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Bioremediation of Kraft Lignin in Wastewater: Optimization of Temperature and Isolation of Valuable Compounds

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ABSTRACT

Conventional methods for the treatment of complex lignin found in the pulp and paper mill effluent is a strenuous and expensive process. Microbial degradation is a promising technique to degrade lignin efficiently due to its adaptability and rapid growth of microbes in diverse environments. This study is focused on the isolation of potential ligninolytic bacterial strains from agricultural soil and decomposed wood sources for successful degradation of lignin present in the wastewater. Initially, 15 ligninolytic strains were identified and three dominant species, *Staphylococcus lentus* (SB5), *Bacillus megaterium* (RWB15) and *Pseudomonas geniculata* (RWP9) were isolated as per their growth tolerance pattern with different lignin concentrations. The lignin degradation study was carried out at different temperatures (25-45°C) with 1000 mg/l of feed lignin concentrations. The optimum degradation temperature of all the strains fell within the range 30-35°C. The maximum lignin degradation of SB5, RWB15 and RWP9 was identified as 89, 77 and 90% at the end of the 6th, 3rd and 7th day of biodegradation, respectively. There was a steep reduction of COD by all three microbes before attaining the stationary phase and thereafter COD reduction was sluggish due to the death phase. Besides, the microbes used in this study showed the transformation of lignin into valuable low-molecular by-products, vanillin, vanillic acid and adipic acid with their concentration being quantified as 28, 101 and 130 mg/l, respectively. This study shows the successful degradation of waste lignin and the recovery of valuable byproducts from waste streams.

Keywords: Biodegradation; COD; Lignin; Ligninolytic strains; Valorization.

1. INTRODUCTION

Pulp and paper mills are the major lignin-producing sectors and release annually 50 to 70 million tons of lignin (Bruijnincx *et al*. 2015; Xu *et al.* 2018; Haq *et al*. 2020). Lignin is mostly discharged as a waste by-product in the effluent during paper production. Globally, pulp and paper mills utilize 906 million m^3 of water and generate 696 million m^3 of dark brown effluent with lignin (Haq *et al*. 2020). The discharge of huge quantities of lignin and its derivatives in the water bodies creates aesthetic issues and causes the death of aquatic animals and plants. Hence, the lignin from the industrial effluent must be appropriately treated (or) removed with efficient techniques. The lignin-bearing wastewater is effectively treated by various physicochemical methods. However, these methods are uneconomical and produce more residues during the treatment process (Haq *et al*. 2020). Also, the lignin sludge produced from the conventional treatment methods is used as fuel or discarded by a landfilling process causing environmental pollution and resource depletion. Biodegradation can be an alternative and affordable method to handle the huge quantity of lignin-containing effluent. Biodegradation of lignin can be achieved by different fungi, yeast and bacteria. There are many studies reported on fungi degradation, specifically with white-rot and brown-rot fungi. Wong (2009) reported that fungi employ extracellular oxidative mechanisms to break down the complex lignin polymers. *Phaenerochaete, Ganoderma* spp. and white-rot fungi contain lignin-degrading enzymes (Leonowicz *et al.* 2001; Bugg *et al.* 2011). The mixed culture of *Trichosporn* yeasts with *Arthrobacter*, *Chromobacterium* genera and *Pseudomonas* bacterial species has been reported for 50% degradation of calcium lignosulfonate within 24 hours (Asina *et al.* 2017). However, the variations in the physicochemical parameters such as pH, electron donating groups, lignin concentration, carbon and nitrogen supplementation can significantly affect the degradation performance of lignin.

Among various biodegradation techniques, bacterial degradation has emerged as a promising method due to the adaptability, rapid growth and diverse metabolic capabilities of bacterial strains (Haq *et al.*2021; Blasi *et al.* 2023). *Streptomyces viridosporus*, *Pseudomonas putida, Bacillus subtilis*, *Rhodococcus jostii* and *Comamonas testosteroni* are some of the notable bacterial strains reported with lignin-degrading capabilities (Ramachandra *et al.* 1988; Zimmermann 1990; Masai *et al.* 2007; Ahmad *et al.* 2011). *Bacillus* sp.

LD003 has significant ligninolytic potential for decolourization of ligninolytic indicator dyes. *Pseudomonas putida* KT2440, a genetically modified bacterial species, is used to enhance the production of specific products from aromatic compounds. *Sphingomonas paucimobilis* SYK-6 is another strain that exhibited the capability to metabolize β-aryl ether lignin dimer compounds by specific enzymes (Asina *et al.* 2017). The identification of efficient ligninolytic strains offers several advantages for biotechnological applications including scalable growth, convenience for molecular genetics and possible isolation of thermophilic strains (Hasan *et al.* 2006). Naturally, the bacteria with lignin degradation ability can be identified in various environments like compost, agricultural and rainforest soil and eroded bamboo slips. Lignin-degrading bacteria are also identified in the paper mill sludge and intestines of wood-feeding insects (Mathews *et al.* 2016; Xu *et al.* 2018). The ligninolytic strains also transform waste lignin into low-molecular-weight value-added products during the valorization process. However, in this context, merely a few studies have reported the formation of valuable products. Hence, there is a need for extensive studies to identify the specific conditions to produce the specific bioproducts. The broad spectrum of such studies may harness lignin-degrading bacteria for both environmental remediation and industrial applications.

This study specifically focuses on the isolation and characterisation of ligninolytic bacterial strains that can efficiently degrade lignin and simultaneously produce value-added compounds like vanillin, vanillic acid and adipic acid during the valorization. The optimization of process temperature and feed concentration has been carried out, which is essential for efficient degradation of complex lignin molecules. Biochemical characterization of bacterial strains was done to identify the potential ligninolytic strains. The quantification of valorized compounds was also performed to estimate the concentration of bioproducts formed during the degradation process.

