Degradation of lignin by *Bacillus altitudinis* SL7 isolated from pulp and paper mill effluent

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ABSTRACT

Lignin is a major by-product of pulp and paper industries, which is resistant to depolymerization due to its heterogeneous structure. Degradation of lignin can be achieved by the use of potential lignin-degrading bacteria. The current study was designed to evaluate the degradation efficiency of newly isolated *Bacillus altitudinis* SL7 from pulp and paper mill effluent. The degradation efficiency of *B. altitudinis* SL7 was determined by color reduction, lignin contents, and ligninolytic activity from degradation medium supplemented with alkali lignin (3 g/L). *B. altitudinis* SL7 reduced color and lignin contents by 26 and 44%, respectively, on 5th day of the incubation, as evident from the maximum laccase activity. Optimum degradation was observed at 40 °C and pH 8.0. FT-IR spectroscopy and GC-MS analysis confirmed lignin degradation by emergence of the new peaks and identification of low molecular weight compounds in treated samples. The identified compounds such as vanillin, 2-methoxyphenol, 3-methyl phenol, oxalic acid and ferulic acid suggested the degradation of coniferyl and sinapyl groups of lignin. Degradation efficiency of *B. altitudinis* SL7 towards high lignin concentration under alkaline pH indicated the potential application of this isolate in biological treatment of the lignin-containing effluents.

Key words: alkaline pH, *Bacillus* sp., degradation products, laccase, lignin depolymerization

HIGHLIGHTS

- *Bacillus altitudinis* SL7 isolated from effluent could efficiently degrade lignin under alkaline conditions.
- Degradation efficiency was determined by analyzing lignin content, color reduction, and ligninolytic enzyme activity.
- Extracellular laccase from *B. altitudinis* SL7 can play a significant role in the depolymerization of lignin.
- Various low molecular weight lignin degradation products were determined through GC-MS.

INTRODUCTION

The pulp and paper (P & P) industry is among the largest fast-growing industries of the world which utilize lignocellulosic biomass for paper production. It consumes an enormous amount of fresh water and inorganic compounds during pulping, bleaching and washing processes and generates highly contaminated wastewater (Kumar et al. 2015). The lignocellulosic biomass utilizing industries are a major threat to the environmental health and the magnitude of problem is indicated by lignocellulosic waste generation from these industries: agricultural waste (200 billion tons/year), food industry (1.3 billion ton/year), P & P industry (effluent 150–200 m³/ton) and sugarcane molasses-based distilleries (effluent 15 lit/1) (Chandra et al. 2011; Kharayat 2012; Kadam et al. 2013; Ravindran & Jaiswal 2016; Taha et al. 2016). The P & P industry generates effluent which is characterized as dark brown with fluctuating pH (generally alkaline), and high chemical oxygen demand (1,110–1,272 mg/L), suspended solids (1,160–1,380 mg/L), dissolved solids (1,043–1,293 mg/L) and lignin contents (Singh 2015). The concentration of lignin in effluent depends on the type of lignocellulosic biomass used for production of pulp. Disposing off untreated effluent accounts for undesirable coloration of aquatic resources along with deterioration of aquatic flora and fauna by obstructing the passage of sunlight. Lignin derivatives such as chlorolignin affects the reproductive system of fishes by causing delayed maturity, lower sex hormone, and reduction in gonad size (Singh & Chandra 2019). In the terrestrial...
ecosystem, these contaminants enter the food chain and have carcinogenic and genotoxic effects on humans and other animals (Savant et al. 2006).

