



Enzyme-Mediated Biodegradation Of Pulp And Paper Industry Effluent By Indigenous Bacterial And Fungal Isolates

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ABSTRACT

Effluents released from pulp and paper industries are characterized by high organic load, intense color, and the presence of lignocellulosic and aromatic compounds that pose serious environmental challenges. The present study investigated the isolation, purification, screening, and biochemical characterization of indigenous microorganisms from pulp and paper industry effluent, with emphasis on their enzymatic potential and wastewater treatment efficiency. Physicochemical analysis of the effluent revealed alkaline pH and elevated biochemical oxygen demand (BOD) and chemical oxygen demand (COD), indicating substantial pollution strength. A total of twenty-four microbial isolates were recovered, including eighteen bacterial and six fungal strains, reflecting considerable microbial diversity. Primary screening identified eight effluent-tolerant isolates capable of sustained growth at high effluent concentrations. Secondary screening demonstrated that selected isolates produced extracellular cellulolytic, amylolytic, and ligninolytic enzymes, with isolates affiliated to *Bacillus* and *Aspergillus* exhibiting the highest enzymatic activity. Laboratory-scale treatment experiments showed a strong correlation between enzyme production and pollutant removal efficiency. The most effective isolate achieved significant reductions in BOD and COD, highlighting its capacity to degrade both biodegradable and recalcitrant organic fractions. Biochemical characterization further confirmed the metabolic versatility of dominant isolates, supporting their adaptability to industrial wastewater conditions. Overall, the findings demonstrate that pulp and paper effluent harbors functionally robust microorganisms with considerable potential for biological wastewater treatment. Exploitation of such indigenous enzyme-producing strains offers a sustainable and cost-effective alternative to conventional physicochemical treatment methods for managing pulp and paper industry effluents.

Keywords: Pulp and paper effluent; extracellular enzymes; biodegradation; microbial characterization; wastewater treatment

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1. INTRODUCTION

The pulp and paper sector is globally acknowledged as one of the most resource-intensive manufacturing industries, consuming substantial quantities of freshwater and releasing complex wastewater streams

into the environment (Pokhrel & Viraraghavan, 2004; Bajpai, 2018). Effluents generated during wood preparation, pulping, bleaching, and sheet formation are chemically heterogeneous and often exhibit dark coloration, strong odor, and elevated pollutant loads

(Hubbe et al., 2016). These wastewaters typically contain lignin fragments, cellulose and hemicellulose residues, resin and fatty acids, extractives, suspended fibers, and chlorinated organic compounds formed during bleaching operations (Ali & Sreekrishnan, 2001; Kamali & Khodaparast, 2015).

The discharge of inadequately treated pulp and paper effluent poses serious ecological threats due to its high biochemical oxygen demand (BOD) and chemical oxygen demand (COD), which can rapidly deplete dissolved oxygen in receiving water bodies (Ashrafi et al., 2015). Such oxygen stress negatively affects aquatic organisms and disrupts trophic interactions within freshwater ecosystems (Singh et al., 2019). Moreover, bleaching-derived chlorophenols and other halogenated compounds exhibit mutagenic and toxic properties, contributing to long-term environmental persistence and bioaccumulation (Vidotti et al., 2011). These concerns have intensified regulatory pressure on pulp and paper mills to adopt efficient and sustainable wastewater treatment technologies.

Traditional physicochemical treatment strategies, including sedimentation, chemical coagulation, advanced oxidation, and adsorption, are widely applied to reduce pollutant load; however, these approaches often involve high operational costs and generate large quantities of secondary sludge (Hubbe et al., 2016). In addition, such methods may prove insufficient for complete mineralization of complex aromatic polymers derived from lignin (Bajpai, 2018). As a result, attention has progressively shifted toward biologically driven treatment processes that exploit the metabolic potential of microorganisms for pollutant transformation (Rintala & Puhakka, 1994).

Microbial systems offer several advantages over conventional technologies, including lower energy demand, reduced chemical input, and the ability to adapt to fluctuating effluent composition (Leonowicz et al., 2001). Bacteria and fungi possess diverse enzymatic machineries capable of catalyzing the depolymerization and oxidation of recalcitrant organic substrates commonly present in pulp and paper wastewater (Sharma et al., 2014). Enzymes such as cellulases, amylases, laccases, lignin peroxidases, and manganese peroxidases play a critical role in the breakdown of structural polysaccharides and aromatic compounds into assimilable units (Madlala & Whiteley, 2020).

