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Research Article

Optimizing Coconut Coir Pith as a Substrate for Kraft Lignin Production: Insights into the Lignin-Degrading Potential of *Pusillimonas noertemannii*

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Abstract
Coconut coir pith (CCP), a lignocellulosic waste from the coconut industry, offers opportunities for environmental management through kraft lignin (KL) production and the isolation of lignin-degrading bacteria. This study evaluated KL, obtained via alkaline pretreatment of CCP, as a substrate for bacterial growth and lignin degradation. Bacterial isolates were cultured in mineral salt medium (MSM) supplemented with KL, revealing laccase activity indicative of effective lignin degradation. The effects of KL concentrations (1000 to 16000 mg/L) on bacterial growth and degradation efficiency were assessed. Moderate KL levels (2000 mg/L) significantly enhanced bacterial growth and achieved a 92.03% degradation rate, while higher concentrations diminished efficiency, suggesting an inverse relationship.
Color reduction of up to 94.12% was noted at 4000 mg/L, indicating strong decolorization capabilities. Molecular characterization identified the most promising isolate as *Pusillimonas noertemannii*, demonstrating its efficacy in degrading complex lignin structures and facilitating the recovery of valuable byproducts. The study highlights the importance of optimal KL concentrations for maximizing microbial performance. This research confirms the potential of CCP for KL production and positions *P. noertemannii* as a viable candidate for biotechnological lignin biodegradation applications, contributing to sustainable waste management and environmental conservation.

Key words: coir pith**,** kraft lignin, Bio degradation, *Pusillimonas noertemannii*, Waste management

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Introduction

Coir pith is residue remainders getting from coir fiber is extracted from the coconut husk it has consisted of lignin, hemicellulose & cellulose The product contained is largely lignin which fabricated for slow decomposition and give chances of waste disposal and sustainable environment. This plant fiber differs from other plant fiber in different ways in that; lignin content of alkali treated summers plant fiber is of about 37-42%, cellulose content of about 27-36% and hemicellulose that is of about 17-23% [1]. It is a phenolic polymer mostly found in cell walls of higher plants which is comprises of phenyl propane units of coumaryl, guaiacyl and syringyl units which are β-aryl ether coupled to each other and is responsible for about 35% of coir pith. It is associated with the stiffness of the coir and is inherent in the fiber. The manufacture of carbohydrates is believed to be responsible for lignin production [2]. The technical lignin, generated by methods for collecting the carbohydrates from lignocellulose via pre-treatment are categorized into physical, chemical and the ones which are both physical and chemical in nature. They include kraft, lignosulfonate, soda, organosolv and hydrolyzed lignin processes which involving sodium sulfide (Na2S), sodium hydroxide (NaOH), at temperatures of 150-180°C to cleave lignin's ether bonds [3]. Compared to the lignin obtained from this particular kind of alkaline pretreatment, the resulting lignin possess various characteristics in terms of molecular weight, polydispersity, and chemical composition and structure, purity, as well as others [4,5]. Kraft lignin (KL) is high in polyphenols as a result of defibring, lengthy retting in salt water, or its discharge from paper and pulping generation [6,7], that often cause toxic compounds to seepage into the hubs of disposal [8]. The current research seeks to determine the possibility of utilizing KL in the degradation of coir pith toxicity for environmental conservation. Some of the fungi involved in the degradation of lignin are *Trametes versicolor*[9],*Serpulalacrymans*, *Gloephyllum trabeum* and *Pleurotussp*[10] and these fungi are reported to cause brown and soft rot. Earlier, researchers tended to think that bacteria played a minor and an ambiguous role in the degradation of lignin. Nevertheless, research carried out in the recent past has shown that different species of bacteria are able to break down lignin through attacking its subunits [11]. Delignification has been reported to be achieved with either individual bacterial species and mixed cultures [12,13]. But bacteria have several advantages compared to fungi: high speed of proliferation, the formation of complexes of enzymes multi-functional, better adaptation to extreme conditions in the environment [14,15]. these microorganisms are the vital sources of the valuable metabolites as well as the enzymes; besides, they create the basis of the genetic flexibility that might contribute to the biotechnological uses. Significantly, actinomycetes are home to many mundane genera with unutilized prospect in biotechnology and industry [16]. *Aeromonas, Flavobacterium, Pseudomonas, Bacillus, Alcaligenes,* and *Nocardia* are identified as agents that degrade lignin [17]. To establish effective and permanent strategies for the use of bio-resources in biotechnology it is important to know more about the variety of structures and the properties of bacteria degrading lignin. Lignin is broken down by microbial enzymes with special focus on bacterial and fungal lignolytic enzymes; including lignin peroxidases, manganese peroxidases, and laccases. This enzyme has displayed excellent ligninolytic property in rainforest soil, rumen, and termite gut [18]. For the present investigation, coir pith samples were sourced from dumping sites of the region; they were subjected to chemical treatment to get KL and supplemented with alkaline lignin as the sole carbon source. Lignin degradation potential of bacterial isolates were determined by biochemical assays and molecular identification by gene sequencing of 16S rRNA gene. The obtained bacterial isolates were further investigated for their ability to produce the laccase, lignin peroxidase, and manganese peroxidase enzymes.

