BATCH AND REACTOR STUDY ON BIOREMEDIATION OF LIGNIN RICH PULP AND PAPER MILL EFFLUENT WITH *BACILLUS CEREUS* BACTERIAL ISOLATE

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Paper mills generate colored effluents that are rich in lignin and its derivatives. In the present study, an indigenous ligninolytic bacterium, RJH-50, was isolated from paper mill sludge and identified as *Bacillus cereus* using 16S rRNA sequencing. The bioremediation efficiency of the bacterial isolate was evaluated in terms of lignin degradation, chemical oxygen demand (COD) reduction and color removal using the shake flask method in batch mode and in reactors under semi-continuous mode at different retention times (RT). In batch mode, the bacterial isolate reduced COD, color, and lignin by 57%, 51% and 48%, respectively, after 72 h. In semi-continuous mode, the same parameters were reduced by 58%, 49% and 46%, respectively at a RT of 48 h. The bacterium was able to effectively treat lignin-rich effluent using cost-effective ammonium sulphate as N and phosphoric acid as P source at a relatively low retention time. Thus, it could be considered a promising candidate for treating paper mill effluents.

Keywords: lignin degrading bacteria, color, COD, treatment efficiency, pulp and paper mill effluent

INTRODUCTION

The pulp and paper industry is one of the oldest industries in India.[1] It is considered one of the most polluting industries worldwide.[2-4] The effluents produced pose a serious environmental hazard as the industry discharges large volumes of water rich in organic and inorganic compounds.[5,6] The discharged water is dark brown in color due to the presence of lignin and its derivatives.[7-9] If this untreated water is released into water bodies without proper treatment, it can inhibit photosynthesis in aquatic systems by reducing solar radiation penetration, thereby affecting the aquatic ecosystem.[10] As a result, scientists and environmentalists around the world have turned their attention to the biodegradation of this highly polluted wastewater.

Although some effective chemical and physical methods exist for removing lignin from wastewater, their high operating costs and operational unreliability make them less practical for widespread industrial use.[11-14] Therefore, microbial degradation is considered a more effective and sustainable alternative for lignin removal. White rot fungi have been extensively studied for their ability to remove color and degrade lignin in pulp and paper mill wastewater. These fungi produce ligninolytic enzymes, however, their effectiveness is limited under high pH and low oxygen conditions, which are typical of pulp and paper mill effluents.[15-17] Consequently, bacteria may be a better option than fungi for this purpose.[18] Bacteria can play a leading role in lignin degradation, as they are more tolerant of environmental variations, oxygen limitations and pH extremes compared to fungi.[19]

Several bacterial species, including *Comamonas* sp.,[20] *Bacillus* sp.,[21-24] *Azotobacter* sp.,[25] *Pseudomonas* sp. (*P. veronii, P. fluorescens* and *P. putida*),[26-28] as well as *Bacillus cereus*, *Serratia marcescens*[29] and *Paenibacillus* sp.,[30] are well known for their ability to solubilize and degrade lignin. A recent study has focused on the

effective bioremediation of pulp and paper mill wastewater using *Bacillus cereus*.[31] For efficient biodegradation of pulp and paper mill wastewater, it is crucial to identify and isolate indigenous bacterial strains capable of degrading lignin, reducing chemical oxygen demand (COD), and decolorizing effluents rapidly and cost-effectively. This study aims to isolate and identify an efficient bacterial strain capable of utilizing lignin as the sole carbon source for treating pulp and paper mill effluent, thereby ensuring compliance with the Central Pollution Control Board (CPCB) discharge norms.