Microorganisms indigenous to industrial effluents are of particular interest because prolonged exposure to toxic

and nutrient-rich environments often selects for strains with enhanced tolerance and catabolic efficiency (Kumar et al., 2020). Such native populations frequently outperform externally introduced cultures, as they are physiologically acclimatized to extreme pH conditions, high salinity, and inhibitory compounds present in untreated wastewater (Kamali & Khodaparast, 2015). Previous investigations have reported the prevalence of bacterial genera such as *Bacillus*, *Pseudomonas*, and *Enterobacter*, along with filamentous fungi including *Aspergillus* and *Penicillium*, in pulp and paper effluent ecosystems due to their robust enzyme secretion capabilities (Ali & Sreekrishnan, 2001; Sharma et al., 2014).

Despite the recognized biodegradative potential of effluent-adapted microbial communities, comprehensive studies focusing on their systematic isolation, purification, functional screening, and biochemical profiling remain relatively limited (Ashrafi et al., 2015). Detailed characterization of these microorganisms is essential to elucidate their metabolic attributes, enzyme production patterns, and pollutant removal efficiency under controlled conditions (Bajpai, 2018). Such information is crucial for designing effective biological treatment systems and for integrating microbial processes into existing effluent treatment plants.

In this context, the present investigation aims to isolate and purify microorganisms from pulp and paper industry effluent, followed by screening for effluent tolerance and enzymatic activity relevant to wastewater remediation. Furthermore, biochemical characterization of selected isolates is undertaken to assess their physiological diversity and treatment potential. By exploring the functional capabilities of indigenous microorganisms, this study seeks to contribute toward the development of sustainable, low-cost, and environmentally compatible strategies for industrial effluent management.

2. MATERIALS AND METHODS

2.1 Sample collection

Effluent samples were collected from the terminal discharge channel of an operational pulp and paper manufacturing facility to ensure that the wastewater reflected the cumulative impact of all processing stages, including pulping, bleaching, and paper formation (Pokhrel & Viraraghavan, 2004; Bajpai, 2018). Sampling was conducted during regular production hours to avoid dilution effects associated with shutdown

periods or maintenance operations (Hubbe et al., 2016). Sterile, airtight high-density polyethylene containers were employed to prevent microbial contamination and chemical interaction with container surfaces (APHA, 2017).

Immediately after collection, samples were placed in insulated containers containing ice packs and transported to the laboratory to minimize physicochemical alteration and microbial succession (Kamali & Khodaparast, 2015). Upon arrival, wastewater was stored at 4 °C and processed within 24 h, a timeframe considered optimal for preserving indigenous microbial populations and enzymatic activity (Ashrafi et al., 2015). Prior to microbiological analysis, samples were gently homogenized to ensure uniform distribution of suspended solids and microorganisms (Bajpai, 2018).

Physicochemical characterization of the effluent was conducted to assess pollution intensity and establish baseline conditions influencing microbial growth (Singh et al., 2019). Parameters including pH and temperature were measured using calibrated digital meters, while biochemical oxygen demand (BOD), chemical oxygen demand (COD), and total dissolved solids (TDS) were determined using standard analytical procedures (APHA, 2017). These measurements provided essential context for interpreting microbial tolerance and degradation performance (Hubbe et al., 2016).

2.2 Isolation and purification of microorganisms

Isolation of microorganisms was performed using the serial dilution technique, which allows recovery of discrete colonies from complex environmental matrices such as industrial effluents (Cappuccino & Sherman, 2014). Effluent samples were serially diluted up to 10^{-6} using sterile physiological saline to reduce microbial density while maintaining osmotic stability (Prescott et al., 2017). Aliquots from appropriate dilutions were spread onto nutrient agar for general heterotrophic bacteria, minimal salt agar supplemented with 10 percent (v/v) effluent to enrich pollutant-adapted strains, and potato dextrose agar amended with antibiotics for selective fungal growth (Sharma et al., 2014).