2. MATERIALS AND METHODS

2.1 Chemicals

In this study, the low sulfonate alkali kraft lignin (KL) is used as lignin source. It was procured from Sigma Aldrich USA and HPLC-grade solvents were purchased from Merck (India). All the media and other solutions were prepared using deionized/double-distilled water. The modified Luria-Bertani (LB) media ($NaNO_3$, KH_2PO_4, $MgSO_4$, NaCl and $CaCl_2$) in the presence of kraft lignin was used for the isolation of ligninolytic microbes. The pH of the media was adjusted to 7. Agar powder (15 g/l) was added to solidify the media for plating purpose. All the chemicals and reagents used in this study were of analytical grade.

2.2 Screening, Purification, and Identification of Prominent Bacterial Strains with Ligninolytic Capabilities

Here, the focus is to identify the potential lignin-degrading bacterial strains from the soil source for efficient degradation of lignin by using nutrition enrichment technique. Soil samples were collected from diverse environments including agricultural soil (S1), decayed wood (S2) and a combination of decayed wood and soil (S3). Subsequently, 5 g of each sample was added into a 100 ml fed-batch shaker system, and the experiment was conducted in triplicate. Seed cultures from all three soil samples were cultivated and prepared in 1-litre LB media and 1 ml of the sample was added to each flask for incubation at 30°C and shaken at a speed of 150 rpm for 48 hours. Subsequently, serial dilutions were performed with S1, S2 and S3. The diluted solution was spread onto modified LB agar plates containing 100 mg/l of kraft lignin. Phenotypically distinct colonies were isolated and marked for further analysis. The isolated single colonies underwent a series of three consecutive restreaking process to ensure the acquisition of a homogeneous colony for both strain identification and the characterisation of ligninolytic activity.

2.3 Bacterial Growth and Identification of Dominant Strain

In the KL nutrition enrichment technique, the concentration of KL in LB agar plates was increased from 100 to 1000 mg/l followed by inoculation. The plates were incubated at 30°C and the biochemical characterization of the selected bacterial strains was carried out. The strain which has rapid growth with increased lignin input was identified as the predominant strain.

2.4 Plate Assays for Ligninolytic Enzyme Activity

The degradation of lignin is significantly influenced by ligninolytic enzymes. To evaluate the ligninolytic enzyme activity of the identified bacterial strains, the strains were inoculated in a dye decolourization plate assay system. Phenol red manganese peroxidase (MnP) and azure B lignin peroxidase (LiP) along with guaiacol for laccase activity (LA) were used as substitutes or analogues for lignin assessment. These compounds served as mimicking agents with lignin to identify the respective enzymes. The reduction of colour in the plates indicated both bacterial growth and ligninolytic enzyme activity. The plate assay method was performed by using inoculation of a 1% culture and pre-cultured for 24 hours in a dye agar plate containing specific components. Subsequently, the plates were incubated at 30 °C for 168 hours and monitored daily to observe the sample decolourization for assessing

the growth and ligninolytic enzyme activity (Chandra and Yadav, 2017).

2.5 Optimization of Temperature for Lignin Degradation

The optimization of bacterial growth temperature with respect to KL degradation was carried out. In a 250 ml Erlenmeyer flask, 100 ml of KL-LB broth was autoclaved for 20 minutes at 121 ℃ by maintaining a pH of 7.0±0.2 to avoid the cross contamination. The KL concentration was fixed as 1000 mg/l. After reaching the room temperature, the media was inoculated with 1% of a pre-cultured inoculum. For analysis, a triplicate setup was prepared and placed in an orbital incubating shaker (Make: REMI; Model: CIS-24 Plus) at 100 rpm. The incubation time spanned twelve days with varying temperatures ranging from 25 to 45℃ for all three different samples. The unseeded flasks were maintained as controls for daily setups. The samples collected were centrifuged at 10,000 rpm for 10 minutes followed by COD analysis with the help of closed reflux titrimetric method. The daily monitoring of COD reduction and bacterial growth indicate the amount of KL degradation.

2.6 Lignin Reduction Assay

The residual lignin content was assessed based on the methodology outlined by Chandra *et al*. (2007). The supernatant was collected, and the absorbance of the lignin solution was measured at 280 nm using a UV-visible spectrophotometer. The growth rate of the bacterial strains was assessed by measuring optical density of the sample at 600 nm by UV-visible spectrophotometry. The degradation efficiency (%) was calculated using the following formula:

$$Degradation\ efficiency(\%) = \frac{Initial\ absorbance - final\ absorbance}{Initial\ absorbance} \times 100$$

2.7 Metabolite Production and Analytical Techniques

The metabolites produced during the lignin degradation were analysed by Agilent 1260 Series HPLC system with a Poroshell 120 EC C-18 column and a 2487 UV/VIS-DAD detector. The quantification of vanillin, vanillic acid, and adipic acid was achieved by using distinct solvent systems and conditions. Vanillin was analyzed with acetonitrile and water (70:30, v/v) at a 0.8 ml/min flow rate at 280 nm. Vanillic acid was assessed by methanol and water (50:50, v/v) at 0.8 ml/min, with peak identification at 250 nm. Adipic acid was analysed with the help of 0.1% trifluoroacetic acid in water (Eluent A) and adipic acid-acetonitrile (Eluent B, 40:60, v/v) at 1 ml/min by identification of peak at 200 nm. The method was effective in efficient separation and detection of lignin metabolites, ensuring a reliable analysis of metabolite production.

3. RESULTS AND DISCUSSION

3.1 Isolation of Ligninolytic Bacteria and Identification of Dominant Strains

To isolate the ligninolytic bacteria from soil samples, three soil samples were collected and three different setups were made. In the first setup with agricultural soil (S1) sample, 15 distinct colonies were identified and isolated in which five strains show positive growth at less lignin concentration and these SB2, SB3, SB5, SB8 and SB11 were successfully isolated (Table 1), whereas SB2, SB3 and SB5, SB8 and SB11 emerged as standout strains due to the efficient degradation of KL when lignin concentration increased from 100 to 1000 mg/. These strains exhibit characteristics of *Staphylococcus* spp. Interestingly, SB5 showed the rapid utilization of KL as a carbon source and it also exhibited significant traits, including motility, aerobic behaviour, Gram-positive nature, and catalase activity.