Materials and Methods

Coirpith sample collection, culture enrichment and alkaline pretreatment.

A sample of coir pith (CP) was collected from a dumping site in Tumkur, Karnataka, India (13.349437N 77.103471E). The sample was placed in sterile polythene bags and stored at 4°C for subsequent analysis. A modified protocol was followed to gel KL from CP [19]. Briefly, 10 grams of coir pith were powdered and treated with 5 ml of 1% sulfuric acid. This mixture was then heated in a hot air oven at 80°C for 30 minutes, allowed to cool, and treated with 100 ml of 4% sodium hydroxide before boiling for 30 minutes. The resulting filtrate was autoclaved and kept in an airtight container.The lignolytic bacteria were isolated from the dumped raw coir pith material with enrichment method [20]. Briefly, 5 grams of coir pith were inoculated into a lignin Minimal Salt Media-Lignin (MSM-L), which was prepared with 4.44 grams of K2HPO4, 0.533 grams of KH2PO4, 0.5 grams of MgSO4, 5 grams of NH4NO3, 0.5 grams of peptone, and 1% kraft lignin in 250 ml Erlenmeyer flasks. These flasks were incubated in the dark at 35°C for 7 days on a rotary shaker set at 120 rpm. To isolate pure cultures, serial dilutions were performed and plated on MSM-L agar plates.

Bacterial isolation and Biochemical studies

The lignolytic capabilities of bacterial isolates from pure cultures were evaluated by observing decolorisation zones on MSML agar plates and the decolorisation of MSML solution [21].

Molecular characterization of bacteria

The DNA from bacteria was isolated by the lysis method [22]. The 16S rRNA gene sequencing was performed using a reaction mixture containing 1 µl of DNA, 20 µl of reaction buffer (Kappa SA), 3 mM MgCl2, a dNTP mix, 0.25 mMTaq DNA polymerase, 0.05 U of primer 1 picomol, and 50 ng of template DNA. Sterile nuclease-free water was used as a negative control. The PCR amplification involved 35 cycles: denaturation at 94°C for 1 minute, annealing at 57°C for 40 seconds, and extension at 72°C for 2 minutes. The primers used were forward primer (16sFP) 5'-AGAGTTTGATCCTGGCTCAG-3' and reverse primer (16sRP) 5'-AAGGAGGTGATCCAGCCGCA-3', amplifying a 1500 bp fragment. The sequence data obtained were analyzed phylogenetically using the Basic Local Alignment Search Tool (BLAST) to compare with previously reported sequences. The sequence was deposited in the GenBank Database with accession number PP528199.1

Lignolytic Enzyme Activity

Laccase activity

The ABTS (2-2’-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)) assay for measuring laccase activity was used in this study [23] which involves mixing 100 µl of culture supernatant with 2.8 ml of 0.1 mM sodium acetate buffer (pH 4.5) and 0.5 mM ABTS. After the mixture is incubated for five minutes, absorbance is recorded at 420 nm using a spectrophotometer, with a suitable blank as reference. Laccase activity is expressed in units, where one unit corresponds to the oxidation of one micromole of ABTS per minute.