Inoculated plates were incubated aerobically at 30 ± 2 °C, reflecting ambient conditions commonly encountered in biological treatment systems (Rintala & Puhakka, 1994). Bacterial colonies were observed after 24–48 h, whereas fungal growth was monitored for up to 5 days to allow adequate sporulation and

morphological differentiation (Barnett & Hunter, 1998). Colonies displaying distinct morphological attributes such as pigmentation, elevation, margin configuration, and surface texture were selected to maximize taxonomic diversity (Madigan et al., 2018).

Purification of isolates was achieved through repeated streaking on fresh agar plates until uniform colony morphology was obtained, indicating axenic cultures (Cappuccino & Sherman, 2014). Pure cultures were maintained on agar slants at 4 °C for short-term storage and periodically subcultured to retain viability and metabolic activity (Prescott et al., 2017).

2.3 Primary screening for effluent tolerance

Purified isolates were subjected to primary screening to evaluate their tolerance to pulp and paper wastewater under controlled laboratory conditions (Kamali & Khodaparast, 2015). Individual cultures were inoculated into nutrient broth supplemented with graded concentrations of effluent (25, 50, and 75 percent, v/v), simulating increasing environmental stress levels encountered in treatment systems (Kumar et al., 2020). Incubation was carried out at 30 °C for 48 h with constant agitation to maintain aeration and uniform exposure to pollutants (Rintala & Puhakka, 1994).

Microbial growth was monitored spectrophotometrically by measuring optical density at 600 nm, a widely accepted proxy for biomass accumulation in liquid cultures (Madigan et al., 2018). Isolates exhibiting sustained growth at higher effluent concentrations were considered tolerant and selected for further functional evaluation, as tolerance is a prerequisite for effective wastewater bioremediation (Ashrafi et al., 2015).

2.4 Secondary screening for enzymatic activities

Isolates demonstrating significant tolerance were further screened for extracellular enzyme production associated with the degradation of complex organic constituents present in pulp and paper effluent (Leonowicz et al., 2001). Cellulolytic potential was assessed using carboxymethyl cellulose agar, where enzymatic hydrolysis of cellulose was visualized following Congo red staining and subsequent destaining with sodium chloride solution (Teather & Wood, 1982). Amylolytic activity was examined on starch agar plates, with iodine solution used to detect zones of starch degradation (Prescott et al., 2017).

Ligninolytic capability was evaluated on lignin-modified agar media, where oxidation and depolymerization of lignin-related compounds resulted

in visible clearance or discoloration around colonies (Madlala & Whiteley, 2020). The diameter of hydrolysis zones was measured to provide a comparative estimate of enzyme production efficiency among isolates (Sharma et al., 2014). These qualitative assays served as an initial indicator of the isolates' potential role in effluent detoxification.

2.5 Biochemical characterization

Bacterial isolates exhibiting prominent enzymatic activity were subjected to comprehensive biochemical profiling to determine their physiological and metabolic attributes (Cappuccino & Sherman, 2014). Gram staining was performed to differentiate cell wall structure, followed by a series of biochemical assays including catalase and oxidase reactions, citrate utilization, indole production, methyl red, Voges–Proskauer tests, nitrate reduction, and carbohydrate fermentation patterns (Prescott et al., 2017). Results were interpreted using standard taxonomic keys to assign isolates to probable genera (Bergey & Holt, 1994).

Fungal isolates were characterized based on macroscopic features such as colony texture, growth rate, pigmentation, and sporulation pattern (Barnett & Hunter, 1998). Microscopic examination was carried out using lactophenol cotton blue staining to visualize hyphal septation, conidiophore arrangement, and spore morphology, enabling tentative identification of genera such as *Aspergillus* and *Penicillium* (Sharma et al., 2014).

2.6 Laboratory-scale effluent treatment study

To evaluate pollutant removal efficiency, selected bacterial isolates were tested in laboratory-scale effluent treatment experiments (Kumar et al., 2020). Actively growing cultures were inoculated individually into Erlenmeyer flasks containing untreated wastewater and incubated at 30 °C for 7 days under shaking conditions to enhance microbial–substrate interaction (Rintala & Puhakka, 1994). Control flasks containing uninoculated effluent were maintained under identical conditions to account for abiotic changes (Ashrafi et al., 2015).