Besides, in S2 soil sample (decomposed wood), another 15 ligninolytic isolates named RWB1 to RWB15 were obtained. Among these, RWB2, RWB4, RWB6, RWB7, RWB9, RWB11, RWB12 and RWB15 exhibited faster growth and were also capable for KL degradation with an increase in KL concentration from 100 mg/l to 1000 mg/l (Table 1). These isolates are also with similar characteristics of *Bacillus* spp. From the above strains, the RWB15 strain was considered superior for subsequent studies on lignin degradation. The pursuit for ligninolytic bacteria was also extended with sample 3 (S3), where 12 strains, RWP1 to RWP12 were isolated. The strain RWP5 emerged dominant with increasing KL concentration, and it was directly proportional to the growth of microbes in the KL atmosphere. At 1000 mg/l KL, RWP5 was identified and showed rapid KL degradation. This is aerobic, Gram-negative, motile by nature, contains rod-shaped chained bacterial cells and is positive for catalase activity. Further biochemical tests confirmed similarities with *Pseudomonas* spp. underscoring the potential of RWP5 as a key player for lignin degradation. The extensive literature survey depicted that the potential ligninolytes isolated and identified in this study may produce some of the valuable flavouring compounds, such as vanillin, vanillic acid, and adipic acid (Vardon *et al*. 2015; Asina *et al.* 2017; Zuo *et al.* 2022).

Table 1 outlines the growth tolerance patterns and conditions of different bacterial isolates in the presence of various KL concentrations. SB5 in Sample 1, RWB15 in Sample 2 and RWP5 in Sample 3 are the rapidly growing dominant strains at high concentrations of KL. This provides insights into the adaptability and

growth characteristics of each isolate under different KL conditions and provides the valuable information for understanding their response to lignin-related substrates. *Staphylococcus, Bacillus* and *Pseudomonas* show the adaptability to a wide range of environmental conditions which allow their survival across diverse ecosystems. Despite commonalities, each genus possesses unique characteristics. The individual species within each genus exhibit considerable variations in the physiology and ecological behaviour of the strains.

Table 1. Growth tolerance and conditions of various bacterial isolates with different KL concentrations

Bacterial Strain Source	Sample 1				Sample 2				Sample 3			
	Lignin concentration											
		100mg/l	500 mg/l	1000 mg/l		100 mg/l	500 mg/l	1000 mg/l		100 mg/l	500 mg/l	1000 mg/l
Name of the Bacterial Strains and Growth tolerance patterns at different KL concentrations in aerobic condition	SB1	−	−	−	RWB1	−	−	−	RWP1	+++	+++	++
	SB2	+++	++	++	RWB2	+++	+++	++	RWP2	+++	+++	++
	SB3	+++	++	++	RWB3	−	−	−	RWP3	−	−	−
	SB4	−	−	−	RWB4	+++	+++	++	RWP4	−	−	−
	SB5	+++	+++	+++	RWB5	−	−	−	RWP5	+++	+++	+++
	SB6	−	−	−	RWB6	++	+	−	RWP6	−	−	−
	SB7	−	−	−	RWB7	+	−	−	RWP7	−	−	−
	SB8	+	−	−	RWB8	−	−	−	RWP8	+++	++	+
	SB9	−	−	−	RWB9	+++	+++	+	RWP9	+++	++	++
	SB10	−	−	−	RWB10	−	−	−	RWP10	−	−	−
	SB11	+	−	−	RWB11	+++	++	++	RWP11	+	−	−
	SB12	−	−	−	RWB12	+	−	−	RWP12	+	−	−
	SB13	−	−	−	RWB13	−	−	−	−	−	−	−
	SB14	−	−	−	RWB14	−	−	−	−	−	−	−
	SB15	−	−	−	RWB15	+++	+++	+++	−	−	−	−

Note: *(-) No growth; (+) Slow growth; (++) Fast growth; (+++) Very fast growth*

Table 2. Biochemical profile of screened dominant bacterial strains with Kraft Lignin (KL)

Characterisation	Dominant strains from the sample 1,2 and 3		
	SB5	RWB15	RWP9
Cell Shape	*Road (arranged in clusters)*	*Road*	*Short Rod (arranged in chain)*
Gram stain	+*ve*	+*ve*	−*ve*
Growth in air (Aerobic)	+*ve*	+*ve*	+*ve*
Growth in KL	+++	+++	+++
Growth at 27ºC	++	+++	+++
Growth at 40ºC	++	++	+++
Anaerobic	−*ve*	−*ve*	−*ve*
Oxidase	−*ve*	+*ve*	+*ve*
Growth at NaCl 5%	+*ve*	+*ve*	+*ve*
Growth at pH 9−12	−*ve*	−*ve*	−*ve*
Growth at pH 4−8	+*ve*	+*ve*	+*ve*
Catalase	+*ve*	+*ve*	+*ve*
Gelatin Hydrolysis	*NA*	+*ve*	−*ve*
Nitrate Reduction	+*ve*	+*ve*	+*ve*
Citric acid utilization	+*ve*	+*ve*	+*ve*
Urease	+*ve*	+*ve*	+*ve*
Coagulase Test	−*ve*	*NA*	*NA*
Motility	+*ve*	+*ve*	+*ve*
Indole	−*ve*	−*ve*	−*ve*
Phenol degradation	+*ve*	+*ve*	+*ve*
Growth at agar plate	+++	+++	+++

***Note**: (G +ve) = Gram positive; (-) No growth; (+) Slow growth; (++) Fast growth; (+++) Very fast growth NA (Not Applicable)*

