Development of a biofilm-forming bacterial consortium and quorum sensing molecules for the degradation of lignin-containing organic pollutants

Sangeeta Yadav[a b,] *, Sonam Tripathi[c], Diane Purchase[d], Ram Chandra [c,] *

[a] Department of Microbiology, Dr. Shakuntala Misra National Rehabilitation University, Lucknow-226017(Uttar Pradesh), India

[b]Department of Botany, Vaishno Devi Prashikshan Mahavidyalaya, Godahi, Kunda, Pratapgarh, (Uttar Pradesh), India

[c]Department of Environmental Microbiology, School for Environmental Sciences, Babasaheb Bhimrao Ambedkar University (A Central University), Vidya Vihar, Raebareli Road, Lucknow-226025 (Uttar Pradesh), India

[d]Department of Natural Sciences, Faculty of Science and Technology, Middlesex University, The Burroughs, London, UK.

*Corresponding authors

E-mail: dr.sangeeta.bbau@gmail.com ; sangeeta_biotech12@rediffmail.com

prof.chandrabbau@gmail.com

Received 26 September 2022, Revised 18 February 2023, Accepted 3 March 2023, Available online 14 March 2023.

Highlights

- Quorum sensing (QS) induced biofilm formation.
- Biofilm formation was maximum by bacterial consortium.
- Lignin (900 ppm) reduction was maximum 73% by consortium.
- Treatment of lignin by QS based biofilm lead an effective approach to treat effluent.

Abstract

The presence of lignin along with other pollutants makes effluent more complex when it is discharged from Pulp and paper mills. The present study investigates the use of biofilm-forming bacteria isolated from pulp paper mill effluent contaminated sites (PPMECSs) for lignin degradation. Isolated biofilm-forming and lignin-degrading bacteria were identified as *Bacillus subtilis*, *Enterobacter cancerogenus,* and *Bacillus licheniformis* by 16S rRNA gene sequencing. Thin liquid chromatography (TLC) analysis showed that the consortium of bacteria produced acyl-homoserine lactone (AHL) as quorum sensing molecules and extracellular polymeric substances (EPS) that protect the bacterial consortium under unfavorable conditions. The potential consortium was able to reduce lignin (900 ppm) by 73% after 8 days of incubation in a minimal salt medium containing kraft lignin and glucose at pH 7.0 and 37°C as compared to individual strains. The degradation by-products were identified as amides, alcohols, and acids. The major organic pollutants in the effluent were reduced after treatment of the constructed consortium, thus confirming active biotransformation and biodegradation of the lignin. Microscopic examination also indicated the presence of lignin induced biofilm formation. Hence, the constructed biofilm-forming bacterial consortia based on quorum sensing offered a sustainable and effective solution to treat lignin-containing complex pollutants.

Keywords: Acyl-Homoserine Lactone, Biofilm, Lignin degradation, Pulp and paper mill, Signaling molecules, Quorum sensing

Abbreviations

AHL- Acyl-homoserine lactone

AOX- adsorbable organic halides

BFB- biofilm-forming bacteria

EPS- Extracellular polymeric substances

FTIR-Fourier transform infrared spectroscopy

GC-MS-Gas chromatography/mass spectrometry

HPLC-High-performance liquid chromatography

MSM: Mineral Salt Media

PBS-Phosphate buffer saline

PPM - Parts per million

PPMECSs- Pulp paper mill effluent contaminated sites

OD: Optical density

QS: Quorum Sensing

SD-Standard Deviation

SEM: Scanning Electron Microscopy

TLC- Thin liquid chromatography

V/V: Volume/Volume

W/V: Weight/Volume

Introduction

The pulp and paper industry plays an important role as one of the world's most important industrial sectors. It is, nevertheless, a large consumer of natural resources and energy (fossil fuels, electricity), as well as a significant polluter. The pulp and paper industry is the third-largest wastewater producer, and the sixth largest polluter producing air emissions, wastewater, and solid wastes; their effective treatment and proper disposal are essential to safeguard the environment and protect human health. Lignin is the most prevalent contaminant in the pulp bleaching process, and it is released in the effluent after pulp washing (Yadav and Chandra, 2018). Lignin and its derivatives are highly persistent that resulting in the dark brown color in

the effluent. This wastewater has a high biochemical oxygen demand (BOD) and chemical oxygen demand (COD), as well as elevated levels of chlorinated compounds measured by adsorbable organic halides (AOX), cellulose, hemicellulose, suspended solids, fatty acids, tannins, resin acids, sulfur, and lignin and its derivatives, if not properly treated (Singh and Tripathi, 2020). In addition to the naturally occurring wood extractives like tannins, resin acids, lignin, cellulose, and hemicellulose; the effluent also contains acute or even chronic toxins contaminants and endocrine disruptive chemicals (Damjan et al., 2022). There is increased concern about the potential adverse effects of genotoxicants that are mutagenic on aquatic flora and fauna, and human health through water reuse for the irrigation of crops (Haq et al., 2017). Therefore, various physical, chemical, and biological strategies are being developed to protect and sustain the environment. Many bacterial species e.g. *Pseudomonas aeruginosa, Staphylococcus aureus* and *Bacillus sp*. can produce chemicals that play critical roles in cell signaling processes or regulate the expression of certain genes in response to changes in population density that imitate their ability to treat wastewater biologically and achieve a high level of cooperation (Anburajan et al., 2021; Yong et al., 2015).

Many bacteria can cooperate and coordinate in adverse conditions through quorum sensing (QS). Many biological processes like bioluminescence, antibiotic synthesis, mobility, siderophore formation, horizontal gene transfer, virulence factor production and biofilm formation are governed by QS. Bacteria are known to produce cell aggregates called mats, flocs, or biofilms, which are necessary for their survival. The extracellular matrix, also known as EPS, is a collection of polymers to which cells aggregate and adhere. It is one of the most important components of biofilm. Polysaccharides, proteins, nucleic acids, lipids, and humic substances are all components of EPS, which affect the biofilm's hydrophobicity, biodegradability, and adsorption characteristics. Dispersion processes in the biofilm matrix are influenced by the biofilm's capacity to absorb water and its movement. Biofilm-mediated

bioremediation is an efficient and safe alternative to planktonic microorganism-mediated bioremediation. As these cells are shielded by the polysaccharide matrix in a biofilm, they have a higher chance of adapting and surviving during stressful conditions. Biofilms are employed in industrial plants to aid in the immobilization and breakdown of organic pollutants because of the tight, mutually advantageous physical and physiological interactions between bacteria in biofilms. This study proposed a novel biofilm-forming bacterial consortium to treat pulp paper wastewater and aimed to understand the lignin degradation mechanisms via comparative analysis of planktonic and biofilm-mediated lignin degradation. This work shows that it is possible to effectively bioremediate the organic and inorganic contaminants found in pulp and paper mill effluent by using adapted microbial consortia in wastewater containing lignin.

2. Materials and methods

2.1. Study site

PPMECSs sludge samples were collected from Tamil Nadu Newsprint and paper limited (TNPL), Chennai, India (13.0113°N, 80.21916°E). This site is highly polluted with complex pollutants discharged from the paper industry. To isolate the bacterial population PPMECS samples were collected from disposal sites which were contaminated with pulp paper mill effluent. Sludge was collected at approximately 1-6 cm depth from the soil surface and collected into sterile containers (Perera et al., 2019). The temperature of the collection site was 40 °C and the sample pH was 8.5. Collected samples were stored at 4 °C before analysis.

