



ISSN Print: 2664-9926
 ISSN Online: 2664-9934
 NAAS Rating (2026): 4.82
 IJBS 2026; 8(7): 253-261
www.biologyjournal.net
 Received: 20-05-2026
 Accepted: 24-06-2026

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Isolation, screening and characterization of ligninolytic bacteria from paper mill effluent for bioremediation applications

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DOI: <https://www.doi.org/10.33545/26649926.2026.v8.i7c.950>

Abstract

Paper mill effluent contains high concentrations of lignin and other recalcitrant organic pollutants that pose serious environmental concerns. This study aimed to isolate, screen, and characterize indigenous ligninolytic bacteria from paper mill sludge for potential bioremediation of lignin-rich wastewater. Physicochemical analysis of effluent collected from four paper mills in Chhattisgarh, India, revealed high levels of lignin, colour, BOD, COD, TDS, phenols, sulphate, and heavy metals. Thirty-one bacterial isolates were obtained using lignin mineral salt medium, of which fifteen utilized lignin as the sole carbon source. Five efficient isolates exhibiting growth at 1% lignin concentration were selected for further investigation. Ligninolytic activity was confirmed by gallic acid oxidation and Remazol Brilliant Blue R (RBBR) decolorization assays, with Isolate 15 showing the highest dye decolorization (8.3%). During effluent treatment, Isolate 15 achieved the highest colour (55%) and lignin (48%) removal, whereas Isolate 10 and Isolate 03 exhibited maximum reductions in BOD (42%) and COD (38%), respectively. Morphological, biochemical, and VITEK® 2 Compact analyses identified the isolates as *Sphingomonas paucimobilis*, *Acinetobacter baumannii*, *Bacillus cereus*, and *Pseudomonas aeruginosa*. These indigenous ligninolytic bacteria demonstrate significant potential as eco-friendly biocatalysts for the sustainable treatment of lignin-rich paper mill wastewater.

Keywords: Ligninolytic bacteria, bioremediation, VITEK® 2 compact system

1. Introduction

The pulp and paper industry is one of the largest industrial sectors worldwide, with an estimated market value exceeding US\$200 billion and a significant contribution to global economic development (Thomas J. Rainey, 2016) [23]. However, it is also recognized as one of the most resource-intensive industries, accounting for nearly 6% of global industrial energy consumption and approximately 2% of direct industrial CO₂ emissions (B. K. Dylan D. Furszyfer Del Rio, 2022) [7]. The increasing demand for paper products has consequently intensified environmental concerns associated with wastewater generation, greenhouse gas emissions, and solid waste disposal (Liang *et al.*, 2023; Manli Yang, 2023) [16, 29].

Paper manufacturing involves several sequential processes including raw material preparation, pulping, washing, bleaching, screening, chemical recovery, and papermaking. During these operations, enormous quantities of water and chemicals are consumed, generating large volumes of highly polluted wastewater. Paper mill effluent contains elevated concentrations of lignin, cellulose residues, hemicellulose, chlorinated organic compounds, resin acids, phenolics, suspended solids, and various recalcitrant organic pollutants that contribute to high biochemical oxygen demand (BOD), chemical oxygen demand (COD), dark coloration, unpleasant odor, and toxicity (D. Pokhrel, 2004; Lokeshwari N., 2013; Rajni Hood, 2015; Abhay Raj, 2007) [22, 18, 10, 1].

Among the pollutants present in pulp and paper wastewater, lignin and its degradation products represent one of the greatest environmental challenges. Lignin is a highly heterogeneous aromatic polymer that resists natural degradation and imparts a characteristic dark brown colour to industrial effluents. Its persistence reduces light penetration into aquatic ecosystems, suppresses photosynthetic activity, decreases dissolved oxygen levels, and adversely affects aquatic organisms. Furthermore, bleaching processes introduce chlorinated lignin derivatives, adsorbable organic halides (AOX), dioxins, furans, and other

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xenobiotic compounds that exhibit toxic, mutagenic, and carcinogenic properties (Monika and Indu, 2010; Kartik Patel, 2021; Singh and Tripathi, 2020) [21, 27].

Conventional wastewater treatment methods such as physical and chemical treatments are commonly employed to reduce pollutant loads; however, these techniques often suffer from high operational costs, incomplete degradation of recalcitrant compounds, sludge generation, and secondary pollution (Singh and Tripathi, 2020; D. V. Savant, 2006) [27, 24]. Consequently, biological treatment has emerged as a sustainable and environmentally compatible alternative. Bioremediation utilizes naturally occurring microorganisms to convert hazardous pollutants into less toxic or harmless products through enzymatic degradation pathways (EPA, 2016; ThandapaniGomathi, 2020) [28].