Lignolytic activity

Samples from bacterial isolates were analyzed for lignin degradation, total substrate reduction, and color change [24]. The growth of bacterial cells in liquid media was monitored at 620 nm using various concentrations of KL, including 2%, 4%, 6%, and 8%. To assess color reduction and residual KL, samples were centrifuged at 8000g for 30 minutes. Absorbance measurements were taken at 465 nm for color change and at 280 nm for lignin degradation, after diluting 1 ml of the supernatant with 3 ml of phosphate buffer (pH 7.6) using a UV-visible spectrophotometer. To determine the total substrate loss, biomass-free samples were

Results and Discussion

Coconut coir pith utilization and KL production

Coconut coir pith (CCP) is a lignocellulosic waste from the coconut industry that hasn't gotten much attention [25]. The necessity for KL degradation by bacteria stems from various industrial and environmental concerns regarding

acidified to a pH of 1-2 with concentrated H_2SO_4. The resulting precipitate was collected, dried in centrifuge tubes at 50–60°C for 48 hours, and weighed after centrifugation at 8000g for 30 minutes.

the lignin-resistant byproduct of kraft pulping [26]. Because microorganisms can break down this complex material into less hazardous forms or possible commodities, they can play a significant role in waste treatment and pollution reduction[27].In this study the CCP is used as raw material for the degradation study.Coir pith pretreated with NaOH produces a dark brown liquid known as black liquor or KL(Figure 1).

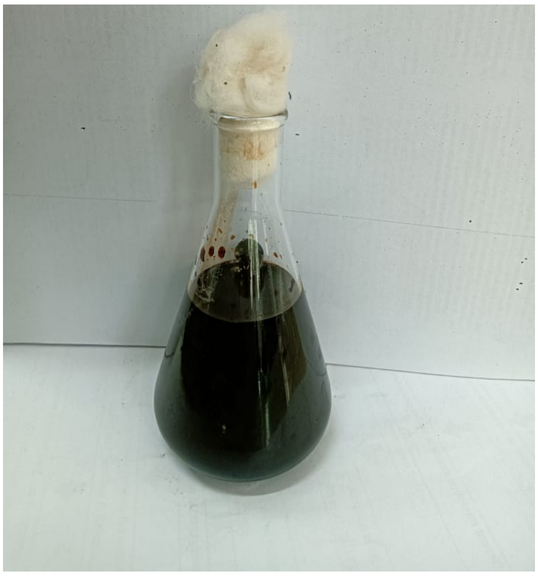

Figure 1-Crude Kraft lignin extracted from raw coir pith

which is rich in polyphenols and lignin monomers [7,28]. Kraft lignin is essentially a polyphenolic compound with a high thermal decomposition temperature, characterized by various aromatic functional groups, including hydroxyl (phenolic or alkyl), conjugated double bonds, methoxyl, and sulfonate groups [29]. These phenolic structures have notable radical scavenging properties due to the resonance stabilization of the phenoxy radical induced by the aromatic nucleus [30].

Isolation of lignin degrading bacteria in MSML

In the study, KL was used as a carbon source in MSML media to maximize the number of bacterial isolates with lignolytic capabilities and their ability to decolorize KL(Figure 2).

Figure 2- Bacterial growth on MSM-L media

After incubating the isolates at 35°C with agitation at 120 rpm in dark conditions for 7 days, bacterial was obtained.Initial biochemical classification was carried out with bacterial isolate obtained from the MSML media (Table 1).

