.0. [7] The strain *Serratia liquefaciens* effectively reduced pollution parameters (color 72%, lignin 58%, COD 85% and phenol 95%) of real effluent after 144 h of treatment at 30 ◦C, pH 7.6 and 120 rpm [8]. An anaerobic kraft lignin (KL)-degrading bacterial strain was isolated from sludge of a pulp and paper mill. It was characterized as *Acetoanaerobium* sp. WJDL-Y2by 16S rRNA gene sequencing. The maximum KL degradation capability of strain Y2 was determined to be 24·9% on a COD basis under an optimal condition with temperature of 31·5°C, initial pH of 6·8 [9].

II. MATERIAL AND METHODS

2.1 Sampling location and collection

The effluent samples were collected from Madhya Bharat Pulp and Paper Mill, Champa, Chhattisgarh, India. The effluent was collected in sterile plastic container were brought to the laboratory and immediately stored of 4°C until used for further analysis.

2.2 Isolataion of lignin degrading bacteria

To isolate mixed bacterial cultures that were capable of Alkali Kraft lignin (KL) decolorization /degradation selective nutrient enrichment techniques were used. Bacteria were isolated from effluent by enrichment culture technique [10]. An aliquote of sample (one ml) 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; $NiCl_2.6H_2O$, 50; $CuCl_2.2H_2O$, 25; $NaMoO_4.2H_2O$, 50; $NaSeO_3.5H_2O$, 26; $NaVO_3.H_2O$, 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 from flasks exhibiting decolourization were serially diluted and spread on L-MSM agar plates and incubated in dark at 30°C for 6 days. The different colonies on plates were further purified on KL-MSM agar plates. The bacterial strain was named as PMB3. This bacterium was stored at 4 °C for further analysis [10].

2.3 Screening of selected isolates

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The presence of lignin degrading enzymes was analyzed through screening method. Decolorization of lignin mimicking dyes were assessed in agar plates. The substrate used for Manganese Peroxidase (MnP) was phenol red, for lignin Peroxidase (LiP) was Azure B (.002%) while Laccase activity was detected in the presence of guaicol as substrate in B & K agar medium contain dextrose; 1%, peptone;0.5%, NaCl; 0.5%,beef extract 0.3% and $CuSo_4$ (1 mM). Bacterial cultures was inoculated into agar plate, incubated at 30^oC and monitored daily for three days. A visible change in the color of the substrate indicates the presence of lignin degrading enzymes [11].

2.4 Kraft lignin decolorization/ degradation studies

For the decolorization/ degradation studies 2% (v/v) overnight grown suspension of having inoculums size $100*10^5$ cells were transferred aseptically to 250 ml flask containing 95ml L-MSM amended with glucose (1% w/v) and peptone 3 % (w/v) (pH 7.6). The inoculated flasks were incubated at 30^o C for 6 days. During the decolorization period samples were taken at every 24 hr interval period analyzed for pH, Color removal and Lignin degradation.

2.4.1 Color reduction

The intensity of the KL color, before and after incubation was determined according to (12). The samples were centrifuged at 10,000 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. The absorbance values were then transformed into color units (CU) according to the following formula.

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 =color units of uninoculated

B = color units of inoculated sample

2.4.2 Lignin Degradation

The lignin content present in effluent was estimated according to the method of Pearl and Benson.21 In this method, 1mL CH3COOH (10%) and 1mL NaNO2 (10%) were added to a 50mL of sample. After 15 min, 2mL of NH4OH was added then the mixture was left sfor 5 min and the absorbance measured at 430 nm. For the blank, 1mL CH3COOH (10%) was added to 50mLdistilledwater and 2mLNH4OH.After 15 min, 1mL of NaNO2 (10%) was added. After 5 min, the absorbance was measured at 430 nm. The absorbance value was transformed into lignin content (ppm) [13].