Among biological agents, bacteria have attracted considerable attention because of their rapid growth, metabolic diversity, adaptability to harsh environmental conditions, and ability to degrade complex lignocellulosic compounds. Several bacterial species including *Bacillus cereus*, *Serratiamarcescens*, *Pseudomonas syringae*, *Dysgonomonas* sp., and *Brevibacillusparabrevis* have demonstrated significant potential for reducing lignin, COD, BOD, phenolic compounds, colour, and chlorinated pollutants present in paper mill effluents (Ram Chandra, 2008; Jing Duan, 2016; RajniHooda, 2018; Madan, Sachan and Singh, 2018; Ismail, 2015) [5, 8, 20, 12]. Their biodegradation capability is primarily mediated through extracellular ligninolytic enzymes such as laccases, lignin peroxidases, and manganese peroxidases, which catalyse oxidative depolymerization of lignin into low-molecular-weight compounds that can be further mineralized.

Recent advances in molecular biology have substantially improved the identification and characterization of environmentally important microorganisms. Molecular techniques, particularly 16S rRNA gene sequencing, enable accurate taxonomic identification of bacterial isolates and facilitate the discovery of novel ligninolytic species with enhanced biodegradation capabilities. Integrating conventional microbiological screening with molecular characterization provides a reliable approach for selecting efficient bacterial strains suitable for industrial wastewater treatment and environmental restoration.

Therefore, the isolation, screening, and molecular characterization of indigenous ligninolytic bacteria from paper mill effluent represent an important strategy for developing efficient, eco-friendly, and economically viable bioremediation technologies. Native bacterial isolates are often better adapted to the physicochemical characteristics of industrial effluents and possess superior degradation efficiency under local environmental conditions. The identification of such microorganisms may contribute significantly to sustainable wastewater management, reduction of environmental pollution, and advancement of biological treatment technologies for the pulp and paper industry.

2. Materials and Methods

2.1 Chemicals and Reagents

All analytical-grade chemicals and reagents used in this study were procured from Merck (India). Distilled water was used for the preparation of culture media, buffers, stock solutions, and reagents. Laboratory glassware including Petri dishes, conical flasks, culture tubes, test tubes, beakers,

and micropipette tips were sterilized before use. The major laboratory equipment employed included a laminar airflow cabinet, incubator, centrifuge, autoclave, pH meter, COD digester, hot air oven, refrigerator, electronic balance, and water bath.

2.2 Sampling Site and Collection of Paper Mill Effluent

Paper mill effluent and sludge samples were collected from different paper mills located in Chhattisgarh, India, during 2023. Effluent samples were collected aseptically in sterile jerry cans, while sludge samples were collected in sterile sampling bottles. Samples were transported to the laboratory under refrigerated conditions and stored at 4°C until further analysis and bacterial isolation.

2.3 Culture Media

2.3.1 Enrichment and Isolation Medium

Lignin mineral salt medium (LMSM) was prepared using K₂HPO₄ (0.5 g/L), KH₂PO₄ (0.5 g/L), MgSO₄·7H₂O (0.5 g/L), agar (20 g/L), sludge infusion (400 mL), and distilled water (600 mL). Lignin was incorporated at concentrations ranging from 0.1-1.0% (w/v) as the sole carbon source for enrichment and selective isolation of ligninolytic bacteria.

2.3.2 Nutrient Agar

Nutrient agar containing beef extract (3 g/L), peptone (5 g/L), sodium chloride (5 g/L), and agar (15 g/L) was used for maintenance of bacterial isolates.

2.3.3 Nutrient Broth

Nutrient broth containing beef extract (3 g/L), peptone (5 g/L), and sodium chloride (5 g/L) was used for bacterial cultivation. The pH was adjusted to 7.0 before sterilization at 121°C for 15-20 min.

2.4 Physicochemical Analysis of Paper Mill Effluent

Raw paper mill effluent was characterized by determining pH, colour, lignin content, biological oxygen demand (BOD), chemical oxygen demand (COD), total solids (TS), total dissolved solids (TDS), phenol, sulphate, and heavy metal concentrations according to the analytical procedures described in the thesis.

2.5 Isolation and Screening of Ligninolytic Bacteria

Ligninolytic bacteria were isolated from sludge samples using the enrichment culture technique. Sludge samples were inoculated into lignin-amended mineral salt medium, where lignin served as the sole carbon source. Cultures were incubated at 30°C under shaking conditions (180 rpm) for seven days to selectively enrich lignin-utilizing microorganisms.