An appropriate treatment of wastewater from P & P mills is an important issue worldwide and needs to be addressed. Different physicochemical methods such as, membrane filtration, sedimentation, chemical oxidation and ozonation have been reported but they are not suitable for the treatment of effluent due to operational cost and environmental problems. Biological treatment is an efficient, cost-effective alternative to achieve it. Intensive research has been carried out to decontaminate the effluent from P & P industries using fungi, bacteria and their enzymes (Ebanyenle et al. 2016; Mathews et al. 2016). Most of the research on detoxification of lignocellulosic waste involved brown rot and white rot fungal species (Karp et al. 2012; Karim et al. 2016; Schmidt et al. 2016) but the usage of fungal species for effluent detoxification have growth limitations. Fungus requires acidic pH for the production of ligninolytic enzymes, usually, the pH of P & P mills effluent tends to be alkaline so the practice of fungal system for effluent treatment requires pH adjustment which adds extra cost to the treatment process. While bacteria are gaining interest because of environmental adaptability and biochemical versatility as compared to fungi (Mathews et al. 2016). The bacteria can consume simple aromatics to complex lignocellulosic biomass for their growth and production of ligninolytic enzymes involved in degradation (Rinaldi et al. 2016).

Few bacterial species have been investigated for detoxification of lignin and production of ligninolytic enzymes. Bacteria such as Bacillus sp. and Paenibacillus sp. have been isolated from P & P mill sludge, their lignin degradation potential was confirmed by analysis of degradation products (Chandra et al. 2007). Laccase-producing bacteria like Azotobacter, B. megatarium, and Serratia marcescens isolated from soil, are capable of lignin depolymerization, and their degradation activities are correlated with the production of laccase (Xu et al. 2018). Elsalam & Bahobail (2016) reported lignin degradation efficiency of B. licheniformis and B. subtilis towards 0.6 and 0.7 g/L of lignin in 7 days, respectively. However, the lignin reduction rates of reported bacterial species are much lower as compared to fungi, and there is also a gap in knowledge about ligninolytic enzymes involved in lignin degradation. There is a need of time to search for more efficient lignin-degrading bacteria as well as their characterization for maximum lignin reduction. Therefore, the current study is focused on isolation and identification of efficient lignin-degrading bacteria from P & P mill effluent, and optimization of growth conditions for lignin degradation. The degradation efficiency of newly isolated Bacillus altitudinis SL7 was analyzed by its ability to reduce lignin contents and decolorize degradation medium. Furthermore, the lignin degradation products were analyzed by gas chromatography-mass spectrometry (GC-MS). Degradation efficiency of B. altitudinis SL7 towards a high concentration of lignin could attract more attention for detoxification of the lignin-contaminated sites.

MATERIALS AND METHODS

Materials

Purified synthetic alkali lignin, 2-Methoxyphenol (Guaiacol), and Azure B dye were purchased from Sigma-Aldrich (St. Louis, MO, USA), COD Cell Test kit (25–1,500 mg/L) from Merck. co (Darmstadt, Germany) and Genomic DNA purification kit, pGEM-T Easy cloning vector and plasmid DNA extraction kit from Promega (Madison, WI, USA). All other chemicals used in this study were of highest commercial grades.

Collection of samples

The paper industry utilizes sulfite process for pulping of raw material and chlorine for bleaching of the pulp. To assess the pollutant load, samples were collected from different units of effluent treatment plant (ETP) of Century Paper and Board Mills Limited, Punjab, Pakistan. These units included; inlet point, aeration tank I, aeration tank II, secondary sedimentation tank, and final sedimentation tank. Effluent samples were collected carefully to make them as representative as possible of the whole water. Three grab samples having a volume of 1 L were collected, at uniform time intervals over the sampling period and mixed in 5 L sterile plastic bottles to give a composite sample of effluent. While sludge samples were collected in sterile zipper bags from the aeration tank of effluent treatment plant (ETP). The samples were kept in an ice-chest under 4 °C and transported to Applied, Environmental and Geomicrobiology laboratory at Quaid-i-Azam University, Islamabad, and processed for analysis within 24 hours of collection.

Physicochemical characteristics of wastewater

All the effluent samples were subjected to physicochemical analysis and analyzed for pH, total suspended solids (TSS), total dissolved solids (TDS), nitrates (NO₃) and sulphates (SO₄) as per the standard methods provided by American Public Health
Association (APHA) (Rice et al. 2017) and chemical oxygen demand (COD) was determined by COD Cell Test kit according to the instructions provided by the manufacturer. Lignin concentration and color were determined by the methods of Pearl and Benson (Brauns & Brauns 1960), and CCPA (Canadian Pulp and Paper Association) (CPPA 1974), respectively.