2.2. Purification and characterization of biofilm-forming bacteria (BFB)

For isolation of biofilm-forming bacteria through serial dilution techniques. The morphology of isolated strains was examined according to the standard biochemical protocol (Barrow and Feltham, 1993). The isolate was further identified using 16S rRNA gene sequencing. The CLUSTAL W software was used to align the first ten consensus sequences of the 16S rRNA gene (Tamura et al., 2013). Subsequently, the colonies with diverse morphology were selected

and purified on lignin (400 ppm) amended MSM plate [composition in % (g L^{-1}); glucose 1.0%; peptone 0.1%; K_2HPO_4 0.1% and $MgSO_4.7H_2O$ 0.05%] and incubated at 35±2°C for 24 h. to obtain a pure culture. The bacterial isolates were screened for their ability to form biofilm by test tube and microtiter plate assay as per the method described by Priyadarshanee and Das (2021). In this process, 10 µL of overnight bacterial suspension was inoculated into 2 mL TSB broth and incubated at 37°C for 24 h without agitation. The culture was then removed, and tubes were washed thrice with phosphate buffer saline (PBS, pH=7.2). The adhering biofilm was fixed for 10 min with 2 mL methanol, air-dried for 30 min, and then stained for 10-15 min with 2 mL crystal violet (1 %, v/v). The unattached stain was removed by washing with PBS and air-dried for 30 min in an inverted position. The attached dye was removed by the addition of 2 mL of 33% v/v glacial acetic acid. In a UV-VIS spectrophotometer, the optical density (OD) of samples was evaluated at 620nm (Thermo Electron; Model IRIS Intrepid II XDL, USA (Stepanovic et al., 2004). Scanning electron microscopy (SEM) was used to analyze the biofilm architectures per the described methods by Dey and Paul (2018).

$$Biofilm\ formation\ (BF) = (OD_{620}\ of\ bacteria\ containing\ tube\ with\ crystal\ violet - OD_{620}\ control\ tubes\ with\ crystal\ violet)$$

Equation (1)

2.3. Lignin degradation by planktonic biofilm-forming bacteria

The bacterial biofilms and planktonic cells from the consortium were examined for their ability to reduce different concentrations of lignin: 100, 300, 500, 700, 900, and 1100 ppm (w/v) in mineral salt media (MSM). The pH of the broth initially is alkaline to dissolve lignin in the MSM broth. Further, the pH of the broth was adjusted to 7.2 after the lignin was dissolved. The selection of the maximum limit of lignin degradation by the consortium was based on the ability of the bacteria to utilize lignin. Bacterial strains showed growth up to 900 ppm lignin-containing MSM media. Hence, this concentration was used for further study. The biofilm was obtained from an overnight culture growing in lignin (400 ppm) containing MSM medium.

The planktonic cell culture was carefully transferred to a sterile flask without damaging the biofilm. The attached biofilm on the flask wall was washed using PBS. A 200 mL lignin-containing fresh MSM was added to the flask. The planktonic cells were harvested by centrifugation (6000 x g for 15 min), and the cell pellets were transferred to fresh lignin-containing MSM media. The flasks were incubated at the optimized condition in a shaker (New Brunswick Scientific, Excella E24) for 8 days. A spectrophotometer (Chemito Instruments UV 2600, India) was used to measure bacterial growth at 620 nm. The amount of lignin in the broth was calculated using the Pearl and Benson method (1990).

2. 4. Estimation of extracellular polymeric substances and characterization

The EPS produced during lignin degradation was examined. The EPS was first extracted using EDTA and precipitated by pre-chilled ethanol (Nisha and Thangavel, 2014). Lowry method was used to measure the EPS compositions such as carbohydrate and protein (Dey and Paul, 2018) and Fourier Transform Infrared Spectroscopy (Thermo scientific, Nickolet-6700, USA) was used to characterize the EPS chemical structure.

2.5. Biofilm assays and confocal laser scanning microscopy (CLMS)

CLSM was used to examine the biofilm load in the presence of lignin (Dasgupta et al., 2013). Briefly, the isolated strains were grown on a tilted glass slide in 100 mL of MSM containing lignin in a 250 mL flask. Flasks were inoculated at 35±2 °C for 24 h. After incubation, the slides were washed with 1x PBS three times and stained with 0.005% acridine orange (w/v) for 5 minutes in dark.

2.6. Identifying N-acyl homoserine lactone signal molecule by thin-layer chromatography

The samples from the lignin degradation experiment (section 2.3.) were removed by centrifugation for 5 min at 6000x g, and the supernatant was extracted twice with an equal volume of ethyl acetate. The extract was pooled and dried over anhydrous magnesium sulfate, then filtered and evaporated to dryness. The residues were dissolved in 50µL of HPLC grade

acetonitrile. A sample (1-4µL) along with a standard of AHL molecules were applied to the TLC plate (200□m layer) and the chromatogram was developed with methanol/water (60:40 V/V). Hexanoyl DL-Homoserine Lactone, Octanoyl Homoserine Lactone and N(3-Oxodecanoyl L-HomoserineLactone were purchased from Sigma Aldrich. The plates were dried and covered with the culture of *Agrobacterium tumefaciens* as an indicator bacterium prepared as per the method described by Paul et al. (1997)

2.7. HPLC and GC-MS analysis

The bacterial biomass and particle matter in the bacterial treated and untreated samples were separated by centrifugation for the degradation research (5000 x g for 10 min). The supernatant was extracted thrice with an equal volume of ethyl acetate and analyzed using HPLC as per the methods described by Yadav and Chandra (2021). HPLC equipped with UV–vis (Waters-2487, Milford, USA) lignin analysis was carried out at 280 nm. The GC–MS analysis (Perkin Elmer, UK) was used to identify the metabolic products after bacterial degradation. The organic pollutants were identified by comparing their mass spectra with that of the National Institute of Standards and Technology (NIST) library available with the instrument and by comparing the retention time (RT) with those of available authentic organic compounds. Microsoft Excel (2010) was used to compute the mean and standard deviation (SD) of the data.

3. Results and Discussion

3.1. Identification of biofilm-forming bacteria

A total of 29 bacterial strains (IITRSY-1 - IITRSY -29) were isolated from the pulp paper mill sludge. These colonies (in triplicate) were subcultured on lignin-containing MSM agar plates and differentiated by their colony morphology and pigmentation. Fourteen isolates that were shown to produce biofilm were selected for further analysis. Of these, 3 bacteria (IITRSY-16, IITRSY-22, and IITRSY-23) showed strong biofilm formation in presence of lignin (Fig. 1)

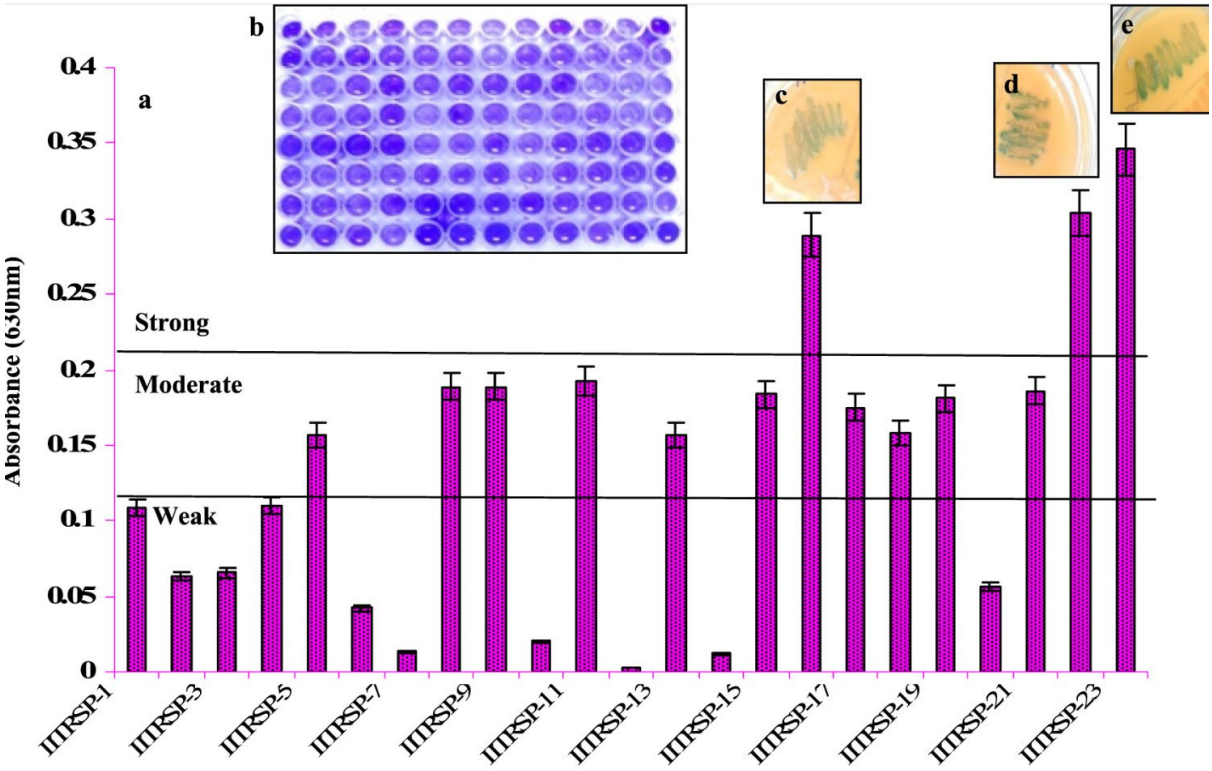

Fig. 1. (a–e). Biofilm formation of isolated bacterial strains (a), Weak biofilm formation (OD630nm < 0.120), M = Moderate biofilm formation (0.11-0.24 OD630nm), S = strong biofilm formation (>0.24 OD630nm). Inserted picture 1st showed the result of 96 well microtiter plate assay (b) and production of quorum sensing signal molecules on the X-Gal containing LB plate (c–e). IITRSP-1 to IITRSP-23 strains correspond to ITRCSPY-16, and ITRCSY-22.