Lignin (ppm) = Absorbance/0.00024

III. RESULTS AND DISCUSSION

3.1 Isolation and characterization of lignin degrading bacteria

Total five degrading bacterial isolates (PMB1-PMB5) were isolated from pulp and paper mill effluent by serial dilution method. Among these five isolates one isolate PMB3 has potentiality to produce all the three

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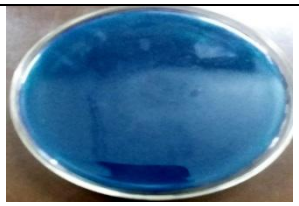
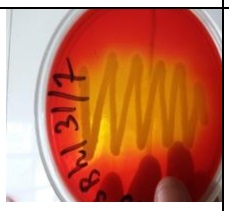
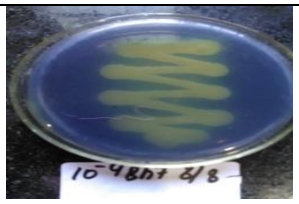
Bacterial Strain	MnP	LiP	Laccase
Control			
Bacillus aryabhattai			

ligninolytic enzymes [Manganese peroxidases (MnP), Lignin Peroxidase (LiP) and Laccase]. The disappearance of the blue color of the media for LiP activity, conversion of dark pink color into yellow for MnP and brown color halos for laccase activity is appearant in Fig (a).

The isolated bacterium was gram positive and rod shaped. The PMB3 was identified and characterized as *Bacillus aryabhattai* by Microbial Type Culture Collection and Gene Bank (MTCC).

Fig. a. Ligninolytic activities of *Bacillus aryabhattai*

3.2 Decolorization/ Degradation of Lignin

The bacterial growth and reduction of color and lignin content during the experiment is shown (Fig b and c) revealing that bacterium achieved good growth initially and growth was maintained upto 72 h of incubation afterwards a decline in growth was observed. Significant color and lignin reduction was observed after 24 h. at 9 h 47% and reduction of color and lignin was noted. The initial color and lignin content was 719CU and 1000ppm respectively but after bacterial treatment it was 379CU and 833ppmonly respectively.

Changes in medium pH during decolorization study was observed and initial pH 7.6 of the medium decreased to pH 5.3 after 48 h, and thereafter gradually increased up to pH 7.2 at the end of the experiment (Fig. d). While in the control flasks the medium pH remained almost constant. The shift in pH towards acidic range during initial phase of decolorization might be due to acetate efflux along with other TCA cycle intermediates [14]. As the simple carbon sources deplete, the bacteria shifts back to utilize the excreted metabolic intermediates including acetate, leading to a gradual increase in pH. This facilitates lignin degradation as lignins are uniformly soluble at high pH.

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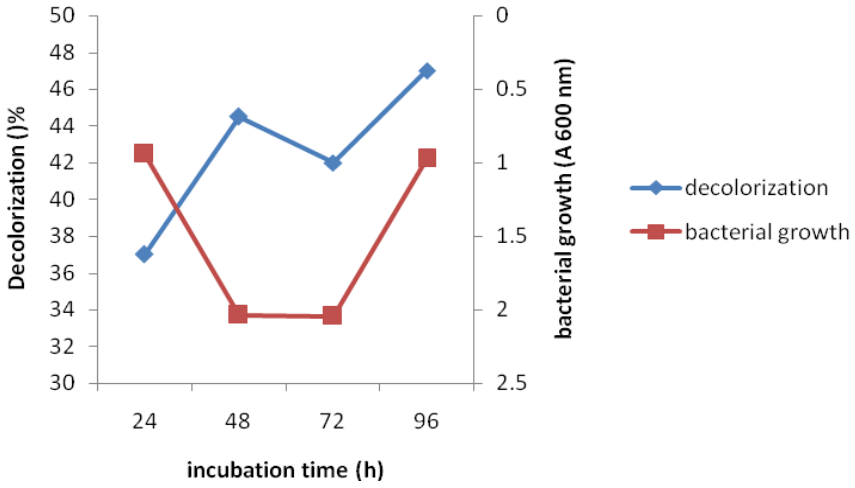