Isolation and screening of lignin-degrading bacteria from sludge

The spread plate method was used to isolate potential lignin-degrading bacteria on alkali lignin amended mineral salt medium (AL-MSM) plates. AL-MSM composed of (g/L): lignin 0.5; Na₂HPO₄, 2.4; NH₄NO₃, 0.1; K₂HPO₄, 0.01; CaCl₂, 0.01; M(16S)arSO₄, 0.01; CaCl₂, agar 20, where lignin provided as a sole carbon source. The sludge sample was serially diluted to 10⁻⁷, and 100 μL of diluted sample was spread on the AL-MSM agar plates and aerobically incubated at 35 °C for 7 days to recover the bacterial population that utilizes lignin as a carbon source. Morphologically distinct colonies were picked and purified after multiple re-streaking on nutrient agar. Stock cultures of pure isolates were prepared in 20% glycerol and stored at −20 °C for further experiments. Primary screening was carried out on AL-MSM plates supplemented with increasing concentrations of lignin, i.e., 0.5–3 g/L. One potential bacterial isolate designated as SL7 exhibited good growth in the presence of maximum concentration of lignin (5 g/L) and was selected for further study.

Screening of ligninolytic activity on agar plate-test

The isolate SL7 was screened for laccase and lignin peroxidase activity on substrate amended nutrient agar plates. Nutrient agar was sterilized by autoclaving at 121 °C for 20 mins and then poured into glass petri plates supplemented with filter-sterilized guaiacol and azure B at the final concentrations of 0.02% and 0.01%, respectively. The bacterial isolate SL7 was spot-inoculated on plates and then observed for the appearance of activity zones after incubation at 35 °C for 7 days.

Identification of ligninolytic bacterial strain SL7

Isolate SL7 was identified by colony morphology, microscopic depiction and 16S rRNA gene sequencing. The isolate was grown overnight in 50 ml nutrient broth at 37 °C and 120 rpm. Bacterial cells were harvested through centrifugation in the late exponential phase and genomic DNA was extracted using a commercially available DNA extraction kit, according to the manufacturer's instructions. The purity and quantity of extracted DNA was analyzed by using NanoDrop (Fisher Scientific) and observed as 10 μg/mL. The 16S rRNA gene was amplified by PCR using universal primers: 27F (5'-ATT CTA GAG TTT GAT CAT GGC TCA -3') and 1492R (5'-ATG GTA CCG TGT G ACG GGC GGT GTG TA-3'). The reaction mixtures contained 0.5 μl of each primer, 12.5 μl of DreamTaq master mix, 10.5 μl of deionized water, and 1 μl DNA template (10 ng/μl). PCR was carried out under the following conditions: denaturation at 94 °C for 2 min, 30 cycles of denaturation at 95 °C for 1 min, annealing at 55 °C for 1 min, extension at 72 °C for 1 min, and a final extension step at 72 °C for 10 min. 16S rRNA gene amplification was confirmed by gel electrophoresis using 1% agarose gel containing 0.5 μg/ml ethidium bromide and analyzed using a Bio-Rad Gel Doc imaging system. The amplified 16S rRNA gene product was cloned into pGEM-T Easy cloning vector and sequenced by Macrogen Company (Netherlands). The obtained sequence was subjected to BLAST analysis using BLASTn program (http://www.ncbi.nlm.nih.gov/BLAST/). All the sequences were aligned, and a neighbor-joining phylogenetic tree was constructed using MEGA-X software (Kumar et al. 2018).

Culture conditions for alkali lignin degradation by bacterial isolate SL7

Degradation experiment was carried out in Erlenmeyer flasks (500 mL) containing 200 mL AL-MSM. The composition of medium was (g/L): Lignin, 3; Na₂HPO₄, 2.4; CaCl₂, 0.01; NH₄NO₃, 0.1; MgSO₄, 0.01; K₃HPO₄, 2.0; peptone (0.25%, w/v) and glucose (0.5%, w/v). The flasks were inoculated with 2 ml of overnight growth culture having an OD₆₀₀ of 0.6. The effect of temperature and pH on lignin degradation by isolate SL7 was investigated by running the experiment at various ranges of temperature (20–50 °C) and pH (6.0–12.0). The experiment was run in triplicates and uninoculated flasks were used as a control. The samples were withdrawn at 24 h intervals up to the maximum of 7 days and processed for measurement of lignin degradation.