Gram staining of potential bacterial strains is shown in Fig. 2a. The PCR products of the biofilm-forming bacteria using universal primers eventually produced ~1700 bp fragment. The partial nucleotide sequence of IITRSY-16 (1539 bp), IITRSY-22 (1587bp), and IITRSY-23 (1606 bp) were compared with the available database (http://www.ncbi.nlm.nih.gov/) using BLAST which showed maximum similarity (98.74%, 98.71%, and 99.12%) with *Bacillus subtilis*, *Enterobacter cancerogenus,* and *Bacillus licheniformis*, respectively. The phylogenetic tree using MEGA-6 and the neighbor-joining method was predicated on the availability of 16S rRNA gene sequences in the GenBank database (Fig. 2b). The 16S rRNA gene sequence investigation of bacterial strains IITRSY-16, IITRSY-22, and IITRSY-23 have shown that these bacterial strains have the closest relatedness with *Bacillus subtilis*,

Enterobacter cancerogenus, and *Bacillus licheniformis*, respectively with accession numbers KX379716, KX379717, and KX379718, respectively (Fig. 2b). A number of Bacillus species have been shown to be able to degrade kraft lignin (Lee et al., 2019). *Bacillus subtilis* and *Bacillus licheniformis* in bioremediation and phytoremediation of heavy metals have also been demonstrated by several workers which can form biofilms and spores under stress conditions (Kalaycı et al., 2021; Mohsin et al., 2021; Yahya et al., 2016). Interestingly, *E. cancergenus* has only previously been reported as a human pathogen (Demir et al., 2014).

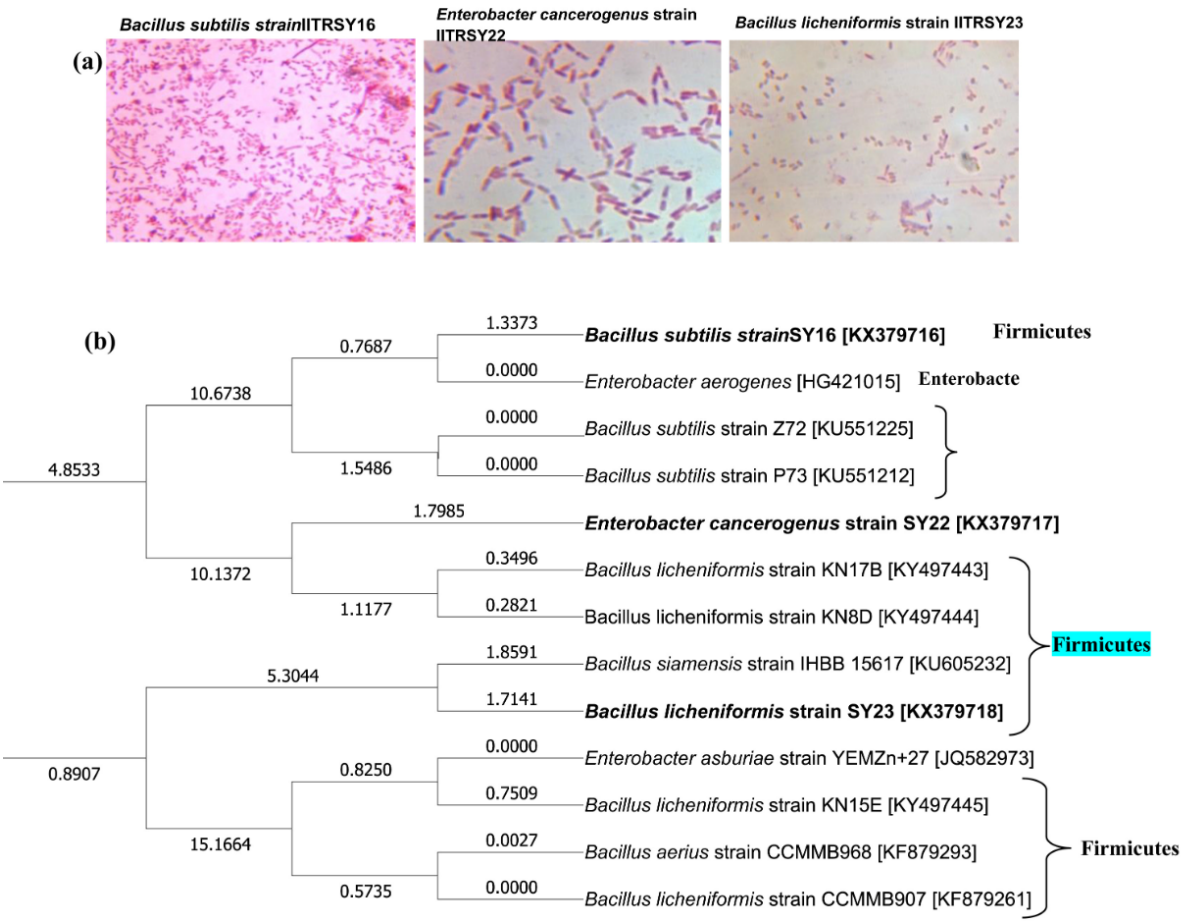

Fig. 2. a The microscopic examination of the isolates after gram staining. **2b.** Molecular phylogenetic analysis by maximum likelihood method. The analysis involved 13 nucleotide sequences. codon positions included were 1st+2nd+3rd + noncoding. All positions containing gaps and missing data were eliminated. There were a total of 1507 positions in the final dataset. Evolutionary analyses were conducted in MEGA6.

3.2. Assessment of lignin degradation using bacterial consortia

Fig. 3 shows comparative bacterial growth and biofilm development during lignin degradation. *Enterobacter cancerogenus* (OD: 2.55 ± 0.01), *Bacillus subtilis*, (OD: 2.01 ± 0.01), and *Bacillus licheniformis* (OD: 1.86 ± 0.01) were noted for maximum planktonic growth. The pattern of biofilm formation was like the growth pattern i.e. *Enterobacter cancerogenus* (OD: 2.56 ± 0.01), *Bacillus subtilis*, (OD: 2.54 ± 0.01), and *Bacillus licheniformis* (OD: 2.32 ± 0.01). Moreover, the consortium of these three isolates showed greater development of plankton and biofilms than pure culture (Fig. 3). This finding has been corroborated with previous findings which showed microorganisms acting in a consortium can have higher biodegradation efficiencies than individual strains (Cao et al., 2022).