Following enrichment, serial dilutions up to 10⁻⁹ were prepared, and aliquots from appropriate dilutions were spread onto lignin mineral salt medium (LMSM) plates according to the method described by Virendra Kumar (2012) [15]. All procedures were carried out aseptically in a laminar airflow cabinet.

Thirty-one morphologically distinct bacterial colonies were isolated and purified by repeated streaking. The isolates were subsequently screened on LMSM containing increasing lignin concentrations (0.1-1.0%). Only isolates capable of sustained growth at higher lignin concentrations were selected for further characterization. Based on colony morphology and lignin utilization efficiency, five bacterial isolates were selected for subsequent studies.

2.6 Evaluation of Bacterial Treatment of Paper Mill Effluent

The selected ligninolytic bacterial isolates were cultivated in nutrient broth at 35°C for 24 h. Cells were harvested by centrifugation at 8000 rpm for 30 min at 4°C, washed with 50 mM sodium phosphate buffer, and inoculated into 100 mL of paper mill effluent.

The inoculated effluent was incubated at 35°C for 45 h with continuous shaking at 160 rpm. Following incubation, samples were allowed to settle for two hours, and the supernatant was collected for subsequent physicochemical analyses.

2.7 Morphological and Biochemical Characterization of Bacterial Isolates

Five selected bacterial isolates were characterized using standard morphological and biochemical methods according to Barrow and Feltham (1993) [2].

The following biochemical tests were performed:

- Gram staining
- Motility test
- Catalase test
- Oxidase test
- Urease test
- Indole test
- Citrate utilization test
- Nitrate reduction test
- Hydrogen sulphide (H₂S) production test
- Starch hydrolysis test
- Oxidative/Fermentative (O/F) test

The principles, media composition, procedures, and interpretation of each test were performed as described in the original methodology.

2.8 Molecular Identification Using VITEK® 2 Compact System

The selected bacterial isolates were identified using the VITEK® 2 Compact automated microbial identification system. Pure colonies were suspended in sterile saline, and the inoculum density was adjusted to 0.5 McFarland. The standardized suspension was inoculated into identification (ID) and antimicrobial susceptibility testing (AST) cards according to the manufacturer's instructions.

The inoculated cards were incubated automatically at 35.5±1°C, and biochemical reactions were monitored by the VITEK® 2 Compact system. The generated biochemical profiles were analyzed using the integrated Advanced Expert System database, providing rapid identification of bacterial isolates within 5-8 h.

3. Results

3.1 Physicochemical Characteristics of Paper Mill Effluent

The physicochemical characteristics of paper mill effluent collected from four paper industries were analyzed prior to bacterial isolation and treatment. Considerable variation was observed among the effluent samples with respect to pH, colour, total dissolved solids (TDS), lignin, phenol, biological oxygen demand (BOD), chemical oxygen demand (COD), total solids (TS), sulphate concentration, and heavy metal content. Among the investigated industries, the effluent obtained from Aananya Paper Mill exhibited the highest pollution load, with colour (1975±11.86 Pt-Co

units), lignin (287±8.64 mg L⁻¹), BOD (1953±17.20 mg L⁻¹), and COD (1954±20.55 mg L⁻¹). The acidic pH of the Ishwar and Sukraft mill effluents further indicated the need for biological treatment. Heavy metal analysis confirmed the presence of cadmium, lead, zinc, and nickel in varying concentrations. These observations demonstrated that the paper mill effluent contained a high organic and lignin load suitable for the isolation of ligninolytic microorganisms. Table 1 summarizes the physicochemical characteristics of the collected effluent samples.

Table 1: Characteristics of wastewater

Parameters	Ishwar Mill	Aananya Mill	Ajanta Mill	Sukraft Mill
pH	5.50±0.22	7.27±0.12	6.80±0.54	5.47±0.39
Color	1108±8.50	1975±11.86	1811±2.05	1171±1.70
TDS	1580±1.25	1852±3.86	1647±4.90	1447±13.14
Lignin	122±2.94	287±8.64	173±10.27	135±10.20
Phenols	78±3.40	86±4.78	83±4.03	75±3.68
BOD	1340±13.47	1953±17.20	1766±20.55	1325±14.08
COD	1320±8.16	1954±20.55	1835±20.55	1215±4.97
TS	1316±17.00	1971±24.94	1211±8.98	1817±9.74
Sulfate	363±28.6	411±10.5	389±7.85	404±2.94