EXPERIMENTAL

Chemicals

Analytical grade reagents and chemicals were used throughout the study. Distilled water was used to prepare all required solutions. All tests were conducted according to standard microbiological methods for the examination of water and wastewater (APHA 2005).[32]

Collection of sludge samples

Sludge samples were collected from the effluent treatment plants (ETPs) of leading Indian pulp and paper mills located in Haryana, Uttar Pradesh, Uttrakhand and Orissa (India). These sites were specifically chosen based on the assumption that the resident microbial communities were naturally adapted to harsh conditions characterized by low dissolved oxygen (DO) levels (<2 mgL^{-1}), high chemical oxygen demand (COD) (>1000 mgL^{-1}), alkaline pH (8.5-9.5), and elevated lignin concentrations (>500 mgL^{-1}). Such selective environmental pressure is expected to enrich bacterial populations with enhanced ligninolytic capabilities, thereby increasing the likelihood of isolating efficient lignin-degrading strains. The sludge samples, which were semi-solid, dark-colored, and rich in moisture content, were collected aseptically in sterile polythene bags. They were immediately stored at 4 ± 1 °C to preserve microbial viability and prevent the degradation of organic matter until further microbiological analysis and experimentation. These pre-adapted bacterial communities can potentially be exploited for the bioremediation of pulp and paper mill effluents.

Effluent source and its characteristics

The wastewater used in this study was prepared to simulate the effluent discharged from a pulp and paper mill into an effluent treatment plant (ETP). Specifically, wastewater samples were collected from the modified chlorination stage (a combination of chlorine with chlorine dioxide, C_D) and the alkali extraction stage using oxygen and hydrogen peroxide (E_{OP}) of a pulp and paper mill located in Yamuna Nagar, Haryana, India. The C_D and E_{OP} samples were mixed in a 2:1 ratio to create a composite wastewater.[33] To achieve a pollutant profile representative of actual pulp and paper mill effluent, the composite wastewater was supplemented with starch and black liquor to increase chemical oxygen demand (COD) and color. The proportions of these components were carefully calculated to attain target COD, color, and adsorbable organic halides (AOX) values of approximately 500±25 mg O_2/L, 1000±50 Pt-Co Unit and 15±1 mg/L, respectively.[34,35] The pH of the wastewater was adjusted to 7.0 ± 0.2 using sodium hydroxide (NaOH) and sulfuric acid (H_2SO_4) solutions. Freshly prepared wastewater, as described above, was used throughout the study to ensure consistency and to minimize changes in its characteristics over time. To simulate real-world ETP conditions, the wastewater was not autoclaved, thereby preserving its natural microbial and chemical composition. The simulated wastewater served as the feed for both batch and semi-continuous experiments, providing a realistic representation of the pollutants and conditions typically found in pulp and paper mill ETPs.

Isolation of bacteria

Bacterial strains were isolated from sludge samples stored at 4 °C, as previously described, using an enrichment method.[30,34] For this, 5 g of sludge was added to 100 mL of mineral salt medium (MSM) with an initial pH of 7.5 ± 0.1. The MSM was supplemented with 100-400 ppm of indulin (black liquor), a byproduct of the Kraft pulping process in the pulp and paper industry, as the sole carbon source. The mixture was incubated at 37 ± 1 °C and agitated at 150 rpm for 10 days in an incubator shaker.

Enrichment process

A 10 mL aliquot of the incubated microbial consortium was aseptically transferred to 90 mL of sterile mineral salt medium (MSM) supplemented with lignin (100-400 ppm, derived from black liquor) and incubated for an additional 96 h at 37 ± 1 °C and 150 rpm in an incubator shaker. To selectively enrich lignin-degrading microorganisms, the culture underwent four consecutive serial transfers, each at 96 hour intervals, into fresh MSM supplemented with lignin. This iterative enrichment process effectively selected and enhanced the population of lignin-degrading bacteria within the consortium, promoting the predominance of microorganisms exhibiting high ligninolytic activity.