At the end of the incubation period, treated samples were analyzed for reductions in BOD and COD using standard protocols (APHA, 2017). Percentage removal efficiencies were calculated to assess biodegradation performance and compare treatment efficacy among isolates (Bajpai, 2018).

2.7 Statistical analysis

All experimental procedures were conducted in triplicate to ensure reproducibility and minimize experimental error (Zar, 2010). Results were expressed as mean $\pm$ standard deviation to reflect variability among replicates. Statistical significance of differences among treatments was determined using one-way analysis of variance (ANOVA), with $p < 0.05$ considered indicative of meaningful variation (Ashrafi et al., 2015).

3. RESULTS

3.1 Physicochemical characteristics of effluent

The untreated pulp and paper industry effluent exhibited a dark brown coloration with a characteristic odor, reflecting the presence of lignin-derived chromophoric compounds. The wastewater was moderately alkaline, and elevated organic and dissolved solids content indicated a substantial pollution load. High biochemical oxygen demand (BOD) and chemical oxygen demand (COD) values suggested the dominance of biodegradable as well as recalcitrant organic matter. Considerable concentrations of suspended solids and color intensity further confirmed the complex nature of the effluent matrix.

Table 1 presents the physicochemical profile of the collected wastewater. The COD/BOD ratio was greater than 2.5, indicating the presence of slowly degradable organic fractions in addition to readily biodegradable substrates.

Table 1. Physicochemical characteristics of pulp and paper industry effluent

Parameter	Value
pH	8.3 $\pm$ 0.1
Temperature (°C)	32 $\pm$ 1
Color (Pt–Co units)	1860 $\pm$ 85
BOD (mg L ⁻¹)	840 $\pm$ 40
COD (mg L ⁻¹)	2280 $\pm$ 95
COD/BOD ratio	2.71
Total suspended solids (mg L ⁻¹)	610 $\pm$ 28
Total dissolved solids (mg L ⁻¹)	1520 $\pm$ 65
Lignin content (mg L ⁻¹)	410 $\pm$ 22

These characteristics confirm that the effluent represents a chemically stressed environment capable of selectively enriching metabolically versatile microbial populations.

3.2 Isolation and purification of microorganisms

Microbiological analysis of the effluent resulted in the recovery of twenty-four distinct isolates, comprising eighteen bacterial strains and six filamentous fungi. Bacterial colonies displayed marked heterogeneity with respect to size, pigmentation, margin configuration, and surface texture. Several isolates exhibited mucoid or rough appearances, suggesting differences in extracellular polymer production.

Fungal growth was characterized by rapid mycelial expansion and pigmentation ranging from white to dark green. Microscopic examination revealed septate hyphae and diverse sporulation patterns. Repeated subculturing resulted in stable, axenic cultures suitable for further screening. The predominance of bacterial isolates indicated that bacteria constituted the major cultivable microbial fraction in the effluent.

3.3 Primary screening for effluent tolerance

All purified isolates were initially evaluated for their ability to grow in effluent-amended liquid medium. Growth responses varied considerably among isolates, particularly at higher effluent concentrations. At 25 percent effluent supplementation, most isolates showed moderate growth; however, increasing the concentration to 50 percent imposed selective pressure, allowing only a subset of strains to maintain substantial biomass accumulation.

Eight isolates demonstrated strong tolerance at 50 percent effluent concentration, with optical density values at 600 nm ranging from 0.84 to 1.38 after 48 h of incubation. Among bacterial strains, isolate B7 exhibited the highest growth, followed by B15, indicating superior adaptability to effluent-associated stress. Fungal isolates also showed consistent growth, although optical density values were generally lower compared to bacterial counterparts due to filamentous growth patterns.

Table 2. Growth response of selected isolates in effluent-amended medium

Isolate code	OD ₆₀₀ at 50 percent effluent
B1	1.14 ± 0.05
B3	0.99 ± 0.04
B7	1.38 ± 0.06
B11	0.91 ± 0.03
F2	0.96 ± 0.05
F4	1.10 ± 0.04
F5	0.84 ± 0.03

B15	1.26 ± 0.05
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Isolates exhibiting optical density values above 1.0 were considered highly tolerant and were selected for further functional characterization. The observed variation in growth performance reflects differences in metabolic flexibility and stress resistance among effluent-derived microorganisms.