TDS - Total dissolved solids, BOD - Biological oxygen demand, COD - Chemical oxygen demand, TS - Total Suspended

Heavy Metals

Cd(mg/l)	0.005	0.11	0.9	0.008
Pd(mg/l)	<0.005	<0.025	<0.025	<1.00
Zn(mg/l)	0.08	0.09	0.05	0.10
Ni(mg/l)	0.14	0.23	0.17	0.41

3.2 Isolation and Screening of Ligninolytic Bacteria

Following enrichment of sludge samples in lignin mineral salt medium, 31 morphologically distinct bacterial isolates were recovered. Preliminary screening on lignin mineral salt medium supplemented with lignin as the sole carbon source revealed that only 15 isolates exhibited sustained growth. These isolates were subsequently evaluated for their ability to tolerate increasing lignin concentrations ranging from 0.1 to 1.2%.

Among the screened isolates, Isolates 03, 04, 07, 10, and 15 demonstrated consistent growth up to 1% lignin, whereas the remaining isolates exhibited poor or no growth at higher lignin concentrations. Growth decreased progressively with increasing lignin concentration, indicating differential lignin tolerance among the isolates. Based on their growth characteristics and lignin utilization capacity, these five isolates were selected for further investigation. The growth response of the isolates at different lignin concentrations is presented in Table 2.

3.3 Confirmation of Ligninolytic Activity

The ligninolytic potential of the selected bacterial isolates was confirmed on gallic acid-amended medium. Formation of clear zones surrounding the bacterial colonies indicated extracellular degradation of lignin-derived phenolic compounds (Figure 1).

The selected isolates were further evaluated for their ability to decolorize Remazol Brilliant Blue R (RBBR), a model lignin analogue dye. Dye decolorization varied among the isolates. Isolate 15 exhibited the highest decolorization efficiency (8.3%), followed by Isolate 03 (7.9%), Isolate 07 (7.1%), Isolate 10 (6.8%), and Isolate 04 (4.2%). The higher

decolorization percentage observed in Isolate 15 indicated greater ligninolytic enzyme activity compared with the remaining isolates (Figure 2).

Table 2: LMSM plates devoid of an external carbon source were used to grow bacterial isolate, and their tolerance was examined across different lignin concentrations

Isolates	Lignin Concentration (g/L)					
	0.1%	0.2%	0.5%	0.8%	1%	1.2%
Culture 1	+++	++	+	-	-	-
Culture 2	+++	++	+	-	-	-
Culture 3	+++	+++	+++	++	+	-
Culture 4	+++	+++	+++	++	+	-
Culture 7	+++	+++	+++	++	+	-
Culture 10	+++	+++	+++	++	+	-
Culture 11	++	+	-	-	-	-
Culture 12	++	++	+	+	+	-
Culture 15	+++	+++	+++	++	+	-
Culture 18	+++	++	+	+	-	-
Culture 20	+++	++	+	-	-	-
Culture 22	+++	+++	++	+	-	-
Culture 25	+++	+++	++	+	-	-
Culture 28	+++	++	-	-	-	-
Culture 29	++	+	-	-	-	-

+++; Very fast growth

++; Fast growth

++; Slow growth

-; No growth



Fig 1: Decolorization of lignolytic indicator (Gallic Acid)

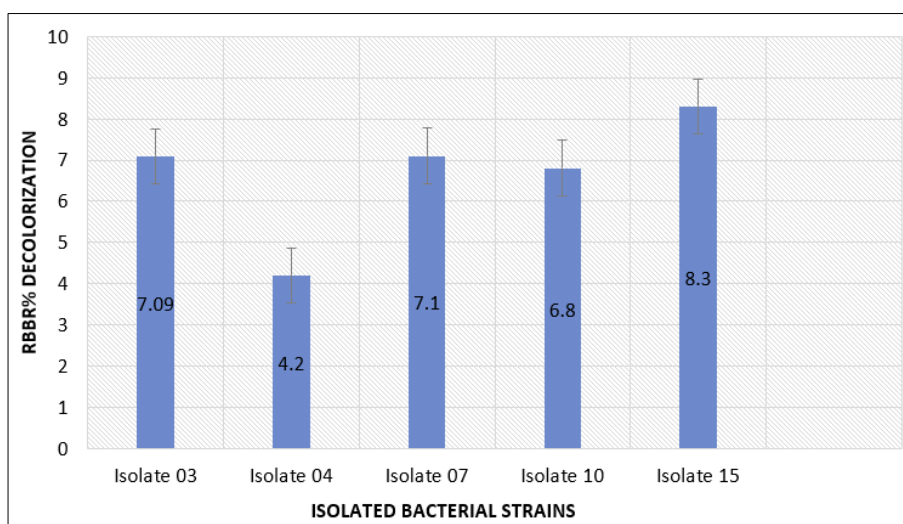


Fig 2: Maximum dye decolorization was observed by Isolate 15 (8.3%) followed by Isolate 03 (7.9%), Isolate 07 (7.1%), Isolate 10 (6.8%), Isolate 04 (4.2%)