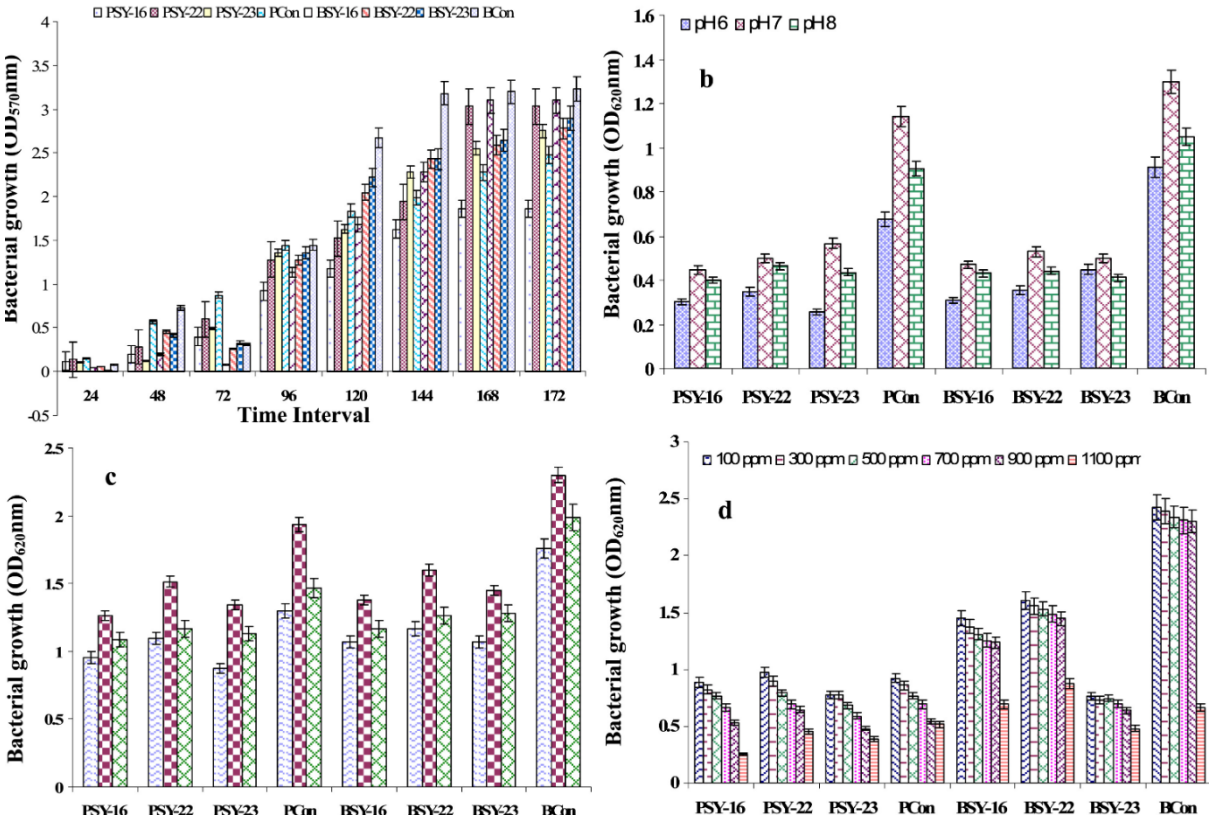

Fig. 3. Comparative growth evaluation of planktonic and biofilm formation at a different time intervals **(a)**, pH **(b)**, temperature **(c)**, and various concentrations of lignin **(d)**. The values are presented as the mean ± SD (n = 3) of triplicate experiments. PSY16, 22, 23: planktonic growth of strain IITRSY16, IITRSY22, IITRSY23; BSY16, 22, 23: biofilm-forming bacterial growth of strain IITRSY16, IITRSY22, IITRSY23; Con-consortium.

The affinity of bacterial strains in the consortium was also checked through Gram staining and found that the growth of *Enterobacter cancerogenus* was dominant, this might be due to the survival potential of the strains (Dhanya et al., 2021). *E. cancerogenus* has been reported as a cellulose degrader and exopolysaccharides (EPS) producer (Dhanya et al, 2021; Flimban et al., 2019). The EPS produced by *E. cancerogenus* in this study could have contributed to the biofilm formation and enhanced their survival. The consortium was found to be most effective in biofilm production, this might be due to synergistic induction and co-metabolism of multispecies biofilm formation via quorum sensing (Jing et al., 2020; Nikolina et al., 2018). Fig. 3a showed the biofilm production at different time intervals. All three bacterial isolates demonstrated unique planktonic growth and biofilm formation over the incubation period of 1 to 8 days. The planktonic growth of all tested strains started after 24 h, while biofilm formations for these strains started after 48 h of incubation. Maximum planktonic growth in *Bacillus subtilis* and *Bacillus licheniformis* was observed on day 3, while the highest development of biofilms was recorded on day 4. *Enterobacter cancerogenus* showed a lag in optimal planktonic growth with biofilm development, the highest level was noted on days 4 and 5, respectively. Interestingly, the consortium demonstrated a further lag where optimal planktonic growth with biofilm development on days 5 and 6 days, respectively. This is probably reflecting the strains were competing for the same nutrient resources which impacted their optimal growth. Effects of pH, temperature, and lignin concentration were also evaluated on the planktonic and biofilm growth. Results showed that these parameters played a key role in planktonic and biofilm development. All three isolates showed pH 7 was optimal for planktonic growth levels and biofilm development (Fig. 3b). Earlier studies also found that this is favorable pH for biofilm formation for most bacterial strains (Schultze et al., 2021; Morohoshi et al., 2018). Neutral pH might be effective for mRNA expression to produce autoinducers in isolated potential bacterial strains. Schultze et al. (2021) showed that acidic and alkaline pH is also favorable for several

bacteria. Bacterial community composition and AHL level that led to biofilm formation are significantly affected by the pH of the medium (Liu et al., 2021). In addition, 37°C was found best temperature for biofilm formation (Fig. 3c), this agrees with the previous finding of Zhang et al. (2021a) where the best biofilm formation was found between 25–37°C.The bacterial growth under different concentrations of lignin is presented in Fig. 3d. Compared to the control, the presence of lignin appeared to limit the planktonic growth of individual strains but encouraged biofilm formation (increased by 25-30%). The formation of biofilm by the consortium similarly increased by about 34% but there was no adverse effect on the planktonic growth. The increased concentration of lignin decreased bacterial growth and 900 ppm of lignin was found to be the maximum concentration that could be tolerated by the consortium (Fig. 3d). Hence, 900 ppm lignin concentration was chosen for further study. Degradation of wastewater discharged by various industries by biofilm-forming bacteria has been reported by several researchers, but in pulp paper mill effluent and lignin degradation are completely unknown (Dey and Poul, 2018; Kumari et al., 2016). Zhang et al (2021b) showed lignin (0.5 g/L) degradation by a microbial consortium of *Shinella sp*., *Cuprividus sp.* and *Bosea sp.* only up to 54% after 48 h of incubation. While in the present study, lignin degradation was observed in the MSM broth amended with 900ppm lignin. Results showed that the maximum degradation achieved by the bacterial consortium was 73% as recorded by the consortium on day 6 of incubation. The axenic culture *Bacillus subtilis*, *Enterobacter cancerogenus,* and *Bacillus licheniformis* could decolorize lignin up to 36, 39, and 34%, respectively (Fig. 4a). Moreover, there was a lag in the reduction in color and lignin content in the initial 48 h incubation period. This might be due to the acclimatization of bacterial strains at the initial stage of their growth, having exhausted the simple carbon and nitrogen source such as glucose and peptone and switched to the subsequent utilization of the come complex lignin as a nutrient. Thereafter, a sharp reduction in lignin content was observed up to day 6 and this reduction might be

attributed to the lignin depolymerization by oxidizing enzyme and biofilm formation. This significant reduction in color was observed at the end of the experiment (i.e., after day 8) indicating a co-metabolism process for lignin degradation as reported by Raj et al (2007). Similarly, in a previous study, heavy metals and various pollutants triggered more biofilm formation by potential strains (Maddela et al., 2019). EPS matrix is a promising candidate for pollutant reduction and adsorption. The production of a biofilm by isolated potential strains in a lignin stress environment showed that these isolates could be used in a biofilm bioreactor to remediate pulp mill wastewater.