Lignin degradation by strain SL7

Color reduction assay

The color of degradation medium was determined according to the CPPA standard methods (CPPA 1974). 5 mL sample was collected at 24 h intervals and centrifuged at 8,000 rpm for 30 min. The pH of the supernatant was adjusted to 7.6 with 2M NaOH and its absorbance was measured at 465 nm by UV-visible spectrophotometer. Absorbance values were converted into
color units (CU) by using the following formula:

\[ CU = \frac{500 \times A_2}{A_1} \]

where \( A_1 \) is the absorbance of standard solution (platinum–cobalt), and \( A_2 \) is the absorbance of the sample.

### Lignin reduction assay

The residual lignin contents were estimated by the method as previously described by Chandra et al. (2007). The pH of the supernatant was adjusted to 7.6 by phosphate buffer and its absorbance was measured at 280 nm by UV-visible spectrophotometer. Degradation efficiency (%) was calculated as follows:

\[
\text{Degradation efficiency (\%)} = \left( \frac{\text{Initial absorbance} - \text{final absorbance}}{\text{Initial absorbance}} \right) \times 100
\]

The growth rate of strain SL7 was determined by measuring optical density at 600 nm (OD\text{600}) using UV-visible spectrophotometer.

### Ligninolytic enzymes assay from strain SL7 during the time course of degradation

#### Laccase assay

The extracellular laccase activity was determined by measuring the oxidation of guaiacol by the method as previously reported by Barapatre et al. (2017). The culture-free supernatant was obtained by centrifugation of samples at 8,000 rpm for 10 min. The assay mixture contained: 1 mL supernatant; 10 mM sodium acetate buffer (pH 4.5); and 2 mM guaiacol, incubated for 15 min at 30 °C. The laccase activity was measured at 450 nm by UV-visible spectrophotometer. The activity was expressed in terms of the International Unit (IU) and described as the amount of laccase required to oxidize 1 μM of guaiacol per minute under standard assay conditions. The laccase activity in U/ml was calculated by the following formula:

\[
E.A = \frac{A}{V/t \times e \times v}
\]

where: \( E.A \) = Enzyme activity, \( A \) = Absorbance, \( V \) = Total mixture volume (ml), \( v \) = enzyme volume (ml), \( t \) = incubation time, \( e \) = extinction coefficient for guaiacol (0.6740 μmol/cm).

#### Lignin peroxidase assay

The lignin peroxidase activity was determined by monitoring the decolorization of azure B using the method as previously described by Archibald (Menon & Hartz-Karp 2019). The reaction mixture consisted of 500 μL supernatant; 100 mM sodium tartrate buffer (pH 3.0); 32 μM azure B; and the reaction was initiated by adding 1 mM H\text{2}O\text{2}. The enzyme activity was measured at 651 nm by UV-visible spectrophotometer. Lignin peroxidase activity was expressed in terms of International Unit (IU) and described as the amount of lignin peroxidase required to oxidize 1 μM of azure B per minute under standard assay conditions.

### Analysis of lignin degradation

#### Determination of structural modification by FTIR

Fourier Transform Infra-Red (FT-IR) spectroscopy was performed to analyze changes in the functional group of heteropolymeric lignin during the degradation experiment. 2 mg of vacuum-dried sample was mixed with 200 mg of potassium bromide (KBr), mixture was homogenized and compressed under continuous pressure of 40 MPa to form thin pellets. These pellets were analyzed by FT-IR spectra (Perkin Elmer Spectrum One FT-IR, Waltham, USA) within the range 4,000 to 400 cm\textsuperscript{-1}, and the time for analysis of each sample was set as 60 sec.