Isolation and purification of bacterial isolates

Following the final transfer, the enriched sample was serially diluted in autoclaved distilled water and spread onto MSM agar plates containing 300-400 ppm black liquor as the sole carbon source. The plates were incubated at 35 ± 1 °C for 4-7 days. Bacterial isolates were selected based on their colony morphology, Gram staining, and microscopic observations. The selected isolates were then purified through repeated sub-

culturing on nutrient agar plates to obtain single, pure cultures.

Preservation of pure cultures

The purified bacterial isolates were preserved on Luria-Bertani (LB) Agar slants containing (in g/L): casein enzymatic hydrolysate (10), yeast extract (5), sodium chloride (5), and agar (15). The preserved cultures were stored for future use.

Screening of potential ligninolytic bacteria

The purified bacterial strains were further screened for the presence of ligninolytic enzymes – lignin peroxidase, Mn peroxidase and/or laccase – using the plate assay method.[36] Bacterial isolates were streaked on MSM agar plates containing Azure B (0.02 g/100 mL, Phenol red 0.01 (g/100 mL), and Ramazol brilliant blue (0.04 g/100 mL) to test for lignin peroxidase, Mn peroxidase and laccase activity, respectively.[37] The plates were incubated at 37 °C and observed for decolorization. The isolates showing positive results were selected for wastewater degradation studies under batch mode (Fig. 1).

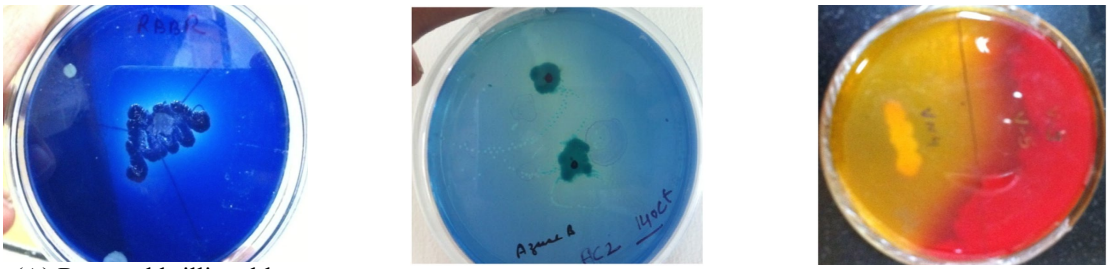
Shake flask study in batch mode

A loopful of bacterial isolates exhibiting positive ligninolytic enzyme activity was aseptically transferred to 20 mL of sterile Luria-Bertani (LB) broth and incubated for 48 hours to promote optimal growth. Following incubation, the culture was centrifuged, and the resulting pellet was aseptically transferred to a 250 mL flask containing 100 mL of wastewater supplemented with a standardized nutrient mixture comprising nitrogen (N) and phosphorus (P) sources. The nutrient formulation was adjusted to achieve a COD:N:P ratio of 100:5:1, as previously established by Reddy *et al*. (2005).[38] The flasks were incubated in an incubator shaker at 150 rpm and 37±1 °C. A control flask or reference flask (containing the same volume of wastewater without bacterial inoculation) was also maintained under identical experimental conditions. Treated wastewater samples were withdrawn at regular time intervals and analyzed for reductions in color, COD and lignin content using standard protocols. Lignin and color were analyzed spectrophotometrically by measuring absorbance at 280 nm and 465 nm, respectively, as per the methods described earlier.[39] To determine color and lignin, treated wastewater samples were collected, and the pH was adjusted to 7.4±0.2. The samples were then centrifuged at 8000 rpm for 30 min, and the supernatant was collected. The supernatant was diluted threefold, and color reduction and lignin degradation were determined as described earlier.[40,41] The color was expressed in Platinum-Cobalt units, and lignin concentration in ppm.