Table 1 – Biochemical test of the bacterial isolates

Sl no	Bacterial isolates	Gram stain	Motility	Indole	Methyl red test	Voger-proskauer	Citrate	Cata lase	Oxidase	Urease
1	L001	-	+	+	+	-	-	+	-	-
2	L002	-	+	-	-	-	+	+	-	-
3	L003	-	+	-	-	-	+	+	-	+
4	L004	-	-	-	-	-	+	+	-	-
5	L005	-	+	+	+	+	+	+	+	-
6	L006	-	+	-	-	+	+	+	-	-
7	L007	-	+	-	-	-	+	+	+	-
8	L008	-	+	+	+	-	-	+	-	+
9	L009	+	-	-	-	-	-	+	+	-
10	L010	-	-	+	+	+	-	+	+	-

The analysis of bacterial isolates for their lignin-degrading capabilities revealed distinct variations in enzyme activities and efficiency in lignin degradation, placing it in the middle range of effectiveness [31]. In the assessment of bacterial isolates grown in mineral salt medium (MSM) supplemented with KL, the presence of laccase activity was evaluated. The results demonstrated that the bacterial isolates exhibited laccase positivity, indicating their capability to produce this enzyme in the lignin-rich environment (Figure 3A and 3B).

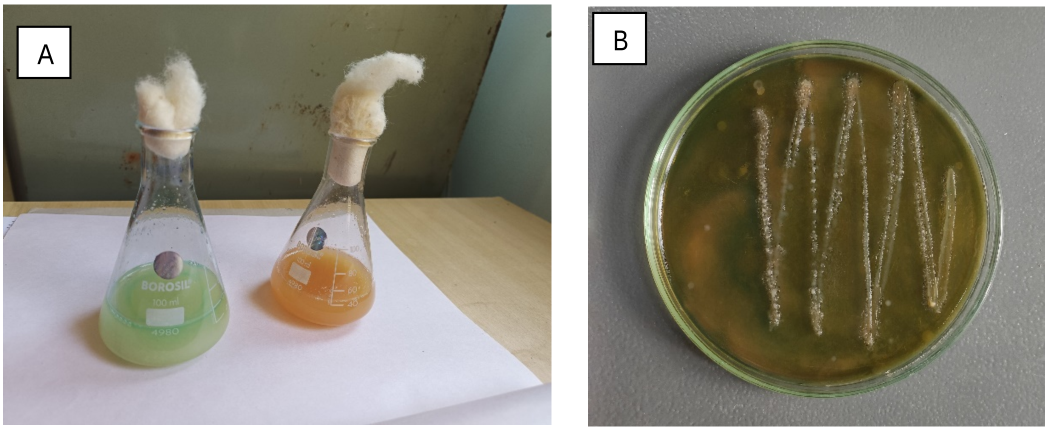

Figure 3-A and B, Laccase positive on MSML broth and Agar plate

This enzymatic activity was confirmed through a colorimetric assay, which revealed a distinct color change associated with laccase-mediated oxidation of substrate compounds in the media [32]. The presence of laccase in these isolates suggested that they could effectively participate in the biodegradation of lignin, facilitating the breakdown of its complex phenolic structures[33].

Effects of KL on bacterial growth

In a study examining the effects of Kraft lignin concentration on cell growth, measured at an optical density of 600 nm (O.D. 600), various concentrations were evaluated (Figure 4). At 1000 mg/L, cell growth was recorded at 31%, reflecting a modest increase in response to lignin. When the concentration was raised to 2000 mg/L, cell growth increased to 40%, indicating a beneficial effect of the higher lignin levels. However, at 4000 mg/L, cell growth stabilized at 40%, suggesting that this concentration did not provide any additional growth benefits, likely due to factors such as toxicity or nutrient limitations[34].