3.4 Evaluation of Bacterial Treatment of Paper Mill Effluent

The five selected ligninolytic bacterial isolates were evaluated for their efficiency in reducing major pollution parameters of paper mill effluent following incubation.

Among the tested isolates, Isolate 15 achieved the highest colour reduction (55%) followed by Isolate 10 (28%), Isolate 03 (20%), Isolate 07 (14%), and Isolate 04 (5%) (Figure 3).

Similarly, lignin degradation was highest in Isolate 15 (48%), followed by Isolate 07 (29%), Isolate 03 (17%), Isolate 10 (12%), and Isolate 04 (9%) (Figure 4).

Reduction in biological oxygen demand (BOD) ranged from 19.9% to 42%, with Isolate 10 showing the maximum reduction (42%), followed by Isolate 15 (40%), Isolate 07 (31%), Isolate 03 (22%), and Isolate 04 (19.9%). Likewise, chemical oxygen demand (COD) reduction ranged from 12% to 38%, where Isolate 03 exhibited the highest reduction (38%), followed by Isolate 10 (33%), Isolate 07 (30%), Isolate 15 (22%), and Isolate 04 (12%) (Figure 5).

Overall, all selected isolates demonstrated the ability to reduce the pollutant load of paper mill effluent, with Isolate 15 showing superior colour and lignin removal efficiency, while Isolate 10 and Isolate 03 were more effective in reducing BOD and COD, respectively.