Reactor study in semi-continuous mode

To evaluate the degradation efficiency of the promising isolate, a semi-continuous reactor study was conducted using custom-fabricated bioreactors with a total capacity of 2 liters, operated at varying retention times (RT) under controlled aeration and agitation conditions. The reactor setup used for this lab-scale semi-continuous study is illustrated in Figure 2. The bioreactors were equipped with a glass tube immersion heater, regulated by a proportional-integral-derivative (PID) controller, to maintain a consistent temperature. Airflow was precisely controlled using an air rotameter positioned at the bottom of each bioreactor. Additionally, a magnetic stirrer was used to ensure uniform mixing. A control reactor, without any added culture, was maintained under identical experimental conditions to serve as a reference. Throughout the experiment, the reactors were continuously aerated to maintain a dissolved oxygen (DO) level of 1-2 ppm and agitated at 200 rpm to prevent settling of microbial biomass at the base of the reactor.

Inoculum preparation involved growing the culture in LB media, followed by centrifugation and resuspension of the pellet in wastewater. This suspension was then added to a 1 L reactor containing wastewater supplemented with nitrogen and phosphorus maintaining a COD:N:P ratio of 100:5:1. The culture was allowed to acclimatize for 7 days under controlled conditions of temperature, aeration, and agitation. To minimize treatment costs, no additional nutrient sources (*e.g*., glucose, sucrose, or peptone) were added to the wastewater throughout the study, in alignment with previous findings.[42-44]



(A) Ramazol brilliant blue (B) Azure B (C) Phenol red

Figure 1: Screening of ligninolytic bacteria by the dye decolorization method (zone of decolorization – positive for enzyme activity; no zone of decolorization – no enzyme activity)

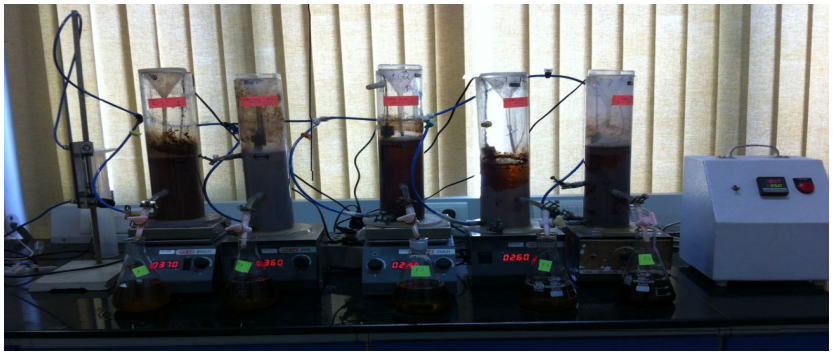


Figure 2: Semi-continuous reactor study setup with controlled aeration and agitation

Table 1
Daily addition of fresh feed and removal of treated wastewater for semi-continuous reactor study

S. No	RT, h	Volume of fresh feed added and treated wastewater removed based on RT, mL		
		Total volume	After 16 h	After 8 h
1	96	250	83.3	166.7
2	48	500	166.7	333.3
3	32	750	250	500
4	24	1000	333.3	667.7

*RT = V/Q, where V = volume of effluent (mL), Q = flow rate (mL/h)

Following acclimatization, the treatment efficiency of the bacterial strains was evaluated daily by monitoring reductions in color, COD, and lignin degradation. The reactor's retention time (RT) was progressively reduced from 96 h to 24 h. To maintain a consistent RT, a semi-continuous feeding strategy was employed, where treated wastewater was withdrawn in two stages (one-third and two-thirds of the total volume were removed after 16 h and 8 h, respectively) and replaced with an equal volume of fresh feed (Table 1).