#### Determination of lignin degradation products by GC-MS

100 mL of test and control samples were centrifuged at 10,000 rpm for 15 min. The cell-free supernatant was acidified to pH 1.0–2.0 by adding concentrated HCL and acidified supernatant was thoroughly extracted with three-volume ethyl acetate. The organic layer was collected and dehydrated over anhydrous Na\textsubscript{2}SO\textsubscript{4}, the residues were dried in a rotary vacuum evaporator. The dried residues were derivatized by adding dioxane (100 μL) and pyridine (10 μL), followed by silylation with 50 μL.
trimethylsilyl (TMS). The silylated mixture was heated at 60 °C for 15 min with periodic shaking to dissolve the residues. 5 μL of silylated sample was injected into the GC-MS injector port (Shimadzu Corporation, GC-2014C, Japan), equipped with DB-FFAP capillary column (30 m × 0.25 μm × 0.25 mm) (Agilent Technologies, Wilmington, DE, USA). The column temperature was set to 120–280 °C (10 °C per min increase) with a flow rate of 1.5 mL per min. A solvent removal time was set to 3.0 min and electron ionization mass spectra in the range of 50–750 (m/z). The detection of lignin degradation products was accomplished by comparing the retention time and mass spectra of products in test sample with available mass spectra in the National Institute of Standards and Technology (NIST) library.

RESULTS AND DISCUSSION

Physico-chemical characteristics of effluent

The characteristic features of wastewater are good indicators of toxicity level in it. The effluent sample was dark brown and alkaline in nature (pH 8.0) with high COD (994 mg/L), lignin (7,416 mg/L), TDS (440 mg/L), TSS (600 mg/L), sulphate (440 mg/L), nitrates (144 mg/L) and color (954 CU). The effluent was treated through an aerated lagoon system at industrial site that reduced pollutant concentration but still, it remained beyond the permissible limits as recommended by EPA (USEPA 2002) (Table 1). Lignin is recalcitrant in nature and major constituent of lignocellulosic biomass, presence of lignin and its derivatives possibly contributed to the dark brown color of effluent and high concentration of COD (Rice et al. 2017). The source of sulfate in the effluent might be sodium sulfite, which is used during pulping process (Singhal & Thakur 2009).

Isolation and screening of lignin-degrading bacteria

The sludge samples were collected from ETP of P & P mill. The area for collecting the samples was selected by assuming the presence of potential lignin-degraders in the area that contains lignin and other toxic chemicals. Several researchers have reported the isolation of lignin-degrading bacteria from lignin contaminated sites that were successfully applied for remediation of P & P mills effluent (Barapatre et al. 2017; Menon & Hartz-Karp 2019). In this study, 08 bacterial strains designated as strains S1–S8 were isolated from sludge samples on L-MSM agar plates, only strain SL7 showed remarkable ability to grow well in the presence of a high concentration of lignin (3 g/L), utilizing lignin as a sole carbon source (Table 2). Only potent bacteria can survive in the presence of a high concentration of lignin because lignin-derived aromatics have harmful effects and causes cell death by membrane disruption, DNA damage, and enzyme inhibition (Zeng et al. 2014). Strain SL7 was further screened for ligninolytic activity (laccase and peroxidase) on nutrient agar plates amended with respective substrates. Strain SL7 was found an efficient laccase producer, indicated by the appearance of brown color around bacterial growth on guaiacol agar plates (Figure 1(a)). The oxidation of guaiacol is one of the most suitable substrates for laccase assays produced by bacteria. Hence, the formation of the brown color is induced by laccase due to the oxidative polymerization of guaiacol (Kumar et al. 2020). Peroxidase activity was not detected as no decolorization zone around bacterial growth was observed on azure B agar plates (results are not shown).