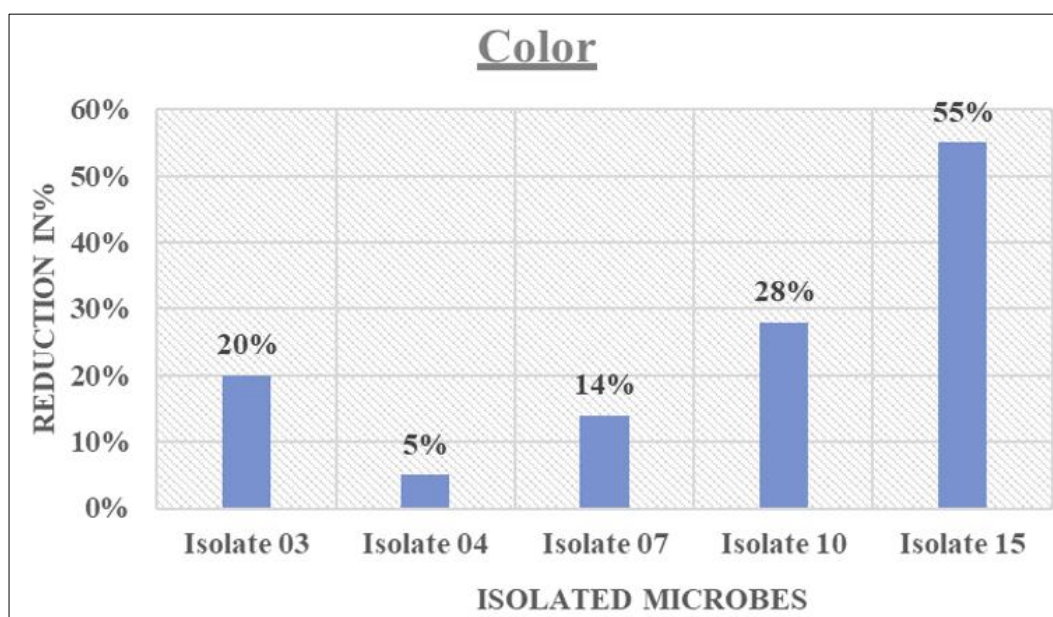


Fig 3: Reduction of color by different isolates on effluent

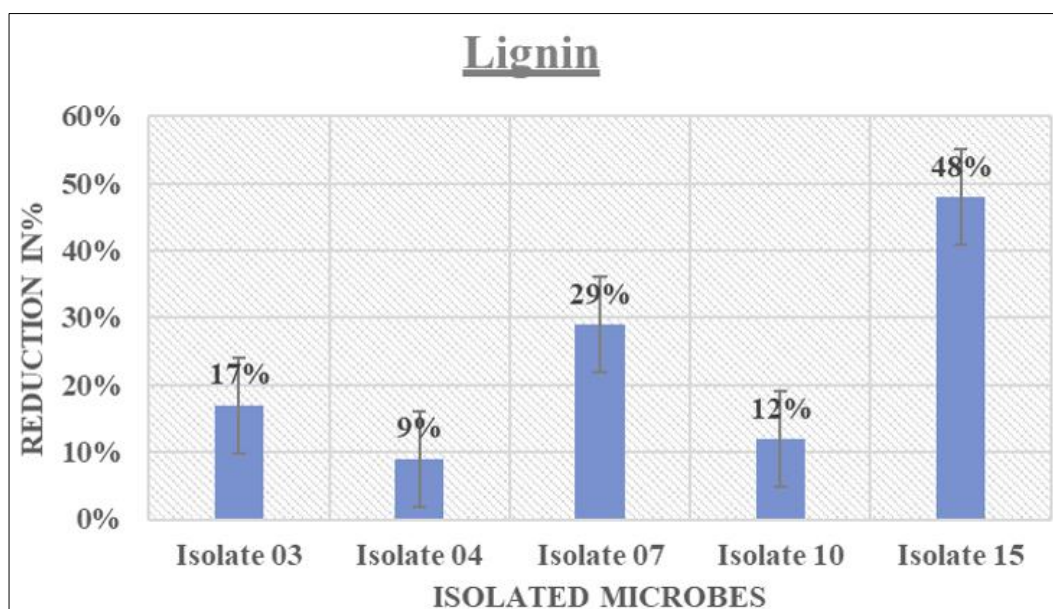


Fig 4: Reduction of lignin by different isolates on effluent

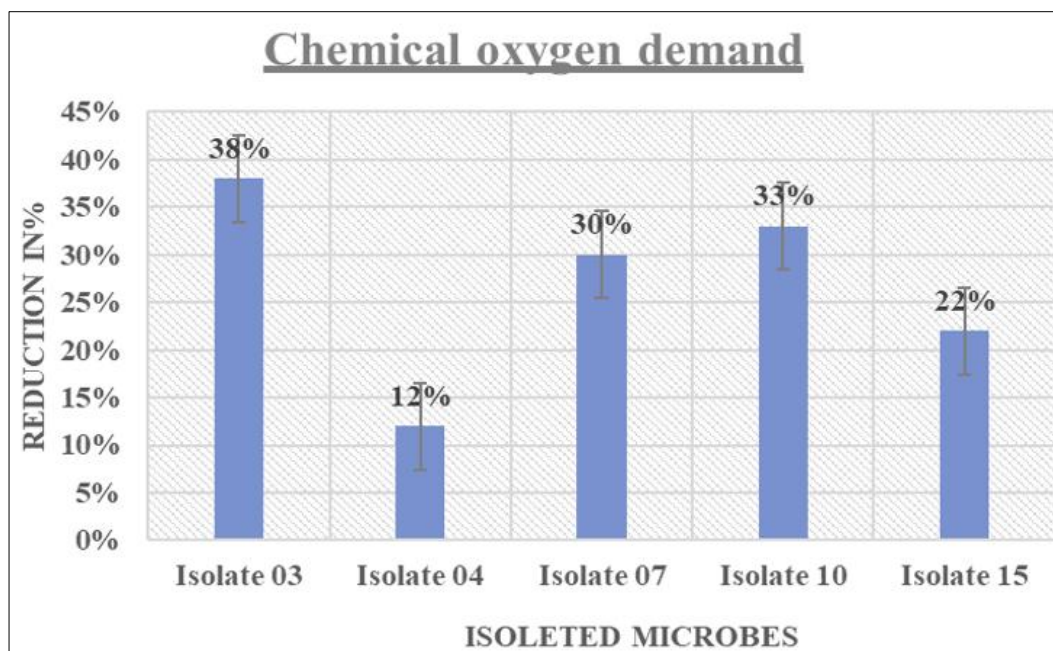


Fig 5: Reduction of COD by different isolates on effluent.

3.5 Morphological and Biochemical Characterization of Selected Isolates

Five efficient ligninolytic bacterial isolates were subjected to morphological and biochemical characterization. The isolates exhibited distinct colony morphology, pigmentation, Gram reaction, motility, and biochemical characteristics. Variations were observed in oxidase activity, citrate

utilization, nitrate reduction, starch hydrolysis, and carbohydrate utilization, whereas all isolates were positive for catalase activity. These biochemical characteristics facilitated preliminary identification of the selected bacterial isolates. The biochemical profile of each isolate is presented in Table 3.