3.4 Secondary screening for enzyme production

Secondary screening revealed that all selected isolates were capable of producing at least one extracellular hydrolytic or oxidative enzyme associated with pulp and paper effluent degradation. However, the extent and spectrum of enzyme secretion varied markedly among isolates. Clear zones of substrate hydrolysis surrounding colonies confirmed enzymatic activity, with differences in diameter reflecting relative production efficiency.

Among bacterial isolates, B7 exhibited the highest overall enzymatic performance, producing prominent clearance zones on carboxymethyl cellulose, starch, and lignin-modified media. The fungal isolate F4 demonstrated superior ligninolytic and cellulolytic potential, as indicated by extensive decolorization and clearance on lignin-containing agar. In contrast, isolate B1 showed moderate cellulase and amylase activity but comparatively lower ligninolytic capability.

Table 3 summarizes the extracellular enzyme activity of selected isolates. Ligninolytic activity exceeded cellulolytic and amylolytic responses in isolates B7 and F4, highlighting their specialization toward degradation of aromatic polymers commonly present in pulp and paper wastewater.

Table 3. Extracellular enzymatic activity of selected isolates (zone diameter in mm)

Isolate	Cellulase (mm)	Amylase (mm)	Ligninolytic (mm)
B1	13 ± 1	11 ± 1	9 ± 1
B7	17 ± 1	15 ± 1	20 ± 2
B15	14 ± 1	12 ± 1	11 ± 1
F4	18 ± 2	13 ± 1	23 ± 2

The broader enzyme spectrum displayed by isolates B7 and F4 suggests enhanced metabolic versatility, which is advantageous for treatment of chemically complex industrial effluents.

3.5 Biochemical characterization

Biochemical profiling of selected bacterial isolates revealed clear physiological distinctions. Isolates B7 and B15 were Gram-positive, rod-shaped, catalase-positive, and capable of hydrolyzing starch, indicating strong similarity to members of the genus *Bacillus*. These isolates also demonstrated nitrate reduction and citrate utilization, reflecting their respiratory flexibility and adaptability to nutrient-rich environments.

Isolate B1 was identified as Gram-negative and oxidase-positive, with positive citrate utilization and glucose oxidation, suggesting affiliation with the genus *Pseudomonas*. Such traits are consistent with aerobic, metabolically versatile bacteria known for their role in biodegradation of industrial pollutants.

Fungal isolates exhibited fast-growing, septate mycelia with abundant conidiation. Microscopic examination revealed globose to radiate conidial heads and unbranched conidiophores in one isolate, characteristic of *Aspergillus*, while brush-like conidiophore structures with chains of conidia in another isolate were indicative of *Penicillium*. These genera are widely recognized for their extracellular enzyme secretion and resilience under stressful environmental conditions.

3.6 Effluent treatment efficiency

The biodegradation potential of selected isolates was further evaluated through laboratory-scale effluent treatment experiments. All tested microorganisms achieved measurable reductions in organic load; however, treatment efficiency varied among isolates. Bacterial isolate B7 achieved the highest pollutant removal, demonstrating substantial reductions in both BOD and COD after 7 days of incubation.

Fungal isolate F4 also exhibited notable treatment performance, particularly in COD reduction, likely due to its strong ligninolytic enzyme activity. Isolate B15 showed moderate removal efficiency, reflecting its narrower enzymatic profile compared to B7 and F4.

Table 4 presents the percentage reduction in BOD and COD following microbial treatment. The COD/BOD reduction trend suggests that isolates with higher ligninolytic activity were more effective in degrading recalcitrant organic compounds.

Table 4. Reduction of organic load after microbial treatment

Isolate	BOD reduction (%)	COD reduction (%)
B7	61 ± 3	55 ± 2
B15	48 ± 2	43 ± 2

F4	57 ± 3	51 ± 2
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The observed reduction in organic parameters confirms the biodegradation potential of effluent-derived microorganisms. In particular, isolate B7 demonstrated superior treatment efficiency, highlighting its suitability for further development in biological wastewater treatment applications.