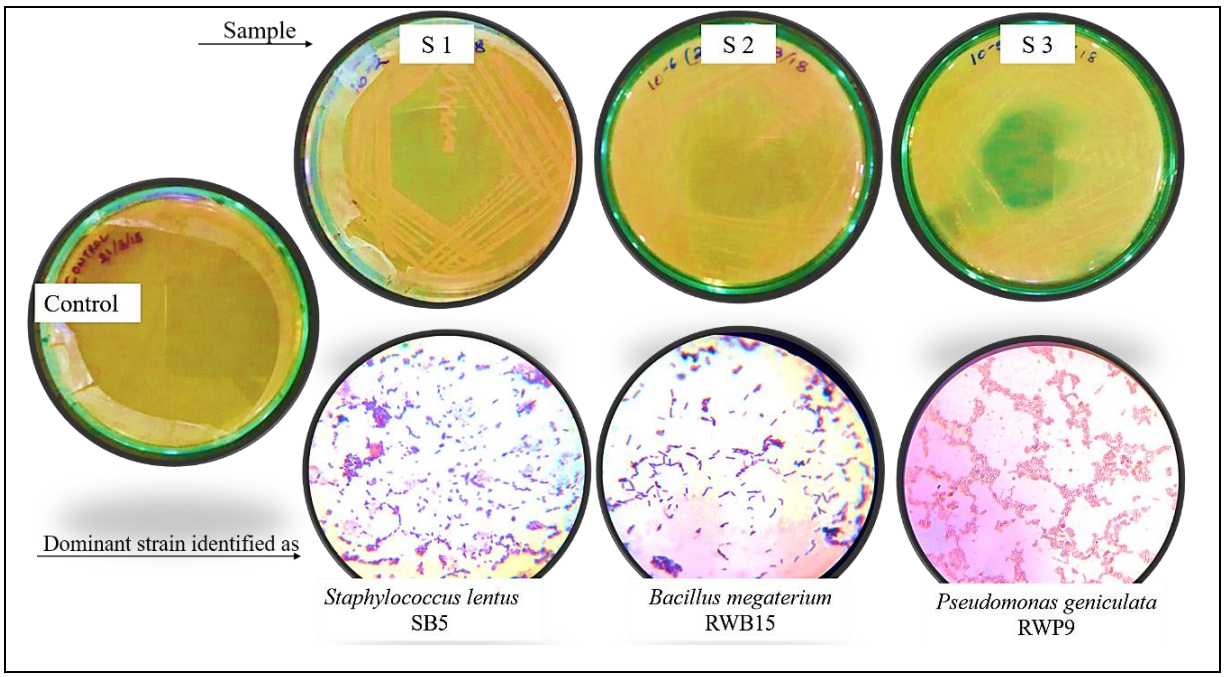

Fig. 1: Ligninolytic bacteria isolation and identification of dominant strain

3.2 Biochemical and Physiological Characterization of Dominant Strains

The biochemical and physiological characterisation of dominant strains, SB5, RWB15 and RWP5 was performed in the presence of KL. This investigation assessed various metabolic, biological and morphological profiles and physiological traits of the strains with the presence of KL in a lignin-rich environment (Table 2). The strains SB5 and RWB15 exhibit characteristic traits of Gram-positive and motile rod-shaped bacterial cells under aerobic conditions. They demonstrate positive catalase activity and display moderate reduction in nitrate and phenol. In contrast, RWP9 is Gram-negative characterized by rod-shaped cells in chains. Besides, the isolates SB5, RWB15 and RWP5 were subjected to 16S rRNA gene sequencing and Vitek 2 technique was employed to identify the specific strain. Based on the analysis of 16S rRNA gene sequence, it was determined that these isolates show 99% match with *Staphylococcus lentus* SB5, *Bacillus megaterium* RWB15 and *Pseudomonas geniculata* RWP9. Consequently, these three isolates are recognized as *Staphylococcus lentus* (SB5), *Bacillus megaterium* (RWB15) and *Pseudomonas geniculata* (RWP9) (Fig. 1).

3.3 Ligninolytic Enzyme Activity of Dominant Strains

The identification of enzymatic activity of the isolated strains will uncover their roles on KL degradation. A plate assay method was employed to evaluate manganese peroxidase (MnP) activity, lignin peroxidase (LiP) activity, and laccase activity (LA) with the screened strains. The assay utilized a modified synthetic medium (MSM) with substrates like phenol red (0.1%) for MnP and azure B (0.002%) for LiP to assess the enzymatic activity. The positive MnP activity was observed through the conversion of dark pink to yellow and positive LiP activity was characterized by the disappearance of the blue colour around the bacterial colonies. The positive laccase activity was confirmed by the formation of brown colour halos near the bacterial colonies after 72 hours of incubation. Notably, LiP responsible for the oxidative depolymerization of phenolic lignin substances exhibited positive result for all the isolated dominant bacterial strains (SB5, RWB15 and RWP9).

Similarly, MnP activity was observed in the form of brown colour halos indicating the peroxide-based oxidation of Mn^{2+} to Mn^{3+}. Furthermore, the positive laccase activity near the bacterial colonies suggested the capabilities of SB5, RWB15 and RWP9 to produce the ligninolytic enzymes. It emerges as the dominant species in ligninolytic enzyme activity among the lignin-degrading bacteria isolated from samples 1, 2 and 3. This confirmation underscores its ability to attack the robust aromatic ring structure of lignin during the degradation. Ligninolytic enzymes are crucial for breaking down and detoxifying the lignocellulosic waste in the environment (Hofrichter *et al.* 2010).