Fig.b. Time course of kraft lignin decolorization and bacterial growth

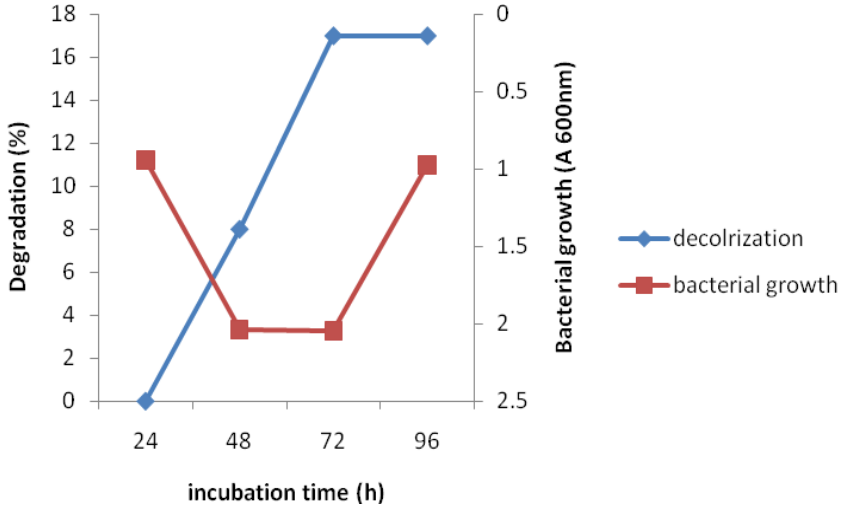

Fig.c.Time course of kraft lignin degradation and bacterial growth

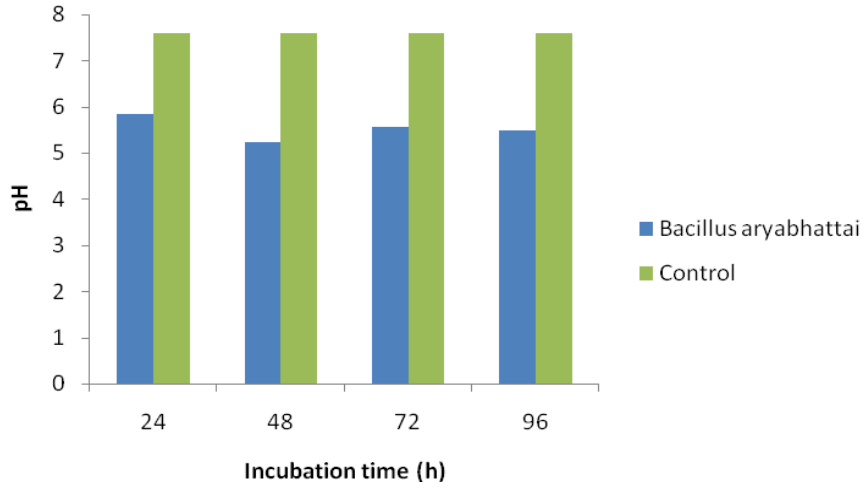

Fig. d. The relationship between the change in the acidity of the growth medium (pH) and the incubation time (h) by *Bacillus aryabhattai*.

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IV. CONCLUSIONS

A potential ligninolytic strain was isolated from an effluent contaminated site and characterized as *Bacillus aryabhattai*. The bacterium, significantly reduced color and lignin content of the medium. The bacterium was capable of producing LiP, MnP and Laccase while growing in basal media components and growth associated enzyme production was observed. The decrease of color and lignin content was observed as the growth progressed. On the basis of its decolorization and degradation activity it is a potential candidate for further pulp and paper mill waste water treatment studies.

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