4. DISCUSSION

The present investigation demonstrates a clear functional relationship between extracellular enzyme production and the efficiency of organic load reduction in pulp and paper industry effluent. Wastewater originating from this sector contains a complex mixture of polymeric carbohydrates and aromatic compounds that require coordinated enzymatic action for effective biodegradation (Pokhrel & Viraraghavan, 2004; Bajpai, 2018). The elevated biochemical oxygen demand (BOD) and chemical oxygen demand (COD) observed in untreated effluent reflect the dominance of both readily biodegradable substrates and recalcitrant fractions, particularly lignin-derived molecules (Hubbe et al., 2016).

Isolates exhibiting broad enzymatic spectra demonstrated superior treatment performance, emphasizing the importance of metabolic versatility in industrial wastewater remediation (Ashrafi et al., 2015). Among the tested microorganisms, isolate B7 showed the highest reductions in BOD and COD, which corresponded with its pronounced cellulolytic, amylolytic, and ligninolytic activities. Cellulases and amylases contribute to the hydrolysis of structural polysaccharides and starch residues, releasing soluble sugars that can be rapidly assimilated and mineralized (Prescott et al., 2017). This enzymatic conversion directly supports microbial growth while simultaneously lowering biodegradable organic content in the effluent. Ligninolytic enzymes play a particularly critical role in pulp and paper wastewater treatment, as lignin and its derivatives are major contributors to effluent color, toxicity, and chemical oxygen demand (Leonowicz et al., 2001; Madlala & Whiteley, 2020). The strong ligninolytic activity observed in isolates B7 and F4 explains their enhanced COD reduction efficiency, as oxidative depolymerization of aromatic structures facilitates subsequent microbial metabolism (Sharma et al., 2014). Such oxidative enzymes, including laccases and peroxidases, are known to initiate cleavage of

complex phenolic bonds that are otherwise resistant to conventional biological treatment (Vidotti et al., 2011). The comparatively high performance of isolate B7, tentatively affiliated with the genus *Bacillus*, is consistent with earlier reports describing the resilience and enzyme-producing capacity of *Bacillus* species in industrial effluents (Kumar et al., 2020). Members of this genus are known for rapid growth, endospore formation, and secretion of extracellular hydrolases, attributes that confer stability under fluctuating environmental conditions (Cappuccino & Sherman, 2014). These traits likely contributed to sustained biodegradation activity throughout the treatment period. Fungal isolate F4, identified as belonging to the genus *Aspergillus*, also demonstrated substantial pollutant removal, particularly with respect to COD. Filamentous fungi possess an extensive hyphal network that enhances substrate contact and enzyme diffusion within complex wastewater matrices (Leonowicz et al., 2001). Moreover, fungal ligninolytic systems are highly effective in transforming high-molecular-weight aromatic compounds, supporting the observed reduction in recalcitrant organic load (Sharma et al., 2014).

In contrast, isolates exhibiting narrower enzymatic profiles showed moderate treatment efficiency, highlighting that single-enzyme dominance is insufficient for comprehensive effluent detoxification (Kamali & Khodaparast, 2015). Effective biological treatment of pulp and paper wastewater requires synergistic enzymatic pathways capable of degrading both carbohydrate-based and aromatic constituents (Hubbe et al., 2016). The COD/BOD reduction patterns observed in this study further support the role of ligninolytic activity in addressing slowly degradable organic fractions.

The strong association between enzyme production and treatment efficiency underscores the importance of selecting indigenous microorganisms for bioremediation applications (Kumar et al., 2020). Native strains are inherently adapted to site-specific stressors, including alkaline pH, elevated salinity, and inhibitory compounds, enabling superior performance compared to non-native inoculants (Ashrafi et al., 2015). Harnessing such organisms may reduce reliance on costly physicochemical processes and improve sustainability of effluent treatment systems.

Overall, the findings indicate that microorganisms capable of producing multiple extracellular enzymes, particularly those involved in lignin and cellulose

degradation, are well suited for application in pulp and paper industry wastewater treatment. Integration of such isolates into biological treatment frameworks could enhance pollutant removal efficiency while minimizing operational complexity and environmental impact.

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