Molecular characterization of RJH-50

16S rRNA gene sequencing is a widely used method for the identification of bacterial strains.[45] Genomic DNA was isolated, and the 16S rRNA gene was amplified using universal primers. The forward primer used was 5'-AGAGTTTGATCCTGGCTCAG-3' and the reverse primer was 5'-ACGGCTACCTTGTTACGACTT-3'. PCR amplification was carried out using a Mastercycler® thermocycler (Eppendorf, Germany). The ~1500 bp PCR product was purified using a PCR purification kit (Norgen Biotek, Canada) to remove unincorporated dNTPS and primers prior to sequencing. Both strands of the amplified rDNA region were sequenced using an automated DNA sequencer (3037xl DNA analyzer, Applied Biosystems) and the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). The resulting sequences (forward and reverse strands) were aligned using DNA Baser software before performing the bioinformatics analysis. Dendrograms were generated using Sequence Analysis Software version 5.2 (Applied Biosystems). The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model. The tree with the highest log likelihood (-1028.7715) is presented. Initial tree(s) for the heuristic search were generated automatically by applying the Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) method, followed by selection of the topology with the highest log likelihood value. The analysis involved 11 nucleotide sequences. Codon positions included were 1st, 2nd, 3rd, and noncoding regions. All positions containing gaps and missing data were eliminated, resulting in a final dataset comprising 737 positions. Evolutionary analyses were conducted using MEGA version 5.

RESULTS AND DISCUSSION

Isolation of bacteria and screening of ligninolytic bacteria

A total of 54 distinct bacterial strains were isolated on lignin-based mineral salts medium (MSM) agar plates. Subsequent screening using the plate assay revealed that 15 isolates exhibited ligninolytic enzyme activity, with some showing activity for one or two specific enzymes (Table 2). These 15 isolates were further evaluated for their wastewater degradation potential in batch mode experiments. Key parameters – including wastewater decolorization, lignin degradation, pH variation, and chemical oxygen demand (COD) reduction – were monitored at regular intervals (24, 48, 72, 96, and 120 h), and the results were compared to a control. Among all tested isolates,

one named as RJH-50 exhibited the highest degradation potential.

Table 2
Bacterial isolates screened for the presence of ligninolytic enzymes by dye decolorization plate assay

Lab name of bacterial isolate	Azure B (LiP)	Phenol red (MnP)	Ramazol brilliant blue (Laccase)
AV-1	+	-	+
AC-2	+	-	-
AV-3	-	-	+
AV-4	+	+	-
AV-5	+	-	+
BL-1	-	-	+
BL-6	-	+	+
BL-10	-	-	+
RJH-12	-	-	+
RJH-50	+	-	+
IN-1	+	-	+
IN-3	-	+	-
IN-4	-	+	-
IN-6	+	-	-
IN-7	+	-	+

* + zone of decolorization, positive for enzyme activity; - no zone of decolorization, no enzyme activity

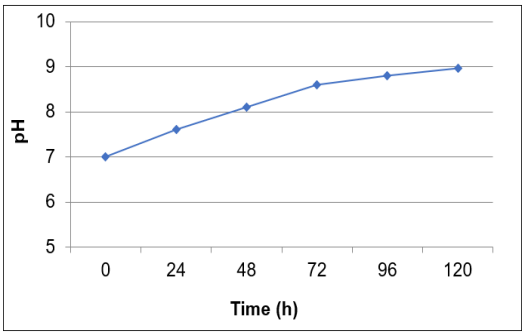


Figure 3: Change in pH of the wastewater treated with RJH-50 during the batch study

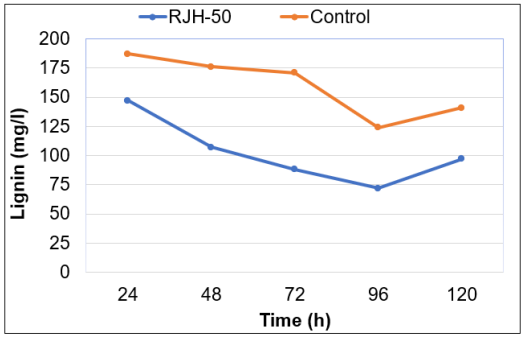


Figure 4: Lignin degradation during the batch study by lab isolate RJH-50 (relative to control)

pH of treated wastewater

During the batch study, the pH of the treated wastewater gradually increased, reaching a max. of 8.96 after 120 h of incubation (Fig. 3). Similar observations of increase in pH during lignin degradation were reported by El-Hanafy *et al.* (2008) and Crawford *et al*. (1982).[46-47]