Table 3: Biochemical tests of bacterial strains isolated from sludge sample

	Isolate 03	Isolate 04	Isolate 07	Isolate 10	Isolate 15
Source	Sludge	Sludge	Sludge	Sludge	Sludge
Colony Color	Yellow pigmented	White	Yellow pigmented		
Gram Staining	-	-	-	+	-
Bacterial Motility Test	Motile	Non motile	Motile	Motile	Motile
Catalase Test	+	+	+	+	+
Oxidase Test	+	-	+	+	-
Urease Test	-	-	-	-	-
Indole Test	-	-	-	-	-
Citrate Test	-	+	-	+	+
Nitrate Reduction Test	-	-	-	+	+
Hydrogen Sulfide Production (H ₂ S)	-	-	-	-	-
Starch Hydrolysis Test	-	-	-	-	+
Oxidative/ Fermentation (OF) Test	Non fermentative	Non fermentative	Non fermentative	Non fermentative	Fermentative

3.6 Identification of Ligninolytic Bacteria Using the VITEK® 2 Compact System

The taxonomic identity of the selected bacterial isolates was confirmed using the automated VITEK® 2 Compact microbial identification system. The analysis identified Isolate 03 as *Sphingomonas paucimobilis* with a probability of 97%, Isolate 04 as *Acinetobacter baumannii* (99%),

Isolate 07 as *Sphingomonas paucimobilis* (91%), Isolate 10 as *Bacillus cereus* (87%), and Isolate 15 as *Pseudomonas aeruginosa* (89%). The high probability values confirmed the identity of the selected ligninolytic bacteria and validated their suitability for paper mill effluent bioremediation applications. Figure 6 presents the VITEK® 2 identification results.