| Parameters       | Inlet point | Aeration tank (1) | Aeration tank (2) | Secondary sedimentation tank | Final sedimentation tank | Permissible limit (USEPA, 2002) |
|------------------|-------------|-------------------|-------------------|-------------------------------|-------------------------|-------------------------------|
| pH               | 8.0         | 7.3               | 7.3               | 7.4                           | 7.6                     | 5–9                           |
| COD (mg/L)       | 994         | 997               | 962               | 650                           | 407                     | 120                           |
| Sulphate (mg/L)  | 440         | 540               | 670               | 620                           | 740                     | 252                           |
| TDS (mg/L)       | 440         | 439               | 440               | 426                           | 408                     | –                             |
| TSS (mg/L)       | 600         | 700               | 700               | 400                           | 200                     | –                             |
| Color (CU)       | 1,954       | 2,636             | 2,272             | 2,136                         | 1,909                   | –                             |
| Nitrates (mg/L)  | 144         | 200               | 188               | 224                           | 64                      | 10                            |
| Lignin (mg/L)    | 7,416       | 7,958             | 5,916             | 7,790                         | 279                     | 0.05                          |

*–(–): not specified.
Identification of bacterial isolate SL7

Strain SL7 was an aerobic, motile, Gram-positive and rod-shaped bacterium. The DNA fragment of 1,500 bps was cloned into pGEMT Easy vector and sequenced. The sequencing results were analyzed by comparing the nucleotide sequences obtained from NCBI database and a phylogenetic tree was constructed. The 16S rRNA gene sequence of strain SL7 exhibited 100% similarity with Bacillus altitudinis strain SGAir0031 (CP022319) (Figure 2), thus the bacterium was identified as Bacillus altitudinis strain SL7. The nucleotide sequence reported here can be obtained from the NCBI nucleotide database under accession number MZ400969.

Effect of temperature and pH on lignin degradation by B. altitudinis strain SL7

The effect of temperature and pH on lignin degradation by B. altitudinis strain SL7 was determined in shake flask experiment. The experiment was run in triplicates and control with each experiment was set up in a separate flask without inoculum. The maximum reduction in lignin content (44.2%) was observed at temperature 40 °C and pH 8.0 (Figure 3(a) and 3(b)). Strain SL7 showed the ability to degrade lignin under alkaline pH 7.0–11.0, with optimum pH 8.0. In a lignin degradation system, the pH value has been reported to be an important factor. Pulp and paper industries utilize alkaline chemicals
during pulping to dissolve lignocellulosic biomass; hence the effluent released from industries carries high pH (8.0–12.0). Therefore, bacterial strains that could degrade lignin under alkaline conditions would be the suitable choice for detoxification of such effluent (Fang et al. 2018). Several researchers have reported bacteria such as Aneurinibacillus aneurinilyticus, Comamonas sp, Bacillus strain, Rhodococcus pyridinivorans and Streptomyces strain with optimum degradation pH ranges from 7.0–7.5 (Chen et al. 2012; Shi et al. 2013; Chong et al. 2018). The lignin degradation potential of Bacillus altitudinis SL7 under alkaline pH (7.0–11.0) could make it a suitable candidate for application on sites with lignin contamination in soil and water.

**Bacterial growth and lignin degradation**

Relationship between the growth and lignin degradation by *B. altitudinis* SL7 was investigated during the degradation experiment. Figure 4(a) shows the growth curve of isolate SL7 during lignin degradation over 7 days of incubation. *B. altitudinis* SL7 grew at a fast rate during the initial 2 days of incubation and achieved maximum growth at day 5. A significant reduction in lignin was observed after 3 days of incubation with maximum reduction (44%) on day 5. In contrast, the color reduction
started after 24 h of incubation, while maximum reduction (26%) achieved on day 5 followed by a gradual decrease till day 7 (16%) (Figure 4(b)). Due to fast bacterial growth during the initial days of incubation, a significant reduction in color and lignin contents was achieved on day 5, which indicates the phenomenon of co-metabolism adopted by *B. altitudinis* SL7. It could be possible that bacteria utilized glucose and peptone as carbon and nitrogen source to initiate its growth followed by the utilization of lignin as a co-substrate. Similar to this study, co-metabolism in bacteria and fungi for lignin degradation has also been reported by various authors (Singhal & Thakur 2009; Singh & Chandra 2019). While considering the initial concentration of lignin, the degradation efficiency of *B. altitudinis* SL7 was higher than the previous reports (Table 3).