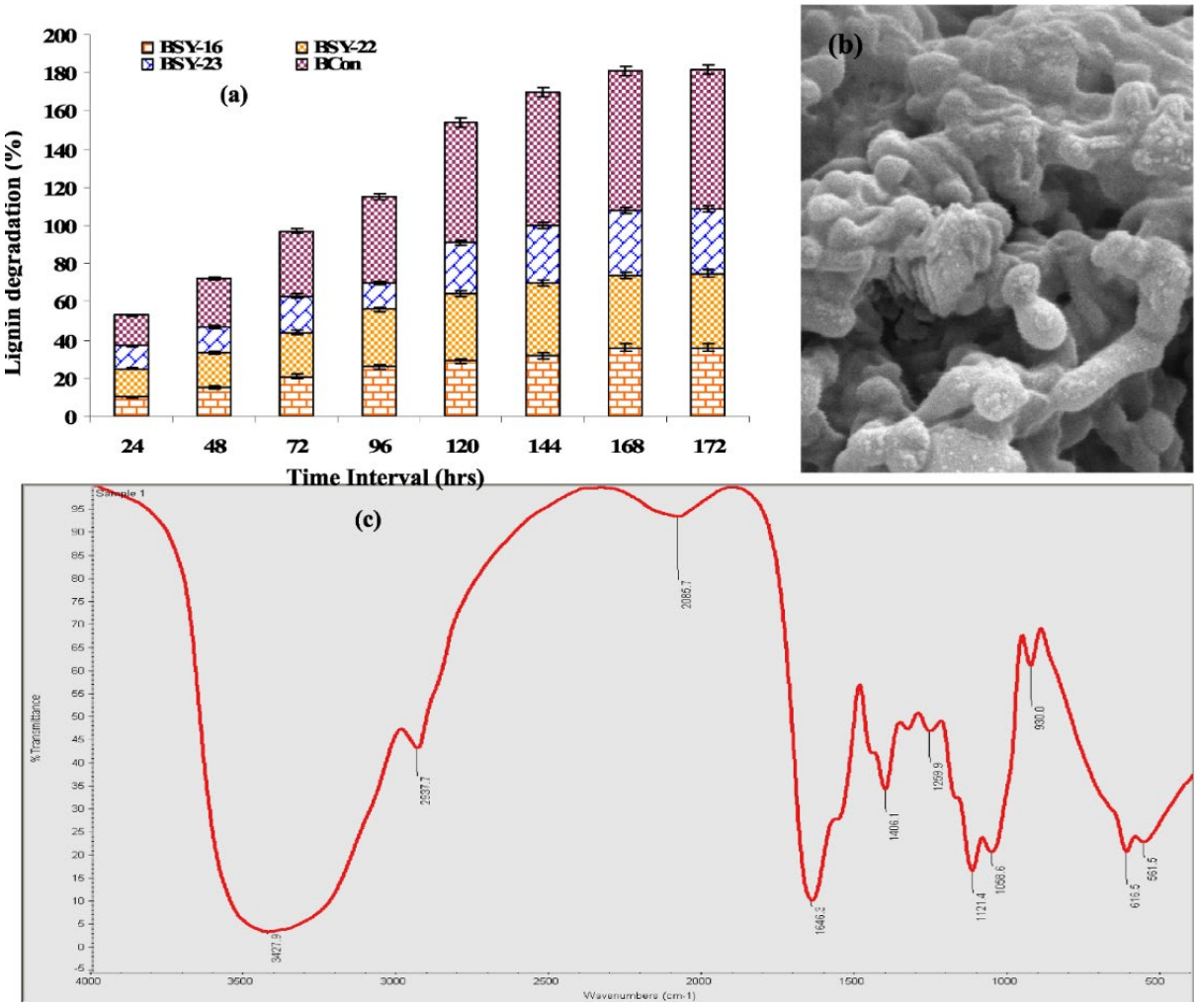

Fig. 4. (a–c). Showing lignin degradation by biofilm-forming potential bacterial strains **(a)**, SEM view of consortium **(b)** and FTIR spectra of EPS extract **(c)** during lignin degradation.

3.3. Morphological view, functional groups of EPS molecules

SEM investigation validated the consortium's production of biofilm on the glass slide surface during lignin breakdown. Results showed individual rod-shaped cells surrounded by a matrix of polymeric substances and connection between the cells (Fig. 4b). The attachment of bacteria to the surface via cell surface proteins, pili, and flagella begins the construction of a biofilm, which is followed by micro colonization and the production of a mature biofilm (Das and Samal, 2018). It was observed that EPS and carbohydrate production by the consortium was the maximum at day 4 of the lignin degradation experiment ($3x10^2$ and $55x10^2$ gm mL^{-1}, respectively) as compared to individual strains (data not shown). EPS plays an important role in microbial aggregation, prevention of pollutants entry into the cell and pollutants degradation. FTIR spectra of lignin degradation by the consortium showed stretching at 3427, 2937, 2085, 1646, 1406, 1255, 1121, 1058, 930, 616 and 561 cm^{-1} (Fig. 4c). Results showed several different functional groups present on the bacterial cell surface for lignin binding. The IR peaks found at 3427, 2937, and 2085 cm^{-1}in spectra of consortium are noticed to be the -OH stretching of the alcoholic or phenolic group, alkenes stretch, and NH_3 structure, respectively which plays an active role in pollutants binding as suggested by earlier workers (Mishra et al., 2021; Kalola and Desai, 2020). The IR peaks were observed at wave numbers 1646 cm^{-1} in spectra of the sample assigned to the amide I group (C-O stretch) and these peaks have the capacity to bind with metals, hence these consortia have the potential to also remove the metals in the wastewater. IR absorption peaks were observed at 1406 cm^{-1} in the spectra of the consortium, indicating the presence of carbonate binding. Wavelength 930 cm^{-1} indicates C-O and 561 cm^{-1} indicates alkyl halide. These findings showed the complex nature of EPS extracted during lignin degradation by the biofilm-forming consortium. Overall, the results showed that the amide, carboxyl, phenyl, hydroxyl, and carbonyl groups are active surface functional groups that interact with lignin (Mishra et al., 2021). Cell surface functional groups play a very

important role during the degradation or utilization of any pollutants present in the surrounding environment. To confirm biofilm formation during lignin degradation, confocal microscopy was carried out and the results are presented in Fig. 5. Results showed that bacterial aggregation was found higher in presence of lignin, while aggregation was absent without lignin. It might be due to lignin induced the production of autoinducer to lead to biofilm formation due to quorum sensing.

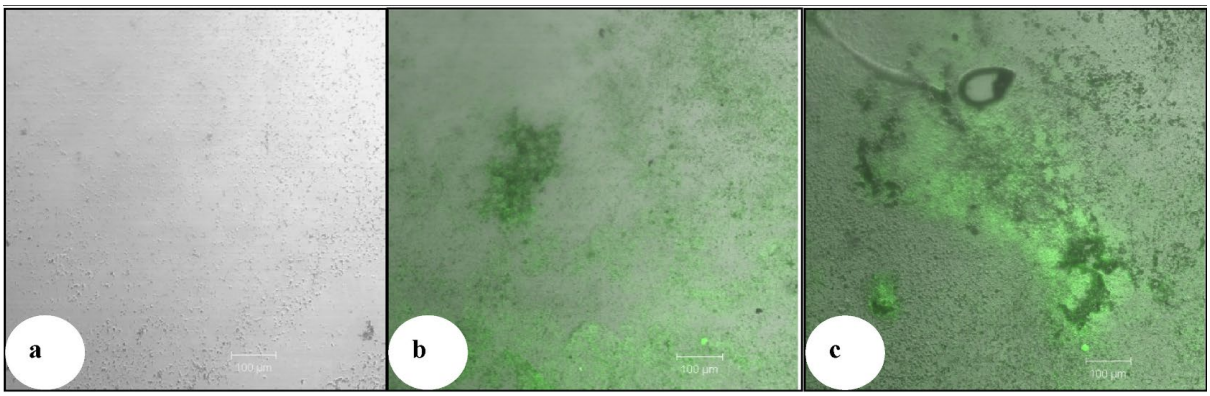

Fig. 5. Confocal laser scanning microscopy image of biofilm formation during lignin degradation by consortium. Without lignin **(a)**, lignin degradation after 4 days incubation **(b)** and lignin degradation after 8 days incubation **(c)**.

3.4. Identification of quorum sensing molecules

To understand the mechanism of lignin degradation TLC observation of AHL molecules secreted by the isolates were analyzed and results are shown in Fig. 6. All three isolates *Bacillus subtilis, Enterobacter cancerogenus* and *Bacillus licheniformi* produced high molecular weight AHL Octanoyl Homoserine Lactone, N (3-Oxodecanoyl L-Homoserine Lactone and Hexanoyl DL-Homoserine Lactone, respectively. This observation strengthens the hypothesis that quorum sensing induced biofilm formation improved the degradation of pollutants and is corroborated by the finding of other researchers (Sarkar et al., 2020; Maddela et al., 2019). Avery interesting observation has been observed in this study that Gram-positive bacteria produce AHL as autoinducers, while AHL is mostly reported for Gram-negative bacteria. Only

a few studies showed that Gram-positive bacteria release AHL as an autoinducer (Biswa and Doble, 2013). This is completely new information in the field of bioremediation.