Lignin degradation by RJH-50 during shake flask study in batch mode

Over the incubation period, the lignin concentration in the treated samples decreased steadily, while the control flask, containing only indigenous microorganisms, exhibited slower degradation. In comparison to the control, RJH-50 achieved lignin degradation rates of 21±7%, 39±9%, 48±9%, 41±7%, and 38±9% after 24, 48, 72, 96, and 120 h, respectively. The maximum degradation rate was observed at 72 h (Fig. 4), indicating that this time point represents the optimal duration for lignin degradation by RJH-50.

Color reduction by RJH-50 during shake flask study in batch mode

A gradual reduction in color was observed in both the control and inoculated flasks during the batch study. The control flask, containing indigenous microflora, exhibited some color reduction due to the activity of native microorganisms. Isolate RJH-50 achieved color reduction rates of 17±9%, 41±9%, 52±9%, 50±8%, and 49±11% after 24, 48, 72, 96, and 120 h, respectively. These results indicate a significant increase in color reduction up to 72 h, with the

maximum reduction (52±9%) occurring at that time point (Fig. 5).

COD reduction by RJH-50 during shake flask study in batch mode

During the batch study, COD reduction by the lab isolate RJH-50 after 24, 48, 72, 96 and 120 h of incubation was 29±8%, 48±9%, 57±6%, 52±9%, and 46±10% respectively. Since non-autoclaved wastewater was used throughout the study, a slight reduction in COD was also observed in the control flask due to indigenous microbial activity. The results indicate that the maximum COD reduction by RJH-50 was achieved at 72 h (Fig. 6). The subsequent increase in COD after 72 h may be attributed to a decline in bacterial activity over time, possibly due to the accumulation of complex or toxic intermediate compounds.

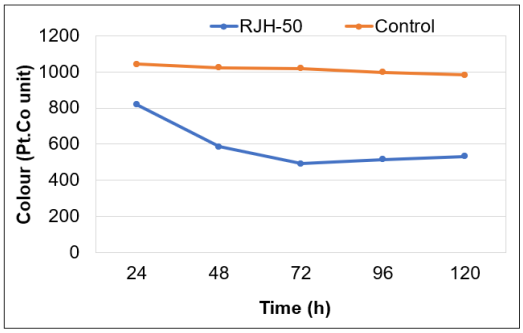


Figure 5: Reduction in colour during the batch study by lab isolate RJH-50 (relative to control)

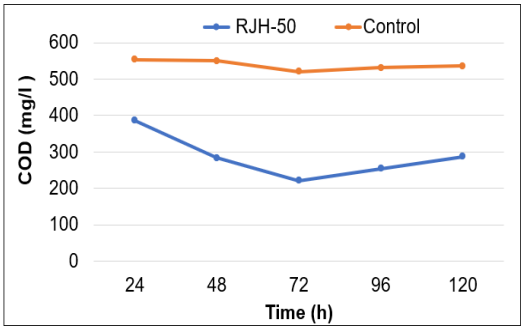


Figure 6: COD reduction by RJH-50 during the batch study (relative to control)

Reactor study in semi-continuous mode

The degradation efficiency of the bacterial isolate RJH-50 was further evaluated in a reactor study, demonstrating its effectiveness in treating wastewater under various retention times (RT). As the RT decreased from 96 h to 48 h, the percentage reductions in Chemical Oxygen Demand (COD), color, and lignin (relative to the control reactor) increased. This improvement in degradation can be attributed to the effective breakdown of complex organic compounds (such as lignin and its derivatives) as well as inorganic substances present in the effluent.