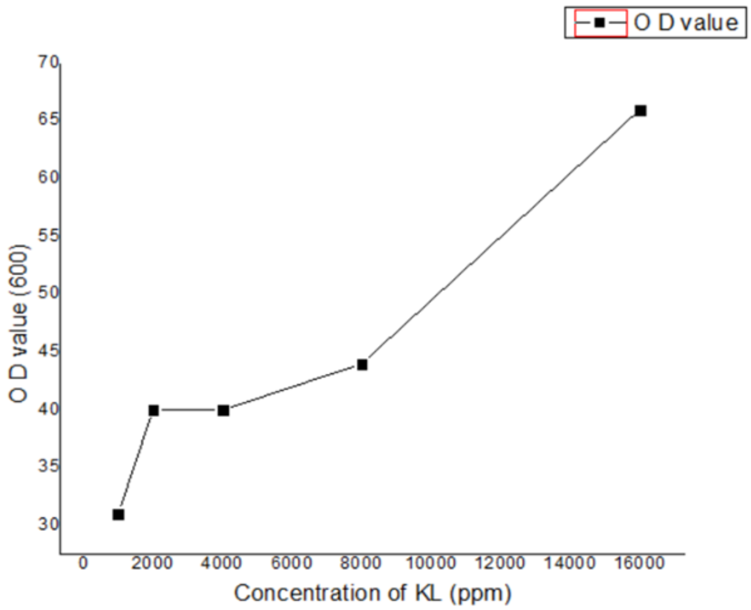

Figure 4. Effects of different concentration of KL on bacterial growth

A further increase to 8000 mg/L led to a slight enhancement in cell growth to 44%, suggesting that elevated concentrations could still foster growth. Ultimately, at 16000 mg/L, the most significant growth was observed, with cell growth reaching 66%. This finding illustrated a complex relationship between lignin concentration and cell growth, highlighting both positive and negative effects at the various tested levels[35].The findings of this study shed light on the relationship between KL concentration and cell growth, as measured by optical density at 600 nm. Initially, the modest growth observed at 1000 mg/L suggests that low lignin concentrations can be advantageous, likely by providing essential nutrients or enhancing metabolic processes. The notable increase to 40% growth at 2000 mg/L further supports the idea that moderate lignin levels positively affect cellular functions.However, the plateau seen at 4000 mg/L, where growth remained at 40%, raises significant questions. This stabilization indicates that higher concentrations of lignin may reach a point where growth ceases to improve, potentially due to toxicity or a lack of other necessary nutrients [36]. This observation underscores the importance of balancing lignin concentrations in scenarios where it is used as a growth enhancer.The slight increase to 44% growth at 8000 mg/L suggests that, despite the earlier plateau, there may be conditions under which higher concentrations can still foster growth, possibly reflecting an adaptive response of the cells to increased lignin levels. The most pronounced growth at 16000 mg/L, reaching 66%, underscores the complexity of this relationship, implying that at elevated concentrations, specific mechanisms may enable cells to effectively utilize lignin, thus overcoming previous inhibitory effects. these results demonstrate a complex interaction between Kraft lignin concentration and cell growth, revealing both positive and negative influences depending on the concentration range [37].

Impact of KL on degradation efficiency of bacteria

In this study assessing the effects of Kraft lignin concentration on degradation, various concentrations were analyzed, and the results were measured at an optical density of 280 nm (O.D. 280). At a concentration of 1000 mg/L, the optical density was recorded at 0.41, corresponding to a degradation percentage of 71.21% (Figure 5).

This indicated a relatively high level of degradation, suggesting that lower concentrations of lignin facilitated effective breakdown [38]. When the concentration increased to 2000 mg/L, the O.D. dropped significantly to 0.122, reflecting a remarkable degradation rate of 92.03%. This finding demonstrated that moderate lignin concentrations could significantly enhance the degradation process. However, at 4000 mg/L, the O.D. increased again to 0.376, resulting in a degradation percentage of 75.45%. This decrease in degradation efficiency indicated that higher lignin concentrations may hinder the degradation process, possibly due to factors such as the formation of more complex lignin structures that are less accessible for breakdown. As the concentration further increased to 8000 mg/L, the O.D. rose to 0.548, with a degradation percentage of 64.22%, highlighting a continued decline in degradation efficiency. At the highest concentration of 16000