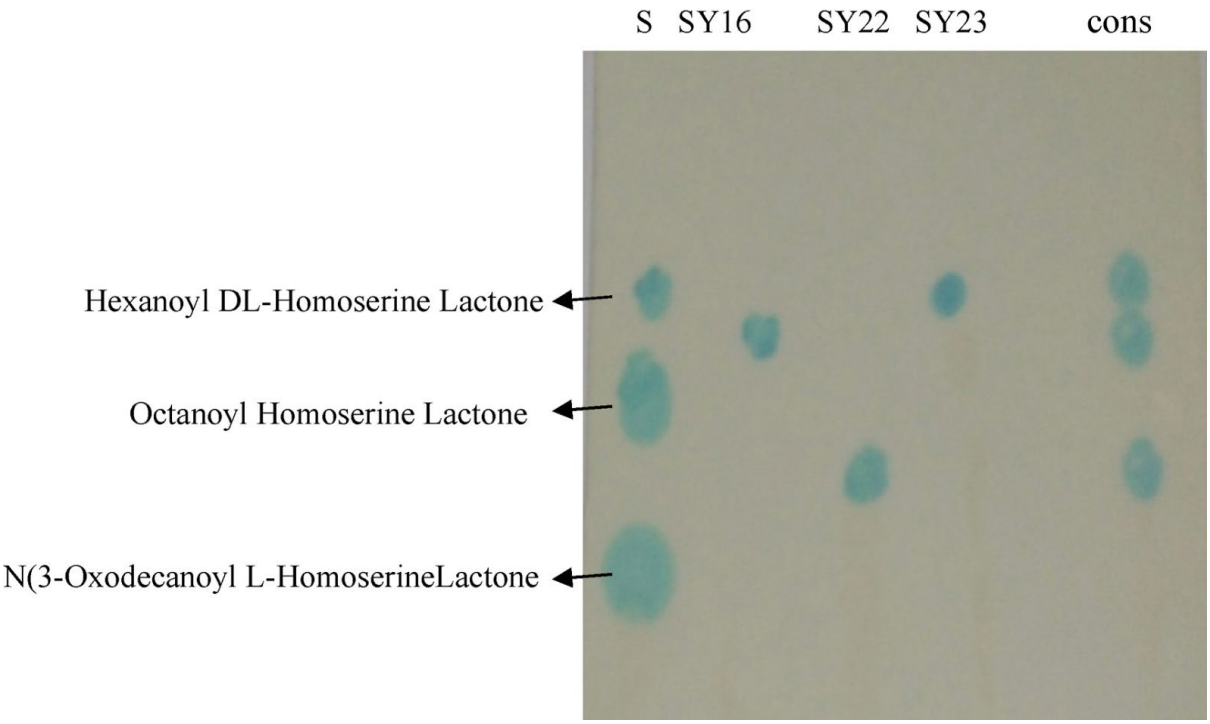

Fig. 6. TLC analysis of autoinducer molecules produced by biofilm-forming bacterial strains during degradation of lignin. S: Standard of different AHL molecules, Cons: consortium.

3.5. HPLC analysis of bacteria during lignin degradation

The degradation of lignin by the bacterial consortium was confirmed by the HPLC analysis (Fig. 7). Results indicated the capability of the bacterial consortium to decolorize and degrade lignin by its enzymatic action. *Bacillus subtilis* and *Bacillus licheniformis* have been explored by researchers for their lignocellulosic enzymes in many biotechnological applications (Da Silva et al., 2021; Malik and Jayed, 2021). Several enzymes have been reported to be involved with lignin degradation in Bacillus species, for example, the dye-decolorizing peroxidase DyP, in *B. subtilis* (Min et al., 2015) and multi-copper oxidase (laccase-like multicopper oxidase LMCO), CotA, in *B. subtilis* (Ihssen et al., 2015) and *B. licheniformis* (Koschorreck et al., 2008). Although *Enterobacter* sp. has been reported to produce a lignocellulosic enzyme

(Chukwuma et al., 2021), the ability of *Enterobacter cancerogenus* to degrade lignin has not been reported or investigated. This work presents new information to further explore the biodegradation potential of this bacterial strain. In addition, compared to the untreated sample, the HPLC chromatogram after day 8 showed peak shifting, a decrease in peak area height, and the creation of several new peaks. This demonstrates that a created bacterial consortium with biofilm-forming potential can biotransform and biodegrade lignin into different metabolites.

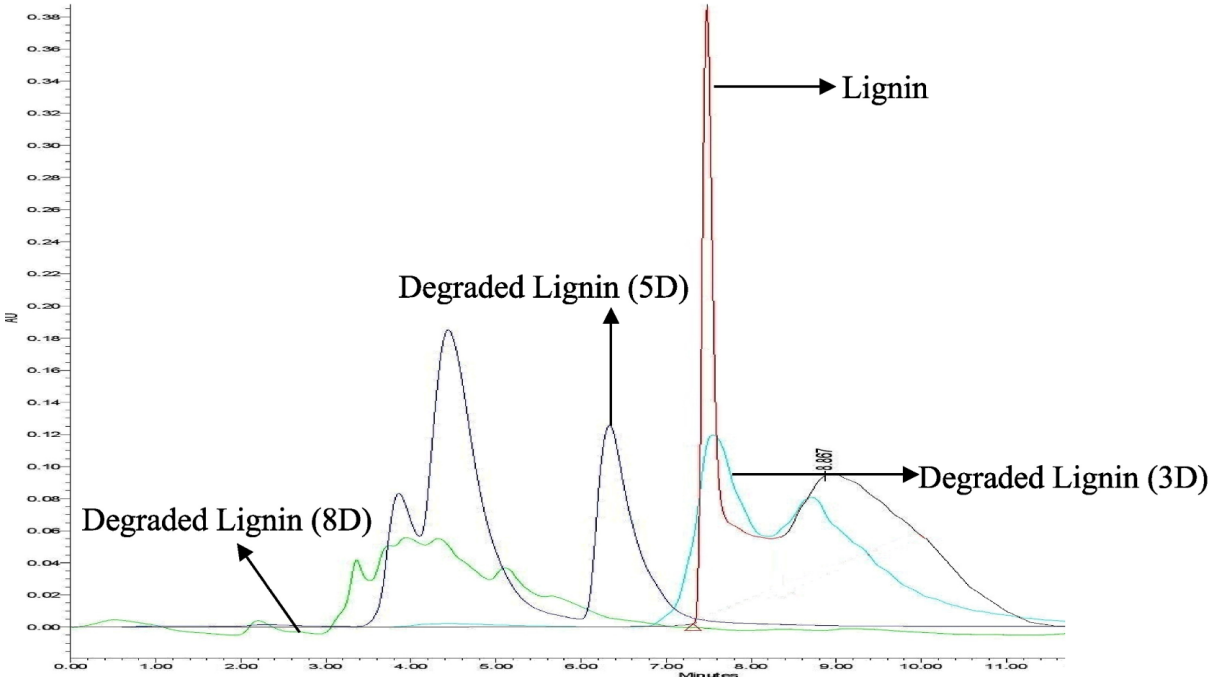

Fig. 7. Comparative HPLC chromatograph of control (lignin, 900 ppm) and lignin degraded by biofilm-forming potential bacterial strains.

3.6. Organic pollutant identification

The GC-MS spectrum of ethyl acetate extracted from lignin degraded sample exhibited several major and minor peaks at retention times 22.30, 23.13, 23.90, 24.70, 25.53, 25.80, 26.07, 26.99, 27.17, and 27.94 minas compared to control with a major signal peak at 22.67, 23.99, 25.48 and 33.08 (Fig 8 and Table 1). At RT 22.67, 23.99, 25.48 and 33.08 compounds were obtained which show 97% similarity with Tetrabromo-2-(3-hydroxy-1,2-dihydro-quinol-2-ylidene)-2,3-dihydro-1H-benz[f]indene; βdgalactofuranose;1,2 Benzene dicarboxylic acid, butyl octyl ester and Francheline, respectively. This study showed that the control sample showed the presence

of several compounds that are highly toxic organic pollutants, if untreated and disposed of improperly, they could cause significant damage to body organs and systems. RT 22.30 showed a similarity with 7,10,13-hexadecatrienoic acid methyl ester (90.08%), and RT 24.70 showed a similarity (92.76%) with hexadecenoic acid. Hexadecenoic acid was the most abundant, thus in agreement with the findings of Morrison and Akin (2001), researchers discovered palmitic acid to be the most abundant fatty acid in fiber extracts from several flux varieties.