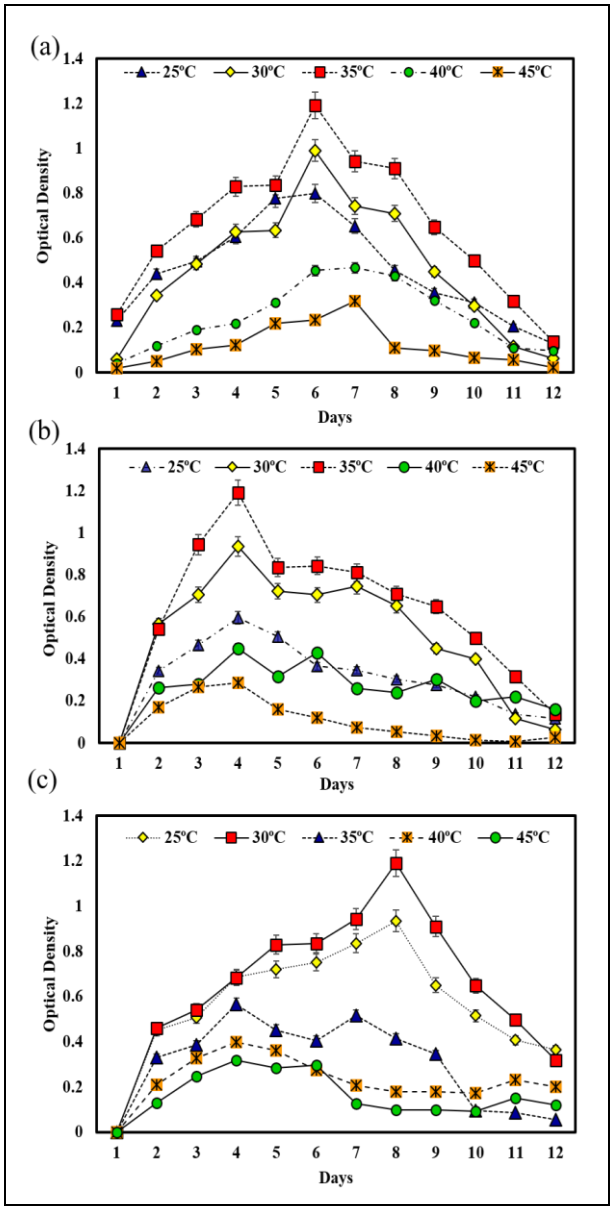

Fig. 2: Optimization of temperature on bacterial growth (a) SB5, (b) RWB1 and (c) RWP9 [Co = 1000 mg KL/l, RPM = 120, initial pH = 7.0, λ = 600 nm]

3.4 Optimization of Temperature on Bacterial Growth for Better Degradation of Lignin

This study focuses on optimizing the temperature conditions of bacterial strains to achieve higher KL degradation. The different temperatures within a specified range were assessed to determine the most convenient environment for the microbial breakdown of KL. Temperature fluctuations significantly influence the microbial life cycle, affecting population growth, degradation processes and product formation. In the temperature optimization study, an investigation was carried out to study the impact of bacterial incubation temperature on KL degradation with different temperatures ranging from 30°C to 45°C. The results indicate that strains SB5 and RWB1 show higher lignin degradation at 35°C which stands out as the most favourable temperature (Fig. 2a and 2b). For strain RWP9, the optimum temperature was 30°C for better growth of microbes with high KL degradation efficiency (Fig. 2c). However, temperatures exceeding 45°C have a detrimental effect on microbial enzyme activity which directly leads to enzyme denaturation.

These findings show the importance of the best suitable temperature for the screened strains. Also, they highlight the requirement of setting the incubation temperature for good lignin degradation efficiency. It is essential to consider the specific and unique requirements for each strain to achieve the maximum performance of ligninolytic activities. Specifically, the optimization of temperature contributes to higher lignin degradation and enhances the enzyme mechanism by accelerating molecular movements, thereby promoting increased catalytic activity. Achieving optimal biological processes involves a balance between improved enzymatic activity and temperature induced stability. The elevated temperature generally denatures and reduces the enzyme activity of microbes (Wong, 2009).

3.5 Effect of Incubation Time on Lignin Degradation and Cod Reduction

When KL is broken down, reduction of COD occurs due to conversion of complex lignin structures into simple and more soluble compounds. The products from KL degradation are generally more prone to microbial and oxidation processes which leads to decrease in the overall COD of the system.

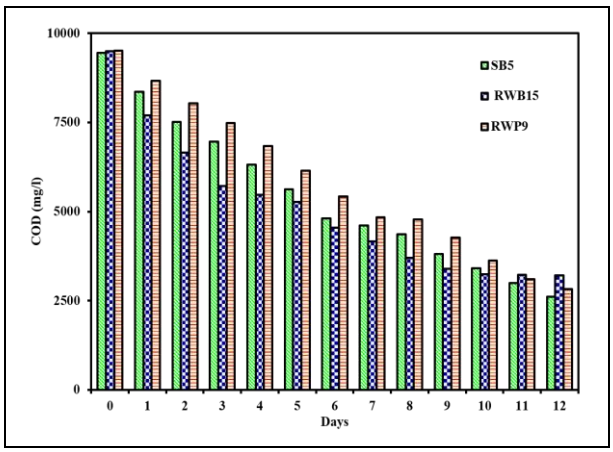

Fig. 3: Effect of incubation time on COD and KL reduction [Co = 1000 mg KL/l, RPM = 120, initial pH = 7.0]

The assessment of the KL degradation can be accurately determined by monitoring the reduction of COD level. At the initial stage of degradation process, the control sample showed COD value of 9500 mg/l. The reduction of COD by SB5, RWB15 and RWP9 strains during the 12 day-degradation process is shown in Fig. 3.

For SB5, a rapid reduction of COD was observed till 6th day and similarly for RWB15 and RWP9, COD reduction was very fast till 3rd and 7th day of KL degradation and thereafter degradation was stable for couple of days and then slowly reduced during the stationery and death phase, respectively. This phenomenon is attributed to the high synthesis of lignin peroxidase (LiP), manganese peroxidase (MnP) enzymes and laccase (Grgas *et al.* 2023). The increased activity of these enzymes indicates their effectiveness in breaking down the complex lignin structures and facilitating for efficient COD reduction in the early stages of KL degradation. The enzymes LiP and MnP are involved in the oxidative reactions that facilitate the cleavage of chemical bonds in lignin making it more prone to microbial degradation. Rapid reduction of COD during the initial days of degradation indicates the effectiveness of these enzymes on KL degradation. Imam *et al.* (2022) reported that the overall reduction in COD is attributed to the synergistic effect of LiP, MnP and extracellular laccase till the last day of degradation. The COD reduction rates are recorded as 72%, 66% and 70% for SB5, RWB15 and RWP9, respectively (Fig. 3).