At an RT of 48 h, a notable COD reduction of 58% was observed, followed by a 56% reduction at 32 h. Lignin degradation reached 42% and 46% at 32 and 48 h RT, respectively. In terms of color removal, RJH-50 achieved a 47% reduction at 96 h RT, which increased to 49% at 48 h. However, further decreasing the RT to 24 h resulted in reduced efficiency, with COD, lignin, and color reductions dropping to 33%, 21%, and 27%, respectively. These findings indicate that the maximum wastewater degradation by the bacterial isolate, RJH-50, was achieved at an RT of 48 h (Figs. 7, 8, 9). Consistent with batch study observations, the pH of the treated effluent gradually increased during the 7-day acclimatization period, becoming alkaline due to bacterial metabolic activities. Throughout the reactor study, the pH remained consistently alkaline, within the range of 8.2 ± 0.4.

The bioremediation of pulp and paper mill wastewater using *Bacillus* species has been well documented in previous research studies.[23,24,29,48,49] Many of these studies supplemented the treatment process with additional carbon (C) and nitrogen (N) sources. For example, Chandra *et al.* (2009) reported 45-52% color reduction, 30-42% lignin degradation, and 50-60% Chemical Oxygen Demand (COD) reduction over 7 days using *Bacillus cereus* (ITRC-S6) and *Serratia marcescens* (ITRC-S7), with the addition of glucose and peptone.[29] Similarly, another *Bacillus* sp. achieved 61% color reduction, 53% lignin degradation, and 78% COD reduction within 6 days with glucose and peptone supplementation.[49] On the other hand, *Bacillus subtilis* showed a significant 94.7% COD reduction over 9 days under agitated conditions without any added growth factors.[50]

Bacillus species have also been recognized as effective degraders and detoxifiers for the remediation of land contaminated with pulp and paper mill black liquor.[44,51] Notably, *Bacillus cereus* demonstrated bioremediation potential for pulp and paper industry waste, by achieving 90.6% decolorization of effluent and 61% reduction in COD with 0.5% sucrose and 1% ammonium sulfate.[52] In contrast, the present study highlights

the ability of *Bacillus cereus* RJH-50 to achieve a 58% COD reduction, 49% color reduction, and 46% lignin degradation within just 48 h in a semi-continuous reactor system without the need for expensive co-substrates, demonstrating its potential as a cost-effective and efficient agent for pulp and paper mill effluent treatment.

Sequence analyses of lab isolate RJH-50

The phylogenetic tree illustrating the evolutionary relationship of RJH-50 with other *Bacillus* species is shown in Figure 10. The analysis of the 16s r RNA gene sequence revealed that RJH-50 is a strain of *Bacillus cereus*. It exhibited 97% similarity with *Bacillus cereus* strain JCM 2152 and 96% similarity with *Bacillus cereus* strains CCM 2010, NBRC 15305, ATCC 14579 and with *Bacillus thuringiensis*.

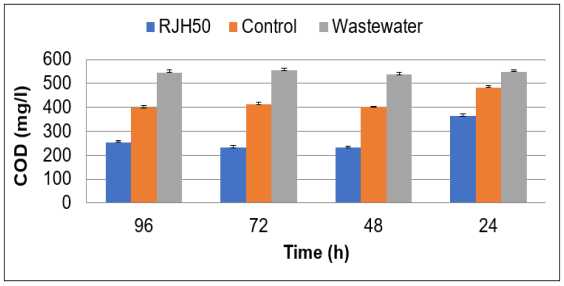


Figure 7: COD reduction by RJH-50 during the semi-continuous reactor study when RT is reduced from 96 h to 24 h