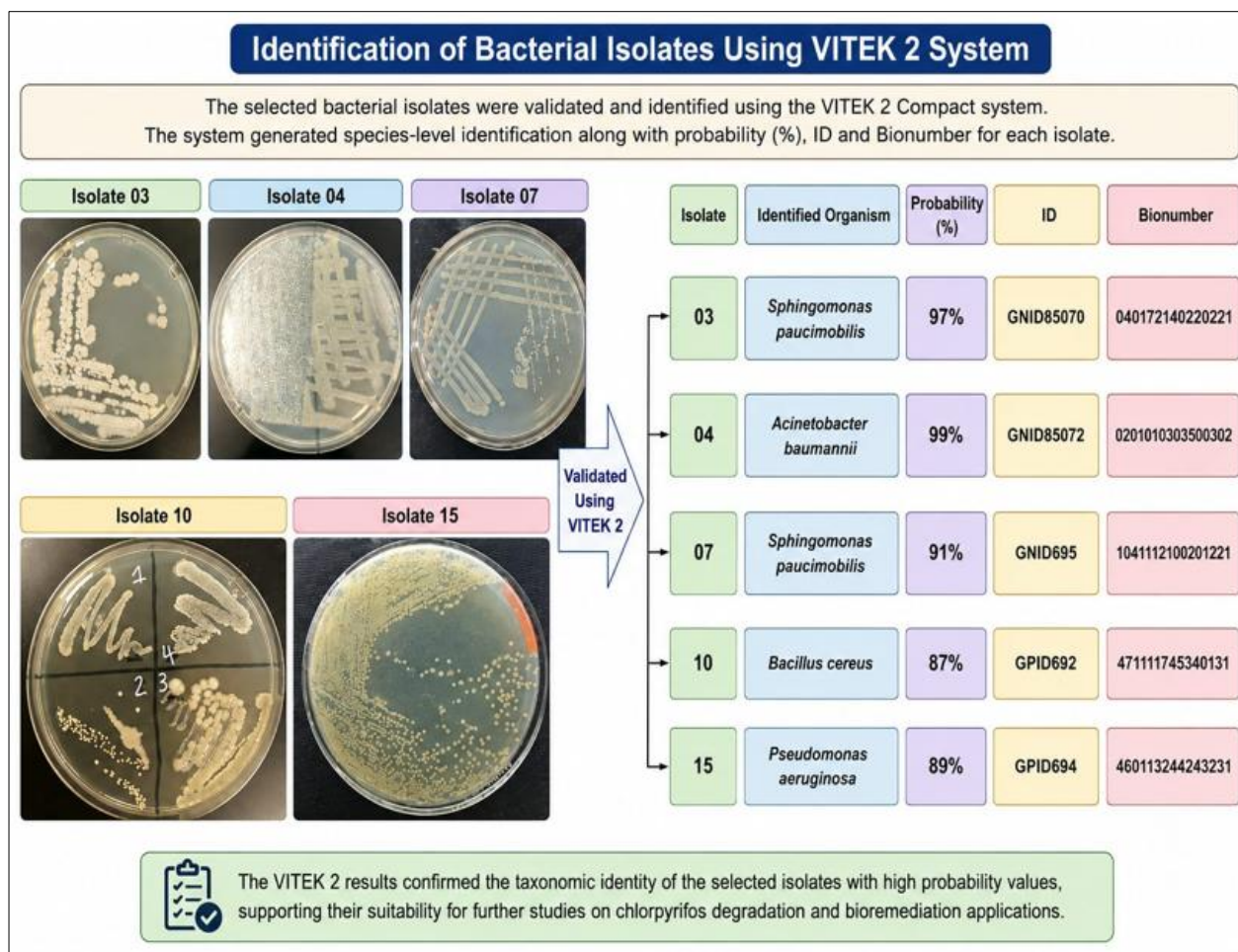


Fig 6: VITEK® 2 identification results for different isolates

4. Discussion

The physicochemical analysis revealed that the collected paper mill effluents contained high concentrations of colour, lignin, BOD, COD, total dissolved solids, and heavy metals, confirming their highly polluted nature. The highest pollution load observed in the Aananya Paper Mill effluent indicates the necessity for efficient biological treatment. Similar characteristics of pulp and paper mill wastewater have been reported previously (Lindholm-Lehto *et al.*, 2015) [19].

Isolation from paper mill sludge yielded thirty-one bacterial isolates, of which only fifteen were able to utilize lignin effectively. Further screening identified five isolates capable of growing at 1% lignin concentration, suggesting their adaptation to lignin-rich environments and their potential for lignin biodegradation.

Gallic acid oxidation and RBBR decolorization assays confirmed the ligninolytic nature of the selected isolates. The highest RBBR decolorization shown by Isolate 15 indicates greater production of ligninolytic enzymes involved in the degradation of aromatic lignin compounds, consistent with previous reports using RBBR as an indicator of ligninolytic activity.

All selected isolates significantly reduced major pollutants in paper mill effluent, although their efficiencies differed. Isolate 15 exhibited maximum colour (55%) and lignin (48%) removal, whereas Isolate 10 achieved the highest BOD reduction (42%) and Isolate 03 showed maximum COD reduction (38%). These variations suggest differences in the metabolic and enzymatic capabilities of individual isolates for degrading complex organic pollutants.

Morphological and biochemical characterization demonstrated considerable diversity among the selected isolates in colony morphology, Gram reaction, motility, and biochemical properties. These characteristics facilitated preliminary identification and provided a basis for subsequent molecular characterization. Overall, the study demonstrates that indigenous ligninolytic bacteria isolated from paper mill sludge possess significant potential for the biological treatment of lignin-rich paper mill effluents in an environmentally sustainable manner.

5. Conclusion

This study successfully isolated and characterized indigenous ligninolytic bacteria from paper mill sludge with significant potential for the biological treatment of lignin-rich industrial wastewater. Among the thirty-one bacterial isolates obtained, five demonstrated high lignin tolerance and effective ligninolytic activity, confirmed through gallic acid oxidation and RBBR dye decolorization assays. The selected isolates significantly reduced key pollution parameters, including colour, lignin, BOD, and COD, with Isolate 15 showing the highest colour (55%) and lignin (48%) removal, while Isolate 10 and Isolate 03 exhibited superior BOD (42%) and COD (38%) reduction, respectively. Morphological, biochemical, and automated VITEK® 2 Compact identification confirmed the presence of environmentally important bacterial species including *Sphingomonas paucimobilis*, *Acinetobacter baumannii*, *Bacillus cereus*, and *Pseudomonas aeruginosa*. The study highlights the adaptability and biodegradation efficiency of indigenous bacteria under lignin-rich conditions,

demonstrating their suitability as environmentally friendly alternatives to conventional wastewater treatment methods. These findings provide a strong foundation for developing microbial consortia and scaling up biological treatment processes for sustainable management of pulp and paper industry effluents. Future studies should focus on optimization under pilot-scale conditions, enzyme characterization, genomic analysis, and evaluation of long-term treatment performance in industrial wastewater systems.

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