### Ligninolytic enzymes produced by *B. altitudinis* SL7 during degradation

#### Laccase assay

The enzyme laccase activity was measured during the course of lignin degradation by *B. altitudinis* SL7. A gradual rise in laccase activity was observed from day 2 and reached up to the maximum (1.3 U/mL) till day 5, followed by a decrease in activity. Lignin degradation is mainly related to enzymes secreted by *Bacillus* (Mei et al. 2020). Laccase is a copper-dependent enzyme that can oxidize lignin phenolic hydroxyl groups, thereby destroying the stability of the aromatic ring (Niu et al. 2021). Previously, laccase-producing *Bacillus megatarium* and *Serratia marcescens* have been reported for lignin degradation (Xu et al. 2018). *B. altitudinis* SL7 did not produce lignin peroxidase, as the enzyme activity was not detected in the degradation medium.

### Table 3 | Summary of lignin degradation by bacterial strains

| Bacteria strain                  | Lignin load (g/L) | pH | Degradation time (Days) | Degradation (%) | References                  |
|----------------------------------|-------------------|----|-------------------------|-----------------|-----------------------------|
| *Bacillus altitudinis* SL7       | 3                 | 8  | 5                       | 44              | This study                  |
| *Rhodococcus pyridinivorans*     | 0.06              | 7  | 5                       | 30              | Chong et al. (2018)         |
| *Bacillus* sp. strains CS-1 & CS-2 | 0.05              | 6  | 2                       | 61              | Chang et al. (2014)         |
| *Citrobacter freundii* (FJ581026) | 0.6               | 8  | 6                       | 49              | Chandra & Bharagava (2013)  |
| *Novosphingobium* sp. B-7       | 3                 | 7  | 7                       | 30              | Chen et al. (2012)          |
| *Streptomyces* sp. F-6           | 2                 | 7  | 12                      | 37              | Yang et al. (2012)          |
| *Bacillus* sp.                   | 1                 | 7  | 6                       | 81              | Abd-Elsalam & El-Hanafy (2009) |
| *Aneurinibacillus aneurinilyticus* | 0.25              | 7.6| 6                       | 43              | Raj et al. (2007)           |

**Figure 4** | Time course of bacterial growth and lignin degradation at 40 °C, over 7 days of incubation. Data represented as a means of triplicate experiments. (a) *B. altitudinis* SL7 growth and lignin degradation (b) Color reduction.
FT-IR spectroscopy

The samples were analyzed for changes in the polymeric structure of lignin after treatment with *B. altitudinis* SL7 through FT-IR spectroscopy. The spectra of test samples showed a decrease in absorbance around wavenumber 3,500–3,000 cm\(^{-1}\) that attributed to the stretching frequency of -OH bonds of alcohol and phenol in lignin, which indicates lignin degradation.

**Figure 5** | FT-IR spectra of lignin after incubation in degradation medium with *Bacillus altitudinis* SL7 for 7 days. (a) Control; (b) Test sample.
Figure 6 | GC chromatographs of control and lignin degraded sample. Degradation products were identified according to the retention time and mass spectra at specific retention time (a) Control (b) Lignin degraded sample by Bacillus altitudinis SL7.
B. altitudinis SL7 not only caused the oxidation of side chain but also transformed and degraded the aromatic skeleton of lignin. There is a marked difference observed in the fingerprint region between 1,800 and 600 cm\(^{-1}\). The absorbencies and shapes of the peaks in the region of 1,600–1,400 cm\(^{-1}\) has been changed, which corresponds to the stretching of C=C bonds in the aromatic skeleton of lignin, meaning that aromatic ring was destroyed during degradation (Wang et al. 2021). The ligninolytic enzymes specifically attack C=C bonds in aromatic skeleton of lignin that leads to enzymatic depolymerization of lignin structure (Zeng et al. 2014). Two new peaks appeared at 1,079 and 1,045 cm\(^{-1}\) while some peaks disappeared from the spectrum around 1,375–1,260 cm\(^{-1}\) indicating C-O stretching. These new bands indicate that the guaiacyl (G) and syringyl (S) groups transformed and converted into simpler compounds, such as ethers, phenol and alcohols (Kumar et al. 2015). Increase in intensity of peaks represent the production of high amount of lignin degradation products (Sonkar et al. 2019). It could be inferred from the findings that B. altitudinis SL7 chemically modified and degraded lignin.