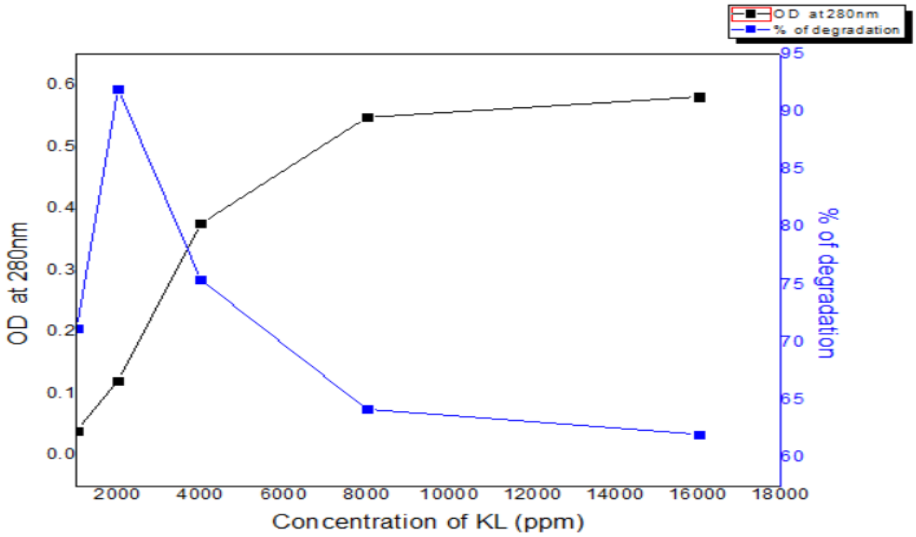

Figure 5. Effects of different concentration of KL on bacterial degradation

mg/L, the O.D. reached 0.581, and the degradation percentage fell to 62.07%. This finding suggested a clear inverse relationship between lignin concentration and degradation efficiency, with optimal degradation occurring at moderate concentrations [39]. The findings emphasized the necessity of balancing lignin concentrations to achieve maximum degradation effectiveness. The observed decline in degradation rates at higher concentrations likely pointed to the challenges posed by lignin's complex structure, which could limit its breakdown[40].

Kraft Lignin Concentration Effects on Color Reduction by the bacteria

In the evaluation of Kraft lignin's impact on color reduction, various concentrations were tested, with optical density measured at 465 nm (O.D. 465). At a concentration of 1000 mg/L, the recorded optical density was 0.49, corresponding to a color reduction of 74.07%. This significant reduction suggested that lower lignin concentrations effectively facilitated decolorization, likely by breaking down color-producing compounds in the solution. However, increasing the concentration to 2000 mg/L led to a rise in O.D. to 0.7, which resulted in a decreased color reduction of 62.96% (Figure 6).

Figure 6. Decolorisation of media with different concentration of Kraft lignin

This decline implied that higher lignin concentrations might inhibit the color removal process, contrary to initial expectations. At 4000 mg/L, a significant change occurred, with the optical density dropping sharply to 0.111, yielding an impressive color reduction of 94.12%. This substantial improvement indicated that a moderate increase in lignin concentration significantly enhanced the effectiveness of color removal, likely due to optimal interactions between lignin and the colorant molecules. In contrast, at 8000 mg/L, the O.D. slightly increased to 0.213, with a color reduction of 88.73%. This finding suggested that while the lignin concentration remained effective, the efficiency of color removal was starting to stabilize and slightly decline. At the highest concentration of 16000 mg/L, the O.D. remained consistent at 0.211, and the color reduction percentage was 88.83% (Figure 7).