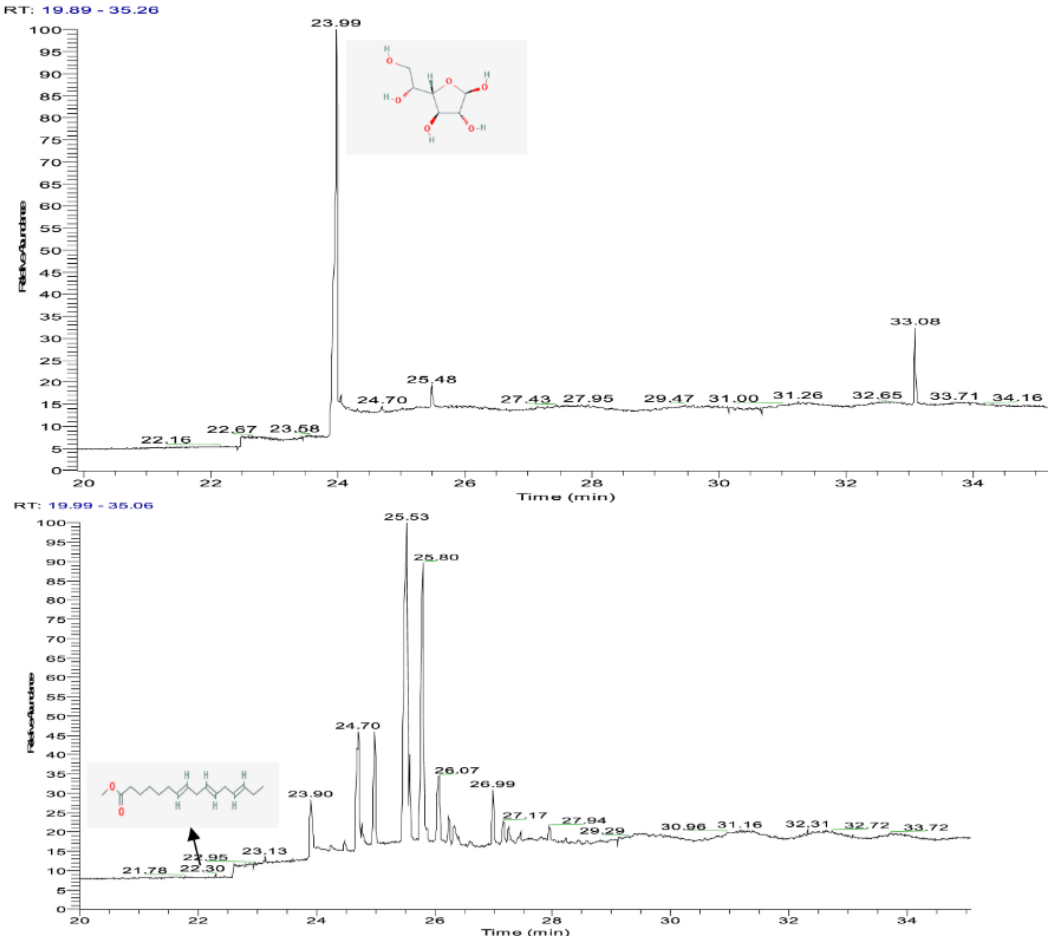

Fig. 8. Total ion chromatogram (TIC) of lignin as (top) control and (bottom) degraded lignin by biofilm-forming bacterial consortium.

Hexadeconoic acids were reported in the pulp paper mill effluent by several researchers (Singh and Chandra, 2019). The RT 25.53 showed similarity with benzyldehyde, 4-(acetyloxy)-3-methoxy (82.36), 25.80 showed similarity with1,2-Benzenedicarboxylic acid, dibutyl ester. These compounds have been might be produced during the different stages of paper production

such as wood digestion, bleaching and pulping. They may also be the result of biodegradation of the persistent organic pollutants presnt in the wastewater (Kumar and Chandra, 2021). Moreover, some minor peaks were obtained in degraded lignin at RT 26.07 (2,2-Dimethylbutanoic acid, 3- trimethylsilyloxy-, ether ester), RT 26.99 (2Hydroxyisocaproic acid, trimethyl ether, trimethylsilyl ester), and RT 27.17 (2-Hydroxyisocaproic acid, trimethyl ether, trimethylsilyl ester). Tetrasiloxane, 1, 1, 3, 3, 5, 5, 7, 7-octamethyl is the only compound that is detected in control and not degraded even after being treated by the biofilm-forming consortium.

Table 1. Organic compounds were identified by GC-MS analysis extracted with ethyl acetate from untreated (control) and treated lignin with the biofilm-forming bacterial consortium. RT-retention time.

S. N.	RT	Control	Biofilm degraded	Compound	Abundance (%)	Toxicity
1.	21.78	-	+	Quercetin 7,3',4'-Trimethoxy	<10	Not confirmed
3.	22.30	-	+	7,10,13-hexadecatrienoic acid methyl ester	<10	Not confirmed
4.	22.67	+	-	Tetrabromo-2-(3-hydroxy-1,2-dihydro-quinol-2-ylidene)-2,3-dihydro-1H-benz[f]indene	<10	Not confirmed
5.	22.95	-	+	Alkane hydrocarbon	>10	central nervous system depression, metabolic acidosis, and arrhythmia (Tormoehlen et al., 2014)
6.	23.99	+	+	β-d-galactofuranose	100	Not confirmed
7.	24.70	+	+	Hexadecanoic acid	>10	Endocrine-disrupting chemicals (Singh and Chandra 2019)
8.	25.48	+		1,2-Benzenedicarboxylic acid, butyl octyl ester	>15	LD50 >63 mL/kg in rat (Gesler, 1973)
9.	25.53	-	+	Octadecanoic acid	>90	
10.	25.80	-	+	Elemol	>90	
11.	26.07	-	+	1,2-Benzenedicarboxylic acid, dibutyl ester (CAS)	>30	Not confirmed
12.	26.99	-	+	3-Carene	>25	
14.	27.43	+	-	Benzene acetic acid, à, 3, 4-tris [(trimethylsilyl) oxy]-, trimethylsilyl ester	>10	Not confirmed
15.	27.95	+	+	Tetrasiloxane, 1, 1, 3, 3, 5, 5, 7, 7-octamethyl-	>15	Hyperlipdemias and weight loss in mice (Samar et al., 2022)
16.	29.29	-	+	Pentacosane	>10	Aspiration hazard (Hazardous Substances Data Bank)
17.	33.08	+	-	Francheline	>25	Not confirmed

4. Conclusions

Bacillus subtilis, Enterobacter cancerogenus, and *Bacillus licheniformis* isolated from pulp and paper wastewater were able to produce high molecular weight quorum-sensing signal molecules (AHL) during the degradation of 900 ppm lignin. Production of AHL leads to biofilm formation which enhanced the lignin degradation up to 900 ppm. These findings were confirmed by analyzing the biofilm load in presence of lignin under confocal laser scanning microscopy. Notably, the lignin degradation rate of biofilm forming consortium was 34-39%

higher than that from axenic culture *Bacillus subtilis*, *Enterobacter cancerogenus*, and *Bacillus licheniformis*. Moreover, GC-MS spectrum exhibited bond cleavage and diminish the functional groups after treatment of lignin by biofilm-forming bacterial consortium. Hence, the present study provides an advantageous method for lignin degradation which would be useful for the remediation of lignin-containing complex pollutants.

Acknowledgements

This work was supported by Project funding from DST (SERB) grant number YSS/2015/000768.

Author credit

Sangeeta Yadav: Supervision, conceptualization, data curation, investigation, and writing-original manuscript and revision. **Sonam Tirpathi, Diane Purchase:** Reviewing, revising & editing. **Ram Chandra:** Supervision and conceptualization of data

Compliance with ethical standards.

Conflict of Interest: - The authors assert no conflicting, contending, or fiscal interests in any capacity.

Ethical Approval: - This article does not contain any studies with human participants or animals performed by any of the authors

Acknowledgements

This work was supported by Project funding from DST (SERB) grant number YSS/2015/000768.

Data availability

Data will be made available on request.

References

Anburajan, P., Kim,Y., Scott, A. et al., 2021. Bacterial signaling and signal responses as key factors in water and wastewater treatment. J. Water Process Eng. 44, 102434.

Barrow, G., Feltham, R. (Eds.). (1993). Cowan and Steel's Manual for the Identification of Medical Bacteria (3rd ed.). Cambridge: Cambridge University Press. doi:10.1017/CBO9780511527104

Biswa, P., Doble, M., 2013. Production of acylated homoserine lactone by Gram-positive bacteria isolated from marine water. FEMS Microbiol. Lett. 343(1), 34–41.

Cao, Z., Yan, W., Ding, M., et al., 2022. Construction of microbial consortia for microbial degradation of complex compounds. Front. Bioeng. Biotechnol 10, 1051233

Chukwuma, O. B., Rafatullah, M., Tajarudin, H. A. et al., 2021. A Review on Bacterial Contribution to Lignocellulose Breakdown into Useful Bio-Products. Intern. J. Environmen. Res. Public Health 18(11), 6001.

Da Silva, R. N., Melo, L., Luna Finkler, C.L., 2021. Optimization of the cultivation conditions of *Bacillus licheniformis* BCLLNF-01 for cellulase production. Biotechnol. Reports (Amsterdam, Netherlands), 29, e00599.

Damjan, B., Metka, F., Aleksandra, K. et al., 2022. Genotoxic activity of endocrine disrupting compounds commonly present in paper mill effluents. Sci. Total Environ. 794, 148489.

Das, P.K., Samal, S., 2018. Microbial Biofilms: Pathogenicity and Treatment strategies. Pharma.Tutor. 6,16-22.

Dasgupta, R., Mallik, A., Tsuno, K. et al., 2013. Carbon-dioxide-rich silicate melt in the Earth's upper mantle. Nature 493, 211-215.

Demir, T., Baran, G., Buyukguclu, T. et al., 2014. Pneumonia due to *Enterobacter cancerogenus* infection. Folia Microbiol (Praha). 59(6), 527-30.