**Analysis of lignin degradation products by GC-MS**

The degradation of alkali lignin by *Bacillus altitudinis* SL7 was determined by analyzing degradation products through GC-MS. Figure 6 shows Total Ion Chromatograph (TIC) of low molecular weight compounds extracted with ethyl acetate from acidic supernatant of both test and control samples. The compounds were identified on basis of their retention time (RT) and mass to charge ratio (Table 4). About 9 low molecular weight products were detected in test sample, identified as glutamic acid (RT-2.27), oxalic acid (RT-2.53), succinic acid (RT-2.65), vanillin (RT-3.62), 3-methylphenol (RT-6.38), 2-methoxyphenol (RT-8.12), ethyl vanillin (RT-10.15), ferulic acid (RT-10.52) and styrene (RT-12.21). Detection of phenolic compounds was clear indication of lignin degradation because these compounds were considered as the basic structural units of the lignin polymer. The presence of various phenolic and chlorinated compounds in lignin degradation medium were previously reported by several researchers (Sonkar et al. 2019). Formation of acidic compounds during lignin degradation was possibly due to the enzymatic degradation of phenolic side chain into ketones, which were further degraded through Cα–Cβ cleavage and forms acids (Yang et al. 2012). Raj et al. (2007) reported the identification of vanillin, guaiacol, gallic and ferulic acid during lignin degradation by *Bacillus* sp. These results confirmed the oxidation of coniferyl (G) and sinapyl (S) groups of lignin polymer. Previously, it has been reported that during fungal degradation of lignosulfonate and bacterial degradation

| Retention time (Min) | Product name       | Product structure | m/z |
|----------------------|--------------------|-------------------|-----|
| 2.275                | Glutamic acid      | ![Glutamic acid structure](image) | 147 |
| 2.53                 | Oxalic acid        | ![Oxalic acid structure](image) | 91  |
| 2.65                 | Succinic acid      | ![Succinic acid structure](image) | 119 |
| 3.629                | Vanillin           | ![Vanillin structure](image) | 148 |
| 6.381                | 3-methylphenol     | ![3-methylphenol structure](image) | 104 |
| 8.12                 | 2-methoxyphenol    | ![2-methoxyphenol structure](image) | 129 |
| 10.15                | Ethyl vanillin     | ![Ethyl vanillin structure](image) | 167 |
| 10.32                | Ferulic acid       | ![Ferulic acid structure](image) | 190 |
| 12.2                 | Styrene            | ![Styrene structure](image) | 169 |
of lignin, the above degradation compounds were also detected (Jiang et al. 2020). The peak at RT 5.09 was observed both in test and control samples, it could be due to lignin that was not detected on mass spectrometer because of its high molecular weight and out of the range of m/z ratio.

CONCLUSION

The study was aimed to explore the potential of lignin-degrading bacteria from the sludge of pulp and paper mill. Present study confirmed the degradation and decolorization of lignin by Bacillus altitudinis SL7 at mesophilic temperature and alkaline pH. FT-IR analysis confirmed the changes in functional groups of lignin treated by bacteria. GC-MS analysis of control and bacterial treated samples showed metabolization and transformation of alkaline lignin into low molecular weight compounds such as, glutamic acid, oxalic acid, succinic acid, vanillin, methoxyphenol and ferulic acid. B. altitudinis SL7 has the potential to degrade lignin under alkaline pH ranges from 7.0–10.0 as compared to other reported lignin degrading bacterial species, therefore no additional adjustment of pH is required during lignin degradation. The presence of extracellular laccase enzyme favors the idea of enzymatic degradation of lignin. It is finally concluded that Bacillus altitudinis SL7 could be of interest for the degradation of high load of lignin from pulp and paper mills effluent.

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CONFLICT OF INTEREST

I hereby confirm that this manuscript is our original work. It has neither been published before in any form nor is under consideration by another journal at the same time as in this journal. All the authors have no financial as well as commercial conflict of interest regarding this manuscript and have approved its submission to Water Science and Technology.

DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

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