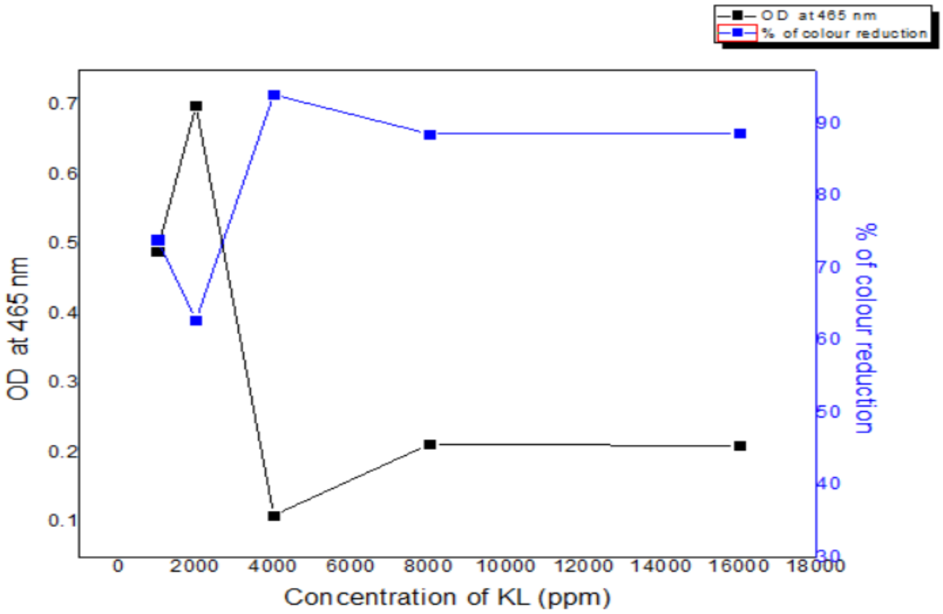

Figure 7. Effects of different concentration of KL on decolorisation

This stability indicated that color removal efficiency had plateaued, showing that additional lignin did not significantly enhance decolorization. Overall, these findings highlighted a complex relationship between Kraft lignin concentration and color reduction. Initially, lower concentrations showed effective color removal, but the impact varied significantly with increased concentration. The optimal performance at 4000 mg/L suggested a potential threshold for effective color removal, beyond which further increases in lignin did not result in notable improvements[41]. This study underscores the importance of carefully considering lignin concentrations in practical applications focused on color removal.

Molecular identification of bacteria

Bacterial isolate L008 was further analyzed for 16sRNA gene sequencing and phylogenetic analysis it was identified as *Pusillimonas noertemannii3-5-1*. It was deposited in the NCBI data bank with ID PP528199.1. In this study, *P. noertemannii* has displayed potential in breaking down KL, a complicated and resistant byproduct of the kraft pulping procedure in the paper sector. KL from CCP, known for its complex aromatic composition and durability against decay, poses a notable environmental issue when discarded as a form of industrial waste. This study confirmed that *P. noertemannii* has the ability to efficiently break down KL by utilizing its enzymatic properties, leading to the conversion of the lignin into easier to handle compounds. Previous research reported that [41] isolated this bacterium very first time from waste water surface and hypothesized that this bacterium could perform biodegradation. Our study confirmed that this bacterium could degrade the bio-complex material into simpler one. Earlier [43] it was reported that *P. noertemannii* gen. nov.sps isolated from the river water was identified as a new member of the *Alcaligenaceae* family, also the study demonstrated the capability to degrade substituted salicylates. In this study, *P. noertemannii* very first time proved to be a potential bacterium to degrade the KL from coir pith. The enzymes produced by the bacteria help break down lignin's complex phenolic structures, potentially leading to a decrease in lignin waste volume and the chance to retrieve valuable byproducts[44]. As a result, *P. noertemannii* presents a feasible biological method for breaking down KL, promoting sustainable waste disposal methods and potentially aiding in the creation of valuable products from industrial waste.

Conclusion

This study highlights the significant potential of utilizing CCP as a substrate for the production of KL and the subsequent isolation of lignin-degrading bacteria, specifically *P. noertemannii*. The findings revealed a complex relationship between KL concentration and its effects on bacterial growth, degradation efficiency, and color reduction. Moderate concentrations of lignin enhanced both degradation rates and color removal effectiveness, while higher concentrations led to diminished results, suggesting a threshold for optimal lignin utilization [45]. The enzymatic capabilities of *P. noertemannii*, confirmed through 16S rRNA sequencing, demonstrate its potential for effective lignin biodegradation, offering a promising biological approach to managing lignin waste in an environmentally sustainable manner. Overall, these results underscore the importance of optimizing lignin concentrations and leveraging microbial properties for effective waste treatment and resource recovery in industrial applications.

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Conflict of Interest

None to declare

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