Dey, S., Paul, A.K., 2018. Influence of metal ions on biofilm formation by Arthrobacter sp. SUK 1205 and evaluation of their Cr(VI) removal efficacy, Intern. Biodeter. Biodegrad. 132,122-131

Dhanya, B. E., Athmika, Rekha, P. D., 2021. Characterization of an exopolysaccharide produced by *Enterobacter* sp. YU16-RN5 and its potential to alleviate cadmium induced cytotoxicity in vitro. 3 Biotech, 11(12), 491.

Flimban, S., Oh, SE., Joo, J.H. et al., 2019. Characterization and Identification of Cellulose-degrading Bacteria Isolated from a Microbial Fuel Cell Reactor. Biotechnol Bioproc E 24, 622-631.

Gesler, R.M., 1973. Toxicology of di-2-ethylhexyl phathlate and other phthalic acid ester plasticizers. Environ. Health Perspect. 3, 73-79.

Haq, I., Kumar, S., Raj, A. et al., 2017. Genotoxicity assessment of pulp and paper mill effluent before and after bacterial degradation using *Allium cepa* test. Chemosph. 169, 642-650.

Ihssen, J., Reiss, R., Luchsinger, R. et al., 2015. Biochemical properties and yields of diverse bacterial laccase-like multicopper oxidases expressed in *Escherichia coli*. Sci. Rep. 5, 10465.

Jing, S., Zhiping, D., Jianfeng, L. et al., 2020. Co-metabolism for enhanced phenol degradation and bioelectricity generation in microbial fuel cell. Bioelectrochem. 34,107527.

Kalaycı, K.A., Fakıoglu, O., Kotan, R. et al. 2021. The investigation of bioremediation potential of Bacillus subtilis and B. thuringiensis isolates under controlled conditions in freshwater. Arch. Microbiol. 203, 2075-2085.

Kalola, V., Desai, C., 2020. Biosorption of Cr(VI) by *Halomonas sp.* DK4, a halotolerant bacterium isolated from chrome electroplating sludge. Environ. Sci. Pollut. Res. 27, 27330–27344.

Koschorreck, K., Richter, S. M., Ene, A. B. et al., 2008. Cloning and characterization of a new laccase from *Bacillus licheniformis* catalyzing dimerization of phenolic acids. Appl. Microbiol. Biotechnol. 79, 217–224.

Kumar, A., Chandra, R., 2021. Biodegradation and toxicity reduction of pulp paper mill wastewater by isolated laccase producing *Bacillus cereus* AKRC03. Cleaner Eng. Tech. 4, 100193.

Kumari, S., Mangwani, N., Das, S., 2016. Synergistic effect of quorum sensing genes in biofilm development and PAHs degradation by a marine bacterium. Bioeng. 7(3), 205-211.

Lee, S., Kang, M., Bae, J.H., Sohn, J.H., Sung, B.H., 2019. Bacterial Valorization of Lignin: Strains, Enzymes, Conversion Pathways, Biosensors, and Perspectives. Front. Bioeng. Biotechnol. 7, 209.

Liu, Q., Jinfeng, W., Hongqiang, R., 2021. Bacterial assembly and succession in the start-up phase of an IFAS metacommunity: The role of AHL-driven quorum sensing, Sci. Total Environ. 777, 145870.

Maddela, N. R., Binbin, S., Shasha, Y. et al., 2019. Roles of quorum sensing in biological wastewater treatment: A critical review. Chemosph. 221, 616-629.

Malik, W. A., Javed, S., 2021. Biochemical Characterization of Cellulase From *Bacillus subtilis* Strain and its Effect on Digestibility and Structural Modifications of Lignocellulose Rich Biomass. Front. Bioeng. Biotechnol. 9, 800265.

Min, K., Gong, G., Woo, H. M. et al., 2015. A dye-decolorizing peroxidase from *Bacillus subtilis* exhibiting substrate-dependent optimum temperature for dyes and β-ether lignin dimer. Sci. Rep. 5, 8245.

Mishra, S., Shaohua, C., Ganesh, D. et al., 2021. Reduction of hexavalent chromium by *Microbacterium paraoxydans* isolated from tannery wastewater and characterization of its reduced products. J. Water Process Eng. 39,101748.

Mohsin, M., Omer, R., Huang, J. et al., 2021. Advances in engineered Bacillus subtilis biofilms and spores, and their applications in bioremediation, biocatalysis, and biomaterials. Synthetic and Systems Biotechnology. 6. 180-191.

Morohoshi, T., Oi, T., Aiso, H. et al., 2018. Biofilm formation and degradation of commercially available biodegradable plastic films by bacterial consortiums in freshwater environments. Microbes Environ. 33(3), 332-335.

Nikolina, A. X., Eleni Nikouli, P.B., Konstantinos, A. K., 2018. Bacterial biofilm development during experimental degradation of Melicertuskerathurus exoskeleton in seawater. AIMS Microbiol. 4(3), 397-412.

Nisha, P., Thangavel, M., 2014. Isolation and Characterization of Biofilm Producing Bacteria from Arabian Sea. Res. J. Rec. Sci.132-136.

Paul, D., Shaw, G.P., Sean, L. et al., 1997. Detecting and characterizing N-acyl-homoserine lactone signal molecules by thin layer chromatography. Proceedings of the National Academy of Sci. 94 (12), 6036-6041.

Pearl, I. A., Benson, H. K., 1990. The determination of lignin in sulphide pulping liquor. Paper Trade J., 111(18), 35–36.

Perera, M., Wijayarathna, D., Wijesundera, S. et al., 2019. Biofilm mediated synergistic degradation of hexadecane by a naturally formed community comprising *Aspergillus flavus* complex and *Bacillus cereus* group. BMC Microbiol. 19, 84.

Priyadarshanee, M., Das, S., 2021. Bioremediation potential of biofilm forming multi-metal resistant marine bacterium *Pseudomonas chengduensis* PPSS-4 isolated from contaminated site of Paradip Port, Odisha. J. Earth Syst. Sci. 130, 125.

Raj, A., Reddy, M. K., Chandra, R. et al., 2007. Biodegradation of kraft-lignin by *Bacillus* sp. isolated from sludge of pulp and paper mill. Biodegradation 18, 783–792.

Sarkar, D., Kasturi, P., Nishchay, V. et al., 2020. Bacterial quorum sensing in environmental biotechnology: a new approach for the detection and remediation of emerging pollutants, Editor(s): Maulin P. Shah, Susana Rodriguez-Couto, S. Sevinç Şengor, Emerging Technologies in Environmental Bioremediation, Elsevier, 151-164.

Schultze, L.B., Maldonado, A., Lussi, A. et al., 2021. The Impact of the pH Value on Biofilm Formation. 29, 19–29.

Singh, A.K., Chandra, R., 2019. Pollutants released from the pulp paper industry: Aquatic toxicity and their health hazards. Aquat Toxicol. 211, 202-216.

Singh, U.S., Tripathi, Y.C., 2020. Characteristics and Treatment of Pulp and Paper Mill Effluents -A Review. Internat. J. Eng. Tech. Res. 10, 19-26.

Stepanovic, S., Cirkovic, I., Ranin, L. et al., 2004. Biofilm formation by *Salmonella spp.* and *Listeria monocytogenes* on plastic surface. Lett. Appl. Microbiol. 38, 428-32.

Tamura, K., Stecher, G., Peterson, D. et al., 2013. MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. Mol. Bio. Evol. 30, 2725-2729.

Yadav, S., Chandra, R., 2018. Detection and assessment of the phytotoxicity of residual organic pollutants in sediment contaminated with pulp and paper mill effluent. Environ. Monit. Assess. 190(581), 1-15.

Yahya, A. W., Biji, S., Saif, A. B. et al., 2016. Bioremediation Potential of *Bacillus licheniformis* in Heavy Crude Oil Polluted Soil. Int'l J. Adv. Chem. Engg. Biol. Sci., 3, 44-45.

Yong, Y.C., Wu, X.Y., Sun, J.Z. et al., 2015. Engineering quorum sensing signaling of *Pseudomonas* for enhanced wastewater treatment and electricity harvest: A review. Chemosph. 140, 18-25.

Zhang, S., Zhong, Q., Jiang, Y. et al., 2021a. Temperature-induced difference in microbial characterizations accounts for the fluctuation of sequencing batch biofilm reactor performance. Biodegradation 32, 595-610.

Zhang, W., Ren, X., Lei, Q. et al., 2021b. Screening and Comparison of Lignin Degradation Microbial Consortia from Wooden Antiques. Molecules 26, 2862.