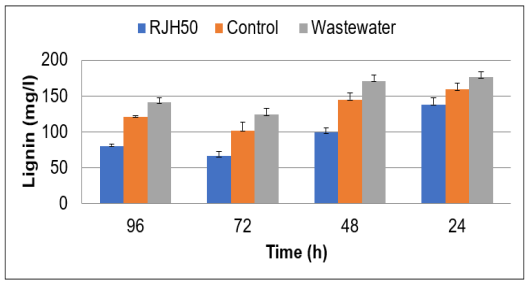


Figure 8: Lignin degradation by lab isolate RJH-50 during the semi-continuous reactor study when RT is reduced from 96 h to 24 h

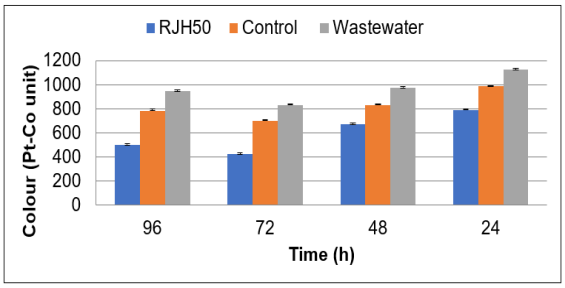
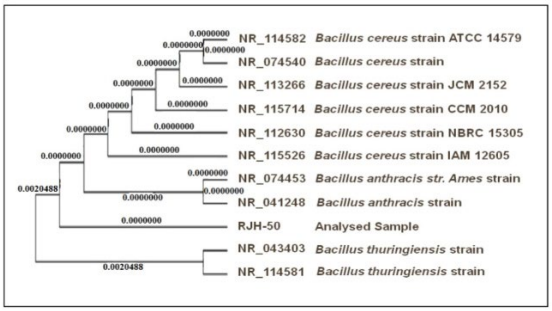


Figure 9: Colour reduction by lab isolate RJH-50 during the semi-continuous reactor study when RT was reduced from 96 h to 24 h

AGTCGAGCGAATGGATTAAGAGCTTGCTCTTATGAAGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAA
CCTGCCCATAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATAACATTTTGAACCGCATGGT
TCGAAATTGAAAGGCGGCTTCGGCTGTCACTTATGGATGGACCCGCGTCGCATTAGCTAGTTGGTGAGGT
AACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACG
GCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCC
GCGTGAGTGATGAAGGCTTTCGGGTCGTAAAACTCTGTTGTTAGGGAAGAACAAGTGCTAGTTGAATAAG
CTGGCACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAG
GTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGCAGGTGGTTTCTTAAGTCTGATGTGAAAG
CCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGAGACTTGAGTGCAGAAGAGGAAAGTGGAATTCC
ATGTGTAGCGGTGAAATGCGTAGAGATATGGAGGAACACCAGTGGCGAAGGCGACTTTCTGGTCTGTAAC
TGACACTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTC

a)



b)

Figure 10: (a) Aligned sequence RJH-50; (b) Phylogenetic dendrogram based on the results of 16S r RNA sequence comparison

CONCLUSION

In the present study, *Bacillus cereus* RJH-50 demonstrated a remarkable ability to efficiently degrade lignin, the primary color-imparting constituent in pulp and paper mill effluent. The bacterial isolate effectively utilized ammonium sulfate as a nitrogen source and phosphoric acid as a phosphorus source, both of which are cost-effective and widely used in paper mills. This characteristic makes *B. cereus* RJH-50 a promising candidate for the bioremediation of lignin-rich effluents from the pulp and paper industry.

The results revealed that *B. cereus* RJH-50 achieved a 58% reduction in chemical oxygen demand (COD), 49% reduction in color, and 46% degradation of lignin at a retention time (RT) of just 48 h in a semi-continuous reactor study. These substantial reductions in pollutants highlight the potential of this bacterial isolate to help paper mills comply with discharge norms and improve environmental sustainability.

Thus, the ability of *B. cereus* RJH-50 to treat lignin-rich effluents effectively, using affordable and readily available nutrients, underscores its value as a sustainable and practical solution for minimizing the environmental impact of pulp and paper mill operations.

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