3.6 Bacterial Growth and Lignin Degradation

In this study, the bacterial growth and lignin degradation efficiency of SB5, RWB15 and RWP9 were monitored with fixed concentration of KL at 120 rpm and 7.0 pH. Bacterial population and lignin degradation efficiency have a codependent relationship. Bacteria depend on lignin molecules as a growth substrate. Breakdown of lignin supplies carbon and energy to the microbes.

This symbiotic interaction benefits both the bacteria and the ecosystem by aiding in the decomposition of lignin, releasing the nutrients, and providing the bacteria with the essential energy source (Grgas *et al.* 2023). Fig. 4a illustrates the growth curve of SB5 over a 12-day incubation period. The SB5 strain exhibited rapid growth for the first 5 days reaching maximum growth on day 6. Remarkably, a substantial reduction (89%) of lignin content was observed after 5 days of degradation, reaching its peak value on day 6. The initial rapid growth of SB5 during the first 5 days shows an efficient utilization of available nutrients, which is due to an increased bacterial population. The attainment of maximum growth on day 6 suggests that the bacterium entered a stationary phase, wherein the rate of cell division matches the rate of cell death. For RWB15 (Fig. 4b), the accelerated growth was prominent during the three days of degradation with a maximum growth on day 3. The maximum lignin removal (77%) was achieved on day 3 followed by an abrupt decline in the bacterial growth. The rapid decline in the microbial growth is attributed to the release of enzymes by the strain which influences both bacterial growth and lignin degradation processes causing a shift in metabolic focus from growth to lignin degradation. On the other hand, RWP9 (Fig. 4c) confirmed robust bacterial growth in the first 6 days of incubation, achieving its peak growth on the 7th day of KL degradation. A significant removal (90%) of lignin content was achieved on 7th day, reaching its maximum reduction by the end of 7th day. These findings emphasize the dynamic relationship between bacterial growth and lignin degradation (Khan *et al.* 2022).

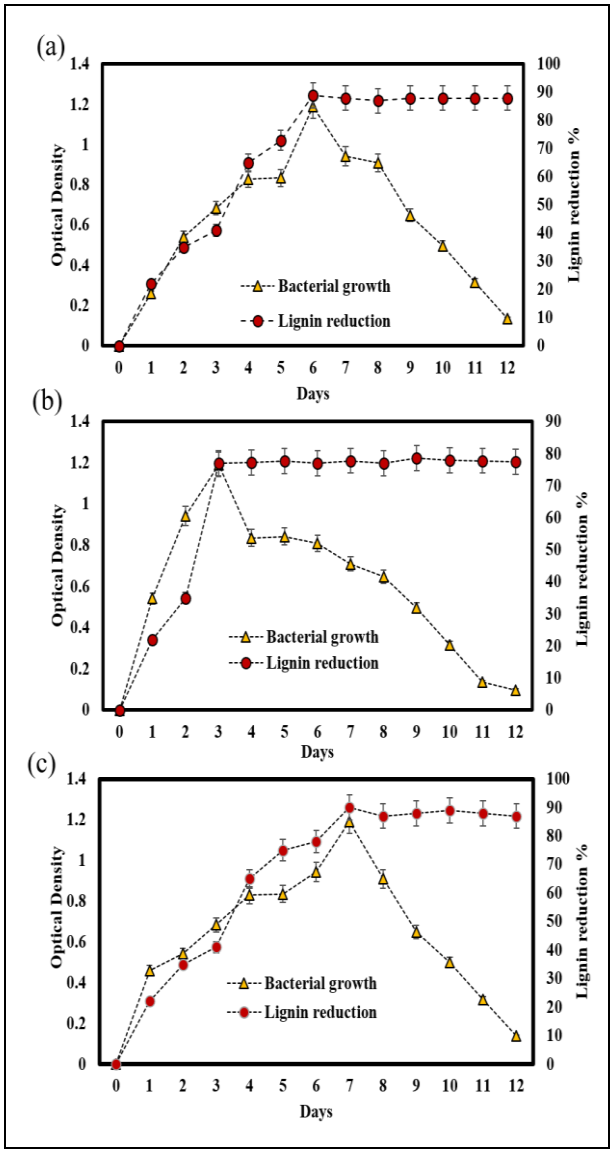

Fig. 4: Lignin degradation with bacterial growth pattern [Co = 1000 mg KL/l, RPM = 120, initial pH = 7.0, λ = 600 nm]

Pseudomonas species are known for their metabolic versatility and adaptability. The strain might have taken some time to adapt to the lignin-rich environment which led to an initial lag phase followed by the rapid lignin degradation and bacterial growth. The variations in lignin degradation patterns among SB5, RWB15 and RWP9 highlight the diverse degradation paths/routes followed by the bacteria in adapting to lignin-rich environments. The strain SB5 depicted the efficient and sustained lignin degradation. The strain

RWB15 exhibited a focused lignin degradation phase coinciding with accelerated growth. The strain RWP9 showed an extended period of lignin degradation due to its metabolic versatility. The straight-line pattern in higher lignin degradation is attributed to the factors such as enzyme kinetics, nutrient dynamics, metabolic equilibrium, feedback regulation, microbial community dynamics, optimal conditions and potential experimental constraints. This sustained KL degradation trend emphasizes the intricate dynamics of microbial processes involved in breaking down lignin-rich substrates (Mei *et al*. 2020; Singh *et al.* 2021). Furthermore, the correlation between the highest lignin degradation and the high concentration of value-added products/fractionates underscores the direct link between lignin breakdown and the production of valuable compounds which emphasize the ecological and biotechnological significance of bacterial strategies in lignin-rich environments.

3.7 Production of Valuable Compounds During KL Degradation

The KL degradation study was performed with SB5, RWB15 and RWP9 strains separately under specific conditions to estimate the production of vanillin, vanillic acid and adipic acid during the biodegradation process in three distinct setups each containing individual strains. The KL concentration was systematically adjusted to 1000 mg/l and all other process parameters were kept constant for maintaining the consistency. This investigation was carried out with only dominant species of SB5, RWB15, and RWP9 as potential ligninolytic bacterial strains to obtain the bioproducts. The highest lignin degradation was achieved on the 7th, 3rd and 6th day of degradation by SB5, RWB15, and RWP9, respectively during 12 days of incubation. The variations in lignin degradation and product formation on different days highlight how the dynamic nature of microbial activity and enzyme mechanisms contribute to the complexity of lignin degradation processes (Khan *et al.* 2022).

The process of lignin depolymerization under these environmental conditions led to the production of value-added green chemicals. The production of specific bioproducts is dependent on the enzymes released by the respective microbes. As shown in Fig. 5, on 6th day of degradation, SB5 strain shows enhanced production of vanillin reaching the maximum concentration of 28 mg/l of vanillin. Furthermore, the RWB15 isolated from the soil source 2 exhibited efficacy of lignin degradation and resulting the production of maximum vanillic acid on 3rd day with the concentration of 101 mg/l. The strain RWP9 shows the maximum degradation of KL on 7th day, and it produced 130 mg/l adipic acid as maximum amount of bioproduct during the lignin fractionation (Fig. 5). Microbial activities often follow specific biological phases wherein certain microbes, particularly dominant strains, exhibit increased metabolic and enzymatic activity under the specific environmental conditions (Singh *et al.* 2021). These findings emphasize efficient degradation of ligninolytic microbes and producing the valuable chemical compounds/fractionates having commercial importance.

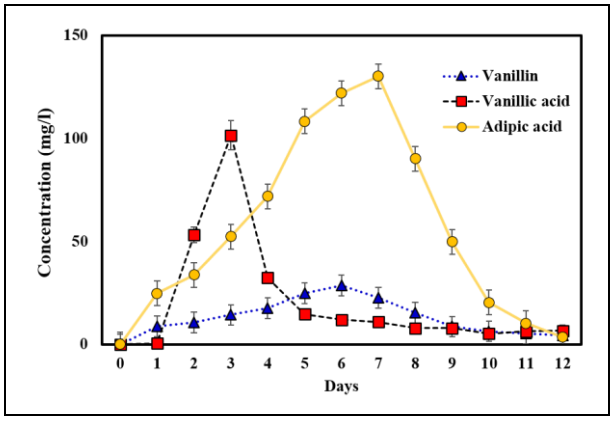

Fig. 5: Formation of valuable compounds after lignin degradation [Co = 1000 mg KL /l, RPM = 120]

4. CONCLUSION

In this study, fifteen ligninolytic bacterial strains were isolated and three dominant strains (SB5, RWB15 and RWP9) were identified as potential microbes to degrade the complex lignin and produce vanillin, vanillic acid and adipic acid. The optimum temperature of all the strains was in the range 30-35°C. The maximum degradation of lignin by SB5, RWB15 and RWP9 was found to be 89, 77 and 90%, respectively. There was a sharp increase in lignin degradation during the log phase that remained stable for a few days during the stationary phase. The COD concentration was linearly reduced by all three microbes before attaining the stationary phase and thereafter COD reduction was sluggish due to the attainment of the death phase. The maximum concentration of vanillin (28 mg/l), vanillic acid (101 mg/l) and adipic acid (130 mg/l) were achieved on the 6th, 3rd and 7th day of degradation, respectively. The outcomes of this study emphasize the potential applications of isolated bacterial strains for efficient degradation of lignin and formation of valuable byproducts as well as commercially important bioproducts.

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CONFLICTS OF INTEREST

The authors declare that there is no conflict of interest.

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REFERENCE

Ahmad, M., Roberts, J. N., Hardiman, E. M., Singh, R., Eltis, L. D. and Bugg, T. D., Identification of DypB from Rhodococcus jostii RHA1 as a lignin peroxidase, *Biochem.*, 50(23), 5096-5107 (2011). https://doi.org/10.1021/bi101892z

Asina, F. N. U., Brzonova, I., Kozliak, E., Kubátová, A. and Ji, Y., Microbial treatment of industrial lignin: Successes, problems and challenges, *Renewable Sustainable Energy Rev.*, 77, 1179-1205 (2017). https://doi.org/10.1016/j.rser.2017.03.098

Blasi, A., Verardi, A., Lopresto, C. G., Siciliano, S. and Sangiorgio, P., Lignocellulosic agricultural waste valorization to obtain valuable products: An overview, *Recycl.*, 8(4), 61 (2023). https://doi.org/10.3390/recycling8040061

Bruijnincx, P. C., Rinaldi, R. and Weckhuysen, B. M., Unlocking the potential of a sleeping giant: lignins as sustainable raw materials for renewable fuels, chemicals and materials, *Green Chem.,* 17(11) (2015). https://doi.org/4860-4861. 10.1039/C5GC90055G

Bugg, T. D., Ahmad, M., Hardiman, E. M. and Singh, R., The emerging role for bacteria in lignin degradation and bio-product formation, *Curr. Opin. Biotechnol.*, 22(3), 394-400 (2011). https://doi.org/10.1016/j.copbio.2010.10.009

Chandra, R., Raj, A., Purohit, H. J. and Kapley, A., Characterisation and optimisation of three potential aerobic bacterial strains for kraft lignin degradation from pulp paper waste, *Chemosphere*, 67(4), 839-846 (2007). https://doi.org/10.1016/j.chemosphere.2006.10.011

Chandra, R., Yadav, S. and Yadav, S., Phytoextraction potential of heavy metals by native wetland plants growing on chlorolignin containing sludge of pulp and paper industry, *Ecol. Eng.,* 98, 134-145 (2017). https://doi.org/10.1016/j.ecoleng.2016.10.017

Grgas, D., Rukavina, M., Bešlo, D., Štefanac, T., Crnek, V., Šikić, T. and Landeka Dragičević, T., The Bacterial Degradation of Lignin - A Review, *Water*, 15(7), 1272 (2023). https://doi.org/10.3390/w15071272

Haq, I. U., Qaisar, K., Nawaz, A., Akram, F., Mukhtar, H., Xu, Y. and Choong, T. S. Y., Advances in valorization of lignocellulosic biomass towards energy generation, *Catal.*, 11(3), 1-25 (2021). https://doi.org/10.3390/catal11030309

Haq, I., Mazumder, P. and Kalamdhad, A. S., Recent advances in removal of lignin from paper industry wastewater and its industrial applications–A review, *Bioresource Technol.*, 312, 123636 (2020). https://doi.org/10.1016/j.biortech.2020.123636

Hasan, F., Shah, A. A. and Hameed, A., Industrial applications of microbial lipases, *Enzyme Microb. Technol.*, 39(2), 235-251 (2006). https://doi.org/10.1016/j.enzmictec.2005.10.016

Hermosilla, E., Schalchli, H., Mutis, A. and Diez, M. C., Combined effect of enzyme inducers and nitrate on selective lignin degradation in wheat straw by Ganoderma lobatum, *Environ. Sci. Pollut. Res.* 24, 21984–21996 (2017). https://doi.org/10.1007/s11356-017-9841-4

Hofrichter, M., Ullrich, R., Pecyna, M. J., Liers, C. and Lundell, T., New and classic families of secreted fungal heme peroxidases, *Appl. Microbiol. Biotechnol.*, 87, 871-897 (2010). https://doi.org/10.1007/s00253-010-2633-0

Imam, A., Suman, S. K., Kanaujia, P. K. and Ry, A., Biological machinery for polycyclic aromatic hydrocarbons degradation: A review, *Bioresour. Technol.*, 343, 126121 (2022). https://doi.org/10.1016/j.biortech.2021.126121

Khan, S. I., Zarin, A., Ahmed, S., Hasan, F., Belduz, A. O., Çanakçi, S. and Shah, A. A, Degradation of lignin by Bacillus altitudinis SL7 isolated from pulp and paper mill effluent, *Water Sci. Technol.*, 85(1), 420-432 (2022). https://doi.org/10.2166/wst.2021.610

Leonowicz, A., Cho, N., Luterek, J., Wilkolazka, A., Wojtas-Wasilewska, M., Matuszewska, A., and Rogalski, J., Fungal laccase: properties and activity on lignin, *J. Basic Microbiol.,* 41(3-4), 185-227 (2001). https://doi.org/10.1002/1521-4028(200107)41:3/4<185::AID-JOBM185>3.0.CO;2-T

Lin, L., Cheng, Y., Pu, Y., Sun, S., Li, X., Jin, M. and Yuan, J. S., Systems biology-guided biodesign of consolidated lignin conversion, *Green Chem.*, 18(20), 5536-5547 (2016). https://doi.org/10.1039/C6GC01131D

Masai, E., Katayama, Y. and Fukuda, M., Genetic and biochemical investigations on bacterial catabolic pathways for lignin-derived aromatic compounds, *Biosci. Biotechnol. Biochem.*, 71(1), 1-15 (2007). https://doi.org/10.1271/bbb.60437

Mathews, S. L., Grunden, A. M. and Pawlak, J., Degradation of lignocellulose and lignin by Paenibacillus glucanolyticus, *Int. Biodeterior. Biodegrad*, 110, 79-86 (2016). https://doi.org/10.1016/j.ibiod.2016.02.012

Mei, J., Shen, X., Gang, L., Xu, H., Wu, F. and Sheng, L., A novel lignin degradation bacteria-Bacillus amyloliquefaciens SL-7 used to degrade straw lignin efficiently, *Bioresour. Technol.,* 310, 123445 (2020). https://doi.org/10.1016/j.biortech.2020.123445

Nguyen, L. T., Phan, D. P., Sarwar, A., Tran, M. H., Lee, O. K. and Lee, E. Y., Valorization of industrial lignin to value-added chemicals by chemical depolymerization and biological conversion, *Ind. Crops Prod*., 161, 113219 (2021). https://doi.org/10.1016/j.indcrop.2020.113219

Ramachandra, M., Crawford, D. L., and Hertel, G., Characterization of an extracellular lignin peroxidase of the lignocellulolytic actinomycete Streptomyces viridosporus, *Appl. Environ. Microbiol.,* 54(12), 3057-3063 (1988). https://doi.org/10.1128/aem.54.12.3057-3063.1988

Singh, A. K., Bilal, M., Iqbal, H. M., Meyer, A. S. and Raj, A., Bioremediation of lignin derivatives and phenolics in wastewater with lignin modifying enzymes: Status, opportunities and challenges, *Scien of Total Environ*., 777, 145988 (2021). https://doi.org/10.1016/j.scitotenv.2021.145988

Vardon, D. R., Franden, M. A., Johnson, C. W., Karp, E. M., Guarnieri, M. T., Linger, J. G. and Beckham, G. T., Adipic acid production from lignin, *Energy Environ. Sci*., 8(2), 617-628 (2015). https://doi.org/10.1039/C4EE03230F

Vijayalakshmi, S., Disalva, X., Srivastava, C., and Arun, A., Vanilla-natural vs artificial: a review, *Res. J. Pharm. Technol.*, 12(6), 3068-3072 (2019). https://doi.org/10.5958/0974-360X.2019.00520.1

Wong, D. W., Structure and action mechanism of ligninolytic enzymes. *Appl. Biochem. Biotechnol*, 157, 174-209 (2009). https://doi.org/10.1007/s12010-008-8279-z

Xu, Z., Qin, L., Cai, M., Hua, W. and Jin, M., Biodegradation of kraft lignin by newly isolated Klebsiella pneumoniae, Pseudomonas putida, and Ochrobactrum tritici strains, *Environ. Sci. Pollut. Res.*, 25, 14171-14181 (2018). https://doi.org/10.1007/s11356-018-1633-y

Zimmermann, W., Degradation of lignin by bacteria, *J. Biotechnol*., 13(2-3), 119-130 (1990). https://doi.org/10.1016/0168-1656(90)90098-V

Zuo, K., Li, H., Chen, J., Ran, Q., Huang, M., Cui, X. and Jiang, Z. Effective biotransformation of variety of guaiacyl lignin monomers into vanillin by Bacillus pumilus, *Frontiers Microbiol.,* 13, 901690 (2022). https://doi.org/10.3389/fmicb.2022.901690